Interomic analysis of pre- and post-menopausal facial skin and the effects of niacinamide treatment

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Abstract

Menopause occurs to females aged 45 to 55. The decline of estrogen can lead to accelerated skin aging conditions.

Our study aimed to investigate the changes in skin from pre-menopausal (PreM) to post-menopausal (PoM) volunteers at the molecular and microbial level using metabolomics, lipidomics and microbiome analysis. Additionally, on PoM skin we evaluated the effects of niacinamide (NA) known to offer numerous benefits.

PreM and PoM skin had similar hydration and TEWL values before and after placebo treatment but NA significantly reduced TEWL and improved hydration. Skin homeostasis seemed to get out of balance at PoM indicated by elevated levels of NMF, histidine catabolic intermediates, and decreased FFA. NA treatment clearly recovered cytosine level that was down in PoM as potential marker for DNA damage. During the observation, the ceramides generally decreased in all groups except EOdS in PreM. More changes are shown in the fatty acid profiles. The NA group had less reduction in ceramides with significantly higher levels for ceramide classes AH, NdS and NS. For microbes, qPCR showed high individual variability with higher absolute abundance of *C. acnes* species on PreM skin. In summary NA can help at issues of PoM skin and conserves the microflora.

Keywords

(pre/post-menopause; TEWL; metabolomics; lipidomics; microbiome; niacinamide).

Introduction.

Menopause typically occur to female age of 45 to 55, during this time, the primary female sex hormones such as estrogen and progesterone, decline significantly. The decline of estrogen can lead to changes in skin conditions, often including dryness, thinning, decreased elasticity, and increased risk of wrinkles and age spots. Accelerated skin aging has been reported after menopause [1]. Skin, as the largest organ, protects us against pathogens, homeostatic regulation, dehydration, and provides immune surveillance [2]. With prolong life expectancy, skin aging is becoming a more prevalent heath concern, which contributes to deep wrinkles, reduced elasticity, pigmentation, and neoplasms.

Stratum corneum (SC) is the outermost layer of the epidermis and is the topmost layer of the skin. It is composed of dead skin cells known as corneocytes and serves as a protected barrier against external environment and prevent trans epidermal water loss (TEWL). Desquamation, shedding of SC, is a natural renewal process to maintain a healthy and fresh skin appearance. The rate of desquamation takes 28-40 days and can be influenced by age, genetics, and environmental conditions. Niacinamide, as a form of vitamin B3, is often used in skin care products to improve moisturization, skin tone and texture by building skin barrier function [3]. Niacinamide also is found to increase keratin, filaggrin, involucrin and desquamation rate to improve SC hydration [4-5]. Its antioxidant property also protects the skin from free radicals and premature aging, and therefore improves skin elasticity. However, there are very few studies of niacinamide effect on post-menopausal skin. A clinical trial is designed by sampling SC using skin strip to understand the metabolomic and performance

differences between pre- and post-menopausal facial skin and evaluate the beneficial effect of niacinamide to post-menopausal skin.

Materials and Methods.

1) Clinical trial setup:

The clinical trial was conducted by Eurofins DERMSCAN Gdansk, Poland. Total 99 subjects were split into three research groups (Table 1) with six superimposed D'Squame tape strip taken from one cheek side of the face at Day 0 and Day 28 of the study. Five percent of niacinamide was used in the treatment group 3 (Table 2). TEWL was measured with Tewameter, hydration rate was measured with Corneometer, protein optical density in collected skin strip samples was measured with SquameScan®, and the skin strip samples were stored in -80°C until analyzed with untargeted metabolomic protocol at Columbia MD, USA or lipidomic analysis by Lipotype (Germany) or microbiome analysis at Bio-Me (Norway)

Table 1 Clinical trial design. 5% niacinamide was used as active ingredient in the product treatment.

Subject group	Description	Treatment	Number of subjects	Average age
1	Pre-menopausal	placebo	30	48.9 (between 42-57)
2	Post-menopausal	placebo	32	59.5 (between 48-65)
3	Post-menopausal	product	32	58.1 (between 48-65)

Product formulation composition with INCI Names:

65.2% AQUA, 10% OCTYLDODECANOL, 8% CAPRYLIC/CAPRIC TRIGLYCERIDE, **5% NIACINAMIDE**, 4% CETEARYL OLIVATE; SORBITAN OLIVATE, 2% PROPANEDIOL, 2% DIMETHICONE, 1.5% CETEARYL ALCOHOL, 1% PHENOXYETHANOL/ ETHYLHEXYLGLYCERIN, 0.4% HYDROXYETHYL ACRYLATE/SODIUM ACRYLOYLDIMETHYL TAURATE COPOLYMER, 0.28% CITRIC ACID, 0.2% CHLORPHENESIN, 0.2% XANTHAN GUM, 0.2% SODIUM GLUCONATE

In the placebo formulation the niacinamide was replaced with water.

