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Six Amino Acids among Natural Moisturizing Factors Responsible for Skin Hydration: Improvement and Anti-Aging of Skin by *Galactomyces* Ferment Filtrate-Pitera™ Containing Skin Moisturizer

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Abstract

Background: Natural moisturizing factors (NMFs) are filaggrin-derived components in the cornified layer that are critical for maintaining healthy skin moisturization and barrier function. However, studies have reported conflicting findings on the relationship between NMF levels and aging, while few studies have investigated this relationship clinically. To fill this research gap, we determined the levels of major NMF components such as free amino acids, pyrrolidone carboxylic acid, and urocanic acids, and individually verified their relationships with skin hydration, barrier function, age, and skin aging. **Purpose:** The objective of this study was to clinically investigate the relationship between NMF components' levels and skin aging in facial skin. The main NMF components were obtained from facial skin and quantified. We then selected NMF components showing strong relationships to skin hydration, and analyzed the relationships of the levels of these selected NMF components with signs of skin aging, namely, texture, pores, wrinkles, and dullness (L-value). We also examined the efficacy of treatment with a skin care formula (SK-II Facial Treatment Essence, called SK-II FTE hereafter) including *Galactomyces* ferment filtrate (GFF, Pitera™) on the selected NMF component levels associated with skin hydration and barrier function, and the signs of skin aging of texture, pores, wrinkles, and dullness (L-value). **Method:** We conducted two clinical trials in this research. In Study 1, we measured 23 NMF components using tape-stripped cornified layer to quantify them via an

HPLC method in 196 Asian females aged 20 to 59 (mean ± S.D., 38.6 ± 9.4). Facial visual aging parameters [texture, pores, wrinkles, and dullness (L-value)], as well as elasticity (R7), skin hydration, and TEWL, were quantified using facial skin imaging and skin physical property measurement devices. Study 2 was performed to evaluate whether the facial application of SK-II FTE affects the NMF levels and skin aging parameters in 63 Asian female volunteers aged 20 to 55 (38.4 ± 9.03). During the course of Study 2, 0.6 mL of SK-II FTE was applied to the face twice daily in the morning and afternoon. Skin measurements were performed at the start of the day (baseline) and at week 8. **Results:** In Study 1, we examined the stratum corneum levels of 23 NMF components comparing to the skin hydration status in 196 female subjects. The subjects were divided into two groups using the median of each measured NMF component. Skin hydration values were compared between the two groups defined for each NMF component. The results showed that subjects with higher levels of six amino acids, alanine, arginine, asparagine, glutamine, glycine, and histidine, exhibited significantly higher skin hydration than those with lower amino acid levels. No significant differences in skin hydration values were found for the other 17 NMF components. We then analyzed whether the sum of these six amino acid NMF components (called 6-AA-NMFs, hereafter) is affected by aging. The 6-AA-NMF level peaked in the subjects aged 25 - 29, and then gradually and significantly decreased with age. Interestingly, the 6-AA-NMF level was significantly correlated with the skin hydration value, but not with TEWL. In addition, the 6-AA-NMF level demonstrated significant correlations with the signs of skin aging of texture, pores, wrinkles, and dullness (L-value). Then, in Study 2, we examined whether the daily application of SK-II FTE affects the 6-AA-NMF level and visual aging parameters in 63 females. SK-II FTE demonstrated significant increases of the levels of 6-AA-NMFs and each of its components associated with hydration and barrier function, and improvements of skin texture, pores, wrinkles, and dullness (L-value) during the 8 weeks of treatment of facial skin. **Conclusion**: These clinical studies with large numbers of subjects across a wide age range revealed that six amino acids as NMF components were highly correlated with facial skin hydration in the stratum corneum. The levels of these six NMF components were also found to decrease at ages after the 30 s and were significantly correlated with major signs of skin aging. Notably, these six NMF components (6-AA-NMFs) were increased by SK-II FTE treatment associated with improvements of skin hydration and signs of skin aging, namely, texture, pores, wrinkles, and dullness (L-value). These studies were limited by the lack of investigation of why some NMF components were not associated with skin hydration. More clinical trials examining various NMF components and their relationship with aging are anticipated.

