Summary of the 2-photon fluorescence microscopy studies performed by Neurotar Ltd, Helsinki

Two-photon Fluorescence Microscopy Studies of the Effect of Dermatopoietin on the Collagen and Elastin Content in Human Volunteer Forearm Skin

Introduction

Neurotar is a spin-off of the University of Helsinki, Finland, and a leading company specialising on contract research based on the new imaging technique called 2-photon fluorescence microscopy. This method allows the 3-dimensional visualization of subcellular structures *in vivo* to a depth of about 250 µm with a resolution of less than 1 µm ('non-invasive optical biopsy').

Neurotar performed a study on forearm skin to explore the effect of Dermatopoietin on collagen deposition in 2011 (Experiment 1), followed by two additional studies in 2012 (NT11-0183 and NT12-0222, Experiments 2 and 3, respectively) to investigate the dose-dependency of the effect of Dermatopoietin.

The present report summarises the long and detailed reports of Neurotar which are on file.

Method

The forearm skin of human volunteers, aged 63 and 64 years, was analysed longitudinally using 2-photon fluorescence microscopy. Autofluorescence (green) and second harmonic generation (SHG, red) image stacks were collected in two separate channels. Stacks are series of images obtained at increasing depths from 0 to about 210 µm. They can be considered as vertical scans of skin. Prior to the application of test products, baseline image stacks were obtained on Day 0.

Excitation: Laser pulses of 70 – 80 fs at 80 MHz and a wavelength of 800 nm.

<u>Experiment 1</u>
Placebo (product without Dermatopoietin) and verum (same product with Dermatopoietin) were applied twice daily to the left and right forearm, respectively, during 4 weeks. After 7, 14, 28 and 60 days image stacks were acquired from both forearms. The total number of time points was five, including imaging at baseline (Day 0). The last measurement on Day 60 was made 32 days after the last product application to study the fading of the effect. All imaging parameters were kept identical between imaging sessions and the same skin spot was targeted each time. Statistical analysis was performed using Student's paired t-test.

<u>Experiment 2 and 3</u>
Test products were applied twice daily to the left and right forearm, respectively, during 2 weeks. On Day 0 and Day 14 image stacks were acquired from both forearms. The total number of time points was two. All imaging parameters were kept identical between imaging sessions and the same skin spot was targeted each time. Statistical analysis was performed using Student's paired t-test.

Test products

<u>Experiment 1</u>
Placebo: Dermatopoietin Hair Protection System Serum Day 5-14, containing no Dermatopoietin and no Hexadeltine

Test product 1: Dermatopoietin Hair Protection Serum Day 1 – 4, containing 150 ug/L Dermatopoietin (interleukin-1 alpha) and 200 mg/L Hexadeltine (Tyr-D-Ala-Gly-Phe-Leu-Asp)

Experiment 2
Placebo applied to the left forearm: Dermatopoietin Hair Protection System Serum Day 5-14, containing no Dermatopoietin and no Hexadeltine

Test product 2 applied to the right forearm: Dermatopoietin Hair Protection Serum Day 1 – 4, diluted 1/5 with placebo, containing 30 ug/L Dermatopoietin (interleukin-1 alpha) and 40 mg/L Hexadeltine (Tyr-D-Ala-Gly-Phe-Leu-Asp)

Test product 3 applied to the right forearm: Dermatopoietin Hair Protection Serum Day 1 – 4, diluted 1/25 with placebo, containing 6 ug/L Dermatopoietin (interleukin-1 alpha) and 8 mg/L Hexadeltine (Tyr-D-Ala-Gly-Phe-Leu-Asp)

Experiment 3
Test product 4 applied to the right forearm: Dermatopoietin Hair Protection Serum Day 1 – 4, diluted 1/10 with placebo, containing 15 ug/L Dermatopoietin (interleukin-1 alpha) and 20 mg/L Hexadeltine (Tyr-D-Ala-Gly-Phe-Leu-Asp)

Test product 5 applied to the left forearm: Dermatopoietin Hair Protection Serum Day 1 – 4, diluted 1/15 with placebo, containing 10 ug/L Dermatopoietin (interleukin-1 alpha) and 13.3 mg/L Hexadeltine (Tyr-D-Ala-Gly-Phe-Leu-Asp)

Results

Test product with 150 ug/L Dermatopoietin and 200 mg/L Hexadeltine led to a steady increase of collagen and elastin content in the dermis compared to placebo and baseline by 80 – 90% during the application period. Maximum collagen and elastin content is reached after about 2 – 3 weeks.

Post-treatment: After withdrawal of Dermatopoietin the collagen and elastin content normalized slowly reaching about +20% above baseline after 4 to 5 weeks.

Test product with 30 ug/L Dermatopoietin and 40 mg/L Hexadeltine show identical efficacy as the reference product with 150 ug/L Dermatopoietin and 200 mg/L Hexadeltine.

Lower concentrations of active ingredients show no significant efficacy compared to baseline.

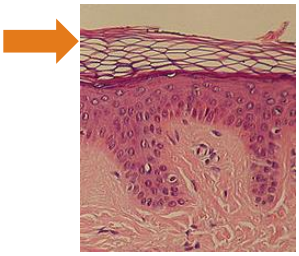
Conclusion

Dermatopoietin at concentrations of 30 – 150 ug/L (in combination with Hexadeltine at 40 – 200 mg/L) showed a unique replenishment of collagen and elastin in the dermis by 80 – 90% within about 2 - 3 weeks. This effect most probably underlies the strengthening and rejuvenation of aging skin observed by ultrasonography and cutometry, the effect against cellulite skin and dark circles around the eyes and the reported anti-wrinkle effect.

