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ORIGINAL RESEARCH

Immunohistochemical Evaluation of Venous Leg Ulcers Before and After Negative Pressure Wound Therapy

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Abstract: Venous leg ulcers represent a medical challenge. Whenever possible, therapy should be causal and include compression therapy and surgery. Negative pressure wound therapy (NPWT) has been successfully used in several phases of venous leg ulcer treatment. Positive effects of NPWT, such as reduction of edema, drainage of wound exudate, and acceleration of granulation tissue formation, are reasons for recommending NPWT in order to improve healing rates. *Aim.* The main goal of this study was to evaluate, using immunohistochemical markers, the efficacy of NPWT in terms of neoangiogenesis and granulation tissue promotion in the treatment of hard-to-heal venous leg ulcers. *Methods.* Thirty patients with hard-to-heal venous leg ulcers were included. The patients were divided into two groups: one group treated with NPWT, polyurethane foam, and four-layer bandaging system, and the second group with moist wound dressings and four-layer bandaging system. Patients were monitored before and after 1 week of treatment with multiple biopsies taken from the wound bed and wound edge. Immunohistochemical evaluation included markers for angiogenesis (CD31), lymphatic vessels (D240), macrophages (CD68), and lymphocytes (CD3). *Results.* All patients included in the NPWT group, after 1 week, showed a significant improvement in terms of angiogenesis, lymphatic vessels, and macrophage and lymphocyte proliferation, compared to the control group. *Conclusion.* This study objectively demonstrated the efficacy of NPWT in hard-to-heal venous leg ulcers via immunohistochemical findings. In particular, the results showed rapid granulation and neoangiogenesis promotion. In the authors' opinion, NPWT must be included as an adjuvant to standard venous leg ulcer therapy.

Wound healing is an interactive process comprised of four defined phases: coagulation, inflammation, tissue formation, and tissue remodeling.[1,2] On a molecular level this comprises formation of a fibrin-rich clot followed by rapid migration of keratinocytes across a provisional matrix and activation of fibroblasts to form granulation tissue. All of these cellular events are accompanied by a robust inflammatory response and are coordinated by a wide variety of cytokines, growth factors (GF), chemokines, and adhesive molecules.[3,4] Chronic wounds have different etiologies with more than 90% falling into three categories: venous leg ulcers, pressure ulcers, an
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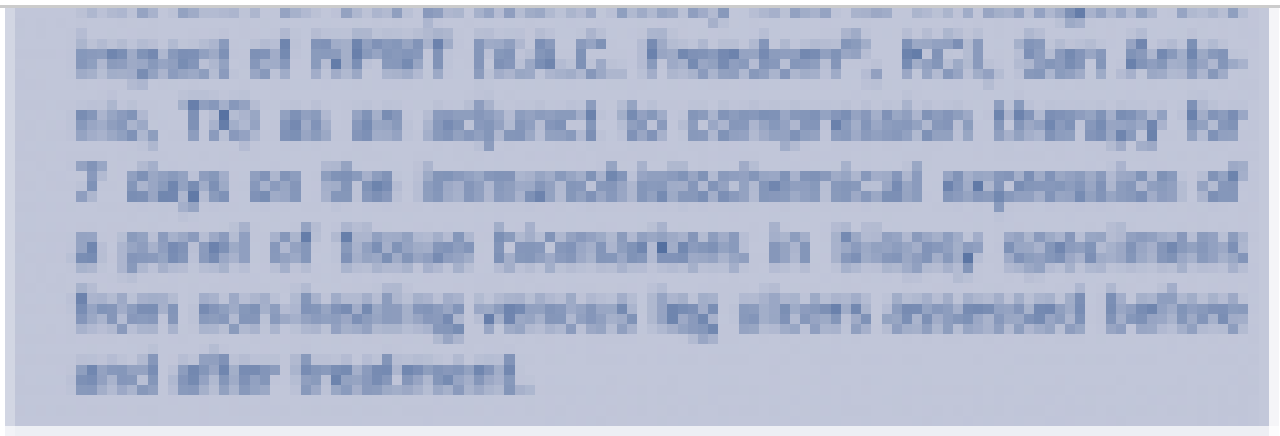
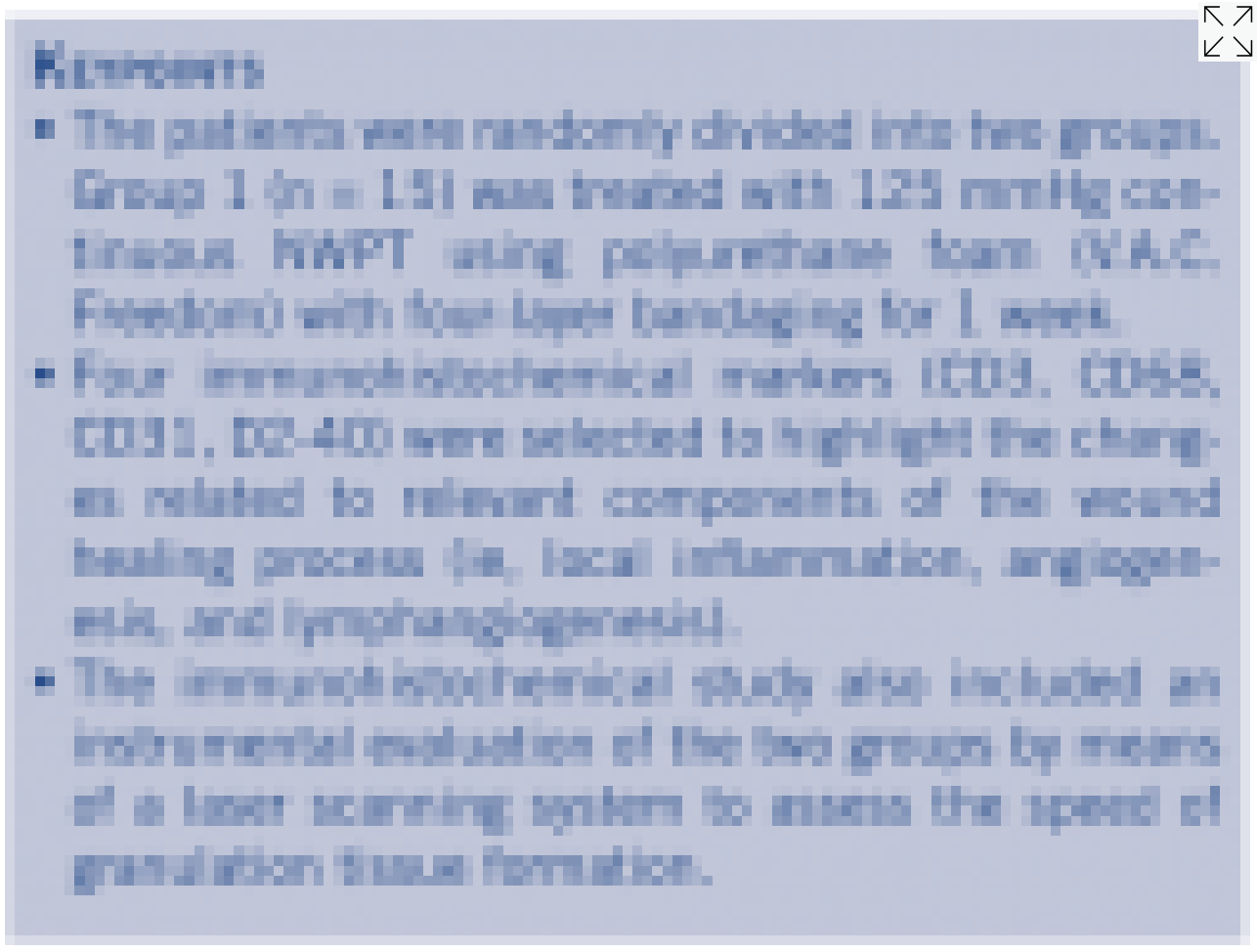


