

Pilot Evaluation of a Novel Topical Formulation Containing High Level, Cholesterol-Dominant, Physiological Lipids for Specific Targeting of Skin Barrier Deficits in Aging Skin

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ABSTRACT

Background: Topical products with epidermal lipids in a ceramide-dominant ratio were originally developed to address atopic dermatitis. They have been used by extension to address the barrier deficits of aging skin, and after laser resurfacing and other barrier-disrupting procedures. The purpose of this study was to evaluate efficacy, tolerability, and safety of a novel formulation containing high levels of physiological barrier lipids, in a unique cholesterol-dominant ratio hypothesized to directly address the specific epidermal lipid imbalances of aging skin.

Methods: An 8-week, single-center study was conducted on females aged 55 to 75 with mild to moderate fine lines and wrinkles, and other stigmata of facial photodamage. Evaluations were performed at baseline, immediately after first use of the cholesterol-dominant test formulation, and at weeks 4 and 8. Efficacy evaluations comprised blinded expert grading of 9 skin attributes, bioinstrumental measurements of trans-epidermal water loss (TEWL) and stratum corneum hydration, standardized digital imaging, and self-assessment questionnaires. Tolerability was scored objectively and subjectively, and by grading of skin dryness. Adhesive tape extracts were analyzed biochemically for epidermal lipids, and by double-staining to detect mature vs. immature corneocytes. In a separate study, the effects of pre- or post-treatment with the test formulation on barrier recovery following traumatic tape stripping of forearm skin were assessed via measurements of TEWL and stratum corneum hydration.

Results. Statistically significant improvements occurred in all facial skin attributes (weeks 4 and 8), TEWL (week 8), and stratum corneum hydration (weeks 4 and 8). There were significant increases in total ceramides and cholesterol, including ceramide isoforms not present in the test formulation itself; and a statistically significant increase in the ratio of mature to immature corneocytes at week 8. Forearm skin treated with the test formulation after traumatic tape stripping recovered barrier function significantly faster than untreated skin. Pre-treatment for 1 week prior to traumatic tape stripping had a significant protective effect. Tolerability and safety of the test formulation were excellent.

Conclusions. This cholesterol-dominant lipid formulation offers a new paradigm of primary skin barrier repair specifically targeted toward aging skin. Correction of age-related epidermal lipid deficits directly addresses the underlying cause and the profound sequelae of barrier dysfunction in aging skin. This may also be of utility to optimize skin tolerance, response and recovery from aesthetic procedures and other topical treatments, and for dermatoses associated with barrier dysfunction, such as rosacea. The formulation achieved sustained improvements in barrier function throughout the course of the 8-week study, had a protective effect when applied prior to barrier insult, and mediated more rapid recovery when applied after barrier insult. Significant increases in total epidermal ceramides, cholesterol and triglycerides, and in mature versus immature corneocytes, indicated both structural and functional normalization of the skin barrier, and possibly a broader-based stimulation of ceramide synthesis. The formulation had excellent tolerability and safety profiles, even in subjects with sensitive skin.

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INTRODUCTION

Skin aging is characterized by profound functional changes. In the epidermis, these include reduced capacities for protection against mechanical and chemical insults, maintenance of hydration and osmotic balance, immunological defense, and toxin elimination.[1] While there are multifactorial etiologies for these changes, age-related deterioration of the skin barrier is a primary cause. Skin barrier abnormalities can be attributed, in large part, to reduced delivery of secreted lipids to the stratum corneum, and a resultant decrease in the number of extracellular lamellar bilayers it contains.[2,3] Although these bilayers are healthy and intact, the extracellular matrix may be more permeable in aged than in young epidermis. If aged skin

becomes dehydrated or damaged, its dysfunction may be exacerbated and the barrier more easily compromised. Studies of transepidermal water loss (TEWL) demonstrate that the barrier of aged skin recovers significantly more slowly than young skin after damage from repeated tape stripping.[2]

Substantial research has focused on the development of topical products to address skin barrier abnormalities. It was previously reported that an emulsion containing ceramides 1 and 3, together with cholesterol and fatty acids, provided cumulative improvements in skin hydration and barrier function when applied to sodium lauryl sulfate-irritated skin for a period of 30 days.[4] Notably, the combination of ceramides 1 and 3 provided a strong synergistic benefit, in comparison to emulsions containing either ceramide alone plus cholesterol and fatty acids. Elias and colleagues have shown in human and animal studies that a topical mixture dominant in any of the three main physiological stratum corneum lipids can accelerate barrier recovery.[5,6] However, it appears to be more efficacious to deliver specific lipid ratios (a critical mole ratio), based on the underlying nature of the barrier defects, which are influenced by factors such as age, environment and disease state.[7] In a key study on chronologically-aged skin, Zettersten reported the benefits of one week of treatment with a cholesterol-dominant molar formulation (1:3:1:1 ratio of ceramide s:cholesterol:palmitate:linoleate) for accelerating barrier recovery following injury induced by tape stripping.[8]

For skin affected by atopic dermatitis, topical formulations with a ceramide-dominant lipid ratio have been developed, and shown to provide good efficacy for barrier repair. A number of over-the-counter and prescription products with a ceramide-dominant ratio of barrier lipids are currently marketed.[9-13] By extrapolation, these products have also been used for aging skin and for post-procedural recovery after laser resurfacing, chemical peels, and other aesthetic interventions. No topical formulation containing all three types of physiologically relevant lipid (ceramides, cholesterol, fatty acids) in a ratio optimal for aging skin has been available.

