UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA DOCTORAL SCHOOL DOCTORAL THESIS

DOCTORAL THESIS

CUTANEOUS ASSESSMENT METHODS IN IMUNE MEDIATED CONJUNCTIVE VASCULAR DISEASES

ABSTRACT

PhD Advisor: Prof. Univ. Dr. CIUREA PAULINA LUCIA

PhD Candidate: CIRSTEA CLAUDIA

CRAIOVA 2015

TABLE OF CONTENTS

Keywords: Systemic sclerosis, Systemic lupus erythematosus, Dermatomyositis, Matrix metalloproteinases.

STATE OF THE ART

Skin lesions are a fundamental modification in the diagnosis of immune-mediated conjunctive vascular disease, both for dermatologist and rheumatologist and often are a modality of debut diseases. Moreover, the evolution pattern skin damage is an indicator of disease activity, being directly interrelated with the severity of the systemic and organ extension. The skin lesions play a determinant rol in quality of life. Patient monitoring involves the use of appreciating extension and severity scores for cutaneous manifestations of various diseases, such as SLE or SSc.

Cutaneous manifestations can be specific, pathognomonic for certain diseases, and the

histopathological aspects accompanied is typical and in some cases is necessary for the diagnosis.

It belongs to several pathological entities, non-specific; most often, cutaneous manifestations conjunctival vascular diseases of immune-mediated are multiple, specific and nonspecific lesions associating.

Regarding the evaluation methods of skin manifestations and microvascular involvement Nailfold capillaroscopy is a non-invasive, safe, reproducible method used to evaluate microvascular lesions in vivo in order to objectification peripheral microangiopathy. This method has been used as a diagnostic auxiliary in diseases such as scleroderma, dermatomyositis, systemic lupus erythematosus and mixed connective tissue disease. It has also been used to differentiate between active and non active diseases, especially dermatomyositis, and to distinguish between primary and secondary Raynaud's phenomenon. Most reports of nailfold capillaroscopy are qualitative and semi-quantitative.

Matrix metalloproteinases (MMP) are a family of structural and functional related endopeptidases, calcium-dependent, zinc-containing which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. MMPs are regulated by hormones, growth factors, and cytokines, and are involved in ovarian functions. Currently, are known 23 families of matrix metalloproteinase. They are classified in six categories: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others.

The aim of this study is the dual approach, clinical and paraclinical of skin manifestations in a group of patients with immune-mediated conjunctive vascular disease, studying the most common three entities lupus, systemic scleroderma and dermatomyositis. The paper aims were to systematize clinical, bio-fluids, imaging and histopathological aspects and to achieve concrete evaluation of skin damage. Personal research oriented towards the study of correlations between skin manifestations and capillaroscopic, histopathological and immunohistochemistry aspects.

The study objectives are:

• Evaluation of the cutaneous manifestations in patients with immune-mediated conjunctive vascular disease.

Correlation of the clinical appearance of skin manifestations severity with visceral manifestations seen in these patients.

• Establish the risk and predictors factors of visceral damage implicitly based on the skin extension lesions.

• Evaluation of microvascular involvement through Nailfold capillaroscopy (NC).

• Correlation between microcirculatory changes, clinical expression of the disease cutaneous and systemic extension.

• Histopathological assessment on skin and muscle biopsy fragments.

• Expression of matrix metalloproteinases 3 (MMP-3) and matrix metalloproteinase 9 (MMP 9) on skin biopsy fragments from the study cohort and the control group.

• Immunohistochemical expression on systemic implications.

MATERIAL AND METHOD

We performed a prospective study on a total of 106 patients with conjunctival vascular diseases mediated immune disease 47with lupus, 39 systemic sclerosis and 20 patients with dermatomyositis. The study was conducted over a period of four years in rheumatology and dermatology clinics of Emergency County Hospital Craiova in the period November 2011 - January 2015. Biological and immunological investigations were performed in the Laboratory Emergency County Hospital Craiova and the immunohistochemical in in the same hospital in Pathology and Cytology Laboratory.The study design involved the inclusion and assessment of patients with immune-mediated conjunctive vascular disease, diagnosed according to the criteria , in terms of the duration of the disease, disease progression, tests of inflammation and immunological examination microvasculature peripherals through capilaroscopiei periunghiale and histopathological and immunohistochemical examination is carried out.