Keywords

NMF, Amino Acid, Visual Aging Parameter, Hydration, Aging, Texture, Pore, Wrinkle, Dullness, *Galactomyces* Ferment Filtrate, Pitera™, SK-II Facial Treatment Essence

1. Introduction

The skin is a vital organ that protects the bodies of terrestrial animals from the effects of dry harsh environments [1] [2]. Skin hydration is a primary biological function to maintain the healthy skin, and varies by the intrinsic and extrinsic factors such as keratinocyte differentiation and proliferation, and life style and body dependent factors [3] [4]. The barrier function of human skin is mainly provided by its outermost epidermal layer, the stratum corneum or cornified layer [1] [2]. Corneocytes are the major components of the stratum corneum. However, other biological materials such as extracellular lamellae of lipids and various natural moisturizing factors (NMFs), including free amino acids, pyrrolidone carboxylic acids, and urocanic acids, are pivotal in maintaining a healthy skin-water balance [1] [2] [4]. In parallel with this, the level of NMFs is decreased in the dry skin of patients with atopic dermatitis [5] [6] [7]. During the differentiation process, keratinocytes sequentially produce epidermal differentiation complex proteins such as filaggrin [8]. The degradation of filaggrin by proteolytic enzymes, such as caspase-14, is essential for the production of NMFs [1] [2] [8] [9] [10]. *Galactomyces* ferment filtrate (GFF) is known to increase the expression of filaggrin and caspase-14 [11] [12] [13]. It was also reported that the daily application of GFF-containing moisturizer increased the hydration of facial skin [14] [15], while also alleviating or completely restoring mask-induced skin damage [15].

Conflicting results have been reported on the relationship between skin aging and stratum corneum NMF levels [16] [17] [18]. The intensity and speed of the aging process differ markedly between individuals [19]. Aging of facial skin is generally evaluated using visual aging parameters, such as rough texture, pore dilation, hyperpigmented spots, wrinkles, dull skin tone (L-value), and decreased elasticity [19] [20] [21]. Dry skin is also associated with facial skin aging, which is assessed by identifying decreased skin hydration and increased transepidermal water loss (TEWL) [19] [20] [21]. However, it remains unknown whether the NMF levels are correlated with these skin aging parameters.

In this study, we measured the levels of NMFs (20 amino acids, 2-pyrrolidone carboxylic acid, trans-urocanic acid, and cis-urocanic acid) of tape-stripped facial cornified middle layer, as well as visual and hydration-related parameters of skin, in 196 female volunteers of various ages. Notably, the group with high hydration showed significantly higher levels of six amino acids (alanine, arginine, aspartic acid, glutamine, glycine, and histidine) than the group with low hydration. It was reported these six (6) amino acids are about 20% of all measured NMF components, that are the second largest NMF component group followed by trans and cis-urocanic acid (about 30%) [22]. The total sum of these six amino acids peaked in subjects aged 25 - 29 and then significantly decreased with aging. Moreover, the total sum of these six amino acids was significantly correlated with visual aging parameters, namely, rough texture, pore dilation, wrinkles, dullness, and decreased elasticity. Furthermore, significant improvements in the levels of these six amino acids and visual aging parameters were observed after 8 weeks of daily application of GFF (Pitera™)-containing moisturizer, SK-II Facial Treatment Essence (SK-II).

2. Materials and Methods

2.1. Study 1 and Study 2

In Study 1 (CT22002-S), we measured 23 NMF components using tape-stripped cornified layer by reverse-phase high-performance liquid chromatography (HPLC) in 196 Asian female subjects aged 20 to 59 (mean ± S.D., 38.6 ± 9.4). We also assessed facial visual aging parameters [texture, pores, wrinkles, and dullness (L-value)], as well as elasticity (R7), skin hydration, and TEWL.

