Original Paper

Dermatology

Dermatology 2010;220:114–120
DOI: 10.1159/000277380

Received: July 16, 2009
Accepted after revision: November 16, 2009
Published online: January 26, 2010

Wound Stimulation by Growth-Arrested Human Keratinocytes and Fibroblasts: HP802-247, a New-Generation Allogeneic Tissue Engineering Product

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Key Words
HP802-247 · Wound healing · Growth factors · Keratinocytes · Fibroblasts

Abstract
Background: HP802-247 is a new-generation, allogeneic tissue engineering product consisting of growth-arrested, human keratinocytes (K) and fibroblasts (F) delivered in a fibrin matrix by a spray device. ***Objective:*** To identify the preferred dose of HP802-247 based on cell concentration and K/F ratio. ***Methods:*** A multicenter, randomized, double-blind, placebo-controlled, explorative phase II study of 6 different doses of HP802-247 administered once per week for 12 consecutive weeks in chronic venous leg ulcers. ***Results:*** HP802-247 was safe and well tolerated and showed increasing efficacy dependent on cell concentration and K/F ratio, in line with in vitro growth factor release data. The mean complete closure rate at week 12 for all patients treated with HP802-247 was 40%, and for placebo it was 33%. In contrast to placebo, all HP802-247 dose groups improved from week 12 to 24. ***Conclusion:*** As an integral part of a rational tissue engineering product development, this explorative trial identified the preferred dose of HP802-247 for further clinical studies.

Introduction

Tissue engineering provides a source of advanced therapies for chronic skin wounds, a relevant and costly problem in modern health care [1, 2]. To treat skin defects with cultured cells, there are two main approaches, replacement of lost tissue by permanent grafting of proliferative autologous cells or regeneration by stimulation of residual cells in the wound using temporarily applied allogeneic or xenogeneic cells. In the last decade, several tissue engineering products of both types have been tested in partly controlled clinical trials, focusing on chronic wounds such as vascular leg ulcers and diabetic foot ulcers [3]. Product launches, however, have been rather disappointing so far [4]. The 'tailor-made' autologous products are expensive due to costly production, quality assurance and logistics. Hence their clinical use will be restricted to wounds that are refractory to conventional treatment, as illustrated by Epidex™, an autologous epidermal equivalent from follicular outer root sheath keratinocytes [5]. Reduced costs of goods and the option of long-term storage should render the allogeneic 'off-the-shelf' products less expensive, thus allowing for repeated application in a larger segment of hard-to-treat chronic

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wounds. Today, discussions on applying either fetal or adult stem or progenitor cells are far from clinical reality since there are complex aspects to be addressed, including immunogenicity and carcinogenesis [6, 7].

To date, two allogeneic, keratinocyte- and fibroblast-based products are on the market for the treatment of chronic leg ulcers, Apligraf® and Orcel® [8–11]. We describe the development and clinical testing of the new-generation, two-cell-type, allogeneic tissue engineering product HP802-247 (previously named Allox). The two cryopreserved components of HP802-247 consist of fibrinogen and of growth-arrested, allogeneic, human keratinocytes and fibroblasts suspended in thrombin. After thawing, these components are sprayed sequentially on the wound bed to form a thin fibrin matrix containing two types of living, but not proliferating, human skin-derived cells that interactively produce growth factors relevant for wound healing during the subsequent several days. In a multicenter, open phase I study, HP802-247 applied once per week for 12 consecutive weeks was safe and well tolerated by 14 patients with chronic venous leg ulcers not responding to standard treatment [12]. Complete closure at week 12 was observed in 10 patients. This formed the rationale to conduct a multicenter, randomized, double-blind, placebo-controlled, explorative phase II study comparing 6 different doses of HP802-247 in the treatment of chronic venous leg ulcers, with the objective to identify a preferred dose level of HP802-247 based on keratinocyte/fibroblast (K/F) ratio and cell concentration for use in future clinical studies.

