Bio-derived hydroxystearic acid ameliorates skin age spots and conspicuous pores

R. Schütz*, A.V. Rawlings†, E. Wandeler*, E. Jackson*, S. Trevisan†, J.-M. Monneuse†, I. Bendik*, M. Massironi‡ and D. Imfeld‡

*DSM Nutritional Products Ltd., Kaiseraugst, Switzerland. †AVR Consulting Ltd., Northwich, UK. ‡Newtone Technologies, Lyon, France. ©Cutech Srl, Padova, Italy

Received 11 December 2018. Accepted 3 April 2019

Keywords: age spots, chemical synthesis, computer modelling, hydroxystearic acid, pores, skin physiology/structure

Abstract
INTRODUCTION: We report on the preparation and efficacy of 10-hydroxystearic acid (HSA) that improves facial age spots and conspicuous pores.

METHODS: The hydration of oleic acid into HSA was catalyzed by the oleate hydratase from Escherichia coli. Following treatment with HSA, collagen type I and type III were assessed in primary human dermal fibroblasts together with collagen type III p53 protein levels and sunburn cells (SBC) after UVB irradiation (1 J cm⁻²) by immunohistochemistry on human ex vivo skin. UVB-induced expression of matrix metalloprotease-1 (MMP-1) was determined from full thickness skin by RT-qPCR. Modification of the fibroblast secretome by HSA was studied by mass-spectrometry-based proteomics. In a full-face, double blind, vehicle-controlled trial HSA was assessed for its effects on conspicuous facial pore size and degree of pigmentation of age spots in Caucasian women over an 8-week period.

RESULTS: HSA was obtained in enantiomeric pure, high yield (≥80%). Collagen type I and type III levels were dose-dependently increased (96% and 244%; P < 0.01) in vitro and collagen type III in ex vivo skin by +57% (P < 0.01) by HSA. HSA also inhibited UVB-induced MMP-1 gene expression (83%; P < 0.01) and mitigated SBC induction (−34% vs. vehicle control) and reduced significantly UVB-induced p53 up-regulation (−46% vs. vehicle control; P < 0.01) in irradiated skin. HSA modified the fibroblast secretome with significant increases in proteins associated with the WNT pathway that could reduce melanogenesis and proteins that could modify dermal fibroblast activity and keratinocyte differentiation to account for the alleviation of conspicuous pores. Docking studies in silico and EC50 determination in reporter gene assays (EC50 5.5 × 10⁻⁹ M) identified HSA as a peroxisomal proliferator activated receptor-α (PPARα) agonist. Clinically, HSA showed a statistically significant decrease of surface and volume of skin pores (P < 0.05) after 8 weeks of application and age spots became significantly less pigmented than the surrounding skin (contrast, P < 0.05) after 4 weeks.

CONCLUSION: HSA acts as a PPARα agonist to reduce the signs of age spots and conspicuous pores by significantly modulating the expression of p53, SBC, MMP-1 and collagen together with major changes in secreted proteins that modify keratinocyte, melanocyte and fibroblast cell behavior.
Hydroxystearic acid reduces age spots and pores

CONCLUSION: (R)-HSA agit comme un agoniste du PPARα pour réduire les signes de taches de vieillesse et l’apparence des pores par une modulation significative de l’expression de la protéine p53, des SBC, de la MMP-1 et du collagène avec des changements majeurs dans les protéines sécrétées qui modifient le comportement cellulaire des kéatinocytes, des mélanocytes et des fibroblastes.

Introduction
As we age, our skin develops visible signs of ageing [1]. In contrast to other body organs, the skin as our first line of defence is constantly exposed to challenges from the environment. Ultraviolet (UV) irradiation, and to some extent, visible light, climate changes and pollution are the major external environmental contributory factors, whereas psychological stress, fatigue and dietary habits are internal stress factors that also contribute to the skin ageing exposure [2]. Recent global consumer surveys on the primary cosmetic concerns of women have identified that not only are the presence of wrinkles and uneven skin tone perceived as major signs of ageing but also the presence of conspicuous skin pores (unpublished data). Moreover, there are ethnic differences in frequency of expression of such skin problems, with the presence of enlarged pores and age spots being of major cosmetic concern for Asian women [3–5].

Visible skin pores are enlarged funnel-shaped or cylindrical openings of the pilosebaceous follicles that become more dilated and more conspicuous caused by the continuous hydrostatic pressure induced by sebum on the pilosebaceous duct in the presence of decreased skin elasticity or slackening in the surrounding areas of the duct caused by ageing [6]. Morphological changes in the dermoepidermal junction (DEJ) are also clearly observed in subjects with conspicuous pores in vivo [5]. Moreover, abnormal keratinocyte differentiation has also been reported with the accumulation of parakeratotic nucleated corneocytes, clearly indicative of UV light irradiation damage to the epidermal keratinocytes [7].

Age spots are benign flat spots of dark pigmentation on the skin occurring especially among older people [8–10]. Prolonged and chronic exposure to UV light and pollutants can accelerate the production of melanin by hyperactive melanocytes [11–13]. However, evidence indicates that the bulk of melanin is inherited only by the non-differentiating daughter cell post mitosis in progenitor keratinocytes via asymmetric organelle inheritance. Moreover, this preferred pattern of melanin distribution can switch to a symmetric or equal daughter cell inheritance mode under conditions of stress [14].

Secretion of melanogenic paracrine factors can also be derived from both keratinocytes and fibroblasts as a result of UV-induced oxidative stress [15,16]. Downregulation of genes involved in epidermal differentiation has also been reported in age spots as well as decreased expression of filaggrin and involucrin [17]. Increased expression of the basal type keratins 5 and 10 also indicates poor keratinocyte differentiation in age spots [18]. Increased p53 levels also contribute to defective epidermal differentiation [19]. The most recent hypothesis is that the increased proliferation of basal keratinocytes combined with decreased turnover of suprabasal keratinocytes puts a backward pressure on the DEJ leading to an exaggerated formation of rete ridges, similar to that in age spots, which also leads to reduced melanin processing upwards from the basal layer of the epidermis [18]. The poor turnover of the suprabasal cells is also suggested by increased numbers of cell layers in the stratum corneum in age spots.

Here, we report on a sustainable source and synthesis of chiral (R)-10-hydroxystearic acid (HSA) that reduces the appearance of both age spots and conspicuous pores in vivo. From in vitro and ex vivo studies we also show that non-UV absorbing HSA mitigates the negative effects of UV stress on skin by reducing p53 activation and MMP-1 (MMP-1) levels. In addition, it increases collagen type I and III synthesis. Proteomic analysis of proteins secreted from fibroblasts corroborates mechanisms that reduce melanogenesis, increase fibroblast activity and stimulate keratinocyte differentiation. These proteins will counter abnormal keratinization, increased melanogenesis and pilosebaceous pore wall slackening associated with age spots and conspicuous pores. HSA is also identified through in situ approaches and reporter gene assays to be a peroxisome proliferator-activated receptor-α (PPARα) agonist.

Materials and methods
Synthesis of 10-HSA
In brief, 10% vegetable oleic acid (81%; KLK OLEO, Emmerich, Germany) and 10% cell-free extract containing regio- and enantioselective oleate hydrolase (EC 4.2.1.53; DSM Chemical Technology, Geleen, The Netherlands) were added to phosphate buffer (1.6 L, 100 mM, pH = 6.5) in a bioreactor and stirred at constant pH and 37°C until >95% conversion was reached (approx. 24 h). Ethyl acetate was added to the mixture and heated to 60°C while gently stirring followed by crystallization. Filtration and drying of the obtained, white solid material resulted in the enantiopure (R)-10-hydroxyoctadecanoic acid (HSA) in 81% yield and 99.8% purity (% w/w, GC-FID, HP-5MS MS: Agilent, Basel, Switzerland). The enantiomeric excess was found to be >99% determined by chiral HPLC analysis (Chiracel OD-H; Daicel, Ilkirch, France) after derivatization as methyl ester. The melting point of the resulting white powder was 86.5°C. Commercial material (DSM Nutritional Products Ltd, Kaiseraugst, Switzerland) is available with the INCI name HSA.

Experimental strategy
To determine the effect of HSA on age spot and conspicuous pore reduction we examined extracellular matrix marker production (Collagen type I and III) together with degradative enzymes (MMP-1), reduction of markers of UV damage [sunburn cells (SBC) and p53], the secretome of fibroblast markers that may influence both keratinocytes and melanocytes and clinically. Moreover, the likely mechanistic target of action was identified as PPARs from in silico and transactivation assays.