Figures

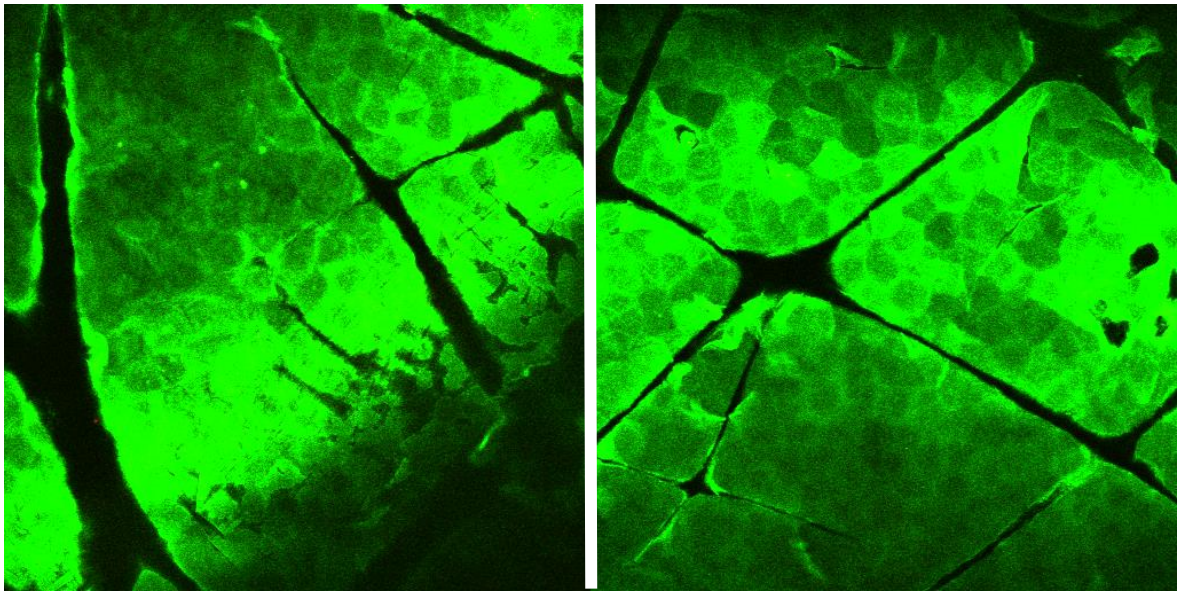
Experiment 1

Scan of 2-photon microscopic pictures at different skin depths. Comparison of Dermatopoietin *versus* Placebo after 4-week treatment. Pictures were obtained from the same skin spot. Clearly visible is the increase of collagen (red) and elastin (green) in the dermis. Dermatopoietin concentration: 150 µg/L

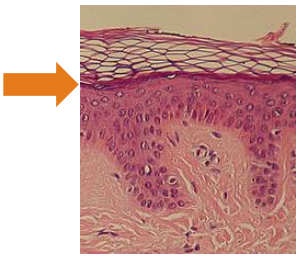


Stratum corneum, 5 µm: Keratinocytes

Placebo Dermatopoietin



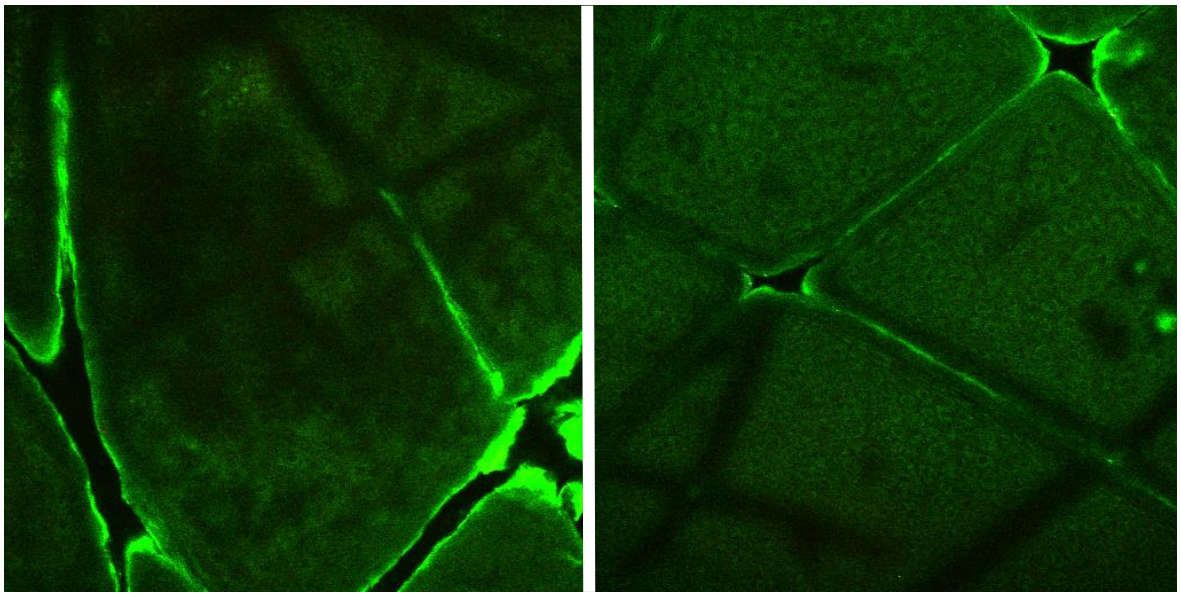
Legend
The strong green fluorescence of the epidermis is due to keratin and favoproteins in the keratinocytes.

