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SUMMARY TEZOFTHE TEST OF DOCTORATION:

MORFOPATOLOGICAL STUDY OF FIBRILLAR MATRIX IN NONTUMORAL CUTANATE LESIONS

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I. Introduction

The present study has proposed to address one of the most complex and controversial themes of the moment, namely changes in dermal fibrillation proteins in non-tumorale skin diseases, in order to help many practical cases in which a diagnosis of certainty and differential is very difficult..

Studies carried out in this thesis have confirmed observations currently existing in the dedicated literature, such as the presence of sclerosis bands, various types of sclerosis, the reduction or absence of elastic tissue in diseases like morphea, lichen sclerosus, annular granuloma or cutis laxa.

In addition, I tried to find specific and differential diagnostic elements using a complementary method, relatively simple and inexpensive, like polarized microscopy, and I found that this method can be of great help in cases where it is necessary to confirm the hyalinization or necrobiosis of collagen, but also in situations where the assessment of the age of the lesion is important in establishing the final diagnosis.

II. ACTUAL STADIUM OF KNOWLEDGE

II.1 Extracellular matrix

II.1.1 General, definitions

Extracellular space is composed mostly of extracellular matrix (ECM) and interstitial fluids. ECM is in turn composed of major biomolecules: glycosaminoglycans (GAGs) and fibrillar proteins (collagen, elastin, fibrillin, fibronectin and laminin). All these components are locally secreted and organized in a network called extracellular matrix (ECM)

Connective tissue is a matrix composed of ECM, cells (mainly fibroblas) and the fundamental substance.

The fundamental substance is a complex of GAGs, proteoglycans and glycoproteins (mainly laminin and fibronectin)

In most connective tissues the matrix constituents are secreted mainly by fibroblasts but, in certain specialized connective tissues, such as bone and cartilage, osteoblasts and condroblasts respectively..

ECM is not only absolutely necessary for the interconnection of cells to form tissues, but it is also the substrate in which cellular migration is guided during embryogenesis and tissue healing/repair. It is also responsible for releasing enviromental signals to the cellular surface

There are 3 majore classes of biomolecule:

1. Structural proteins: collagen, fibrillin and elastin
2. Proteins specialized: fibronectin, laminine, integrine
3. Proteoglycans: compound protein core to which long channels of repetitive disaccharide units are attached, forming GAGs (complex components with high molecular weight[2].

Collagens are the most common proteins found in the animal kingdom, and the prrincipal constituent of human skin. Represents the major protein structure of the ECM. There are 44 different collagen genes within the human genome. These 44 genes generate proteins that combine in various ways and create 28 different types of collagen, identified by roman numerals. Of all, the most common are types I, II, III, V, which belong to the fibrilllar group and type IV collagen, belonging to the non-fibrillary group. Of these types of collagen, type I

is the most common, representing approx. 90% of all collagen of the human body and dermal collagen (85-90%). Type III represents 8-11% of dermal collagen, and type V 2-4%.

Ii. 2 ELASTIC TISSUE

II.1.1 Generalities, definitions, composition

Elastic fibers make up less than 4% of the dry mass of the skin, forming an extensive network in the dermis and representing the most important mobile component of connective tissue.
The components of elastic fibres are:

1 elastin – the main component, in proportion of 90% in mature elastic fiber – and its precursor, tropoelastin,
2 microfibrils, having as its main component fibrillins
3 integrinele
4 lamininele
5 Fibronectin
6 glucozaminoglicans (GAGs)[58]

II.3 POLARIZED MICROSCOPY AND HIS APPLICATIONS IN THE DERMAL COLAGEN STUDY

Collagen lesions can also be studied with special microscopic techniques, such as: classical linear polarized microscopy', 'quantitative polarized microscopy, laser microscopy with confocal scanning, 'microscopy with light divided into small angles, and multiphotonic microscopy, 'of which, obviously, the cheapest method is linear polarized microscopy.
In the medical field, polarized microscopy can detect allars of collagen fibers that determine different refractive indices. In histological sections, the intensity of light depends on the orientation of the collagen fibers below the viewing angle.
The birth of collagen tissue depends to a large extent on the properties of collagen fibers, respectively: density, orientation, form of storage

Iii. PERSONAL CONTRIBUTION

III.1 Work map

The cases studied were represented by the following pathologies: lichen plano-pilar (2 cases), lichen scleros (13 cases), morphea (10 cases), annular granuloma (13 cases), lupus erythematosus discoid (10 cases), cutis laxa (1 case).
These cases were selected from the archive of the Constanta University Emergency Clinical Hospital, for a period of 5 years, between 2015 and 2019.