Measurement of total cellular collagen type I and III contents in normal human fibroblasts
Human dermal fibroblasts (HDF) obtained from adult skin were seeded into 96-well plates (4000 cells per well) and cultured in Dulbecco’s Modified Eagle’s Medium high glucose (DMEM, Gibco Invitrogen, Basel, Switzerland) containing 10% fetal calf serum (FCS; Amimed BioConcept, Allschwil, Switzerland) and 1% penicillin/streptomycin (P/S; Invitrogen, Basel, Switzerland) at 37°C with 5% CO2 for 24 h. Subsequently the cells were starved in DMEM low glucose containing 0.2% FCS and 1% P/S for 2.5 days. Starvation medium was then refreshed together with the addition of the solubilized test compounds and incubated for another 48 h. Thereafter, HSA was diluted in the culture medium from a 10 mM dimethylsulphoxide (DMSO) stock solution. Transforming growth factor- 

© 2019 The Authors. International Journal of Cosmetic Science published by John Wiley & Sons Ltd on behalf of Society of Cosmetic Scientists and the Société Française de Cosmétologie

International Journal of Cosmetic Science, 41, 240–256
factor beta 1 (TGF-β1; PeproTech, Hamburg, Germany) was used as positive control. After compound incubation, cells were fixed in Dulbecco’s Phosphate-Buffered Saline (DPBS; Gibco Invitrogen, Basel, Switzerland) containing 4% formaldehyde (Life Technologies, Zug, Switzerland) for 15 min and permeabilized with 0.1% Triton-X100 (Sigma Aldrich, Buchs, Switzerland) in DPBS for 90 s. Collagen type I or III was detected using mouse anti-human collagen I antibody (Millipore, Schaffhausen, Switzerland) and rabbit anti-human type III collagen (BioTrend, Cologne, Germany), respectively, followed by an AlexaFluor 488-conjugated secondary antibody (goat anti-mouse or anti-rabbit IgG, Life Technologies, Zug, Switzerland). We counterstained the nuclei with 4’,6-diamidino-2-phenylindole (Sigma Aldrich). Image acquisition and quantitative analysis was performed using an ArrayScan® VTI HCS imaging system (Thermo Scientific, Waltham, MA, USA) with 49 pictures per well with 10× objective. Collagen was measured intracellularly, and the fluorescence intensity values were normalized to the cell count. Data are based on a minimum of three independent measurements and were represented as mean values ± standard error of mean (SEM); Student-t test, significance P < 0.01 vs. medium control.

Ex vivo skin sampling and treatment

Human skin from abdominal plastic surgery was obtained from healthy Caucasian donors after their informed consent and was used for the analysis of collagen type III, SBCs, MMP-1 and p53. Skin samples of 8 × 3 mm (diameter × thickness) were maintained in an air-liquid interface in contact with culture medium (modified Williams’ E medium: Thermo Fisher Scientific) up to 6 days. Six skin samples for each treatment were cultured to perform the collagen, SBC and p53 analyses and two samples for the skin viability and MMP-1 expression. For MMP-1, SBCs and p53 analysis the test compound incubation, cells were fixed in DMSO as vehicle on the skin biopsies (8 mm diameter) 1 and 24 h prior to UVB irradiation (1 J cm⁻²) and covered with 7 mm diameter delivery membrane (CoTran, 3M, Italy). The sensor-controlled BIO-SUN irradiation system (Vilber Lourmat, Germany) equipped with two T-30.M tubes (30 W, intensity 3 mW cm⁻²) was used to irradiate the skin with UVB light. The UV emission spectrum ranged from 280 to 400 nm, of which 6.2% emission was in the UVB range (280–320 nm, peak maximum 312 nm) and elongated in the UVA range (320–400 nm).

Ex vivo skin analyses

Skin viability

After 6 days of incubation, two skin punches were weighted and, if necessary, reduced in the dermal portion, to have approximately the same weight for all samples. Samples were processed with methylthiazolyldiphenyl-tetrazolium bromide (MTT) according to supplier’s instructions (Roche Applied Science, Rotkreuz, Switzerland). Skin viability was measured with a plate reader at a wavelength of 570 nm.

Type III collagen quantification

Two sections (n = 12) from six skin samples were immunostained with monoclonal mouse anti-collagen III (Sigma-Aldrich, cat#C7805) and the Alkaline Phosphatase/RED Detection System (Dako #K5005; Agilent Technologies, Glostrup, Denmark). The amount of the antigen present in each slide was evaluated by the intensity and the distribution of the red staining within a selected area of the dermis using ImageJ (NIH, Bethesda, MD, USA).

Quantification of MMP1 expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from full-thickness skin using RNeasy mini kit for fibrous tissues (Qiagen, Hilden, Germany) following the manufacturer’s instructions. After quantification, 400 ng of total RNA was retro-transcribed using random hexamers and Superscript III (Invitrogen, Darmstadt, Germany). The cDNA was used to perform a real-time PCR (SybrGreen protocol) with specific primers for the evaluation of MMP1 expression. Ubiquitin and YWHAZ (Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein Zeta) were used as reference genes for data normalization. Data acquisition and statistical analysis of RT-qPCR were performed on RotorGene thermal cycler (Corbett Life Science, Mortlake, Vic, Australia) and REST 2009 V2.0.13 software (Qiagen, Darmstadt, Germany).

SBC quantification

Haematoxylin-Eosin (H&E) staining was performed on skin sections to count SBC or apoptotic cells per millimeter of epidermis after analysing twelve different sections per treatment.

p53 protein quantification

Skin sections were stained with the monoclonal mouse anti-p53 antibody (#ab7757; Abcam, Cambridge, MA, USA). The p53-positive cells were counted, and the obtained values were divided upon the area covered by epidermis. Two slides of each skin sample were processed by image acquisition and related analysis (i.e. 12 images for each test treatment).

Statistical analysis of and ex vivo data

All quantitative data were summarized in terms of the mean score and SEM for each treatment. Differences between groups were evaluated by one-way ANOVA with permutation test followed by pairwise post-hoc comparisons Dunnett’s permutation test and pairwise post-hoc comparisons-Tukey’s HSD permutation tests.

Mass-spectrometry-based proteomics of fibroblast secretome

Human dermal fibroblasts from a biopsy of an adult female donor were maintained in DMEM, 10% FCS and 1% P/S. Cells were cultivated at 37°C, 5% CO₂-air atmosphere. HDF were seeded in 6-well plates (120 000 cells per well) and cultivated for 24 h in DMEM 10% FCS 1% P/S, then starved in DMEM low glucose, 0.2% FCS and 1% P/S for 2.5 days. Starvation medium was then replaced, 5 μM HSA was added and incubated for 48 h. The preparation of the extracellular secreted proteins from the conditioned media was performed, using 20 mM ammonium hydroxide and stringent washing with water [20]. Secreted protein isolates obtained from the cell culture conditioned media were dissolved in 600 μL of 50 mM ammonium bicarbonate buffer containing 0.5 μg of trypsin culture plate wells. Plates were incubated for 1.5 h at 37°C. Total protein content was determined using the BCA kit from Thermo Scientific (Ilkirch, France). Lysates from same sample were pooled and acidified to block trypsin activity. Lysates were evaporated to dryness (SpeedVac; Thermo Scientific). Proteins were then reduced (DTT) and alkylated (IAA) and trypsin added a second time to complete protein digestion. For all samples, peptides were purified by SPE chromatography (C18), dried and solubilized in 100 μL
0.1% formic acid aqueous solution. Peptide digests (500 mg per run) were analysed by Eksigent Ultra Plus nano-LC 2D HPLC coupled to a TripleTOF 5600 (AB Scienx, Framingham, MA, USA) mass spectrometer interfaced to a nano-spray III source, according to the method described by Voegeli et al. [21]. The absolute signal of peptide or protein was calculated by summing the extracted area of all unique fragment ions as described in Gillet et al. [22].

PPAR transactivation assays and EC50 determination
A one-hybrid-system, using hybrid constructs of GAL4 DNA-binding part coupled to PPARγ. PPARγ or PPARα ligand binding domains together with a luciferase reporter and a renilla-expressing plasmid, was applied. For transient transfections, white 96-well cell culture plates with clear bottom (Corning, Basel, Switzerland) were used. As many as 7.5 x 10^4 HEK293 cells per well were plated in minimum essential medium (Eagle) at 37°C in 5% CO2 without phenol red supplemented with 10% charcoal-treated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA). The cells were transiently transfected at 70–80% confluence by polyethylene-mine-based transfection for 5 h at 37°C. 5% CO2, which was followed with respective stimulations of the applied compounds dissolved in DMSO (0.45% final DMSO concentration in the wells). The GW7647 compound was used as a reference for human PPARα. Stimulations lasted 16 h according to established protocols. Transfection efficiency was adjusted to renilla expression (Promega AG, Dübendorf, Switzerland). All concentrations were tested in three biological replicates. The ‘dose response for one site’ was applied in a curve-fitting model according to the formula: y = A + (B – A)/(1 + ((10C/xD)), where A is the minimum y-value, B the maximum y-value, C log EC50 and D the slope factor. The data were fitted by XLfit (http://www.idbs.com) using the Levenberg Marquardt algorithm.