angiogenesis,[7–9] and removes excess wound fluid that harbors inhibitory factors and microorganisms.[10,11] A recent study showed that NPWT also acts upon lymphatic vessels. NPWT induces initial proliferation of lymphatics, reduces interstitial edema, and improves loco-regional perfusion.[12] The aim of this study was to investigate the impact of NPWT (V.A.C. Freedom®, KCI, San Antonio, TX) as an adjunct to compression therapy for 7 days on the immunohistochemical expression of a panel of tissue biomarkers in biopsy specimens from non-healing venous leg ulcers assessed before and after treatment.



Materials and Methods

Four immunohistochemical markers (CD3, CD68, CD31, D2-40) were selected to highlight the changes related to relevant components of the wound healing process (ie, local inflammation, angiogenesis, and lymphangiogenesis). CD3 was chosen as a pan T-cell marker, which is relevant to the healing process. Previous studies have shown that wound healing is impaired in the absence of gamma-delta T cells.[13] Furthermore, epidermal T cells from patients with healing wounds are activated and secrete growth factors. CD68 is a marker for monocytes and macrophages. These cells are important because they infiltrate the wound after the polymorphonuclear cells and modulate wound angiogenesis by releasing angiogenic factors, such as

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Highlighted Cases From the Mechanically Powered NPWT Case Compendium Contest

Inclusion of Single-Use Negative Pressure Wound Therapy for the Prevention of Closed-Incision, Surgical Site Complications in Multispecialty, Perioperative Care Bundles: Insights From an International Panel

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University of Pisa. The histopathologic and immunohistochemical analysis (processing, staining, and evaluation of the slides) was performed by the Dermatopathology Lab of the Department of Dermatology and Cutaneous Surgery at the University of Miami, Florida. Thirty patients with the clinical diagnosis of hard-to-heal venous ulcers were included after fulfilling the criteria for positive venous Doppler scan, Ankle Brachial Index (ABI)[3] 0.8, failure to heal > 6 months despite standard treatment, ulcer size[3] 20 cm^2, and no previous treatment with NPWT. The patients were randomly divided into two groups. Group 1 (n = 15) was treated with 125 mmHg continuous NWPT using polyurethane foam (V.A.C. Freedom) with four-layer bandaging for 1 week. This group included 8 female and 7 male subjects with a mean age of 72 years, ulcer duration of 32 months (average), and mean ulcer size of 89 cm^2. The control group (n = 15) received only compression treatment for 1 week with the four layer bandaging system. The control group included 6 female and 9 male patients with a mean age of 65 years, ulcer duration of 47 months (average), and mean ulcer size of 72 cm^2. **Immunohistochemical analysis.** For the purpose of this study, a 4-mm punch biopsy was obtained from the wound bed or the wound edge at the baseline visit (day 0) and after 1 week (day 7) in all patients, accounting for a total of 60 tissue samples. However, only 26 samples (12 samples from 6 patients in the NPWT group and 12 samples from 6 patients in the control group) met the selection criteria to enter the immunohistochemical study: 1) site matched specimens from days 0 and 7 obtained from the wound edge (24 samples) or the wound bed (2 samples); 2) sufficient macroscopic tissue (at least 0.3 cm x 0.3 cm). All specimens were fixed in 10% formalin, processed as paraffin-embedded tissue, and stained for hematoxylin and eosin (H&E), CD3, CD68, CD31, and D2-40 (Abcam, Cambridge, MA). At least 4 consecutive sections of all specimens were evaluated in a blinded fashion by two independent dermatopathologists. Edema was graded on the H&E sections as mild (1+) referring to pale papillary and upper dermis, moderate ([2+], pale papillary dermis with Gossamer's stands and diffuse slight separation of the collagen bundles throughout the reticular dermis), and marked or severe ([3+], sub-epidermal cleft/bulla with more pronounced separation of the collagen bundles throughout the reticular dermis). CD3 and CD68 were assessed by scanning tissue sections under low magnification (x2) to identify the hot spot. Within the hot spot, the number of cells within three adjacent high-power fields (x40) was counted and the mean number for each slide was established. The CD31 stain was assessed in a similar way by counting the number of vessels in a hot spot. In an attempt to eliminate false positive results (as histiocytes also stain positive for CD68), a vessel was defined as the presence of three contiguous cells staining for CD31. The number of D2-40 positive lymphatics was calculated per unit area, which was done by counting all the vessels (a lymphatic vessel was defined as at least three contiguous cells staining for D2-40). The width and depth of each section was measured with an ocular micrometer at 2x (Olympus WH10x/22 eye micrometer), and the measurements were expressed in micrometers. The number of lymphatics was calculated per 1000 micrometers/tissue, which is equivalent to 1 mm. The depth of each section was defined as the distance from the dermo-epidermal junction to subcutaneous fat. If no fat was present in the sections, the depth of the entire specimen was obtained.