Nailfold capillaroscopy was performed using a video capilaroscop Video DS Medica 3.0 by the same examiner. To avoid artifacts on image and obtaining inconclusive, it is necessary that on the surface is not applied nail varnish and not cut cuticles at least 7 days prior to examination.

Histopathological and immunohistochemical study exam. Fragments of skin and muscle biopsy were processed using conventional histopathological techniques for paraffin embedding and histological staining were stained initially with hematoxylin-eosin usual for examination. For immunohistochemistry of serial sections were made which were immunolabeled with anti-MMP 3 and anti-MMP-9. Immunohistochemical method used in the study was one of the soluble enzyme immunoassay methods based on complex called LSAB / HRP (streptavidin-biotin Labelled). The putty was used DAKO LSAB 2 System HRP (DAKO Universal Labbeled Strepta¬vidin Biotin two Sys¬tem Horseradish Peroxidase). LSAB method (streptavidin biotin)

is a method called ABC (avi-of biotin complex), which substitutes avidin and streptavidin is based on direct conjugation of avidin with enzyme molecules.

RESULTS AND DISCUSSION

Cutaneous manifestations are an essential in current practice for diagnostic and the skin pattern is an indicative directly interrelated with disease activity and with severity of systemic extension.Cutaneous manifestations can be specific, pathognomonic for a certain condition and accompanied by typical histopathological aspects.

Evaluation of patients with SSc skin manifestations of the study cohort, both with dSSc and/or lSSc revealed the presence of Raynaud syndrome on the entire group. Telangiectasias were found in 9 of 13 patients with dSSc (69.23%) and 20 of 26 subjects with lSSc (76.92%). Although we found that patients with higher proportion lSSc shows telangiectasia, compared to patients with dSSc difference is not statistically significant ($p = 0.522$). Digital ulcers were found in 61.45% (8) patients with dSSc, significantly higher percentage compared with lSSc (26.92%), $p = 0.041$.

Nailfold Capillaroscopy, essential for assessing microvasculature changes in patients with SSc, identified the presence"early" pattern in 40% of cases, both for limited and to diffuse forms, an "active pattern" in 46% and "late pattern" in 1 patient with dSSc and 4 with lSSc.

Histopathological exam, essential for ScS diagnosis revealed dense collagen sclerosis in 9 of 13 patients with dSSc (69.23%) and in 18 of 26 subjects with lSSC with no statistically significant differences between the two groups ($p = 0.648$) .

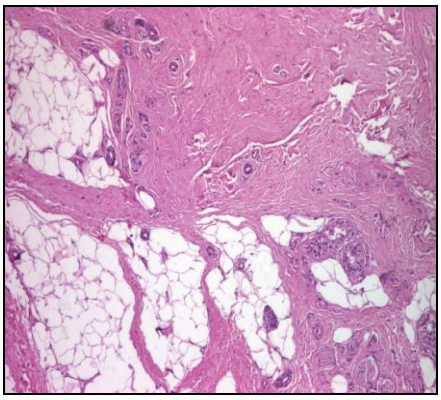

Fig. 1 Colagen sclerosis HE X40

Correlation analysis between the presence of cutaneous manifestations in both lots with dSSc and in those with lSSc revealed many statistically significant data, differentiated by their type. Thus, ulceration digital (UD), in terms of duration of disease, we found a moderate correlation, $r = 0.435$, statistically significant, $p = 0.006$, to diffuse form, similar data to those reported for the limited form of the disease ($r = 0.405$, $p = 0.006$). For the 2 groups we showed a significant link between smoking and the presence or absence UD, 43.75% of smokers

presenting ulcers compared with 39,13% of smoking, statistically significant p = 0.732.

