Research article

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Ultrastructure study of skin fibroblasts in patients with Ehlers-Danlos Syndrome (EDS): preliminary results

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Abstract

Aim of the study. To investigate, *in vivo* and *in vitro*, the fibroblast-to-myofibroblast transition in patients with hypermobile Ehlers-Danlos Syndrome (EDS). To analyze the dermis of patients with classical form of EDS (cEDS) and with hEDS, to identify qualitative and/or quantitative differences in ECM component and ultrastructural changes in collagen.

Materials and methods. Seven subjects, aged over 18, two with cEDS and five with hEDS underwent two skin biopsy. One sample was prepared for transmission electron microscopy (TEM), the other for immunofluorescence. The diameter of collagen fibers was measured with TEM. Fibrils were analyzed in four patients: the two with cEDS and two with hEDS. For each patient, the diameter of n=250 collagen fibrils was measured. αSMA was used as specific marker for myofibroblast to highlight their presence *in vivo* in the skin of patients with hEDS.

Results. IF observation could not assess an increased expression of αSMA in hEDS patients, which showed no statistical difference compared to classic form patients. The major result from the analysis of TEM images is the clear difference in ECM composition between the two forms of EDS: ECM in hEDS is optically more dense and more prominently composed of elastic fibers.

Conclusions. Our study provides the following important evidence: 1) the absence *in vivo* of dermal fibroblasts in patients with hEDS, demonstrated by αSMA negativity; 2) the presence of statistically significant changes in the diameter of collagen fibrils between the classic and the hypermobile forms. ***Clin Ter 2020; 171 (5):e431-436. doi: 10.7417/CT.2020.2253***

Key words: *Ehlers-Danlos Syndrome (EDS), Fibroblast, Myofibroblast, α-Smooth Muscle Actin (αSMA), Skin biopsy*

Introduction

The term Ehlers-Danlos Syndrome (EDS), defines a clinically and genetically heterogenous group of hereditary connective tissue disorders (HCTDs), characterized by joint laxity, cutaneous hyper-elasticity and tissue fragility.

The 2017 International Classification identifies 13 typologies (1). Diagnosis is supported by major and minor clinical criteria and confirmed by genetical-molecular identification of the causative mutation in the respective gene. On the other hand, the diagnosis for the hypermobile form (hEDS) is based exclusively on the clinic phenotype. (2) Several skin signs have been reported in pazients with hEDS and include cutaneous hyperextensibility, soft/velvety/silky skin, ecchymoses, bruising, and scarring, as well as other less frequent manifestations (3).

hEDS has an estimated prevalence of 1/5000 and is probably the most common HCTD, accounting for 80-90% of all EDS cases.

The disease has autosomal dominant inheritance, with variable penetrance and a F:M ratio of 8:1. The main clinical features of hEDS are joint hyper-laxity, variably associated with luxation and/or joint dislocation, chronic muscle-skeletal pain, chronic asthenia, cutaneous and wound healing alterations.

Multiple studies have demonstrated hEDS being a phenotypically and genetically heterogenous disorder (4), whose molecular basis is still largely unknown (5). TNXB (6), COL3A1 (7) and LZTS1 (8) identified in a minority of cases are candidate genes responsible for the hypermobile phenotype; however, the exact physiopathology of the disease is still poorly understood.

The main differential diagnosis for hEDS is joint hypermobility syndrome (JHS). Specific diagnostic criteria allow to distinguish the two entities; however, patients have been reported to show characteristics compatible with JHS and hEDS without meeting diagnostic criteria for either one. These cases are included under the term of hypermobility spectrum disorders (HSD) (9).

Several studies documented ultrastructural changes in the diameter and morphology of collagen myofibrils from the derma of EDS patients (10,11), but no association was shown between histologic modifications and clinical phenotypes.

A recent study concerning patients with hEDS and HSD, diagnosed according to current international diagnostic criteria, provided a novel phenotypical character-

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istic of fibroblasts. Fibroblasts, isolated from patient skin biopsy samples and cultured in vitro, showed a typical transition to myofibroblast phenotype in hEDS and HSD forms that was not observed in the other studied variants of EDS (12).