Skin hydration was measured using a Corneometer® CM825 (Courage + Khazaka Electronic GmbH, Cologne, Germany). TEWL was measured using a Vapometer® (Keystone Scientific, Kuopio, Finland). Skin mechanical elasticity (R7, Ur/Uf) was measured with a Cutometer (Courage + Khazaka).

Study 2 (CT22002-F) was performed to evaluate whether the facial application of SK-II FTE affects the NMF levels and skin aging parameters in 63 Asian female volunteers aged 20 to 55 (38.4 ± 9.03). During the course of Study 2, 0.6 mL of SK-II FTE was applied to the face twice daily in the morning and afternoon. Skin measurements were performed at the start of the day (baseline) and in week 8.

The two study protocols were approved by the Ethical Committee of Global Product Stewardship at P & G Innovation Godo Kaisha. Written informed consent was obtained from all subjects prior to their enrollment in each study.

2.2. Analysis of NMF Components Using Tape-Stripped Cornified Layer

The tape-stripped cornified middle layer was obtained four times from the same site on the left cheek using 2.2 cm D-squam tape in each subject. The third tape-stripped cornified layer was used for the analysis of 23 NMF components, namely, alanine, arginine, aspartic acid, glutamine, glycine, histidine, asparagine, glutamic acid, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, as well as 2-pyrrolidone carboxylic acid, trans-urocanic acid, and cis-urocanic acid, by reverse-phase HPLC using a Waters Atlantis T3 column (2.1 × 50 mm, 3-μm particles). Detection and quantitation were carried out by tandem mass spectrometry operating under multiple reaction monitoring conditions. Calibration standards of known NMF components were used for qualification control. The nominal range of quantitation was 20 to 20,000 ng/tape strip for the NMF components. The concentration of each NMF component was determined in acid extract and then converted into mass/strip by multiplying by the extraction volume. The assay required the total protein content found on the tape strips and extracts to be determined and the final results were reported as total mass of analyte (ng or μg) per total weight of protein (μg) per sample. Specimen concentrations were determined by back-calculation using Wagner or weighted (1/×2) quadratic regression of a calibration curve generated from spiked matrix standards, following normalization by the molecular weight of each NMF component and the area of the stripped tape (3.8013 cm^2).

2.3. Facial Optical Imaging

The subjects washed their faces using the prescribed cleansing foam and then spent 15 min becoming accustomed to the environment of the measurement room, which was maintained at a constant temperature and humidity (room temperature 20˚C ± 2˚C, relative humidity 50% ± 5%). None of the subjects underwent any type of esthetic treatment such as laser cosmetic procedures during the study period. Each subject's face was photographed using a portable image capture system (Magic Scan) illuminated by a number of 5600-K light-emitting diodes mounted on both left and right sides of the system (**Figure 1**) [23]. A high-resolution, complementary-symmetry, metal–oxide–semiconductor digital camera, capable of generating 1980 (vertical) × 1024 (horizontal) effective picture elements (pixels), was also mounted in the imaging module. A series of deep-learning-based post-calibration algorithms was carried out to adjust to the consistent brightness, hue, and saturation after capturing the facial images. This enabled the captured images to be controlled to ensure reproducible collection under the different external optical conditions. A neutral 8.0 gray color board (GretagMacbeth GmbH, Munich, Germany) was used for white balancing of the camera.

Figure 1. Magic Scan facial imaging system. Each subject's face was photographed using Magic Scan.

2.4. Objective Image Analysis for Texture (Roughness), Wrinkles, Pores, and Dullness (L)

The region of interest (ROI) of the images was from the outer edge of the eye to the cheek, and the following characteristic objects were extracted by measuring the contrast in the shape and pixels using an image analysis algorithm [15] [19]. Wrinkles were defined as ≥5 mm in length, perimeter/length ratio ≤ 2.5, and circularity (perimeter^2/area) ≥ 34. Total wrinkle area fraction was quantified as follows: total wrinkle area (pixels)/ROI (pixels). As an index of skin surface roughness (texture), total texture area fraction [total texture area (pixels)/ROI (pixels)] was quantified as ≤3 mm^2 in area, aspect ratio ranging from 0.5 to 2, and color contrast delta E ≥ 1.5. Facial skin dullness (L-value) was also measured in the ROI. Pores were detected using an image analysis algorithm to detect circular pore-like shapes via edge-enhanced binary images at the cheek region. The total area of detected pores was then calculated.