Table 1. Correlations between variables digital ulcers in patients

	Disease duration		Smoking		Pulmonary fibrosis		Pulmonary hypertension		mRSS		ACA		Scl70	
	dSSc	lSSc	SSc	lSSc	dSSc	lSSc	dSScd	lSSc	dSSc	lSSc	dSScd	lSSc	dSSc	lSSc
r	0.453	0.405	0.012	0.230	0.536	0.570	0.381	0.536	0.753	0.503	0.149	0.003	0.219	0.352
p	0.006	0.006	0.230	0.122	0.008	0.002	0.99	0.058	0.002	0.008	0.646	0.882	0.471	0.103

Within our group, **nailfold capillaroscopy** identified the presence of "early" pattern (some giant or dilated capillaries, rare bleeding without avascular areas) to 6 of the 13 patients with dSSc (46.15%) and in 11 of 26 patients with lSSc (42.31%), "active pattern" (frequent giant capillary and bleeding, moderate loss of capillaries, mild disorganization of the architecture capillaries) in 6 patients with dSSc (46.15%) and 12 of those with limited form (46.15%), and "late" pattern in 1 patient with dSSc and 4 with lSSc.

Regarding the group of patients with **lupus,** a condition which recognizes the predominance of the female sex, observation attributed in part to the estrogen effect , in our group of 31 patients with Systemic lupus erythematosus (SLE) have been a predominance of females (29 vs 2), consistent with previously published data. In our group we have identified changes in 29 of the 31 patients with SLE and in 5 of 16 patients with SCLE.

Table 2. Analysis of changes in inter-relationship score actives capilaroscopice disease quantified by SLEDAI revealed the following data:

	Spearman corelation coeficient	p
tortuous capillaries	0.490	0.0001
enlarged capillaries	0.816	<0.0001
giant capillaries	0.726	0.020
proeminent subpapilary plex	0.546	0.008
Scleroderma-like Pattern	0.069	0.757

For the lot with **dermatomyositis** evaluation microvasculature peripherals through nailfold capillaroscopy in patients with DM in the study show modifications on 18 (90%) , represented by disorganization capillary network, footprint of neoangiogenesis, 10 patients (55.55%), dilated capillary loops 10 (55.55%), branching capillaries 11 (61.11%), microbleeds 11 subjects (61.11%), and decreased capillary density with avascular areas in 9 patients (50%).

MMP-3 and 9 expressions in patients with SSc lot from this study showed that cells that expressed the most frequent and more intense both MMP-3 and MMP-9 are fibrocits and fibroblasts.

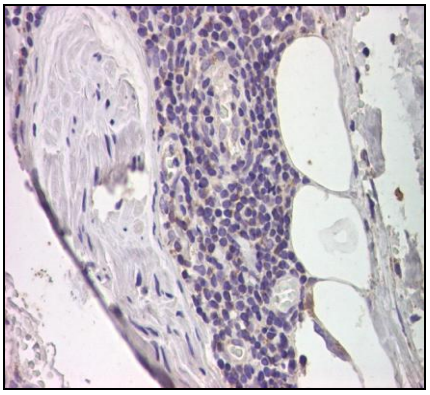

Fig. 2 MMP 3 intense positive in inflammatory cells x100

Our study showed an increase in the keratinocyte expression of MMP-3 and MMP-9 in the absence of levels in patients with SLE.In addition sweat glands, had marked intensively expression in both MMP studied, they are present in the fibroblasts, fibroblasts, macrophages and dermal dendritic cells. As particular aspects, inflammatory cells were negative for both metalloproteinases and MMP-9 was intensely expressed in endothelium of blood vessels from the superficial dermis.

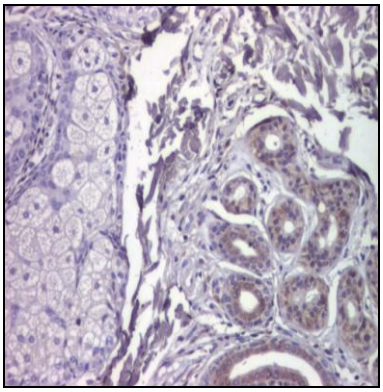

Fig. 3 MMP 3 intense positive in sweat glands x 100

In the present study, the patients group with DM, MMP-3 and MMP-9 presented positive expression in the inflammatory lymphocyte infiltrate with greater intensity for MMP-3. Both MMP were negative in normal muscle fibers in patients with DM and PM. The blood vessels were positive in both MMP with moderate level of medial smooth muscle cells. MMM-9 appears to be intense expressed in lymphocytes from inflammatory infiltrate rather than in the

sarcoplasm of degenerate skeletal muscle fibres. MMP-3 is intense expressed in both in lymphocytes and in the sarcolemma of muscle altered fibers in patients DM and PM.

