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α-Linolenic acid and linoleic acid modulate the lipidome and the skin barrier of a tissue-engineered skin model

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) play an important role in the establishment and the maintenance of the skin barrier function. However, the impact of their derived lipid mediators remains unclear. Skin substitutes were engineered according to the self-assembly method with a culture medium supplemented with 10 μM of both α-linolenic acid (ALA) and linoleic acid (LA). The supplementation with ALA and LA decreased testosterone absorption through a tissue-engineered reconstructed skin model, thus indicating an improved skin barrier function following supplementation. The exogenously provided fatty acids were incorporated into the phospholipid and triglyceride fractions of the skin substitutes. Indeed, the dual supplementation increased the levels of eicosapentaenoic acid (EPA) (15-fold), docosapentaenoic acid (DPA) (3-fold), and LA (1.5-fold) in the epidermal phospholipids while it increased the levels of ALA (>20-fold), DPA (3-fold) and LA (1.5-fold) in the epidermal triglycerides. The bioactive lipid mediator profile of the skin substitutes, including prostaglandins, hydroxy-fatty acids, *N*-acylethanolamines and monoacylglycerols, was next analyzed using liquid chromatography-tandem mass spectrometry. The lipid supplementation further modulated bioactive lipid mediator levels of the reconstructed skin substitutes, leading to a lipid mediator profile more representative of the one found in normal human skin. These findings show that an optimized supply of PUFAs via culture media is essential for the establishment of improved barrier function *in vitro*.

Statement of significance

Supplementation of the culture medium with 10 μM of both α-linolenic acid (ALA) and linoleic acid (LA) improved the skin barrier function of a tissue-engineered skin model. The exogenously provided fatty acids were incorporated into the phospholipid and triglyceride fractions of the skin substitutes and further modulated bioactive lipid mediator levels, including prostaglandins, hydroxy-fatty acids, *N*-acylethanolamines and monoacylglycerols. These findings highlight the important role of ALA and LA in skin homeostasis and show that an optimized supply of polyunsaturated fatty acids via culture media is essential for the establishment of improved barrier function *in vitro*.

1. Introduction

Skin barrier function is provided by the outermost layer of the epidermis, the *stratum corneum* (SC), which is composed of cor-

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neocytes embedded in a lipid matrix [1]. The SC is produced during a multistep process of differentiation of the epidermal keratinocytes in which lipid metabolism plays an essential role, both at a structural and a homeostatic level. First, fatty acids in the skin can be synthesized *de novo* or can be taken up from the diet or extracutaneous sites, with the exception of short-chain polyunsaturated fatty acids (PUFAs) such as α-linolenic acid (ALA) and linoleic acid (LA), which cannot be synthesized in the skin and need to be obtained exclusively through dietary sources [2–4]. Fatty acids then enter various metabolic pathways such as phospholipid synthesis. This pathway is particularly active in the basal layer of the epidermis where cells are proliferating [5]. During the differentiation process of the skin, the catabolism of phospholipids is observed and keratinocytes tend to produce more neutral lipids such as triglycerides and ceramides [6]. The newly synthesized lipids will finally form the SC lipid matrix, specifically composed of a mixture of 45% ceramides, 30% cholesterol and 15% free fatty acids. The contribution of LA to the skin barrier is crucial, since it is incorporated into ω-hydroxylated ceramides [7]. These ceramides are covalently bound to the corneocytes and serve as a scaffold for other lipids, allowing an optimal organization of the lipid matrix. The epidermal differentiation is highly regulated by different signaling systems, involving many different actors such as kinases and also a large number of bioactive lipid mediators derived from PUFAs, which include prostaglandins (PGs), hydroxylated fatty acids (HFAs), leukotrienes (LTs), endocannabinoids (eCBs), *N*-acylethanolamines (NAEs) and monoacylglycerols (MAGs) (Fig. S1) [8,9].

The complex structure of the SC still represents a challenge to replicate *in vitro*. The tissue-engineered bilayered human skin model is the model that currently best represents normal human skin morphology [10–12]. It is produced using both primary fibroblasts and keratinocytes and includes culture steps at the air-liquid interface, which allows complete differentiation of the reconstructed epidermis. Bilayered skin models can be produced by various approaches, including collagen gel, collagen sponges and self-assembled matrix [13]. Among these, tissue-engineered skin substitutes produced according to the self-assembly method have been comprehensively characterized over the past decade [14–17]. For instance, transmission electron microscopy and biomechanical analyses showed that the reconstructed skin substitutes displayed a similar skin barrier physiology to native human skin, presenting lamellar bodies filled with lipids in the epidermis and optimal dermal-epidermal adherent strength [18,19]. Many factors can influence the formation of the barrier function in the reconstructed skin model, one of them being the fatty acid composition of the media used during long-term culture [15,20]. Because of their essential nature, PUFAs must therefore be added to the culture media [21,22]. We have shown in a previous study that a supplementation of the culture media with ALA improved the skin barrier function of the reconstructed skin model, while an equivalent supplementation of the culture media with LA did not affect the skin barrier function [21]. These results were unexpected, since LA is the most abundant PUFA present in the epidermis and is well known to influence skin permeability through its incorporation into ω-hydroxylated ceramides [23–25]. On the other hand, ALA has never been documented as influencing skin permeability, although it is recognized as being implicated in skin homeostasis, since it is the precursor of long-chain n-3 PUFAs that produce eicosanoids and resolvins [26]. Additionally, ALA is a preferred substrate for beta-oxidation and carbon recycling, and it possesses potential anti-inflammatory action in diverse diseases [27]. Therefore, these previous results highlight the importance of the n-3 to n-6 PUFA ratio in cutaneous homeostasis [21]. In the present study, we expanded these studies and assessed the impact of a combined supplementation with ALA and LA on the permeability of the skin substitutes. In addition, the impact of such supplementation on the levels of bioactive lipid mediators in the skin substitutes was also analyzed in order to better understand the metabolism of PUFAs within the skin.

2. Materials and methods

2.1. Cell culture

This study was conducted in accordance with the guidelines of the Research Ethics Committee of the CHU de Québec - Université Laval and with the Declaration of Helsinki. For the production of the tissue-engineered skin substitutes, the fibroblasts and keratinocytes were extracted from breast reduction skin biopsies of three Caucasian women aged 18, 46 and 49 years old, using a method based on thermolysin, trypsin and collagenase digestion as described elsewhere [28]. For the lipid analyses, normal human skin (NHS) was obtained from skin biopsies of 5 Caucasian women (breast reduction) aged 26, 31, 47, 48 and 55 years old, and a Caucasian man (liposuction) aged 21 years old.

2.2. Tissue-engineered skin substitute reconstruction

Skin substitutes were produced according to the self-assembly method previously described by Simard et al. [21]. For each analysis, at least 3 skin substitutes were reconstructed using matching fibroblasts and keratinocytes from 3 different donors (biological replicates). The number of skin substitutes produced per donor (technical replicates) is specified for each analysis in the result section. Human primary fibroblasts (passage 6) were cultured for 25 days in 6-well plates (1×10^4 cells/cm^2) with Dulbecco's modified Eagle's medium (DME) (Gibco, Life Technologies, New York, NY, US) supplemented with 10% Fetal Calf premium Serum (FCS) (Wisent Inc., St-Bruno, QC, CAN), 50 μg/ml ascorbic acid (Sigma, Oakville, ON, CAN) and antibiotics; 60 μg/ml penicillin G (Sigma, Oakville, ON, CAN) and 25 μg/ml gentamicin (Gemini Bio-Products, Sacramento, CA, US). Two of the resulting fibroblast sheets were superimposed and were cultured for 3 days in a 100 mm Petri dish to form the dermal equivalents. Matched human primary keratinocytes (passage 2) were then seeded on the dermal equivalents (1.2×10^6 cells per dermal equivalent). The skin substitute was cultured in submerged conditions for one week in DME mixed with Ham's F12 medium (3:1) (DME-HAM) (Gibco, Life Technologies, New York, NY, US) supplemented with 5% FetalClone II serum (Hyclone, Logan, UT, US), 5 μg/ml insulin (Sigma, Oakville, ON, CAN), 0.4 μg/ml hydrocortisone (Galenova, St-Hyacinthe, QC, CAN), 10^{-10} M cholera toxin (Sigma, Oakville, ON, CAN), 10 ng/ml human epidermal growth factor (EGF) (Ango Inc., San Ramon, CA, US), 60 μg/ml penicillin and 25 μg/ml gentamicin. The skin substitutes were raised to the air-liquid interface and cultured for three additional weeks in DME-HAM medium supplemented with 5% FetalClone II serum, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 60 μg/ml penicillin and 25 μg/ml gentamicin. Cells were incubated at 37°C in an 8% CO_2 atmosphere.

ALA and LA were purchased from Sigma (Oakville, ON, CAN). Individual stock solutions were produced by dissolving ALA and LA in 99% ethanol (EtOH) (Greenfield Global, Brampton, ON, CAN). Culture media were supplemented with the corresponding amount of both stock solutions to reach a final concentration of 10 μM ALA and 10 μM LA. During the preparation of the culture media, PUFAs were first incorporated into the serum containing naturally abundant bovine serum albumin, which increases fatty acid solubility. Skin substitutes were produced either with all culture media supplemented with ALA and LA (Substitute$^{ALA+LA+}$) or with culture media supplemented with the corresponding volume of EtOH

(Substitute$^-$) during the entire culture period, including culture media used for both fibroblasts and keratinocytes (Fig. S2). Culture media were changed three times a week.