2.5. Statistical Analysis

All statistical analyses were performed using JMP® Pro 16.1.0 (SAS Institute Inc., Cary, NC, USA). For each outcome variable, data were analyzed by fitting a linear mixed-effects model with timepoint as the fixed effect and subject as the random effect to account for within-subject variation. Pearson's correlation coefficients (r) between the following variables were examined: between NMF components and chronological age, skin hydration, TEWL, elasticity, and four skin optical parameters [image analysis data on texture, wrinkles, pores, spots, and dullness (L)] in Study 1. Quantitative comparisons were also performed for the following variables: 1) skin hydration of the two groups with values above or below the median of each NMF parameter in Study 1, and 2) NMF parameters and image analysis data on roughness (texture), wrinkles, spots, and skin tone (L), as well as hydration, elasticity, and TEWL at the baseline versus at week 8, by the treatment of SK-II FTE, a GFF-containing skin care formula in Study 2 using two-way ANOVA. A P-value of less than 0.05 was considered to reflect statistical significance.

3. Results

As NMF components play a crucial role in maintaining skin hydration [22] [24] [25], in this study we first examined which NMF components are significantly correlated with skin hydration status. We analyzed the associations with skin hydration of the stratum corneum levels of 23 NMF components by dividing the 196 female subjects into two groups using the median value of each NMF component. As shown in **Figure 2**, significant differences in skin hydration were evident for six amino acids, alanine, arginine, asparagine, glutamine, glycine, and histidine. Subjects with higher levels of these amino acids exhibited significantly higher skin hydration than those with lower amino acid levels. The other 17 NMF components did not show significant associations with skin hydration.

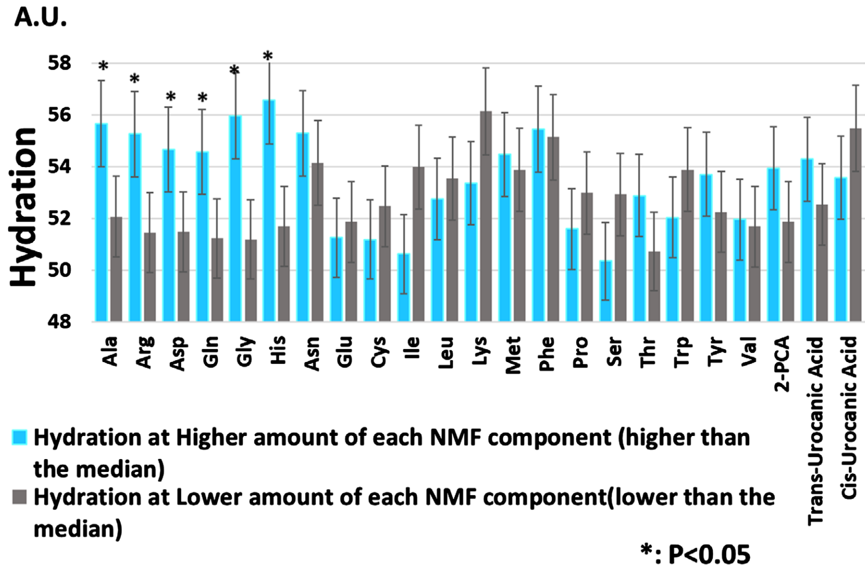

Figure 2. Comparison of skin hydration between the two groups divided by the median of each NMF component. Stratum corneum levels of 23 NMF components in 196 female subjects were measured.

We then analyzed whether the sum of these six amino acid NMF components (6-AA-NMFs) is affected by aging. The 6-AA-NMF level peaked in subjects aged 25 - 29, and then gradually and significantly declined with age (Figure 3, Table 1).

Interestingly, the 6-AA-NMF levels were significantly correlated with the skin hydration values, but not with TEWL (Figure 4, Table 1).

