

Effect of the multifunctional cosmetic ingredient sphinganine on hair loss in males and females with diffuse hair reduction

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Abstract: Sphingolipids are well known to promote keratinocyte differentiation and to induce ceramide production. In addition, they show anti-inflammatory and antimicrobial activities. Thus, the aim of this study is to investigate the potential effect of sphinganine on prolonging the hair anagen rate and improving the overall hair quality and scalp health. The inhibitory potential of sphinganine toward 5- α -reductase was studied using an in vitro assay. The stimulation of the antimicrobial peptide HBD2 by sphinganine was measured by real-time polymerase chain reaction and immunostaining. Sphinganine bioavailability was studied ex vivo using a pig skin model. A placebo-controlled, double-blind study was designed to evaluate the efficacy of sphinganine on hair loss and hair/scalp quality in vivo. In vitro results showed that sphinganine is a potent inhibitor of 5- α -reductase type I that prevents the conversion of testosterone to dihydrotestosterone, a key factor of androgenetic male baldness. In vivo results demonstrated efficacy in reducing non-illness-related hair loss among males. In terms of expert rating, all hair quality and scalp parameters improved after application of sphinganine. Improved scalp health might be linked to the observed increase of the antimicrobial peptide HBD2. Thus, sphinganine is well suited as a topical alternative for the improvement of scalp health and hair quality and anti-hair loss application.

Keywords: sphinganine, 5- α -reductase inhibition, HBD2, hair loss, anagen rate, TrichoScan[®]

Introduction

Sphinganine is a naturally occurring, skin-identical sphingoid base, a representative of the class of amino alcohols, or long chain bases (LCBs). Sphingoid bases can be synthesized chemically in a complex and cost-intensive way. An alternative process is based on the fermentation of yeast¹ and ensures the distinct stereochemical configuration of the two asymmetrical carbon atoms of the molecule, which is also found in nature and in human skin.

LCBs are stereochemically complex molecules, also called sphingolipids, which are found in the membranes of nearly all eukaryotic cells. A sphingoid base amidated with a fatty acid makes up a ceramide. The structures and functions of sphingolipids and their ceramide derivatives are extremely diverse, ranging from being essential components of membranes to ensure cellular integrity, to acting as second messengers in signaling.² It could be demonstrated in a confluence-induced differentiation model of normal human keratinocytes that several LCBs are potent promoters of keratinocyte differentiation as judged by transcriptome analyses,³ and are able to induce ceramide production.⁴ They have also shown evidence of anti-inflammatory⁵ as well as antimicrobial activities.⁶⁻⁸

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Based on the assumption that such keratinocyte stimulation also takes place in the hair matrix keratinocytes, which causes the formation of the different epithelial layers forming the mature hair, it was reasoned that sphinganine application could potentially affect hair fiber production. In particular, the stimulation of differentiation and ceramide production might influence cuticle formation. The cuticle, like the stratum corneum, has a protective function and determines the surface properties of the hair.⁹ Considering also its anti-inflammatory and antimicrobial properties, sphinganine stimulation could eventually lead to improvements in hair quality.

These aspects were the subject of investigation of the present study.

Methods

Materials

dl-Erythro-sphinganine produced by fermentation of the yeast *Wickerhamomyces ciferrii* was provided by Evonik Nutrition & Care GmbH (Essen, Germany).

5- α -reductase type I cell-free inhibition assay

5- α -reductase assays were conducted by Vivacell Biotechnology GmbH (Denzlingen, Germany). Human embryonic kidney cells recombinantly expressing 5- α -reductase isoenzyme type I¹⁰ were cultivated at 37°C in Dulbecco's Modified Eagle's Medium (pH 7.4) supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/mL and 100 μ g/mL), and 0.5 mg/mL of Geneticin-418-sulfate in a humidified 5% CO₂ atmosphere. The assay was conducted basically as described,¹¹ with minor modifications.

Incubations were performed at 37°C in a Tris-HCl ethylenediamine tetraacetic acid buffer incubation mixture (assay buffer) with a final volume of 250 μ L containing 0.24 mM NADPH, 250 nM androstenedione (AD), 100 μ g/mL cell homogenate, and sphinganine dilutions from a 1 mg/mL stock solution in 1,2-pentanediol. Final concentrations of 33.0, 8.3, and 2.1 μ M sphinganine were used for the determination of IC₅₀ values. The reference compound finasteride served as internal positive control.^{10,11} Finasteride was dissolved in ethanol and further diluted in Tris-HCl ethylenediamine tetraacetic acid buffer to final concentrations of 750 and 1,250 nM. Solvent-treated controls were treated the same way and contained 1% 1,2-pentanediol or 1% ethanol. Enzyme reactions and product extraction were performed as described.¹¹

The compounds were separated by high-performance liquid chromatography on a Gemini C6 Phenyl, 3 μ m, 50 \times 2.0 mm

(Phenomenex, Torrance, CA, USA) analytical column using acetonitrile/0.1% (v/v) formic acid and water/10 mM ammonium formate/0.5% formic acid. Mass spectrometry was performed on a TSQ Quantum Discovery Max triple quadrupole mass spectrometer with APCI interface (Thermo Fisher Scientific, Waltham, MA, USA) in positive mode.

Inhibition rates were calculated out of the mean AD-to-dihydroandrostenedione conversion rates determined via peak area ratios from experiments with and without inhibitor. IC₅₀ values were calculated by linear interpolation of the concentrations of test compounds and the corresponding percentage of inhibition. The experiment was done in duplicate and was reproduced in an independent experiment under identical conditions, summing up to n=4.

In vitro *HBD2* gene expression

Cell culture experiments

Primary human epidermal keratinocytes (HNKs) were prepared from neonatal foreskin and maintained in culture under serum-free conditions using the defined keratinocyte growth medium Keratinocyte SFM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with bovine pituitary extract (Thermo Fisher Scientific) and recombinant epidermal growth factor (Thermo Fisher Scientific). Cells were propagated up to passage 2 or 3 at 37°C and 5% CO₂. For induction of differentiation, HNKs were seeded in six-well plates and grown up to confluence. Sphinganine was dissolved in dimethyl sulfoxide (0.1% final concentration). Cells were incubated for 72 hours with sphinganine prior to RNA extraction. Written informed consent was obtained from the patient for the use of the HNKs.

