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Analysis and immunohistochemistry of decorin in localized scleroderma before and after treatment with UVA1

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CHAPTER 1: MORPHEA

1.1: DEFINITION

Localized scleroderma (LS) (skleros: hard - derma: skin) or morphea is a chronic disease that affects mainly the skin[(1)]. It is distinguished from the systemic form, Systemic Scleroderma (SS) or Progressive Systemic Sclerosis (PSS), because is not accompanied by Raynaud's phenomenon, or by acral sclerosis and does not involve internal organs. Although the pathogenesis has not been fully explained, it is believed that, at the base of this disease, there is an increased deposition of collagen and extracellular matrix consequent to an alteration of the autoimmune system, just as in the SS. In fact, despite the existence of various clinical forms of SL, the unifying factor is the presence of a thickening of the skin. The prognosis is generally good, though, next to relatively mild and self-limiting forms, there are some others which are difficult to manage.

1.2: HISTORY

The first descriptions that can be reported to scleroderma are very old and date back to the first century B.C. In fact, in the treatise "Of epidemics" of Hippocrates it is described the case of an Athenian patient whose skin was "..... so hardened that it cannot be raised in folds ....". Many centuries later, in 1753, the Neapolitan doctor Carlo Curzio described in detail the clinical case of a seventeen year old girl, treated at the Hospital of the Incurables in

Naples, "..... whose skin was so hard and inelastic that it was difficult to move the limbs, as well as to open and close the mouth and the eyelids ..... ". Regardless of these observations, the term "scleroderma" has been used for the first time in 1836 by the Italian physician Giovanni Battista Fantonetti (Pavia), although in science, has been commonly used only from 1847 thanks to the French physician Elie Gintrac (Bordeaux) who used this term to describe a condition characterized by increased consistency of the skin. Many years later, in 1945 Goetz, in order to clarify the terminology and to point out a possible visceral involvement, proposed to replace the generic term "scleroderma" with "progressive systemic sclerosis". Although Goetz has laid the groundwork for this important nosographic clarification, the term scleroderma is still used today, especially by non-experts, as synonymous with systemic sclerosis. In fact, despite of the similarity of some morphological and histopathological aspects and the possibility of intermediate forms, Localized Scleroderma (LS) and Systemic Sclerosis (SS) are kept distinct from time, both on the clinically-prognostic level and on the therapeutic one.

1.3: EPIDEMIOLOGY

Although LS is a rare disease, studies have reported an incidence between 0.4 and 2.7 every 100,000 inhabitants. It seems that there is a high prevalence in Caucasians (72.7% -82%), especially in females, with a ratio F: M of 2.4-4.2:1. In addition, it is much more common in childhood than in adulthood. About 90% of affected children are aged between 2 and 14 years, while the average

age in adults is around 40 years. As regards to the morphology, the form in plaques constitutes the vast majority of diagnosis in adults. In children, however, the linear form is more frequent. LS is not a hereditary disease, so that the majority of affected patients has no relative with the disease. It is not uncommon, however, to document a familiar history of other autoimmune diseases.

1.4: CLINICAL FEATURES OF SL

Localized scleroderma (LS) is characterized by fibrotic lesions that are usually confined to the skin although it could involve the underlying tissues of the fibrotic area such as muscles, nerves and even the bone. Rarely, when morphea is located at the face and / or at the head, it may be involve the underlying central nervous system, as in some cases of scleroderma "en coup de sabre" (ECDS). Unlike the systemic form, LS is not accompanied by Raynaud's phenomenon or acral capillaropathy and there is no visceral involvement. In addition, although patients with morphea often present systemic symptoms such as fatigue, arthralgia and myalgia and a positive serology for autoantibodies, they have generally a good prognosis. Localized Scleroderma is a complex disease characterized by an initial inflammatory phase followed by a slow transformation, circumscribed or diffuse, of the skin that looks sclerotic and atrophic. The lesions initially appear as intensely erythematous macules or plaques that soon take on a red-violet color. In time the center of the lesion assumes a sclerotic aspect, white or ivory in color,

surrounded by a typical purple ring, the so called "lilac ring". After this active phase, it remains a translucent, atrophic and sclerotic plaque dotted by white/nacreous areas and a post-inflammatory hyperpigmentation or persistent depigmentation. The atrophy affects the epidermis, the dermis and/or the subcutaneous tissue causing wrinkling and depression of the skin surface. The plate is glabrous and anhidrotic for the excessive deposition of collagen that destroys both the hair follicles and the skin appendages, and dry skin and itching appear.

1.5: CLASSIFICATION

In recent years, many authors have attempted to classify LS but no one of the classification proposed up to now has been validated. This is the reason why, even today, one of the most widely used classification is the clinical-morphological one, proposed by Peterson in 1995 [(2)] that distinguishes five variants:

A. **Localized morphea (in plaques)** characterized by one or a few rounded plaques with an ivory center and a violet edge. In this group are included atrophoderma of Pasini-Pierini, the guttate form, the keloid one and lichen sclerosus et atrophicus.

Guttate morphea comes with little plaque <1 cm located mainly on the trunk which become yellowish, hard, hyper- or hypo-pigmented.

Atrophoderma of Pasini-Pierini is a rare form that affects mostly children with superficial lesions distributed symmetrically to the trunk. The lesions occur in depressed areas of the skin, and present pointed edge and gray-brown pigmentation. The keloid or nodular morphea is rare and characterized by keloid-like nodules in patients with previous or coexisting plaques of morphea mainly in the upper part of the trunk.

Lichen sclerosus et atrophicus mainly affects women between 40 and 50 years. The most typical seats are external genitalia and para-genital region. In the male the foreskin or the urinary meatus may be involved with a constraint that can lead to stenosis.

B. **Generalized morphea** with involvement of two or more areas and frequent muscle involvement

C. **Bullous morphea** with the appearance of blisters due to blockage of the lymphatic vessels by the dermal sclerosis. The superficial dermis, on the floor of the bubble, is lymphangectasic.

D. **Linear morphea** characterized by unilateral linear lesions affecting the limbs, especially the lower ones, with possible involvement of the underlying muscle. The distribution can be dermatomal but it has been hypothesized that it follows the lines of Blashcko (post zygote mosaicism). When this form affects the face, it is possible to observe a

clinical picture called scleroderma "en coup de sabre" (ECDS) or a progressive facial hemiatrophy.

E. **Deep morphea**; in this form, sclerosis affects the subcutaneous tissue, the adipose tissue and the superficial fascia. The skin is stretched and bonded to the floors below. The lesions are often symmetrical and bilateral and involve the upper and the lower limbs. In this group are also included the subcutaneous form, pansclerotic morphea and eosinophilic fasciitis. The last one involves predominantly the fibrous septa of the subcutaneous tissue and the deep band of the extremities, but it spares, generally, hands and feet.

In time have been proposed other classifications, especially since Peterson's classification has been considered, by many authors, extremely controversial because it includes pathologies which are not uniform (as atrophoderma of Pasini-Pierini or eosinophilic fasciitis) and it does not consider that 15% of patients who present mixed forms.

Among all, currently the most accepted and probably the most suitable to describe all clinical forms of morphea, is that proposed by Laxer and Zulian (Table II), which identifies five variants[3]:

1) **Localized morphea**. Is the most common form and occurs usually with a maximum of 3 hardened plaques located on the trunk. It is further divided into a superficial and a deep variant. The first one affects only

the dermis and the epidermis while the deep form strikes, however, the dermis and the subcutaneous tissues, including muscles and fascia. Adult patients with localized scleroderma often develop plaques in areas of pressure (as if there were an underlying isomorphism of Koebner). In women, in fact, the most affected regions are the hips and the breast area around the line of the bra, usually saving the nipples. Although some people report periods of quiescence, many other denounce the occurrence of new lesions over time (Fig. 1).

2) **Generalized morphea**. We talk about generalized morphea when there are more than 4 plaques with a diameter >3 cm involving 2 or more body regions, without the involvement of the face and / or hands. The most affected sites are the trunk, the root of the limbs and the lower back. The plates are often symmetrical and can feed. It occurs in 7-9% of patients with morphea. As in the localized form, the lesions are usually limited to the dermis although rarely, there may be an involvement of the subcutaneous tissues. Generally, patients have a positive serology for autoantibodies (ANA) and accuse as systemic symptoms: myalgia, arthralgia, and asthenia.

3) **Linear morphea**. It has been documented as the most common form in children (41.8-67%). Some authors have suggested, as pathogenetic mechanism, a genetic mosaicism since the strong topographic correlation with the lines of Blaschko. Epidemiological studies have documented a

bilateral involvement in 5-25% of cases. The most common form described are morphea ECDS (en coup de sabre), the progressive hemifacial atrophy and linear morphea of the limbs, which are always associated with the atrophy of the underlying tissue. Morphea ECDS generally occurs at the level of the paramedian frontal region with involvement of the underlying ocular structures and the central nervous system. The lesions may follow two different paths: one runs vertically from the forehead to the side of the nose; the other one from the top continues laterally along the front to head medial to the inner canthus. The progressive hemifacial atrophy, also known as Parry-Romberg syndrome (PRS), is due to the atrophy of the subcutaneous tissues with minimal sclerotic manifestations of the overlying skin. It starts from the forehead and often involves cheeks, tongue and jaw, causing facial asymmetry, especially when it arises in childhood. The average age of onset is 13.6 years with a F: M ratio of 2:1. Tollefson and many other authors have suggested that morphea ECDS and PRS are two variants of the same disease. Linear morphea can also affect the limbs, causing muscle atrophy, reduced muscle strength and contraction of the joints (Fig. 2).

4) **Pansclerotic morphea**. It is the most disabling form of the disease as it affects form the subcutaneous structures to the bone. The disease is manifested by muscle atrophy, junctional contracture and ulcers that fail to heal. Patients present a higher risk of developing squamous cell carcinoma.

5) **Mixed form**. It is found in 15% of patients and is the combination of two or more of the ways previously described.

1.6: SYSTEMIC MANIFESTATIONS

The extracutaneous manifestations generally include myalgia, arthralgia and easy fatigue. Despite these are generally present only in patients with generalized forms but in a retrospective study of 750 children suffering from morphea ECDS has been demonstrated that 22% of them presented extracutaneous manifestations. Of them, about 4% had neurological manifestations such as seizures, headache, peripheral neuropathy, vascular malformations and CNS vasculitis, while 3.2% had ocular involvement with inflammation of the anterior segment, unilateral and asymptomatic uveitis. Many other also had arthritis, gastroesophageal reflux and arrhythmia. Both children and adults presented, with a high percentage, a correlation with autoimmune diseases, with major depressive episodes and anxiety disorders [(4)].

1.7: HISTOPATHOLOGY

The histopathological characteristics of the skin in localized scleroderma depend on the clinical stage of the disease. In the early stages of morphea as well as of systemic sclerosis, in 2/3 of the lower reticular dermis and between the interlobular septa of the subcutaneous adipose tissue, it is possible to see a predominantly perivascular infiltrate consisting mainly of lymphocytes and macrophages, with rare plasma cells, and eosinophils. The collagen fibers are

swollen and edematous particularly in the reticular dermis and shall run parallel to the skin surface. In later stages, the skin becomes non-vascularized and the inflammatory infiltrate disappears. The collagen fibers become thickened, intertwined and uniform. Edema is reabsorbed and the skin takes on a homogeneous and compact aspect (hyalinization) with a reduction in interfibrillar spaces. The elastic fibers are preserved but may present distortions and fragmentation. The eccrine glands become atrophic and surrounded by hypertrophic collagen with little pots around. The subcutaneous adipose tissue is "trapped" in the dermis due to the expansion of collagen in the subcutaneous tissues[5].

1.8: ETIOPATHOGENESIS

Although the etiopathogenesis of morphea remains still unknown, it is thought that many factors can promote its development, such as trauma / radiation, medications, infections, autoimmunity and microchimerism.

- Trauma

  Important retrospective studies have documented in several cases, a previous traumatic event even if the amount is not significant[6]. There are several case reports after injections of vitamin B12 or vitamin K. It is believed that trauma due to the injection may induce a healing process so exuberant that can lead to a fibrosis. It has been also documented several cases of morphea resulting in various vaccinations

(measles, rubella, mumps, diphtheria, tetanus, pertussis, hepatitis B, bacillus Calmette-Guérin). Because of the variety of vaccines involved, it is believed that preservatives or trauma injective could induce morphea rather than vaccines themselves.

- Radiation

Women undergoing radiotherapy in the treatment of breast cancer have an increased risk to develop morphea. The documented incidence is 1 in 500 patients. This process differs from chronic radiodermatitis for its clinical and histological presentation. Post-radiation morphea manifests clinically a clear contraction of the thoracic region involving the breasts. Histologically, it is characterized by the presence of swollen collagen fibers (that are carried upwards towards the epidermis and downwards towards the subcutis), by elastosis, superficial vessels dilated and absence of cellular atypia. Generally this form not necessarily develops at the site of irradiation [(7)].

- Drugs

In several case reports certain drugs such as bisoprolol, ibuprofen, bleomycin, D-penicillamine, bromocriptine, L-5-hydroxytryptophan in combination with carbidopa, pentazocine were considered responsible for the appearance of morphea. The onset of the lesions is very different as latency periods have been registered, ranging from 1 to 30 months

after the intake of the drug. In rare cases, the withdrawal of the drug led to an improvement of the lesions.

- Infections

There is little evidence that relates morphea to Borrelia infection, although some studies have shown a strong correlation between the disease and a positive serology for this parasite. Even CMV was studied as a causative agent because it is able to infect and damage endothelial cells, to recall macrophages, to regulate the formation of TGF-β and to lead to the formation of antibodies against B cells [(8)].

- Autoimmunity

In children, in a rate of between 2% and 5%, was demonstrated a positive correlation with autoimmune diseases (vitiligo, diabetes mellitus, Hashimoto's thyroiditis, Graves' disease and ulcerative colitis). In adults the percentage came up to 30% especially in correlation with psoriasis. In patients with morphea it is possible to document, especially in the early stages of the disease, the presence of autoantibodies, especially ANA, anti-histone antibodies and rheumatoid factor. Recently it has been also documented a significant increase of antipolymerase II alpha (76% of patients with morphea, 85% with systemic form) [(9)].

- Microchimerism

Morphea and systemic sclerosis have clinical and histological features similar to chronic GVHD (graft versus host disease). These similarities raise the interest in the possible pathophysiological role of chimeric cells (non-self-cells transferred from the fetus to the mother during pregnancy or in the opposite direction in the uterus). However, further studies are needed to determine the role of chimeric cells in the pathogenesis of morphea [10].

