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# Action of topical amino-acid mixtures in chronic pressure sore repair in spinal-cord injured patients: study of modulation in inflammation, angiogenesis, and cellular proliferation

CARLA PEZZUTO

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## SUMMARY

*Aim. The aim of this study was to assess the efficacy of local application of sodium hyluronate + aminoacids (VULNAMIN) in wound healing. Pressure sores in patients with spinal cord injuries become chronic due to local and general causes. Many of these patients need to continue a normal familiar, social, sportive and scholar life, so they can accept limitation to their activities but not to lay in bed all day long. Compromise between ulcer status and necessity of everyday life determine a continuous pressure and a delayed heal. There was also fundamental alteration in the skin sensitive nervous system with deep changes in cutaneous tropism. We evaluated histological changes in the first 15 days correlated to clinical improvement of granulation tissue.*

***Methods.****10 patients were recruited and they accepted to give a biopsy to our laboratory. In detail, we took 3 biopsies from each patient at 0 – 7 – 15 days after application of Vulnamin. We had previously carried out another histological study on chronic pressure sores surgically excised. The results in this case were extremely indicative for a hypoxic tissue with different types of local treatment.*

***Results.*** *Histologic changes were evident with immunoistochemical methods. A new angiogenesis, persistence of macrophage components and a significant population of fibroblast with increased collagen deposition.*

***Conclusions.*** *Tissue impairment in chronic lesions depends on different causes: cellular population and possibility of obtaining a good flow of oxygen and molecular components are strictly related with progression in wound healing. The possibility in modulating a new angiogenesis and production of collagen with local treatment represents an interesting way to stimulate the granulating tissue.*

*KEYWORDS*
***Aminoacids-Sodium hyaluronate, Pressure sores, skin ulcer***

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## INTRODUCTION

Pressure sores in patients with spinal-cord injuries tend to become chronic as the result of local and general factors [1]. The need to continue a normal family, social, sporting and academic life leads to compromises on the temporary reduction of stress, but too drastic limitations are impossible. Often, a lesion that is stable and not very extensive but continually subjected to stress from pressure is accepted with the resulting tendency for it to become chronic. Further, factors which closely depend on the sensory nervous system of the skin are altered in spinal cord injuries with serious modifications of epidermis trophism [2,3].

The use of advanced medications and recourse to surgical operations have improved the expectations of healing and patients' quality of life. Some patients are candidates for reconstruction using surgical flaps while others can never be considered operable. In both cases, the need to reduce sores as quickly as possible in any way is a priority. Following international guidelines, patients with pressure sores are offered every possible therapeutic alternative for local and general treatment. The stimulation of granulation tissue, true biological scaffolding, remains an essential in healing ulcers. Pro-trophic medicatio ns are an important point in the evolution towards local improvement or healing.

The ongoing research project has led to a study that highlights changes to the inflammatory stage, angiogenesis, and cell proliferation after the topical application of hyaluronic acid and amino-acids to the hypoxic granulation tissue of chronic pressure sores in patients with spinal cord injuries.

## Materials and methods

### ➜ Background

A study conducted previously in co-operation with plastic surgeons and the skin bank had led to interesting conclusions [4].

Based on the study of 10 skin biopsies of pressure ulcers taken from consenting spinal cord injury patients from trauma and 6 healthy skin biopsies, the edge and base of the sores were analysed using immuno-histochemical techniques. The analysis of the sections obtained from the biopsies highlighted four different types of tissue:

- from the histological point of view, the peri-lesional skin was similar to healthy skin as it had a thin epithelium with dermal papillae and a dermis with regular extracellular matrix;

- the edge of the sores had a thickened epithelium with more marked dermal papillae and a dermal compartment with abundant extracellular matrix with disordered collagen fibres;

- the edge-base join, an area of transition, was marked by an epithelium which no longer had its original features and was in the process of flaking;

- the true base of the sore highlighted a dermis with poorly organised fibres and surrounded by a high number of extracellular matrices.

The following were analysed: CD68, IDO, HIF, CD71, FIBRIN, COX-2 and CD31. A fairly homogeneous distribution of vessels was found in the ulcers studied with

immuno-histochemical analysis of the CD31 molecule, both at the edge and base, although they were much larger in size than the controls and flattened by the extracellular matrix.

Therefore, if the ulcer healing process does not take place, there is an increase in angiogenesis but this micro-circulation malfunctions. The complete mechanism appears to be deficient and it can be theorised that the lack of supply of a nutrient creates an anomalous situation where a cell is able to synthesise various proteins but not all those required for its proliferation. It overproduces abundant protein material which is transferred to the extracellular matrix creating an abnormal physiological situation.