RNA isolation and real-time PCR from cell culture material

Total RNA was extracted from frozen cell layers, and gene expression was measured by real-time polymerase chain reaction (RT-PCR) as described.¹² Total RNA was isolated using RNeasy Total RNA Kits (Qiagen N.V., Hilden, Germany). The RNA concentration and purity were determined photometrically using 260/280 ratios (Biophotometer; Eppendorf, Hamburg, Germany). Aliquots of total RNA (100 ng) were applied for cDNA synthesis using the Superscript[®] III First-Strand synthesis system for RT-PCR using random hexamers (Thermo Fisher Scientific). For both genes, *HBD2* (target gene) and *18S rRNA* (housekeeping gene), a specific primer pair was designed by Primer Express[®] 2.0 software (Thermo Fisher Scientific) based on the published cDNA sequence. Primer sequences were as follows: *HBD2*: 5'-TTCCTGATGCCTCTTCCAGTTT-3' (forward primer) and

5'-GGATGACATATGGCTCCACTCTT-3' (reverse primer); *18S rRNA*: 5'-GCCGCTAGAGGTGAAATCTTG-3' (forward primer) and 5'-CATTCTTGGCAAATGCTTTCG-3' (reverse primer). All results are presented as the average obtained from three independent experiments that were performed in duplicate. The PCRs were carried out on Option 1 (MJ Research, Waltham, MA, USA) using SYBR Green[®] PCR Master Mix (Thermo Fisher Scientific). Each sample was analyzed in duplex PCR employing the universal protocol over 46 cycles – in detail, 94°C and 10 minutes of activation of hot start taq polymerase, 95°C and 20 seconds of denaturation, 55°C and 20 seconds of annealing, and 72°C and 30 seconds of extension. For comparison of relative expression in control cells and treated cells, the $2^{-\Delta\Delta C(t)}$ method was used. Statistical analysis and graphical design were performed using SigmaPlot10.0 (Systat, Inc., San Jose, CA, USA).

Ex vivo HBD2 protein expression

HBD2 protein induction by sphinganine treatment was assessed on human living skin explants. The study was conducted by Laboratoire BIO-EC in France. Explants originated from a 43-year-old Caucasian woman. Written informed consent was obtained from the patient for the use of the human living skin explants. In total, 39 explants of an average diameter of 11 mm (± 1 mm) were prepared. They were kept alive in BIO-EC's Explant Medium at 37°C in a humid, 5% CO₂ atmosphere. Experiments were conducted in triplicates. The test formulation contained 0.1% sphinganine dissolved in 89.9% H₂O and 10% 1,2-pentanediol. A formulation without sphinganine was used as vehicle control. On day (D) 0, D2, D5, and D6, 2 mg/cm² of the formulations was applied topically and spread evenly using a small spatula. Control explants did not receive any treatment except medium renewal. Explants were sampled on D0 and on D7. Explants were cut into two parts. One part was fixed in buffered formalin, and the other one was frozen at -80°C.

Histological processing

After fixation for 24 hours in buffered formalin, the samples were dehydrated and impregnated in paraffin using a Leica TP 1010 dehydration automat (Leica Microsystems, Wetzlar, Germany). The samples were embedded using a Leica EG 1160 embedding station (Leica Microsystems). Five-micrometer-thick sections were prepared using a Leica RM 2125 Minot-type microtome (Leica Microsystems), and sections were mounted on Superfrost[®] (Thermo Fisher Scientific) histological glass slides for immunostainings. Frozen samples were cut into sections of 7 μ m thickness using a Leica CM 3050 cryostat (Leica Microsystems). Sections

were then mounted on Superfrost[®] plus silanized (Thermo Fisher Scientific) glass slides.

Immunostaining

HBD2 was stained on frozen sections with a polyclonal anti-HBD2 antibody (Ref sc-20798; Santa Cruz Biotechnology Inc., Dallas, TX, USA), diluted at 1:100 in phosphate-buffered saline (PBS)-containing 0.3% bovine serum albumin and 0.3% Tween 20, for 1 hour at room temperature with an amplifier system, Vectastain RTU Universal VECTOR avidin/biotin (Vector Laboratories Ltd., Burlingame, CA, USA) and revealed with fluorescein isothiocyanate (Ref SA1001; Invitrogen). Nuclei were post-stained with propidium iodide. Fluorescent staining was visualized by using Leica DMLB or Olympus BX43 (Leica Microsystems and Olympus, Tokyo, Japan) fluorescent microscope. Pictures were digitized with a numeric DP72 Olympus camera with CeIID storing software (Leica Microsystems and Olympus, Tokyo, Japan). Degrees of expression of HBD2 protein were assessed by expert grading on a 13-grade scale, ranging from very weak to strong.

Ex vivo bioavailability study

The study was performed with Franz diffusion cells (4G-01-00-15-12-VDOC; PermeGear, Bethlehem, PA, USA) using a pig skin model of 1 mm thickness based on standardized procedures.¹³ Test formulations (15–20 mg/cm²) were applied and left in contact with the skin nonocclusively for 24 hours at 32°C and 50% relative humidity in a climate chamber under cautious stirring of the receptor fluid (2.5% bovine serum albumin in PBS buffer). Each formulation was tested in eight individual skin penetration experiments (n=8), where the skin of two different pigs was used, each in four experiments. Skin integrity was verified by 1% caffeine penetration. An untreated sample served as a blank control (baseline determination).

After the incubation time, residual superficial test product was removed from the skin surface with a cotton swab and extracted with methanol/ethanol (1:1; rinse-off fraction). The outer part of the skin disk, which was not in contact with the receptor medium, was removed with the aid of a stamp (edge fraction). The stratum corneum was removed by cyanoacrylate biopsy.¹⁴ Separation of epidermis and dermis was achieved by ammonia vapor treatment for ~1 hour at room temperature.¹⁵ Individual skin samples were cut into small pieces for extraction with methanol/ethanol (1:1; epidermis and dermis fraction), and quantitative analysis of the active ingredient in the extracts and receptor fluid (receptor fraction) was performed using high-performance liquid chromatography and quantification was done by mass spectrometry. Data were analyzed by common statistical

methods to obtain average values. Active penetration was corrected by the amount which has undergone lateral diffusion into the edge fraction. The percentage of total applied material is reported.

Clinical study

Two identical studies using randomized, placebo-controlled, and double-blind designs with a 16-week treatment period were conducted. The first pilot study was carried out on 32 healthy test subjects of both sexes (male/female and verum/placebo, ratio 1:1) testing a concentration of 0.2% sphinganine. In addition, a follow-up study was done on a total of 80 male test subjects divided into three treatment groups (sphinganine concentrations of 0.1%, 0.2%, and 0.5%) and one control group.

The subjects were selected according to the inclusion criteria: healthy male or female with non-illness-related, light, diffuse hair loss, and fine and lifeless hair. Exclusion criteria were the evidence of skin diseases of the scalp, history of allergic reactions to shampoo, perfumes, or topically applied hair care products, medication affecting the skin response such as antibiotics and anti-inflammatory drugs, pregnancy or breast-feeding, short hair (<5 cm), and the use of substances promoting hair growth during a period of 3 months before the start of the study. All participating test subjects were informed about the objectives and scope of the study and gave written informed consent prior to the study. They accepted not to alter their haircut, style or dye their hair, and not to use any other topical treatment products, oral products, or any nutritional supplementation aiming at improving hair status during the course of the study.