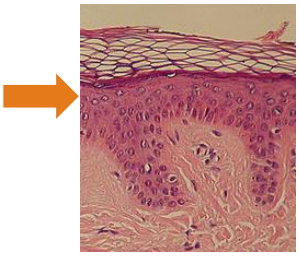


Stratum granulosum, 25 µm: Keratinocytes

Placebo

Dermatopoietin

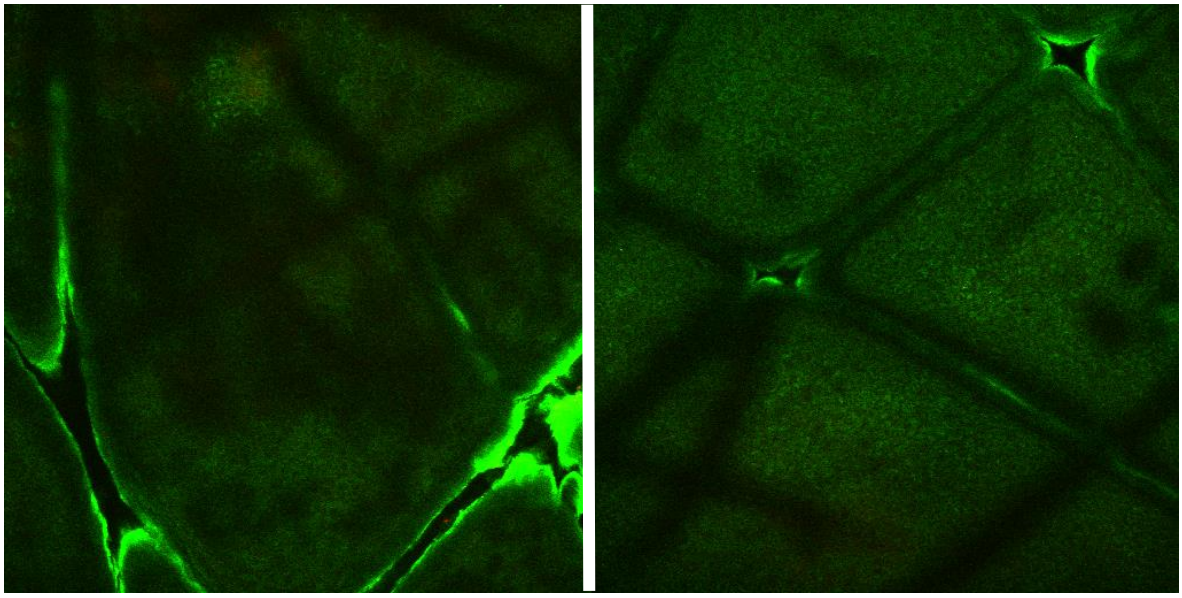




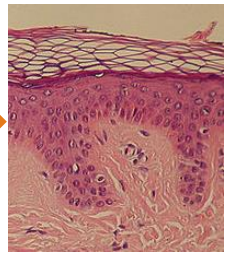
Stratum spinosum, 30 µm: Keratinocytes

Placebo

Dermatopoietin

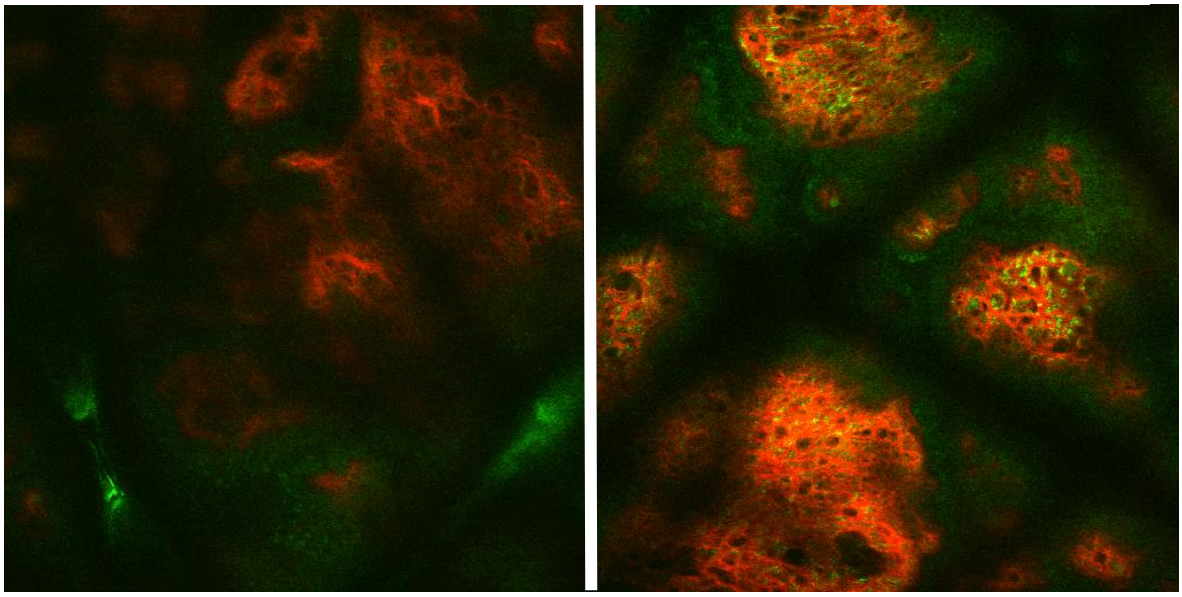


Stratum basale, 50 µm: Keratinocytes, collagen, elastin



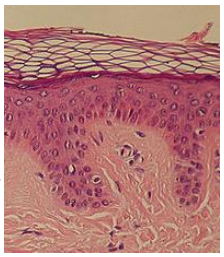
Placebo

Dermatopoietin



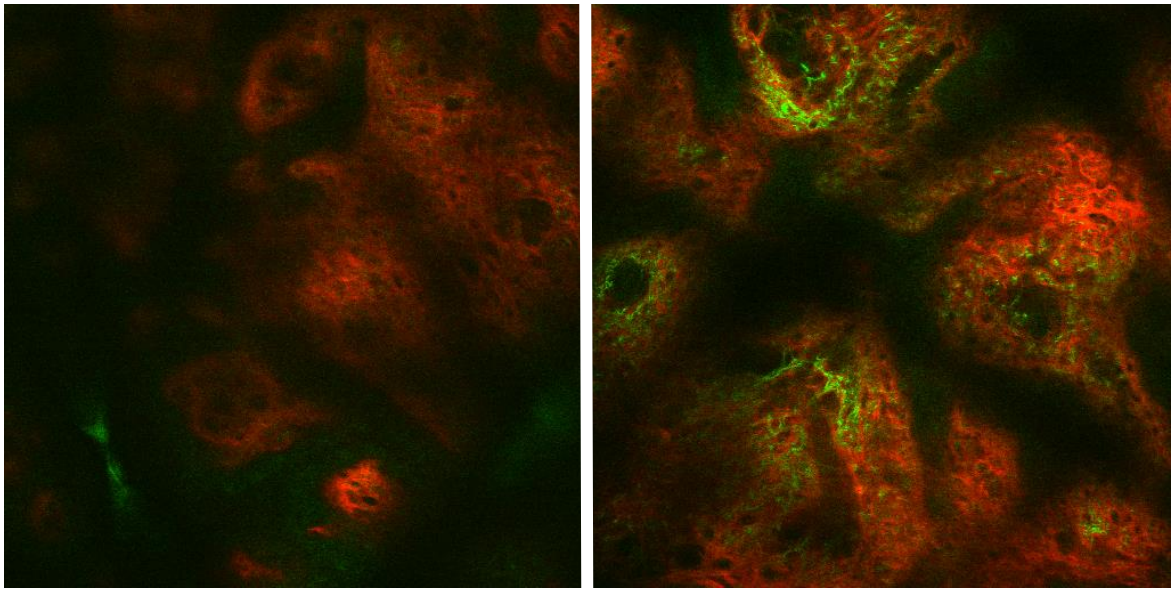
Legend
Visible are reflections of keratinocytes (green), collagen (red) and elastin (light green).