2.3. Histologic and morphometric analyses

Skin substitute biopsies were fixed in HistoChoice solution (AMRESCO, Inc., Solon, OH, US) and embedded in paraffin. Five μm thick sections were cut and were stained with Masson's trichrome. Two substitutes for each of the 3 donors were analyzed (n=6). The thickness of the dermis and epidermis was measured on the stained sections with Image J software (National Institutes of Health, US, http://imagej.nih.gov/ij). Ten measurements on three different sections of each biopsy were made.

2.4. Percutaneous absorption

Percutaneous absorption analyses were performed using the standard Franz diffusion cell technique, as described by Franz, Simard et al. and elsewhere [21,29–33]. Skin substitutes were placed between the giving and the receiving compartment of the Franz cells (LOGAN Instruments Corp., Somerset, NJ, US) and the two compartments were held together using clamps. The receiving compartment was filled with phosphate-buffered saline (PBS) containing 20% ethanol and a stirring bar was used to assure homogeneity. The Franz cells were placed on a heating bench (LOGAN Instruments Corp., Somerset, NJ, US), which kept the receiving compartment at 37°C and the skin sample temperature at approximately 32°C. A fresh solution of 4 mg/ml testosterone (Sigma, Oakville, ON, CAN) in PBS:EtOH (50:50) was prepared before each experiment [34–36]. A volume of 100 μl of the testosterone solution (400 μg) was applied to the skin substitutes in each Franz cell at time point 0. The experiment was performed under occluded conditions by applying paraffin to each giving compartment after testosterone deposition. The contents of the receiving compartment were collected at different time points (1h, 2h, 3h, 4h, 6h, 8h), using a 5 ml syringe with a catheter. Samples were stored at -80°C until analyzed.

Testosterone samples were assayed by an in-house-developed UPLC-UV method (248 nm) using a Waters Acquity UPLC system with a water PDA detector and a thermostatted autoinjector (Acquity UPLC H-Class System, Waters, Mississauga, ON, CAN). Testosterone was separated on a BEH C_{18} Waters column (50 mm x 2.1 mm, 5 μm, ON, CAN) kept at 40°C. The mobile phase was a gradient concentration of acetonitrile (ACN) in water (50% ACN to 60% ACN in three minutes) using a solution buffered with 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.6 ml/min. The injection volume was 5 μl. Under these conditions, testosterone was eluted at approximately 1.50 min. Data collection and peak integration were performed using Empower 2 software (Waters, Mississauga, ON, CAN).

2.5. Gas chromatography (GC-FID)

Gas chromatography was performed as reported elsewhere [21]. The epidermis of skin substitutes was mechanically separated from the dermis using forceps and scalpels, and for human skin (6 Caucasian donors: 2 males and 4 females aged between 26-48 years-old), the epidermis was peeled off using forceps after incubation in water at 60°C for 1 minute. The extraction of the epidermal and dermal lipids was performed using a chloroform-methanol mixture (2:1, vol/vol) according to a modified Folch method [37]. Total PLs were separated by thin layer chromatography. A migration was performed in isopropyl ether/acetic acid (96/4) until the middle of the plate was reached. The fatty acids of isolated phospholipids were methylated. Capillary gas chromatography with a HP5890 gas chromatograph (Hewlett-Packard, Toronto, ON, CAN) equipped with an HP-88 capillary column (100 mm x 0.25 mm internal diameter x 0.20 μm film thickness; Agilent Technologies, Santa Clara, CA, US) coupled with a flame ionization detector was then used to obtain fatty acid profiles, as described elsewhere [38].

2.6. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

For NHS, any trace of adipose tissue was carefully removed from the skin samples and they were then incubated in water at 60°C for 1 min, after which the epidermis was peeled off using forceps. For tissue-engineered skin substitutes, the epidermis was mechanically separated from the dermis using forceps and scalpels. Skin samples were pulverized into a fine powder using a Cryomill MM400 (Retsch®, Newtown, PA, US) and suspended in 500 μl Tris-HCL 50 mM (pH 7) and immediately denatured in one volume of cold (-20°C) methanol containing the internal standards. The extraction of epidermal and dermal lipids was performed using an acidified methanol chloroform technique, exactly as described in [39]. LC-MS/MS was performed as reported elsewhere [40]. Samples were reconstituted in 50 μl of a 50/50 mixture of LC solvent A (H_2O containing 0.05% acetic acid and 1 mM NH_4^+) and solvent B (acetonitrile/H_20, 95/5, v/v, with 0.05% acetic acid and 1 mM NH_4^+). 40 μl of the latter was injected onto a RP/HPLC column (Kinetex C8, 150 $\times$ 2.1 mm, 2.6 μm, Phenomenex) and lipids were separated using the same LC program as described previously [41]. Quantification was done by generating calibration curves using pure standards and analyzing on the LC-MS/MS system three times. The slope was then calculated using the ratio between the peak areas of the compound and its standard (Supplementary Table S1). Because of the acyl migration from the *sn*-2- to the *sn*-1(3) position naturally occurring in MAGs, we present the data as the combination of MAG isomers (1/2-MAG).

2.7. Gene expression profiling

Total RNA was isolated from the complete skin substitutes (dermal and epidermal compartments) using the RNeasy Mini Kit (QIAGEN, Toronto, ON, CAN) and its quality determined (2100 bioanalyzer, Agilent Technologies, Mississauga, ON, CAN) as previously described [42]. The labeling of Cyanine 3-CTP labeled targets, their hybridization on a G4851A SurePrint G3 Human GE 8 $\times$ 60K array slide (Agilent Technologies, Santa-Clara, CA, US) and data acquisition and analyses were all performed as previously reported [42] (GSE120464, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc1/4GSE120464).

2.8. Statistical analysis

Data are expressed as means $\pm$ standard deviation for parametric variables, except when stated otherwise. Statistical analyses of fatty acid lipid profiles and bioactive lipid mediators were performed using ANOVA with Tukey's *post-hoc* test. Only values of $P < 0.05$ were considered significant. All calculations were performed with Prism version 7 software (Graphpad Software, La Jolla, CA, US).

3. Results

3.1. Dual supplementation with ALA and LA increases impermeability of the skin substitutes to testosterone

Tissue-engineered reconstructed skin substitutes were produced according to the self-assembly method with culture media supplemented with 10 μM ALA and 10 μM LA (Substitute$^{ALA+LA+}$)