The study of cases involved the scoring, in each case, of clinical and histopathological data, respectively: clinical suspicion or suspicions, histopathological diagnosis, clinical location of the lesion, type of biopsy (incisional, excisional, punch), patient age, sex of the patient.

All the patients had white skin.

All patients signed the declaration of consent, a protocol included in the patient's clinical observation sheet.

III.2 Working method

Procedures and stages of work:

1. Each biopsied sample was taken up in the Laboratory of Pathological Anatomy in the fixator solution (neutral buffered formalin 10%). After taking over, each biopsy sample was measured,and characteristic aspects were noted, if any, after which the sample followed the conventional stages of processing. The sample-fixing petition ranged from 24 hours to 72 hours.
2. Histopathological processing (dehydration, clarification and paraffin impregnation). In the Laboratory of Pathological Anatomy of the Constanta County Emergency Clinical Hospital, this stage is performed by automatic method in Diapath Donatella Histoprocessor, SN 19807.
3. Inclusion in paraffinblock. It is also done by automatic method in the Diapath Canova paraffin inclusion machine.
4. Microtom sectioning of the sample included in paraffin. The sectioning of paraffin blocks was carried out with the microtom Sakkura Accu Cut SRM 200, SN 1429-1389. The thickness of the microscopic sections was 5 microns.

5. The posting of sections on the blades (obtaining the histopathological preparation uncolored). This stage in our laboratory is performed manually, using gelatin attached to the surface of the blade, and the heat source for correct intimeandation and bonding of the section on the blade.
6. After sectioning and stretching on the blade, the preparations sit in the thermostat room at 70 grd. for 20 minutes, after which they are inserted into the autostainer.
7. Coloring of sections stretched on the microscopic blade - in our laboratory is done automatically for conventional coloration (hematoxylin-eosine) - in Leica Autotainer - and manually for the special colors used in this study, respectively for Van Gieson coloration for elastic tissue and for Picro Sirius Red coloration. The microscopic blades used were conventionalblades.
8. Mounting and covering the sections with the blades – allows the protection and preservation of the colorful histopathological preparation. For this stage we use Canada balm.

Table 7 - Automatic Processing Protocol in Donatella Hisprocessor (Diapath), SN 19807

No.	Reagent	Processing Time (Time)	Temp	Pressure/ Vacuum	Drain Time
1.	Formalina tamp 10%	00:10	23°C	P/V	60 sec.
2.	What	01:30	37°C	P/V	60 sec.
3.	Ottix Shaper	03:00	37°C	P/V	60 sec.
4.	Ottix more	01:10	37°C	P/V	60 sec.
5.	Ottix more	01:10	37°C	P	60 sec.
6.	Ottix more	01:30	37°C	P/V	60 sec.
7.	Paraffin	03:00	37°C	P/V	60 sec.
8.	Paraffin	03:00	52°C	P	60 sec.
9.	Paraffin	03:00	52°C	P	60 sec.

Table 8 - Working protocol for hematoxylin-eosine coloration, automatic method - Leica Autostainer:

Nr crt	Reagent	Timp (min)
1	Ottix more	10
2	Ottix more	5
3	Drying	2
4	Ottix Shaper	3
5	Ottix Shaper	2
6	Water	2
7	Hematoxylin	9
8	Water	10
9	Eozina	4
10	Water	3
11	Ottix Shaper	6
12	Ottix more	5
13	Ottix more	3

Result: nuclei - black; cytoplasm-red

Table 9 - Van Gieson Coloring Manual Coloring Protocol for Elastic Tissue – Van Gieson Elastic kit no. 115974 Merck