Fibroblasts express α-Smooth Muscle Actin (αSMA), which is incorporated into "stress fibers" in order to allow cellular contraction and migration (13,14).

This phenotypical transformation is suggested by these additional changes: reduction of N-cadherin expression, increase and organization of OB cadherin/cadherin 11 and increase of matrix metalloproteinase (MMP), especially of MMP9 (15,16).

Increased levels of MMP signal abnormal tissue homeostasis that translates to a condition of chronic inflammation (17), which is consistent with the symptoms of hEDS and of the broad spectrum of HSD.

In hEDS, as well as in HSD, the triggering event for fibroblast-to-myofibroblast transition consists in the activation of the αvβ3-ILK complex (18,19), an integrin expressed on the cell membrane of endothelia, smooth muscle, myofibroblasts and fibroblasts, capable of binding at least 21 different extracellular matrix (ECM) proteins, including fibronectin and fibrillin (20).

Integrin αvβ3 is involved in several processes, such as adhesion, migration, cell proliferation and survival, angiogenesis, hemostasis, wound repair, inflammation and tissue fibrosis (21,22).

In the previous studies, fibroblast integrin αvβ3 showed only focal expression in control subjects (23), while it is expressed on the entire cell membrane in hEDS patients (24).

The αvβ3 integrin – ILK kinase complex initiates the signal transduction cascade culminating in the activation of transcription factor Snail1, which is responsible for fibroblast-to-myofibroblast transition (12).

Aims of the study

Considering the results of previous studies and the recently established international classification criteria, the aims of this study are the following:

To investigate, *in vivo* and *in vitro*, the fibroblast-to-myofibroblast transition in patients with hEDS and to assess whether this cellular phenotypic characteristic is distinctive of the syndrome

To analyze the dermis of patients with classical form of EDS (cEDS) and with hEDS, to identify qualitative and/or quantitative differences in ECM component and ultrastructural changes in collagen, in order to deepen current understanding on disease etiopathogenesis and direct future molecular investigations.

Materials and methods

Seven subjects aged over 18 years were enrolled in this study: two had a diagnosis of cEDS, confirmed by genetic mutation of COL5A1, and five were diagnosed with hEDS, according to the novel international classification criteria (1).

After providing consent to participate, each patient underwent two 6 mm punch biopsy procedures, in local anesthesia, of the skin on the right and left supra-gluteal regions.

Of the two obtained samples, one was prepared for transmission electron microscopy (TEM) and the other for immunofluorescence (IF) on frozen tissue sections.

Biopsy samples for TEM underwent fixation with a solution of 3% glutaraldehyde in phosphate-buffered saline (PBS). Fixation allows to halt all cellular components, stop phenomena of autolysis and stabilize proteins without denaturation to occur. Following orientation and reduction to optimal size of 1-2 mm^3, samples were post-fixed in 1% osmium tetroxide (OsO4), which fixates lipids and increases electronic contrast.

Prior to the following dehydration phase, the sample was negatively stained with 1% uranyl acetate. During the dehydration phase, all water within the tissue is replaced by an organic solvent compatible with inclusion medium. Dehydrating agent of choice was acetone, which was employed in subsequent steps at increasing concentrations, from 30% to 100%.

The final phase was inclusion in resin, which gradually replaced the organic solvent. Samples were cut with ultramicrotome yielding semithin and ultrathin sections. Semithin sections, with 1 μm thickness, were mounted on microscope slides and stained with toluidine blue. These sections were used as survey sections for selecting areas to be ultrathin sectioned and in light microscopy. Ultrathin section, with thickness of 50-60 nm, were cut with a diamond knife and mounted on a copper grid. Afterwards, staining with 1% tannic acid was performed to contrast elastic fibers and then with 2% uranyl acetate and lead citrate, to artificially improve the limited scattering power of biologic material.