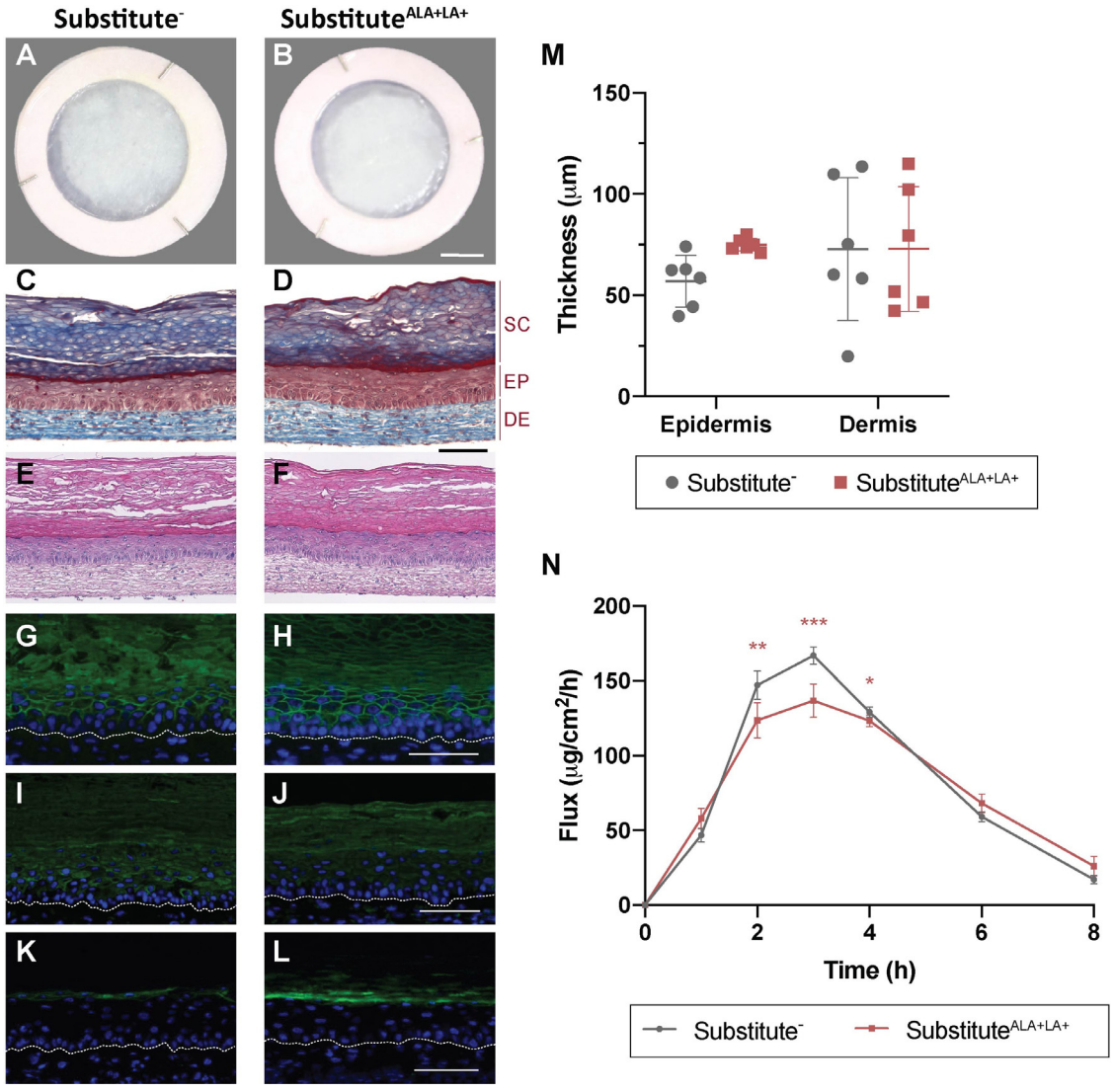


Fig. 1. Impact of supplementation with ALA and LA on skin substitute morphology and functionality. (**A-B**) Macroscopic aspect and (**C-D**) histological cross-sections after Masson's trichrome staining of the skin substitutes. Legend: DE, dermis; EP, epidermis; SC, stratum corneum. Scale bars: A-B) 1 cm, C-D) 100 μm. (**G-H**) Involucrin, (**I-J**) keratin 10 and (**K-L**) loricrin immunofluorescence staining of the skin substitutes. (**M**) Epidermal and dermal thickness quantified from Masson's trichrome-stained sections. (3 donors, 2 skin substitutes per donor). Two-way ANOVA followed by Tukey's *post-hoc* test $^{*}P<0.05$. (**N**) Influence of ALA and LA supplementation on the flux of testosterone absorbed through the skin substitutes. Percutaneous absorption studies were performed in a Franz cell diffusion system. The testosterone solutions were freshly prepared in ethanol/water (1:1), yielding a concentration of 4.0 mg/ml. Testosterone was quantified using a Waters Acquity UPLC. (3 donors, 6 skin substitutes per donor), p-values were derived from Student's t-tests. $^{*}P<0.05$; $^{**}P<0.01$. Abbreviations: ALA, α-linolenic acid; LA, linoleic acid.

or without PUFAs (Substitute^{-}) (Fig. S2). The skin substitutes produced under both culture conditions presented a similar morphology (Fig. 1). They both displayed a dermal layer with a rich collagen matrix as colored in blue on the Masson's trichrome stanned sections (Fig. 1C-D), and an epidermis with proper keratin and differentiation marker expressions (Fig. 1G-J). In addition, they both presented a differentiated epidermis with a SC containing late differentiation markers, such as loricrin (Fig. 1K-L). Moreover, no statistical differences were observed between thickness of the epidermal living layer of Substitute$^{ALA+LA+}$ and Substitute^{-} (Fig. 1M). To evaluate the impact of the dual supplementation with ALA and LA on the permeability of the skin substitutes, the percutaneous absorption of testosterone, a lipophilic compound, was assayed using a Franz cell diffusion system (Fig. 1N). The mean flux of testosterone through Substitute$^{ALA+LA+}$ was significantly smaller than through Substitute^{-} at 2, 3 and 4 hours, showing decreased testosterone absorption through Substitute$^{ALA+LA+}$ (Fig. 1N). Therefore, dual supplementation with ALA and LA stimulates the formation of the barrier function of the skin substitutes.

3.2. Exogenous ALA and LA are incorporated into phospholipids and triglycerides

Testosterone is a lipophilic compound and therefore its absorption through the skin is more susceptible to being affected by a modification of the lipid content of the SC, for which it will have more affinity based on its physico-chemical properties [43]. Therefore, the lipid content of the skin substitutes was further investigated, first in order to identify whether the exogenously added PUFAs were incorporated into phospholipids and triglycerides of the skin samples (Figs. 2 and 3). The epidermis was separated from the dermis and total lipids were extracted from each compartment according to a modified Folch method [37]. Total phospholipids and triglycerides were separated by thin layer chromatography and their fatty acids were analyzed using gas chromatography with a flame ionization detector (GC-FID).

The distribution of fatty acids in the phospholipids of the skin substitutes was compared to that of NHS (Fig. 2A-B). The levels of fatty acids are presented in percentage to allow proper compari-

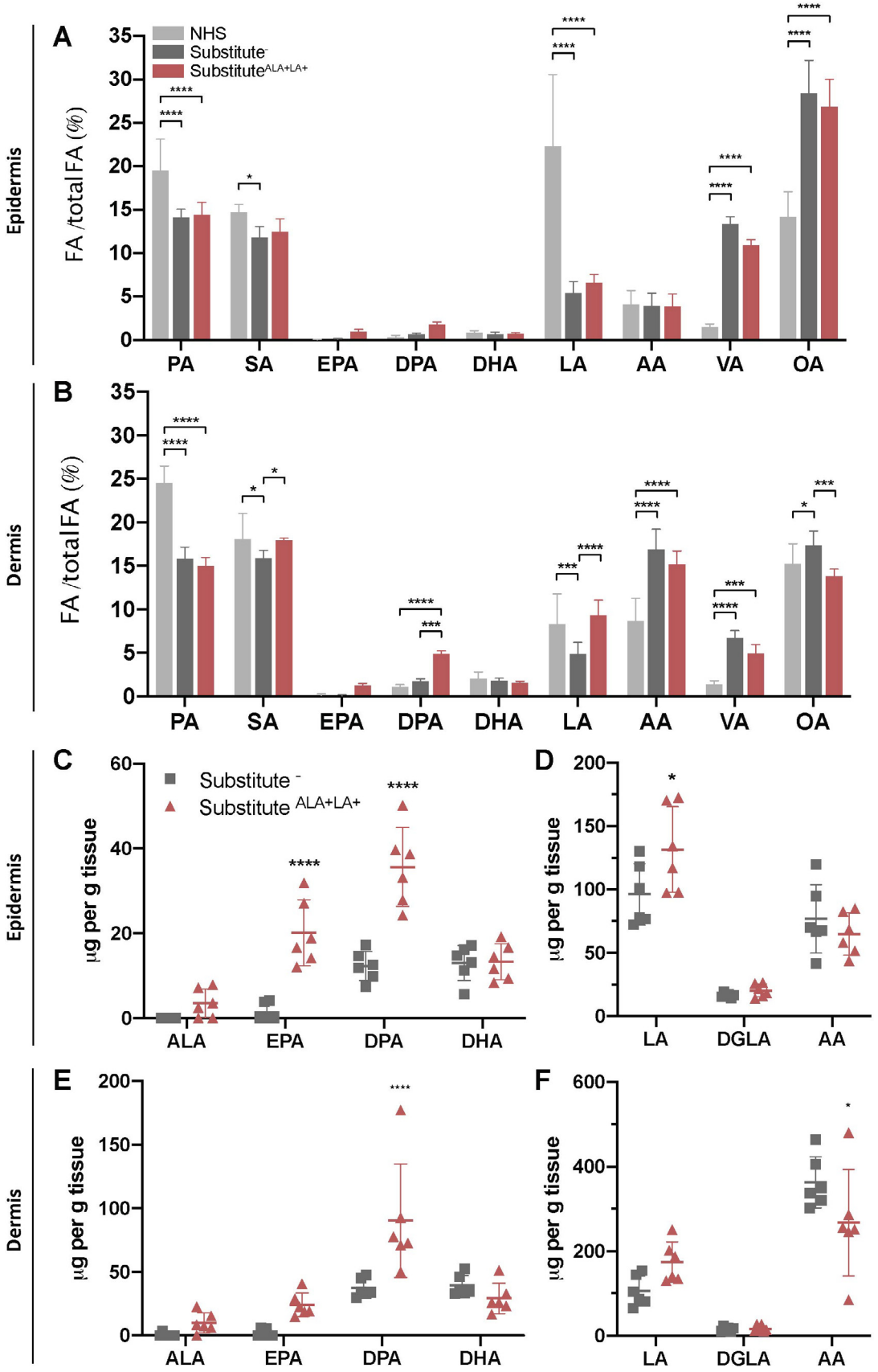


Fig. 2. Incorporation of ALA and LA into the phospholipid fatty acids of the skin substitutes after dual supplementation with ALA and LA. Characterization of epidermal (A, B and E) and dermal (C, D and F) phospholipid fatty acids using gas chromatography. Impact of dual supplementation on (A, C) n-3 PUFAs and (B, D) n-6 PUFAs. Results are expressed as μg per g of tissue. Proportion of various FAs in E) the epidermis and F) the dermis of Substitute^{-}, Substitute$^{ALA+LA+}$ and NHS. Results are expressed as percentages (FA/total FA). For skin substitutes: n=6 (3 donors, 2 skin substitutes per donor); for NHS: n=6. Two-way ANOVA followed by Tukey's *post-hoc* test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; NHS, normal human skin; OA, oleic acid; PUFAs, polyunsaturated fatty acids; VA, vaccenic acid.