Molecular modelling of the interaction of HSA with PPARγ
The ligand binding domain of PPAR alpha was retrieved from the RSCB database (PDB: 3SP6) and prepared for modelling studies using a protein preparation wizard (Maestro version 11.0.015; Schrödinger, LLC, New York, NY, 2016). In short, bond orders were set, hydrogens were added, disulphide bridge was created, all water molecules were removed and hydrogen bonds were optimized using the automated procedure. HSA was docked to the ligand binding domain (Glide, LLC, New York, NY, 2016) and the Standard Precision (SP) setting was used. For docking, multiple conformers of HSA were generated (Conflex, LLC, New York, NY, 2016). Among the top-ranked different binding poses generated, the best pose was selected based on visual inspection. Figures were prepared using the PyMOL Molecular Graphics System, Version 1.8.0.4 Schrödinger, LLC.

Full-face, double-blind, vehicle-controlled, parallel-group in vivo study for the effects of HSA on conspicuous pores and age spots
The study was authorized by the new substance release committee (DSM Nutritional Products, Kaiseraugst, Switzerland) and was conducted in accordance with the Declaration of Helsinki Principles. Written informed consent was collected from all volunteers before enrollment. Before the start of the study, healthy volunteers were advised not to use any cosmetic treatments for 3 days. Two panels with 42 Caucasian females with Fitzpatrick skin phototypes II–III were recruited to apply 1% (33 mM) HSA in a cosmetic product formulation (Table I) or the vehicle formulation, respectively, on the face twice daily for 8 weeks. Thirty-seven completed the study and were included in the final analysis from the product group (aged 38–65, mean 53.4 ± 7.6 years) and 38 in the placebo group (aged 34–65, mean 54.4 ± 7.2 years). No other topical leave-on product was authorized on face during the study and only a specified cleansing milk was allowed (INCI list: AQUA, PARAFFINUM LIQUIDUM, PROPYLENE GLYCOL, ALCOHOL DENAT., GLYCERYL STEARATE SE, POLYSORBATE 60, SORBITAN STEARATE, CAPRYLYL GLYCOL, CARBOMER). Table I describes the product formulation and corresponding vehicle without HSA that was used in the clinical study.

Subjects were acclimatized for 30 min at a temperature of 24 ± 2°C and 35 ± 10% relative humidity before any images were taken. Full-face cross- and parallel-polarized VISIA-CR images (Canfield Scientific, Parsippany, NJ, USA) were assessed at baseline, 4, and 8 weeks during January 19 to March 15, 2016 in Northern Germany. Image analysis and statistics on conspicuous facial pores and solar lentigines were performed with calibrated VISIA images (Newtone, Lyon, France). Three pigmented spots were selected on the chosen profile of each subject from the cross-polarized images with the largest area and the best contrast. The contrast was calculated from the difference of the ITA3 values (individual topological angle based on CIE L × a × b values) of the surrounding skin minus the age spot. For the conspicuous pores analysis, the parallel-polarized images were used to see distinctly the pores and defined areas near the nose on each image for each subject. After segmentation, the identified pores could be analysed in terms of area, depth and volume.

Statistical analysis of in vivo data
For age spots the ΔITA3 were expressed as mean values ± SEM and significance level P < 0.05 product vs. vehicle was determined by Student t-test. For pores the surface and volume differences of conspicuous pores for product and vehicle treatment were compared to T = 0 and expressed in mean ± SEM by Shapiro–Wilks test followed by a Mann–Whitney test using significance level of P < 0.05 product vs. vehicle.

Results
Synthesis and structural characterization
The hydration of oleic acid was catalysed by the oleate hydratase (EC:4.2.1.53) from a crude cell-free extract produced in a proprietary process resulting in the enantiopure (R)-10-hydroxystearic acid in high yield (81%) and purity (99.8%) after crystallization. Reproducibility was shown by five consecutive batches with similar productivity and quality analysed by GC-MS and 1H-NMR (data not shown).

Table I INCI list of formulation (vehicle is minus HSA and replaced by water)

| INCI Name | Description |
|-----------|-------------|
| AQUA | Water |
| HOMOSALATE | Homosalate |
| ETHYLHEXYL SALICYLATE | Ethylhexyl salicylate |
| C12-15 ALKYLDIETHYLAMINOLETHANOLE | C12-15 Alkyldiethylaminolethane |
| BENZOATE | Benzoyl alcohol |
| BUTYL METHOXYDIBENZOYLMETHANE | Butyl methoxydibenzoylmethane |
| OCTOCRYLENE | Octocrylene |
| STEARETH-2 | Steareth-2 |
| BUTYLENE GLYCOL | Butylene glycol |
| CETEARYL ALCOHOL | Cetearyl alcohol |
| HYDROXYSTEARIC ACID | Hydroxystearic acid |
| STEARETH-21 | Steareth-21 |
| PHENOXETHANOL | Phenoxethanol |
| ETHYLHEXYLGLYCERIN | Ethylhexylglycerin |
| XANTHAN GUM | Xanthan gum |
| BHT | Butylated hydroxytoluene |
| ACRYLATES/C10-30 ALKYLCYLATE CROSSPOLYMER | Acrylates/c10-30 alkylacylate crosspolymer |
| DISODIUM EDTA | Disodium EDTA |
| SODIUM HYDROXIDE | Sodium hydroxide |
HSA stimulated collagen type I and type III synthesis in human skin fibroblasts

We assessed the stimulatory effect of HSA on collagen type I and type III on primary HDF after 48 h of incubation. HSA significantly and dose-dependently induced collagen on HDF (Fig. 1). The amount of newly synthesized collagen type I almost doubled compared to the medium control when HSA was added at 5 μM concentration (P < 0.01). In addition, collagen type III synthesis was stimulated up to 244% in HSA (5 μM) treated fibroblasts vs. untreated cells (P < 0.01).

HSA markedly induced ex vivo synthesis of collagen type III

Human skin biopsies from plastic surgery were utilized to verify the modulatory activities of HSA. The test material in DMSO was applied topically and renewed daily for 6 days. The immunohistochemical staining in the prepared skin sections (Fig. 2A) showed that HSA at concentrations between 0.33 and 3.3 mM (=0.1 w/w%) significantly stimulated the formation of collagen type III up to 57% compared to the control at day 6 (Fig. 2B).

UV-induced MMP-1 expression, SBC formation and p53 protein were reduced ex vivo by HSA

The gene expression of MMP-1 in untreated skin explants was analysed 24 h after the UV irradiation (1 J cm⁻²) and was shown to be upregulated by a factor of 3.4 (Fig. 3A) compared to the non-irradiated control. A significant decrease of 83% in MMP-1 expression was observed in ex vivo skin topically treated with 0.33 mM (0.1%) HSA compared with the irradiated vehicle control (P < 0.05). In another experiment, the SBC formation was quantified 24 h post-UVB (1 J cm⁻²) by H&E staining. As expected, UVB irradiation significantly increased the incidence of SBCs more than two-fold vs. non-irradiated control (Fig. 3B). Interestingly, 0.33 mM (0.1%) HSA reduced the formation of SBCs by 49%
compared to the vehicle-treated, UVB-irradiated controls. Next, we tested the effect of HSA on UV-induced upregulation of the stress marker protein p53 when topically applied on human skin biopsies. Indeed, 24 h after UVB irradiation (1 J cm$^{-2}$) immunostained skin sections showed an 80-fold induction of p53 compared to the non-irradiated control skin (Fig. 4). HSA inhibited UV-induced p53 formation by 46% vs. irradiated vehicle control skin.