Histologic samples for IF were fixed in 2% paraformaldehyde and 0,2% glutaraldehyde in solution with PBS. Following orientation and reduction to optimal size of 1-2 mm^3, samples were included in gelatin, first a 6% and then at 12% concentration, cooled in ice and treated with a cryoprotective solution of 2.3 M saccharose. After gelatin inclusion and saccharose infiltration, samples were mounted on particular metal pins and frozen in liquid nitrogen (-180°C). Cryo-ultramicrotome cut semithin sections of 200 nm, retrieved by means of a loop with a solution of 2.3 M saccharose and methylcellulose, and mounted on microscope slides.

Samples were analysed using fluorescence microscopy (indirect immunofluorescence). Murine monoclonal antibodies against the antigens CD34, αSMA, vimentin, fibronectin were used and primary antibodies, while fluoresceinated goat antibodies against antigen-antibody complexes were used as secondary antibodies.

αSMA was used as specific marker for myofibroblast to highlight their presence *in vivo* in the skin of patients with hEDS. Vimentin, which belongs to the family of intermediate filaments, is normally expressed in fibroblasts and was employed as cross control to identify cells undergoing phenotype transition. Fibronectin allowed to define ECM and CD34, a marker that is expressed by several cell types including stem cells, was used to identify endothelia.

All procedures performed in studies involving human participants were accordance with the ethical standards

of the institutional and national research committee. All individuals or their responsible carers signed an informed consent prior to their inclusion.

The ultrastructure analysis of dermal components in patients with hEDS and cEDS began with the study of collagen fibers.

In order to support previous evidence in published literature in consideration of the introduction of the novel classification criteria, the diameter of collagen fibers was the first parameter to be assessed.

Diameters were measured with TEM, analyzing fibrils in the papillary dermis that were in proximity to collagen-producing cells (plausibly fibroblasts or myofibroblasts) and transversally oriented in respect to biopsy sample section.

Fibrils were analyzed in four patients: the two with cEDS and two with hEDS, chosen stochastically. For each patient, the diameter of n=250 collagen fibrils was measured.

Results

For each of the two considered variants of EDS, diameters of n=250 collagen fibers were measured in two patients and the two groups of values were treated as independent and extracted from two populations with unknown variance.

Fisher F test allowed to verify the homogeneity of variances and obtained a $p < 0.01$, indicating non homogeneity between samples.

Then, a t-test performed on the two samples, calculating the degrees of freedom according to the Welch-Satterthwaite formula, showed $p < 0.01$, indicating a statistically significant difference between the two populations (Tab.1; Fig. 1).

IF observation could not assess an increased expression of αSMA in hEDS patients, which showed no statistical difference compared to classic form patients. The only structures that revealed a clear positivity to αSMA in immunohistochemistry were blood vessels, the muscle layer of which normally displays actin within smooth muscle cell cytoplasm (Fig. 2a; Fig.2b).

The major result from the analysis of TEM images is the clear difference in ECM composition between the two forms of EDS: ECM in hEDS is optically more dense and more prominently composed of elastic fibers, that are particularly visible on tannic acid staining (Fig. 3a; Fig. 3b).

Our preliminary data highlights that the collagen fibers in the hypermobile form have a lesser diameter compared to those in the classic form.