1.9: CUTANEOUS SCORES

In patients with scleroderma, the first system of evaluation and measurement used was the Rodnan Skin Score (RSS) and subsequently the Modified Rodnan Skin Score (MRSS) [11]. The latter, still widely used (based on the palpation and the pinching of the skin) is able to analyze with semi-quantitative method the degree of the skin sclerosis in 17 distinct anatomical areas (Fig. 3). The score ranges from 0 to 3 (0 = normal, 1 = mild thickening, 2 = moderate thickening, 3 = severe thickening) and the final score may vary, depending on the degree of thickening, from 0 (no thickening) to a maximum to 51 (severe thickening in all 17 locations). Serial assessments with this Skin Sclerosis Score allow to assess the time course of the disease especially in the course of therapy. On the other hand many authors prefer to use the simplest Modified Skin Score (MSS) that brings the body into seven distinct

anatomical regions (head / neck, trunk, limbs, hands, fingers, legs and feet). A more specific measurement is the Localized Scleroderma Skin Severity Index (LoSSI), later replaced with Localized Sclerodema Skin Damage Index (LoSDI) from the LOCUS (Localized Scleroderma Clinical and Ultrasound Study Group). Some authors have recommended the union of these two systems in order to form the Localized Scleroderma Cutaneous Assessment Tool (LoSCAT). Recently Zulian and others have proposed and used a computerized method based on applying Tegaderm on the lesion and marking with a color the harder components inspected with the fingertips. Even the surrounding erythema is highlighted with a different color. The software is able to recognize the two colors and calculate the total area considering both the inflammatory component and the sclerotic one. By providing height and weight of the patient and using the Interclass Correlation Coefficent Two-Way Random Model (ICC) it is possible to calculate the body surface area, and the percentage occupied by dermatoses.

1.10: NON-INVASIVE METHODS

Currently, the follow-up of patients with localized scleroderma is performed using different measurements obtained through non-invasive methods which use different instruments.

- Hardness Tester: is a portable device that allows us to evaluate the hardness of the skin. The measurements vary considerably in

relation to the location of the lesions, to a possible edema, to sex and age of the patient. However, its use is affected by the lack of a complete awareness.

- Cutometer: is a portable computerized device that measures the elasticity of the skin. It is equipped with a probe that determines the speed with which the skin is stretched and returns to baseline status. In this case, the measurement depends on anatomic location, age, gender and edema.

- Thermography: this is tool which captures images and is based on body temperature. It requires a room at a controlled temperature and patient should stay in said room for 15 minutes in order to reach a temperature equilibrium. The instrument works as an infrared camera that requires trained technicians not only in execution but also in the interpretation of scanned images.

- Ultrasonography: it is a non-invasive technique suitable to evaluate the depth of the lesion. It uses probes with resolutions between 10 and 25 MHz which have a good sensitivity.

1.11: MOLECULAR PATHOGENESIS

To date, the clinical research, aimed specifically at SL, is rather limited. The majority of studies has focused indeed on SS. They start from the concept that

SL presents tissue damage similar to those of the SS and that both are characterized by an increased deposition of collagen and extracellular matrix.

The pathogenesis is not yet fully elucidated but it is supposed that the disease is the result of external triggering factors that act on a genetically susceptible host. It appears that these factors, not yet known, are able to cause damage to the small vessels, with release of several pro-fibrotic cytokines and the consequent alteration of the balance between production and destruction of collagen. It is believed that the basis of vascular injury may be an infection, a specific environmental cause or the formation of antibodies to endothelial cells. The reduction of the density of the capillaries involved and the destruction of the endothelial cells, support this hypothesis. It was also documented an inflammatory infiltrate in the perivascular space and an increased activity of fibroblasts (especially at early stage) that escape the normal control mechanisms producing an excessive amount of extracellular matrix; this mechanism leads to fibrosis. The endothelial damage causes the release of cytokines that increase the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin). Their up-regulation invokes the CD4 + T lymphocytes which produce pro-fibrotic cytokines (IL2, IL4, and IL6) which, in turn, attract eosinophils and macrophages. It produces a real pro-fibrotic cascade which also involves numerous chemokines (CCL2, 5, 7, 17, 22 and 27 and CXCL8) that participate and amplify the tissue fibrosis.

IL-2 is most secreted by lymphocytes, while monocytes / macrophages express its high affinity receptors and, behind this stimulation, synthesize the growth TGF-β that activates fibroblasts [12]. In the skin of patients with scleroderma, it was found an increased production and secretion of TGF-β together with a greater expression of specific receptors (TBRI and II) on the surface of fibroblasts. This increase of TGF-β increases the production of collagen, of tissue inhibitors of metalloproteinases (TIMP) and reduces the formation of metalloproteinase (MMP). This imbalance between production and destruction of collagen translates into an increased production of collagen and in a reduction of activity of matrix metalloproteases that, under normal conditions, are responsible for the degradation of collagen. This is the basis of the fibrosis present in morphea and in the systemic sclerosis [13]. The inhibition of the degradation of collagen, could be partially mediated by antibodies anti-MMP. In fact, in patients with systemic sclerosis and morphea have been documented anti-MMP1 (that have collagenase activity) not present in the control group or in patients with other connective tissue diseases such as SLE or dermatomyositis.

TGF-β is also the only known inducer of CTGF, autocrine mediator that contributes to the maintenance of the "fibrotic phenotype" of these cellular elements that appear to be more resistant to the Fas-mediated apoptosis [14]. The "synthetic phenotype", typical of the advanced stages of the disease, is instead characterized on the one hand by the induction of collagen synthesis

and inhibition of MMPs, and on the other by the transformation of fibroblasts into myofibroblasts. These are able to adhere to the collagen fibers and to contract them. In this way, they exert a traction of the fibers themselves and a retraction of the dermis. It has not yet been clarified whether the myofibroblasts are already present in the tissues or if they differ from certain precursors or pluripotent stem cells. The numerous mast cells seen in the scleroderma skin, would be partly responsible for this change through the release of histamine and the increase of the expression of actin. In patients with morphea, at the level of the damaged skin, is also possible to find an increased expression of IGF (insulin-like growth factor or insulin-like growth factor, also known by the name of somatomedin) which is able to recruit the fibroblasts and to promote the production of collagen and the extracellular matrix deposition. It is supposed that also other cytokines, such as connective tissue growth factor (CTGF), the platelet derived growth factor (PDGF) and monocytes chemo-attractant protein (MCP-1) may play a very important role in fibrogenesis suggesting it is possible to intervene pharmacologically in the fibrotic process, interfering, in this way, the biological responsible mechanisms. For example, imatinib mesylate, an inhibitor of tyrosine kinases (such as c-Abl, c-Kit and those associated with the PDGF), which is used primarily in the treatment of chronic myeloid leukemia (CML), Philadelphia chromosome-positive, works by contrasting BCR-ABL (a fusion oncogene with tyrosine kinase function). Studies have indicated that imatinib mesylate is

able to block the fibrous tissue through the inhibition of c-Abl. Moreover, this prevents the expression of genes for extracellular matrix and abolishes, in the treated cells, the morphological or proliferative changes normally induced by TGF-β. These results are very significant for two reasons: the first is that they allow the identification of c-Abl as a molecular mediator of the intracellular response induced by TGF-β; the second is that they suggest the potential role inactivation of c-Abl in the treatment of fibrosing diseases. Recently, the group of Distler et al. has submitted an abstract in which was described the ability of imatinib [15] to prevent the cutaneous fibrosis induced by bleomycin in an animal with scleroderma [1].

1.12: THERAPY

Although therapeutic protocols, universally valid, has not been defined, one of the most effective treatments appears to be represented by a combination of methotrexate and corticosteroids [16]. Positive results were also described after the use of D-penicillamine, mycophenolate mofetil, cyclosporine, IFN-β, of imiquimod and tacrolimus [17,18]. A systematic review of the literature shows that UVA1 phototherapy is the "gold standard treatment" of many conditions associated with altered metabolism of collagen and represents a viable alternative to conventional therapies. The interest in phototherapy was born in the early 90's with the PUVA therapy and led to a good resolution of the sclerosis. Kerscher later speculated that the psoralens were not needed, because patients treated with UVA1 radiation only, showed a marked

improvement in symptoms [19, 20]. Two subsequent studies have compared the protocols UVA1 a mean dose (70 J/cm^2 for session up to a maximum of 2100 J/cm^2), high-dose (130 J/cm^2 for session for a total of 3900 J/cm^2) and low dose (20 J/cm^2 for session up to 600 J/cm^2 total). Patients treated with protocols at mean dose and low dose showed statistically significant results when compared with placebo, while the high dose groups showed statistically significant changes in skin thickness (measured by ultrasound at 20 MHz and histopathological examination) when compared with those at low doses. Further studies have shown that prolonged treatment with cycles of high-dose UVA1 appears to be effective in adult with localized scleroderma, in children with pansclerotic morphea and in patients with systemic sclerosis. In all patients, there was a progressive improvement of functional parameters related to the activity of the disease (including motility and passive junctional skin elasticity). Finally, immunohistochemical studies have shown a clear improvement of the cutaneous sclerosis for induction of collagenase associated with a reduction of the infiltrate lymphocyte [21].

1 The transforming growth factor-β (TGF-β) is a growth factor protein that stimulates the production of extracellular matrix, regulates proliferation, differentiation and activation of immune cells, inhibits cell growth, promotes apoptosis and rules the proliferation and migration of smooth muscle cells and endothelial cells. TGF-β is secreted in an inactive form, the so-called "small latent complex" (SLC), associated to a propeptide (LAP: Latency Associated Peptide). Thanks to a mechanism of extracellular proteolysis, LAP is separated and TGF-beta is made active. The latter presents itself in the form of a dimer composed of 2 subunits linked by a disulphide bridge and consists of 112 amino acids. Once active, TGFbeta is able to bind to the receptor 2 (TbRII) and forms a stable primary complex that will be able to blind to the receptor 1 (TBRI). The result is a stable secondary complex and the activation of the type I receptor that phosphorylates its specific protein targets, the SMADs, on the serine / threonine domains. These are intracellular effectors that allow the transduction of the signal from the plasma membrane to the nucleus .

CHAPTER 2: UVA1 PHOTOTHERAPY

In recent years the phototherapy had the possibility of using lamps emitting ultraviolet radiation with a wavelength between 340 and 400 nm (UVA1) (Fig. 4).

Even if the equipment able to deliver only this range of electromagnetic waves have been available since 1981, it is only in the last decade that studies have shown that phototherapy has high efficacy and tolerability. This progress has been made possible thanks to the production of devices accompanied by halogen-metal lamps extremely powerful, with high-output, arranged in series and suitably filtered in order to eliminate both the ultraviolet radiation with a wavelength less than 340 nm, and the most of the radiation of the visible and of infrared. Currently the treatment with UVA1 is carried out according to three different regimes: high (between 90 and 130 J/cm^2), medium (between 20 and 90 J/cm^2) and low dose (equal to or less than 20 J/cm^2). Regardless of the phototype and the chosen layout, this method, in general, provides daily radiation at a fixed dose without perform progressive increments, as usually occurs in the other phototherapeutic treatments. Many authors suggest a predefined number of exposures: 15 in atopic dermatitis and 30 in the case of dermal sclerosis.

Unlike UVB and UVA2, UVA1 are able to penetrate deep into the skin until arriving at dermal level with a share of 30-40% of the incident radiation (Fig. 5). Thanks to this ability such radiation may modulate the biological and immunological activities of various cellular elements, both epidermal and dermal, which, for this reason, are able to induce apoptosis of Langerhans cells of the epidermis, of T cells or fibroblasts, and the synthesis of collagen. However UVA1 are not capable of isomerizing the trans-urocanic acid present in the stratum corneum to cis-urocanic and act mainly through photochemical aerobic reactions thanks to the production of reactive oxygen species such as

singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide (ROS). These elements are able to trigger a fast sequence of radical reactions which affect not only the phospholipid bilayers of the cytoplasmic, mitochondrial and nuclear membranes (lipoperoxidation), but also the proteinaceous cellular and extracellular constituents and all the elements of the matrix. Free radicals and ROS, biologically active molecules, induce an oxidative damage of amino acids, in particular tryptophan, tyrosine, histidine, methionine and cysteine, leading to the denaturation of structural proteins, enzymes and receptors. The auto-catalytic process of peroxidation of membrane lipids leads, instead, to the formation of their hydroxyl derivatives and numerous aldehyde compounds, the most important of which is the malonilaldeide. Although the oxidative damage of DNA derive many photoproducts such as 8-hydroxyguanine and 5-hydroxycytosine, their mutagenic activity is low. In fact, the enzymatic system of excision and repair of the bases (BER) is able to act quickly and effectively. The documented but modest increase in pyrimidine dimers was found to be devoid of biological importance.

The UVA1 is capable of performing an immunomodulation, through the modification of the transcription and the release of numerous cytokines, the increased expression of cell surface receptors and adhesion molecules, as well as the selective induction of apoptosis of immunocompetent cells.

In keratinocytes irradiated with UVA1 can be observed an increase of the expression of m-RNA and IL-1, IL-6, IL-8, IL-10, TNF-α and isoforms of peptides derived from proopiomelanocortin (POMC-derived), including α-MSH. These act by binding to specific receptors present on the surface of melanocytes (MC1-R) whose expression is regulated precisely by the exposure to UV. UVA1 are also able to directly activate the receptor transmembrane for EGF (epidermal growth factor) and KGF (keratinocyte growth factor). The exposure to UVA1 stimulates the expression of nitric

oxide synthase-2 (NOS-2) and the synthesis of leukotrienes and prostaglandins, especially PGE-2 (powerful immunosuppressant which prevents the activation of certain subpopulations of T cells , in particular Th1) from arachidonic acid, released after the activation of phospholipase A2 [22]. The expression of ICAM-1 on the surface of keratinocytes, in contrast to circulating levels of ICAM-1, is increased after irradiation with UVA1 and is mediated through an oxidative mechanism modulated by intracellular levels of glutathione. Furthermore, always according to the state of the redox cell, UVA1 can induce an increase of the transcription factor AP-1, as well as c-Fos, c-Jun and NF-kB. Unlike UVB, UVA1 induce the synthesis of metalloproteinases (in particular MMP-1 or "collagenase", MMP-2 or "gelatinase A", MMP-3 or "stromelysin-1") in human dermal fibroblasts with an autocrine mode in which are involved IL-1 and IL-6. These "collagenase" determine a stromal remodeling through the degradation of triple helical structure of collagen fibrils and its fragmentation into segments with increased urinary excretion of soluble degradation products of collagen. Moreover UVA1 reduce the release of histamine from basophils and mast cells and their number in the dermis. Also in contrast to what observed with UVB, UVA1 have little effect on the number of epidermal Langerhans cells even if they reduce the size of the cells; anyway they increase dermal dendritic cells CD 34^+ and cause their accumulation in draining lymph nodes with a mechanism that requires IL1-β. UVA1 are also able to reduce the functionality and the number of circulating and infiltrating eosinophils and decrease the production of eosinophil cationic protein. UVA1 also have the ability to induce a phenomenon of self-destruction through the activation of apoptosis. This mechanism acts selecting and leading to death the cell clones or autoreactive T cells through both the early mechanisms pre-programmed (pre-PCD), and the late one (PCD). The delayed apoptosis is achieved after 24 hours after irradiation and takes about 2 hours. The cell quickly loses volume and condenses. It is separated from its neighbors losing the specializations of membrane and exposes components of the cell surface

which are generally hidden or poorly expressed. At nuclear level, there is a condensation and fragmentation of chromatin into units of 180-200 bp. Of the latter ones, some reach the plasma membrane where they are surrounded by evaginations of the same. These "blebs" are detached from the cell body carrying the cytoplasm and nuclear material and giving rise to the so-called "apoptotic bodies" that are phagocytized by neighboring cells. The damage suffered by the DNA induces an increase in the expression of p-53 which blocks the cell cycle progression in G1 phase and facilitates the implementation of repair mechanisms. When the damage is wide, p-53 promotes apoptosis of the damaged cells. The triggering of this process can also take place upon the binding of specific molecules-signal to the respective membrane receptors such as Fas, surface protein belonging to the superfamily of receptors TNF-NGF, now classified as CD 95. A few seconds after the binding of Fas with the specific ligand Fas-L, whose expression is increased after UVA1, there is a oligomerization of the receptor that causes, in turn, the recruitment of cytosolic proteins FADD-MORT-1 in correspondence of its "death domain". Caspase 8 binds on it and triggers a cascade of other caspases (cysteinyl proteinase aspartate-specific). The caspase present in an inactive form in the cytoplasm are divided into three main subfamilies:

1) ICE (ICE, CASPASE 4, CASPASE 5)

2) CPP32 o CASPASE 3 (CASPASE 3, 6, 7, 8, 9, 10)

3) Ich-Nedd-2

Each of them is activated from the previous that, in turn, activates the next, until arriving to the hydrolysis of substrates cytosolic and nuclear. Their action is regulated byApaf-1 (Apoptotic Protease Activating Factor 1), composed in reality of 2 factors: Apaf-1 and Apaf-3. The Apaf-2 coincides with the cytochrome c, essential cofactor together to ATP.