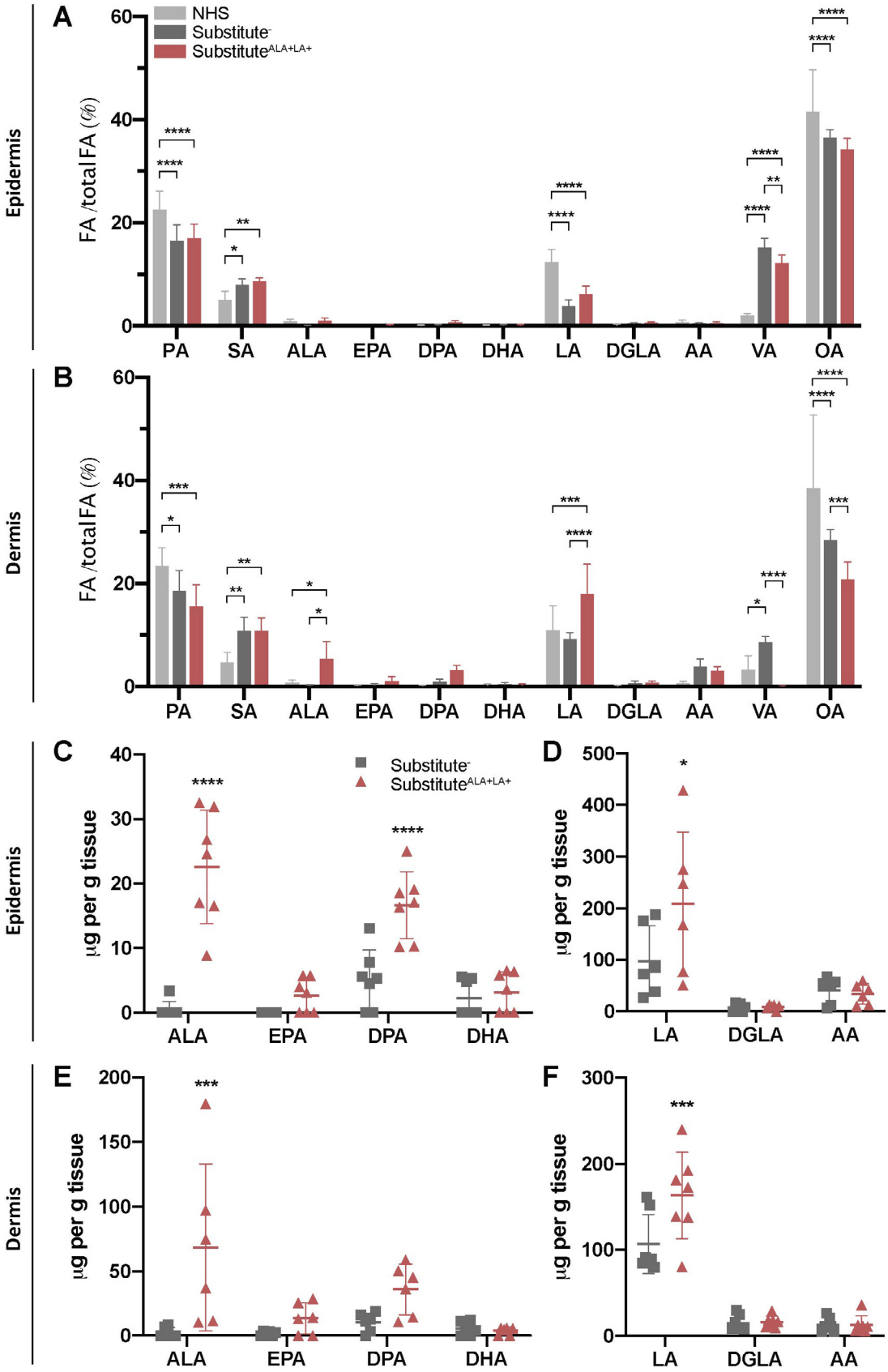


Fig. 3. Incorporation of ALA and LA into the triglyceride fatty acids of the skin substitutes after dual supplementation with ALA and LA. Characterization of epidermal (A, B and E) and dermal (C, D and F) triglyceride fatty acids using gas chromatography. Impact of dual supplementation on (A, C) n-3 PUFAs and (B, D) n-6 PUFAs. Results are expressed as μg per g of tissue. Proportion of various FAs in E) the epidermis and F) the dermis of Substitute^{-}, Substitute$^{ALA+LA+}$ and NHS. Results are expressed as percentages (FA/total FA). For skin substitutes: n=6 (3 donors, 2 skin substitutes per donor); for NHS: n=6. Two-way ANOVA followed by Tukey's *post-hoc* test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; NHS, normal human skin; OA, oleic acid; PUFAs, polyunsaturated fatty acids; VA, vaccenic acid.

son of skin substitutes and NHS, since total levels of fatty acids in the dermis were significantly higher in the skin substitutes than in NHS (Fig. S3). As expected, lower proportions of n-6 PUFAs, notably LA, were found in both the epidermis (-4.1-fold) and dermis (-1.4-fold) of the Substitute^{-} compared with their respective counterparts of the NHS, while in contrast, higher proportions of monounsaturated fatty acids (MUFAs), such as vaccenic acid (VA) and oleic acid (OA), were measured in Substitute^{-} (Fig. 2A-B). Furthermore, PUFAs were efficiently incorporated into epidermal and dermal phospholipids following supplementation with ALA and LA (Fig. 2C-F). Indeed, the dual supplementation increased the amounts of both n-3 and n-6 PUFAs in the epidermis, with higher levels of the n-3 PUFAs eicosapentaenoic acid (EPA, 15-fold) and docosapentaenoic acid (DPA, 2.9-fold), and of the n-6 PUFA LA (1.4-fold) detected in Substitute$^{ALA+LA+}$ than in Substitute^{-} (Fig. 2C-D). Similar results were found in the dermis, as an increase in levels of the n-3 PUFA DPA (2.4-fold) was measured in Substitute$^{ALA+LA+}$ compared with Substitute^{-} (Fig. 2E). Moreover, a 1.7-fold increase in the level of LA was measured in the dermal phospholipids, although it was not significant (Fig. 2F). A decrease in the amount of the n-6 PUFA arachidonic acid (AA, -1.4-fold) was also measured in Substitute$^{ALA+LA+}$ dermis compared with Substitute^{-} dermis, which may suggest decreased LA metabolism following dual supplementation (Fig. 2F). The proportion of LA was still significantly lower in Substitute$^{ALA+LA+}$ epidermis than in NHS epidermis, showing that although it was incorporated into the phospholipids, the LA increase ($\sim$ 55 μg per g of tissue) was not sufficient to reach the proportions of LA found in NHS (Fig. 2A). On the other hand, the proportion of LA was higher in Substitute$^{ALA+LA+}$ dermis than in Substitute^{-} dermis and was not significantly different from that of NHS, indicating that the lipid profile of skin substitute dermis was restored to NHS levels by the dual supplementation with ALA and LA (Fig. 2B). We next investigated whether the fatty acid lipid profiles of the skin substitute phospholipids correlated with the percutaneous absorption of testosterone. Interestingly, LA was the fatty acid the most strongly negatively correlated (r^2=0.9713) with the flux of testosterone at 3 h, clearly showing that LA epidermal levels in the skin substitute phospholipids are of major importance for the establishment of the skin barrier function (Figs. S3 and S4).

The distribution of fatty acids in triglycerides of the skin substitutes was next compared to that of NHS (Fig. 3A-B). As for phospholipids, lower proportions of LA were found in the epidermal triglycerides of the Substitute^{-} compared with those of NHS (Fig. 3A). Moreover, PUFAs were efficiently incorporated into dermal and epidermal triglycerides (Fig. 3). Levels of ALA, DPA and LA were significantly higher in the epidermis of Substitute$^{ALA+LA+}$ than in Substitute^{-} while, levels of ALA and LA were significantly higher in the dermis of Substitute$^{ALA+LA+}$ than in Substitute^{-} (Fig. 3C, F). Of note, the proportion of LA was still significantly lower in Substitute$^{ALA+LA+}$ epidermal triglycerides than in NHS epidermal triglycerides (Fig. 3A). On the other hand, the proportion of LA was higher in Substitute$^{ALA+LA+}$ dermis than in Substitute^{-} and NHS (Fig. 3B). In addition, levels of fatty acids in triglycerides were not significantly correlated with the flux of testosterone at 3 h under our experimental conditions (Fig. S4).

3.3. Characterization of prostaglandins and hydroxy fatty acids of skin substitutes and modulation with ALA and LA supplementation

Supplementation with ALA and LA did not completely restored the fatty acid profile in the phospholipids and triglycerides of the skin substitutes to levels found in NHS. Therefore, we further investigated if the exogenously added fatty acids were used for the synthesis of lipid mediators instead and whether these bioactive lipid mediators can influence skin substitute impermeability. The PGs and HFAs found in the skin substitutes were compared with those in NHS since lipid mediator profiles of reconstructed skin models have not yet been reported to our knowledge (Fig. 4 and Table S1). Regarding PGs, levels of EPA- and AA-derived PGs tended to be lower in the Substitute^{-} epidermis than in NHS epidermis (Fig. 4A-B). Indeed, significantly lower levels were measured for PGD_2, PGE_2 and PGF_{2a} in Substitute^{-} epidermis compared with NHS epidermis (Fig. 4B). These lower levels contrasted with levels of their respective precursor (EPA and AA), which were not significantly different in the epidermal phospholipids of NHS and Substitute^{-}, indicating that enzymes in the biosynthetic pathway of PGs are more likely to be responsible for these lower levels of epidermal PGs. On the other hand, levels of n-6 derived PGs in the dermal compartment tended to be higher in Substitute^{-} than in NHS, notably for PGE_2 whose levels were significantly higher in Substitute^{-} dermis (Fig. 4C-D). Higher levels of PGs in the dermis were consistent with higher levels of AA found in the phospholipids of Substitute^{-}. Dual supplementation with ALA and LA increased levels of EPA-derived PGs, notably PGF_{3a} in the epidermis as well as PGE_3 and PGF_{3a} in the dermis, while it did not affect AA-derived PGs (Fig. 4A-D). These results are therefore representative of the levels of the precursors after dual supplementation, for instance the increase in EPA measured in Substitute$^{ALA+LA+}$ phospholipids.