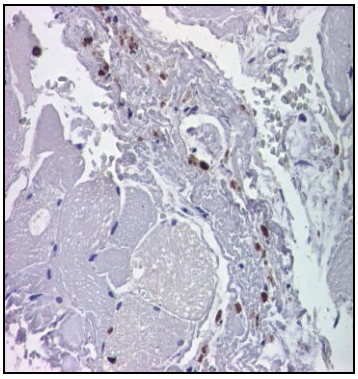

Fig. 4 MMP 9 immunoexpression intense positive in the interstitial inflammatory infiltrate X 200

Table 3 MMP immunoexpression in different cell types in the study groups compared with controls

		fibrocytes	lymphocytes	endothelial cells	keratinocytes	Sweat glands
LOT MARTOR	**MMP 3**	+	Not necessary	+	+	++/+++
	MMP 9	-	Not necessary	+	-	++/+++
ScS	**MMP 3**	+++	+++	+	-	+
	MMP 9	+++	+++	+++	-	-
LES	**MMP 3**	+++	-	+	+++	++/+++
	MMP 9	+++	-	+++	-	++/+++
DM - skin biopsy	**MMP 3**	+++	+++	+	+/++	++/+++
	MMP 9	++/+++	-	+	- /+	+/++
DM/PM muscular biopsy	**MMP 3**	++/+++	+++	++	Not necessary	Not necessary
	MMP 9	++/+++	+++	++/+++	Not necessary	Not necessary

CONCLUSIONS

- Cutaneous manifestations can be specific, pathognomonic for a certain condition and accompanied by typical histopathologic aspects, but most often, the appearance of skin diseases in immune-mediated conjunctive vascular disease is varied associating specific lesions and non-specific aspects.

- Evaluation of patients with SSc skin manifestations of the study cohort, both with dSSc and/or lSSc revealed the presence of Raynaud syndrome on the entire group.

- Nailfold capillaroscopy essential for assessing microvasculature changes in patients with SSc, identified the presence"early" pattern in 40% of cases, both for limited and to diffuse forms, an "active pattern" in 46% and "late pattern" in 1 patient with dSSc and 4 with lSSc.

- Digital ulcers correlated with the active pattern and the presence of telangiectasias was found also significantly higher percentage of these patients.

- Modified Rodnan score (**mRSS),** used to evaluate the extension of skin lesions, was negatively correlated with the capillary density examination found on capilaroscopy.

- Inter-relationship analysis of capilaroscopic astpects and activity score disease measured by the SLEDAI revealed a positive correlation, statistically significant for the presence of elongated capillaries, increased tortuous capillaries, dilated capillaries and prominent subpapilar plexus.

- Immunohistochemical study identified a positive trend of MMP-3 and MMP-9 in the epidermis of patients with SLE and degenerated DM compared with controls. Fibroblasto-fibrociticcells, endothelial and lymphocyte expresses intense MMP 3 and MMP in September than in controls, except for patients with SLE whose lymphocytes are negative for these metalloproteinases.

- In patients with SSc, MMP-3 and MMP-9 are the most highly expressed by fibroblasts and fibroblasts. Increased production of MMP-3 and MMP-9 by the fibroblasts of the dermis suggests that these matrix metaloproteinaze play an important role in the progression of fibrosis in patients with systemic sclerosis skin, most likely through collagen degradation due to insufficient reduction in the activation of MMPs secreted.

- In patients with lupus, like as particular aspect, MMP-3 is intense expressed by epidermal keratinocytes this matrix metaloproteinaze suggesting the involvement in mediating changes in the epidermis in addition to her involvement in dermal tissue remodeling.

- The overexpression of MMP-3 and 9 in the sweat glands, vascular endothelial cells fibroblasts , macrophages and even keratinocytes (MMP 3) in patients with SLE show that this MMP have a rol in the action of proteolytic intense and suggests that the benefits of inhibitors MMP to reduce tissue damage in these patients.

- MMP-3 and MMP-9 immunosuppression in the degenerate muscle fibers may be an important event in the pathogenesis of DM and may have an important role in the development of new therapeutic strategies.