After UVA1, it was also observed an inhibition of the gene coding bcl-2 ("anti-apoptotic protein"). In fact, bcl-2 is the first of a family of genes that includes members with antiapoptotic (bcl-2, bcl-x L, Bag-1, Bcl-w, mcl-1, bid) and pro-apoptotic (Bcl- S x, bax, bad, bak, bik-1) activities. Bcl-2 can bind to other members of his family to form homodimers or heterodimers whose functional significance varies from pro-apoptotic to anti-apoptotic. Therefore, the relationship between the various members is decisive in the cellular response. The protein Bcl-2 would be linked to complexes formed from a dimer of mitochondrial porins VDAC (Voltage Dependent Anion Channel) and two molecules of AdNT (conveyor of adenine nucleotides). These complexes, located at the points of contact between the inner mitochondrial membrane and the external one, give rise to structures called "pores mitochondrial" whose opening is regulated by Ca2 +, Mg2 +, H +, ADP and by family proteins bcl-2. The reduction of d.d.p. and the entry of solutes with swelling of the matrix, which occur when a pore opens, may lead to the rupture of the outer membrane with leakage of proteins localized in the intermembrane space, including its cytochrome C. UVA1 are able to trigger the apoptotic process through a quicker way (early death or pre-programmed) by the direct action of ROS on the membranes. This phenomenon occurs within 4 hours after the irradiation, takes less than 20 minutes and does not require protein synthesis "de novo". This early apoptosis is induced by singlet oxygen, which opens the channels of the mitochondrial membrane (site S mitochondrial mega-pore cyclosporine A-sensitive) with consequent arrest of the cellular respiration. In particular, the release of cytochrome C determines the activation of caspase 9 through the formation, in the presence of dATP, of a complex with the Apaf -1, which in turn induces the activation of caspase 3 able to carry out the process.

CHAPTER 3 THE EXTRACELLULAR MATRIX

3.1: THE EXTRACELLULAR MATRIX

The extracellular matrix is composed of a network of collagen and elastic fibers immersed in a highly hydrated gel, which is able to give to the system viscosity, strength, and consistency and to make it adhesive, lubricant and shock absorber.

While in the past it was considered a relatively inert compartment which could only stabilize the architecture of a tissue, currently it is assigned an active role as a reserve of numerous growth factors and cytokines involved in homeostasis of the tissues and in the modulation of major activities such as cellular survival, growth, proliferation, migration, differentiation and function.

The ECM is composed mainly by two major classes of extracellular macromolecules [23,24]:

1. Fibrous proteins, such as collagen and elastin, with primarily structural function, and non-collagenous glycoproteins, such as fibronectin and laminin, involved in the processes of adhesion and responsible for the elasticity and the resistance which oppose to the tissue tension.

2. Chains of polysaccharide. These are mainly glycosaminoglycans (GAGs) in part linked covalently with the protein in the form of proteoglycans (PGs). They are fundamental in the maintenance of ionic homeostasis, in the stabilization of the whole collagen system and in the regulation of collagen fibrillogenesis. Moreover, thanks to their ability to retain large amounts of water, they make the amorphous fundamental substance ECM like a highly hydrated gel, a colloidal solution very viscous able to resist compression and to optimize the biomechanical properties.

Despite the extracellular matrix appears static, it actually undergoes a slow turnover where the macromolecules are constantly degraded and resynthesized. The matrix components are degraded by extracellular proteolytic enzymes (proteases) secreted from the cells on site. Many of these proteases are metalloproteases and their activity depend on the ability to bind calcium ions or zinc. Some others are serine proteases, which act on a highly reactive serine.

Some metalloproteinases, such as collagenase, are highly selective and specific for certain proteins and limited to these sites; thereby they protect the structural integrity of the matrix. Some others are less specific but, since they are anchored to the plasma membrane, their field of action is limited in space and time

Three basic mechanisms operate to ensure that the protease, which are apt to degrade the components of the matrix, are strictly controlled:

1. Local Activation: Many proteases are secreted as inactive precursors and can be activated locally as needed (i.e. plasminogen).

2. Confinement by surface receptors: many cells have surface receptors that bind proteases, thus confining the enzyme only in sites where it requires its action. The UPA (urokinase-type plasminogen activator) is one such example.

3. Secretion of inhibitors: the action of these protease remains confined to specific areas by specific inhibitors, such as TIMPs (Tissue Inhibitors of metalloproteases - tissue inhibitors of metalloproteases). These inhibitors are protease-specific and able to bind strongly to the enzymes activated, blocking their activities.

3.2: COLLAGEN

Collagen constitutes about 1/3 of the total mass of proteins in vertebrates. It is the most abundant protein in the human body and provides support and tensile

strength to the skin. The collagen fibers are organized in a different way depending on their biological function: in the tendons thy are arranged in parallel bundles linked transversely between them; in the skin the form a braided and irregular network and in the cornea are they arranged in lamina with a woven structure. The collagen fibers in the skin are very resistant to traction but not to elongation. They have a diameter between 1 and of 12 μm, are distributed randomly and appear as wavy beams unless they are under tension where they appear expanse. In histological sections the collagen fibers are acidophilic, weakly PAS positive and poorly argyrophilic. They color in pink with eosin, in red with the method of Van Geeson, in blue with Mallory staining. There are various types of collagen:

- The most known types of fibrillar collagen are:
  - » Type I (tendons, ligaments, skin and bone)
  - » Type II (hyaline cartilage)
  - » Type III reticular fibers (reticular strome of the organs)
- FACIT-collagen (Fibrillar Associated Collagen) or collagen associated with fibrillar collagen
  - » Type IX associated with the type II
  - » Type X and XII associated with the type I
- Collagen with short chain, such as those typical of basement membranes
  - » Type IV, V and VII (basement membranes)

The chemical characterization of collagen has required long periods of study because of the insolubility of its fibers. Collagen, in fact, is resistant to pH neutral to all proteolytic enzymes. The question was solved when it was discovered that collagen could be extracted in a soluble form from young

tissues because the molecule still contained many covalent bonds. The lack of cross-links in collagen immature makes it possible to extract, from tissues, the basic structural unit, the so-called tropocollagen. This has a mass of about 285 KD and consists of three chains of polypeptide of equal size. In type I collagen, two chains have the same primary structure and are referred to as alpha1; the third instead has a different amino acid composition and is called alpha2. The protein is particularly rich in glycine (about 30%) and proline (about 25%). Lysine, hydroxyline and hydroxyproline are also present in considerable quantities. The latter one is contained almost exclusively in collagen and represents, so, the specific amino acid. Thyroxine, tryptophan and residues of sulfur amino acids are very few [25]. The amino acid sequence of collagen is very regular. In the single chain of polypeptide, one residue every three is represented by three glycine and the succession glycine-proline-hydroxyproline is repeated frequently. Since there are no RNAs of transport for the amino acids hydroxyproline and hydroxylysine, these amino acids are formed in the course of post-translational modifications for hydroxylation of proline and lysine, respectively, after being incorporated into polypeptide chains. These amino acids, by virtue of their rotation limits, direct the helical conformation of tropocollagen. Furthermore, the hydroxyproline helps to stabilize the triple helix structure by forming hydrogen bonds between a chain and the other [26,27,28]. Other hydrogen bonds are established between the peptide NH groups of the residues of glycine and groups CO peptide residue of glycine present on other chains.

All this means that the individual chains become wrapped helically in an anti-clockwise verse, and that the three chains become wrapped upon one another to form a cable supercoiled, i.e. a right-handed superhelix. Overall, the molecule has the shape of a long stick around 300 nm with a diameter of 1.5 nm. The tropocollagen, basic structural unit of collagen, is synthesized in the rough endoplasmic reticulum of the fibroblast like bigger precursors

respectively called pro-α1 (I) and pro-α2 (I). These precursors contain additional peptides (propeptides) located in the terminal position, with an amino acid composition that is very different from the rest of the molecule and, for the low content in glycine and hydroxyproline, do not exhibit helical arrangement. These propeptides are a critical step in the formation of collagen as they prevent the premature formation of fibrils; they maintain the molecules of procollagen in solution and favor the correct aggregation and stabilization of the chains [29]. The propeptides, in the later stages of fibrillogenesis, are removed in the extracellular space by specific proteases, i.e. procollagen peptidase. The removal of the N-terminal propeptide precedes that of the C-terminal propeptide and its detachment is accompanied by an increase in the fibrillar diameter that is achieved through the fusion of very thin fibrillar segments [30,31]. The C-terminal propeptide seems then to adjust the successive phases of the assembly of procollagen and its removal promotes the further side growth of the fibrils (Fig. 6). A problem not yet solved is how the ordinate polymerization of tropocollagen molecules is adjusted outside the cell. One suggestive theory hypnotizes that the cell surface of fibroblasts can control the formation and orientation of the fibrils within the extracellular matrix. Recent studies suggest that the conversion of procollagen into tropocollagen takes place in the cytoplasm of the cell by proteolytic cleavage of the N- and C-terminal propeptides [32,33]. In particular the formation of fibrils begin within the secretory compartment post-Golgi; small collagen fibrils with a constant diameter of 28 nm and with a regular period of 67 nm were observed within carriers coated with membrane known as GPCS (Golgi to plasma membrane carriers). These carriers, with a tubulo-saccular form, detach from the trans Golgi compartment (TGN) and shall deliver their content to the plasma membrane [34,35,36]. According to the model proposed by Screen, the conversion of procollagen in tropocollagen would take place just within the GPCS probably following the fusion with vesicles containing the request protease. Within the GPCS would then start the aggregation of

tropocollagen in fibrillar segments and the same carriers would provide to transfer the newly formed fibrils or in any case during assembly, behind peculiar protrusions of the plasmalemma which have been indicated with the term of "fibripositors" (fibril depositors). These are fingerlike evaginations of the membrane having the function to deposit the newly formed fibrils in the ECM and possess a lumen which goes down inside the cell body. In fact at the center of the lumen were observed fibrils of homogeneous diameter which extend in a continuous way into a bundle of fibrils of the ECM. The distal ends of fibripositors open within intercellular peculiar canaliculi of secretion, each of which consists of two showers that are dug in the membranes of two contiguous adjacent cells [32,33]. In the extracellular space these fibrillar segments act as nucleation / propagation structures and grow both in length and in diameter for lateral and / or terminal fusion or through the addition of individual collagen molecules at their ends giving origin to the first thin collagen fibrils [37,38,39,40]. When the collagen fibrils are subjected to staining with heavy metal ions and are examined in the electron microscope, they appear striated transversely. Along their course they have transverse bands showing an axial periodicity every 64 nm. This has placed a fundamental problem: how can the molecules of tropocollagen long 300 nm associate with each other so as to form fibrils with a periodicity of 64 nm? Since the period of the fibril (64 nm) is markedly less than the length of tropocollagen (300 nm), the molecules arranged in adjacent rows cannot be aligned. The first formulated interpretation states that during the formation of fibrils of tropocollagen, molecules align themselves longitudinally head-to-tail and associate parallel with an overlap for a quarter of their length. This offset arrangement of the molecules give rise to transverse striations that are repeated regularly at intervals of 64 nm. However, this hypothesis has not entirely solved the problem, because the latero-lateral deposition of this long molecules of 300 nm with a phase shift of a quarter of their length should generate a period of about 70 nm. A modification of this hypothesis, proposed

by Hodge and Petruska, postulates that the molecules of tropocollagen, arranged linearly along the fiber, are not linked directly to their ends but there is an interposed space corresponding to approximately half a period. This provision makes reason the appearance of fibrils after negative staining: the regions of the fibers that are strongly thickened for the head-to-tail overlap of the tropocollagen molecules are relatively impermeable to the dye and appear clear; regions of the fibers corresponding to the intervals between molecules arranged linearly are more permeable to the dye and appear darker. According to this model, then, the adjacent molecules of tropocollagen are staggered in the longitudinal direction by a distance D and give rise to fibrils with repetitive period consists of a clear zone of overlap and by a dark area of discontinuity.

Initially non-covalent interactions (polar and hydrophobic) between the collagen molecules are responsible for the aggregation of the fibrils but, once this has been formed, it is stabilized by covalent cross-links between the molecules [41,42,43]. The formation of such cross-links or cross-links is therefore essential for the stabilization of the molecules of tropocollagen and to interconnect them within the fibril. The number of these stable bonds increases with age, giving rise to an increase of the resistance. With aging, the covalent bonds (cross-links) within and between units of tropocollagen increase and the collagen fibrils become more rigid and brittle. These cross-links, greatly complex, can be divided into reducible and non-reducible. It would seem that, with age, the reducible cross-links diminish and the non-reducible ones increase (in human skin the maximum reducible cross-links can be demonstrated between 17 and 20 years). The decline of the reducible cross-links would be expression of their slow transformation into non-reducible forms.

With aging also the speed of the catabolism of collagen decreases progressively reducing the speed of the turnover of the molecule. The turnover

of collagen is around 50% every 3 years. With aging decrease also the urinary excretion of hydroxyproline, and of the glycoside of hydroxylysine. The reduction of these catabolites is not only indicative of the decreased speed of degradation of collagen but also of its reduced synthesis. Aging also entails an increase of the caliber of the collagen fibers: this phenomenon appears in connection with the glycosylation of such proteins.

At the electron microscope the fibrils are in turn made up of much finer filaments, the so-called micro fibrils which, as the fibrils, have a regular banding that recurs every 64-70 nm, depending on the degree of hydration [44,45] . The microfibrils may show a thickness varying between 20 and 150 nm, a rectilinear or helical course and, depending on the micro-fibrillar architecture, can be divided into two classes:

- Fibrils of T-type large diameter (150-200 nm), constituted by subfibrills with a slightly spiral course that are wound to form a dextrorotatory helix with a screwing angle of that never exceeds 5 degrees [46,47,48, 49].
- Fibrils of type C of smaller thickness (50-100 nm) characterized by a helical and / or corrugated with a constant angle of screwing of 17 degrees relative to the axis fibrillar [50].