In addition, the 6-AA-NMF levels were significantly correlated with visual skin aging parameters, namely, texture, pores, wrinkles, skin tone (L-value), and elasticity (Figure 5).

We next examined whether the daily application of SK-II FTE affects the 6-AA-NMF level and visual aging parameters in Study 2. SK-II FTE significantly increased the 6-AA-NMF level, and the levels of each of the six amino acids that make up this variable (alanine, arginine, asparagine, glutamine, glycine, and histidine) after the 8 weeks of facial treatment (Figure 6). Skin hydration and elasticity and the skin aging parameters of texture, pores, wrinkles, and dullness (L-value) were also significantly improved in association with the increase in 6-AA-NMFs (Figure 7 and Figure 8).

4. Discussion

NMFs principally comprise hygroscopic amino acids and derivatives that absorb moisture from the surrounding environment, and then serve as the primary humectants of the cornified layer. In this study, by identifying six amino acids among NMF components that were directly related to *in vivo* stratum corneum water content, we were able to demonstrate the relationships of these components with age and skin aging. Other NMF-related studies have reported that the

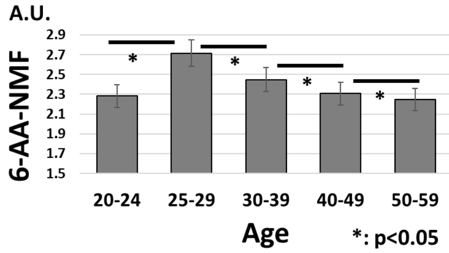

Figure 3. The 6-AA-NMF levels in subjects aged 20 - 24, 25 - 29, 30 - 39, 40 - 49, and 50 - 59.

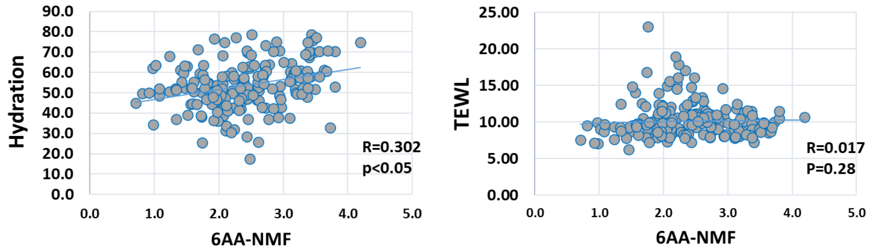

Figure 4. Correlations of the 6-AA-NMF level with skin hydration and TEWL. Study 1, N = 196 Asian females aged 20 - 59 (ave. age 38.6, S.D. 9.40).