1. Conventional deparafination	
2. Conventional rehydration	
3. Application of agent 3 (rezorcin-fucsina solution)	10 min
4. Water washing	1 min

5. Solutie ferica hematoxylin	5 min
6. Washing tap water	1 min
7. Application Agent 4 (picrofucsina solution)	2 min
8. Ethanol 70 %	1 min
9. Ethanol 70 %	1 min
10. Ethanol 96 %	1 min
11. Ethanol 96 %	1 min
12. Ethanol 100 %	1 min
13. Ethanol 100 %	1 min
14. Toluene	5 min
15. Toluene	5 min

Result: nuclei – black; elastic fibers – black; collagen – red; muscle fibers – yellow

The protocol was taken from the kit's accompanying documents

Table 10 - Manual coloring protocol Picro Sirius Red (kit ab. 150681 - kit for research activities) - taken from the kit's accompanying documents

1. Conventional deparafillation
2. Hydration in distilled water
3. Apply soil. Picro-Sirius Red in such a way as to completely cover the section, then incubat60 min
4. Fast switch 2 times in acetic acid solution, with change of solution
5. Spread in alcohol absolut
6. Dehydration in 2 times in absolute alcohol, with the change of solution
7. Clarification
8. Mount

Results:

- In conventional optical microscopy:collagen – red;muscle fibers – yellow
- In polarized microscopy: collagen fibers type I (thick) yellow-orange; collagen fibers type III (thin) green

The microscopic blades were viewed with the Leica DM750microscope, provided with linear polarization kit, the images were transferred to the computer with the Leica ICC 50HDcamera, via the software LAS V6.4.0,for which the license of usewas acquired. .

III.3 RESULTS AND DISCUTS

III.3.1 Study of morphea cases

The cases of morphea studied were morphoe in plaques. Of the 10 cases, 7 cases were recorded in women and 3 in men. The age of the patients was between 48 and 63 years. The localizations were common: 3 abdominal, 2 on the posterior thorax, 2 on the flank, 1 case on the buttock.

From the analysis of the data accumulated in cases of morphea emerges the following::

1. Epidermal atrophy epidermala was observed in half of cases (50%), with the rest of cases presenting a non-atrophicatrofic epidermis (40%) or even hypertrophic (10%) (1 case – representing the case of Sd.. CREST).
2. Hyperkeratosis was observed in all cases of morphea, the variable being represented by the type of hyperkeratosis, respectively compact in 40% of cases and mixed in 60% of cases. Through mixed hyperkeratosis we designated the cases that presented compact hyperkeratosis covered laced hyperkeratosis.
3. The granular layer was attenuated in 60% of cases and normal in 40% of the cases studied.
4. All cases showed diffuse lesions of dermalcollagen, i.e.
5. The number of dermal sweat glands was reduced in all cases, in some viewing only 1 or 2 glandular acins; compression of the sweat glands and demale capillaries was also found in 2 cases.
6. Hair follicles were absent in 4 cases (40%) and rare in the rest of the cases (60%); in 2 cases we were able to capture follicular destruction. It should be noted that none of the biopsies were performed from the acralaregion.
7. The number of capillaries measured in the papillary dermis and the reticular dermis represented an average of the number of capillaries counted in 10 HP fields, ob400x, and wasin 60% of cases of 4 capillaries/1 HPF in the papillary dermis, and 1 capillary/1 HPF in the same cases, being slightly increased to 5 or even 6 capillaries/1 HPF in the dermul papillary dermis, in the rest of the cases. We noticed that an increased number of capillaries in

the papillary dermis correlated with the degree of inflammation (increased) and the absence of epidermalatrophy. In the reticular dermis we found a single case with 2 capillaries /HPF, this is also the caseof Sd. CREST,and associated with a higher degree of dermalinflammation. .