Laser scanner measurement. The immunohistochemical study also included an instrumental evaluation of the two groups by means of a laser scanning system to

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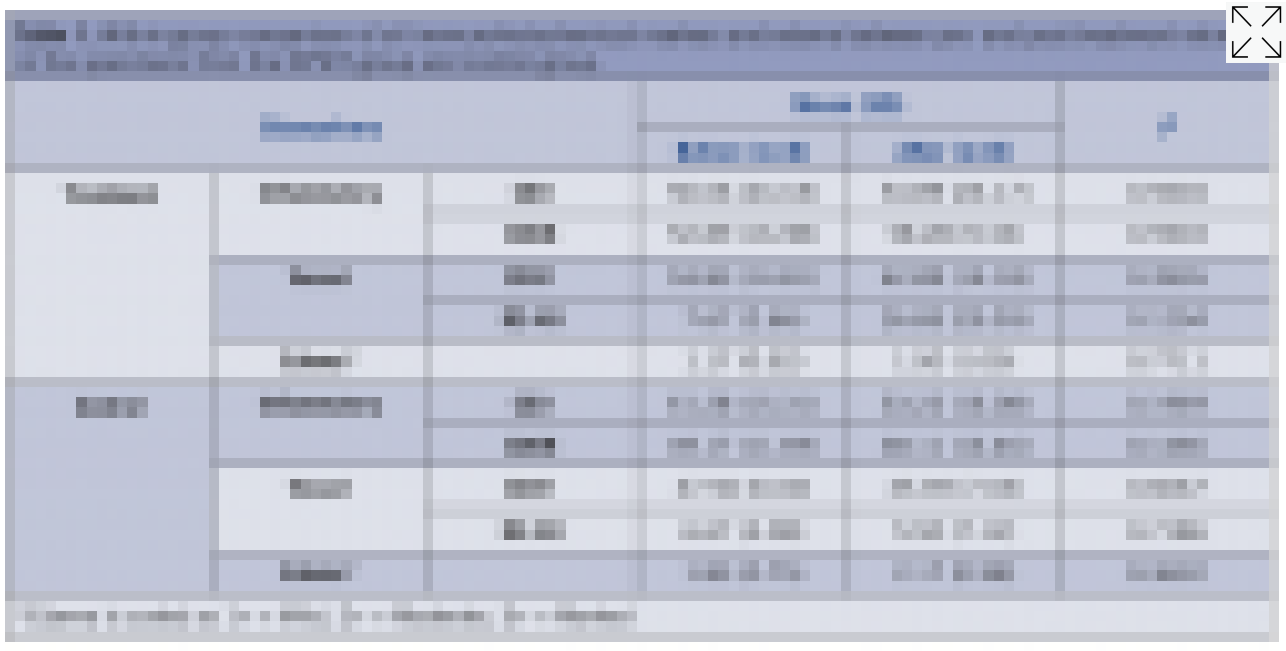
- A slight increase in the number of CD31 positive blood vessels was noted in the NPWT group after treatment, whereas no change or even slight decrease was seen in the number of blood vessels in the control group after compression treatment.
- NPWT treatment appeared to significantly stimulate granulation tissue formation of wound bed compared to control group ($P < 0.001$) at the end of the 1-week evaluation (Table 3).

The statistical analysis was performed by the Division of Biostatistics, Department of Epidemiology and Public Health, University of Miami Miller School of Medicine. Outcome variables, including the expression of the two inflammatory markers (CD3, CD68), the expression of two vessel markers (CD31, D2-40), and pre- and post-treatment mean values, were first compared within control group and within the NPWT group using a paired *t* test. A subsequent independent *t* test was used to compare the value changes between the two groups. Edema was analyzed using conditional logistic regression for paired data in two analyses: comparing mild + moderate versus marked, and then mild versus moderate + marked. All significant results were based on an alpha of 0.05. Differences found between the study groups by using the laser scanner were tested using analysis of variance (ANOVA).