STUDY OBJECTIVE AND DESIGN

The objective of these pilot studies was to evaluate the ability of a novel, cholesterol-dominant topical formulation to improve

TABLE 1.

Ten-Point Visual Analog Scale for Assessment of Clinical Skin Attributes

Assessments were performed by blinded evaluators at pre-treatment baseline, week 4, and week 8. Dryness was used to evaluate tolerability of the cholesterol-dominant test formulation. The other nine attributes were used to evaluate efficacy.

Skin Attribute	Scale									
	0	1	2	3	4	5	6	7	8	9
Dryness	None		Mild			Moderate			Severe	
Tone/evenness (visual)*	Uniform/even		—			—		Uneven/discolored appearance		
Brightness/ radiance/ luminosity*	Bright/radiant/ luminous		—			—		Dull/sallow		
Texture, smoothness (tactile)*	Smooth/even-feeling skin texture		—			—		Rough/ridged-feeling skin texture		
Fine lines/ wrinkles^	None		Mild			Moderate			Severe	
Tone Clarity (visual)*	Clear/ translucent		—			—		Opaque		
Firmness (tactile)*	Firm, tight-feeling skin, resists manipulation		—			—		Loose, lax-feeling skin that moves freely upon manipulation		
Laxity/elasticity (tactile)	Elastic, skin feels toned, dense, resilient		—			—		Lax, skin feels pliable, thin, non-resilient		
Appearance of pore (size)	Small/barely perceptible pores					—		Large/noticeable pores		
Overall appearance	Good/healthy-looking skin					—		Poor/unhealthy-looking skin		

*Subjects required a score of 4, 5 or 6 to qualify for the study.
^Study protocol required at least 30 subjects to be scored 4, 5 or 6.

barrier function, specific indices of skin aging, and global skin quality in subjects up to 75 years old. This cholesterol-dominant formulation contains high concentrations of ceramides (2%), natural cholesterol (4%), and omega fatty acids (2%). It incorporates the synergistic combination of ceramide 1 and ceramide 3. The 2:4:2 weight percent ratio corresponds to the 1:3:2 molar ratio of ceramides, cholesterol, and fatty acids that was previously reported to accelerate barrier recovery in aging skin.

MATERIALS AND METHODS

Facial Skin Study

Study Design

Fifty-nine healthy female subjects aged 55 to 75 (mean age, 64.5 years) were enrolled in an 8-week study to evaluate the efficacy, safety and tolerability of the topical, cholesterol-dominant lipid formulation (Triple Lipid Restore 2:4:2, SkinCeuticals Inc., New York, NY). The number of subjects with Fitzpatrick skin phototypes I, II, III, and IV was 7, 16, 29, and 7, respectively. Thirty of the subjects had self-perceived sensitive skin. The inclusion criteria for the study were a clinical evaluator score of 4 to 6 on a 10-point visual analog scale (0 to 9) for the following characteristics: fine lines and wrinkles (at least 30 subjects), uneven skin tone, lack of skin firmness, skin dullness, skin roughness, and lack of skin tone clarity. All subjects were free of any systemic or dermatological disorders, including a known history of allergies or other medical conditions that could interfere with the conduct of the study or interpretation of results. They agreed to use no topical products or cosmetics other than those comprising the clinical protocol, and to avoid excessive sun exposure and artificial tanning for the duration of the study.

At the screening, pre-treatment visit, subjects who met all inclusion and exclusion criteria were enrolled in the study. Subjects were provided with a facial cleanser (Gentle Cleanser, SkinCeuticals, Inc.), and a broad spectrum sunscreen (Ultimate UV Defense SPF 30, SkinCeuticals, Inc.). They were instructed to wash their faces twice daily at home with the cleanser, followed by application of the sunscreen, in the morning, prior to sun exposure.

Study subjects returned 7 days later for their baseline visit. They spent at least 15 minutes in an environmentally-controlled room, with a temperature of 70 degrees Fahrenheit ± 5 degrees and relative humidity of 45% ± 15%, prior to any clinical assessments or instrumentation measurements. Ten clinical skin attributes (Table 1) were scored on 10-point visual analog scales (0 to 9). Skin irritation was evaluated with tolerability assessments on a four-point scale (0-3). For consistency of scoring, the same clinical evaluator was used throughout the study for a given subject, whenever possible. Clinical evaluators were blinded to previously recorded data. Standardized digital images of each subject were obtained. Subjects then applied the cholesterol-dominant test formulation to their facial skin under staff supervision, and were

FIGURE 1. Study subject questionnaire, administered at week 8 for self-assessment of efficacy of the cholesterol-dominant test formulation.

FACIAL PRODUCT QUESTIONNAIRE
Week 8

Initials _____

Panelist No._____

After using the test product for eight (8) weeks, please read the following statements and check the circle that represents your answer.