The main bioactive lipid mediators found in NHS and Substitute^{-} were HFAs derived from docosahexaenoic acid (DHA), LA and AA. These HFAs were predominantly derived from 15-Lipoxygenase (LOX) and 12-LOX, such as 13-hydroxyoctadecadienoic acid (HODE), 12-hydroxyeicosatetraenoic (HETE), 15-HETE, 14-hydroxydocosahexaenoic acid (HDHA) and 17-HDHA (Fig. 4E-H). The levels of most HFAs in Substitute^{-} epidermis and dermis were similar between Substitute^{-} and NHS, and mostly reflected the levels of their respective precursors found in phospholipids of both skin samples (Fig. 4E-H and Table S2). The main difference encountered between Substitute^{-} and NHS was increased levels of LA-derived 13-HODE in the Substitute^{-} epidermis compared with NHS epidermis (Fig. 4F). High levels of 13-HODE contrasted with low levels of LA found in Substitute^{-} epidermal phospholipids. Therefore, it may support the premise that LA is strongly metabolized into its oxidized derivative in the epidermis of reconstructed skin models and point out high 15-LOX activity in the skin substitutes epidermis. On the other hand, the levels of AA-derived 12-HETE were decreased in both the epidermis and dermis of Substitute^{-} compared with NHS (Fig. 4F, H). This result is not consistent with the levels of AA found in the phospholipids of Substitute^{-} compared with NHS. The dual supplementation with ALA and LA did not significantly stimulate the production of HFAs in the epidermis, although it tended to increase n-3-derived HFAs (Fig. 4E-F). Indeed, the supplementation even decreased the levels of 13-HODE in Substitute$^{ALA+LA+}$ compared with Substitute^{-} (Fig. 4F). This decrease in 13-HODE levels contrasts with the higher levels of its precursor (LA) found in Substitute$^{ALA+LA+}$ and may be the result of increased competition with n-3 PUFAs such as ALA or EPA for 15-LOX. In the dermis, dual supplementation with ALA and LA increased levels of HFAs accordingly with increases of their precursor in phospholipids (Fig. 4G-H). Exogenous provision with ALA and LA increased levels of HFAs derived from DHA (14-HDHA) and LA (9-HODE and 13-HODE), while it had no impact on oxidized metabolites derived from EPA and AA (Fig. 4G-H). The fact that levels of AA-derived HFAs and PGs were low even after supplementation may confirm a low conversion rate of LA to AA. Of note, all signals for the lipid mediators were above the detection limit of the LC-MS/MS and were detected in at least one skin sample. The detailed lipid mediator concentrations expressed in pmol/g of tissue and used to produce Fig. 4 are also available in Supplementary Table S1.

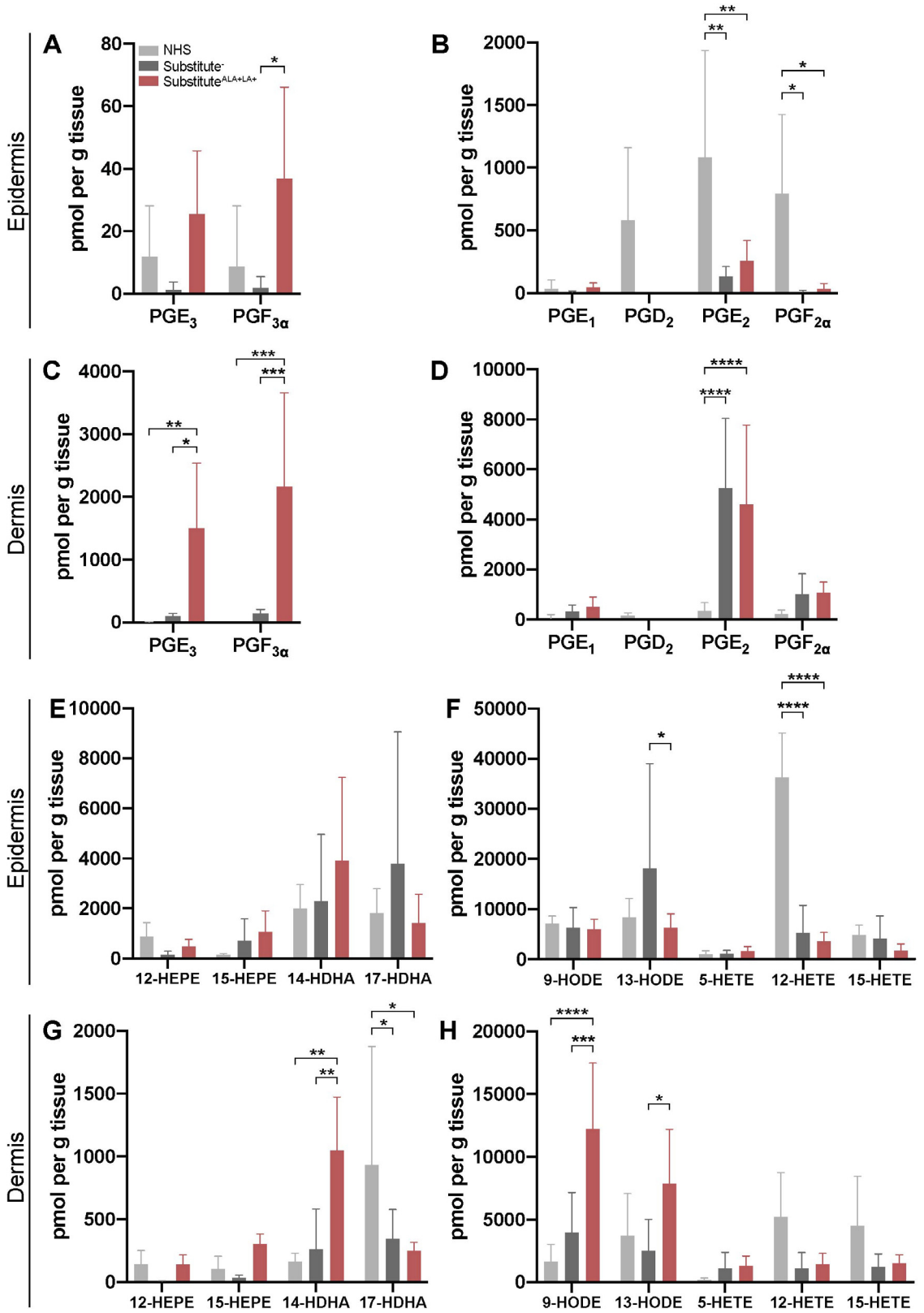


Fig. 4. Impact of a dual supplementation with ALA and LA on the lipid mediator profile of the skin substitutes and NHS. Characterization of epidermal (A, B, E and F) and dermal (C, D, G and H) lipid mediators. Impact of dual supplementation on (A-D) COX metabolites and (E-H) LOX metabolites. For skin substitutes: n=6 (3 donors); for NHS n=6. Two-way ANOVA followed by Tukey's *post-hoc* test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Abbreviations: ALA, alpha-linolenic acid; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroperoxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid; LA, linoleic acid; LT, leukotriene; NHS, normal human skin; PG, prostaglandin.