Dermis, 70 µm: collagen, elastin

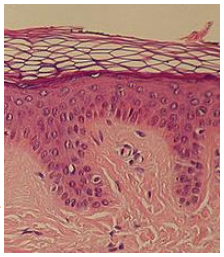


Placebo

Dermatopoietin

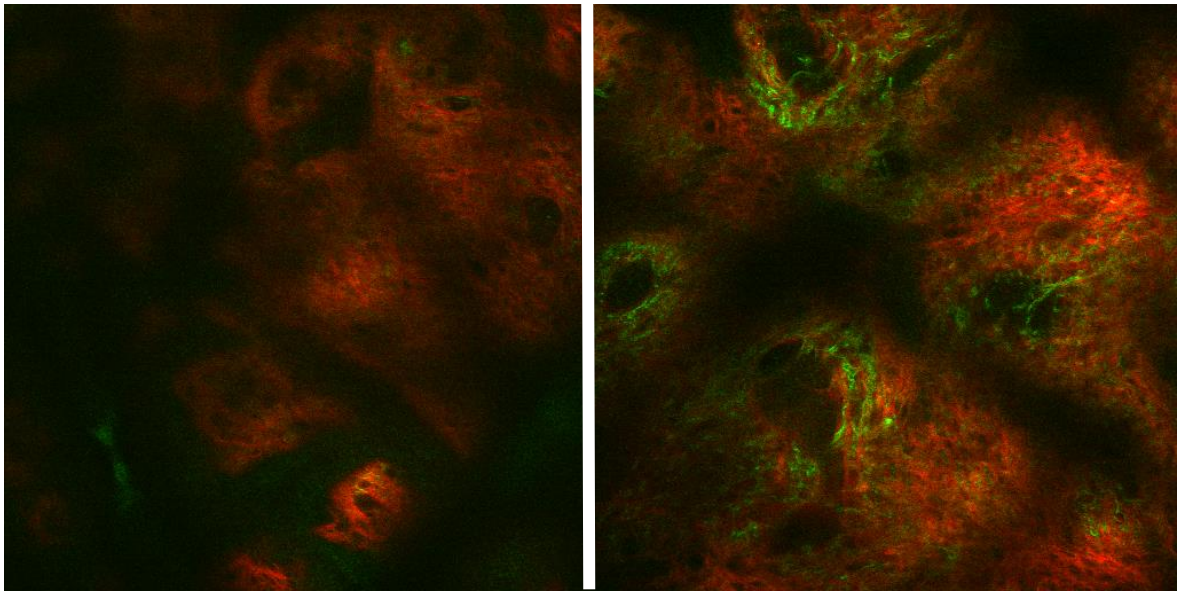


Dermis, 80 µm: collagen, elastin



Placebo

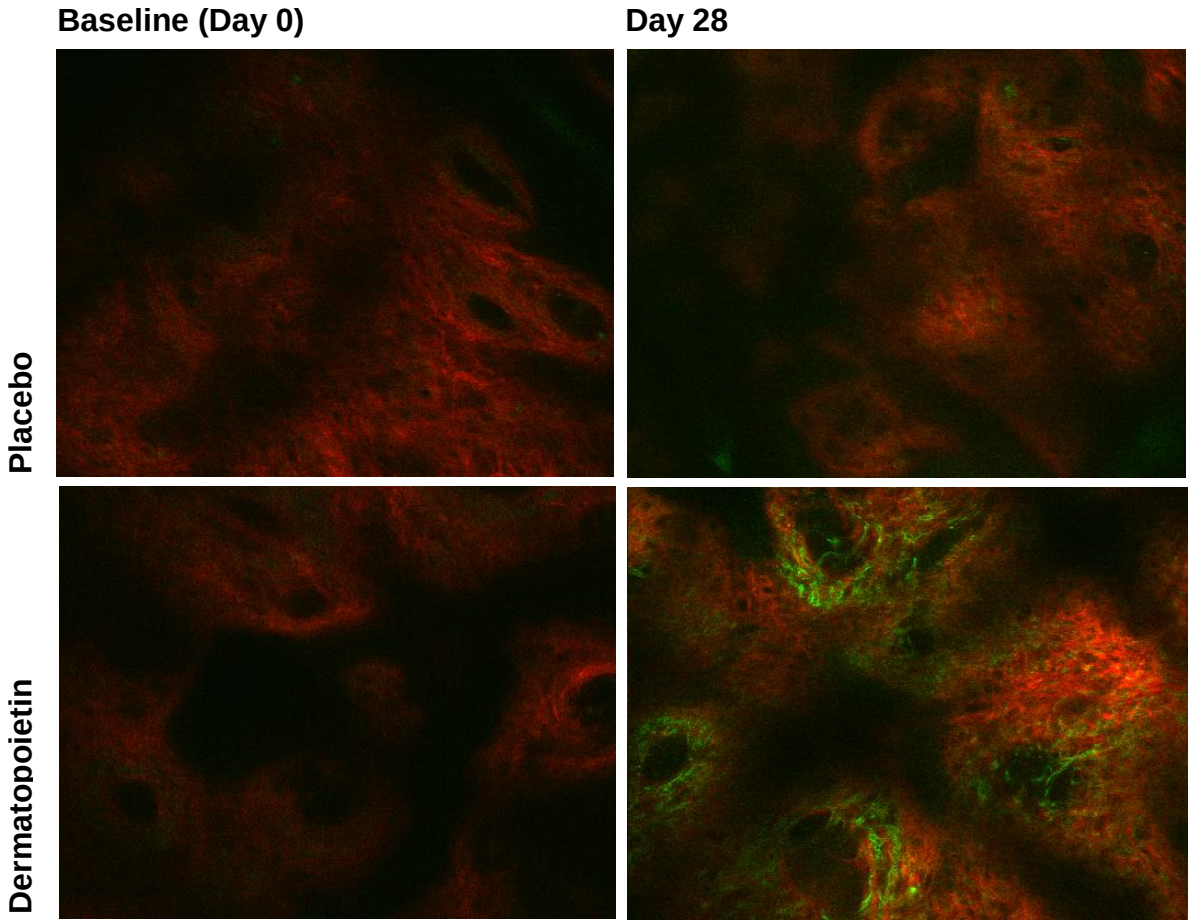
Dermatopoietin



Legend
After 4-week treatment with Dermatopoietin the content of collagen and elastin is greatly enhanced in the dermis compared to placebo.