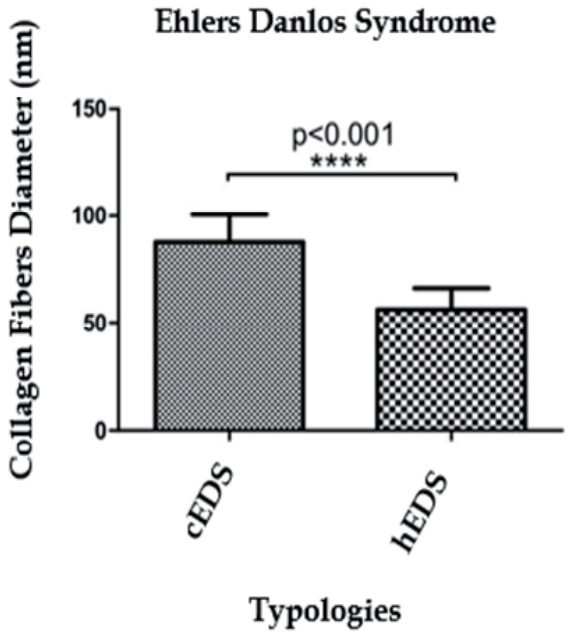

Fig. 1. Statistical analysis

Table 1. Statistical analysis

	hEDS	cEDS	p*
Sample size	500	500	
Mean diameter (± DS)	56,23 nm (± 9,9)	87,02 nm (± 15,3)	<0,01
Maximum diameter (± DS)	101,4 nm (± 9,9)	126,2 nm (± 15,3)	<0,01
Minimum diameter (± DS)	26,19 nm (± 9,9)	52,19 nm (± 15,3)	<0,01
25% Percentile (± DS)	49,95 nm (± 9,9)	76,36 nm (± 15,3)	<0,01
Median (± DS)	86,27 nm (± 9,9)	55,72 nm (± 15,3)	<0,01
75% Percentile (± DS)	62,03 nm (± 9,9)	98,30 nm (± 15,3)	<0,01

*Student Test with Welch-Satterthwaite Correction Values are expressed as averages (DS).