It was also observed that, while the fibrils of type T have a period of 67 nm, the fibrils of type C have a banding that is repeated every 64 nm. This is highly indicative of the helical structure of the fibrils of type C. The fibrillar polymorphism appears to be closely connected to the functional role of the fibrils in the connective tissue: the fibrils of large diameter and with almost rectilinear trend are typically present in tissues subjected to high tensile unidirectional forces (tendons, ligaments, aponeuroses); the fibrils of small diameter, however, are mainly found in tissues subjected to multidirectional forces or reversible changes in shape and size (skin, cornea, tendon sheaths,

sheaths of nerves, blood vessel wall) ensuring, by virtue of the higher surface / volume ratio, a greatest number of interfibrillar bonds.

3.3: ELASTIN

The elastin is found in most connective tissues together to collagen and polysaccharides and is the main component of elastic fibers. It is present in large quantities in the walls of blood vessels and in ligaments. The amino acid composition, even for the elastin, is very special. A third of the amino acid is represented by glycine; it is rich in proline, alanine and other non-polar aliphatic residues but not in hydroxyproline. The mature elastin contains many cross-links that make it insoluble and difficult to analyze. The subunit base of the fibrils of elastin is the tropoelastin that has a PM of about 72,000 and contains about 800 amino acid residues. The tropoelastin differs from tropocollagen because it has many lysine residues but few proline. The tropoelastin assumes a helical structure different from that of collagen. The zones of helix stretch when a voltage is applied and return to their original length when the tension is released. Four groups R of lysine merge and are converted by enzymes into desmosine and in another similar compound, the isodesmosine. These are able to splice chains of tropoelastin in provisions that can be stretched reversibly in all directions.

3.4: GAGS

The glycosaminoglycans (GAG) chains are composed of repeating units of disaccharides and owe their name to the fact that one of the two sugars (with the exception of hyaluronic acid) always consists of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), often combined with a sulfate group. The second sugar is usually an uronic acid (iduronic or glucuronic). The biosynthesis of GAG is made by fibroblasts and mast cells. It starts from glucose which is phosphorylated through ATP, by the enzyme glucokinase (a hexokinase), to G-6-P, which in turn, is isomerized to F-6-P (reaction

catalyzed by the enzyme phosphoglucose isomerase). F-6-P undergoes amination (reaction catalyzed by the enzyme transamidases) with glutamine which acts as a donor of amino groups and energy. Glucosamine-6-P is formed and then acetylated (reaction catalyzed by an acetylase). Acetylglucosamine-6-P is, so, achieved and, by an enzyme mutase, gives rise to the formation of acetylglucosamine-1-P. For the intervention of a phosphorylase is formed acetylglucosamine that gives rise to the formation of hyaluronate, keratan sulfate, heparan sulfate, heparin and, for the intervention of an epimerase and acetylgalactosamin. Starting from acetylglucosamin, there will be the formation of chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate. Glycosaminoglycans are normally classified according to the presence or absence of sulfate groups in non-sulfurated (GAG) such as hyaluronate and sulfurated (GAGs) such as dermatan sulfate, keratin sulfate, heparin, the heparan sulfate, chondroitin-4-sulfate, and the chondroitin-6-sulfate.

Glycosaminoglycans typical of the skin (hyaluronate and dermatan sulfate) are mostly concentrated in dermal connective tissue. In the dermis of young men there is a greater presence of chondroitin-4-sulfate and chondroitin-6-sulfate, while in the dermis of the adult dermatan sulfate predominates, and there is a gradual depletion of hyaluronic acid. Glycosaminoglycans have the ability to solvate themselves with water. The ability to fix the water arises great interest since the dehydration plays essential meanings in skin aging. For their high molecular weight (they are linear anionic polymers that alternate an amino sugar N-acetylated or sulphate and a uronic acid or galactose and sometimes also contain sulfate esters), the high ionic properties (the electronegative charge performs a function of ion uptake in concerns of cations) in addition to the ability to solvate with water, GAGs have the property of conferring a significant viscosity to the connective tissue. This is not only the base of the dermal turgor but it expresses, also, a correct physiology of the microcirculation. In fact GAGs bind strongly cations acting like a kind of ion

exchange resin. The metabolites in the microcirculation must necessarily pass through this gel of mucopolysaccharides, so it follows a certain filtration. So, impairing the biosynthesis of GAGs, it is possible to alter what we might consider the guarantors of the iso-ionic, isotonic and isosmotic properties of the ground substance.

3.5: PGs

The proteoglycans (PGs) are macromolecules composed of a main proteic axis (core) on which are inserted a series of chains of glycosaminoglycans (GAGs). The "core" is synthesized on the ribosomal membrane unlike the polysaccharide chains that are synthesized at the level of the Golgi apparatus. Everything is transported and assembled in the lumen of the endoplasmic reticulum.

There are several classification criteria of these molecules on the basis of the location, composition of the chains and the performed function. However it is impossible to identify a single recurrent structure within this class of macromolecules (Ruoslahti, 1988). Indeed different types of proteoglycans can be modified during cellular expression, according to different biological needs. Furthermore it was found that the proteoglycanic component changes over time, in a characteristic way, both in quality and in quantity, during the aging of tissues. The major structural variations are found at the level of the chains of glycosaminoglycans which may vary in number or length, as well as in the level of sulfation which is highly versatile (Hardingham & Fosang, 1992). The side chains of glycosaminoglycans, composed of repeating disaccharide units, determine the physical properties of the proteins to which they are attached [Evered & Whelan, 1986] and play a fundamental role in the assembly and disassembly of the basal membranes, of the elastic and interstitial matrices (Cattaruzza & Perris, 2005, 2006).

Proteoglycans are able to create an aqueous compartment in the immediate vicinity thanks to the negative charges carried by the sulfate groups and carboxyl groups present on the side chains. Specifically, the polysaccharide chains of PGs have numerous anionic groups that for a repulsion effect between negative charges create a situation where such chains tend to unwind and to distance itself as much as possible in the solvent. In these conditions the polar water molecules assume a definite orientation in respect of the charged groups of the carbohydrate chains. The consequence is that the volume of solvent controlled by proteoglycan is very large and can be even 1000 times higher than that of the molecule.

This determines the characteristic water-proteoglycans gel that gives to the extracellular matrix the resistance to compression forces. Furthermore, the osmotic imbalance caused by the high local concentration of charged species draws water from the surrounding areas, maintaining in this way a well-hydrated matrix (Hardingham & Bayliss, 1990). A further function of these molecules is to act as selective sieves to regulate traffic between molecules and cells depending on their size, their electrical charge, or both. In practice regulate the transport of salts, nutrients and the solvent, while exercising a filter that prevents the diffusion of molecules of high molecular weight or highly charged. In this way the concentration of macromolecules increases in certain districts and this favors all interactions, especially those concentration-dependent. Proteoglycans are therefore important modulators of the organization of the extracellular matrix and processes that reside here (Hardingham & Fosang, 1992). They play a crucial role also in the issue of intercellular biochemical signals. They are capable of binding various molecules secreted by cells, which are "signal" as growth factors, and can promote or inhibit their activity. The proteoglycans bind and regulate the activity even of different proteolytic enzymes (protease) and protease inhibitors. In humans the proteoglycans are encoded by more than thirty genes:

some of these are subject to alternative splicing, thus allowing the generation of a high functional and structural variability within this class of macromolecules. Regardless of whether they are linked to the cell surface or assembled with other components of the matrix, many of the known proteoglycans are involved in interactions with other cells or extracellular molecules, through the core protein or specific domains of the chains of glycosaminoglycans. These interactions can be divided into three main categories:

- Those involved in the binding of proteoglycans with other structural elements of the ECM;
- Those involved in the kidnapping of soluble factors;
- Those that mediate the binding of proteoglycans to cell surface receptors or to other components associated with the membrane. In particular the latter behave as co-receptors for many soluble factors and increase their affinity towards their receptors (Cattaruzza & Perris, 2006).

In the connective matrix, in addition to the major proteoglycans, known as modular PGs, there are the small proteoglycans with leucine-rich sequences at the level of the core protein (SLRPs, Small Leucine-Rich Proteoglycans) [Iozzo, 1998; Yamaguchi, 2000]. They have a molecular weight of about 40 KDa (Heinegard & Oldberg, 1989; Hardingham & Fosang, 1992) and are characterized by the presence of a central domain rich in leucine, to which are linked one or two chains of chondroitin sulphate (CS) or dermatan sulfate (DS) or more chains of keratan sulfate (KS). The leucine-rich sequences allow the molecule to adopt a conformation like a horseshoe that facilitates interactions between protein and protein (Kresse & Schonherr, 2001). They are normally entirely bound to the surface of the collagen fibrils through their core protein and project their side chains into the interfibrillar space, forming an extended three-dimensional network in the matrix.

These molecules are able to control cell proliferation; they are involved in the modulation of fibrillogenesis and, depending on the mechanical load which must bear, in three-dimensional configuration of the collagen fibrils. Several studies have indicated that different SLRPs bind (non-covalently) in different locations on the collagen fibrils and which are important regulators of assembly and remodeling of the collagen fibrils themselves [51,52]. There are many evidences in this sense:

- Knockout mice for the genes of the SLRPs (genetically modified mice in which has been suppressed, for the study, the expression for genes that encode SLRPs) show disorganized collagen fibrils and consequently loss of most of the functions of connective tissues [53, 54]. In vitro experiments have shown that the SLRPs interact with collagen through specific binding sites, delaying the formation of collagen fibrils in the extracellular space [55].

- The most representative and best known of these are the SLRPs decorin, biglycan, fibromodulin and the lumican (Fig. 7).

The decorin (or proteoglycan II) is secreted by fibroblasts and is the predominant proteoglycan in the tendon and connective tissues rich in collagen type I. It contains a leucine-rich protein core to which are linked one or two chains of DS and is localized on the surface of the collagen fibrils, as to decorate them (hence the name) (Fig. 8 and 9). More precisely, according to Scott [56], the decorin binds non-covalently to the collagen fibrils through the core protein, while the chain of DS extends in the direction of a fibril adjacent and joined head-to-tail with the chain of DS (having antiparallel orientation) of a molecule of decorin linked to such fibril. In this way, there will be bridges interfibrillar which help to keep ordered the fibrils and to impart them a parallel alignment (Fig. 10). It has been determined that different portions of collagen I and II interact with decorin and that the interaction is canceled by

N-acetylation of residues Lys / Hyl, while it is modulated by N-methylation of Lys / Hyl, leading to the conclusion that these residues are directly involved in the bond. The interaction of the protein core with the fibrillar surface is therefore one of the limiting factors in the process of fibrillogenesis. In this regard, it is known (both in clinical and in knock-out animal models) as an alteration of this component has obvious repercussions on the three-dimensional architecture of the matrix. The presence of decorin seems in fact to promote the fusion tail-tail between fibrils and, conversely, inhibit the fusion side, thereby regulating both the diameter and the length of the collagen fibrils and then their remodeling in response to the tensile load [57,58]. The decorin is therefore essential in the formation of collagen fibrils, in fact, the lack of this SLRP cause skin fragility and reduced tensile strength of the tissue. Moreover it seems that it is also able to modify the morphology of the fibrils already positioned. Large amounts of decorin have in fact been documented in bone, tendons and skin in growth. Furthermore, this SLRP when bound to collagen is able to sequester various cytokines at the level of the ECM, such as TGF-β. The biglycan (or proteoglycan I) promotes the assembly of large molecules of collagen. Experiments conducted both in vivo and in vitro have shown that biglycan promotes the maturation of collagen fibrils. In fact, the collagen fibrils of the tendons of knock-out mice for the gene of biglycan show immature fibrils, of smaller diameter and with an altered morphology. These alterations appear to be more pronounced in mice knock-out for both biglycan and fibromodulin [59]. Further studies have shown that the alteration in the expression of fibromodulin is accompanied by a compensatory increase in the levels of lumican, which binds to collagen type I in the same region of fibromodulin, substituting it functionally [60]. However, mice lacking lumican produce collagen fibrils with large caliber that form a disorganized matrix in the cornea and into the dermis. Several studies showed that the SRSPs may determine in relation to the content, to the type and to their mutual proportions, the fibrillogenesis, the final size and the orientation of the collagen fibers, by

an interaction between the polyanionic chains and the basic areas of the molecules of tropocollagen [61,62]. Recent studies also suggest a non-secondary role in the growth and tissue differentiation, through a specific action on the cells. It has been found in fact that many proteoglycans can bind some growth factors, modulating their activity. The small decorin appears to play a direct role on the proliferation of many cancer cells through interactions, even if at low affinity, with the EGF receptor (Patel et al., 1998; Iozzo et al., 1999; Santra et al., 2002). It is also able to bind TGF-β blocking its activity (Yamaguchi & Ruoslahti, 1988; Yamaguchi et al., 1990). In detail the decorin, in the extracellular, binds TGF-beta by inhibiting its binding with the receptors TGFR-I and TGRF-II preventing, so, the way TGF/SMAD. The lack of production of decorin favors instead the way TGF/SMAD which, in turn, encourages fibrosis and destruction of the elastic fibers due to the formation and activation of CTGF and versican.

All connective matrices undergo quali-quantitative changes with the age which affect in different degrees the macromolecules that constitute them. At birth collagen represents about 35% of the dry weight of the tissue, while the complex protein-polysaccharides and glycoproteins represent respectively about 5% and 6%. When the body development is completed, the quantity of collagen reaches about 80%, while that of proteoglycans and glycoproteins passes respectively to 2% and 3%. In adulthood there is a further reduction of proteoglycans and glycoproteins [63]. In reference to proteoglycans, Carrino et al. describe a decrease of macromolecular proteoglycans and an increase of small proteoglycans modified in the skin of elderly subjects [64]. In particular, in adult tissues but not in those embryonic, there is the presence of a catabolite of decorin, the "decoran", which has a reduced ability to bind collagen type I and would therefore be responsible for the changes in the elasticity of the skin in the elderly. Similar changes have been found in other tissues, including the tendons. At the same time it also reduces the water content by passing from

80% at birth to about 30% in the elderly. The progressive increase in collagen with the age is a consequence of the major biomechanical needs. The decrease of proteoglycans and glycoproteins, considered their function in the phase of aggregation and stabilization of the collagen fibrils, can be explained by the decreased synthesis of tropocollagen and with the final structuring and stabilizing function that the collagen presents in the adult.

CHAPTER 4: AIM OF THE STUDY

Localized scleroderma is a disease characterized by loss of elasticity and thinning skin resulting in thickening and hardening of the skin and subcutaneous tissue due to an altered and exuberant fibrillogenesis.

Studies carried out on the skin of patients with systemic sclerosis and localized scleroderma have documented that some short chain leucine-rich proteoglycan (as decorin, biglycan and lumican) play a key role in fibrillogenesis and in the modulation of different growth factors with fibrotic activity.