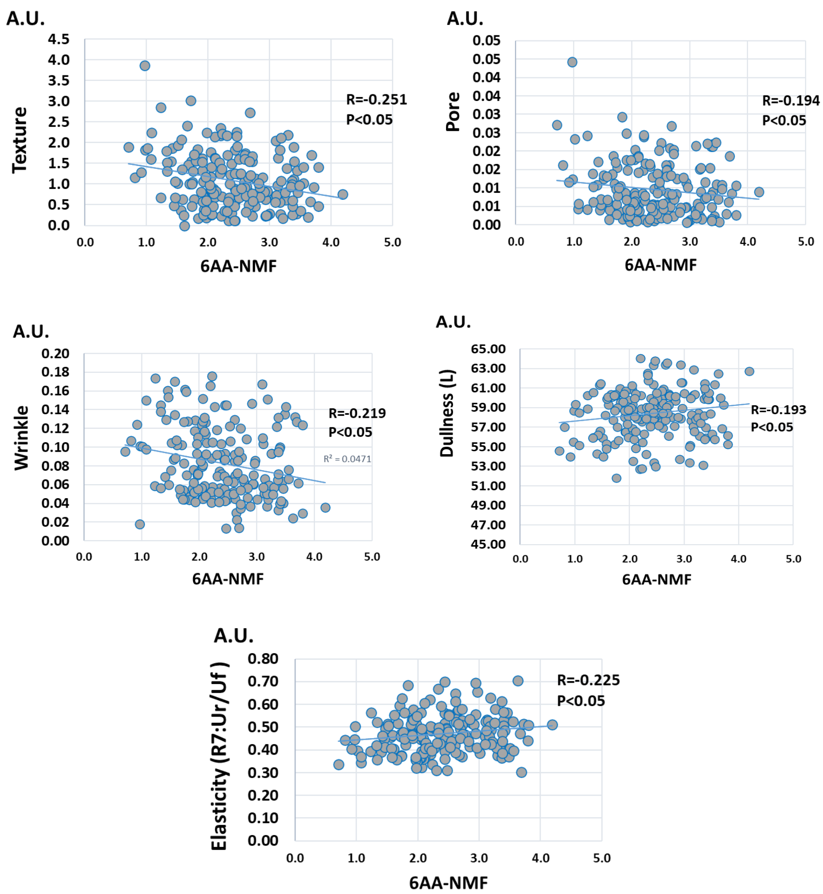

Figure 5. Correlations of 6-AA-NMFs with hydration, TEWL, texture, pores, wrinkles, skin tone (L-value), and elasticity (R7). Study 1, N = 196 Asian females aged 20 - 59 (ave. age 38.6, S.D. 9.40).

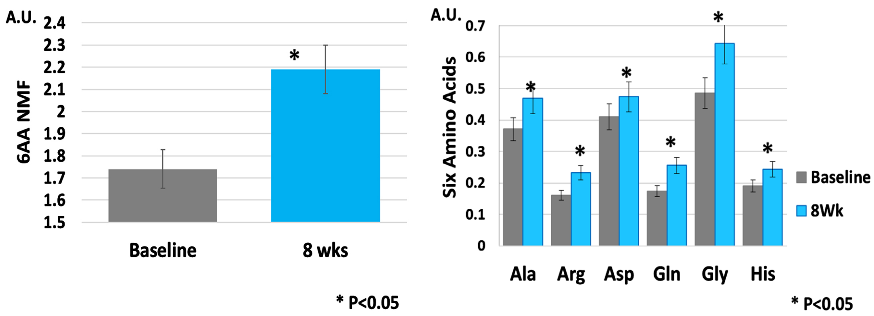

Figure 6. Stratum corneum levels of 6-AA-NMFs (left) and each of the six amino acids making up 6-AA-NMFs at baseline and after 8 weeks of SK-II FTE treatment (right). Study 2, N = 63 Asian females aged 20 - 55 (ave. age 38.6, S.D. 9.03). *Significant difference, P < 0.05.