Furthermore, at the end of the follow-up study, three selected test subjects of each group were asked to participate in the study for a further 9 months. Test subjects selected of the verum groups responded very well to the treatment, whereas test subjects selected of the placebo group showed a deterioration of the hair status over the course of 4 months.

Subjects were instructed to evenly apply 3 mL hair tonic (3.0% TEGINACID® C; 0%, 0.1%, 0.2%, or 0.5% sphinganine; 50% ethanol; 0.1% perfume; ad. 100% water, neutralized with 10% aqueous lactic acid to pH 5.5) to the dry hair using a plastic pipette and to gently massage it into the hair and scalp as well as the hairline twice a day, in the morning and evening. After the completion of the study, data were analyzed before un-blinding.

The study design for noninvasive research was approved by the Ethics Committee of the Witten/Herdecke University, Germany.

The effectiveness of the treatment was quantitatively evaluated after 8 and 16 weeks by TrichoScan® analysis.¹⁶ On the first day, the test field was identified, and the hair was clipped evenly to a length of 1 mm in a measuring field of 18 mm by means of a mini hair trimmer. Images were taken at a 20-fold magnification in order to evaluate the evenness of the clipped measurement area. After 3 days, the test subjects returned to the study site in order to monitor hair growth after hair dying for ~10–15 minutes. For the duration of the study, the test field was always the same for every test subject, and three images of the same area were recorded. Images were analyzed using TrichoScan® Research Edition 3.0 (Tricholog GmbH, Freiburg, Germany) or by manual evaluation for the parameters, telogen and anagen rate (%).¹⁷

In addition, evaluation by an expert on the basis of a questionnaire was performed at baseline and at the end of the study. A five-point scale was used for evaluation of the hair quality parameters shine, volume, and strength, and for the scalp health parameters, dryness, scaling, and general scalp health. Results were expressed as the change between pre- and posttreatment scores.

To objectively measure the responsiveness to hair tonic treatment, a photographic documentation was used. Color photographs of the head were taken of both sides, the front, back, and top using Fotofinder dermascope (Fotofinder Systems GmbH, Bad Birnbach, Germany) under identical environmental conditions, as well as identical conditions of hair color and hairstyling.

For TrichoScan® and the expert assessment, the groups were analyzed statistically. Within the groups, each measuring time was compared to week 0 using the Wilcoxon signed-rank test. In addition, pre–post differences were calculated, and then treatment and placebo were compared by using the Wilcoxon rank sum test. Results were considered statistically significant at the 5% level ($P < 0.05$). A combined analysis was done including the data on males of study 1 and the data of study 2.

Results

Sphinganine inhibits 5- α -reductase activity

The enzyme 5- α -reductase directly causes hair loss in both males and females.¹⁸ Thus, to determine whether sphinganine has an inhibitory potential toward 5- α -reductase type I, IC_{50} values were determined in a cell-free assay using cell homogenates isolated from stably transfected human embryonic kidney cells. The IC_{50} for sphinganine, determined in two independent experiments, 6.5 ± 0.33 and 6.8 ± 0.1 μ M, while

the control finasteride showed an IC_{50} of $0.42 \pm 0.02 \mu\text{M}$. The results indicate that sphinganine is a suitable ingredient to inhibit 5- α -reductase and thus prevent the conversion of testosterone to dihydrotestosterone (DHT).

Sphinganine induces the innate immunity marker HBD2 in vitro and ex vivo

In light of the pro-differentiating activities described for sphingoid bases,³ it seems likely that components of skin's innate immunity system might be induced by these molecules as well. To address this possibility, we first analyzed the influence of sphinganine treatment on *HBD2* gene expression in an in vitro keratinocyte culture model. Primary HNKs

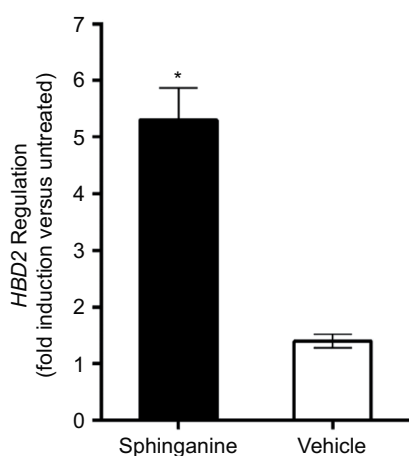


Figure 1 *HBD2* gene expression in normal human epidermal keratinocytes after 72-hour treatment with 0.00002% sphinganine measured by qRT-PCR.

Notes: Results are shown as fold induction compared to vehicle (0.1% DMSO) treatment. * $P < 0.05$ versus vehicle.

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; DMSO, dimethyl sulfoxide.

were treated with 0.00002% sphinganine before cells were harvested, and regulation of *HBD2* gene expression was determined by RT-PCR analysis. *HBD2* gene expression was readily induced by a factor of 5.3 (mean ± 0.57 standard error) (Figure 1).

Next, we analyzed HBD2 protein induction by sphinganine treatment on human living skin explants, representing a skin model far closer to the in vivo situation compared to a monolayer cell culture. A solution containing 0.1% sphinganine was repeatedly applied on the explants for a period of 7 days. Subsequently, explants were collected and incubated with an anti-HBD2 antibody followed by microscopical immunofluorescence analysis. Representative photos are shown in Figure 2. HBD2 protein was markedly induced by sphinganine compared to vehicle treatment. Semiquantitative assessment of HBD2-staining intensities by expert grading revealed a quite clear expression (grade 7) of sphinganine-treated skin compared to weak expression in the vehicle control (grade 3) on a 13-grade scale. These results further corroborate the findings from the in vitro cell culture experiment.

Sphinganine is readily bioavailable from a hair tonic application

Skin absorption was determined from an ethanolic hair tonic, which was also used in the clinical studies described. After topical application and incubation for 24 hours, >40% of the applied sphinganine was found in the skin. The major part was recovered from the stratum corneum ($23.9\% \pm 5.3\%$) and viable epidermis ($17.1\% \pm 0.99\%$), and to some extent also from the dermal tissue ($3.1\% \pm 0.53\%$).