**Fibroblast secretome modified by HSA**

Considering factors that influence melanogenesis and thereby the skin lightening activity of HSA (Table II). As shown in Table III, HSA appears to modulate many of the proteins in the Wingless-related Integration site (WNT) signalling pathway that would normally increase melanogenesis. Typical of these are reduced levels of secreted frizzled-related protein 1 (sFRP1; 0.77X) and increased antagonist proteins such as insulin-like growth factor-binding protein 3 (IGFBP3; 15.07X) and Angiopoietin-related protein 4 (ANGPTL4; 2.9X). However, Dickkopf-related protein 1 (DKK1) also a WNT antagonist had slightly lower levels following treatment (0.78X). Protein CYR61 also inhibits the melanocyte growth and increased levels (1.45X) were observed with HSA treatment. Gremlin-1 (0.48X), a bone morphogenic binding protein that increases melanogenesis, was also decreased as was adrenomedullin (0.36X), a melanocyte dendrite branching factor. Semaphorin 3A (Sem3A; 1.35X) was increased, whereas the 3B/3D isoforms (0.72X; 0.63X) were decreased and their changes influenced nerve growth and melanogenesis. Similarly, IGFBP3, fibroblast growth factor 5 (FGF5) and nestrin-1 increased in pigmentation disorders in melanoma and their decreases observed with HSA are consistent with a skin lightening effect (0.90X, 0.76X and 0.71X). As TGF$\beta$ is normally associated with decreased skin pigmentation then reductions in Latent-TGF$\beta$-binding protein 2 (LTBP2) (0.66X; Table IV) caused by HSA will help this. Also, increased levels of gremlin-2 (1.74X; Table IV) probably inhibit certain BMP isoforms, e.g. BMP6 to reduce melanogenesis [23]. A disintegrin and metalloproteinase with thrombospondin motifs 23 (ADAM 23), MMP3 and serine peptidases are known to be increased in solar lentigo’s and we observed many decreases in proteases that may be associated with decreased melanogenesis or increased protease inhibitors (Tables V and VI). Alpha-2-antiplasmin (3.90X) is of interest as plasmin destruction of the DEJ is associated with increased melanogenesis. Increased Tissue...
Hydroxy-stearic acid reduces age spots and pores

R. Schütz et al.

Inhibitor of metalloproteinase 3 (TIMP3; 1.66X) may also control the excessive proteolysis and melanogenesis activation. Moreover, MMP14 (0.65X) controls melanocyte migration and the reduction by HSA can account for decreased age spot expression.

Considering factors that influence keratinocyte differentiation and thereby the strengthening pore wall elasticity activity of HSA. As shown in Table III, changes in the IGF-1/IGFBP signalling pathway were observed with lower levels of IGFBP3 (0.90X) following HSA treatment but also dramatic increases in another IGF-1 binding protein IGFBP2 (15.07X) consistent with changes in pores. Moreover, increases in the levels of Midkine (15.74X), the highest secreted protein, ANGPTL4 (2.90X), TGFBI (1.41X) and Sema3A (1.35X) may be associated with increased keratinocyte differentiation and isoform delta of stromal cell-derived factor 1 (SDF1A; 3.94X) with keratinocyte proliferation. Adrenomedullin was decreased (0.36X).

Considering factors that influence fibroblasts and ECM production and thereby the strengthening pore wall elasticity activity of HSA. The above-reported changes in midkine and TGFBI can improve fibroblast migration and proliferation together with glycosaminoglycan synthesis. Increased levels of connective tissue growth factor (CTGF; 2.29X) will increase procollagen production to help with pore wall structure and reductions in complement C1q tumour necrosis factor-related protein 3 (C1QTNF3; 0.34X) will help as this factor inhibits TGFβ effects on fibroblasts and ECM production. Many extracellular matrix proteins were increased by HSA treatment: Fibronectin type III domain-containing protein (FNDC1; 19.64X), vitronectin (VTN; 5.33X), hyaluronan-binding protein 2 (HABP2; 5.09X), proteoglycan 4 (PRG4; 2.91X), tenascin (TNC; 1.75X), matrilin-2 (MATN2; 1.47X). Versican core protein (VCAN; 1.38X), elastin microfibril interface 1 (EMILIN-1: 1.30X) and collagen alpha chains-2 and -3 (VI) (COL6A2/6A3: 1.31 and 1.22X), which will all help with strengthening the pore wall structure (Table IV).

HSA is a transactivating ligand of PPARα

In a transient transactivation assay, both HSA forms (R-enantiomer and racemate) were tested for ligand binding and subsequent transactivation using human peroxisome proliferator-activated receptor proteins, PPARα, PPARβ and PPARγ. Only PPARα was positive for HSA. The half-maximal effective (EC_{50}) concentration for the R-enantiomeric HSA was determined to be 5.43 ± 0.18 µM, whereas the racemate was 11.81 ± 0.39 µM, indicating that only (R)-10-HSA and not the (S)-10-HSA is a PPARα agonist (Table V).

In silico mechanistic evidence for the binding of HSA to PPARα

Docking of HSA into PPARα revealed that the carboxylic acid moiety of HSA forms H-bonds with Tyr 314(H5), His 440(H11) and Tyr 464(H12) and Ser 280 (H3), which also forms a H-bond to the hydroxy group in HSA (Fig. 5). This hydrogen bond network is key in stabilizing the active conformation of PPARα required for heterodimerization with retinoid receptor (RXR) [24]. This heterodimer promotes coactivator recruitment that increases transcriptional activity [25].

Clinical effects of HSA reduced age spots and conspicuous pores

Conspicuous facial pores and age spots on healthy subjects were significantly reduced by a 1% (33 mM) HSA formulation compared to the vehicle treatment. The image analysis of both pore parameters, volume and surface, showed statistical significance (P < 0.05) of the product vs. the vehicle treatment after 8 weeks of treatment (Figs 6 and 7). Age spots became significantly lighter than the surrounding skin by product compared to vehicle application when analysing contrast by the differences in ITA° angles between the surroundings and the pigmented spot after 4 weeks of treatment (Figs 8 and 9).

Discussion

Consumer research indicates that the expression of age spots and pores is of major concern to consumers globally. Both skin problems

Figure 3 Comparison of ex vivo treatments vs. vehicle [dimethylsulphoxide (DMSO)] 24 h after UVB irradiation. (A) UVB-induced matrix metalloproteinase-1 (MMP-1) gene expression from skin sections by RT-qPCR. (B) Sunburn cells quantification on Haematoxylin-Eosin-stained skin sections. Mean values ± SEM, n = 12; *P < 0.05 vs. vehicle control (DMSO) + UVB.
are induced by oxidative stress to the skin and have some similarities in their pathophysiology. Poor keratinocyte differentiation and aberrations to the DEJ are characteristic to both [5,7,17,18,26–29]. As a result, agents that improve epidermal differentiation or reduce the negative effects of UV irradiation on keratinocyte differentiation are likely to be beneficial. Proteases are also involved in their formation: MMPs are involved in the destruction of the dermal matrix in conspicuous pores and ADAMs, a type of MMP, induce melanogenesis [6,30]. Consequently, agents that reduce the levels of MMPs will be advantageous. Moreover, agents known to improve the levels of collagen will likely counteract the pilosebaceous pore wall slackening that occurs in conspicuous pores. In addition, paracrine signalling molecules induced by UV irradiation on keratinocytes and fibroblasts induce melanogenesis [16,17]. Also, the effects of UV on increasing sebaceous gland activity should not be underestimated in the expression of skin pores also [31]. Here we identified the monohydroxy fatty acid HSA to counteract many of these issues and to reduce facial age spots and conspicuous pores in vivo. In this respect, improvements in reduction of conspicuous skin pores and age spots were observed as early as 4 weeks clinically, although the effect of HSA on age spots was slightly less at 8 weeks.

To clarify mechanisms of action, several in vitro, ex vivo and in silico studies were performed. HSA was shown to reduce the negative effects of UV irradiation on the epidermis in vitro (reduced SBC formation and p53 levels). These effects are consistent with an improvement in keratinocyte differentiation capacity and will help with the poor keratinization of keratinocytes in age spots and the surrounding epidermis around skin pores [17–19]. Furthermore, HSA was also shown to increase collagen type I and III synthesis and reduce MMP levels, which will help to improve the pilosebaceous pore wall slackening in conspicuous skin pores. Relatively higher increases in collagen type III are indicative of extracellular

![Figure 4](image1.png)

**Figure 4** (A) Immunohistochemical-stained skin section for p53 analysis. (B) Image analysis of p53-stained skin sections. Mean values ± SEM, n = 12. **P < 0.01 significance vs. vehicle (dimethylsulphoxide) control. HSA, hydroxystearic acid.