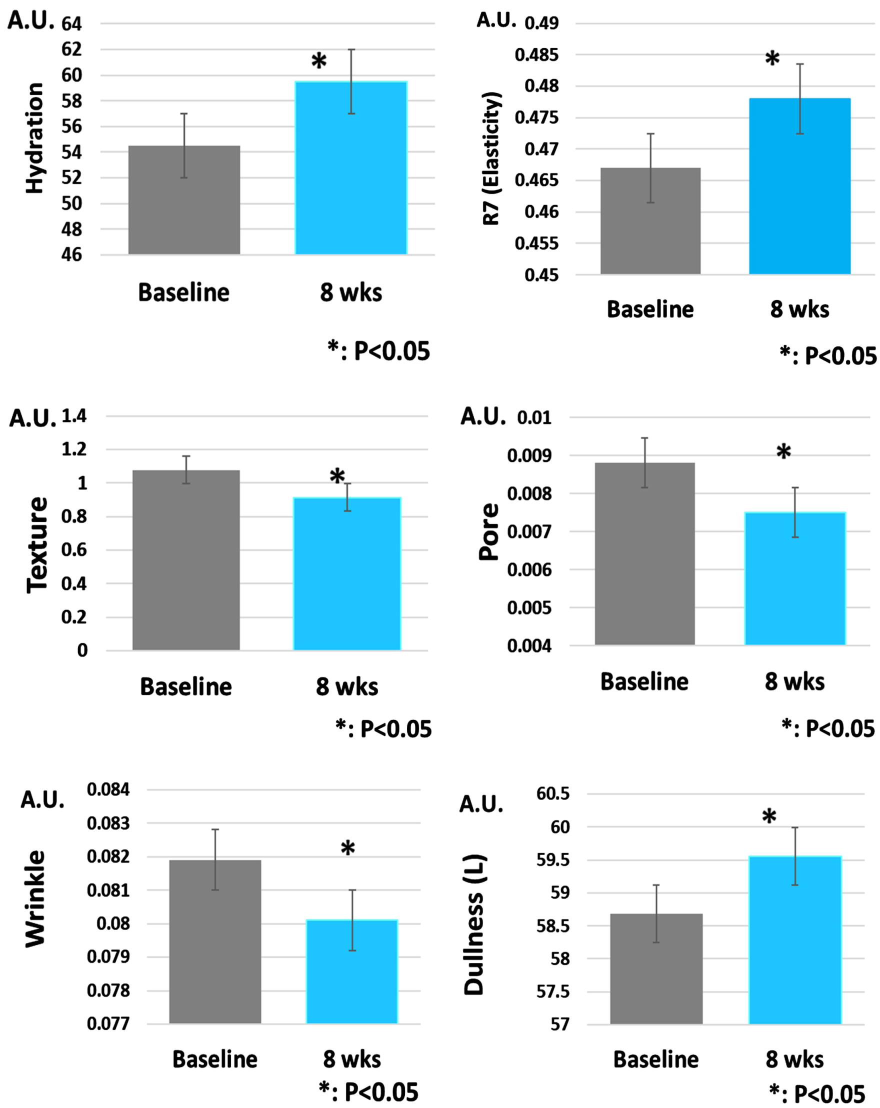

Figure 7. Skin parameters of hydration, elasticity (R7), texture, pores, wrinkles, and dullness (L) at the baseline and week 8 of treatment with SK-II FTE, GFF (Pitera^{TM})-containing skin care formula. Study 2, N = 63 Asian females aged 20 - 55 (ave. age 38.6, S.D. 9.03). *Significant difference, P < 0.05.

Figure 8. Facial skin examples at the baseline and week 8 of treatment with SK-II FTE, GFF (Pitera^{TM})-containing skin care formula, in Study 2. A pair of facial images at the baseline (upper left) and 8 weeks (upper right) at age 20. Another pair of facial images at the baseline (lower left) and 8 weeks (lower right) at age 30.

Table 1. Levels of 6-AA-NMFs and skin physical properties (hydration, TEWL, and elasticity) and skin aging parameters (texture, pores, wrinkles, and dullness) of the face by age group in 196 Asian females.

Age Group	Age	Number of Subjects	6-AA-NMFs	Hydration	TEWL	Elasticity (R7)	Texture	Pores	Wrinkles	Dullness (L-value)
20 - 24	21.778 ± 1.665	24	2.282 ± 0.773	53.684 ± 7.274	10.588 ± 2.454	0.582 ± 0.085	0.601 ± 0.381	0.00794 ± 0.00456	0.0642 ± 0.0232	58.945 ± 2.223
25 - 29	27.920 ± 0.640	26	2.716 ± 0.580*	54.878 ± 7.388	10.251 ± 1.576	0.569 ± 0.063*	1.012 ± 0.406*	0.0131 ± 0.00712*	0.0628 ± 0.0365	59.387 ± 2.739
30 - 39	35.000 ± 2.539	48	2.448 ± 0.690†	55.114 ± 8.035	10.229 ± 2.003	0.488 ± 0.049*†	1.136 ± 0.783*†	0.0105 ± 0.00870*†	0.0644 ± 0.0273	59.011 ± 2.185
40 - 49	45.948 ± 2.513	50	2.307 ± 0.704†	52.132 ± 8.378†	10.501 ± 2.968	0.418 ± 0.062*†	1.161 ± 0.629*†	0.00877 ± 0.00629*†	0.101 ± 0.0367*†	57.705 ± 2.396*†
50 - 59	54.833 ± 3.689	48	2.246 ± 0.883†	51.418 ± 2.471*†	9.500 ± 1.021*†	0.415 ± 0.037*†	1.174 ± 0.684*†	0.00572 ± 0.00425*†	0.111 ± 0.0362*†	57.489 ± 2.749*†