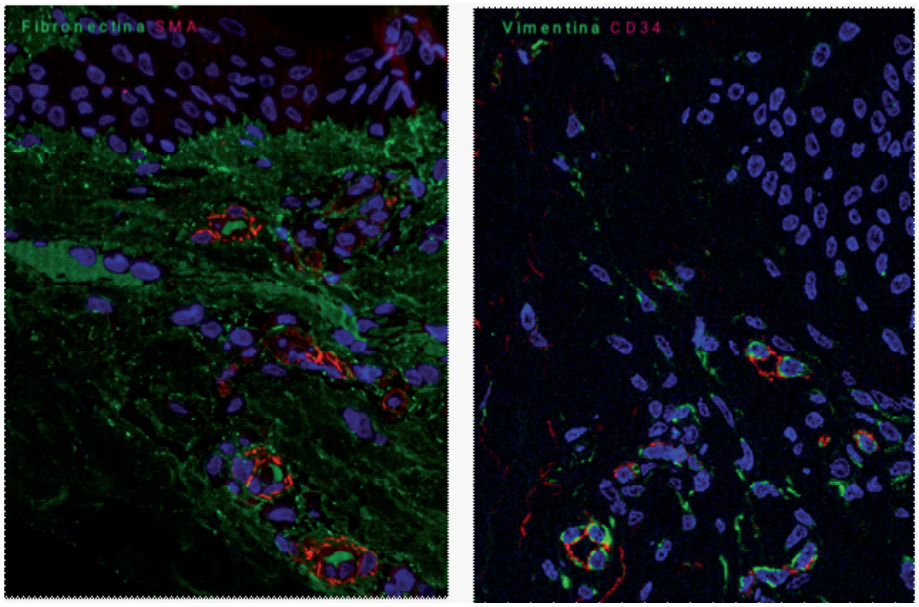

Fig. 2a. Immunofluorescence in hEDS

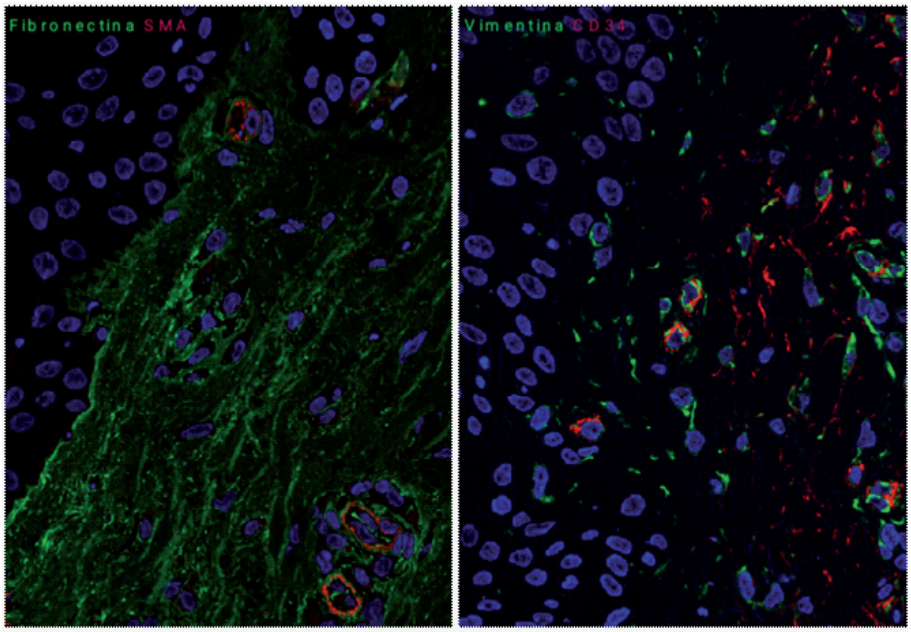

Fig. 2b. Immunofluorescence in cEDS

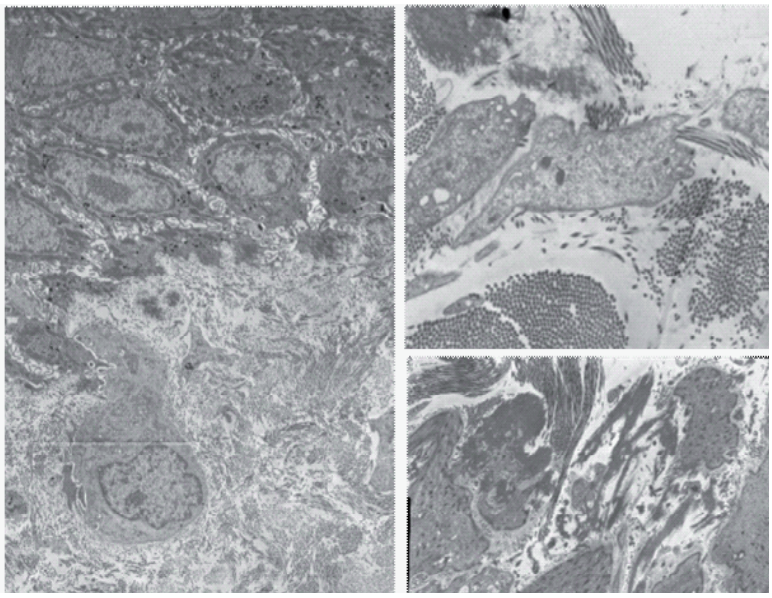

Fig. 3a. TEM in hEDS

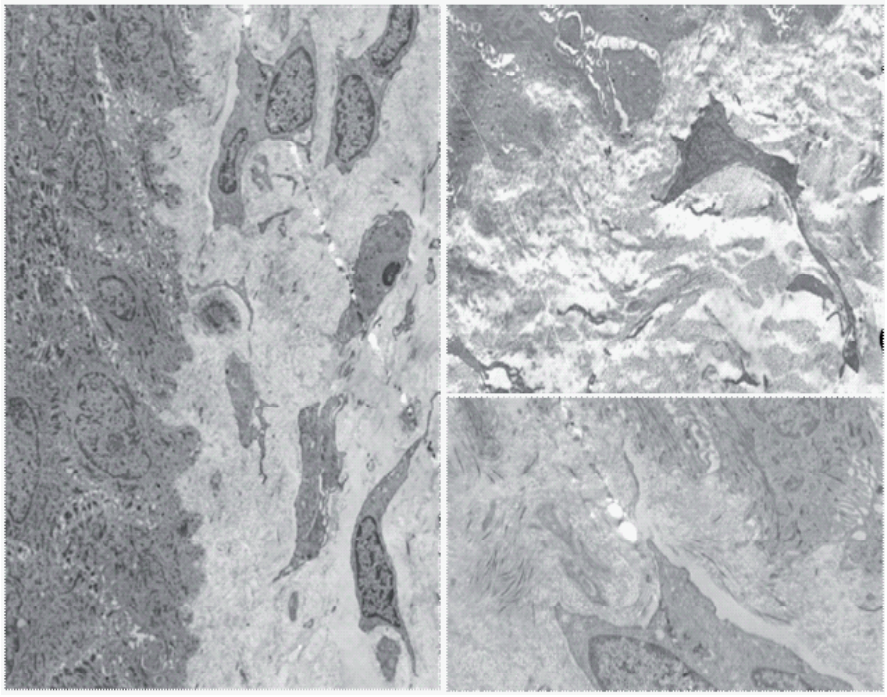

Fig. 3b. TEM in cEDS

Conclusions

Our investigations allowed the study of the components of the dermis and the ECM of patients with hEDS and cEDS.