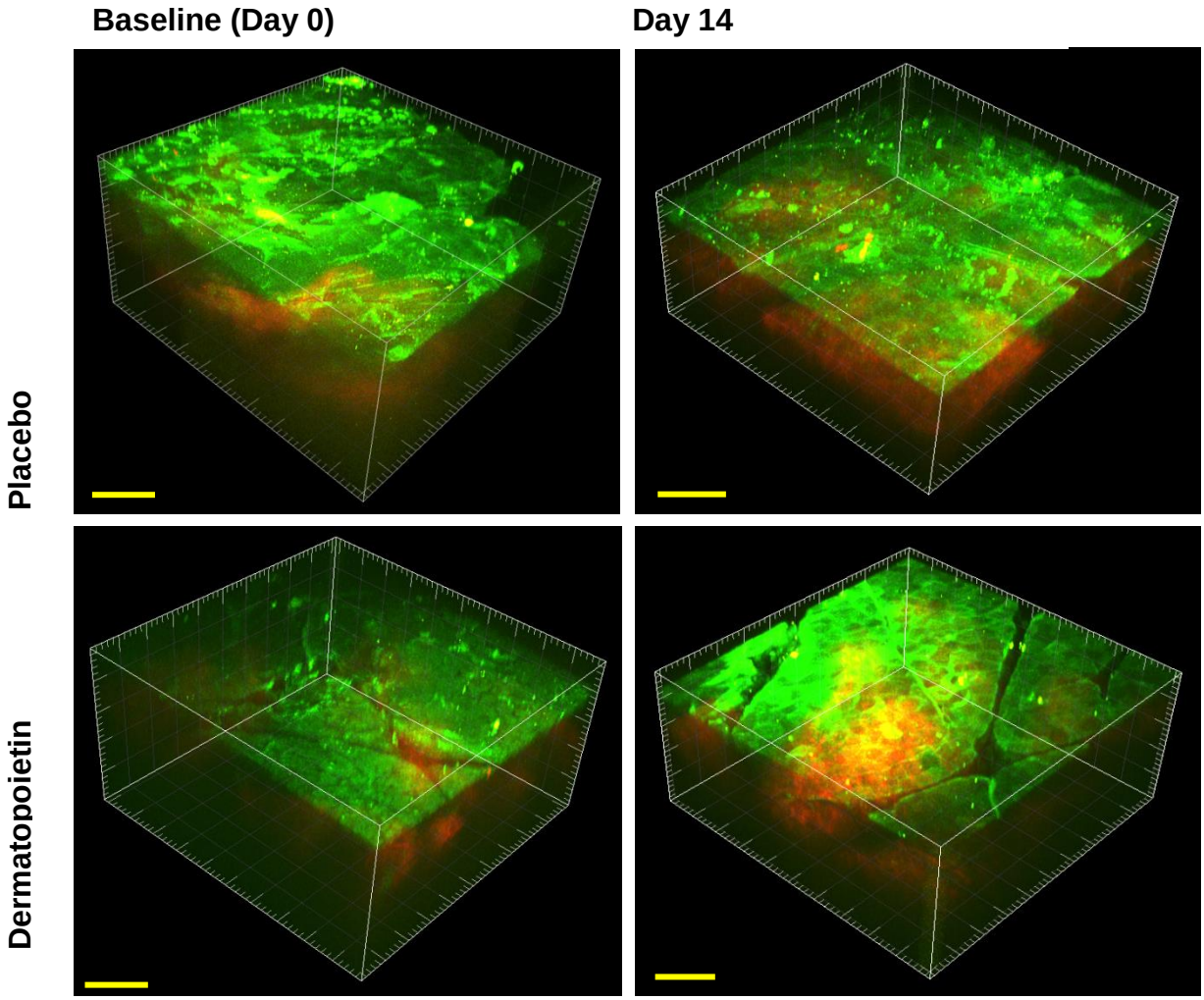
Experiment 1

Two-photon microscopic pictures from the dermis at 80 µm skin depth. Comparison of Dermatopoietin *versus* Placebo and Baseline *versus* 4-week treatment. Pictures were obtained from the same skin spot. Clearly visible is the increase of collagen (red) and elastin (green). Dermatopoietin concentration: 150 µg/L