Results



The results of the pre- and post-treatment mean values of each marker in the NPWT group and the control group are shown in Table 1. The value changes for each

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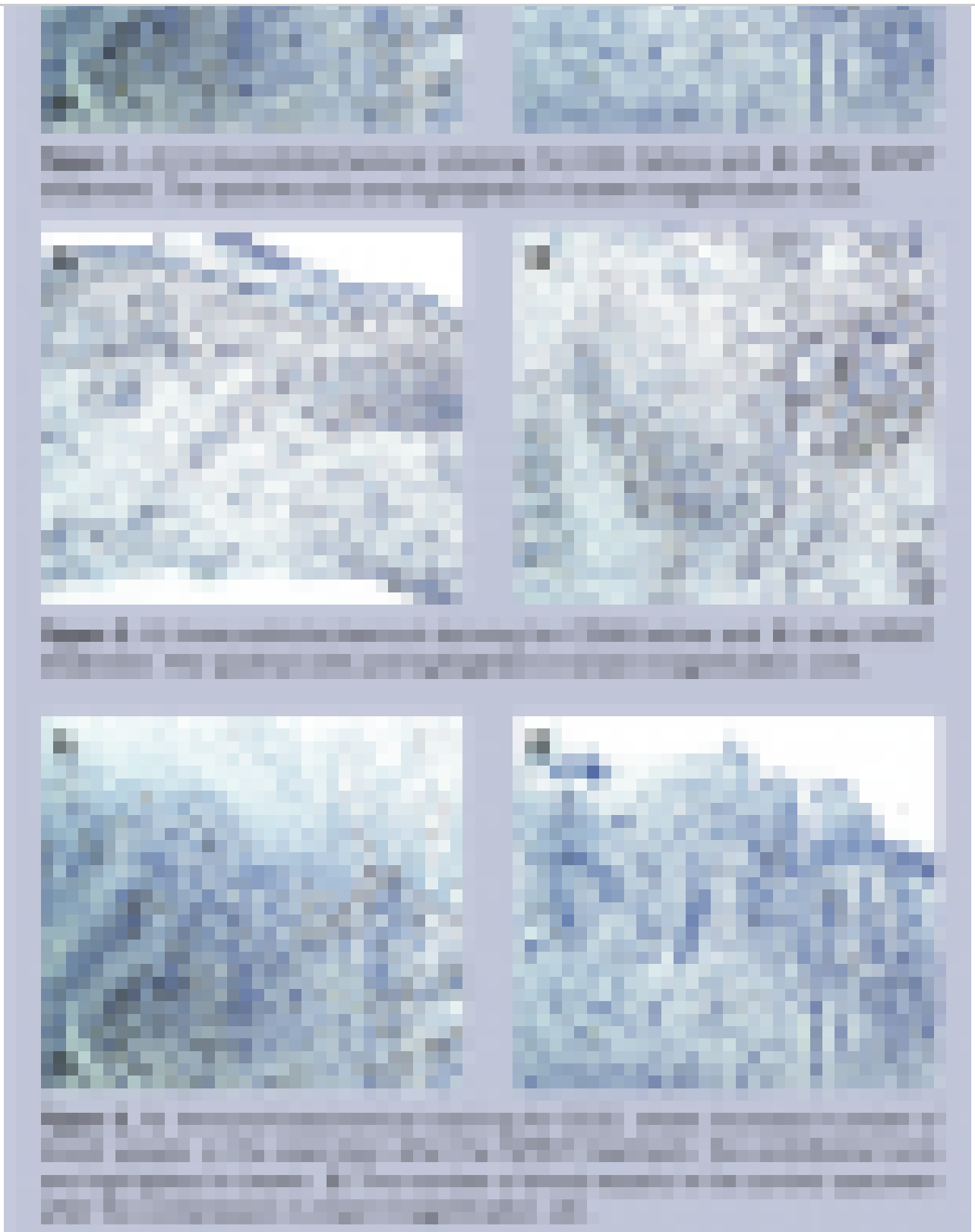

NPWT-treated wounds was 56.0 (SD ± 24.18) versus 54.89 (SD ± 23.17) post treatment. Similar results were obtained in the control group in which the mean number of CD3 cells in the venous ulcers on day 0 was 61.28 (SD ± 15.31) and decreased slightly to 53.22 (SD ± 16.08) after compression treatment. The mean difference in the CD3-positive cells at baseline versus after treatment was -1.17 (SD ± 36.33) in the NPWT group versus -8.06 (SD ± 13.26) in the control group. The T cell population remained stable in the NPWT-treated wounds, whereas in the control group, the T cell number decreased after compression (Figure 1A, B). T cells are very important for the release of growth factors and cytokines and their number should remain high enough during all phases to accelerate the granulation process, as shown in the NPWT-treated samples. However, the results did not reach statistical significance ($P = 0.67$) due to the small number of samples (6 in each group) and large standard deviations. CD68 in the NPWT-treated wounds showed a mean baseline value of 52.89 (SD ± 16.98) and a post-treatment value of 58.28 (SD ± 9.33). In the control group, the mean baseline value for CD68 was 49.17 (SD ± 11.69) and