Note how the edges of the ulcer are made up of fibroblasts, inflammatory cells, and a high number of mainly occluded vessels in a matrix of dense bundles of collagen.

A high number of leucocytes was highlighted in the biopsies we analysed, especially macrophages where tests were made for the IDO enzyme. The results obtained showed a significant increase in the production of this enzyme by the macrophages compared to that of healthy skin. As a result, the lack of normal proliferation and function of the fibroblasts could also be due to a lack of amino-acid consumed by the IDO enzyme and not just because of poor supply.

HIF is a protein induced by the low levels of $pO_2$ and is expressed in hypoxic tissues with a compromised vascular system. In the sections of the edge, particularly in the dermis compartment, the tissue had significant marking with the direct monoclonal antibody against HIF. The fact that we found HIF in the case of bedsores is significant and describes, above all, a tissue that is suffering where the nutrients essential for healing are missing. Also in this case, both the edge and base of the lesion show a profile considerably different from that found in the controls, where positivity to this antibody is almost zero. Further, one of the genes induced to transcription by HIF is VEGF, which causes microvascular hyperpermeability to plasma proteins. In detail, what is found in bedsores is an extravasation of fibrinogen that coagulates into fibrin, which operates as a provisional matrix, promotes angiogenesis, and supports proliferating cells in normal conditions.

In the case of the bedsores we studied, there was an abnormal situation as there was an effective increase in the vessels which, however, malfunction and lack normal cell proliferation following the lack of nutrients.

## ➜ Objective of the work

The study of granulation tissue in chronic lesions arose after noting the clinical improvements (multi-centre study) [5] and experimental study in rats which highlighted local change in the first 15 days post-application [6]. The research project on granulation tissue was developed respecting the directives of the Ethics Committee and informing the patients of the sampling procedure.

The patients, between 35 and 55 years of age, are all affected by a spinal cord injury and had one or more pressure sores in the sacral, ischial, and trochanteric regions. Supplements were not added systemically. A total of 10 patients were enrolled, biopsies carried out of a diameter of 6 mm of granulation tissue taken from the base of the lesion at Time (T) 0, 7 days and 15 days after the application of a hyaluronic acid + amino-acids mixture 3 times a week.

The sections of tissue analysed with immunohistochemical techniques were compared with a reference sample, only medicated with hydrogel or other substance. The CD3 and CD8 antibodies were used for the analysis of the cellular component for the lymphocytes, the anti-CD68 antibody for the macrophages, the anti-CD20 antibody for lymphocytes B, the anti-CD57 for NK cells and the anti-CD31 for the endothelium of the vessels.

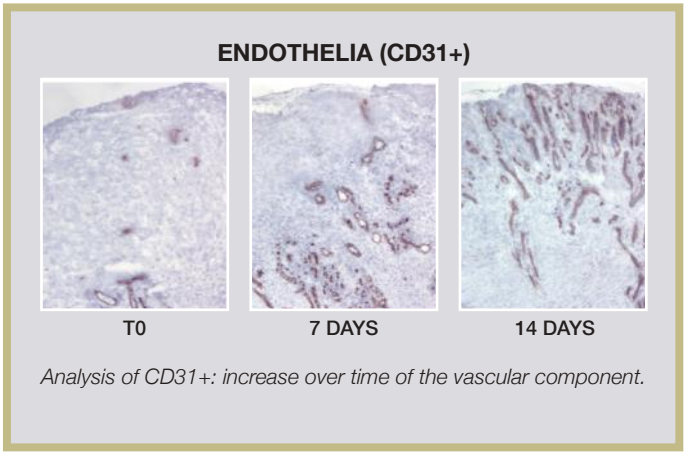
The following factors were studied for the evaluation of the tissue hypoxia:

- HIF: polyclonal antibody which recognises the transcription factor inducible by HIF2a hypoxia. The lack of an adequate blood flow associated with prolonged pressure at tissue level causes local ischaemia in bedsores;

- IDO specific for the Indolamine 2,3-dioxygenase enzyme involved in the degradation of tryptophan to N-formylkynurenine and produced by macrophages and dendritic cells. It increases in certain pathophysiological conditions such as some viral lung infections or some tumour pathologies;