1. Skin feels smoother:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

2. Skin feels softer:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

3. Skin feels firmer:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

4. Skin feels comfortable:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

5. Skin feels hydrated/moisturized:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

6. Skin feels more elastic/has more bounce back:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

7. Skin appears radiant/illuminated:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

8. Skin tone appears more even:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

9. Fine lines appear minimized/reduced:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completel

10. Do you have enlarged/visible pores?

____Yes
____No (go to 12)

11. Visible pores appear minimized/reduced:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

12. Wrinkles appear minimized/reduced:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

13. Skin appears healthier:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

14. Skin appears smoother:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

15. Overall, skin quality appears improved:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

16. Overall, skin feels improved:

O O O O O O O O O
Disagree Completely — Neither agree Nor Disagree — Agree Completely

17. Overall, how satisfied are you with the test product?

O O O O O O O O O
Extremely Dissatisfied — Neither satisfied Nor Dissatisfied — Extremely Satisfied

18. What, if anything, did you LIKE about the test product?

_____

_____

19. What, if anything, did you DISLIKE about the test product?

_____

TABLE 2.

Statistical Analysis of Changes in 9 Clinical Attribute Scores for Facial Skin, from Baseline to 3 Study Time Points: Immediately After the First Application of the Test Formulation (3 attributes scored), Week 4 and Week 8

The lower (more negative) the value of the mean change from baseline, the greater the improvement. Values in **boldface** are statistically significant with $P \leq 0.001$. Values for standard deviation of the mean (SD) are included.

Skin Attribute	Mean Change from Baseline (±SD)		
	Immediate	Week 4	Week 8
Overall skin brightness/radiance	—	**-0.71 ± 0.66**	**-1.17 ± 0.60**
Overall skin texture/smoothness	**-1.72 ± 1.08**	**-1.96 ± 0.84**	**-2.85 ± 0.75**
Skin tone clarity	—	**-0.47 ± 0.75**	**-1.08 ± 0.72**
Skin tone evenness	—	**-0.61 ± 0.69**	**-0.94 ± 0.67**
Pore appearance	**-0.52 ± 0.74**	**-0.87 ± 0.98**	**-1.29 ± 0.93**
Fine lines/wrinkles	—	**-0.26 ±0.57**	**-0.81 ± 0.69**
Overall skin firmness	—	**-0.93 ± 0.84**	**-1.82 ± 0.77**
Skin laxity/elasticity	—	**-0.97 ±0.91**	**-1.77 ± 0.80**
Overall appearance of skin	0.03 ± 0.38	**-0.38 ± 0.54**	**-1.02 ± 0.52**

evaluated immediately afterwards for skin dryness, texture/smoothness, pore prominence, and overall appearance. Objective and subjective tolerability assessments were also performed. Measurements of trans-epidermal water loss (TEWL) and skin conductance were obtained as described below. D-Squame adhesive tape specimens were collected and analyzed as described below.

After completing this baseline visit, subjects were provided with the cholesterol-dominant test formulation for use at home. They continued use of the cleanser twice daily, followed by application of the test formulation, and then the sunscreen every morning.

Subjects returned for a first follow-up visit at 4 weeks. After skin equilibration in the same manner as at the baseline visit, the same clinical evaluations, digital imaging, and tolerability assessments were performed. Measurements of trans-epidermal water loss and skin conductance were obtained as described below. D-Squame adhesive tape specimens were collected and analyzed as described below.

Subjects returned for a second and final follow-up visit at 8 weeks. All evaluations, imaging and assessments that had been performed at the week 4 visit were repeated at week 8. Measurements of trans-epidermal water loss and skin conductance were also obtained as described below. D-Squame adhesive tape specimens were collected and analyzed as described below. Subjects then exited the study.

Standardized Digital Imaging

Subjects had standardized digital photographs taken at the pre-treatment baseline, week 4, and week 8 time points using the VISIA-CR (Canfield Scientific, Fairfield, NJ) imaging system. Full frontal, left, and right profile views of the face were obtained, under visible (Standard 1, Standard 2), crossed-polarized, and parallel-polarized light.

Measurement of Transepidermal Water Loss and Stratum Corneum Hydration

At pre-treatment baseline, week 4 and week 8, each subject had measurements of transepidermal water loss (TEWL) and skin conductance on either the left or right side of the face, as determined by computer-generated randomization. TEWL measurements were taken in duplicate from the center of the cheek, using a Tewameter 300® (Courage-Khazaka, Köln, Germany). Conductance measurements, reflecting stratum corneum hydration, were taken in triplicate from the center of the cheek, using a Corneometer® CM 825 (Courage-Khazaka, Köln, Germany). To ensure standardization of sampling at baseline and at week 8, the site of testing on the cheek was a constant distance in millimeters from the angle of the mouth. TEWL data were expressed in $g/m^2/h$ and conductance data were expressed in arbitrary units (AU).

Analysis of Epidermal Lipids With D-Squame Adhesive Tape Extracts

D-Squame tape analysis was performed at pre-treatment baseline and at week 8, on the contralateral cheek to the one used for measurement of TEWL and skin conductance. To ensure standardization of site sampling, the distance in millimeters from the angle of the mouth to the center of the cheek was recorded and kept constant. The resultant tape samples were stored at -80°C prior to analysis.