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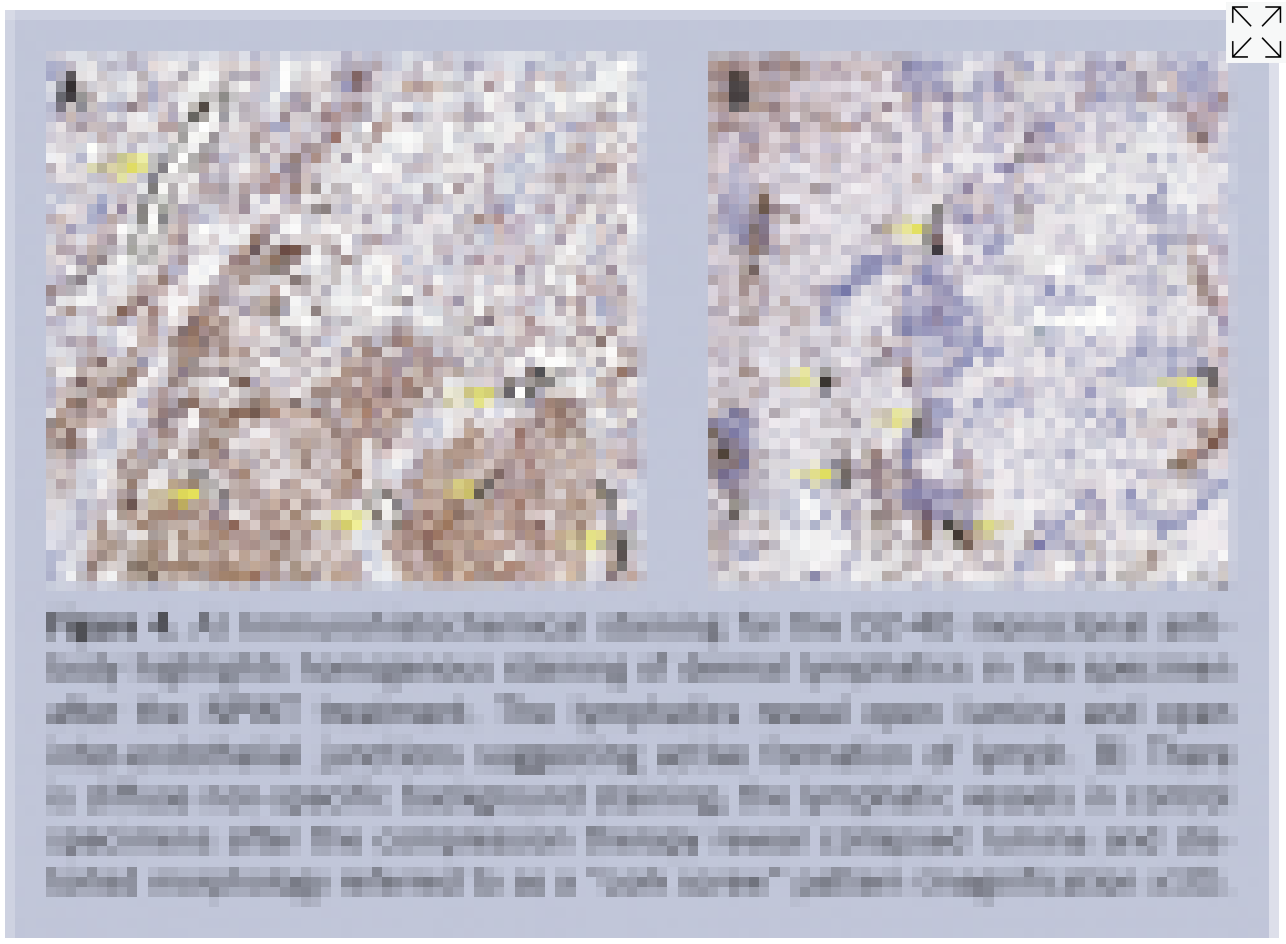
± 7.05 on day 7, [Figure 3A, B]). Only vessels composed of at least three continuous cells positive for CD31 were counted. This is important because CD31 also stained individual cells in the stroma. However, to avoid false positive results, the individual cells in the stroma were not counted, as they could be either macrophages or single endothelial cells giving rise to new blood vessels (neo-angiogenesis). Although the mean difference between the number of vessels in the specimens before and after the NPWT treatment was positive in the NPWT group (9.06, SD ± 23.76) and negative in the control group (-2.11, SD ± 9.42), the comparison between the two groups did not reach statistical significance (P = 0.3).



The number of lymphatic vessels highlighted by the D2-40 monoclonal antibody increased in the specimens from wounds treated with the NPWT system from 7.67 (SD ± 2.66) on day 0 to 26.83 (SD ± 25.93) on day 7. In the control group, the baseline number of lymphatics in the wound specimens before the compression therapy was similar (6.67, SD ± 4.08). The number remained stable and was slightly lower after the treatment (5.50, SD ± 7.12). Most lymphatic vessels in the specimens after NPWT treatment showed open lumina and occasionally displayed open inter-endothelial junctions suggesting active lymph formation (Figure 4A). Conversely, D2-40 staining in the control specimens tended to be less dense, less homogeneous, and varied in thickness. Furthermore, the vessels showed more disorganized architecture and more often they showed collapsed lumina than in the specimens from the NPWT group (Figure 4B). The mean difference between the number of lymphatics on day 0 and day 7 in the NPWT-treated group showed a positive mean increase of 19.17 (SD ± 25.10), whereas the mean difference in the control specimens was negative (-1.17, SD ± 7.49). Despite this impressive difference, the results were not statistically significant (P = 0.1) most likely due to the small sample of specimens and considerable SD (up to ± 25.10). **Edema.** Edema did not change in specimens before and after treatment in either group (the baseline values of 1.17, SD ± 0.52 (mild edema) in the NPWT group remained stable after 7 days of NPWT therapy: 1.50, SD ± 1.05 (mild edema). In the control group, the baseline value of 1.83 ([SD ± 0.75], mild to moderate edema) showed very little increase to 2.17 ([SD