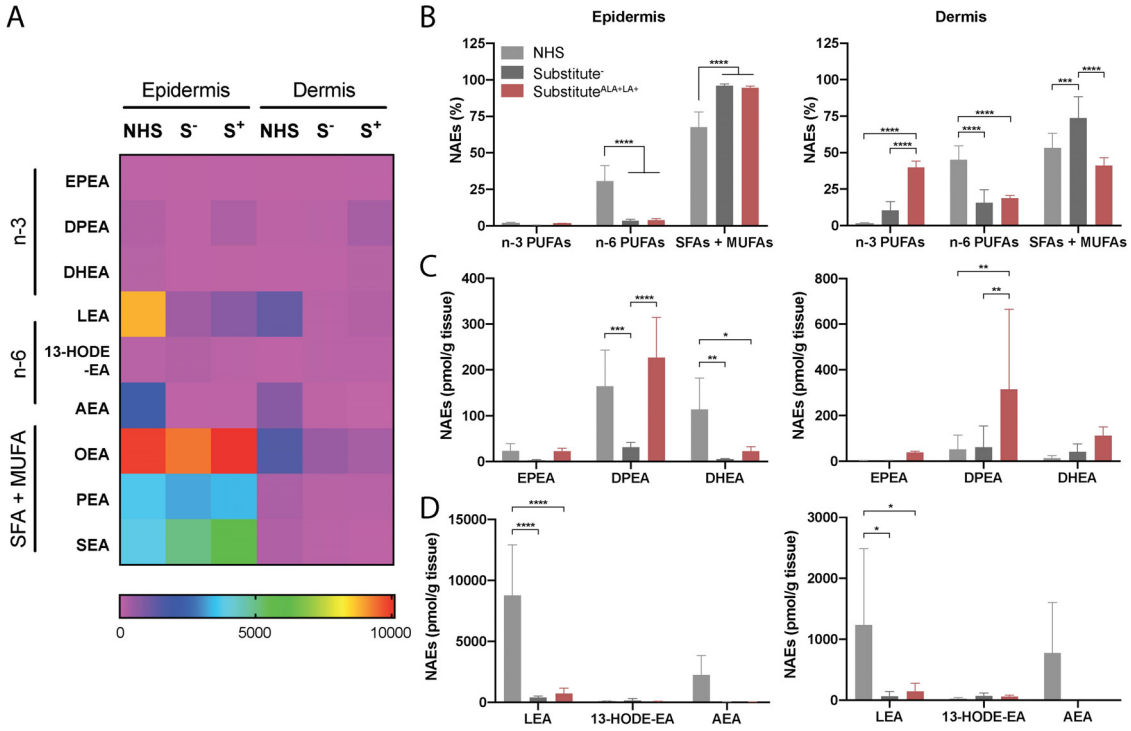


Fig. 5. *N*-acyl-ethanolamine (NAE) content of the skin samples quantified using LC-MS/MS analysis. (**A**) Heatmap of the eight NAEs measured in NHS, Substitute^{-} and Substitute$^{ALA+LA+}$ (S+). (**B**) Proportion of NAEs derived from n-3 PUFAs, n-6 PUFAs or MUFAs and SFAs. NAE classes are shown as a percentage of total NAEs. NAEs derived from (**C**) n-3 PUFAs and (**D**) n-6 PUFAs found in NHS, S^{-} and S^{+}. For human skin: n=6 donors; for skin substitutes: n=3 donors. Two-way ANOVA followed by Tukey's *post-hoc* test $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Abbreviations: AEA, *N*-arachidoyl-ethanolamine; DHEA, docosahexaenoyl-ethanolamine; DPEA, *N*-docosapentaenoyl-ethanolamine; EPEA, *N*-eicosapentaenoyl-ethanolamine; HDHA, hydroxydocosahexaenoic acid; HDPA, hydroxydocosapentaenoic acid; LEA, *N*-linoleoyl-ethanolamine; MUFA, monounsaturated fatty acid; NAE, *N*-acyl-ethanolamine; NHS, normal human skin; OEA, *N*-oleoyl-ethanolamine; PEA, *N*-palmitoyl-ethanolamine; PUFA, polyunsaturated fatty acid; S, substitute; SFA, saturated fatty acid.

3.4. Identification of main NAEs of the skin and investigation of their biosynthesis in reconstructed skin following supplementation with ALA and LA

To investigate the potential involvement of eCBs, arachidonoyl-ethanolamide (AEA), and eCB-like compounds from the NAE class in the formation of the barrier function of the skin, eight NAEs were quantified in NHS, Substitute^{-} and Substitute$^{ALA+LA+}$ (Fig. 5). The NAE profile of NHS was described here since it has not been extensively documented yet. The main NAEs measured in NHS were *N*-oleoyl-ethanolamine (OEA), *N*-linoleoyl-ethanolamine (LEA), *N*-palmitoyl-ethanolamine (PEA), *N*-stearoyl-ethanolamine (SEA) and AEA, respectively accounting for 37.4%, 25.2%, 19.1%, 11.0% and 4.9% of total NAE species in NHS (Fig. 5A). Similarly, the main NAEs in NHS dermis were also OEA (39.5%), LEA (28.5%), AEA (15.8%), PEA (7.5%) and SEA (6.3%) (Fig. 5A). Low levels of NAEs were derived from n-3 PUFAs in NHS (Fig. 5A-B). Although similar NAEs were identified in both the epidermis and the dermis of NHS, the epidermal compartment displayed significantly higher levels of NAEs than the dermal compartment (Fig. S6). The NAE profile of NHS found in the present study was comparable to the one depicted by Kendall and co-workers, a slight difference being higher levels of SEA and lower levels of OEA and LEA reported by Kendall than were found in the present study [44].

Next, the NAE profile of the skin substitutes were compared to that of NHS. All NAEs detected in NHS were also found in Substitute^{-} (Fig. 5A). However, lower levels of NAEs derived from n-6 PUFAs were found in both compartments of Substitute^{-} compared with their respective counterparts in NHS (Fig. 5B). This deficiency in n-6 PUFA-containing NAEs was mainly the consequence of lower levels of LEA (-17.1-fold) in Substitute^{-} than in NHS (Fig. 5D). Similarly, the levels of n-3 PUFA-containing NAEs, specifically docosapentaenoyl-ethanolamine (DPEA) and docosahexaenoyl-ethanolamine (DHEA), were significantly lower in Substitute^{-} epidermis than in that of NHS, showing a slight n-3 PUFA deficiency in Substitute^{-} epidermis (Fig. 5C). These lower NAE levels can be linked to the lower levels of n-6 PUFAs and n-3-PUFAs found in other lipid fractions of the skin substitutes, thus showing that this lipid class were also affected by the n-6 and n-3 PUFA deficiency [21,45]. NAEs biosynthesis in the skin substitutes was next studied after dual supplementation with ALA and LA. Supplementation with ALA and LA induced significant increases in levels of the n-3 PUFA-containing NAE DPEA in both the epidermis (7.3-fold) and dermis (5.1-fold) of Substitute$^{ALA+LA+}$ (Fig. 5C). Supplementation with ALA and LA tended to increase LEA levels (1.8-fold) in Substitute$^{ALA+LA+}$, although this increase was not statistically significant (Fig. 5D). The levels of NAEs seem to correlate with the levels of their precursors found in phospholipids (Table S2).

3.5. Identification of main MAGs of the skin and investigation of their biosynthesis in reconstructed skin following supplementation with ALA and LA

The eCB 2-arachidonoyl-glycerol (2-AG) and 6 other MAGs were next quantified using LC-MS/MS to first identify the species found in NHS (Fig. 6). The major MAGs in NHS were 1/2-palmitoyl-glycerol (2-PG), and 2-oleoyl-glycerol (2-OG), and 1/2-linoleoyl-glycerol (2-LG), accounting for 78.2%, 14.0% and 6.4%, of total MAG species in the epidermis (Fig. 6A). Low levels of MAGs were derived from n-3 PUFAs in NHS (Fig. 6A). Although similar MAGs were identified in both the epidermis and the dermis of NHS, the epidermal compartment displayed significantly higher levels of MAGs than the dermal compartment (Fig. S7). These results sug-

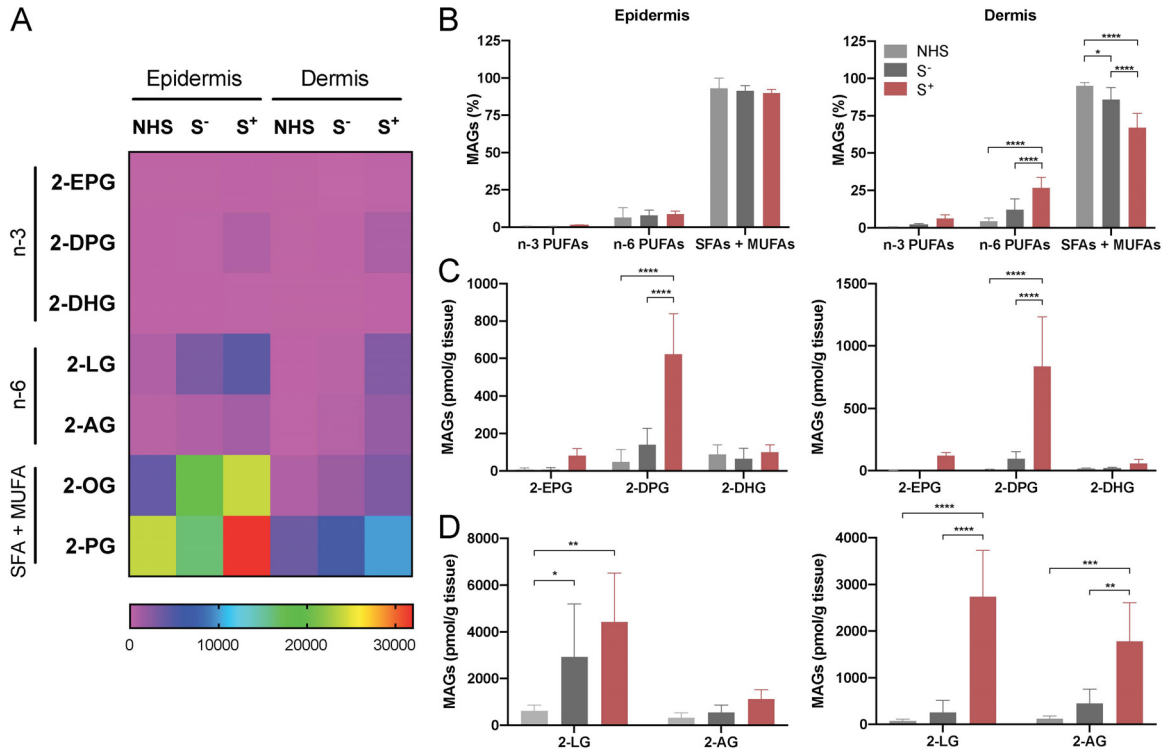


Fig. 6. Quantification of monoacylglycerols in the skin samples using LC-MS/MS analysis. (**A**) Heatmap of the seven MAGs measured in NHS, Substitute^{-} and Ssubstitute$^{ALA+LA+}$ (S+). (**B**) Proportion of MAGs derived from n-3 PUFAs, n-6 PUFAs or MUFAs and SFAs. MAG classes are shown as a percentage of total MAGs. MAGs derived from (**C**) n-3 PUFAs and (**D**) n-6 PUFAs found in NHS, S^{-} and S^{+}. For human skin: n=6 donors; for skin substitutes: n=3 donors. Two-way ANOVA followed by Tukey's *post-hoc* test $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Abbreviations: 1/2-AG, 1/2-arachidonoyl-glycerol; 1/2-DHG, 1/2-docosahexaenoyl-glycerol; 1/2-DPG, 1/2-docosapentaenoyl-glycerol; 1/2-EPG, 1/2-eicosapentaenoyl-glycerol; S, substitute; 1/2-LG, 1/2-linoleoyl-glycerol; MAG, monoacylglycerol; MUFA, monounsaturated fatty acid; NHS, normal human skin; 1/2-OG, 1/2-oleoyl-glycerol; 1/2-PG, 1/2-palmitoyl-glycerol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