Patients and Methods

Product Manufacturing

The cells originated from human skin biopsies, under informed consent guidelines and extensive donor testing for infectious pathogens. The fibroblasts were derived from discarded tissue from a breast reduction surgery and the keratinocytes from one skin biopsy obtained from an organ and tissue donation. HP802-247, previously named Allox, was developed and manufactured at Isotis SA (formerly Modex SA, Lausanne, Switzerland) according to Good Manufacturing Practice requirements. DFB Pharmaceuticals Inc. (Fort Worth, Tex., USA) acquired HP802-247 in 2004. The cell banks were negative for a large number of pathogens by molecular, biological and culture testing. After amplification the cells were growth arrested by low-dose gamma irradiation (80 Gy). Keratinocytes and fibroblasts were subsequently mixed in a defined ratio and cell concentration into a Hanks' buffered salt solution containing thrombin and cryoprotectants (glycerol and human serum albumin). Final cell suspensions were frozen using a controlled-rate freezer and stored at –80°C. Cell viability after cryopreservation as well as spraying was measured by the trypan blue exclusion assay [13].

In vitro Analysis of Growth Factor Release

Either on freshly prepared material or after thawing of the frozen product, stored for up to 6 months, a spray pump was attached to the vials containing fibrinogen and cell suspension, and their content was delivered into culture dishes by sequential spraying of the two components. Cell culture medium was added, and the dishes were placed in an incubator at 37°C and 5% CO_2. Medium samples as well as cell-containing fibrin matrices were collected, and the secretion of the growth factors – vascular endothelial (VEGF), basic fibroblast (bFGF), keratinocyte, hepatocyte growth factors and transforming growth factor β – and cytokines – granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins 1 and 18 – was measured using various cell concentrations (2.5, 5, 10, 20 and 40 × 10^6/ml) and ratios. Initial studies have shown a cell-ratio-dependent relationship in the growth factor secretion with an optimum release for the K/F ratio of 1:9. The experiments presented hereafter were performed with the 1:1 and 1:9 K/F ratios.

Clinical Study Design

This multicenter, randomized, double-blind, placebo-controlled, explorative phase II study was designed to identify the preferred dose of HP802-247, administered once per week for 12 consecutive weeks, for further efficacy studies on the basis of the safety and tolerability and the effect on wound healing in patients with chronic venous leg ulcers.

At study entry each eligible patient was centrally randomized to one of the 7 treatment groups, 6 doses of HP802-247 and placebo. Patients were treated with the investigational agent once a week for 12 consecutive weeks or up to complete wound closure, whichever came first. The investigational agent was applied to the ulcer bed after careful, protocolized preparation, including mechanical debridement. Wound dressings without active components were selected according to the severity of exsudation. All patients received standard compression treatment throughout the study [14]. Patients were followed up once per month until week 24.

This study was performed under International Conference on Harmonization Good Clinical Practice requirements and in accordance with the Declaration of Helsinki, and was approved by the local ethics committee and/or institutional review board and the relevant competent regulatory authority.

Patients

The study was conducted in 20 centers in the Netherlands (10), Hungary (6), Netherland Antilles (1), Aruba (1) and Switzerland (2), between November 2002 and June 2004. All patients gave their written informed consent.

All eligible patients had an established diagnosis of a venous leg ulcer that had existed for 1–12 months. The ulcer surface area, as calculated by 0.8 × largest width × largest length, had to be between 1 and 48 cm^2. A duplex report within the 12 months preceding study start was accepted to quantify the venous insufficiency. The ankle brachial pressure index was not ≤0.7. The major exclusion criteria were presence of diabetic, rheumatoid or vasculitic ulcers, severe peripheral neuropathy, as well as fascia or tendon exposure. Patients were also excluded for uncontrolled edema, clinical suspicion of ulcer bed infection or prior use of cell-based treatments.

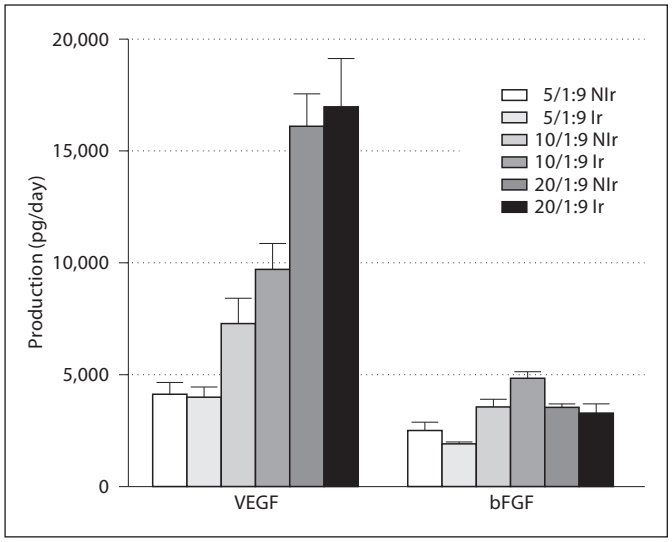

Fig. 1. Effect of growth-arresting cells by irradiation on VEGF and bFGF production. Comparison of growth factor secretion in vitro (pg/ml, means ± SEM) by HP802-247 using either irradiated (Ir) or non-irradiated (NIr) cells in 3 cell concentrations (5, 10 and 20 × 10^6/ml) with a 1:9 K/F ratio.