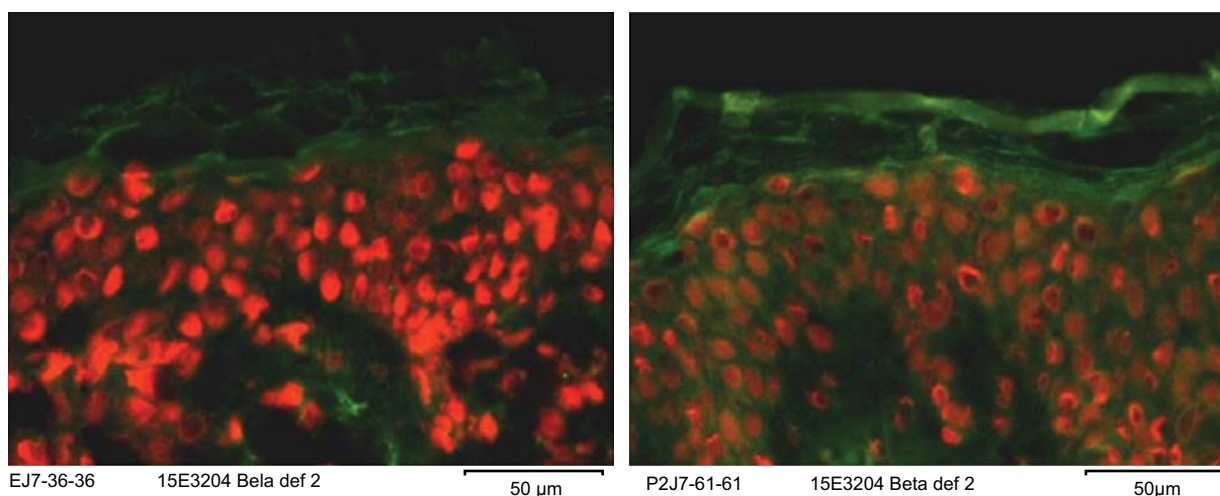


Figure 2 HBD2 protein expression in human living skin explants.

Notes: Either the vehicle (left) or a test solution containing 0.1% sphinganine (right) was repeatedly applied during an overall cultivation period of 7 days. Following histological processing and immunostaining with an anti-HBD2 antibody, HBD2 protein (green color) was visualized by fluorescence microscopy. Nuclei (red color) were stained with propidium iodide.

Sphinganine shows in vivo activity

First of all, a pilot study on female and male test subjects treated with 0.2% sphinganine was performed. In this pilot study, the mean anagen rate at baseline was similar in the female verum group (mean value 75.1%) and the female placebo group (mean value 74.9%). At the 8-week follow-up, mean anagen rate in the female verum group was increased by 3.6% (mean value 77.9%) and in week 16 by 2.7% (mean value 77.2%) compared to the baseline. In contrast, the mean anagen rate in the female placebo group was unchanged after 8 weeks of usage (mean value 74.8%) and slightly increased by 1.1% (mean value 75.8%) after 16 weeks of usage. However, none of these differences were statistically significant (Figure 3A and C).

Among males, the mean anagen rate at baseline was higher in the verum group (mean value 75.7%) than in the placebo group (mean value 71.5%). At the 8-week follow-up, the verum group showed a significantly improved mean anagen hair rate increased by 4.3% to a mean anagen hair rate of 78.9% ($P=0.0391$). In contrast, the mean anagen rate in the male placebo group significantly decreased by 6.0% to a mean anagen rate of 67.2% ($P=0.0234$). Analysis of the change of the mean anagen hair

rate within the verum group compared to the placebo group after 8 weeks of usage showed a statistically significant result in favor of the verum group ($P=0.0027$). At 16 weeks after hair tonic usage, the mean anagen hair rate was even more improved, by 5.7% compared to baseline, but narrowly missed statistical significance ($P=0.1172$). However, in the male placebo group, the anagen hair rate was almost unchanged (70.6%) compared to baseline (Figure 3B and C). A statistical analysis of the pre–post difference between the groups showed no statistical significance.

In this pilot study, males responded better to the sphinganine treatment than females. Thus, in a follow-up study, the efficacy of different concentrations of sphinganine on males was investigated.

The characteristics of the male test subjects including age range, mean hair density, and mean anagen rate of the verum groups and controls, including the follow-up study with 80 male test subjects, are shown in Table 1. In the 0.1% sphinganine group, the anagen rate was increased, but not statistically significant, by 1.7% from an anagen rate of 71.0% at baseline to 72.2% after 8 weeks of usage (Tables 1 and 2). At end point, no further improvement

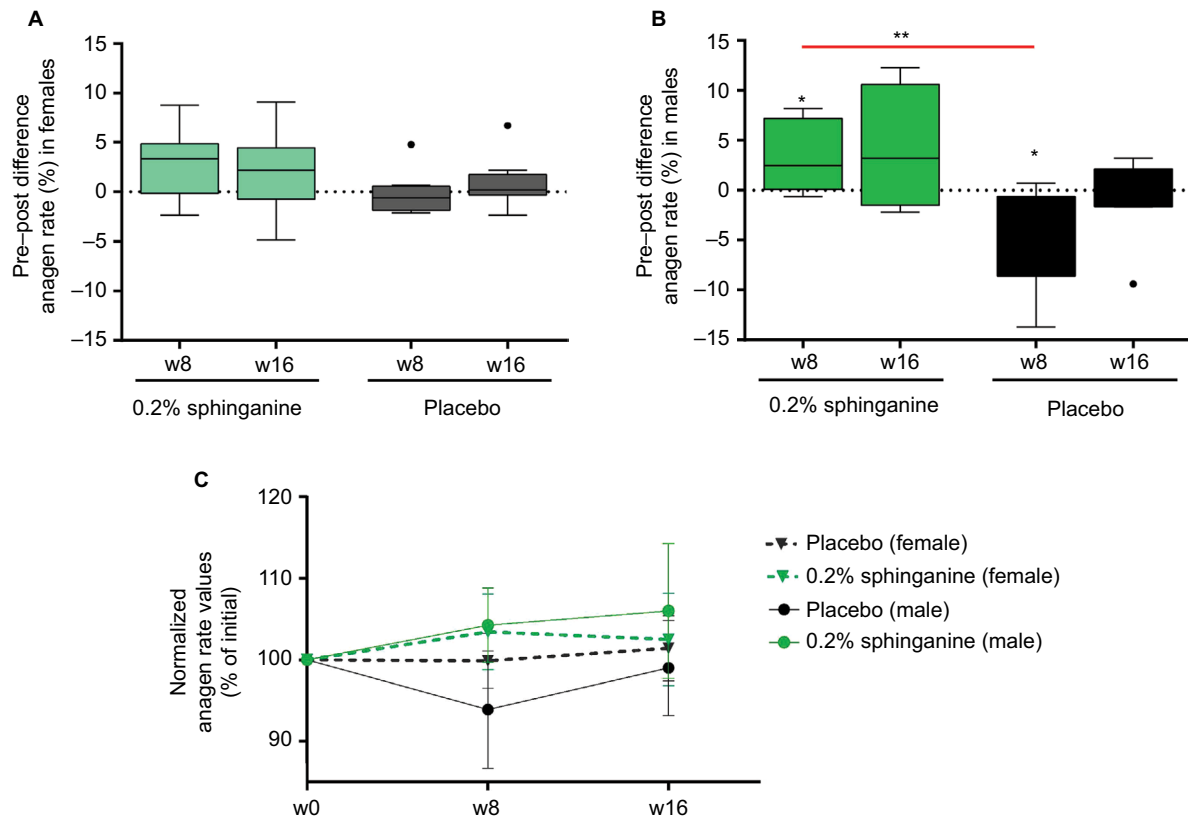


Figure 3 Effect of sphinganine on hair loss in males and females.