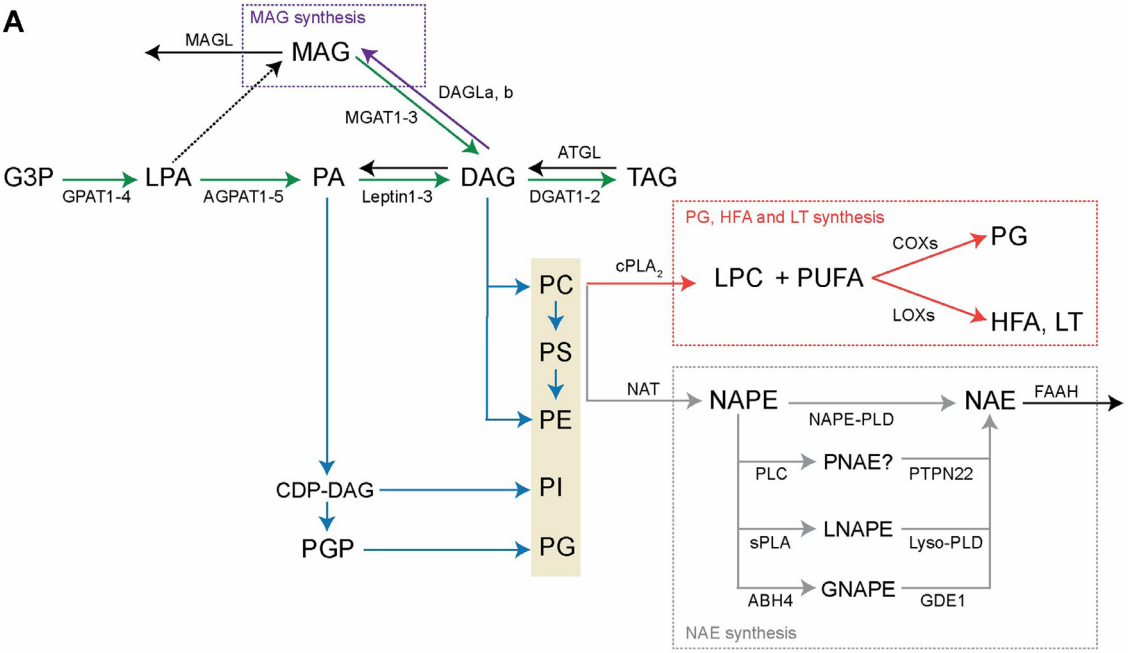
gest that the epidermis has a greater endocannabinoidome biosynthetic capacity than the dermis and possibly that some eCB-like lipids (NAEs and MAGs) are more important in the epidermis than in the dermis [44]. The present study is to our knowledge the first to report the levels of various MAGs in human skin.

The MAGs identified in NHS were also found in Substitute^{-} (Fig. 6A). In contrast with phospholipid, triglyceride and NAE fractions, the MAGs in Substitute^{-} did not displayed a deficiency in n-6 PUFAs compared with that of NHS (Fig. 6A-B). Indeed, the levels of MAGs were similar in both skin samples, except for 2-PG, which was decreased in Substitute^{-} compared with NHS (-3.3-fold) (Figs. 6A and S6). MAG biosynthesis in the skin substitutes was next studied after dual supplementation with ALA and LA. Supplementation of the culture media with ALA and LA increased the total amount of MAGs in the epidermis and the dermis of Substitute$^{ALA+LA+}$, although the increase was only significant in the dermis (Fig. S6). More specifically, increased levels of 2-docosapentaenoy-glycerol (2-DPG) (4.8-fold) were measured in Substitute$^{ALA+LA+}$ dermis and epidermis compared with Substitute^{-} (Fig. 6C). Furthermore, tremendous increases in the quantities of 2-LG and 2-AG were measured in the dermis of the Substitute$^{ALA+LA+}$ compared with Substitute^{-} (Fig. 6D). These results suggest that n-3 and n-6 PUFAs tend to accumulate as MAGs in the skin substitutes and thus may explain the deficiency observed in other lipid fractions even after supplementation.

The pathways for MAG synthesis and degradation are still not well documented, which makes these unexpected results difficult to explain. Phosphatidylinositol and phosphoinositides have been postulated to be the precursors of 2-AG, and therefore the biosynthesis of MAGs was thought to involve 1) hydrolysis of phosphatidylinositol by the Phospholipase C (PLC) and 2) further hydrolysis of the resulting diacylglycerol (DAG) by the DAG lipase α and β (DAGL) to generate MAGs (Fig. 7A). However, a recent study revealed that AA stimulated 2-AG biosynthesis in leukocytes independently of the PLC/DAGL or the PLD/DAGL pathway, since low levels of DAGL were found in these cells and DAGL inhibitors did not decrease the AA-stimulated biosynthesis of 2-AG [46]. The resulting hypothesis was that 2-AG synthesis would involve lysophosphatidic acid (LPA) as an intermediate, which was found to increase upstream of 2-AG after AA administration [46]. Interestingly, the expression of *DAGLA* and *DAGLB* was not detected in the skin substitutes under our culture conditions, therefore supporting the premise that MAG synthesis does not rely on DAGL activity (Fig. 7B). Furthermore, among the genes encoding proteins involved in the biosynthetic pathways of phospholipids and triglycerides, the expression of *GPAT, AGPAT1-3* and *DGAT1-2* was detected in the skin substitutes, supporting the conclusion that these biosynthetic pathways are active in keratinocytes and fibroblasts (Fig. 7A-B). MAGs could thus be important intermediates produced during the biosynthesis of phospholipids and triglycerides. Therefore, perhaps the supplementation of the culture media with MAGs, such as 2-LG, could equilibrate the lipid metabolism of the skin substitutes, leading to a better distribution of PUFAs.

4. Discussion

Tissue-engineered skin substitutes represent promising tools for the study of lipid mediator signaling and functions in the skin. These models made it possible to dissect the metabolism of various subsets of cells in order to identify specific mechanisms. The present report is to our knowledge the first to study the lipidome of a tissue-engineered human skin model and its impact on the skin barrier function. As expected, the reconstructed skin substitutes displayed lower proportions of n-3 and n-6 PUFAs and higher



B

Gene symbol	Gene name	Linear signal
GPAT2	Glycerol-3-phosphate acyltransferase 2	109
AGPAT1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	877
AGPAT2	1-acyl-sn-glycerol-3-phosphate acyltransferase beta	7684
AGPAT3	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	860
AGPAT4	1-acyl-sn-glycerol-3-phosphate acyltransferase delta	79
AGPAT5	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	304
AGPAT6	Glycerol-4-phosphate acyltransferase 4	188
AGPAT9	Glycerol-3-phosphate acyltransferase 3	27
MOGAT1	2-acylglycerol O-acyltransferase 1	7.7
MOGAT2	2-acylglycerol O-acyltransferase 2	6.8
MOGAT3	2-acylglycerol O-acyltransferase 3	73.9
DGAT1	Diacylglycerol O-acyltransferase 1	264.3
DGAT2	Diacylglycerol O-acyltransferase 2	450.5
DAGLA	Diacylglycerol lipase alpha	4.8
DAGLB	Diacylglycerol lipase beta	66.6
MGLL	Monoacylglycerol lipase	1726.7
ABHD6	Monoacylglycerol lipase ABHD6	553
ABHD12	Monoacylglycerol lipase ABHD12	230

Fig. 7. Biosynthetic pathways of phospholipids, triglycerides and lipid mediators. (**A**) Schematic overview linking phospholipid, triglyceride and lipid mediator biosynthetic pathways. Triglycerides (TAG) are synthesized *de novo* (lipogenesis, green arrows) from glycerol-3-phosphate (G3P). Phospholipids can also be synthesized *de novo* (blue arrows). In regard to lipid mediators, prostaglandins (PGs, orange box), hydroxy fatty acids (HFAs, orange box), *N*-acyl-ethanolamines (NAEs, grey box) and monoacylglycerols (MAGs, purple arrows and purple box) are produced mainly from phospholipids. (**B**) Profiling of genes encoding the main proteins involved in the biosynthetic pathways of lipids presented in panel A.

proportions of monounsaturated fatty acids compared with NHS. These results are consistent with other studies, in which a deficiency of n-6 PUFAs was found in tissue-engineered human skin produced with culture media that were not supplemented with PUFAs [20,45]. Dual supplementation with ALA and LA decreased the percutaneous absorption of testosterone and modulated the phospholipid, triglyceride and lipid mediator profile of the skin substitutes. Therefore, the exogenous provision of ALA and LA had a similar impact on the barrier function of the reconstructed skin substitutes as dietary supplementation with essential fatty acids *in vivo* [24,25,47]. These results showed that the metabolism of the n-3 and n-6 polyunsaturated fatty acids remains functional in the skin model produced *in vitro*.