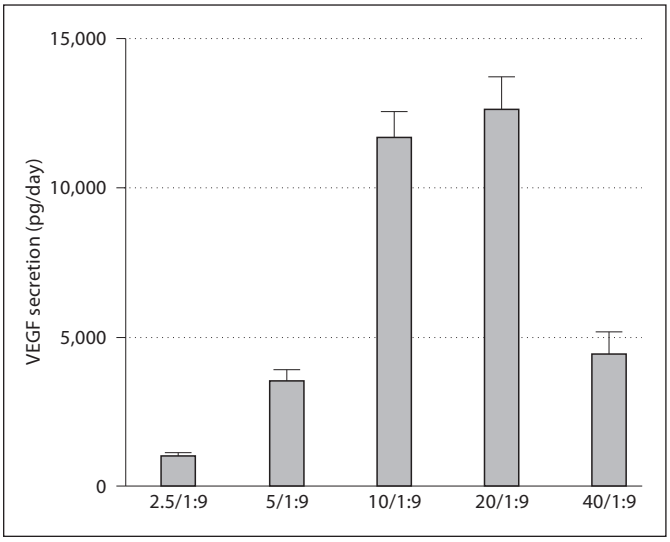

Fig. 2. Effect of cell concentration (×10^6 cells/ml) on VEGF production (pg/day, means ± SEM) in vitro.

Investigational Treatment

The investigational treatment, HP802-247 or placebo, was presented as two separate vials, vial 1 containing fibrinogen and vial 2 containing allogeneic, growth-arrested, human keratinocytes and fibroblasts in thrombin. The two sterile vials were packaged into a sealed pouch with two disposable, sterile, screw-on spray pumps separately packaged. One spray of HP802-247, i.e. 1 spray of each vial 1 and 2, equating 260 μl, was to cover 12 cm^2 of ulcer surface area, when sprayed at a distance of 10 cm from the wound bed. Six doses were tested, 2 different K/F ratios (1:1 and 1:9) each at 3 cell concentrations, 2.5, 5 and 10 × 10^6 cells/ml, respectively. A dose is further described by an abbreviation, e.g. 5/1:9 (cell concentration/cell ratio). Placebo consisted of the same sprays without the cells. The investigational treatment was shipped and stored at –80°C, to be thawed for use within 30 min by placing the packed vials in a 37°C water bath for 5 min. Study treatment was sprayed sequentially to cover the whole surface area of the ulcer bed.

Statistical Analysis

The analysis is descriptive by nature as this was an explorative study with sample size not based on a power calculation. All 118 patients randomized to treatment were included in the safety analysis, reporting all adverse events with particular emphasis on wound infection, parameters included in the reference ulcer examination, local tolerance, laboratory values and vital signs. The primary efficacy endpoint was the proportion of patients with complete closure of the reference ulcer at week 12. Secondary endpoints included reduction of the ulcer surface area at week 12, time to complete ulcer closure and the wound healing rate. All patients were followed up until 12 weeks after the treatment period, i.e. week 24. Prior to breaking the codes the data monitoring board evaluated the blinded data and excluded 8 patients with major protocol violations, evenly distributed over the 7 study groups, from the efficacy analysis. Efficacy analysis, using the 'last observation carried forward' principle, was performed on all patients randomized to treatment on the intent-to-treat (ITT, n = 110) data set and on all patients who received at least 3 consecutive treatment applications, the per-protocol (PP, n = 74) data set.