Notes: Box plot graphic illustrating the difference (pre–post) of anagen rate after 8 and 16 weeks of treatment compared to baseline in females (A) and males (B). Median, upper, and lower quartile and whiskers are illustrated. Statistical differences in the anagen rate between baseline and after 8 and 16 weeks of treatment as well as statistical differences in the anagen rate between verum groups and placebo group are indicated by asterisks: * $P<0.05$ and ** $P<0.01$ pre–post (Wilcoxon signed-rank test). Normalized anagen rates after 8 and 16 weeks of treatment are shown for males and females (C).

Abbreviation: w, week.

Table 1 Test subjects' characteristics at inclusion and descriptive statistics of anagen hair rates, in percentage, of dose-dependent efficacy study

Baseline data	Time (weeks)	Minimum	25th percentile	Median	75th percentile	Maximum	Mean \pm SD
0.1% sphinganine							
Total number: 20	0	48	65.1	73.4	78.9	82	71.0 \pm 9.3
Age range (years): 30–60, mean: 48.9	8	55	67.2	74.8	77.8	88	72.2 \pm 9.6
Mean hair density (hair/cm ²): 167.75	16	56	63.3	76.2	78.4	88	72.3 \pm 9.9
0.2% sphinganine							
Total number: 28	0	50	67.3	73.2	79.7	88	72.5 \pm 9.3
Age range (years): 28–60, mean: 45.4	8	45	68.8	74.7	81.2	93	74.0 \pm 10.7
Mean hair density (hair/cm ²): 169.96	16	41	68.3	75.7	80.9	94	74.4 \pm 10.6
0.5% sphinganine							
Total number: 20	0	37	56.2	72.4	79.2	82	67.1 \pm 13.7
Age range (years): 30–60, mean: 44.7	8	43	57.6	73.8	81.4	89	69.8 \pm 14.2
Mean hair density (hair/cm ²): 158.04	16	44	58.8	73.2	80.2	87	69.8 \pm 14.0
Placebo							
Total number: 28	0	52	60.4	71.6	79.2	85	69.9 \pm 10.2
Age range (years): 25–59, mean: 45.7	8	43	58.7	70.6	80.1	89	68.3 \pm 12.3
Mean hair density (hair/cm ²): 161.44	16	43	57.9	70.4	80.4	89	68.8 \pm 13.1

Abbreviation: SD, standard deviation.

Table 2 Anagen hair rates, in percentage, for each individual test subject of dose-dependent efficacy study

Test subject	0.1% sphinganine			0.2% sphinganine			0.5% sphinganine			Placebo		
	w0	w8	w16	w0	w8	w16	w0	w8	w16	w0	w8	w16
1	67.1	66.5	76.1	55.3	44.9	41.3	44.7	46.2	44.1	79.1	81.6	80.7
2	79.7	76.0	77.1	70.0	74.4	64.0	58.6	58.4	67.4	54.9	56.9	45.2
3	66.5	76.7	78.2	80.1	86.4	87.1	71.2	75.0	73.3	61.7	62.2	42.8
4	73.4	75.2	82.2	74.6	74.8	68.6	79.0	85.6	87.1	79.1	79.9	80.0
5	82.3	85.8	55.8	79.7	81.2	76.9	65.4	72.6	73.0	71.6	76.0	75.7
6	70.4	69.1	76.7	77.6	83.9	83.0	78.8	78.3	81.0	80.3	80.3	80.8
7	76.8	76.8	57.7	76.0	76.1	75.7	59.2	59.3	51.1	61.4	59.4	62.2
8	75.4	57.5	59.3	67.4	64.9	67.2	76.7	77.9	78.4	64.4	51.9	50.7
9	69.9	71.1	67.9	77.9	68.9	68.0	52.1	55.4	51.3	55.7	50.0	61.3
10	63.7	67.9	76.2	70.4	58.1	77.3	73.6	68.0	63.3	72.5	59.1	55.6
11	78.9	74.4	65.8	50.0	58.6	61.6	81.4	89.3	86.6	71.6	73.1	70.4
12	61.2	68.5	80.3	85.0	87.7	82.9	82.0	81.1	79.4	57.9	63.0	53.5
13	78.9	79.3	63.3	63.2	74.5	65.1	36.7	54.0	44.5	78.3	76.7	79.2
14	58.5	54.6	61.9	56.8	71.7	59.9	81.7	81.6	85.1	59.3	63.1	59.0
15	58.1	58.9	86.6	72.9	63.6	75.3	56.8	63.3	67.4	59.0	50.9	69.4
16	81.7	84.9	88.1	71.1	75.4	81.3	73.9	76.8	77.1	82.1	89.4	88.6
17	80.6	87.5	78.4	72.7	65.1	70.3	79.4	84.4	84.9	78.4	80.7	71.1
18	73.3	78.7	78.4	60.6	73.0	75.6	55.5	43.1	54.3	73.9	82.2	85.8
19	75.9	76.0	64.5	81.3	81.1	76.5	79.4	88.2	75.6	62.1	58.3	67.3
20	48.1	57.6	–	81.1	82.7	84.5	55.3	56.7	71.7	82.5	80.9	81.6
21				76.1	75.5	74.1				52.4	43.2	55.6
22				67.1	68.7	71.3				79.9	77.7	78.2
23				84.9	93.0	94.0				70.4	69.9	70.3
24				79.6	80.3	79.5				85.0	71.3	83.5
25				73.4	73.3	71.2				56.2	54.8	56.9
26				69.3	77.5	80.4				79.3	72.5	78.7
27				87.9	92.7	90.1				80.5	81.2	83.1
28				67.2	70.5	79.5				68.2	67.2	58.8
Mean	71.0	72.2	72.3	72.5	74.0	74.4	67.1	69.8	69.8	69.9	68.3	68.8
SD	9.3	9.6	9.9	9.3	10.7	10.6	13.7	14.2	14.0	10.2	12.3	13.1

Abbreviation: SD, standard deviation; w, week.

was achieved. In the 0.2% sphinganine group, the anagen rate was increased by ~3%, from an anagen rate of 72.5% at baseline to 74.0% and 74.4% after 8 and 16 weeks, respectively. Analysis of the pre–post difference showed no statistical improvement after 8 and 16 weeks. Application of 0.5% sphinganine resulted in an increased anagen rate of 4%, from 67.1% at baseline to 69.8% after 8 and 16 weeks. A statistically significant difference between pre- and post-treatment was observed after 8 weeks ($P=0.0266$), whereas statistical significance was narrowly missed after 16 weeks ($P=0.0708$). In contrast, the mean anagen hair rate in the placebo group was decreased by ~2% during the course

of the study (Tables 1 and 2). Analysis of the change of the mean anagen rate in the verum groups and the placebo group after 8 weeks showed a statistically significant result in favor of the 0.2% sphinganine group ($P=0.0421$) and of the 0.5% sphinganine group ($P=0.0148$). Statistical significance was narrowly missed after 16 weeks in the 0.5% sphinganine group ($P=0.0883$) (Figure 4A and B). Our results demonstrated a dosage-dependent effect on hair growth, with a sphinganine dose of >0.2% being the most effective.