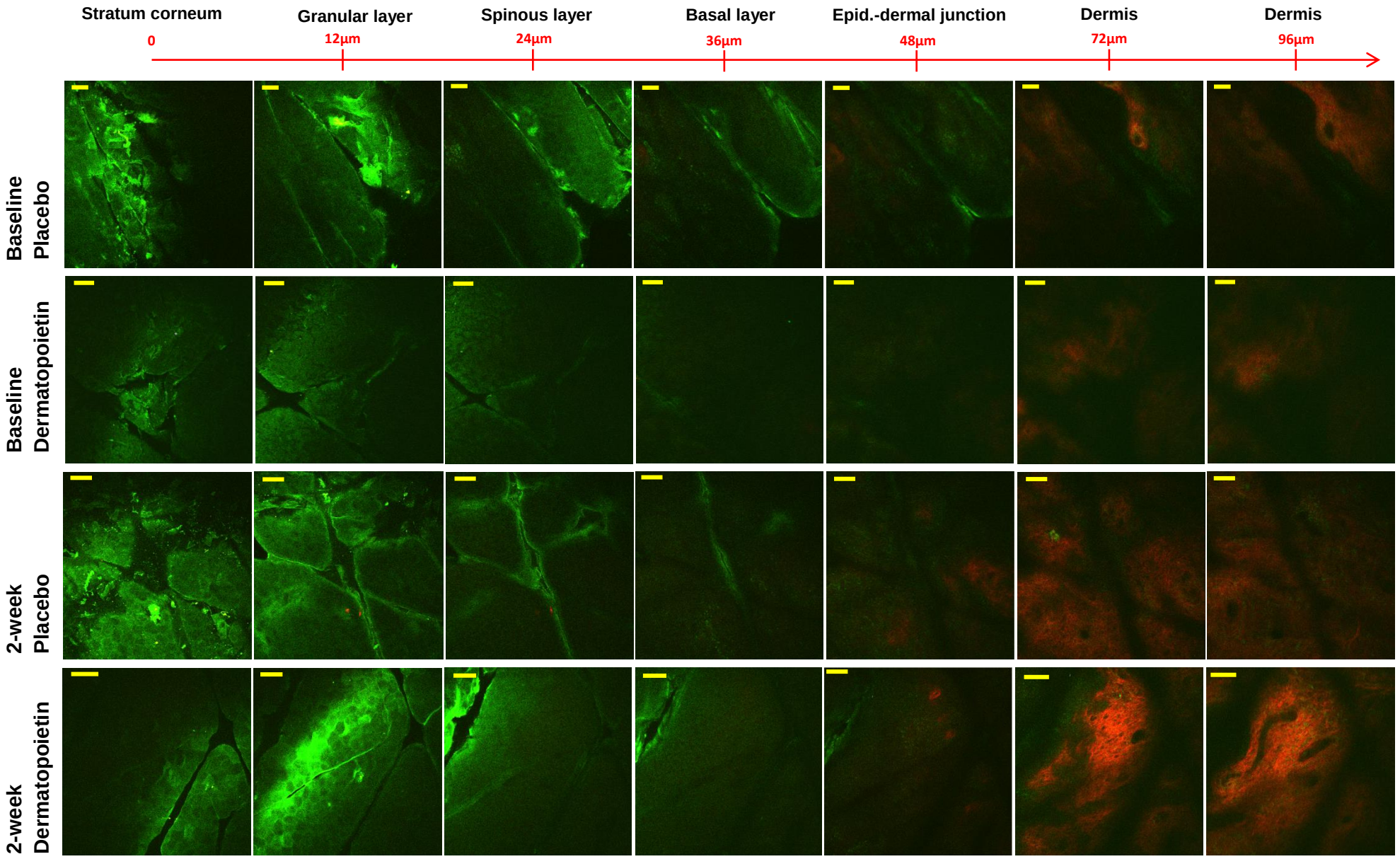


Experiment 2

Three-dimensional 2-photon microscopic composite pictures showing the green autofluorescence in the epidermis and dermis as well as collagen (red, second harmonic generation) in the dermis. Clearly visible is the increase of collagen after two weeks in the skin of the forearm treated with Dermatopoietin at a concentration of 30 µg/L.

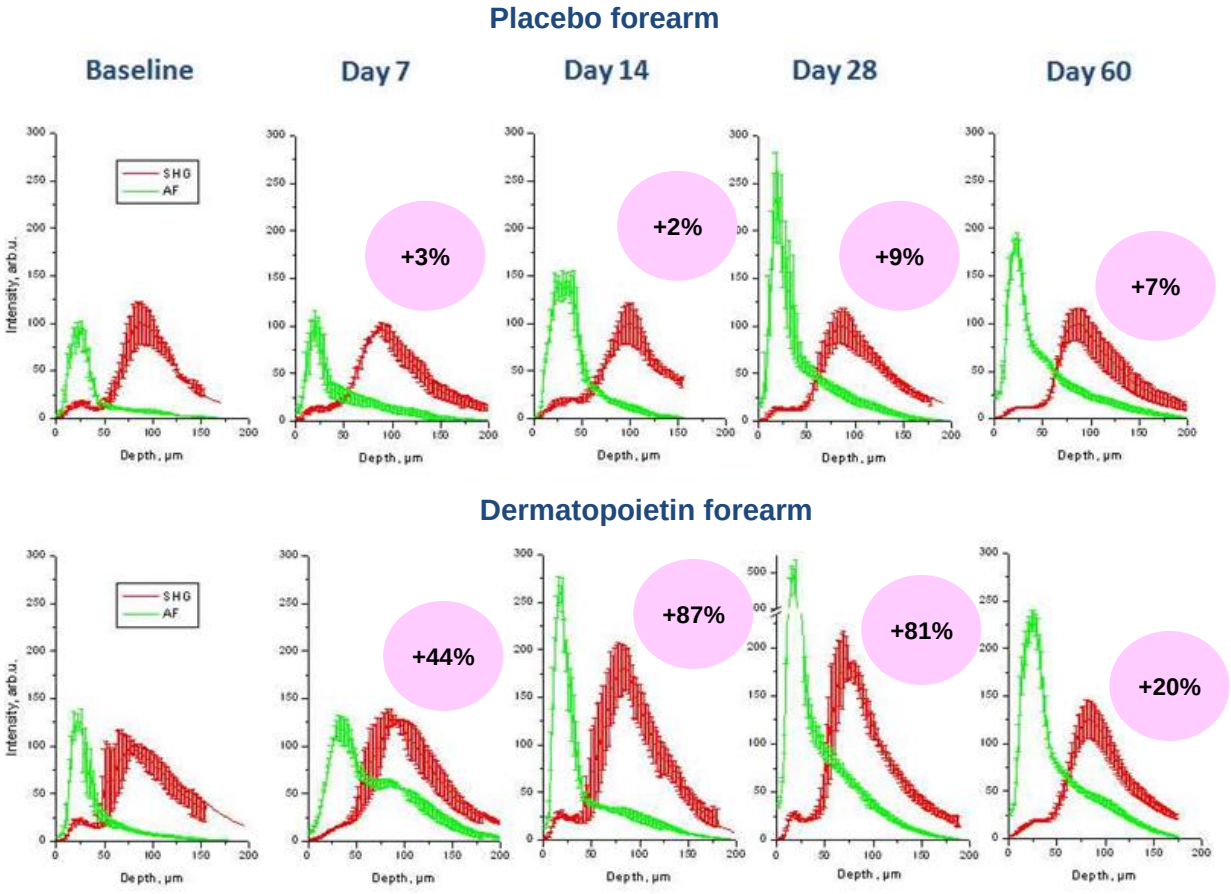


Experiment 2: Two-photon microscopic pictures at different skin depths at Baseline (Day 0) and after 2-week treatment with Placebo or 30 µg/L Dermatopoietin. Only Dermatopoietin led to a strong replenishment of collagen and elastin in the dermis compared to Placebo and Baseline.



Time course of the Dermatopoietin effect

Experiment 1



Legend
Time dependence of the effect of Dermatopoietin. The graphs show the intensity of the fluorescence and SHG signal as a function of skin depth at Baseline (Day 0) and after 7, 14, 28 and 60 days. Epidermis: 0 – 50 µm, Dermis: 50 – 200 µm

The green autofluorescence reflects keratin and flavoproteins in the epidermis and elastin in the dermis. The red signal is the second harmonic generation (SHG) and stems from collagen in the dermis. The origin of the red signal in the epidermis is not known.