Results

In vitro Studies

HP802-247 made from freshly trypsinized as well as irradiated (80 Gy) keratinocytes and fibroblasts secreted a vast array of different growth factors and cytokines. The production of VEGF, bFGF and GM-CSF appeared to be independent of whether the cells were irradiated or not. This demonstrates that the cells remain active after irradiation as illustrated by VEGF and bFGF (fig. 1). Increasing cell concentrations led to a dose-dependent increase in the growth factors measured. This effect plateaued at a cell concentration of 20 × 10^6/ml (as illustrated for VEGF only in fig. 2). Stability studies performed on the final product stored for 6 months at –80°C showed that cell viability after thawing remained higher than 70% and that growth factor secretion (VEGF and bFGF) remained stable for at least 7 days. Quality control testing performed on all batches used for the clinical study confirmed the dose-effect relationship between cell concentration and growth factor release and showed a similar

Table 1. Main baseline characteristics of patients with chronic venous leg ulcers in the ITT data set (n = 110)

	Placebo	HP802-247						Overall
K/F ratio:		1:9			1:1			
Concentration, $\times 10^6$ cells/ml:		2.5	5	10	2.5	5	10	
Number	15	14	17	15	17	16	16	110
Median leg ulcer history, rounded months	124	122	118	69	83	125	156	114
History of DVT, rounded %	27	43	41	20	29	44	13	31
Reference ulcer duration, weeks	22.9 ± 14.3	18.4 ± 13	15.2 ± 12.5	20.1 ± 9.1	19.9 ± 13.6	20.7 ± 11.8	19.9 ± 15.3	19 ± 12.5
Reference ulcer surface area, cm^2	12.4 ± 11.1	13.7 ± 14	13.4 ± 12.8	7.2 ± 6.4	7 ± 6.6	11.4 ± 12.7	11.4 ± 12.7	10.6 ± 11.3

Reference ulcer duration and surface area expressed as means ± standard deviation. DVT = Deep vein thrombosis.

Table 2. Percentage (rounded) of patients with a complete closure of the reference leg ulcer at week 12 and 24 in the PP data set (n = 74)

	Placebo	HP802-247						Pooled HP802-247 groups					
K/F ratio:		1:9			1:1			1:9	1:1	2.5	5	10	all
Concentration, $\times 10^6$ cells/ml:		2.5	5	10	2.5	5	10						
Number	8	11	11	10	11	11	12	32	34	22	22	22	66
Week 12, %	38	46	36	50	55	46	42	47	47	50	45	45	47
Week 24, %	50	64	73	60	55	45	67	66	56	59	59	64	61

release profile of the 1:1 and the 1:9 K/F ratio. The spray device delivered a thin fibrin film with evenly distributed viable cells.

Characteristics of the Patients

A total of 118 patients, the safety data set, were randomized to the 7 treatment groups; 62.7% were female and 37.3% male, with a mean age of 69.2 years. The demographic, limb and reference ulcer characteristics at baseline were similar across treatment groups in the ITT data set (table 1). The mean reference leg ulcer area was numerically lower in the 2.5/1:1 and 10/1:9 HP802-247 dose groups as compared to other treatment groups. For the other groups these characteristics were similar to that of the placebo group. Treatment exposure was similar for all treatment groups (between 19.3–88.8 days in the HP802-247-treated group and 19.5–79.8 days in the placebo group).

Efficacy

The mean complete closure rate at week 12, the ITT data set, of all patients treated with HP802-247 (40%) was higher than that of placebo (33%). The complete closure rate over the subsequent 12-week follow-up period remained the same for the placebo group, while the pooled HP802-247 group improved to 53.7% (fig. 3). The K/F ratio 1:9 appeared to be more effective in complete ulcer closure at week 12 than the ratio 1:1 (43.5 vs. 36.7%, respectively; fig. 3). This effect was improved during the 12-week follow-up for both ratios in contrast to placebo ($p = 0.008$, χ^2 test). Very few differences were observed between the 3 HP802-247 groups for cell concentration independent of cell ratio. However, at week 24 the HP802-247 dose 5/1:9 appeared to be the most effective with a complete closure rate of 64.7%. The mean complete closure rate at week 12 and week 24 in the pooled HP802-247 groups was consistently higher for patients treated with at least 3 consecutive HP802-247 applications as compared to placebo, the PP data set (table 2). At week 24, the HP802-247 dose of 5/1:9 appeared to be the most effective with a complete closure rate of 72.7% in comparison to 50% in the placebo group.