UVA1 increase the production of collagenase, reduce the production of TGF-β (profibrotic cytokine), increase the production of IFN-γ (antifibrotic cytokine) and represent the therapeutic gold standard of all dermatoses characterized by dermal fibrosis.

With this study we aimed to evaluate the effects induced by UVA1 in decorin. In particular, we evaluated the immunohistochemical expression of this proteoglycan before and after treatment with UVA1 to see if its photo-induced modulation exerts a key role in the progression of the dermatoses.

4.1: MATERIALS AND METHODS

4.1.1: CASE STUDY AND CONVENTIONAL HISTOPATHOLOGY

In the first phase of the study, we selected patients with localized scleroderma following the classification criteria proposed by Laxer and Zulian; the clinical

diagnosis was confirmed histologically. Of all selected patients, we acquired data, anamnesis and written informed consent excluding those presenting an absolute contraindication to treatment with UVA1 (Table 3). So we have recruited ten subjects, all female and aged between 40 and 70 years, with localized scleroderma that had lasted for a minimum of 9 months to a maximum of 5 years.

All subjects in the study, after a period of pharmacological washout of 30 days, have been dispensed a sub-erythematous fixed dose of 50 J/cm^2 of UVA1 first in the supine position and then in the prone one. The regimen included irradiation daily for five days, from Monday to Friday, and for six consecutive weeks, up to a maximum of 30 irradiations, with a global dose of 1500 J/cm^2.

As phototherapy device has been used a bed of UVA1 of Cosmedico, equipped with 24 lamps (F72T12/BL9/HO) each of one at high pressure from 500 watts (Fig. 11). This device, thanks to a system of panels made of acrylic material and filters for both the infrared and the light spectrum, is able to emit a band of non-ionizing REM comprised between 340 and 400 nm together with a negligible share of radiation with a wavelength up to 530 nm and infrared (780-3000 nm). The irradiance emitted by this equipment was constantly measured and monitored with a optoelectronic spectroradiometer with a diffraction grating, able to acquire the UV radiation through a fiber optic probe and transmit the recorded data to a PC through an interface with a standard parallel port (Spectramedtm Flyby srl Livorno-Italy) (Fig. 12). The measurements have been performed continuously placing the sensor at the level of the skin surface in the sternal area, during the treatment, every time the patient was in a supine position.

Before starting the sessions of phototherapy and at the end of the provided treatment, in all patients enrolled was calculated, by palpation and the pinching of the skin, the value of the Rodnan Modified Skin Score (MRSS).

In addition, at the level of a target lesion previously identified, was made:

1) An ultrosonographic examination with an ultrasound equipped with a probe of 10 MHz;

2) An elastomeric examination by the use of a non-invasive device, the USB Dermalab of Cortex Technology. The principle of action of this instrument is based on the possibility of measuring, by optoelectronic sensors, the time required to raise the skin of 1.5 mm within a probe in which is created a vacuum not exceeding 700 mBar. The results of each determination were recorded as a graph of the cutaneous deformation where the height in mm of the skin drawn into the probe is indicated in the ordinates and on the abscissa there is the time in which the height parameter is detected. The computerized elastomeric evaluation of the skin allowed us to study the deformability of the skin after 7-10 cycles of suction and release allowing to analyze three different parameters: tensile distensibility (ability of a tissue to change its shape in response to an external stimulus; it is related to the distensibility of the collagen fibers), resilience (ability of a tissue to regain its original shape after a deformation; it is related to the distensibility of the elastic fibers) and hysteresis (characteristic of a system to react to the stress applied and to return to the previous state)

3) A withdrawal of a skin by punch biopsy of 4 mm in diameter under local anesthesia on which were performed both histopathological examination and immunohistochemistry. The biopsy material used was fixed in 10% buffered formalin, embedded in paraffin and processed for conventional histology. The histological sections stained with hematoxylin and eosin were evaluated by microscopy.

4.1.2: IMMUNOHISTOCHEMICAL PROCEDURES

The entity of the immune-expression was evaluated with immunohistochemistry on deparaffinized sections of 5 microns thick, immersed in citric acid (pH 6.0), using a Rabbit Anti Decorin Polyclonal Antibody (Abcam). The sections underwent several phases of washing and incubation; at the end of the process, the sections are stained with Mayer's hematoxylin at 10% for 3-4 minutes, washed with water for 10 minutes and then added with glycerol-gelatine. The immunohistochemical evaluation was performed using a semi-quantitative method which allowed us to identify the cytoplasmic positivity and that of cytodiagnosis and of the membrane (+ = weakly positive; + + = moderate positivity; + + + = intense positivity). For each section, it was calculated a H-SCORE by adding the percentages of the stained cells and then multiplying it for the valuated intensity of the signal (i.e. H-SCORE = Σ P x (I+1); "P" is percentage of stained cells while "I" is the intensity of the signal).

CHAPTER 5: RESULTS

All patients included in the study completed the expected scheme of phototherapy. During its execution, only two patients reported the onset of a moderate itching associated with a mild xerosis responsive, inter alia, the application of emollients. In one patient, however, there was the occurrence of a relapse of labial herpes infection.

The irradiance value registered with the spectroradiometer was an average of 95 ± 5 mJ/cm^2. The median age of patients enrolled in the study was 54 years (corresponding to the 50th percentile), while the range of values that contains "center" limit of the observed values (interquartile range or IR) was equal to 47.75 and 62.25 (corresponding to 25th and 75th percentile).

As confirmed by the RSSM, it was clinically possible to appreciate a marked improvement of the lesions (Fig. 13, 14, 15) of all subjects treated (Table 5, Graph 1). Under normal conditions, the ultrasound examination is able to highlight an echogenic inhomogeneous band, corresponding to the dermis and correlated to the density and the size of its components. In particular, the hyperechoic areas correspond to the dermal fibers (elastic fibers, collagen fibers type I and type III), while the hypoechoic to the water.

In the study, it was carried out a quantitative analysis of the ultrasound before and after therapy by evaluating the echogenicity and measuring the thickness of the dermis, ie the space between the two more hyperechoic lines (in binary-like aspect): the upper meet the air-stratum corneum interface and the lower one the dermis-hypodermis interface.

The ultrasound examination, performed in all subjects examined before starting phototherapy, showed a thickening of the dermis associated with edema of the subcutaneous tissue (Fig. 16, Table 5, Graph 4). At the end of the intended therapy, the ultrasonic examination has shown an higher intensity of the ultrasound correlated to a reduction of the maximum thickness of the skin, while the echostructure was unchanged (Fig. 17, Table 5, Graph 4). These data indicate that the variation of the ultrasound after UVA1-therapy is related not only to the change and to the remodeling of the fibrous component but also to the simultaneous reduction of the interstitial edema. Comparing the elastomeric graphs obtained before (Fig. 18) and after (Fig. 19) the phototherapy, it was possible to record a clear improvement of all parameters in the study: resilience (0.03> 0.05), hysteresis (0.02> 0.06) and tensile distensibility (0.08> 0.19) (Table 5, Graph 3). In histological sections obtained from the skin of patients with scleroderma before treatment phototherapy, it was possible to appreciate with the EE a lymphocytic infiltrate, predominantly perivascular, associated with wall thickening and ectasia of the vessels and a full-thickness dermal-hypodermic dermal fibrosis with homogenization of

collagen (Fig. 20) . The immunohistochemistry (Table 5, Graph 2) showed inhomogeneous positivity in the papillary dermis with positive areas which were alternated with areas with low positivity, as in normal skin. In the reticular dermis, however, it was observed a reduced positivity characterized by collagen fiber bundles weakly positive or entirely negative, mixed to other, generally smaller, and strongly positive. The epidermis appeared mostly negative and the basement membrane was almost entirely negative. At the end of the treatment, in preparations with EE, it was possible to find a considerable reduction of the mononuclear infiltrate and of the vascular lesions and a conspicuous decrease of fibrosis, especially at the level of the reticular dermis where the collagen fibers bundles appeared more widely spaced (Fig. 21). The immunohistochemical results showed a positive zone in the suprabasal and basal layers of the epidermis and mild, sometimes moderate, positive in the papillary dermis. In the reticular dermis, however, it was observed the presence of collagen fibers mixed with other intensely positive or moderately positive, with an appearance very similar to the normal skin.

5.1: STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS V.20 for Windows statistical package (SPSS Inc, Chicago, IL, USA). Data were presented as median and interquartile range (IR) for descriptive analysis, as shown in the table. The Wilcoxon test (Table 4) for two paired samples was chosen for inferential analysis and groups comparisons. A p-value less than 0.05 was considered significant.

CHAPTER 6: DISCUSSION

Although the pathogenesis of localized scleroderma has not been fully understood, several studies have confirmed the existence of complex biological mechanisms at the base of fibrotic damage, even not of immune nature. The fibrillogenesis of collagen is, in fact, an articulated and complex

process which must be finely and continuously controlled in order to avoid a high and / or altered accumulation of collagen. Under physiological conditions, this task is performed by decorin that, by binding to specific sites on the collagen fibers, controls their volume, the equality of their diameter and their regular arrangement. In fact this proteoglycan, anchoring to the side surface of the fibrils between the gaps existing between collagen molecules consecutive, prevents their excessive assembling and packing. The interaction of the core protein of decorin with the fibrillar surface is, therefore, one of the limiting factors in the process of fibrillogenesis and it is known that a reduction or its alteration has obvious repercussions on the three-dimensional architecture of the matrix. Another important function of decorin is its ability to bind and, therefore, to inhibit the pro-fibrotic action of TGF-β. In fact, according to some authors, when it binds to free TGF, decorin seizes it and blocks its access to the membrane receptors. Other authors, however, argue that the antifibrotic action is not the result of a direct physical interaction with TGF-β (Hildebrand et al 1994; Schonherr et al 1998), but rather that decorin is able to interfere in the signaling cascade that leads to the activation of this pro-fibrotic factor. Finally the decorin also plays a role in regulating the apoptotic processes thanks to its capacity to activate the caspase 3 and 8. As reported in the literature, despite the fact that the mechanism of action has not yet been fully elucidated, UVA1 are able to improve the phase of fibrotic connective tissue disease and related disorders by modifying the cytokine pattern and growth factors involved. This results in a shutdown of the maturation thrust of fibroblasts but, above all, in an increased production of collagenase and in a decrease of the inhibitors of metallo-protease-tissue. All this results in a lower production of collagen I and III with a lower mechanical tension of the extracellular matrix. This situation triggers a "boomerang" because fibroblasts, which are no more subjected to tensional stress, not only reduce the activities of synthesis, but must also undergo apoptosis. The clinical-instrumental results of our study, in agreement with what reported in the literature, confirm that

UVA1 phototherapy is a valid treatment of localized scleroderma regardless of the duration of the disease, of the extent of the involved skin and the age of the subject. In fact, with the Modified Rodnan Skin Score, that evaluates the severity of fibrosis, we have documented a significant reduction of the "score" post-UVA1 phototherapy, although in some parts of the body (hands, forearms and chest) were recorded changes more sensitive than other parts of the body. Histopathological and immunohistochemical investigations carried out before treatment at the level of the damaged skin, made it possible to detect full-thickness dermal-hypodermal fibrosis and significantly reduced levels of decorin. After phototherapy, however, we found a significant decrease in fibrosis, with increased positivity of decorin in the papillary dermis, but especially in the reticular one (Fig. 22). Furthermore, regardless of the phototherapy, it was also observed that the levels of decorin in the healthy skin of patients with localized scleroderma were not significantly different from those highlighted in the skin from healthy control subjects. This indicates that the localized scleroderma is a disease characterized by a down-regulation of decorin. Moreover, the antifibrotic properties of the decorin and its ability to block or attenuate the action of TGF-β have been exploited also in the experimental field to draw genetic therapies for pulmonary fibrosis or glomerulonephritis. Our results confirm that if on one hand the irradiation of the skin with UVA1 is able to affect the fibrillogenesis of collagen, on the other it also gives the proof of the regulatory role played by decorin in the process of fibrillogenesis. It remains to be clarified whether radiation UVA1 are able to directly induce gene expression of decorin or if the increased expression of this is linked to a restoration of the normal architecture of collagen after phototherapy with UVA1. To confirm these data are still needed long-term multicenter studies involving large cohorts of patients. Finally, in order to adjust the process of fibrillogenesis and to reduce the radiation dose of UVA1 necessary to control the fibrosis, it would be interesting to evaluate

the results obtainable with a therapy based on peptides capable of mimicking the activity of decorin (such as the decorinyl ®) associated with UVA1.