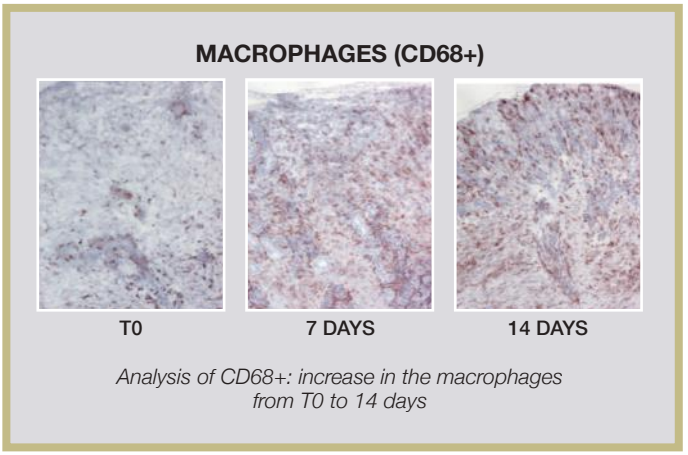
- CD 71 specific for the transferrin receptor essential for the transport of iron in proliferating cells; the expression of the receptors depends on the availability of iron by the cells. A high amount of iron corresponds to a limited number of receptors and a low content can increase the number by up to seven times.

Figure 1.



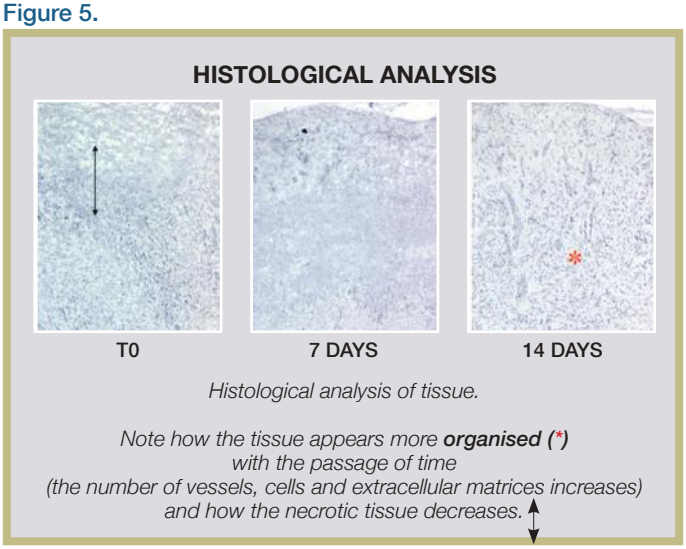

*Analysis of CD31+: increase over time of the vascular component.*

Figure 2.




*Analysis of CD68+: increase in the macrophages from T0 to 14 days*

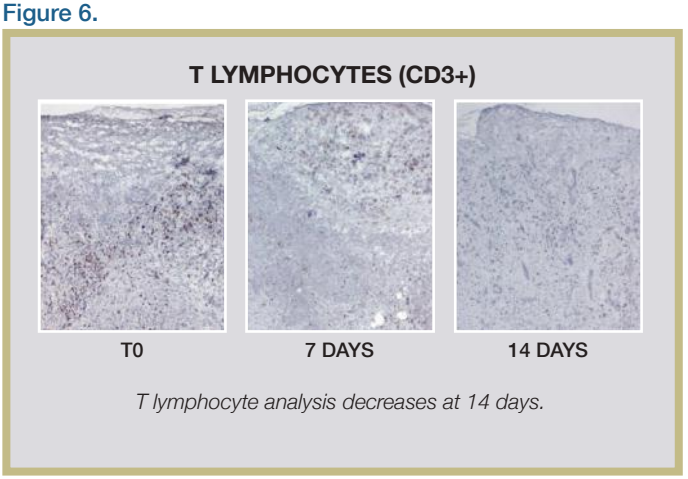
Figure 5.




*Histological analysis of tissue.*

*Note how the tissue appears more **organised** (*) with the passage of time (the number of vessels, cells and extracellular matrices increases) and how the necrotic tissue decreases.*

Figure 6.




*T lymphocyte analysis decreases at 14 days.*

## Results

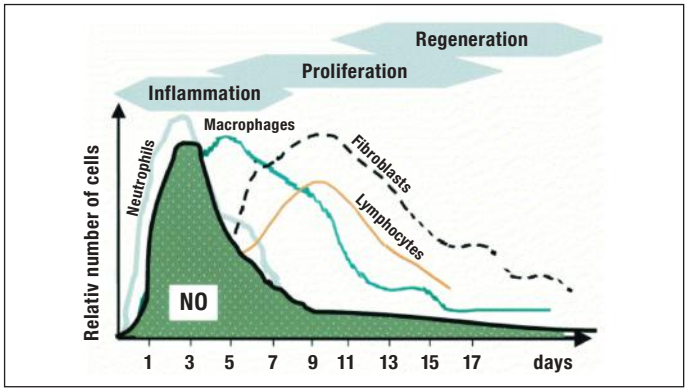
The study cannot be said to be complete and the comparative and statistical analysis will be processed when at least 20 cases are reached; we reserve the right to investigate the mechanisms that may influence the final outcome and continue tissue analysis beyond 15 days.