On the basis of data in the literature showing that fibroblasts, obtained from patients with the hypermobile form of EDS and cultured *in vitro*, have a marked disarrangement of ECM and transition to a myofibroblast phenotype (12), we assessed the same alterations *in vivo*, together with the presence of other abnormalities.

Our preliminary results could not identify a transition to myofibroblast pattern in patients with the hypermobile forms; actually, we could not stain in any biologic sample from hEDS patients on IF αSMA positive cells, with the exception of smooth muscle cells of vascular tunica media, which were regarded as positive controls in this case.

The physiopathology of EDS is based on inflammatory mechanisms (12) that are able to cause tissue remodeling and alterations of ECM (25,26). A state of chronic inflammation would explain not only the changes occurring to the ECM but also the systemic symptoms that are typically referred by these patients, such as chronic pain.

While in genetically determined forms of EDS the changes in ECM seem consequent to underlying molecular defects, the mechanisms responsible for the hypermobile variant are unknown. It is mandatory to deepen the knowledge on the morphologic and structural alterations related to this variant, that represents 80-90% of all cases of EDS.

According to the preliminary results of our investigation, we can hypothesize that collagen fibrils in hEDS are not only smaller compared to those in cEDS but are also located at the lower limit of normal range.

These data might explain the abnormal mechanical properties of the skin and joint observed in these patients.

Our study provides the following important evidence:

The absence *in vivo* of dermal fibroblasts in patients with hEDS, demonstrated by αSMA negativity;

The presence of statistically significant changes in the diameter of collagen fibrils between the classic and the hypermobile forms.

The latter result is particularly significant in relation to hEDS as it may correlate with the typical clinical features of the syndrome and potentially carry diagnostic implications.

Small sample size and our encouraging preliminary results demand that our study be continued to include further selected subjects and a greater number of patients with classic, hypermobile and vascular form of the disease, in order to confirm our observations and to perform additional morpho-structural analyses aimed an effective early diagnosis.

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References

1. Malfait F, Francomano C, Byers P, et al. The 2017 international classification of the Ehlers-Danlos syndromes. Am J Med Genet C Semin Med Genet. 2017;175(1):8-26
2. Sulli A, Talarico R, Scirè CA, et al. Ehlers-Danlos syndromes: state of the art on clinical practice guidelines. RMD Open. 2018 Oct 18;4(Suppl 1):e000790
3. Castori M, Dordoni C, Morlino S, et al. Spectrum of mucocutaneous manifestations in 277 patients with joint hypermobility syndrome/Ehlers-Danlos syndrome, hypermobility type; American journal of medical genetics. Part C, Seminars in medical genetics, 2015; 169C(1):43–53