![Figure 5](image2.png)

**Figure 5** Molecular modelling of docking of hydroxystearic acid (HSA) to peroxisome proliferator-activated receptor-α (PPARα). Docking of HSA (blue tubes) into PPARα (PDB: 3SP6, shown in green).
Table II  Total number of proteins identified in fibroblast secretome 15 and % of proteins that were modulated by hydroxystearic acid (HSA) and transforming growth factor beta (TGF-\(\beta\))

|                        | 0.23 nM TGF vs control | 5 mM 10-HSA vs control |
|------------------------|-------------------------|------------------------|
| Total proteins         | 352                     | 352                    |
| % Regulated proteins, \(P\)-value < 0.05 | 141 (40%)              | 151 (43%)              |
| % Non-regulated proteins, \(P\)-value < 0.05 | 211 (60%)              | 201 (57%)              |
| % Down-regulated proteins, \(P\)-value < 0.05, fold change < 0.5 | 14 (4%)                | 18 (5%)                |
| % Down-regulated proteins, \(P\)-value < 0.05, fold change 0.5 to 1 | 65 (19%)               | 54 (15%)               |
| % Up-regulated proteins, \(P\)-value < 0.05, fold change 1 to 1.5 | 40 (11%)               | 39 (11%)               |
| % Up-regulated proteins, \(P\)-value < 0.05, fold change > 1.5 | 22 (6%)                | 40 (11%)               |

Table III  Paracrine/autocrine growth factors and cytokines that modulate keratinocyte differentiation and ECM production to influence pore wall elasticity and melanogenesis in age spots

| Name                              | Gene names                  | Fold change | \(P\)-value | Potential function                                                                 |
|-----------------------------------|-----------------------------|-------------|-------------|-----------------------------------------------------------------------------------|
| Midkine                           | MDK MK1 NEGF2               | 15.74       | 0.0027      | Involved in keratinocyte differentiation                                           |
| Insulin-like growth factor-binding protein 2 | IGFBP2 BP2 IBP2            | 15.07       | 0.0001      | Insulin-like growth factor I binding to improve pores, reduce sebum, decrease fibroblast migration and decrease melanocyte growth. |
| Isoform Delta of Stromal cell-derived factor 1 | CXCL12 SDF1 SDF1A SDF1B   | 3.94        | 0.0002      | Involved in melanocyte/fibroblast migration and keratinocyte proliferation. Enhances wound healing. |
| Angiopoietin-related protein 4   | ANGPTL4 ARP4 HFARP PGAR PP1158 PSEC0166 UNQ171/PRO197 | 2.90        | 0.0004      | WNT signalling antagonist to reduce melanogenesis. Also improves keratinocyte differentiation. |
| Connective tissue growth factor   | CTGF CCN2 HCS24 IGFBP8     | 2.29        | 0.0001      | Insulin-like growth factor binding (as above). Increases procollagen production to help pore wall structure. |
| Protein CYR61                    | CYR61 CCN1 GIG1 IGFBP10    | 1.45        | 0.0007      | Extracellular matrix binding. Inhibits melanocyte growth but increases MMP's |
| Transforming growth factor-beta-induced protein ig-h3 | TGFBI BIGH3               | 1.41        | 0.0000      | Promotes fibroblast growth and keratinocyte differentiation. |
| Semaphorin-3A                  | SEMA3A SEMAD               | 1.35        | 0.0332      | Inhibits inflammation (reduces melanogenesis and ECM destruction) and decreases TEWL (keratinocyte differentiation) via neurophilin-1 receptor that protects against UVB apoptosis. Increased levels in conspicuous pores |
| Insulin-like growth factor-binding protein 3 | IGFBP3 IBP3               | 0.90        | 0.0384      |                                               |
| Growth/differentiation factor 15 | GDF15 MIC1 PDF PLAB PTGFB | 0.78        | 0.0078      | Transforming growth factor beta receptor binding [GO:0005160]. Involved in keratinocyte differentiation. GDF9 increases CTGF? |
| Dickkopf-related protein 1      | DKK1 UNQ492/PRO1006        | 0.78        | 0.0050      | Low-density lipoprotein particle receptor antagonist activity and reduces melanogenesis |
| Secreted fizzled-related protein 1 | SFRP1 FRP FRP1 SARP2      | 0.77        | 0.0050      | Frizzled binding reduces melanogenesis. Increased levels found in age spots |
| Fibroblast growth factor 5      | FGF5                       | 0.76        | 0.0483      | Fibroblast growth factor receptor binding and elevated levels in melanoma |
| Semaphorin-3B                  | SEMA3B SEMA5 SEMAA         | 0.72        | 0.0015      | Causes growth cone collapse of sensory neurons may help with itch |
| Netrin-1                        | NTN1 NTN1L                 | 0.71        | 0.0329      | Proinflammatory and promotes melanoma invasiveness Causes growth cone collapse of sensory neurons may help with itch |
| Semaphorin-3D                  | SEMA3D UNQ760/PRO1491     | 0.63        | 0.0127      |                                                                                       |
| Gremlin-1                       | GREM1 CKTSF1B1 DAND2 DRM PIG2 | 0.48        | 0.0011      | BMP binding and transient increases induces melanogenesis. Melanocyte dendrite branching factor, induces keratinocyte and fibroblast proliferation |
| Adrenomedullin                  | GREM1 CKTSF1B1 DAND2 DRM PIG2 | 0.36        | 0.0020      |                                                                                       |
| Complement C1q tumour necrosis factor-related protein 3 | C1QTNF3 CTRP3 UNQ753/PRO1484 | 0.34        | 0.0277      | CTRP3 inhibits TGF-\(\beta\)-induced collagen synthesis, proliferation and migration. Attenuates CTGF production. CTRP3 also attenuated TGF-\(\beta\)-induced Smad3 phosphorylation, nuclear translocation, and interaction with p300 |
Hydroxystearic acid reduces age spots and pores  

R. Schütz et al.

Table IV  Structural proteins of ECM and dermoepidermal junction to influence pore wall elasticity and melanogenesis in age spots

| Protein Name | Description | F | ECM protein |
|--------------|-------------|---|-------------|
| Fibronectin type III domain-containing protein 1 | FNDC1 FNDC2 | 19.64 0.0000 | ECM protein |
| Vimentin | KIAA1866 MEL4B3 | 5.33 0.0000 | Extracellular matrix binding [GO:0050840]; heparin binding [GO:0008021]; integrin binding [GO:0005178]; polysaccharide binding [GO:0030247]; scavenger receptor activity [GO:0005044] |
| Hyaluronan-binding protein 2 | HABP2 HGFAL PHBP | 5.09 0.0345 | Glycosaminoglycan binding [GO:0005539]; serine-type endopeptidase activity [GO:0004252] |
| Proteoglycan 4 | PRG4 MSF SZP | 2.91 0.0290 | Polysaccharide binding [GO:0030247]; scavenger receptor activity [GO:0005044] |
| Pentraxin-related protein | PTPX TNAFAP5 TSG14 | 1.93 0.0014 | Involved in wound healing. |
| Tenasin | TNC XB8 | 1.75 0.0004 | Syndecan binding [GO:0045545] |
| Gremlin-2 | GREM2 CKTSF1B2 | 1.74 0.0005 | Inhibits BMP signaling to reduce melanogenesis |
| Matrilin-2 | MATN2 UNQ193/PRO219 | 1.47 0.0000 | Calcium ion binding [GO:0005509]; carbohydrate binding [GO:0030246]; extracellular matrix structural constituent [GO:0005201]; glycosaminoglycan binding [GO:0005539]; hyaluronic acid binding [GO:0005540]; Scavenger receptor binding [GO:0005044] |
| Versican core protein | VCAN CSPG2 | 1.38 0.0012 | Calcium ion binding [GO:0005509]; carbohydrate binding [GO:0030246]; extracellular matrix structural constituent [GO:0005201]; glycosaminoglycan binding [GO:0005539]; hyaluronic acid binding [GO:0005540]; integrin binding [GO:0005178]; polysaccharide binding [GO:0030247]; scavenger receptor activity [GO:0005044] |
| Collagen alpha-2(VI) chain | COL6A2 | 1.31 0.0041 | Extracellular matrix constituent conferring elasticity [GO:0045545]; integrin binding [GO:0005178]; polysaccharide binding [GO:0030247]; scavenger receptor activity [GO:0005044] |
| COL6A3 | 1.22 0.0103 | Serine-type endopeptidase inhibitor activity [GO:0004867]; platelet-derived growth factor binding [GO:0048407]; protein binding, bridging [GO:0030674] |
| CD44 antigen | CD44 LHR MDU2 MDU3 MIC4 | 0.66 0.0066 | Collagen binding [GO:0005518]; hyaluronic acid binding [GO:0005540]; Scavenger receptor binding [GO:0005044] |
| Latent-transforming growth factor beta-binding protein 2 | LTBP2 C14orf141 LTBP3 | 0.66 0.0007 | Assists TGF beta signaling for matrix production and melanogenesis |
| Collagen alpha-2(I) chain | COL1A2 | 0.59 0.0103 | Extracellular matrix structural constituent [GO:0005201]; identical protein binding [GO:0042802]; metal ion binding [GO:0046872]; platelet-derived growth factor binding [GO:0048407]; protein binding, bridging [GO:0030674] |
| Isoform 4 of Elastin | ELN | 0.59 0.0018 | Extracellular matrix structural constituent [GO:0005201]; identical protein binding [GO:0042802]; metal ion binding [GO:0046872]; platelet-derived growth factor binding [GO:0048407]; protein binding, bridging [GO:0030674] |
| Vimentin | VIM | 0.53 0.0002 | Double-stranded RNA binding [GO:0003725]; glycoprotein binding [GO:0001948]; identical protein binding [GO:0042802]; protein C-terminus binding [GO:0008022]; scaffold protein binding [GO:0097110]; structural constituent of cytoskeleton [GO:0005212]; structural constituent of eye lens [GO:0005212] |
| Prelamin-A/C | LMNA LMN1 | 0.06 0.0100 | Structural molecule activity [GO:0005198] |