The improvement with regard to hair loss can be seen clearly in the photos (Figure 4C).

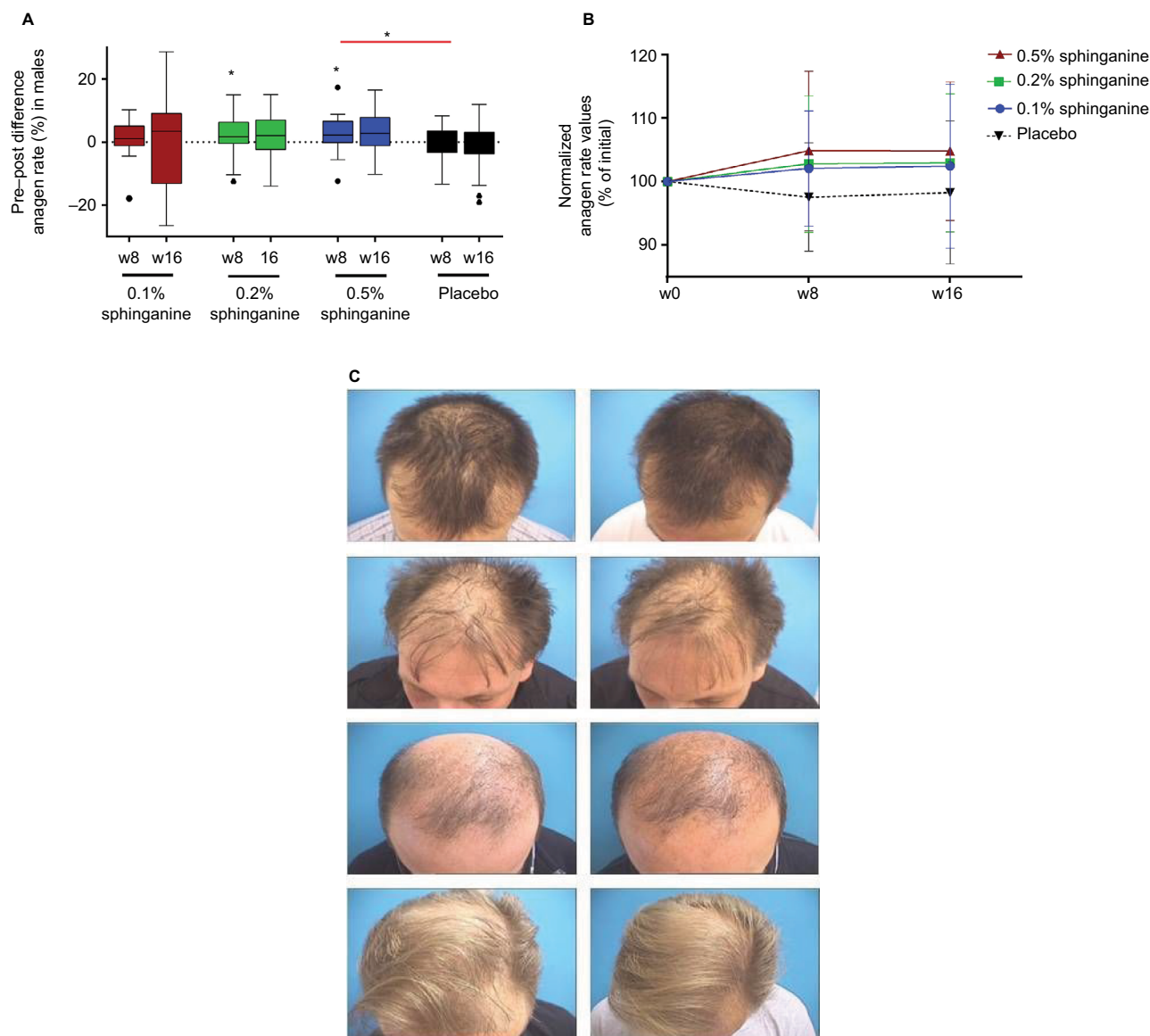


Figure 4 Dose-dependent effect of sphinganine in male.

Note: Box plot graphic illustrating the difference (pre–post) of anagen rate after 8 and 16 weeks of different treatments with different concentrations of sphinganine compared to baseline in males (**A**). Statistical differences in the anagen rate between baseline and after 8 and 16 weeks of treatment as well as statistical differences in the anagen rate between verum groups and placebo group are indicated by asterisks: * $P<0.05$ pre-post (Wilcoxon signed-rank test). Normalized anagen rates after 8 and 16 weeks are shown (**B**). Representative photographs of hair loss improvement of four test subjects taken before (left) and after 16 weeks (right) of application of verum hair tonic are shown (**C**).

12-Month extension study

In order to see whether an extended treatment period with sphinganine has a further positive effect on the hair status, three “high-responder” test subjects of each group were selected. The mean anagen rate was 62.6% in the 0.1% sphinganine group, 60.6% in the 0.2% sphinganine group, 53.0% in the 0.5% sphinganine group, and 70.9% in the placebo group. In the 0.1% sphinganine group, the anagen rate peak was reached after 4 months (73.3%), and in the 0.2% and 0.5% sphinganine group, after 6 months (74.5% and 65.3%, respectively). In the following 6–8 months, the values fluctuated slightly around this peak value but were constantly higher than at baseline (Figure 5). In contrast, the mean anagen rate was lowest in the placebo group after 4–6 months (56%) and did not reach baseline values at study end. Thus, long-term treatment led to a constantly improved anagen rate.