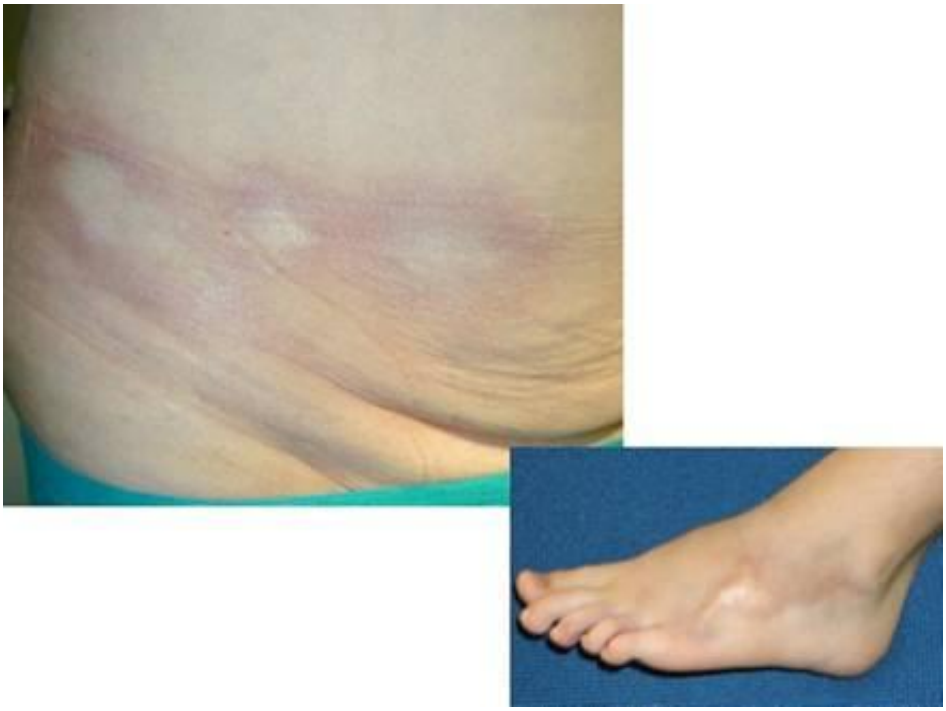


Fig. 1

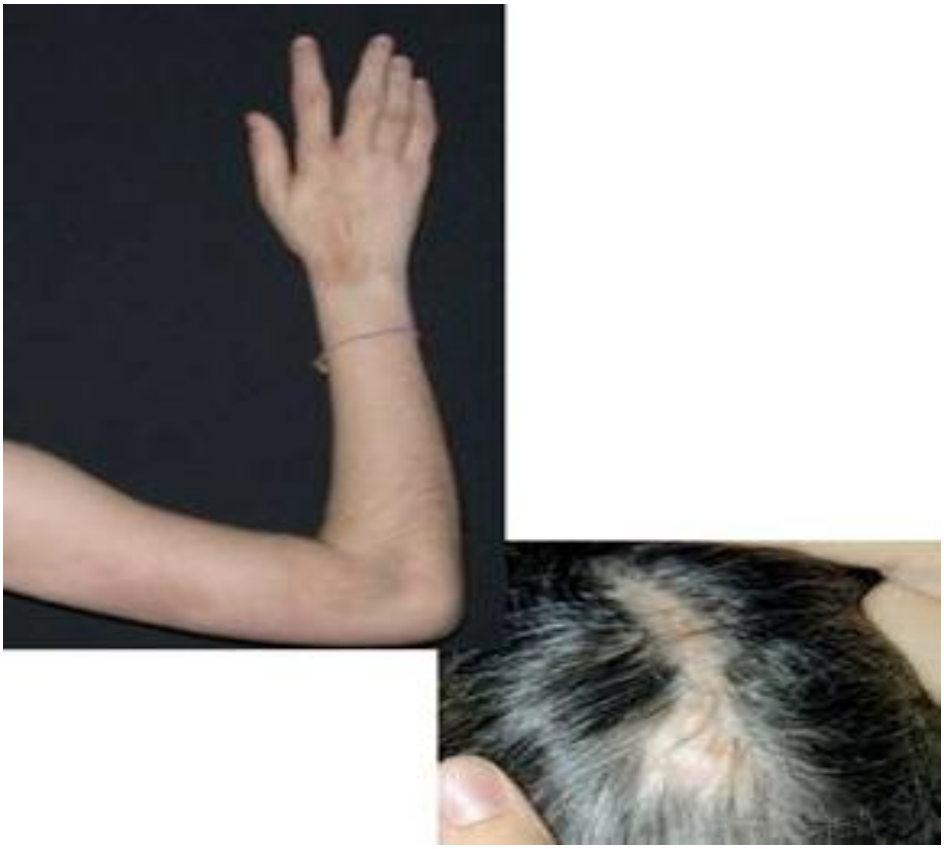


Fig. 2

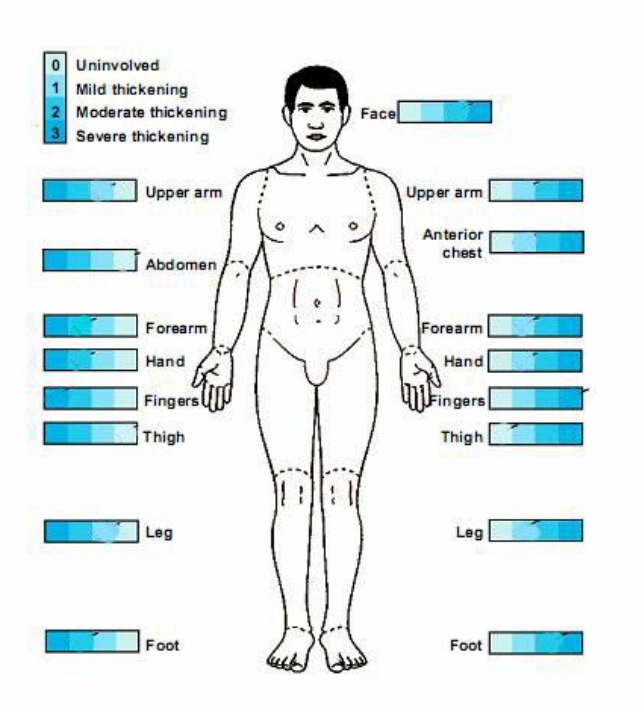


Fig. 3

Emission spectrum of a lamp UVA1

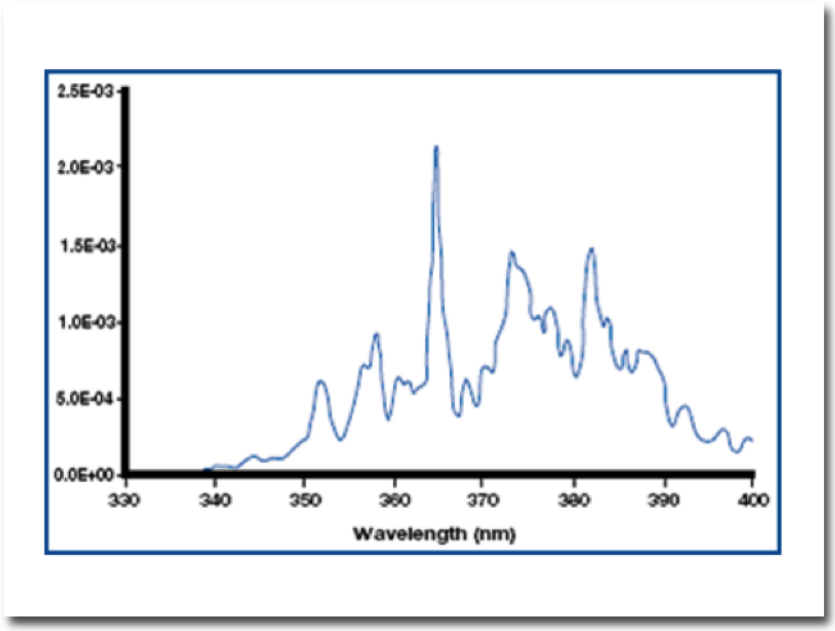


Fig. 4

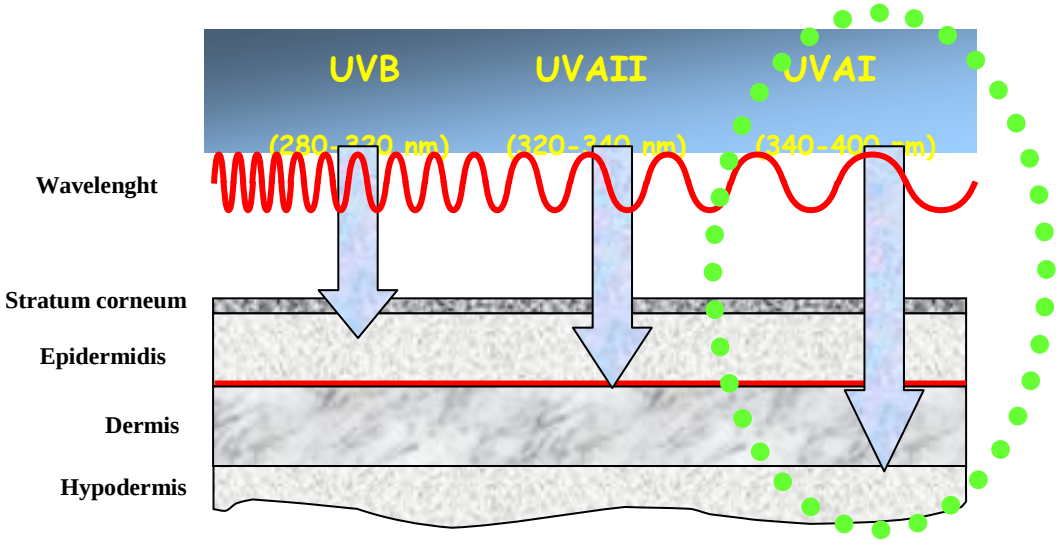
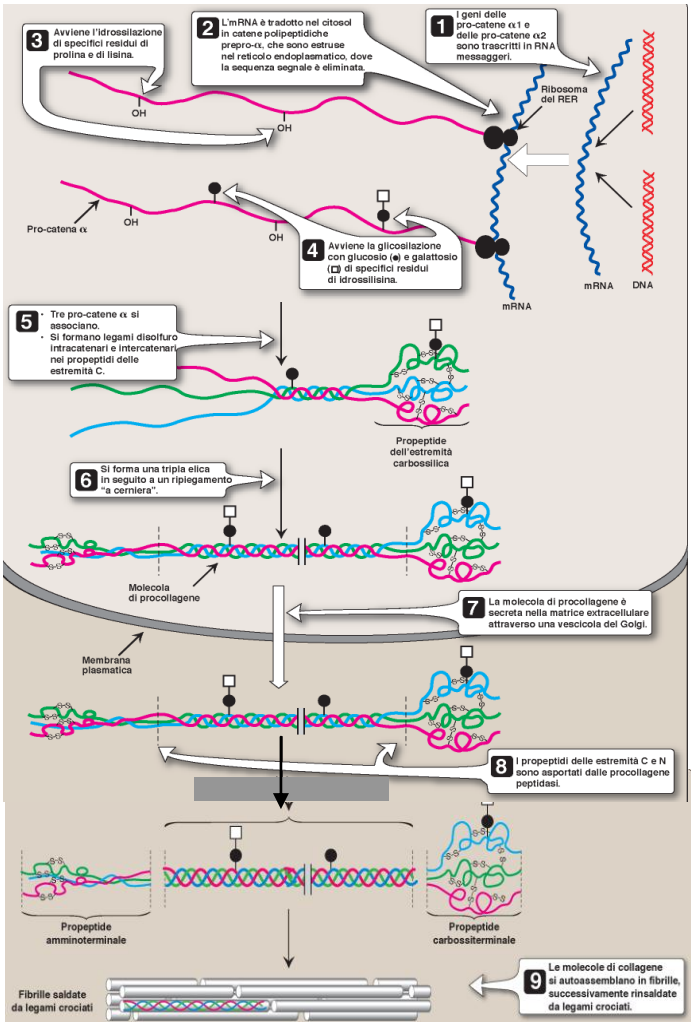

UVB
UVAII
UVAI
Wavelenght
Stratum corneum
Epidermidis
Dermis
Hypodermis

Fig. 5

Fig. 6


1 I geni delle pro-catene α1 e delle pro-catene α2 sono trascritti in RNA messaggeri.
2 L'mRNA è tradotto nel citosol in catene polipeptidiche prepro-α, che sono estruse nel reticolo endoplasmatico, dove la sequenza segnale è eliminata.
3 Avviene l'idrossilazione di specifici residui di prolina e di lisina.
4 Avviene la glicosilazione con glucosio (●) e galattosio (□) di specifici residui di idrossilisina.
5 Tre pro-catene α si associano.
Si formano legami disolfuro intracatenari e intercatenari nei propeptidi delle estremità C.
6 Si forma una tripla elica in seguito a un ripiegamento "a cerniera".
7 La molecola di procollagene è secreta nella matrice extracellulare attraverso una vescicola del Golgi.
8 I propeptidi delle estremità C e N sono asportati dalle procollagene peptidasi.
9 Le molecole di collagene si autoassemblano in fibrille, successivamente rinsaldate da legami crociati.
Ribosoma del RER
mRNA
DNA
Pro-catena α
OH
Propeptide dell'estremità carbossilica
Molecola di procollagene
Membrana plasmatica
Propeptide amminoterminale
Propeptide carbossiterminale
Fibrille saldate da legami crociati

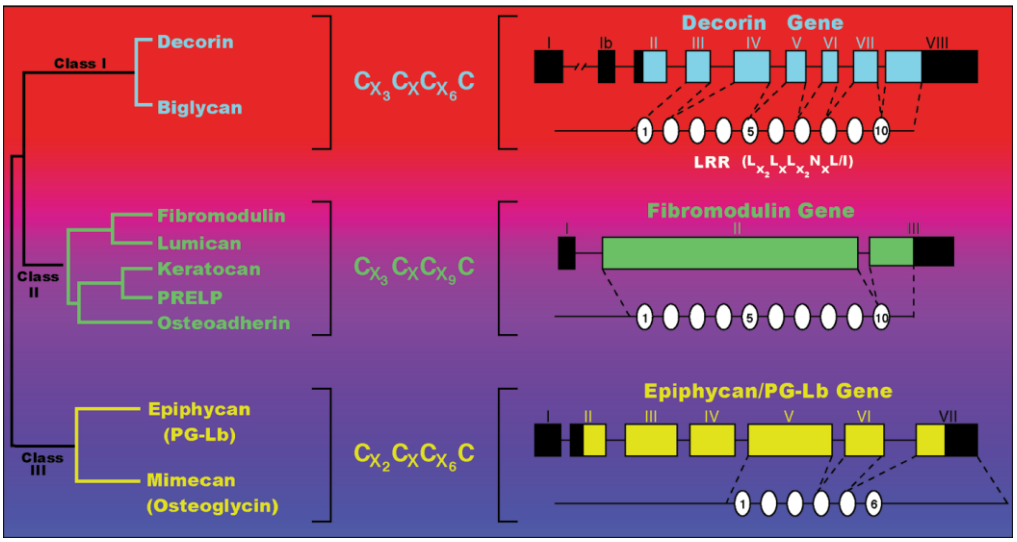


Fig. 7

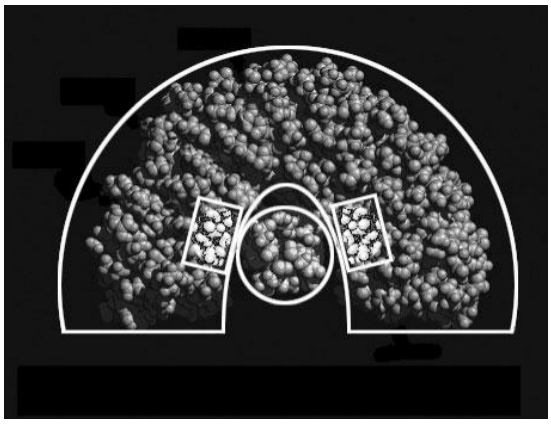


Fig. 8

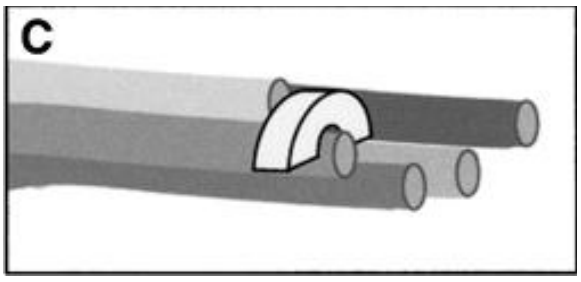
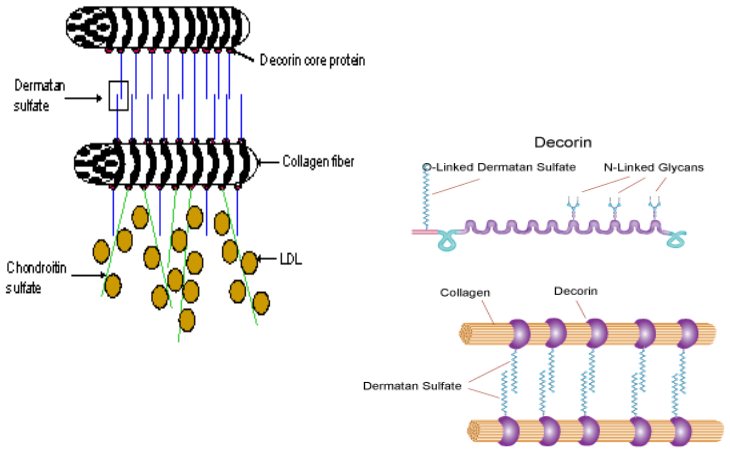