Table V  Proteases and sulphatases that degrade extracellular matrix and modulate melanogenesis via protease-activated receptors to influence pore wall elasticity and melanogenesis in age spots

| Protease Name | Description | F | ECM protein |
|--------------|-------------|---|-------------|
| Prothrombin | F2 | 7.46 0.0000 | Serine-type endopeptidase activity |
| ADAMTS1 KIAA1346 | 2.28 0.0064 | Metalloendopeptidase activity |
| ADAMTS5 ADAMTS11 | 1.78 0.0134 | Metalloendopeptidase activity and modulates proteoglycan synthesis |
| ADAMTS9 | 1.86 0.0066 | Metalloendopeptidase activity and modulates proteoglycan synthesis |
| Procollagenase 2 | CP2 | 1.39 0.0232 | Metalloproteinase activity |
| Prss23 Zsi13 | 1.35 0.0149 | Serine-type endopeptidase activity |
| Unq270/Pror307 | 1.20 0.0033 | Serine-type endopeptidase. Regulates availability of IGF by cleaving IGFBP. Processes LTBP, facilitates TGFβ signaling |
| Htra1 Htra Prss11 | 0.68 0.0250 | Serine-type endopeptidase activity |
| MMP14 | 0.65 0.0048 | Metalloendopeptidase activity |
| Sulf-1 | Sulf-1 | 0.65 0.0011 | N-acetylglucosamine-6-sulfatase activity |
| Thsd4 | 0.64 0.0055 | Metalloendopeptidase activity |
| Unq934/Pror3005 | 0.10 0.0038 | Calcium-dependent cysteine-type endopeptidase activity |

matrix remodelling and a more ‘youthful’ dermis [32]. Moreover, reducing the negative effect of UV on melanogenesis will aid the reduction in expression of age spots.

In an attempt to provide greater insight into the potential mechanism of action of HSA, particularly on the potential paracrine effect of fibroblasts after treatment, on the expression of pore and
age spot problems, we examined changes in secretome of fibroblasts (cell culture conditioned media), determined by mass spectrometry-based proteomics, that might influence the fibroblasts in an autocrine fashion or keratinocytes or melanocytes in a paracrine fashion [15,16]. Dermal fibroblasts play a key role in ECM formation and melanocyte pigmentation [33,34]. Naturally our analysis excludes the impact of the role of other cells in skin.

As many as 352 secreted proteins were annotated. However, first we need to consider gene and protein expression in age spots (and related disorders) and determine if we observe and relatable changes in our fibroblast secretome dataset. The regulation of genes associated with the WNT signalling pathways has been described in age spots as well as melasma. Increased sFRP-1 and FRZB/sFRP3 together with increased WNT1, WNT5a and Frizzled-4 expression were found in age spots [35–37], whereas increased WNT expression increases in WIF1, sFRP2 and WNT5a were observed in melasma [38,39]. We observed decreased levels of sFRP-1 and DKK1 indicating that changes in at least the former of these fibroblast proteins induced by HSA have the potential to reduce melanogenesis and age spot expression. Others that may act via the WNT signalling pathway are IGFBP2 and ANGPTL4 which are both reported to be WNT antagonists by inhibiting lipoprotein receptor-related protein 6 [40,41]. In respect of the former molecule, IGF-1 is reported to be melanogenic and IGFBP2 might also control melanogenesis by reducing its unbound levels [42]. Adrenomedullin, which was also decreased following HSA treatment, is a WNT signalling inhibitor and a melanocyte dendritic branching factor and may therefore decrease melanogenesis [43,44]. The increased levels of Semaphorin 3A may also contribute as it is reported to decrease melanoma motility, invasiveness and proliferation [45]. The reductions in Netrin-1 and FGF5 may also contribute to decreased expression of age spots as they decrease melanocyte migration [46,47]. As we observed a

![Figure 6](image-url)
Concerning conspicuous pores, the IGF/IGFBP pathway appears to be altered [50,51]. Increased levels of free IGF-1 can lead to disturbances in the architecture of the DEJ, pore wall slackening, increased sebum production and hyperproliferation of keratinocytes. IGFBP-3 is a major IGF-binding protein and its loss in the rete ridges leads to keratinocyte hyperproliferation and the associated alterations in the DEJ. Interestingly, its levels are normal at the tips of the dermal papillae. Increased levels of K16 were observed in the conspicuous pores. A small decrease in IGFBP3 was observed in our HSA-treated fibroblast secretome; however, the much larger increases in IGFBP2 may mitigate the negative effects of IGF-1 on the expression of conspicuous pores [52].

Figure 7 Reduction of conspicuous facial pores on subject #20 after treatment with product formulation (1% hydroxystearic acid). VISIA-CR images taken at T = 0, 4, and 8 weeks: Parallel-polarized photos and pore surface segmentation near the nose (as shown as in blue).
Moreover, midkine is known to influence keratinocyte differentiation being expressed before transglutaminase-1 [53]. ANGPTL4, which was also dramatically increased by HSA treatment, is known to also mediate keratinocyte differentiation [54]. BIGH3 also increases keratinocyte differentiation [55]. Levels of semaphorin 3A were also increased that may contribute to the reduction of skin pores by reducing keratinocyte hyperproliferation in the rete ridges via its receptor neuropilin-1 [56]. Via this mechanism it may also prevent UV-induced apoptosis and expression of p53 [57]. Adrenomedullin also increases keratinocyte proliferation and its reduced levels may also have a positive benefit [58]. Conversely, we found increased levels of SDF-1 which is known to promote keratinocyte hyperproliferation and we believe its likely effects are to be countered by all the above other changes in other proteins [59].

Figure 8 Left: projection of pigmentation extent in age spot region: Age spot region becomes lighter than surroundings after treatment. Right: Contrast given by differences between ITA* values of surrounding skin and age spot for T = 4 and 8 weeks compared to baseline. Three pigmented spots per subject were chosen from the profile with the largest area and best contrast. Mean ± SEM; Student t-test *significance P < 0.05 product vs. vehicle.

Cross-polarized images:

![Cross-polarized images](image)

Projection axis using false colors:

![Projection axis using false colors](image)

Figure 9 Cross-polarized photos from selected pigment spot selected of subject #20 which was treated with product formulation (1% hydroxystearic acid) at T = 0, 4, and 8 weeks. Below images encoded along the projection axis using false colours for pigmentation.
Improving ECM production is also key in strengthening pore walls to alleviate the expression of conspicuous pores. Of the factors already discussed BIGH3 promotes the function of fibroblasts interacting with biglycan and decorin to promote collagen aggregation and ANGPTL4 enhances the proliferation and migration of fibroblasts [60–62]. Midkine also stimulates Collagen’s I/III, glycosaminoglycan synthesis and especially hyaluronic acid and this may be reflected in the changes in collagen 6A2 and 6A3 levels and HABP2 levels in our samples [63]. Other proteins that are increased that will help are fibronectin type III repeats, vitronectin and proteoglycan 4 (lubricin) that promote migration of dermal fibroblasts [64–67]. PTX3 also promotes wound healing and fibron remodelling [68]. The increases in tenasin, versican and matrilin-2 are also involved in ECM remodelling [69–71]. Moreover, during extracellular matrix assembly EMLINS, which we found to be increased, are deposited on and co-regulated with fibrillins [72]. LTBP2 is also enhanced in intrinsically aged skin reducing TGFβ activity and its suppression in our samples may aid ECM production [73]. The increases in CYP6P1 are known to increase MMP-1 levels [74]. This might be considered to be negative for skin benefits, but all ECM remodelling requires turnover of the existing ECM [75]. The reduced levels of other proteins following HSA treatment are unclear (CD44, ELN, VIM, LMNA).