Expert assessment on efficacy of hair quality

For the parameter hair volume, the mean score in the sphinganine groups increased by between 0.40 and 0.64, while the mean score in the placebo group decreased by 0.11. Expressed in percentage, 40%–46% of the test subjects in the sphinganine groups were rated by the investigator as having improved hair volume. A deterioration of the hair volume was seen by no more than 5% of the test subjects in the 0.1% and 0.5% sphinganine test group. In contrast, in the placebo group, only 14% of the subjects showed an increase in hair volume, whereas 25% of test subjects showed reduced hair volume. There was a statistically significant improvement in hair volume with all sphinganine concentrations (0.1% sphinganine, $P=0.0049$; 0.2% sphinganine, $P=0.0004$; 0.5% sphinganine, $P=0.0087$) compared to the placebo.

Expert evaluation scores for changes from baseline to week 16 for the parameter hair strength were also significantly improved for all sphinganine groups (0.1% sphinganine, $P=0.0131$; 0.2% sphinganine, $P=0.0345$; 0.5% sphinganine, $P=0.0253$) compared to the placebo. Thus, half of the test subjects in the verum groups were assessed as having more hair strength. A deterioration of the hair strength by only ~5% was observed in the 0.1% and 0.2% sphinganine group. In the placebo group, however, only 21% of the test subjects were found to have improved hair strength.

In addition, the use of 0.2% and 0.5% sphinganine was also associated with statistically improved hair shine (0.2% sphinganine, $P=0.0343$; 0.5% sphinganine, $P=0.0158$) compared to the placebo group. In 36%–45% of the test subjects, more hair shine was noticed, and none of the test subjects showed any deterioration. In contrast, only 14% of the test subjects in the placebo group were assessed as having an improved hair shine, with 7% of the test subjects showing deterioration (Figure 6A).

Expert assessment on efficacy of scalp health

Before application of hair tonic, between 20% and 30% of the test subjects in the individual sphinganine groups and 40% of test subjects in the placebo group were suffering from dandruff (subjects with scores >1). Despite the relatively low number of cases with dandruff, a change in the mean score between 0.21 and 0.40 toward fewer scales was observed in the individual sphinganine groups, while in the placebo group, the status of dandruff only changed slightly (pre–post scoring: -0.07).

With regard to the parameter scalp dryness, the expert noticed that before application of hair tonic, 25%–43% of the test subjects in each sphinganine test group and 50% of the test subjects in the placebo group were suffering from

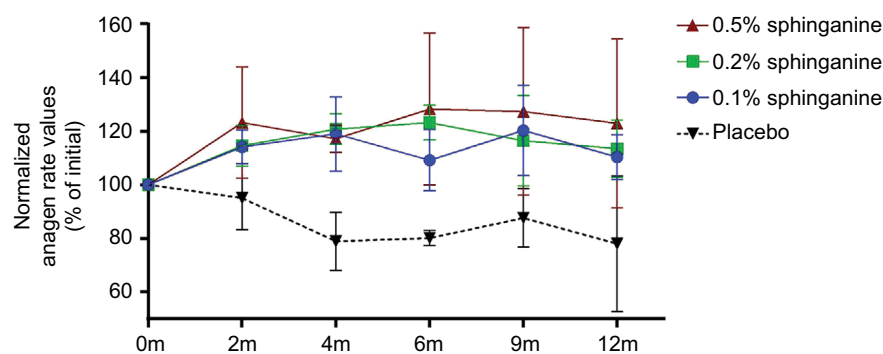


Figure 5 12-Month extension study with three selected “high-responder” test subjects.

Note: Shown are normalized anagen rates after 2, 4, 6, 9, and 12 months of application of hair tonic with different sphinganine concentrations.

Abbreviation: m, month.

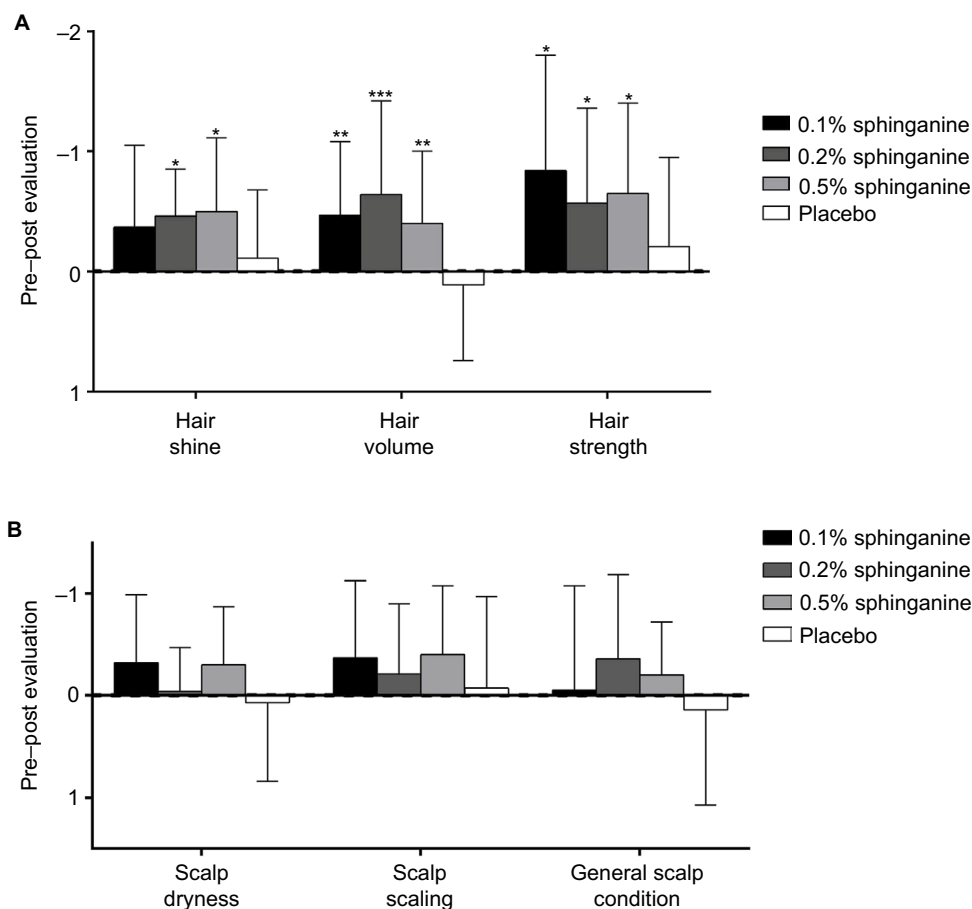


Figure 6 Expert evaluation on hair quality (A) and scalp health (B).

Notes: Mean differences in pre- and post-evaluation scores comparing data from baseline and after 16 weeks are illustrated. Statistical significance in the pre–post difference of the sphinganine groups versus placebo is indicated (Wilcoxon rank sum test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

dry scalp (subjects with scores >1). An improvement was observed in the 0.1% (pre–post difference: -0.32) and 0.5% sphinganine test group (pre–post difference: -0.30). However, dryness of the scalp was only slightly improved in the 0.2% sphinganine group (pre–post difference: -0.04), and worsened in the placebo group (pre–post difference: 0.07).