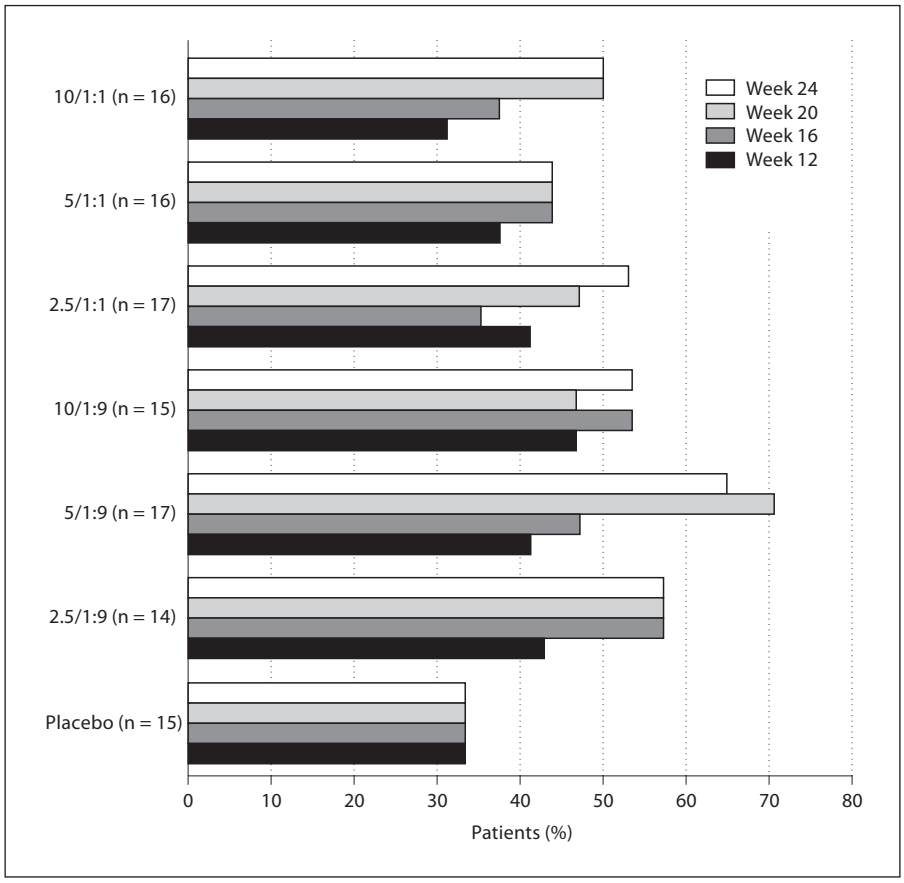

Fig. 3. Complete closure of chronic leg ulcers at week 12–24. Percentage of patients with complete closure of the reference chronic leg ulcer at weeks 12 (end of treatment), 16, 20 and 24 (end of follow-up) by HP802-247 dose groups (ITT data set, n = 110).

Safety

The incidence of adverse events and serious adverse events in the HP802-247 groups was similar to that observed in the placebo group. The incidence of possibly or probably related adverse events tended to be somewhat lower in the HP802-247 groups (29–47%) when compared to the placebo group (63%). No clinically relevant changes in laboratory parameters and vital signs were observed. Most commonly reported adverse events with HP802-247 included local edema, erythema and wound discharge, as well as local pruritus without any cutaneous signs of hypersensitivity. However, reported adverse events were similar to those for the placebo group.

Discussion

The use of single-agent growth factors has failed to promote closure of recalcitrant chronic wounds [15]. In view of the complex pathogenesis of chronic wounds, a sustained, timely scheduled, cell-based stimulation of the healing process acting by interactive secretion of multiple growth factors seems more rational. Since a permanent take of the applied cells is not aimed at, proliferative capacity is not required. In view of a stable, optimal ratio of the two cell types used, growth arrest is mandatory. As far as fibroblasts are concerned, postmitotic fibrocytes were recently claimed to stimulate keratinocyte proliferation to a greater extent than their progenitors [16]. In fact, fibroblasts have been used as feeder cells in keratinocyte culture for two decennia [17]. This most probably depends on significantly different patterns of cytokine release [18], which – as shown here – also varies with numbers and ratio of the cocultured cells. To establish a cell-based therapeutic product with reproducible parameters for dose finding and quality control as requested by regulation [19–22], the definition of cell numbers and ratio in correlation with the amount and pattern of growth factor release as well as clinical efficacy and safety is crucial.