Fig. 9

Fig. 10



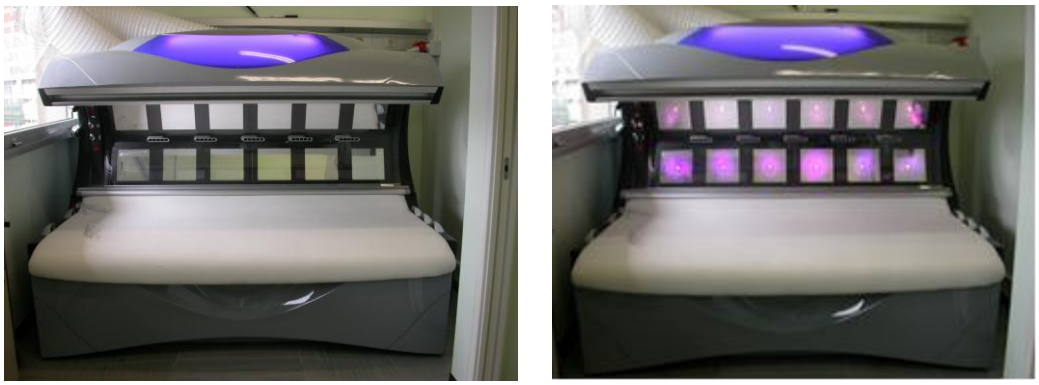


Fig. 11 UVA1 Cosmetico Medizintechnik GP-24H

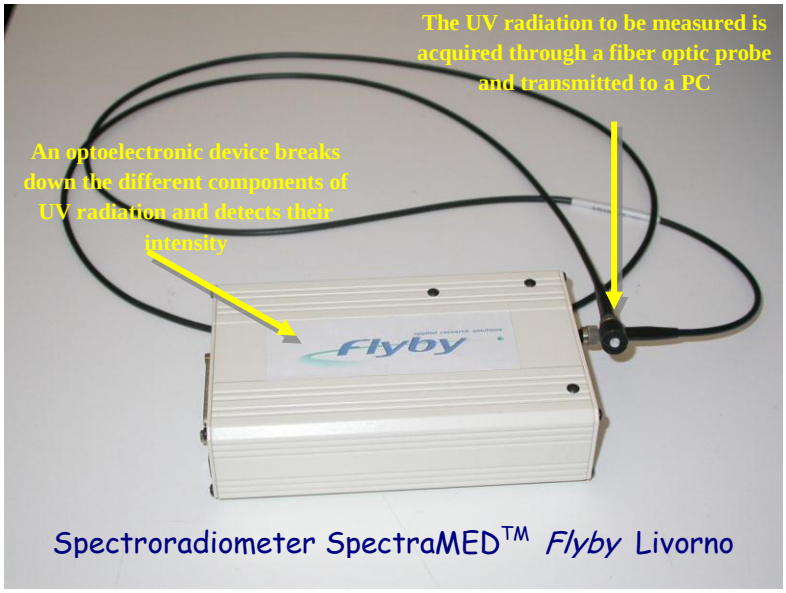


Fig. 12

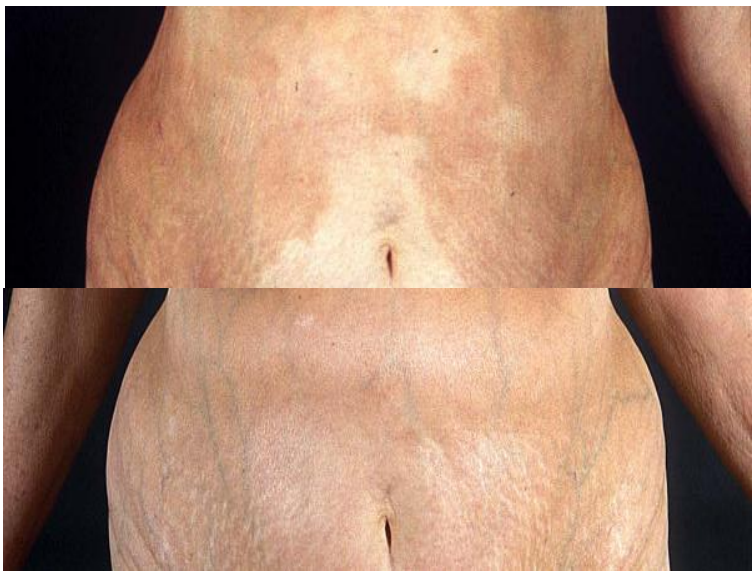


Fig. 13 Pre e Post treatment

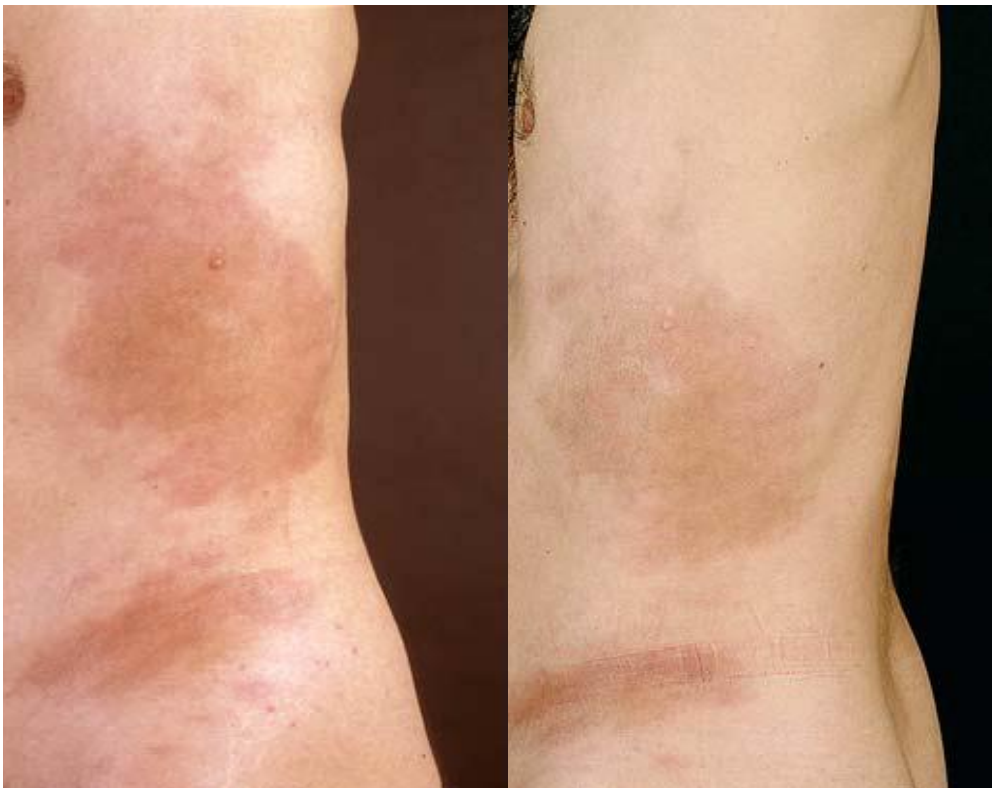


Fig. 14 Pre e Post treatment

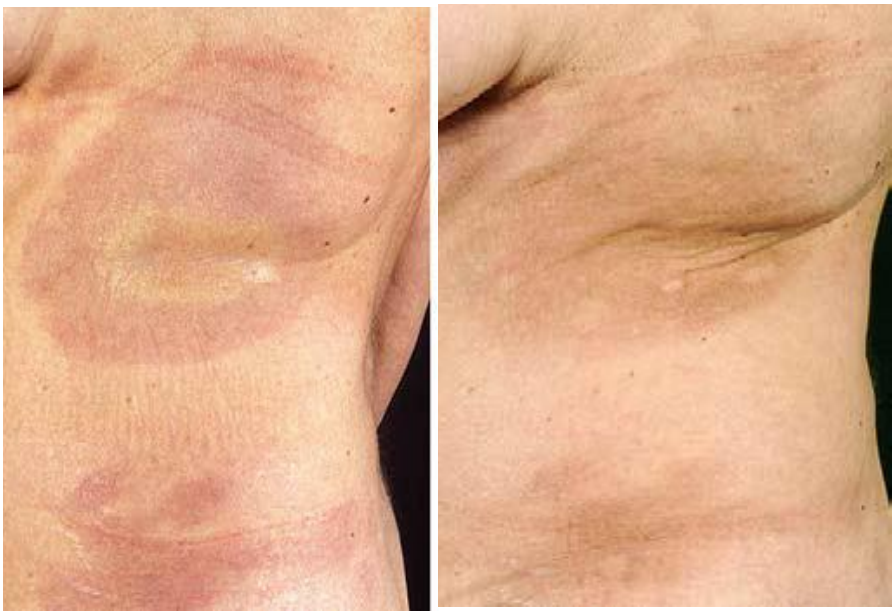


Fig. 15 Pre e Post treatment

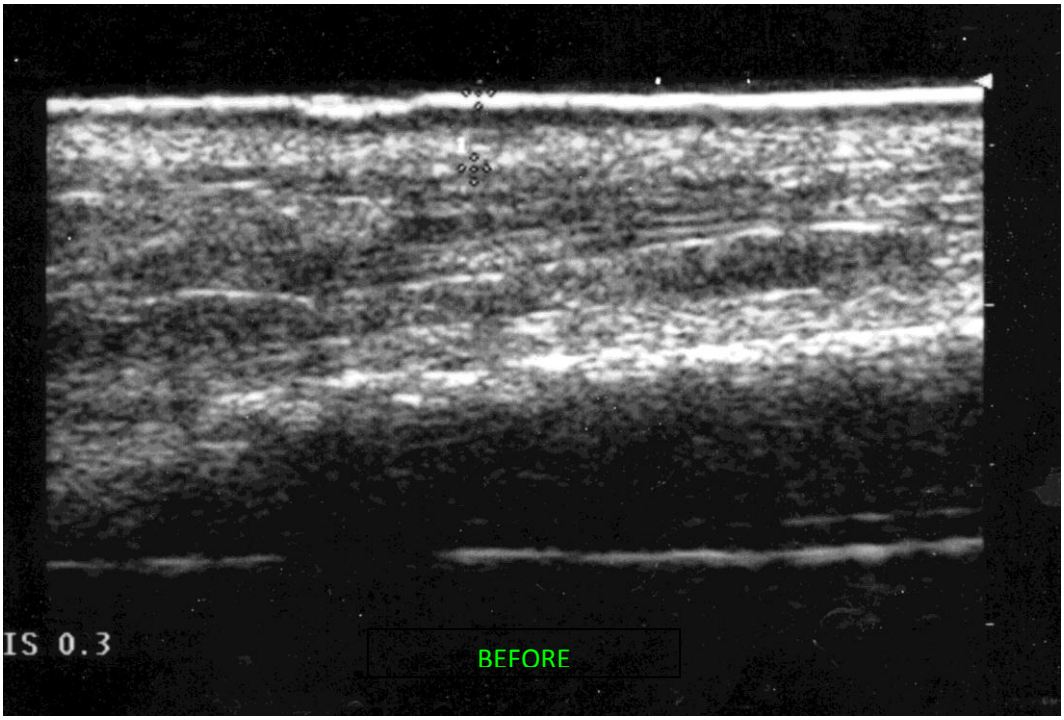


Fig. 16

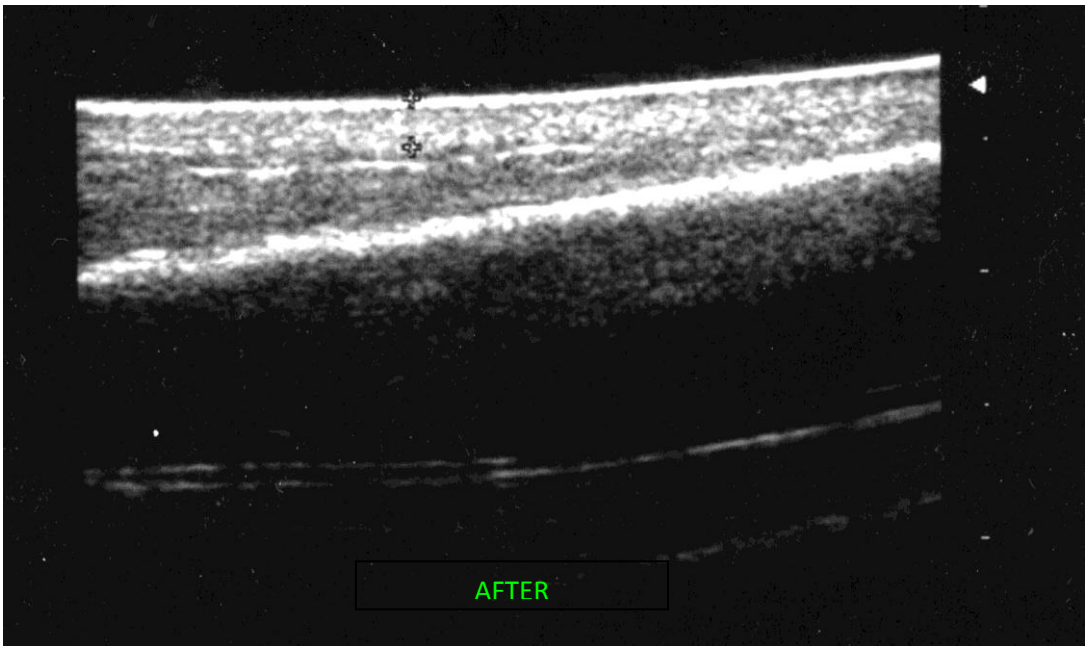


Fig. 17

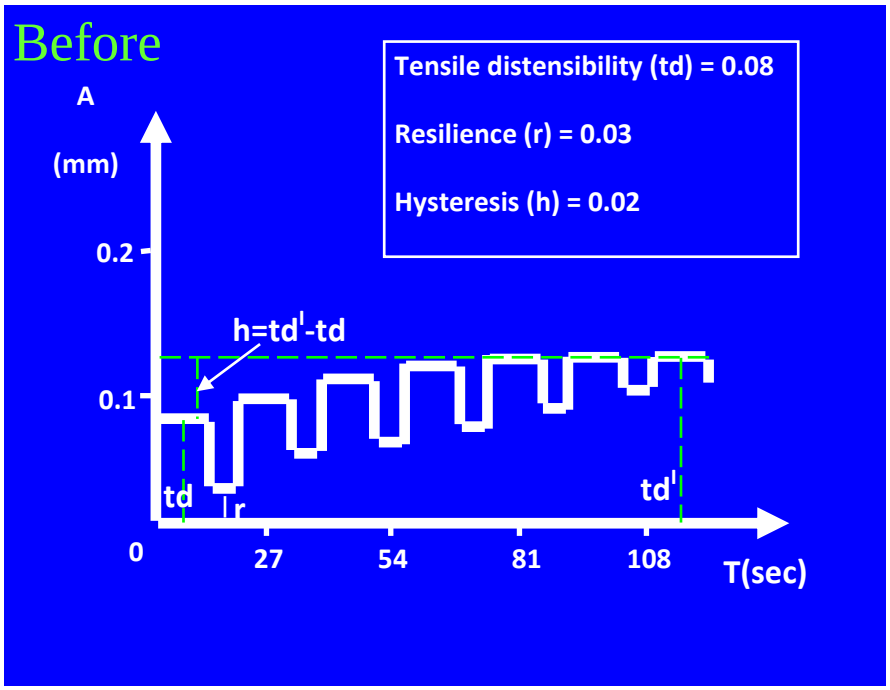

Before
Tensile distensibility (td) = 0.08
Resilience (r) = 0.03
Hysteresis (h) = 0.02
A
(mm)
0.2
0.1
0
h=td'-td
td
r
td'
27
54
81
108
T(sec)