*Significant difference from age group 20 - 24, $P < 0.05$. †Significant difference from age group 25 - 29, $P < 0.05$.

total amount of cornified NMF components increases with age [16] [17] [18], which is known to be explained by thickening of the stratum corneum with age [26] [27]. We also selectively examined cornified NMF components only in the middle layer of the stratum corneum; we consider that the levels of these selected NMF components decline with age and may also be associated with signs of skin aging. The reason why no correlation was observed between 6-AA-NMF components and TEWL in this study is unknown. It is also possible that other NMF components, such as PCA and urocanic acid, which were not selected here, are related to the cornified lamellar structure and its functions, and therefore more greatly influence the barrier function of the skin [17] [28] [29]. Furthermore, in this study, daily application of a GFF-containing skin moisturizer improved these six amino acids, 6-AA-NMFs, which was associated with skin hydration, barrier function, and improvements of the signs of skin aging. GFF (Pitera™) is known to increase the expression of filaggrin, caspase-14, and claudin-1, which may facilitate the production of natural moisturizing factors and strengthen the epidermal tight junction structure [11] [12] [27]. In addition, GFF has been reported to inhibit oxidative stress mediated by proinflammatory cytokines in epidermal keratinocytes [30] [31]. In parallel with this *in vitro* evidence, several clinical studies have revealed that the daily application of GFF-containing moisturizer increased facial skin hydration [14] [15] [20], while also improving mask-induced skin damage [15] and potently reversing the deterioration of visual parameters of facial skin aging [14]. SK-II FTE is a potent moisturizing GFF-containing skin care product [14] [15]. GFF is known to activate the aryl hydrocarbon receptor and upregulate the expression of skin barrier-related proteins [11] [12] [27] [30] [32]. It also increases the production of the anti-inflammatory cytokine interleukin-37 in epidermal keratinocytes [13]. In addition, GFF inhibits oxidative stress by activating the cellular antioxidative system [28] [30]. These multifaceted properties of GFF may facilitate its moisturizing and anti-inflammaging effects [13] [33] [34]. These biological effects may underpin the anti-aging effects of SK-II FTE.

In summary, in this study we selectively collected cornified NMF components from the middle part of the stratum corneum layer on the cheek. By identifying 6-AA-NMF components that are related to skin hydration *in vivo*, we were able to clinically demonstrate their relationships with age and signs of skin aging. We also observed that the daily application of skin moisturizer containing GFF chronically increased the amount of 6-AA-NMFs in association with improvements of skin hydration and signs of skin aging in a clinical study. However, it remains unknown why no relationships between these 6-AA-NMFs and barrier function or between other cornified NMF components and *in vivo* facial skin hydration were observed in this study. Further clinical investigation will be undertaken using improved methods of collecting cornified NMFs and quantification methodologies to elucidate the influences of NMF components on skin hydration and barrier function mechanisms.

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Author Contributions

Conceptualization of this study and clinical investigation were performed by K.M., Y.M., K.F., and W.S. K.M., S.S., and M.F. wrote the first draft of the manuscript, while all authors revised it. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

This study was conducted in accordance with the tenets of the Declaration of Helsinki and approved by P & G Ethics Committee. Data acquisition and analysis were performed in compliance with protocols approved by the Ethical Committee of Global Product Stewardship at P & G Innovation Godo Kaisha (ethical approval numbers CT22002-S and CT22002-F). Written informed consent was obtained from all participants prior to inclusion in the study.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The data presented in this paper are available on request from the corresponding author. The data are not publicly available because of privacy restrictions.

Conflicts of Interest

Masutaka Furue is a consultant of P & G Innovation GK. The other authors are employees of P & G Innovation GK.

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