4. De Wandele I, Rombaut L, Malfait F, et al. Clinical heterogeneity in patients with the hyper- mobility type of Ehlers-Danlos syndrome. Res Dev Disabil 2013; 34:873–881
5. Pacey V, Adams RD, Tofts L, et al. Joint hypermobility syndrome subclassification in paediatrics: A factor analytic approach". Arch Dis Child 2015a; 100:8–13
6. Zweers MC, Bristow J, Steijlen PM, et al. Haploinsuf- ficiency of TNXB is associated with hyper- mobility type of Ehlers-Danlos syndrome. Am J Hum Genet 2003; 73:214–217
7. Narcisi P, Richards AJ, Ferguson SD, et al. A family with Ehlers-Danlos syn- drome type III/articular hypermobility syndrome has a glycine 637 to serine substitution in type III collagen. Hum Mol Genet 1994; 3:1617–1620
8. Syx D, Symoens S. De Paepe A, et al. Ehlers-Danlos syndrome, hypermobility type, is linked to chromosome 8p22-8p21.1 in an extended Belgian family. Dis Mark 2015; 2015:828970
9. Tinkle BT, Bird HA, Grahame R, et al. The lack of clinical distinction between the hypermobility type of Ehlers- Danlos syndrome and the joint hypermobility syndrome (a.k.a. hypermobility syndrome). Am J Med Genet Part A 2009; 149A:2368–2370
10. Carlesimo M, Cortesi G, Gamba A, et al. Ehlers-Danlos syndrome: case report and an electron microscopy study. Rheumatology international, 2012; 32(6):1507–1510
11. Castori M, Celletti C, Camerota F. Ehlers-Danlos syndrome hypermobility type: a possible unifying concept for various functional somatic syndromes. Rheumatology international, 2013; 33(3), 819–821
12. Zoppi N, Chiarelli N, Binetti S, et al. Molecular Basis of Disease. Dermal fibroblast-to-myofibroblast transition sustained by αvß3 integrin-ILK-Snail1/Slug signaling is a common feature for hypermobile Ehlers-Danlos syndrome and hypermobility Spectrum disorders. Molecular basis of disease. 2018; 1010-1023
13. Asano Y, Ihn H, Yamane K, et al. Increased expression of integrin αvβ3 contributes to the establishment of autocrine TGF-β signaling in scleroderma fibroblasts. J. Immunol. 2005; 175:7708–7718
14. Sarrazy V, Koehler A, Chow ML, et al. Integrinsαvβ5 and αvβ3 promote latent TGF-β1 activation by human cardiac fibroblast contraction. Cardiovasc. Res, 2014; 102: 407–41
15. Wang Y, Xu F, Chen J, et al. Matrix metalloproteinase-9 induces cardiac fibroblast migration, collagen and cytokine secretion: inhibition by salvianolic acid B from Salvia miltiorrhiza. Phytomedicine 2011; 19:13–19
16. van den Borne WMS, Diez J, Matthijs Blankesteijn W, et al. Myocardial remodeling after infarction: the role of myofibroblasts, Nat. Rev. Cardiol. 2010; 7:30–37
17. Chiarelli N, Carini G. Zoppi N, et al. Transcriptome-wide expression profiling in skin fibroblasts of patients with joint hypermobility syndrome/Ehlers-Danlos syndrome hypermobility type. PLoS One 2016; 11:e0161347
18. Hinz B, Phan SH, Thannickal VJ, et al. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. Am. J. Pathol. 2012; 180:1340–1355
19. Bhattaram P, Chandrasekharan U. The joint synovium: a critical determinant of articular cartilage fate in inflammatory joint diseases, Semin. Cell Dev. Biol. 2017; 62:86–93
20. Dejana E, Raiteri M, Resnati M, et al. Endothelial integrins and their role in maintaining the integrity of the vessel wall"; Kidney Int. 1993; 43:61–65
21. Horwitz A, Duggan K, Buck C, et al. Interaction of plasma membrane fibronectin receptor with talin—A transmembrane linkage; Nature, 1986; 320:531–533
22. Meredith JE, Fazeli B, Schwartz, M.A. et al. The extracellular matrix as a cell survival factor; Mol. Biol. Cell, 1993; 4:953–961
23. Schaller MD, Paxillin. A focal adhesion- associated adaptor protein; Oncogene, 2001; 20:6459–6472
24. Zoppi N, Chiarelli N, Ritelli M, et al. Multifaced Roles of the αvβ3 Integrin in Ehlers–Danlos and Arterial Tortuosity Syndromes' Dermal Fibroblasts; International journal of molecular science. 2018; 19:982
25. Trial J, Rossen RD, Rubio J, et al. Inflammation and ischemia: macrophages activated by fibronectin fragments enhance the survival of injured cardiac myocytes; Exp. Biol. Med. (Maywood) 2004; 229:538–545
26. Yabluchanskiy A, Ma Y, Iyer RP, et al. Matrix metalloproteinase-9: many shades of function in cardiovascular disease, Physiology (Bethesda) 2013; 28:391–4