Treatment:

- Placebo (left forearm) twice daily for 28 days
- Dermatopoietin (150 µg/L) twice daily for 28 days

The reason for the increase of the signal in the epidermis upon treatment (placebo and verum) is not clear. Only treatment with Dermatopoietin however led to a strong increase (almost doubling of the area-under-the-curve) of elastin and collagen in the dermis after 2 - 4 weeks. On Day 60, 32 days after discontinuation of treatment, the collagen content is back to about 20% above baseline.

Dose dependence of the Dermatopoietin effect

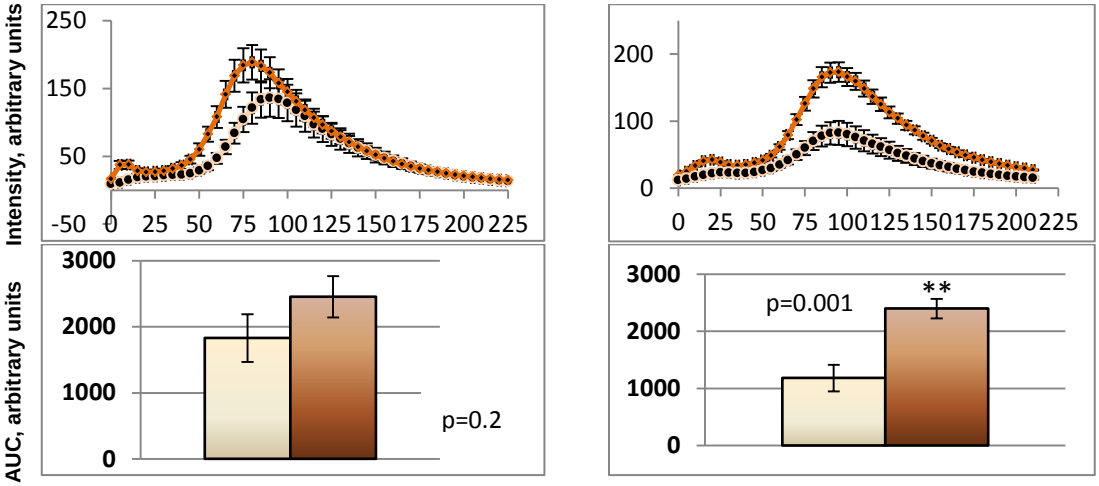
Experiment 1
Twice daily application of a product containing 150 µg/L Dermatopoietin and 200 mg/L Hexadeltine led to an increase of collagen of more than 80% within 2 to 4 weeks (see Figure on previous page).

Experiment 2
Twice daily application of a product containing 30 µg/L Dermatopoietin and 40 mg/L Hexadeltine (1/5 dilution of regular gel) led to a doubling of collagen and elastin content in forearm skin within 2 weeks as shown in the Figures below.

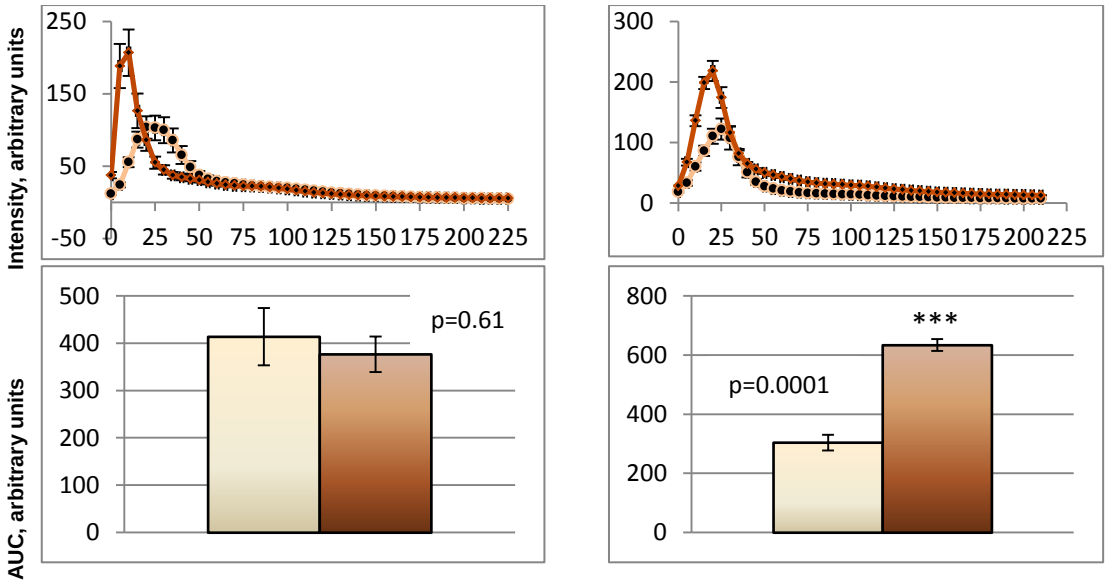
Experiments 2 and 3

Gels with concentrations of 6, 10 and 15 µg/L Dermatopoietin and 8, 13 and 20 mg/L Hexadeltine, respectively, showed no statistically significant effects on the collagen and elastin content of forearm skin (data not shown).

The amount of collagen fibres



The amount of elastin fibres



Legend Intensity profiles and areas-under-the-curve (AUC) in the dermis (50 – 200 µm) for collagen and elastin, respectively, at baseline and after 2-week application of Placebo or 30 µg/L Dermatopoietin / 40 mg/L Hexadeltine in an aqueous gel formulation.
Left: Placebo at baseline (light colour) and after 2 weeks (dark colour)
Right: Dermatopoietin at baseline (light colour) and after 2 weeks (dark colour)