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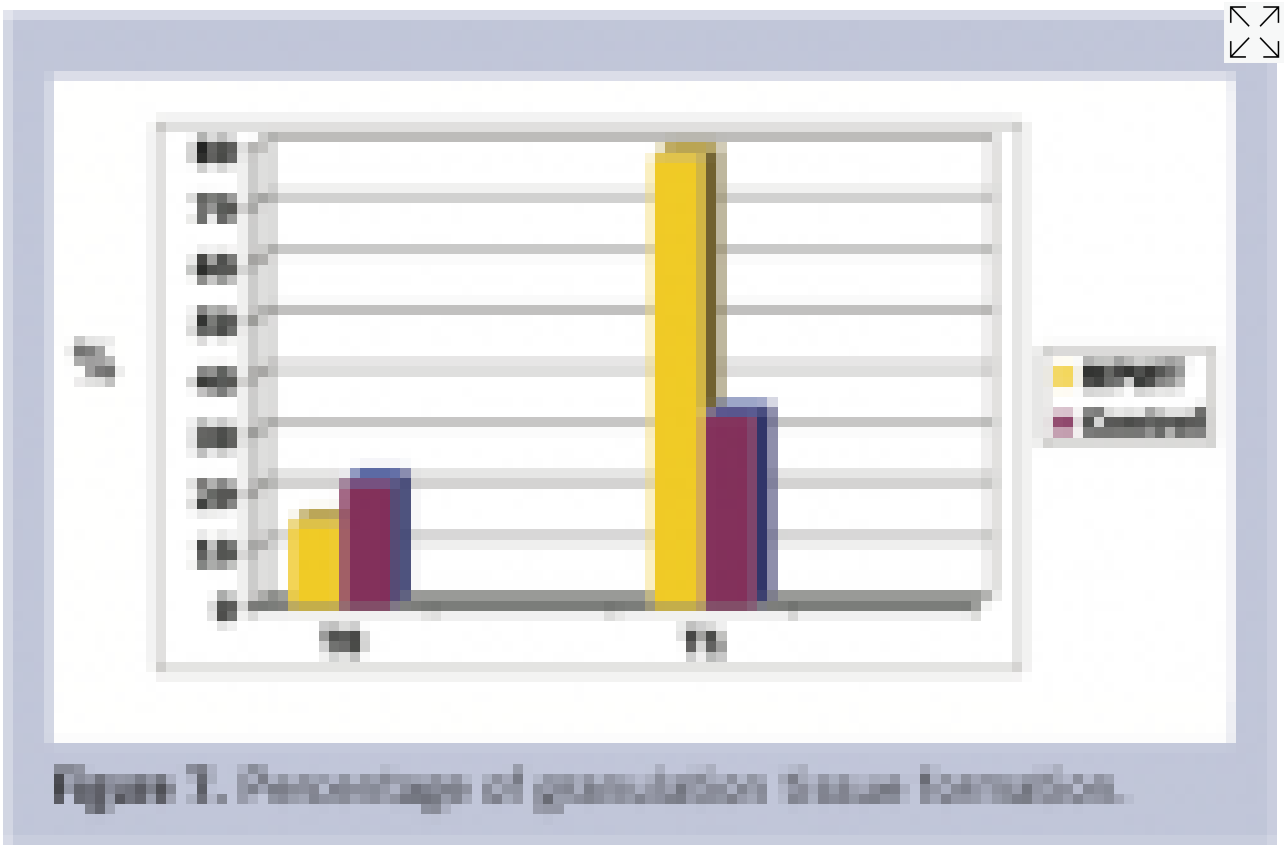
Granulation tissue. NPWT treatment appeared to significantly stimulate granulation tissue formation of wound bed compared to control group ($P < 0.001$) at the end of the 1-week evaluation (Figure 7). At the first dressing change (after 3 days of treatment), the mean percentage of granulation tissue in the NPWT group increased from 14.6% ± 12.8% to 70.5% ± 10.5% and this value remained stable at 1 week of treatment (Figures 5, 6).



Discussion

NPWT is a treatment that has benefited both chronic and acute wounds. This method of exposing a wound to subatmospheric pressure for an extended period to promote debridement and healing was first described by Fleischmann et al[20] in 1993. Morykwas et al[21] investigated the physiological basis of the NPWT (V.A.C.) therapy and suggested that removal of the interstitial fluid decreases localized edema and increases blood flow, which in turn decreases local bacterial load. Although this may be an important mechanism, we have shown no statistically significant difference in the edema score in tissue specimens before and after the

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cells that are allowed to stretch tend to divide and proliferate in the presence of soluble mitogens, while retracted cells remain quiescent. Cells that cannot extend assume a more spherical shape (by retraction), are growth arrested, and undergo apoptosis.[22,23] Saxena et al[7] created a computer model of a wound and simulated NPWT application. In this model, they altered the pressure, pore diameter, and pore volume fraction to study the effects of NPWT-induced material deformation. The morphology of deformation in this wound model was compared to histological sections of wounds treated with NPWT. The results showed that most elements stretched by the NPWT application caused 5%–20% strain, which is similar to the *in vitro* strain levels proven to promote cellular proliferation. The conclusion was that negative pressure stimulates wound healing by promoting cell division, angiogenesis, and local proliferation of growth factors. Consequent studies have confirmed that the NPWT treated wounds manifest on histologic examination increased neo-vascularization as well as increased VEGF.[11] We detected relatively stable number of CD3 positive cells and CD68 positive cells in the specimens of the NPWT treated wounds whereas these populations were slightly decreased in the control group. Although not statistically significant, these results showed a trend of maintaining a favorable healing environment in the NPWT treated wounds, as both CD3 (T-cells) and CD68 (macrophages) are important for the intercellular communications and release of growth factors and cytokines to promote cellular proliferation. We did not assess the number of polymorphonuclear cells as part of the inflammation, as they are numerous and not easily identifiable in histologic specimens of chronic wounds that show signs of neutrophilic karyorrhexis (nuclear dust), diffuse fibrin, and necrosis. In severely ischemic, hypoxic wound conditions, increasing oxygen concentrations results in accelerated wound healing with increased blood vessel growth.[24,25] In addition, clinical and experimental studies have shown that mechanical stress triggers VEGF secretion[26,27] and that NPWT treatment stimulates neovascularisation in traumatic wounds.[11] The present results showed a slight increase in the number of CD31 positive blood vessels in the NPWT group after treatment compared to the number of blood vessels in the control group after compression, which showed a slight decrease. However, the mean difference in the pre- and post-treatment values between both groups was not statistically significant. One possible explanation is that neovascularisation is a very dynamic process and the density of blood vessels in specimens from chronic wounds may depend on the time point of tissue sampling. Jacobs et al[8] found that NPWT treated wounds showed significantly higher mean vessel density by day 3, which was lost by days 5–7. We have not assessed specimens in the process of treatment but only at baseline (day 0) and at the end (day 7). Another possible factor that counteracts with the results may be the fact that only well developed, identifiable blood vessels composed of at least three contiguous cells with/without lumina were counted. In most specimens, particularly those obtained from the NPWT-treated wounds, significant background CD31 staining of individual cells was seen. This might be in part due to macrophages that co-express CD31, but more significantly, might have been tiny individual endothelial cell islands of neo-angiogenesis in the stroma. It has been shown that lymphatic function significantly decreases with increasing severity of chronic venous insufficiency.[28,29] Only one study has looked at the morphology of lymphatics at microscopic level by using the D2-40 monoclonal antibody in specimens from chronic venous ulcers versus normal site matched skin