A subset of 28 study subjects (20 aged 55 to 64 years and 8 aged 65 to 75 years) was randomly selected for epidermal lipid analysis. The number of subjects with Fitzpatrick skin

FIGURE 2. Standardized digital images of study subjects at pre-treatment baseline and week 8, showing typical study results. Improvements in skin tone, texture, radiance, and elasticity were apparent. (A) Subject 1 at baseline. (B) Subject 1 at week 8. (C) Subject 2 at baseline. (D) Subject 2 at week 8.

phototypes I, II, III and IV was 1, 12, 12, and 3, respectively. In these subjects, one D-Squame tape disc was placed in the center of the cheek for approximately 5 seconds, using the D500 D-Squame Pressure Instrument (CuDerm Corporation, Dallas, TX). The tape was removed with tweezers, and then a second tape disc was placed at the site with the same procedure and removed. Neutral lipids and free fatty acids were extracted from the paired D-Squame tape samples of each subject and pooled. These extracts were analyzed by gas chromatography (7890A Series GC System, Agilent Technologies, Santa Clara, CA) coupled to mass spectrometry (5975C series Inert XL EI/CI MSD system, Agilent Technologies). Ceramides were extracted and analyzed by high performance liquid chromatography (HPLC system Ultimate 3000, Thermo Scientific, Waltham, Massachusetts) coupled to mass spectrometry (MSQ+ detector, Thermo Scientific, Waltham, MA).

Analysis of Corneocyte Maturation via Double Staining of D-Squame Adhesive Tape Samples

Twenty-seven subjects (7 aged 55 to 64 years and 20 aged 65 to 75 years) were selected for analysis of corneocyte maturation. The number of subjects with Fitzpatrick skin phototypes I, II, III and IV was 6, 4, 14, and 3, respectively. One D-Squame tape was placed at the center of the cheek as described above, and then removed. The single D-Squame tape samples from each subject were stained with Nile Red to detect mature corneocytes, and with involucrin antibody to detect immature corneocytes. The resultant images were viewed by confocal microscopy. Ratios of mature to immature corneocytes were determined via confocal image analysis. (Apotome, Zeiss Axio-observer HXP120, Oberkochen, Germany).

The simultaneous identification of mature and immature corneocytes via a double staining protocol has been described previously. As keratinocytes migrate from the epidermal basal layer to the stratum corneum, they undergo a maturational process of differentiation that results in formation of the corneocyte lipid envelope.[14,15]. A fully mature corneocyte lipid envelope is hydrophobic. Therefore, it will stain with nile red, but not with involucrin antibody because the involucrin epitope is inaccessible due to the high degree of cross-linking of the envelope.[16] In contrast, an immature corneocyte envelope will stain solely with involucrin antibody because of its lack of cross-linking.

Subject Self-Assessment via Questionnaires and Diaries

Study subject questionnaires were administered and subjects were instructed to keep diaries to aid in efficacy assessment of the test formulation (Figure 1).

Forearm Skin Studies

Assessment of Skin Barrier Recovery Following Tape Stripping

Twenty-seven female subjects aged 55 to 73 years (mean age, 61.74) were enrolled, and sequestered in a climate-controlled environment for the 8-hour duration of this study. Temperature was 21 degrees Celsius ± 1° and relative humidity was 45% ± 5%. Baseline TEWL prior to tape stripping was measured as an indicator of skin barrier function, on both volar forearms of each subject with a DermaLab Evaporimeter (Cortex Technology, Hadsund, Denmark). Epidermal damage was induced by repeated tape stripping on both forearms (Office Depot brand) until TEWL values exceeded 20 g/m^2/hr. The TEWL at this stage was measured and recorded as the initial, post-stripping TEWL. The cholesterol-dominant test formulation was then applied to one randomly selected forearm of each subject. TEWL was measured 1 hour, 2 hours, 4 hours, and 8 hours later on both treated and untreated forearms.

TABLE 3.

Mean Changes from Pre-treatment Baseline in Transepidermal Water Loss (TEWL) as Measured by Tewameter, and in Skin Conductance as Measured by Corneometer

Mean changes are documented at week 4 and week 8. The lower the Tewameter readings and the higher the Corneometer readings, the greater the improvements in TEWL and skin conductance respectively. *P* values are also shown at weeks 4 and 8. (s) indicates statistical significance.

Instrument	Mean Change from Baseline (±SD)		*P* value	
	4 Weeks	8 Weeks	4 Weeks	8 Weeks
Tewameter 300	-0.47 ± 3.37	-1.69 ± 3.38	0.3067	0.0004 (s)
Corneometer	11.94 ± 15.17	10.88 ± 12.51	<0.0001 (s)	<0.0001 (s)

(s) = significant.

Efficacy of the cholesterol-dominant test formulation for skin barrier repair was assessed by comparing TEWL values on treated versus untreated forearms at each post-stripping time point with the initial, post-stripping TEWL measurements.

Assessment of Skin Barrier Protection via Pre-Treatment Followed by Tape Stripping

Twenty-six female subjects aged 55 to 75 years (mean age, 63.88) were enrolled in this study. They applied the cholesterol-dominant test formulation twice daily for 1 week to a 4x4 cm site on one, randomly-selected volar forearm. The untreated forearm served as a control. TEWL was measured on both forearms using a DermaLab Evaporimeter at pre-treatment baseline. Treated and untreated skin sites were then perturbed by 12 consecutive cycles of application and removal of packaging tape strips (Office Depot brand). After 15 minutes, TEWL was measured again at each site. The efficacy of the cholesterol-dominant test formulation in protecting the skin barrier was evaluated by comparing TEWL values at the treated sites versus the untreated sites following tape stripping. These data were correlated with the pre-tape stripping TEWL values after 1 week of test formulation use.