Although other techniques, such as the measurement of transepithelial water loss, can be used to assess membrane permeability, the percutaneous absorption assay is recommended by the OECD, since this sensitive technique allows the detection of small changes in barrier integrity [48,49]. In addition, percutaneous absorption studies offer the possibility of evaluating different pathways of molecule penetration, based on their physicochemical properties [33,50–52]. In the present study, the percutaneous absorption assay revealed that dual supplementation with

both ALA and LA decreased the permeability of the skin substitutes to testosterone, showing an improvement of the skin barrier function after lipid supplementation. Testosterone is a lipophile molecule and thus the assay predominantly evaluates the absorption of molecules through the intracellular pathway, in which molecules passed mainly through the stratum corneum lipid matrix [43]. Therefore, this assay suggests that the lipid supplementation has mainly influenced the lipid content of the skin substitutes. It is important to mention that many factors other than fatty acid composition can influence skin permeability, including skin type, age and body area [53,54]. It is of note that darkly pigmented skin was shown to display a more resistant skin barrier compared with lightly pigmentated skin [53]. Therefore, skin pigmentation could contribute to the suboptimal permeability of the reconstructed skin substitutes since the melanocytes responsible for skin pigmentation are not added to reconstructed tissues. Although these factors did not vary between the supplemented and controlled conditions in the present studies and thus should not have influenced our findings, they are particularly important to take into consideration when comparing the permeability of different models. In fact, there is a variety of models used in the literature and they have been found to have different levels of permeability [55,56]. For instance, it was shown that skin models produced with hyaluronic acid had lower transepidermal water loss than those produced with agarose [57]. Previous studies have shown that individual supplementation with ALA decreased testosterone absorption through the skin substitutes [21]. It was also shown that exogenous provision with ALA increased n-3 PUFA levels in the skin substitutes while supplementation with LA increased n-6 PUFA levels in the skin substitutes. These results showed that although ALA supplementation improved the skin barrier function, it did not restore LA levels, which therefore remained the most deficient fatty acid in the skin substitutes. In the present study, gas chromatography analyses revealed that the exogenously provided ALA and LA were incorporated into the skin substitute phospholipids and triglycerides. The resulting fatty acid profile was enriched in LA, which represents an improvement compared with the individual supplementation with ALA. LA was the fatty acid that was most closely correlated with the decreased absorption of testosterone, thus showing its importance for the establishment of the skin barrier function.

Once incorporated into phospholipids, fatty acids can be hydrolyzed by the phospholipase A2. These liberated fatty acids can then be converted into several bioactive lipid mediators (Figs. 7A and S1). Whether fatty acids will serve in the biosynthesis of either prostaglandins, leukotrienes or endocannabinoids depends on several factors, including the expression and activation of the enzymes responsible for their biosynthesis and the levels of fatty acids in phospholipids [58,59]. Consequently, bioactive lipid profiles vary among tissues and cell types [60]. In the present study, investigation of the PG and HFA content of reconstructed skin revealed that 15-LOX is the predominant active biosynthetic pathway in the epidermis, leading to high levels of 15-LOX products. Higher levels of 13-HODE in the skin substitutes may support the premise that LA is strongly metabolized into its oxidized derivative in the epidermis of reconstructed skin models. This enhanced metabolism could be the result of higher activity of 15-LOX in the skin substitute epidermis than in that of normal human skin, which is in agreement with the overexpression of this enzyme found in the skin substitutes [61]. The dual supplementation decreased the levels of 13-HODE in the skin substitute epidermis, although an increase in LA was measured in the phospholipids, suggesting that the addition of the n-3 PUFA decreased LA metabolization through competition for 15-LOX [62]. It is noteworthy that high production of 13-HODE found in the skin was shown to regulate keratinocyte hyperproliferation [62]. Furthermore, levels of 12-HETE were lower in the skin substitutes compared with NHS. These lower levels are consistent with high amounts of 15-LOX products, such as 15-HEPE, which are known to inhibit 5-LOX and 12-LOX pathways. Moreover, lower levels of PGs were found in the epidermis of the skin substitutes. Since the activity of COX-1 was shown to be similar in the reconstructed skin model (Epiderm^{TM}) produced with primary keratinocytes to that in human skin, the lower levels of PGs found in the epidermis are more likely to be the result of competition with other pathways such as the high activity of LOX in the skin substitutes [63]. Contrary to quantification performed in normal human skin and reported in other studies, the levels of AA-derived PGD_2 were too low to be detected in Substitute$^-$, which confirms that this lipid mediator is mostly produced by other epithelial cells than keratinocytes and fibroblasts. Indeed, PGD_2 is usually known for being produced principally by Langerhans cells and dermal mast cells exerting anti-proliferative and anti-inflammatory activities [26]. On the other hand, the COXs were the predominant active biosynthetic pathways in the dermis, leading to high levels of COX products, notably PGE_2. Higher levels of PGs in the dermis were consistent with the higher levels of AA found in the phospholipids of Substitute$^-$. High levels of PGE_2 in the Substitute$^-$ dermis could be stimulated by cell-cell interactions of keratinocytes and fibroblasts. Indeed, the secretion of proIL-1α by keratinocytes stimulates the production of PGE_2 in fibroblasts by a mechanism in which the activity of COX-2 is increased [64]. It was shown that high amounts of fibroblast-derived PGE_2 stimulate keratinocyte proliferation [64,65]. Under our culture conditions, no correlation was found between lipid mediator levels and the permeability of the skin substitutes to testosterone. It is however important to mention that bioactive lipid mediators have very short half-lives and therefore individual supplementation with bioactive lipid mediators would bring relevant insight into their respective roles in the establishment of the skin barrier function.

The best-known NAE and MAG species are AEA and 2-AG respectively [66]. Indeed, these two bioactive lipid mediators are called endocannabinoids as they can activate the type-1 and -2 cannabinoid receptors [67,68]. Based on their similar structures and functions, other NAEs and MAGs are referred to as endocannabinoid-like mediators [69]. In the present study, the levels of NAEs correlate with the levels of their respective precursors. Therefore, the deficiency in n-3 and n-6 PUFAs was found to affect the levels of NAEs in the skin. Since PEA, SEA, OEA and LEA are present in high amounts in the skin, they may play important roles in that tissue. These NAEs activate several receptors that are found in the skin, such as peroxisome proliferator-activated receptor α (PPARα), transient receptor potential cation channel subfamily V member 1 (TRPV1) and the orphan G protein-coupled receptors GPR119 and GPR55 [69,70]. In a dermatitis animal model, the topical application of LEA on the affected ear skin decreased pro-inflammatory cytokine expression, while inhibiting NF-kB signaling [71]. Therefore, the role of these metabolites in the skin should be more deeply investigated.

Interestingly, we showed in the present study that PUFAs tend to accumulate in the form of MAGs in epithelial cells cultured in a reconstructed tissue, which may explain the deficiency in LA observed in other lipid fractions such as phospholipids, triglycerides and NAEs, even after supplementation. The role of MAGs in the skin has been poorly described and a few studies have pointed out lately that the synthesis of MAGs is still not well understood [46,72]. Indeed, the main pathway for MAG synthesis is mediated by DAGL α and β. However, under our culture conditions the expression of DAGL α and β was not detected in the skin substitutes by microarray analyses, indicating that MAG biosynthesis may not occur through this pathway in the reconstructed skin model. The expression of DAGLs has not been documented extensively in native human skin and whether keratinocytes express DAGLs is, to

our knowledge, unreported. Nevertheless, the expression of DAGL α and β was found in the hypodermis, fibroblasts, melanocytes and the sweat glands [73,74]. Interestingly, Lee and coworkers have reported that MAGs could be involved in triglyceride synthesis in the epidermis of the guinea pig [75]. In contrast, Lin and coworkers showed that decreased levels of MAGs in FATP4$^{-/-}$ epidermis was accompanied by an unaltered level of triacylglycerols [72]. This accumulation of MAGs could be attributed to an unsuccessful attempt to synthesize triglycerides via the monoacylglycerol pathway in epithelial cells.

5. Conclusion

The findings described in the present study show that an optimized supply of essential fatty acids via culture media is essential for the establishment of the barrier function *in vitro*, as it largely affects the lipidome of the reconstructed skin. While individual supplementation with ALA previously pointed toward the importance of n-3 PUFAs in the establishment of the skin barrier, the present study shows that n-6 PUFAs, and more specifically LA, play a crucial role in the skin barrier function and that they need to be used in combination with n-3 PUFAs for optimal incorporation and metabolization by the reconstructed tissue. Since little is known about the individual effects of each lipid mediator in the skin, our study highlights the importance of examining in more detail the effect of bioactive lipid mediators in the development and maintenance of the barrier function. Indeed, several lipid mediators such as 13-HODE, DPEA and 2-AG are known to exert anti-inflammatory properties, and a better understanding of their respective cutaneous metabolisms could make it possible to identify new therapeutic targets for skin disease.

Declaration of Competing Interest

The authors declare no competing interests

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.11.021.

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