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the pre- and post-treatment values was not statistically significant between the two groups due to the small number of samples and large standard deviations, there was a definite trend toward lymhangiogenesis in the NPWT group versus no change in the control specimens. Furthermore, the morphology of the lymphatics displayed homogenous and thick ribbon-like staining, open lumina, and increased inter-endothelial junctions. In the control specimens, the morphology of the lymphatics showed more collapsed lumina (as a possible sign of lymph overload) and a more heterogenous staining pattern. This suggests that many of the otherwise "sufficient" number of lymphatic vessels detected in the control specimens may be dysfunctional or non-functional. Prior to this study, only one study investigated the effect of the NPWT treatment on the lymph vessels in different types of wounds referring to the global and local architecture of lymphatics as well as to deviations of their normal morphology.[12] Specimens from 13 patients with chronic venous ulcers who underwent NPWT treatment for between 8 and 16 days (mean 15 days) were evaluated. The D2-40 monoclonal antibody was used to highlight the lymphatics. The results showed that there was 58%–62% lymph vessel proliferation by the day 4 post the NPWT application. However, 1%–4% regression of the number of the D2-40 positive vessels was observed by day 8 with further 30%–31% regression by day 12. Interestingly, those patients with less than 5 risk factors for non-healing wounds (eg, infection, ischemia, trauma, radiation, diabetes, smoking) did not demonstrate any lymph vessel regression, while the ones with 5–10 risk factors demonstrated lymph vessel proliferation on day 4 after NPWT treatment followed by lymph vessel regression. This may be a valid explanation as to why in the present study the increase of lymphatics in the NPWT specimens on day 7 was not statistically significant compared to the control specimens on same day: 1) we evaluated the specimens only at the baseline and at the end of the treatment; 2) we did not assess and correlate for risk factors. NPWT treatment seems to induce morphological and quantitative alterations in the lymph vessel network in chronic venous ulcers by stimulating lymphangiogenesis more efficiently in the first days of the treatment. This proliferation may not be sustained by the end of the treatment (day 7) because of underlying disease and risk factors that impair wound healing and in particular lead to lymphatic regression. The increase in the lymphatics number in the NPWT group correlated with a better clinical outcome in terms of granulation rate and pain relief.

Conclusion

NPWT applied on stable venous leg ulcers has a positive impact on the microscopic features in tissue specimens of treated wounds versus controls. All biomarkers referring to inflammation, angiogenesis, and lymphangiogenesis showed an absolute positive change in the treatment group, except for CD3, which showed negative absolute change in the treatment group and greater negative absolute change in the control group. The other three biomarkers showed negative absolute change in the control group but positive absolute change in the treatment group. Although some of the differences showed greater absolute magnitude change, there was no statistically significant change ($P > 0.05$) due to the small number of samples collected (6 in each group) and the large standard deviations (mainly in the

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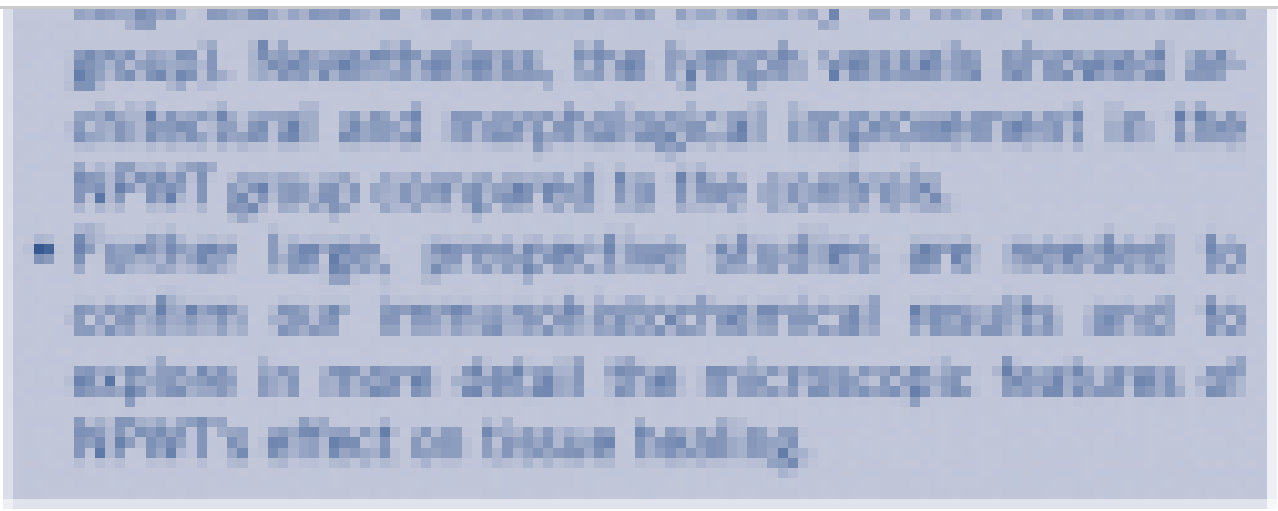

healing, which refers to increased lymphangiogenesis and improved lymph drainage. Further large, prospective studies are needed to confirm our immunohistochemical results and to explore in more detail the microscopic features of NPWT's effect on tissue healing.