Fig. 18

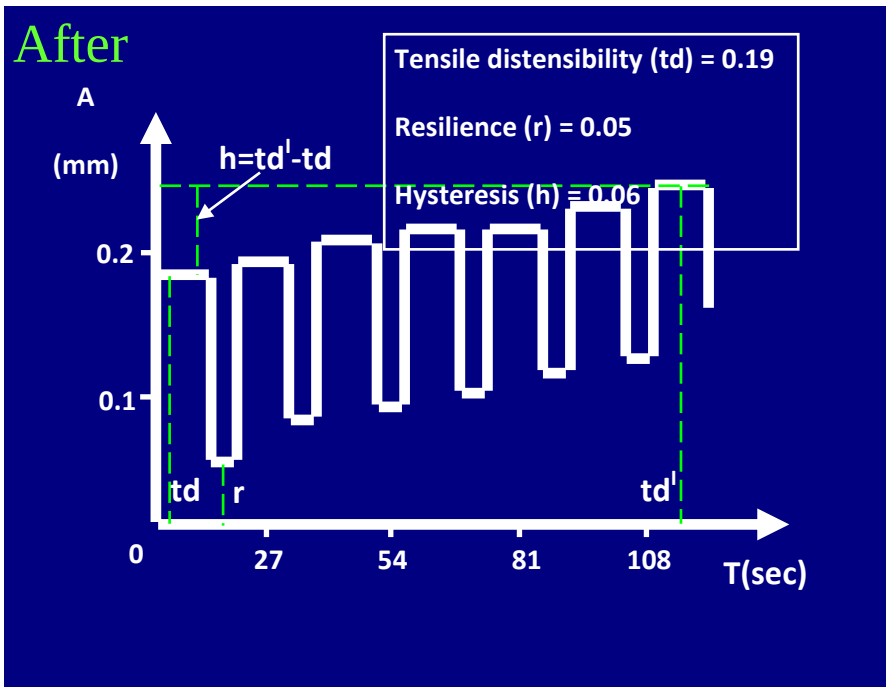

After
Tensile distensibility (td) = 0.19
Resilience (r) = 0.05
Hysteresis (h) = 0.06
A
(mm)
0.2
0.1
0
h=td'-td
td
r
td'
27
54
81
108
T(sec)

Fig. 19

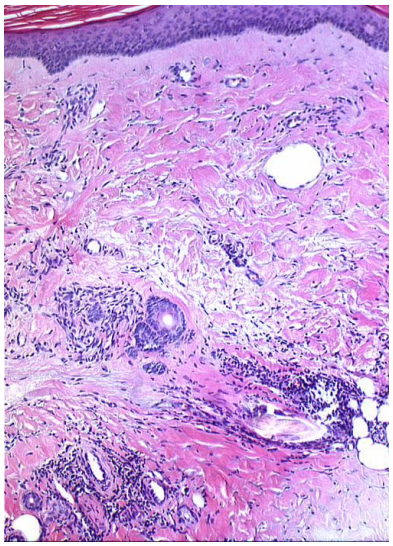


Fig. 20

Histological exam pre-treatment

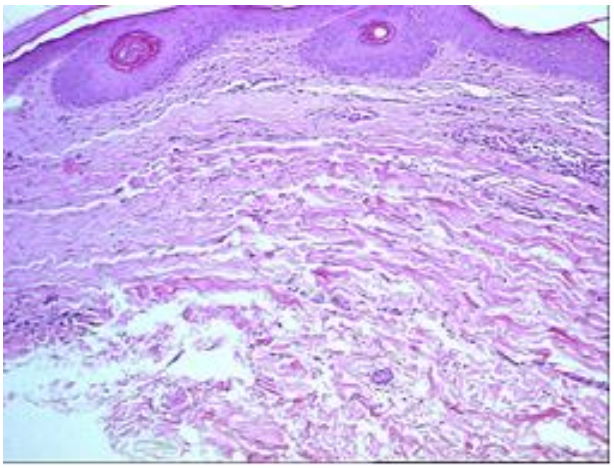


Fig. 21

Histological exam post-treatment

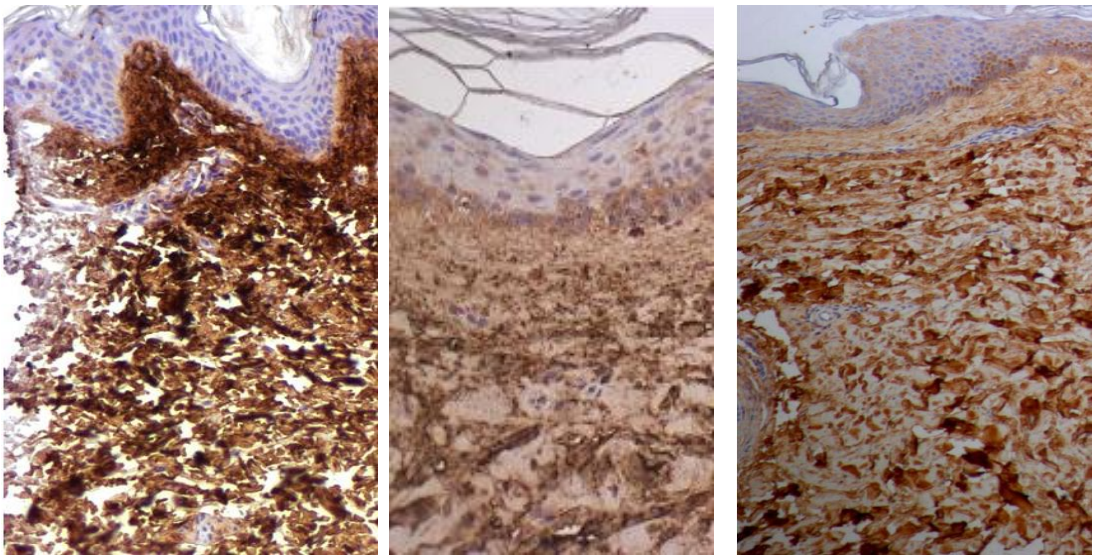


Fig. 22

Normal skin Pathological skin Skin post-treatment

Table 1

Table I. Morphea classification according to Peterson et al*

Classification	Included subtypes
Plaque	Morphea en plaque, guttate, atrophoderma of Pasini and Pierini, keloidal, and lichen sclerosis et atrophicus
Generalized	Defined as involving 2 or more body areas
Bullous	
Linear	Linear morphea of the extremities, en coup de sabre, and progressive facial hemiatrophy
Deep	Morphea profunda, subcutaneous morphea. eosinophilic fasciitis, and pansclerotic morphea

Table 2

Table II. Morphea classification according to Laxer and Zulian*

Classification	Included subtypes	Description
Circumscribed morphea	Superficial variant	Oval areas of induration limited to epidermis and dermis
	Deep variant	Oval areas of deep induration including the subcutaneous tissue (may include fascia and muscle); overlying skin may not be involved
Linear morphea	Trunk/limb variant	Linear induration involving the dermis and subcutaneous tissue (may include muscle and bone)
	Head variant	
	En coup de sabre	Linear induration involving the dermis of the face and scalp (may involve underlying muscle, bone, and central nervous system)
	Parry–Romberg	Loss of dermis, subcutaneous tissue, muscle and bone of the unilateral face
Generalized morphea		Four or more individual indurated plaques >3 cm each, involving ≥ 2 of seven anatomic sites (head-neck, each extremity, anterior trunk, and posterior trunk)
Pansclerotic morphea		Circumferential involvement of limbs involving epidermis, dermis, subcutaneous tissue, muscle, and bone; may affect other areas of the body with full depth sclerosis
Mixed variant morphea		Combination of 2 or more previous subtypes

Table 3

Absolute exclusion criteria for UVA-1 phototherapy:
- Congenital defects in DNA repair;
- Congenital defects of the skin pigmentation;
- Dysplastic nevus syndrome;
- Previous cutaneous melanoma,
- Severe cardiac disease;
- Treatment with photosensitizing drugs.

Table 4
Wilcoxon Test

	RMSS post RMSS pre treatment	Dexp IHC post Dexp IHC pre treatment	Ultrasound skin post Ultrasound skin pre treatment	Elasticity skin post Elasticity skin pre treatment
Z	-2,809[b]	-2,598[c]	-2,809[b]	-2,821[c]
Sig. Asint. a 2 code	,005	,009	,005	,005

b. Based on positive ranks
c. Based on negative ranks

Table 5 Clinical data of ten female patients with localized scleroderma localized

Patient no.	Age	Duration of disease (months)	RMSS score		Decorin expression IHC semiquantitative score		Ultrasound Skin (μ)		Elasticity skin (μ)	
			Pre UVA1	Post UVA1	Pre UVA1	Post UVA1	Pre UVA1	Post UVA1	Pre UVA1	Post UVA1
1	40	9	9	2	2	2	1180	1220	200	220
2	52	24	14	2	1	3	1210	1350	100	300
3	53	30	21	3	1	2	1190	1340	120	160
4	55	42	12	6	1	3	1200	1400	210	250
5	47	13	11	2	1	3	1300	1360	200	210
6	61	43	23	5	2	2	1300	1420	180	220
7	48	36	14	2	1	3	1150	1300	160	190
8	56	18	11	3	1	3	1180	1360	100	220
9	70	60	32	3	2	3	1260	1380	140	180
10	66	52	10	2	2	3	1220	1360	160	240
Median	**54**	**33**	**13**	**2,5[§]**	**1**	**3[§]**	**1205**	**1360[§]**	**160**	**220[§]**
± SD	**8,56**	**16,03**	**6,98**	**1,34**	**0,48**	**0,45**	**48**	**53**	**39**	**37,26**

RMSS: Rodnan Modified Skin Score (0= normal thickening, 1= slight thickening, 2= moderate thickening, 3= thickening serious)

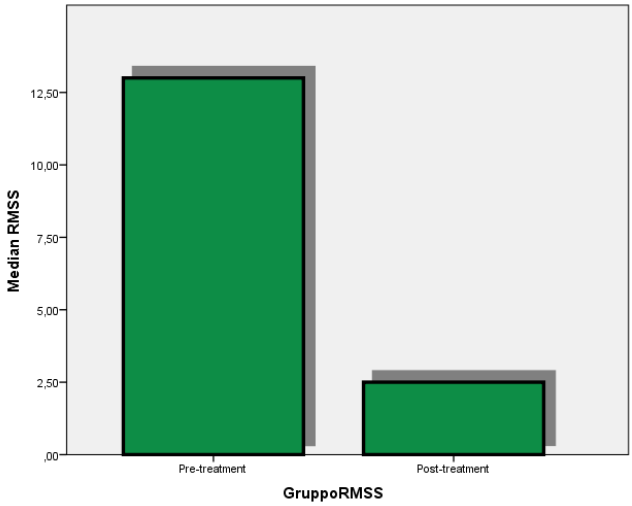
IHC: Immunohistochemistry semiquantitative score (0= none, 1= slight, 2= moderate, 3= strong)

SD: Standard Deviation

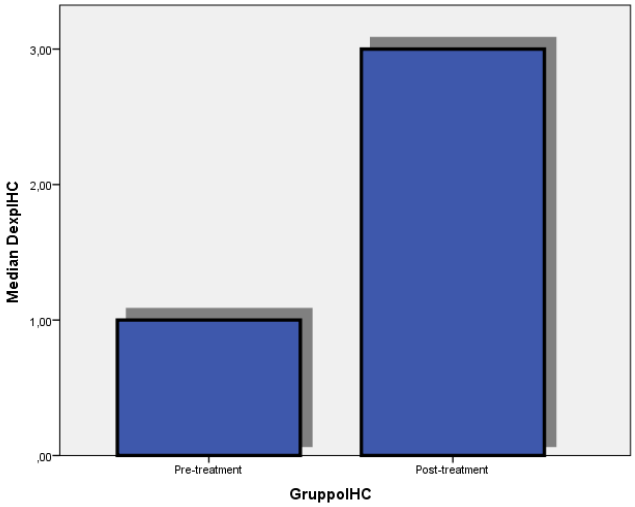
UVA1: Ultraviolet A1 (340-400 nm wavelength)

[§] Significant difference between preUVA1 and post UVA1 (P value RMSS → ,005; P value Decorin expression IHC→ ,009; P value Ultrasound skin → ,005; P value Elasticity skin→ ,005)

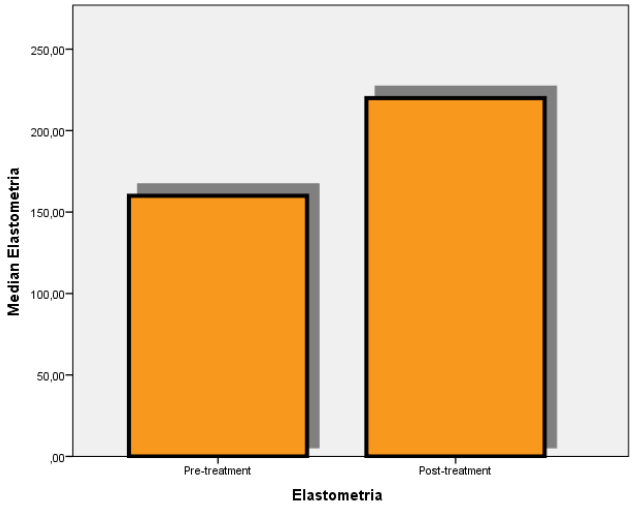
Graph 1: RMSS pre and post treatment



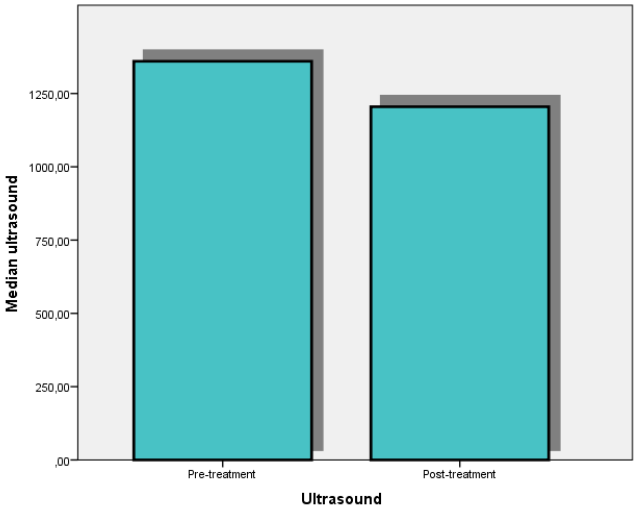
Graph 2: IHC pre and post treatment



Graph 3:Elasticity pre and post treatment



Graph 4: Ultrasound pre and post treatment



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