Further immunohistochemical details will be explained in subsequent publications.

At the time of publication, the histological sections highlighted:

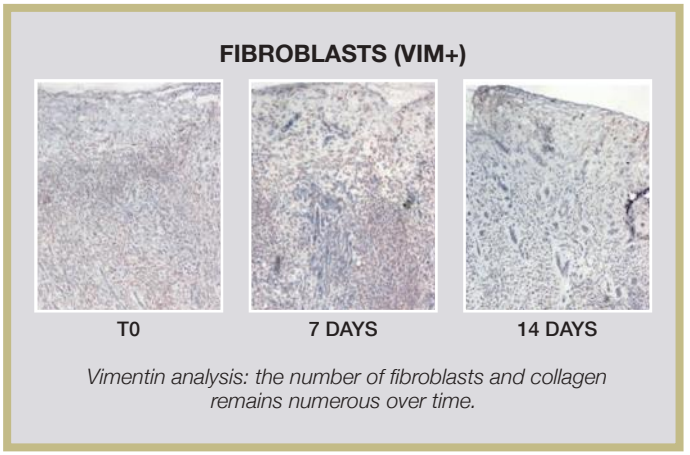
- a significant increase in the vascular component with the increase of vessels in the granulation tissue already seen at the 7th day and a progressive increase at the 15th day **(Fig. 1)**;
- an increased and persistent number of macrophages even at the 10th day with the consequent extension of their local action **(Figs. 2 and 3)**;
- an increase in the fibroblastic quota and the collagen component **(Figs. 4 and 5)**;
- there are many T lymphocytes in the tissue at T0 and, in almost all cases, they decrease at 14 days **(Fig. 6)**;
- a significant positivity of the IDO factor in the samples compared to healthy skin agrees with the hypothesis that the lack of normal fibroblast proliferation and their normal operation could be attributed to not only the lack of nutrients from the blood flow but also the lack of tryptophan 'consumed' by the IDO produced by the macrophages **(Fig. 7)**;
- the analysis of CD71 shows a low progress in iron content from T0 to 14 days thus with a progressive increase in transferrin receptors **(Fig. 8)**;
- lesser tissue hypoxia con with progress of the treatment found through HIF analysis **(Fig. 9)**;
- the number of lymphocytes in pressure sores increased at 7 days and then decreased at 14 days **(Fig. 10)**.

Figure 3.



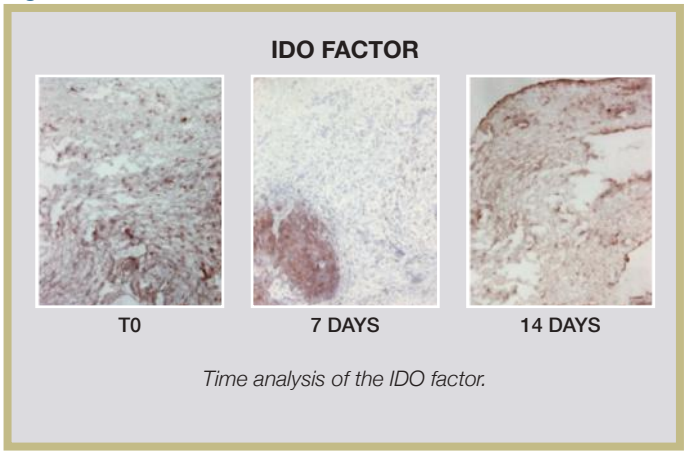

*Evolution in time of the cell populations with increases in macrophages.*

Figure 4.



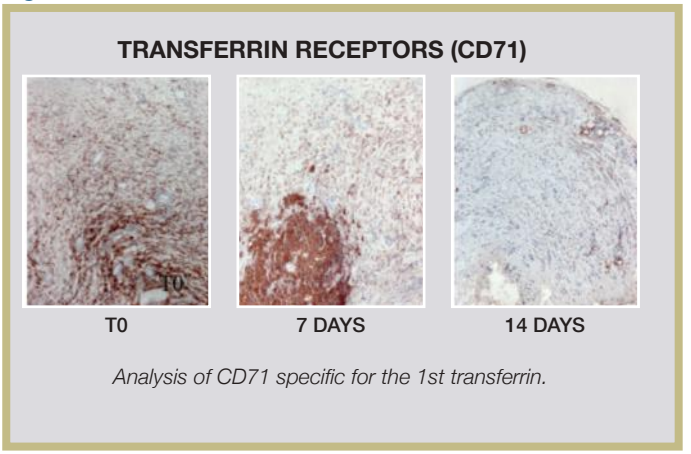

*Vimentin analysis: the number of fibroblasts and collagen remains numerous over time.*

Figure 7.