8. Inflammatory infiltration, lympho-monocytictype, has been reduced or absent in the dermis,a slightly increased degree of inflammation being found in thecase of sd.. CREST.
9. Elastic tissue has been preserved transdermal in all cases of morphea, with reductions or attenuations in 2 cases in the papillary dermis, and absent around the outbreak of skin calcinosis dermul
10. PSR coloration showed aratat an intensely diffuse red reaction of dermalcollagen, with mood in strips, and a homogenized collagen appearance in the papillary and perifolicular dermis in 2 cases
11. Natural birefringence of dermal collagen has been preserved,viewed both in he and VGET colors, especially in the reticular dermis and less in the papillary dermis, and intense transdermal in coloratiile dermul PSR. In PSR we found a prototype of BR, with the predominence of thick fibers (Orange or Red) compared to thin fibers (Green or Yellow).
12. Examination of skin biopsies in PSR allowed observation of the birefringences of the other skin structures, respectively we found that the epidermis, basal membrane, skin appendages and vessels have no birefringence; we also found that the birefringence of the hair (green BR) is different from the epidermal keratin (red BR), and that calcium deposits have no birefringence.

III.3.2. Study of lichen scleros cases

In this study, 13 cases of lichen scleros were analyzed. Of the total of 13 cases, 12 cases had vulvar localization in women (90.8%), and 1 case had posterior thorax localization in men (9.2%). The age of the patients was between 32 and 76 years.

From the analysis of the data obtained from the morphopathological study of cases of lichen scleros, we were able to find the following::

1. 53.8% of cases of lichen scleros had epidermal hypertrophy and 42.8% with epidermal atrophy
2. All cases of lichen scleros had hyperkeratosis, 69.2% of cases had compact hyperkeratosis, 40.8% had mixed hyperkeratosis (compact and laced) and 1 case of mixed hyperkeratosis also showed outbreaks of parakeratosis. parakeratoza.
3. The granular layer was preserved in 7 cases (53.8%) and lost in 6 cases (42.8%).
4. Alteration of the basal layer(vacuolization) was a constant in all 13 cases.
5. Dermal sclerosis was present in all cases, especially objectified by the coloration of VGTE, possible but also seen in conventional coloration (HE), by homogenizing

dermal collagen, dermal both in the papillary dermis and in the reticular dermis. Only one case allowed the visualization of prufunde adipose tissue incorporation into the reticular dermis, due to the biopsytechnique, which provided a specimen with sufficient depth. dermul

6. Dermal inflammatory infiltration was always present in biopsies, in 9 of cases reduced (69.2%), in 3 cases moderately (23%), and 1 case was intense (7.8%).
7. In 5 of the cases studied, in the HE and VGET colors, it seemed possible to develop foci of hyalinization in the papillary dermis, by the "glassy" homogenization of dermal collagen and the sharp reduction or even absence of elastic fibers in the respective foci, but birefringence in PSR was lost in only 2 cases, thus concluding that the real papillary dermal hyalinization was present in only 15.3% of cases. dermul
8. The mean density of dermal capillaries was the same in the papillary dermis and in the reticular dermis, in 2 (15.3%) average cases being 8 capillaries/1HPF, in 5 (38.4%) cases of 6 capillaries/1HPF, in 3 (23%) cases being 5 capillaries/1HPF, in 2 (15.3%) cases of 4 capillaries/1HPF.
9. The average number of capillaries calculated on all 13 cases was 5.7 in the papillary dermis and 5.6 in the reticular dermis
10. Elastic fibers were focally absent in the papillary dermis in 4 cases (30.7%), reduced in 8 cases (61.4%), preserved in 1 case (7.9%). Both cases with the absence of elastic fibers in the papillary dermis presented hyalinization of dermalcollagen, with the absence of its polarization. In the reticular dermis, the elastic fibers were kept in 12 cases (92.3%), 1 case could not be evaluated in the reticular dermis due to the depth of the biopsy.
11. In PSR coloration, in optical microscopy, collagen reaction was red-diffuse, rosu transdermal,fine, without the formation of collagen aggregations. In 2 cases I noticed a condensation of perianal collagen .
12. The birefringence of dermal collagen has been preserved in the papillary and reticular dermis in 11 of the cases, except for the 2 cases with focal papillary dermal hyalinization, in all 3 colors..
13. The birefringence of dermal collagen in PSR coloration showed a constant and diffuse pattern O+++/V+, with 2 exceptions: 1 case in which the perianexial condensedcollagen showed a birefringence O+/V+++, and a case in which green birefringence was relatively equal to orange birefringence in the reticular dermis, type O+/V++. dermul

III.3.3. Study of cases of lichen plano-pilar

The number of cases of lichen plano-pilar found in the archive of Constanta County Clinical Hospital in 2015-2019 was very small, only 2 cases, which is somewhat acceptable if we consider that the plano-pilar lichen is a rare disease.