Several proteases are increased in solar lentigines and their suppression may aid both the increased melanogenesis and ECM destruction in both these and conspicuous pores. However, some may be involved in ECM remodelling. In this respect, we found increases in prothrombin and ADAMTS1 that may be involved in fibroblast chemotaxis and migration, while ADAMTS5 has a role to play in optimal versican content [76]. The latter may also have a role to play in hyaluronic acid metabolism, as it is a HABP. Serpin H1 (Hsp47) is a collagen-specific chaperone and the slight decline in its levels is uncertain but expression kinetics may have a role to play as previously discussed [88].

Some changes in protein levels were consistent with increased PPAR activity e.g. increased levels of ANGPTL4 and IGF/IGFBP responses [54,89–91]. We focussed on PPARα activity as we could not detect any activity in PPARδ/γ in reporter gene assays (>15 μM). Its EC50 of 5.54 μM for the enantiopure HSA was superior to the racemate and other HSA isomers. The in silico docking studies showed that the carboxylic acid moiety of HSA forms H-bonds with Tyr 314(H5), His440(H11) and Tyr464 (H12) and Ser280 (H3) in the PPAR-binding pocket, which also forms a H-bond to the hydroxy group in HSA [24]. This hydrogen bond network is key in stabilizing the active conformation of PPARα required for heterodimerization with its partner, the retinoid receptor (RXR), that is necessary for PPAR activity [25].

We believe targeting PPARα to be important in targeting conspicuous pores and age spots as levels of its mRNA are diminished in photodamaged skin [92–94]. Moreover, its levels were diminished in irradiated fibroblasts. Wy14643, a PPARα agonist, was also shown to limit procollagen diminution and MMP expression induced by UV irradiation in HDF and mouse skin consistent with our findings.

PPARα agonists have, in particular, been shown to improve keratinocyte differentiation [95]. Clearly HSA can directly target the keratinocytes but changes in the fibroblast secretome that then influence keratinocytes in a paracrine fashion are clearly possible but still yet to be proven.

Although controversial, activators for PPARα and γ are also reported to enhance or diminish melanogenesis [96–100]. However, the most recent evidence indicates a potential reduction in melanogenesis for PPARα [101]. Moreover, it is reported that PPARα expression is downregulated in other skin pigmented disorders such as melasma [39]. Although we have not measured melanogenesis in vitro, we anticipate such an effect from HSA as a direct PPARα target in melanocytes and indirectly from its PPARα activity on the fibroblast and keratinocyte secretome as our clinical results were unequivocal. Studies are ongoing to determine the most important potential paracrine signalling molecules in vitro.

In conclusion, HSA is a novel fatty acid for use in cosmetic formulations that we believe binds to PPARα to induce changes in skin cells, particularly fibroblasts, to mitigate the presence of conspicuous skin pores and age spots.

Acknowledgements

This work was fully funded by DSM Nutritional Products. The clinical study was conducted by Institute Dr. Schrader, Holzminden, Germany. AVR is a consultant to DSM.

References

1. Kligman, A.M. Early destructive effect of sunlight on human skin. JAMA 210, 2377–2380 (1969).
2. Krutmann, J., Boulac, A., Sore, G., Bernard, B.A. and Passeron, T. The skin aging exposome. J. Dermatol. Sci. 85, 152–161 (2017).
3. Flament, F., Francois, G., Qiu, H. et al. Facial skin pores: a multiethnic study. Clin. Cosmet. Investig. Dermatol. 8, 85–93 (2015).
4. Hillebrand, G., Levine, M. and Miyamoto, K. The age dependent changes in skin condition in African Americans, Asian Indians, Caucasians, East Asians and Latinos. IFSCC Mag. 4, 259–266 (2001).
5. Sugiyama-Nakagiri, Y., Sugata, K., Hachiyà, A., Osanai, O., Ohuchi, A. and Kitahara, T. Ethnic differences in the structural properties of facial skin. J. Dermatol. Sci. 53, 135–139 (2009).
6. Lee, S.J., Seok, I., Jeong, S.Y., Park, K.Y., Li, K. and Seo, S.J. Facial pores: definition, causes, and treatment options. Dermatol. Surg. 42, 277–285 (2016).
7. Katsuza, Y., Iida, T., Inomata, S. and Yoshida, S. Improving the appearance of...
Hydroxystearic acid reduces age spots and pores

R. Schütz et al
Hydroxystearic acid reduces age spots and pores

R. Schütz et al.

44. Motokawa, T., Miwa, T., Mochizuki, M., Toritsuka, M., Sakata, A. and Ito, M. Adrenergic: a novel melanocyte dendrite branching factor. J. Dermatol. Sci. 79, 107–310 (2015).
45. Chakraborty, G., Kumar, S., Mishra, R., Patil, T.V. and Kundu, G.C. Semaphorin 3A suppresses tumor growth and metastasis in mice melanoma model. PLoS ONE 7, e36313 (2012).
46. Ghassemi, S., Vojdovskyi, K., Sahin, E. et al. IGF is expressed in melanoma and enhances malignancy in vitro and in vivo. Oncotarget 8, 77750–77862 (2017).
47. Kaufmann, S., Kuphal, S., Schubert, T. and Bosserhoff, A.K. Functional implication of Ntrin expression in malignant melanoma. Cell Oncol. 31, 415–422 (2009).
48. Xu, Z., Chen, L., Jiang, M., Wang, Q., Zhang, C. and Xiang, L.F. CCN1/Cyr61 stimulates melanogenesis through integrin alpha6beta1, p18 MAPK, and ERK1/2 signaling pathways in human epidermal melanocytes. J. Invest. Dermatol. 138, 1825–1833 (2018).
49. Yamaguchi, Y., Passeron, T., Hoashi, T. et al. Dickkopf 1 (DKK1) regulates skin pigmentation and thickness by affecting Wnt/beta-catenin signaling in keratinocytes. FASEB J. 22, 1009–1020 (2008).
50. Sugiyama-Nakagiri, Y., Naoe, A., Ohuchi, A. and Kitahara, T. Serum levels of IGF-1 are related to human skin characteristics including the conspicuousness of facial pores. Int. J. Cosmet. Sci. 33, 144–149 (2011).
51. Sugiyama-Nakagiri, Y., Ohuchi, A., Hachiya, A. and Kitahara, T. Involvement of IGF-1/IGFBP-3 signaling on the conspicuousness of facial pores. Arch. Dermatol. Res. 302, 661–667 (2010).
52. Clemmons, D.R. IGF binding proteins and their functions. Mol. Reprod. Dev. 35, 368–374 (1993); discussion 74–75.
53. Monna, F., Honumi, Y., Ikematsu, S., Kawaguchi, M., Kadomatsu, K. and Suzuki, T. Expression of midline in normal human skin, dermatis and neoplasms: association with differentiation of keratinocytes. J. Dermatol. 40, 980–986 (2013).
54. Pal, M., Tan, M.J., Huang, R.L. et al. Angiopoietin-like 4 regulates epidermal differentiation. PLoS ONE 6, e25177 (2011).
55. Oh, J.E., Kook, J.K. and Min, B.M. Beta ig-h3 induces keratinocyte differentiation via modulation of involucrin and transglutaminase expression through the integrin alpha3beta1 and the phosphatidylinositol 3-kinase/Akt signaling pathway. J. Biol. Chem. 280, 21629–21637 (2005).
56. Kurschat, P., Bielenberg, D., Rossignol-Talhamler, M., Stahl, A. and Klugbrunn, M. Neuron restrictive silencer factor. NR5F1 REIST is a transcriptional repressor of neurophin-1 and eliminates the ability of semaphorin 3A to inhibit keratinocyte migration. J. Biol. Chem. 281, 2721–2729 (2006).
57. Riese, A., Ellert, Y., Meyer, Y. et al. Epidermal expression of neuropelin 1 protects murine keratinocytes from UVB-induced apoptosis. PLoS ONE 7, e50944 (2012).
58. Albertin, G., Carraro, G., Parigotto, P.P. et al. Human skin keratinocytes and fibroblasts express adrenomedullin and its receptors, and adrenomedullin enhances their growth in vitro by stimulating proliferation and inhibiting apoptosis. Int. J. Mol. Med. 11, 635–639 (2003).
59. Quan, C., Cho, M.K., Shao, Y. et al. Dermal fibroblast expression ofstromal cell-derived factor-1 (SDF-1) promotes epidermal keratinocyte proliferation in normal and diseased skin. Protein Cell 6, 890–903 (2015).
60. LeBaron, R.G., Beverkov, K.L., Zimber, M.P., Pavelec, R., Skonier, J. and Purchio, A.F. Beta IG-H3, a novel secretory protein inducible by transforming growth factor-beta, is present in normal skin and promotes the adhesion and spreading of dermal fibroblasts in vitro. J. Invest. Dermatol. 104, 844–849 (1995).
61. Reinbold, B., Thomas, J., Hamsen, E. and Gibson, M.A. Beta IG-h3 interacts directly with biglycan and decorin, promotes collagen VI aggregation, and participates in ternary complexing with these macromolecules. J. Biol. Chem. 281, 7816–7824 (2006).
62. Jamil, S., MousaviAZadeh, R., RoshanMoniri, M. et al. Angiopoietin-like 4 enhances the proliferation and migration of tendon fibroblasts. Med. Sci. Sports Exerc. 49, 1769–1779 (2017).
63. Yamada, H., Inuzumi, T., Tajima, S., Muramatsu, H. and Muramatsu, T. Stimulation of collagen expression and glycosaminoglycan synthesis by midline in human skin fibroblasts. Arch. Dermatol. Res. 289, 429–433 (1997).
64. Hintner, H., Dahlback, K., Dahlback, B., Pepys, M.B. and Breathnach, S.M. Tissue thrombin and its receptors in human scars. Comparison by immunohistochemical and northern analyses. Lab. Invest. 72, 662–669 (1995).
65. Halper, J. and Kjær, M. Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. Adv. Exp. Med. Biol. 802, 31–47 (2014).
66. Korpos, E., Deak, F. and Kiss, I. Matrilin-2, an extracellular adaptor protein, is needed for the regeneration of muscle, nerve and other tissues. Neural Regen. Res. 10, 866–869 (2015).
67. Schiavinato, A., Keene, D.R., Wohl, A.P. et al. Targeting of EMILIN-1 and EMILIN-2 to fibrillin microfibrils facilitates their incorporation into the extracellular matrix. J. Invest. Dermatol. 136, 1150–1160 (2016).
68. Langton, A.K., Sherratt, M.J., Griffiths, C.E. and Watson, R.E. Differential expression of elastic fibre components in intrinsically aged skin. Biogerontology 13, 37–48 (2012).
69. Qin, Z., Fisher, G.J. and Quan, T. Cysteine-rich protein 61 (CCN1) domain-specific stimulation of matrix metalloproteinase-1 expression through alpha6beta3 integrin in human skin fibroblasts. J. Biol. Chem. 288, 12386–12394 (2013).
70. Karimipour, D.J., Kang, S., Johnson, T.M. et al. Microdermabrasion with and without aluminum oxide crystal abrasion: a comparative molecular analysis of dermal remodeling. J. Am. Acad. Dermatol. 54, 405–410 (2006).
71. Artuc, M., Hermes, B., Algermissen, B. and Henz, B.M. Expression of prothrombin, thrombin and its receptors in human scars. Exp. Dermatol. 15, 523–529 (2006).
Hydroxystearic acid reduces age spots and pores