In addition, the general scalp health was assessed by the expert. In 7%–20% of the test subjects in the individual sphinganine groups and 18% of the test subjects in the placebo group, there was moderate-to-very poor scalp health at baseline (subjects with scores >2). After 16 weeks, an improved general scalp health was especially observed in the 0.2% (pre–post difference: -0.36) and 0.5% sphinganine group (pre–post difference: -0.20), while in the placebo group, a deterioration was seen (pre–post difference: 0.14) (Figure 6B).

Skin compatibility

None of the participating test subjects prematurely terminated the study due to undesirable effects. In the placebo group, four test subjects (11.1%) complained about burning, itching,

redness desquamation, formation of pimples and/or redness, and desquamation around the eyes. The undesirable effects were mostly of very slight-to-light severity and lasted for the first 4–8 weeks. In the verum groups, one test subject (1.5%) experienced slight itching of the skin, a moderate burning around the eyes, and strong burning of the eyes as well as watery eyes. The undesirable effects lasted for the first 4–6 weeks.

The skin compatibility was rated as very good to good by 95% of the test subjects in all verum groups and by 89% of the test subjects in the placebo groups.

Thus, over the course of the study, verum and placebo were well tolerated by the test subjects.

Discussion

Hair growth occurs over a cycle consisting of growth (anagen), regression (catagen), and resting (telogen) phases.¹⁹ Hair loss is a problem of reduced hair growth caused by an imbalanced hair growth cycle. The underlying processes are complex and not yet fully understood with the most important mechanisms being androgen signaling, a lack of

cellular communication, and insufficient stem cell activity within the hair follicle.²⁰ Available pharmaceutical anti-hair loss treatments include Minoxidil[®], an oral drug that decreases blood pressure. However, topical application stimulates blood microcirculation in the scalp presumably leading to increased hair growth.²¹ Another drug for increasing hair growth is finasteride, which after oral uptake blocks the 5- α -reductase, an important enzyme that plays a key role in the context of the balding scalp.^{18,22} Nevertheless, finasteride has also shown certain detrimental side effects like anxiety, depression, and infertility.^{23,24} The data presented in this study show that sphinganine is a potent inhibitor of 5- α -reductase. These data fit well with the observed efficacy of sphinganine in males. 5- α -reductase enzyme acts as a catalyst to convert testosterone into DHT. DHT has a high affinity for androgen receptors, binds to them, and thus initiates a process of follicular miniaturization. Testosterone is the primary male sex hormone, whereas females have testosterone only in trace amounts. This fact might explain the superior efficacy of topical sphinganine on male compared to female subjects.

However, the biological context as to why a sphingoid base inhibits androgen signaling remains to be clarified. Even if sphinganine has an enzyme inhibition potency which is ten times lower than that of finasteride, due to a topical application, it might well be suited as a cosmetic alternative positively influencing the hair growth cycle by reducing DHT levels in the dermal papilla. This would delay transition into the catagen phase and attenuate follicle miniaturization,²⁵ particularly sex-specific among males.

It was also shown that topically applied sphinganine is efficiently absorbed by the skin out of a cosmetic formulation, which is a mandatory prerequisite to in vivo efficacy. Staining of sphinganine in a histological sample containing part of the hair follicle indicated a penetration into the hair follicle (data not shown). But even though a distinction between an epidermal and follicular penetration route of absorption is not possible,¹⁴ the activity data indicate bioavailability which is a prerequisite for the observed in vivo efficacy of sphinganine in reducing non-illness-related hair loss among males. The positive activity was already observed 8 weeks after start of the treatment and remained constant for up to 1 year. The increase in anagen hair rates in the individual treatment groups (Table 1 and Figure 4A and B) can be expressed as an increase in total number of hairs caused by reduced hair loss. Thus, 4-month application of different concentrations of sphinganine leads to a dose-dependent increase of 4.0–6.0 hairs/cm² relative to

placebo. Even higher efficacies have been reported for the pharmaceutical ingredient Minoxidil[®]; an increase of 16.2 hairs/cm² was reported relative to placebo within 4 months.²⁶ An improvement of scalp blood microcirculation, which is one of the described mechanisms for Minoxidil[®], has not been observed with sphinganine (data not shown). Whether a combination of both actives might lead to an additive or synergistic effect shall be subjected to further research.

It was previously shown that sphinganine has antimicrobial properties,^{6,7} is a potent inducer of keratinocyte differentiation, and can serve as a precursor for the biosynthesis of barrier lipids.³ In addition, the formation of the skin barrier also involves the induction of antimicrobial peptides, in particular HBD2. This peptide is known to play an important role in the cutaneous innate immune response.²⁷ In the context of the present research, it was confirmed in primary HNKS by quantitative RT-PCR that the application of sphinganine resulted in a statistically significant upregulation of *HBD2* mRNA compared to control. This finding was confirmed at protein level using ex vivo skin. Together with its role as last common ancestor of all skin ceramides, sphinganine might play a key dual role in scalp biology. On the one hand, it is strengthening the barrier formation via induction of differentiation and antimicrobial response, and on the other hand, it is reducing sebum production, which is a main nutrient of the scalp microbiome, via inhibition of 5- α -reductase. If this hypothetical mechanism is solely controlled by the amount of sphinganine or if there is a functional antagonist remains to be elucidated.

In the context of the clinical evaluation, it was found that sphinganine also improves the hair quality parameters, hair volume, hair strength, as well as hair shine. It is well known that sphinganine-derived ceramide binds to the hair, strengthens the cohesion between the cuticular cells of the hair, and protects it from weakening.²⁸ This might be one reason for the observed improved hair quality parameters. Secondly, direct absorption of sphinganine by the scalp might strengthen the hair follicle and rebuild the hair from inside. Moreover, a first positive trend toward a reduction of dandruff and dryness as well as an improvement of general scalp health was seen after application of sphinganine.

Dandruff is, among other factors, associated with a heightened secretion of sebum and increased colonialization with the fungus *Malassezia*. We demonstrated that the induction of the antimicrobial peptide HBD2 that is thought to contribute to fungal containment^{29,30} is mediated by sphinganine. Both effects, sebum reduction and HBD2 induction

by sphinganine, could play a role in the reduction of dandruff. However, further studies are needed to confirm these preliminary results.

It is also described in the literature that altered levels of sphingoid bases correlate with skin dryness and disturbed skin barrier function.³¹ These findings fit well to the improvement of general scalp health and reduction of scalp dryness.

Altogether, the reported findings suggest that sphinganine might play a central role in the biology of scalp health and the hair growth cycle.

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Disclosure

The authors report no conflicts of interest in this work.

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