Both cases were recorded in female patients aged 30 and 55 years, respectively.

The 55-year-old patient is in postmenopausalstatus.

The 30-year-old patient is in the sexually active period, but with symptoms of ovarian failure – irregular menstruation, with long periods of absence of menstruation.

Both locations were in the temporal region.

Clinical diagnoses were in both cases of localized alopecia, with suspicion of lichen plano-pilar.

We observed that the only constants in the two cases are the lesions observed in the HE and VgTE colors, both in optical microscopy and in polarized microscopy, namely: the presence of concentric fibrosis and destructive perifolicular inflammation.

The major difference between the two cases is the different birefringence of perifolicular collagen in PSR coloration. Thus, in the first case perifolicular collagen consists of thin collagen fibers type III, while in the second case perifolicular collagen is a mature collagen, represented by thick collagen fibers type I. This finding leads to the assumption of the existence of different lesions in the two lezionale cases, in the sense that in recent lesions perifolicular fibrosis is performed with immature collagen type III, subsequently it is replaced by thick, mature collagen, type I

III.3.4 Study of cases of discoid lupus erythematosus

This study included 10 cases of lupus erythematosus with skin damage.

Of the 10 cases, 6 cases belonged to the female sex, 4 cases to the male sex. Locations of the biopsies evaluated were: scalp (5 cases), forearm (1 case), cervical region (1 cases), anterior thorax (1 case), leg 1 case

The age of the patients was between 11 and 58 years.

Of the 5 cases of skin lupus with localization in the scalp, 2 cases had a clinical diagnosis of scarring alopecia.

Conclusions:

- The lesions of collagen and elastic tissue observed in the colorations performed, in optical microscopy, were uniform.
- All cases showed interfacedermatitis , dermalfibrosis , inflammation and/or perifolicular fibrosis

- In all cases, a reduction in the number of hair follicles was observed
- Collagen birefringence in HE and VGTE colors was of 2 types: reduced DP/kept in DR in 8 cases, and kept in DP and DR in 2 cases
- Collagen birefringence in PSR was of 3 types:
  1. BR predominantly green in DP/BR predominantly orange in DR (6cases),
  2. BR equal orange/green in DP/predominantly orange in DR (1 case),
  3. BR predominantly orange in DP/DR (2 case)

  NA: (1 caz a fost neevaluabil in PSR).

III.3.5 Cutis laxa study

In the study range 2015-2019 we found 1 case of cutis laxa, male, 36 years, type with dominant autosomal transmission, severe systemic manifestations, and skin damage. manifestari

Cutaneous biopsy had as localization the right arm, and histopathological - skin tissue with flattening and epidermal thinning, with the focal disappearance of the rete ridges and the skin appendages, dermal thickening by thickening of the collagen beams; this dermal collagenization extends into the subcutaneous tissue,which appears atrophic; bratul elastic tissueis reduced quantitatively and focally absent, ande the existing elastic fibers appear thinned, shortened, disorganized.

IV. GENERAL CONCLUSIONS AND ORIGINALITY OF THE THESIS

1. The study presented in this doctoral thesis is the first study of this kind conducted nationally.
2. Dermal collagen changes were noted in all cases studied, establishing particular types of augmented and non-augmented birefringence in all types of lesions analyzed.
3. Modifications of the elastic tissue analyzed and classified in the case of each type of lesion analyzed
4. Polarized microscopy has provided important data on the type of collagen fibers in the dermis in the cases studied, and can successfully serve as an adjunctive method of differential diagnosis in difficult cases.