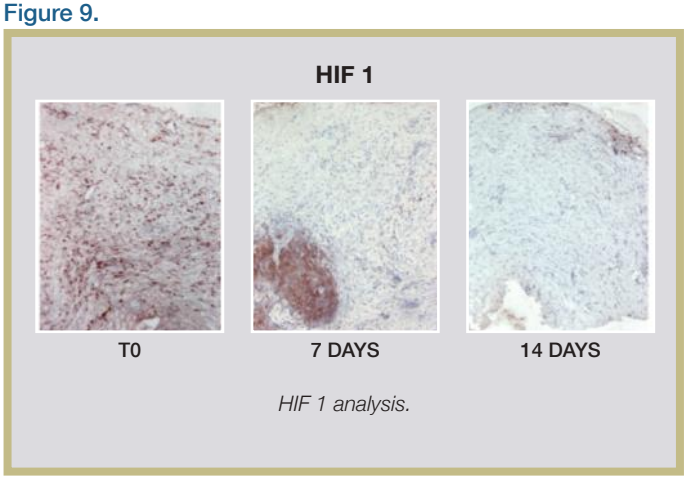

*Time analysis of the IDO factor.*

Figure 8.



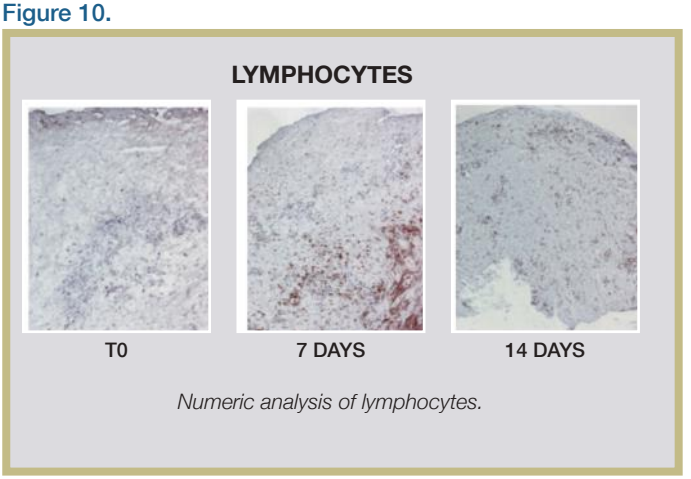

*Analysis of CD71 specific for the 1st transferrin.*

Figure 9.




*HIF 1 analysis.*

Figure 10.




*Numeric analysis of lymphocytes.*

## Discussion

In careful histological analysis, the granulation tissue of chronic sores shows a reduction in vessels with an increase in their calibre and compression by the extracellular matrix.

HIF in chronic sores is indicative of hypoxic tissues, which have a malfunctioning vascular system and lack the essential nutrients for healing.

The topical supply of hyaluronic acid and amino-acids mixtures enables the whole tissue structure of sores with little reaction (uninfected sores) to be modified in the first 15 days of treatment. In detail, the increase in angiogenesis enables the healing process to continue and the sections show this histologically; the improved $O_2$ supply associated with increased, persistent macrophages may justify the fibroblast activity with the production of granulation and collagen tissue.

## Conclusions

The dynamic complex of healing pressure sores leads to different treatment strategies to obtain a progression in the sores in the shortest possible time. Not all cell mechanisms are clear and much remains to investigate [7]. Nevertheless, the ability to act on the improvement in the supply of oxygen and nutritional substances is fundamental in unblocking chronic sores.

The study carried out with histological and histochemical analysis after the application of amino-acids and sodium hyaluronate highlighted a change in the cellular and tissue component with neo-angiogenesis, and increase in and persistence of macrophages which produced TGF-beta1 which, in turn, stimulates fibroblasts in both their number and the deposition of thin collagen fibres.

***Contributions:*** *C.P. conception and design of the study, A.P. analysis of the study, S.L. development of photographic documentation, B.G. recruitment of patients and co-ordination, D.A. analysis of results, I.C. analysis and interpretation of the statistical analyses, C.C. manuscript supervision.*

***Conflicts of interest:*** *the authors state that there are no conflicts of interest.*

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