TABLE 4.

Transepidermal Water Loss (TEWL) from Treated and Untreated Forearm Skin Before and After Treatment With Cholesterol-Dominant Test Formulation

The lower (more negative) the mean change from post-tape stripping baseline, the greater the improvement in skin barrier function. Recovery was calculated as the TEWL at each time point divided by the TEWL at the pre-tape stripping baseline, and expressed as a percentage. Boldface indicates statistical significance with $P \leq 0.001$ for differences between baseline and post-tape stripping values, and for differences between values for treated vs untreated sites.

Time Point	Forearm Skin Site	Mean TEWL (± SD) (g/m²/hr)	Mean Change in TEWL from Post-tape stripping Baseline (± SD)	Mean % Change in TEWL from post-tape stripping Baseline	Recovery as a % of pre-tape stripping Baseline
Baseline	Treated (Pre-tape stripping)	3.82 ± 1.08			
	Untreated (Pre-tape stripping)	3.61 ± 1.17			
	Treated (Post-tape stripping)	23.55 ± 1.70			
	Untreated (Post-tape stripping)	24.09 ± 1.76			
1 hour	Treated	7.31 ± 1.51	-16.24 ± 1.76	**-68.97%**	**-82.34%**
	Untreated	13.43 ± 2.61	-10.66 ± 2.69	**-44.26%**	**-52.05%**
2 hours	Treated	5.84 ± 1.18	-17.71 ± 1.57	**-75.20%**	**-89.77%**
	Untreated	9.59 ± 2.11	-14.51 ± 2.25	**-60.22%**	**-70.82%**
4 hours	Treated	5.38 ± 1.05	-18.17 ± 1.59	**-77.17%**	**-92.12%**
	Untreated	8.18 ± 1.81	-15.91 ± 1.75	**-66.04%**	**-77.67%**
8 hours	Treated	4.86 ± 0.92	-18.70 ± 1.63	**-79.38%**	**-94.76%**
	Untreated	7.34 ± 1.56	-16.75 ± 1.86	**-69.52%**	**-81.76%**

Bold indicates statistical significance with $P \leq 0.001$.

TABLE 5.

Transepidermal Water Loss (TEWL) from Treated and Untreated Forearm Skin, at Baseline, Prior to Tape Stripping, and After Tape Stripping

Baseline values are prior to treatment. Week 1 pre-tape stripping values are after the treated skin had received twice-daily application of the test formulation for 1 week, with the untreated skin serving as a control. Lower (more negative) TEWL values indicate better skin barrier function.

Time Point	Foream Skin Site	Mean TEWL (±SD)	Mean change in TEWL from Baseline*	Mean % change in TEWL from Baseline**	Mean change in TEWL from Pre-Tape Stripping value*	Mean % change in TEWL fromPre-Tape Stripping value**	Difference between Mean % changes in TEWL for treated and untreated skin
Baseline	Treated	5.13 ± 2.66					
	Untreated	4.53 ± 2.20					
Week 1 Pre-Tape Stripping	Treated	4.08 ± 2.45	-1.05 ± 0.49	-20.41%			**-20.58%**
	Untreated	4.54 ± 2.16	0.01 ± 0.24	0.17%			
Week 1 Post-Tape Stripping	Treated	5.26 ± 3.20			1.18 ± 1.43	28.93%	**-30.64%**
	Untreated	7.24 ± 3.99			2.70 ± 2.35	59.58%	

*Mean (week 1) – Mean (baseline).
**Mean (week 1) – Mean (baseline) X 100/Mean (baseline).
Boldface indicates statistical significance with $P<0.05$.

Assessment of Stratum Corneum Hydration

Twenty-seven female subjects aged 55 to 75 years (mean age, 61.74) were enrolled, and sequestered in a climate-controlled environment for the first 8 hours of this study. Temperature was 21 degrees Celsius ± 1° and relative humidity was 45% ± 5%. Conductance was measured on treated and untreated sites on volar forearms using a Corneometer (Courage-Khazaka, Köln, Germany) at baseline and 15 minutes, 1 hour, 8 hours, and 24 hours after initial cholesterol-dominant test formulation application.

Statistical Analysis of Data

All subjects who completed the facial or forearm skin studies were included in the statistical analysis. Data were tested for normal distribution with the Shapiro-Wilk test, using a cut-off value of $P<0.01$ for efficacy, tolerability, and questionnaire data. Efficacy and tolerability of the cholesterol-dominant test formulation were evaluated by comparing the post-treatment data to the pre-treatment baseline data for each attribute. The significance of changes was determined using either a paired t-test for normally distributed data, or a Wilcoxon signed rank test for non-normal data. The cutoff value for significance was $P<0.05$. Instrumental data were evaluated similarly. The mean percent changes from pre-treatment baseline were reported at each time point for each attribute.

RESULTS

Facial Skin Study

59 subjects were enrolled in this study and 55 completed it. Of the 4 subjects who exited the study before completion, 2 were non-compliant with follow-up visits, 1 took an anti-inflammatory medication that was prohibited during the study, and 1 left due to a new diagnosis of diabetes mellitus.