REFERENCES

1. Clements PJ, Lachenbruch PA, Ng SC et al. Skin score. A semiquantitative measure of cutaneous involvement that improves prediction of prognosis in systemic sclerosis. Arthritis Rheum 1990;33:1256–63.
2. Mandana N et al. Epidemiology of systemic sclerosis. Best Parctice&Research Clinical Rheumatology. 24(2010); 857-869
3. Walker UA, Tyndall A, Czirkak L et al. Geographical variation of disease manifestations in systemic sclerosis a report from EULAR Scleroderma trials and Research (EUSTAR)group database.Ann of Rheum Disease.2009: 68:856-62
4. H*esselstrand R, Scheja A, Shen GQ, Wiik A, Åkesson A. The association of antinuclear antibodies with organ involvement and survival in systemic sclerosis.* Rheumatology 2003*;* 42*:*534*–40.*
5. Corlova O, Weng S-F, Ying J et al. Genome-wide association study of systemic sclerosis in a alarge US cohort of over 1500 cases. Arthritis and Rheumatism.2009; 60:S203.
6. Arnett FC, Howard RF, Tan F et al. Increased prevalence of systemic sclerosis in a native American tribe in Oklahoma. Association with an amerindian HLA hallotype. Arthritis and Rheumatism 1996. 39: 1362-1370
7. Le Guern V, Mahr A, Mouthon L et al. Prevalence of systemic sclerosis in a French muti-ethnic county. Rheumatology (Oxford) 2004; 43: 1129-1137.
8. Mayes MD, Lacey JV, Beebe Dimmer J et al. Prevalence, incidence, survival and disease characteristics of systemic sclerosis in a large US population. Arthritis Rheum 2003, 48; 2246-2255.
9. Chifflot H, Fautrel B, Sordet C et al. Incidence and prevalence of systemic sclerosis, a systematic review. Semmin Arthrit Rheum 2008; 37: 223-235.
10. Masi A, Rodnan G, Medsger T. Preliminary criteria for the classification of systemic sclerosis. Arhritis Rheum 1994; 37: 452-462.

11. van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum 2013; 65:2737
12. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988; 15:202.
13. Lonzetti LS, Joyal F, Raynauld JP, et al. Updating the American College of Rheumatology preliminary classification criteria for systemic sclerosis: addition of severe nailfold capillaroscopy abnormalities markedly increases the sensitivity for limited scleroderma. Arthritis Rheum 2001; 44:735.
14. Falanga V, Medsger TA Jr, Reichlin M, Rodnan GP. Linear scleroderma. Clinical spectrum, prognosis, and laboratory abnormalities. Ann Intern Med 1986; 104:849.
15. Rencic A, Goyal S, Mofid M, et al. Bullous lesions in scleroderma. Int J Dermatol 2002; 41:335.
16. Black CM. Measurement of skin involvement in scleroderma. J Rheumatol 1995; 22:1217.
17. Poormoghim H, Lucas M, Fertig N, Medsger TA Jr. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. Arthritis Rheum 2000; 43:444.
18. Mondini M, Vidali M, De Andrea M, et al. A novel autoantigen to differentiate limited cutaneous systemic sclerosis from diffuse cutaneous systemic sclerosis: the interferon-inducible gene IFI16. Arthritis Rheum 2006; 54:3939.
19. Silman AJ, Jones S. What is the contribution of occupational environmental factors to the occurrence of scleroderma in men? Ann Rheum Dis 1992; 51:1322.
20. Systemic sclerosis: current pathogenetic concepts and future prospects for targeted therapy. Lancet 1996; 347:1453.
21. Arnett FC, Eno M, Cnatterjee S et al. Familiar occurerence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis and Rheumatism 2001; 44: 1359-62.
22. Agrarwal SK, Tan FK. Genetics and genomic studies in scleroderma (systemic sclerosis). Rheumatic Disease Clinics of North America 2008; 34:17-40.
23. Assassi S. Mayes MD, Arnett FC et al. Systemic sclerosis and lupus: points in an interferon-mdiated continiuum. Arthritis and Rheumatism 2010; 62:589-98.
24. Dieude P, Guedj M, Wipff J et al. Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. Arthritis and Rheumatism. 2009; 60:225-33.