R. Schütz et al.

77. Dawes, K.E., Gray, A.J. and Laurent, G.J. Thrombin stimulates fibroblast chemotaxis and replication. Eur. J. Cell Biol. 61, 126–130 (1993).
78. Hattori, N., Carrino, D.A., Laufer, M.E. et al. Pericellular versican regulates the fibroblast-myofibroblast transition: a role for ADAMTS5 protease-mediated proteolysis. J. Biol. Chem. 286, 34298–34310 (2011).
79. Krampert, M., Kuenzle, S., Thai, S.N., Lee, N., Irure-Arispe, M.L. and Werner, S. ADAMTS1 proteinase is up-regulated in wounded skin and regulates migration of fibroblasts and endothelial cells. J. Biol. Chem. 280, 23844–23852 (2005).
80. Mead, T.J. and Apte, S.S. ADAMTS proteins in human disorders. Matrix Biol. 71–72, 225–239 (2018).
81. Beaufort, N., Scharrer, E., Kremmer, E. et al. Cerebral small vessel disease-related protease HtrA1 processes latent TGF-beta binding protein 1 and facilitates TGF-beta signaling. Proc. Natl. Acad. Sci. USA 111, 16496–16501 (2014).
82. Amano, S. Characterization and mechanisms of photoageing-related changes in skin. Damages of basement membrane and dermal structures. Exp. Dermatol. 25(Suppl 3), 14–19 (2016).
83. Schafer, B.M., Maier, K., Eickhoff, U., Bechtel, M. and Kramer, M.D. alpha 2-Antiplasmin and plasminogen activator inhibitors in wound healing human skin wounds. Ann. Biomed. Eng. 43, 128–139 (2015).
84. Lu, Z., Wang, F. and Liang, M. SerpinC1/ Antithrombin III in kidney-related diseases. Clin. Sci. (Lond.) 131, 823–831 (2017).
85. Rost, F., Diarra-Mehpour, M. and Martin, J.P. Inter-alpha-trypsin inhibitor proteoglycan family—a group of proteins binding and stabilizing the extracellular matrix. Eur. J. Biochem. 252, 339–346 (1998).
86. Brew, K. and Nagase, H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim. Biophys. Acta 1803, 55–71 (2010).
87. Papareddy, P., Kalle, M., Sorensen, O.E. et al. Tissue factor pathway inhibitor 2 is found in skin and its C-terminal region encodes for antibacterial activity. PLoS ONE 7, e52772 (2012).
88. Ito, S. and Nagata, K. Biology of Hsp47 (Serpin H1), a collagen-specific molecular chaperone. Semin. Cell Dev. Biol. 62, 142–151 (2017).
89. Kang, H.S., Kim, M.Y., Kim, S.J. et al. Regulation of IGFBP-2 expression during fasting. Biochem. J. 467, 453–460 (2015).
90. McMullen, P.D., Bhattacharya, S., Woods, C.G. et al. A map of the PPARalpha transcription regulatory network for primary human hepatocytes. Chem. Biol. Interact. 209, 14–24 (2014).
91. Urbanska, K., Pannizzo, P., Grabacka, M. et al. Activation of PPARalpha inhibits IGF-I-mediated growth and survival responses in medulloblastoma cell lines. Int. J. Cancer 123, 1015–1024 (2008).
92. Kim, E.J., Jin, X.J., Kim, Y.K. et al. UV decreases the synthesis of free fatty acids and triglycerides in the epidermis of human skin in vivo, contributing to development of skin photosaging. J. Dermatol. Sci. 57, 19–26 (2010).
93. Shin, M.H., Lee, S.R., Kim, M.K., Shin, C.Y., Lee, D.H. and Chung, J.H. Activation of peroxisome proliferator-activated receptor alpha improves aged and UV-irradiated skin by catalase induction. PLoS ONE 11, e0162628 (2016).
94. Xue, J., Zhu, W., Song, J. et al. Activation of PPARalpha by clofibrate sensitizes pancreatic cancer cells to radiation through the Wnt/beta-catenin pathway. Oncogene 37, 953–962 (2018).
95. Hanley, K., Jiang, Y., He, S.S. et al. Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha. J. Invest. Dermatol. 110, 368–375 (1998).
96. Chen, J.H., Chung, J.L., Chen, P.R. et al. Inhibition of peroxisome proliferator-activated receptor gamma prevents the melanogenesis in murine B16/F10 melanoma cells. Biomed. Res. Int. 2014, 695797 (2014).
97. Flori, E., Mastrofrancesco, A., Kovacs, D. et al. 2,4,6-Octatrienoic acid is a novel promoter of melanogenesis and antioxidant defence in normal human melanocytes via PPAR-gamma activation. Pigment Cell Melanoma Res. 24, 618–630 (2011).
98. Huang, Y.C., Liu, K.C., Chiou, Y.L. et al. Fenoibrate suppresses melanogenesis in B16-F10 melanoma cells via activation of the p38 mitogen-activated protein kinase pathway. Chem. Biol. Interact. 205, 157–164 (2013).
99. Kang, H.Y., Chung, E., Lee, M., Cho, Y. and Kang, W.H. Expression and function of peroxisome proliferator-activated receptors in human melanocytes. Br. J. Dermatol. 150, 462–468 (2004).
100. Wiechers, J.W., Rawlings, A.V., Garcia, C. et al. A new mechanism of action for skin whitening agents: binding to the peroxi- some proliferator-activated receptor. Int. J. Cosmet. Sci. 27, 123–132 (2005).
101. Grabacka, M., Wieczorek, J., Michalczyk-Wetula, D. et al. Peroxisome proliferator-activated receptor alpha (PPARalpha) contributes to control of melanogenesis in B16 F10 melanoma cells. Arch. Dermatol. Res. 309, 141–157 (2017).