Clinical Evaluation of the Cholesterol-Dominant Test Formulation

Figure 3 shows mean scores for the nine clinical skin attributes that were used to assess efficacy of the test formulation, at pre-treatment baseline, week 4, and week 8. Table 2 presents a statistical analysis of the changes in mean scores, from baseline to immediately after first application of the test formulation (3 attributes were scored at this time), week 4, and week 8. Statistically significant improvements from baseline were observed at both the week 4 and week 8 study time points, for all nine clinical attributes that served as indices of efficacy.

Tolerability and safety of the cholesterol-dominant test formulation were determined objectively by blinded evaluator assessments of skin dryness, erythema, peeling, and edema, immediately after the first application of the test formulation, and at weeks 4 and 8. Tolerability and safety were also determined subjectively by patient assessments of itching, tingling, stinging, and burning. Skin erythema was significantly decreased at week 8 of treatment with the test formulation. The other objective indices of tolerability were unchanged, indicating that the test formulation did not produce skin irritation or inflammation. There were no treatment-related adverse effects during the studies.

Standardized Digital Imaging

Figure 2 shows standardized digital images for two patients at pre-treatment baseline and after 8 weeks of treatment with the cholesterol-dominant test formulation. Improvements in skin tone, texture, radiance and elasticity were observed.

Transepidermal Water Loss (TEWL) and Skin Conductance

Table 3 shows the mean changes from pre-treatment Baseline to weeks 4 and 8 in TEWL and skin conductance. As described

TABLE 6.

Changes in Stratum Corneum Lipid Biomarkers at Baseline and After 8 Weeks of Treatment With the Cholesterol-Dominant Test Formulation

Epidermal Lipid Biomarker	Pre-Treatment Baseline (Mean ± SD)	Week 8 (Mean ± SD)	Mean Change from Baseline (±SD)	Mean % Change from Baseline
Total Ceramides	11.75 ± 1.51	18.39 ± 1.80	**6.64 ± 0.88**	**57%**
Ceramide 3 [NP]	0.85 ± 0.12	4.71 ± 0.42	**3.87 ± 0.42**	**457%**
Ceramide 5 [AS]	5.17 ± 0.62	6.43 ± 0.82	**1.26 ± 0.43**	**24%**
Ceramide 2 [NS]	5.74 ± 0.88	7.25 ± 1.19	**1.51 ± 0.58**	**26%**
Cholesterol	4.04 ± 1.33	6.06 ± 2.48	**2.02 ± 1.84**	**50%**
Triglycerides	37.84 ± 6.19	42.25 ± 5.42	**4.41 ± 5.03**	**12%**

Boldface indicates statistical significance with P≤0.001.

in the Materials and Methods above, TEWL was measured by Tewameter, and skin conductance by Corneometer. There were statistically significant improvements in TEWL at week 8, and in skin conductance at weeks 4 and 8.

Forearm Skin Studies

Skin Barrier Recovery following Tape Stripping

Table 4 shows TEWL values for treated vs. untreated sites on the forearms, in comparison to TEWL values at baseline, before and after tape stripping. There were no significant differences in the TEWL values of treated and untreated sites at baseline, either before or after tape stripping. This indicated no significant variations between the treated and untreated sites prior to product application. At the time points following tape stripping and product application, TEWL values decreased progressively for both treated and untreated sites, reflecting progressive improvements in skin barrier function. TEWL values for treated sites were significantly lower than those for untreated sites at all time points. This indicates that sites treated with the cholesterol-dominant test formulation recovered skin barrier function at a significantly faster rate than untreated sites after tape stripping.

Assessment of Forearm Skin Barrier Protection via Pre-Treatment Followed by Tape Stripping

Table 5 presents TEWL values for treated and untreated forearm skin at pre-treatment baseline, at week 1 pre-tape stripping after the cholesterol-dominant test formulation had been applied twice daily for 7 days to the treated forearms, and at week 1 post-tape stripping after both treated and untreated forearms had been subjected to skin barrier disruption via tape stripping. The week 1 pre-tape Stripping TEWL for treated skin was significantly decreased, by 20.58% in comparison to untreated skin, indicating an improvement on skin barrier function with the treatment of the test formulation. While TEWL values increased as expected after tape stripping for all forearms, there was a 30.64% smaller increase for treated in comparison to untreated skin, which was statistically significant. This indicates that the test formulation provided a protective effect after 1 week of use.

Analysis of Epidermal Lipids

Table 6 shows changes in epidermal lipid biomarkers based on D-Squame tape analysis. In comparison to pre-treatment baseline, there was a significant increase at week 8 in total ceramide content, and in cholesterol and triglyceride levels. Further analysis indicated a significant increase in the specific content of ceramide 3 (NP), ceramide 2 (NS) and ceramide 5 (AS).

Analysis of Corneocyte Maturation

Table 7 shows changes in the ratio of markers for mature vs. immature corneocytes, based on D-Squame tape analysis. In comparison to pre-treatment baseline, there was a significant increase in the mean ratio of Nile Red to involucrin antibody staining at week 8 ($P<0.001$), indicating an increase in the number of mature corneocytes relative to immature ones.

Figure 4 shows images of D-Squame tape strip samples after double-staining for mature and immature corneocytes. The images visually depict the increase in the ratio of mature to immature corneocytes at week 8, in comparison to baseline (Table 7).