In the development of HP802-247, the combination of different cell concentrations and K/F ratios were charac-

terized by in vitro measurement of growth factor production, considered to be essential in the wound healing process, such as VEGF, bFGF, keratinocyte growth factor and GM-CSF. The in vitro data correspond well to the reported clinical efficacy of the different dose levels of HP802-247 in this randomized, double-blind, placebo-controlled, phase II trial performed in recalcitrant venous leg ulcers. The treatment application frequency, once per week, was based on the observation that the in vitro growth factor secretion remained stable for at least 7 days. Every dose level of HP802-247 tested in this explorative clinical study had a higher proportion of patients with complete closure of their reference venous leg ulcer at week 12 as compared to placebo (ITT data set). This observation was further supported by the results of patients who received at least 3 consecutive applications of HP802-247 (PP data set). The data at week 12 do not point to a promotion of wound healing in chronic venous leg ulcers by the diluted fibrin sealant alone (placebo); controlled studies are however not reported in the literature [23]. Between weeks 12 and 24, the proportion of patients with complete closure in the placebo group remained unchanged, while it increased in all HP802-247 groups, despite the fact that patients were only treated until week 12. This may indicate that the biological effect of HP802-247 on wound healing continues for a period of at least 12 weeks after treatment has stopped. HP802-247 appeared to be safe and well tolerated without signs of local hypersensitivity, predisposition for bacterial infection or immunogenicity. Given the explorative nature of the study, based on a descriptive statistical analysis of efficacy parameters, the recommended dose of HP802-247 for subsequent efficacy studies is the 1:9 K/F ratio at 5×10^6 cells/ml.

In line with the literature, cryopreservation, essential for the logistics and the shelf life of this cell-based wound healing product, allowed to maintain stable cell viability and cytokine and growth factor release after thawing [24]. Initially, HP802-247 was applied to the ulcer bed using a double-lumen syringe in a 'proof of concept' study that led to massive bacterial colonization due to the occlusive fibrin matrix [unpubl. observations, data on file]. The sequential fibrinogen and thrombin spray application, which in line with the literature [25] had a limited impact on cell viability in vitro, offers an elegant non-touch approach to evenly deliver the cells to the wounds. Together, the in vitro data and the reported explorative phase II study allow for a rational final product design of HP802-247 to be tested in further clinical studies for its efficacy in wound healing.

Investigations to assess the efficacy in wound healing by applying HP802-247 in animal models were difficult and not reproducible. In addition to the complexicity of an in vivo performance assay (lack of a true chronic wound model, immunogenicity of human cells in an animal), the studies were compromised by bacterial contamination of the wound sites due to repeated wound dressing changes to mimic clinical application. Hence the use of animal models for the translation from in vitro to man of a cell-based wound healing product was considered to be of limited value. The chorioallantoic membrane angiogenesis assay was however positive, indicating that a selected mode of actions can be tested in an appropriate in vitro setting.

In conclusion, the translation of in vitro and explorative clinical data of this novel, allogeneic tissue engineering product, which uses the cells in a nonproliferative, differentiated state, illustrates a rational means of dose finding and quality control, which coincides with emerging requirements of regulatory authorities. HP802-247 is safe and well tolerated for use in the treatment of chronic venous leg ulcers and warrants further efficacy studies at the recommended dose.

Acknowledgements

In addition to the authors, the following investigators participated in the study. *Hungary:* J. Feldmann, Flor Ferenc Hospital, Kerepestarcsa; M. Hüttler, Hungarian Railway Hospital, Budapest; A. Bihari, Kispesti Egeszsegugyi Intezet, Budapest; M. Hernadi, St. Gyorgy Hospital, Szekesfehervar; M. Simola, Jozsefvaros Healthcare Service, Budapest. *The Netherlands:* I. Boersma, Albert Schweitzer Hospital, Zwijndrecht; M. Gaastra, Phlebologic Center Oosterwal, Alkmaar; H. van Gerwen, Skin and Laser Clinic Lievensberg, Bergen op Zoom; D. der Kinderen, St. Antonius Hospital, Nieuwegein; G. Lucker, Atrium Medical Center Parkstad, Heerlen; J. Mekkes, Academic Medical Center, Amsterdam; W. van der Veen, Academic Medical Center, Amsterdam; J. Bolling, Tjongerschans Hospital, Heerenveen. *Switzerland:* F. Angehrn, Piano Hospital, Biel; N. Crivelli-Tingelly, Hôpital de la Vallée, Le Sentier. Biostatistics was conducted by Y. Tal, Technostat Inc., Raanana, Israel.

Conflict of Interest

Modex/Isotis SA sponsored, designed and managed the conduct of the studies. R. Goedkoop was an employee of Isotis SA and has received consulting fees of DFB Pharmaceuticals Inc. E. Rolland was an employee of Isotis SA and is an employee of DFB Pharmaceuticals Inc. T. Hunziker was consultant to Isotis SA.

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