Acknowledgements

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References

1. Gottrup F, Karlsmark T. Leg ulcers: uncommon presentations. *Clin Dermatol*. 2005;23(6):601–611. 2. Broughton G, 2nd, Janis JE, Attinger CE. Wound healing: an overview. *Plast Reconstr Surg*. 2006;117(7 Suppl):1e–S–32e-S. 3. Nickoloff BJ, Bonish BK, Marble DJ, et al. Lessons learned from psoriatic plaques concerning mechanisms of tissue repair, remodeling, and inflammation. *J Investig Dermatol Symp Proc*. 2006;11(1):16–29. 4. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci*. 2004;9:283–289. 5. Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. *Plast Reconstr Surg*. 2006;117(7 Suppl):35S–41S. 6. Mustoe T. Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy. *Am J Surg*. 2004;187(5A):65S–70S. 7. Saxena V, Hwang CW, Huang S, Eichbaum Q, Ingber D, Orgill DP. Vacuum-assisted closure: microdeformations of wounds and cell proliferation. *Plast Reconstr Surg*. 2004;114(5):1086–1096. 8. Jacobs S, Simhaee DA, Marsano A, Fomovsky GM, Niedt G, Wu JK. Efficacy and mechanisms of vacuum-assisted closure (VAC) therapy in promoting wound healing: a rodent model. *J Plast Reconstr Aesthet Surg*. 2009;62(10):1331–1338. 9. Loree S, Dompmartin A, Penven K, Harel D, Leroy D. Is Vacuum Assisted Closure a valid technique for debriding chronic leg ulcers? *J Wound Care*. 2004;13(6):249–252. 10. Venturi ML, Attinger CE, Mesbahi AN, Hess CL, Graw KS. Mechanisms and clinical applications of the vacuum-assisted closure (VAC) Device: a review. *Am J Clin Dermatol*. 2005;6(3):185–194. 11. Labler L, Rancan M, Mica L, Harter L, Mihic-Probst D, Keel M. Vacuum-assisted closure therapy increases local interleukin-8 and vascular endothelial growth factor levels in traumatic wounds. *J Trauma*. 2009;66(3):749–757. 12. Labanaris AP, Polykandriotis E, Horch RE. The effect of vacuum-assisted closure on lymph vessels in chronic wounds. *J Plast Reconstr Aesthet Surg*. 2009;62(8):1068–1075. 13. Havran WL

lymphatic alterations in chronic venous insufficiency ulcers using D2-40. *J Am Acad Dermatol*. 2011;64(6):1123 e1–12. 19. Bertone MS, Dini V, Romanelli P, Rizzello F, Romanelli M. Objective analysis of heterologous collagen efficacy in hard to heal venous leg ulcers. *WOUNDS*. 2008;20(9):245–249. 20. Fleischmann W, Strecker W, Bombelli M, Kinzl L. [Vacuum sealing as treatment of soft tissue damage in open fractures]. *Unfallchirurg*. 1993;96(9):488–492. 21. Argenta LC, Morykwas MJ. Vacuum-assisted closure: a new method for wound control and treatment: clinical experience. *Ann Plast Surg*. 1997;38(6):563–577. 22. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science*. 1997;276(5317):1425–1428. 23. Mochitate K, Pawelek P, Grinnell F. Stress relaxation of contracted collagen gels: disruption of actin filament bundles, release of cell surface fibronectin, and down-regulation of DNA and protein synthesis. *Exp Cell Res*. 1991;193(1):198–207. 24. Hopf HW, Gibson JJ, Angeles AP, et al. Hyperoxia and angiogenesis. *Wound Repair Regen*. 2005;13(6):558–564. 25. Marx RE, Ehler WJ, Tayapongsak P, Pierce LW. Relationship of oxygen dose to angiogenesis induction in irradiated tissue. *Am J Surg*. 1990;160(5):519–524. 26. Brauchle M, Funk JO, Kind P, Werner S. Ultraviolet B and H2O2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem*. 1996;271(36):21793–21797. 27. Bonomo SR, Davidson JD, Yu Y, Xia Y, Lin X, Mustoe TA. Hyperbaric oxygen as a signal transducer: upregulation of platelet derived growth factor-beta receptor in the presence of HBO2 and PDGF. *Undersea Hyperb Med*. 1998;25(4):211–216. 28. Bollinger A, Pfister G, Hoffmann U, Franzeck UK. Fluorescence microlymphography in chronic venous incompetence. *Int Angiol*. 1989;8(4 Suppl):23–26. 29. Bollinger A. Microlymphatics of human skin. *Int J Microcirc Clin Exp*. 1993;12(1):1–15. *Valentina Dini, MD, PhD; Mariastefania Bertone, RN; and Marco Romanelli, MD, PhD are from the Department of Dermatology, University of Pisa, Italy. Maria Miteva, MD; and Paolo Romanelli, MD are from the Department of Dermatology and Cutaneous Surgery, University of Miami Miller School of Medicine, Miami, Florida.* **Address correspondence to:** Marco Romanelli, MD, PhD Dept. of Dermatology University of Pisa Via Roma, 67 56126 Pisa, Italy +39 050 992436 m.romanelli@med.unipi.it

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