DISCUSSION

Data from these studies demonstrate that the cholesterol-dominant test formulation significantly improved baseline skin barrier function after only 1 week of twice-daily application, with sustained improvements throughout the course of the 8-week study. In a clinical model of skin barrier repair, skin treated with the test formulation achieved a significantly more rapid and greater recovery of barrier function than untreated skin. When the test formulation was applied for 1 week prior to skin injury by tape stripping, it provided significant protection against skin barrier damage.

Lipid analysis showed that the test formulation induced significant increases in the epidermal content of total ceramides,

TABLE 7.

Ratios of Mature to Immature Corneocytes at Baseline and After 8 Weeks of Treatment With the Cholesterol-Dominant Test Formulation

Mature corneocytes were stained with Nile red, and immature corneocytes with involucrin antibody, in a double-staining protocol.

Parameter	Time Point	Test Product				
		Number of Subjects (n)	Mean Ratio (±SD)	Mean Change from Baseline (±SD)	Mean % Change from Baseline	*P* value
Ratio of mature to immature corneocytes (Nile Red: Involucrin antibody staining)	Pre-treatment Baseline	26	0.51 ± 0.65			
	Week 8	26	1.56 ± 2.01	1.05 ± 1.84	200.00%	<0.001

cholesterol and triglycerides after 8 weeks of treatment. The increase in ceramide 3 was substantial, and presumably reflects the high concentration of ceramide 3 in the test formulation itself. It is noteworthy that there were also significant increases in ceramides 2 and 5, although these isoforms are not present in the test formulation. This may be indicative of a broader stimulation of ceramide synthesis.

A healthy, normally functioning skin barrier is dependent on overall lipid content, which is known to decrease in chronologically aged skin,[7] and also on an appropriate ratio of physiological lipids. The cholesterol-dominant test formulation may be of value in reinforcing and even repairing the stratum corneum architecture, by replenishing cutaneous lipids that have been lost during skin aging. Support for the concept that the test formulation can actually mediate structural normalization of the aging skin barrier is provided by the study data demonstrating a twofold increase in the ratio of mature to immature corneocytes after 8 weeks of treatment.

The test formulation had excellent tolerability and safety profiles, even in subjects who self-identified as having sensitive skin. One of the authors (HS) has found it to be an appropriate and efficacious intervention for all aging skin, and also for other dermatoses that are characterized by a dysfunctional skin barrier, such as rosacea.

CONCLUSION

This pilot evaluation provides a proof-of-concept for the novel cholesterol-dominant physiological lipid formulation as an active agent for repair and protection of the skin barrier. From an evidence-based perspective, the test formulation was demonstrated to have significant utility for aging skin. Its clinical benefits included significant improvements in skin tone and texture, radiance, clarity, fine lines and wrinkles, firmness, laxity, appearance of pores, and global appearance. The evidence level of these observations would be further optimized by longer term, vehicle-controlled studies, and by comparison of cholesterol-dominant and ceramide-dominant formulations.

The far-reaching structural, functional, and metabolic consequences of barrier dysfunction in aging skin have been discussed previously.[17] Logically, the resultant increase in TEWL, and decrease in water content of the epidermis and dermis, can be ameliorated most effectively by primary repair

FIGURE 3. Mean scores of 9 clinical skin attributes (A,B,C), as indices of efficacy of the cholesterol-dominant test formulation at pre-treatment baseline, and at weeks 4 and 8 after initiating the twice-daily regimen to the face. The lower the score on the ten-point scales, the greater the improvement. Vertical bars show values for standard error of the mean.

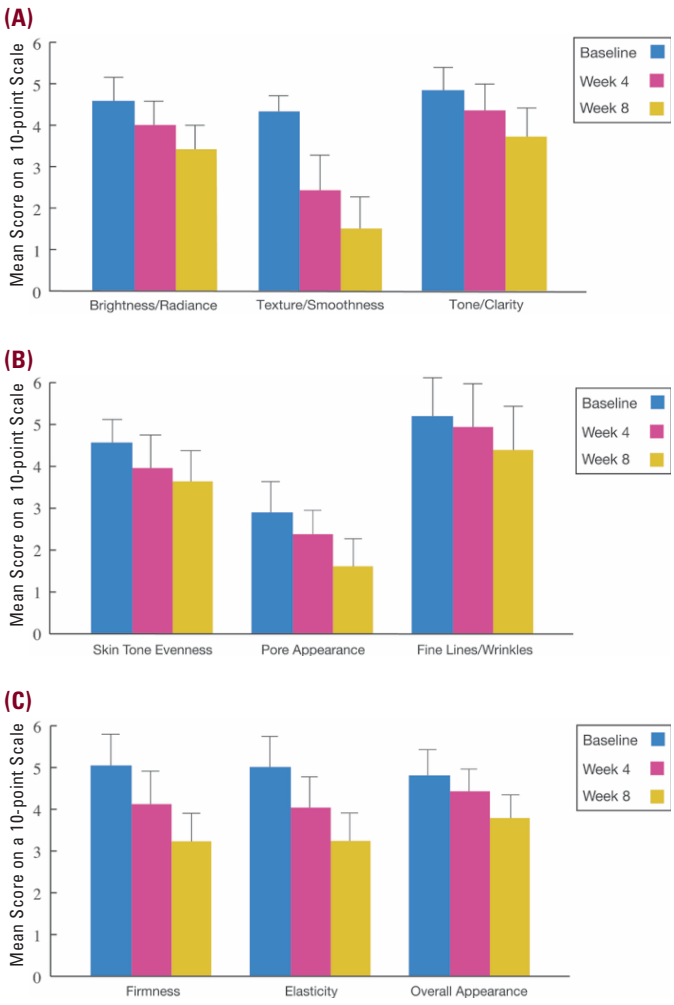
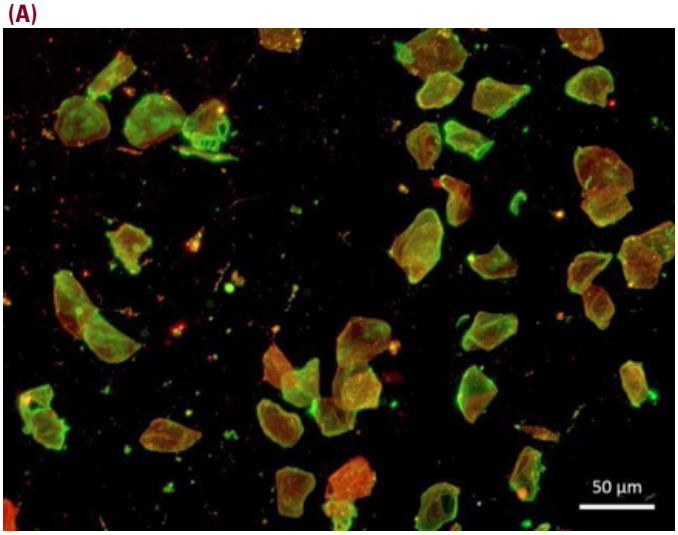
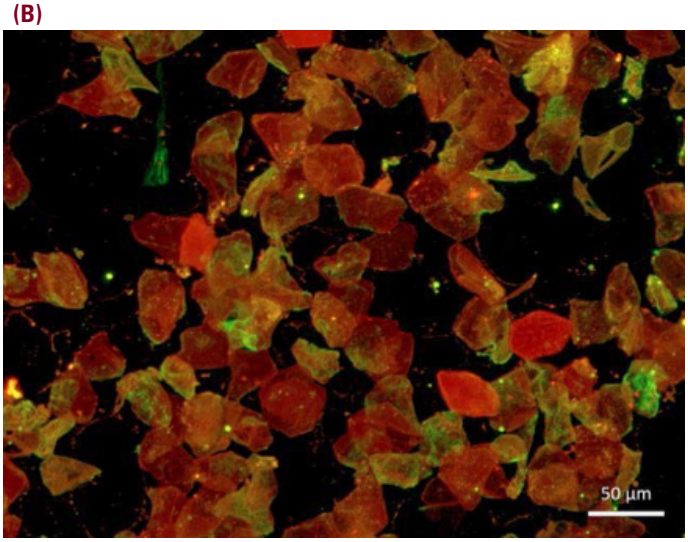


FIGURE 4. Images of D-Squame tape strip samples from the cheek at pre-treatment baseline (A) and week 8 (B), after double-staining for mature and immature corneocytes. Mature corneocytes are stained with Nile red, while immature corneocytes are stained green with involucrin antibody. At baseline, most corneocytes stained green, indicating that they are immature. At week 8 most corneocytes stained red, indicating an increase in in maturation after treatment with the cholesterol-dominant test formulation.

(A)



(B)



of the skin barrier. Primary barrier repair may be considered a pre-requisite for aging skin to tolerate, respond to and recover optimally from all aesthetic procedures and other topical interventions.[17]

As discussed above, barrier repair in aging skin has been accomplished to date with ceramide-dominant lipid mixtures that have been extrapolated from use in atopic dermatitis to aging skin. The cholesterol-dominant formulation that is described in this publication represents a new paradigm of treatment. Its objective is to correct the specific deficits and imbalances of epidermal lipids in aging skin, and thereby to address directly the underlying causes of its impaired barrier integrity and function. From a broader perspective, this individualization of therapy reflects an evolving trend for individualization of all aesthetic interventions, based on growing appreciation of the better clinical outcomes that are achieved when underlying, primary etiologies are addressed rather than their secondary effects.[18]

The studies presented in this publication raise some intriguing questions regarding the nuances of skin barrier repair and protection. Recent research has revealed that the skin barrier is not a static structure but, rather, in a state of constant, dynamic, homeostatic activity. Domains of particular plasticity reside within the extracellular lipid matrix, which is the principal barrier to the passage of water and other molecules across the stratum corneum.[19] Further investigations may help to clarify the mechanisms by which topical application of specific ceramides can stimulate synthesis of unrelated ceramide isoforms. This would provide deeper insight into the complex feedback loops and regulatory mechanisms that control the synthesis, turnover and balance of physiological lipids in the epidermis.

DISCLOSURES

Dr. Sundaram serves as a Clinical Investigator and/or Consultant for cosmeceuticals for Allergan/SkinMedica, Biopelle, Frezyderm, SkinCeuticals, Suneva, and Teoxane.

Ana Du, Margarita Yatskayer, Stephen Lynch, Christian Oresajo are employed by L'Oreal Research and Innovation. Yevgeniy Krol was previously employed by SkinCeuticals.

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