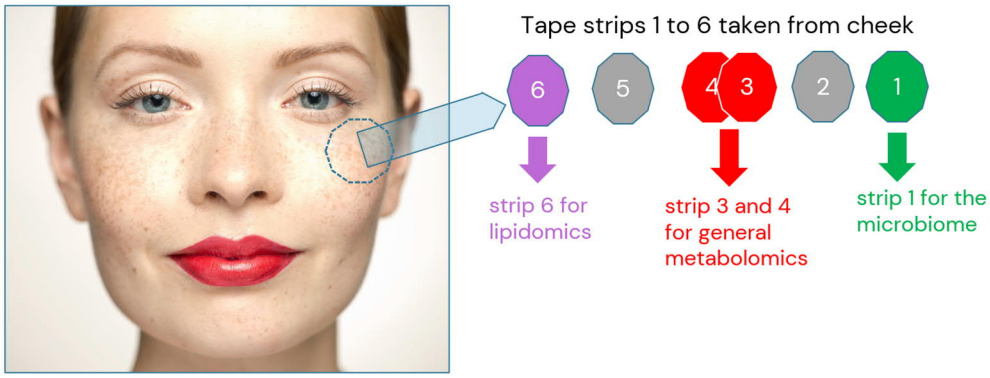


Figure1 Six D-Squame tape strips were taken from the same spot on the cheek.
No 1 used for microbe analysis, no3 and 4 for metabolomics and no6 for lipidomics.

2) Procedure for metabolomics:

Skin strip extraction for metabolomic study: Two skin strips at layers 3 and 4 are combined into one 1.7mL Eppendorf tube and extracted with 0.5ml methanol, vortexed, centrifuged and filtered 0.4μm.

Data acquisition for metabolomic study: The skin strip extracts were analyzed with the untargeted metabolomic platform using a ThermoQ Exactive at a mass resolution of 35000 to cover comprehensive major chemical classes expected in stratum corneum. The skin strip extracts were analyzed with three separate LCMS methods (Polar, free fatty acids (FFA) and Ceramides). The extracts were analyzed with HILIC-LC-MS with positive electrospray ionization (ESI) for polar metabolites and natural moisturizing factors (NMF). Free fatty acids (FFA) and organic acids were analyzed with RP-LC-MS with negative ESI. The extracts were concentrated and analyzed with RP-LC-MS with positive atmospheric pressure chemical ionization (APCI) for ceramides, cholesterol, and lipid analysis. The samples were randomized, and 5 µL extract were injected on column. Pooled samples were run for every 12 samples, methanol blanks were run for every three samples. Amino acids standard mix and free fatty acid mix were run daily for quality control. MSMS data were collected with data dependent acquisition on pooled samples.

Data analysis: The x.raw LCMS data from each LCMS method were analyzed separated using Thermo Compound Discoverer. Compounds were detected with 15 ppm mass tolerance, 1x106 minimal peak intensity, and greater than 10 chromatographic S/N. Detected compounds were grouped with 20 ppm mass tolerance and 0.5 min retention time tolerance. The peaks were filtered with peak rating threshold of 4 in minimal five datafiles. Missing peaks were gap-filled with 15ppm mass tolerance and 10 S/N threshold. Compound annotations were made based on MSMS matches from NIST_2020_MSMS_HR and internal database with 10 ppm precursor mass tolerance, 2 min RT tolerance (adjustment to RT shift) and 50 match factor tolerance. Metabolites were identified either with authentic standards or based on MSMS library match.

Data matrix from all three chromatographic methods were analyzed separately. Before analysis, data reduction was performed. Metabolite features that had peak area greater than $1x10^8$ in methanol blanks, or peak area ratio greater than one between methanol blank average and maximum in all samples (i.e. metabolites appeared to be more abundant in blank than in skin strip samples) were removed. Multivariance analysis, principal component analysis (PCA) and orthogonal partial least square discriminative analysis (OPLS-DA), were performed using SIMCA-P after pareto scaling. Agilent Mass Profiler Professional was used for univariant analysis and plotting Venn diagram. For within group analysis comparing Day 0 and Day 28, pairwise student's t test was used, moderated student t-test was used for between group analysis. Benjamini Hochberg FDR was used for false discovery rate (FDR) correction, when needed. Pearson correlation between metabolites and performance measurement (TEWL and hydration) was conducted using Microsoft Excel.

3) Procedure for the lipid analysis:

Materials: Methanol, propan-2-ol, chloroform, acetyl chloride and ammonium acetate were of analytical grade. Deuterated NS D3 (36:1;2) (cat# 2201) and EOS D9 (68:3;2) (cat# CUS9530) were purchased from Matreya LLC. Deuterated TAG D5 (cat# 110544) and DAG D5 (cat# 110538), and CE(20:0;0) (cat# 110870) were purchased from Avanti Polar Lipids.

Methods: Lipids were extracted from tape strip no 6 using chloroform as described in [6], page 3 of the paper contains comprehensive description of the workflow]. Samples were spiked with lipid class-specific internal standards prior to extraction. After drying and re-suspending in MS acquisition mixture, lipid extracts were subjected to mass spectrometric analysis. Mass spectra were acquired on a hybrid quadrupole/Orbitrap mass spectrometer

equipped with an automated nano-flow electrospray ion source in both positive and negative ion mode. Lipid identification using LipotypeXplorer [7] was performed on unprocessed (*.raw format) mass spectra. For MS-only mode, lipid identification was based on the molecular masses of the intact molecules. MSMS mode included the collision-induced fragmentation of lipid molecules and lipid identification was based on both the intact masses and the masses of the fragments. Prior to normalization and further statistical analysis lipid identifications were filtered according to mass accuracy, occupation threshold, noise and background. Lists of identified lipids and their intensities were stored in a database optimized for the particular structure inherent to lipidomic datasets.

4) Procedure for microbiome analysis:

For the microbiome analysis we used the first tape from the skin surface. The tapes were stored at -80°C and prior to analysis the DNA was extracted according to standard procedures. The composition of the skin microbiota was analyzed by a validated quantitative PCR method (Bio-Me's Precision Microbiome Profiling [PMP™]), based on TaqMan™ technology on OpenArray® format (Thermo Fisher Scientific), that targets 48 skin bacterial taxa and 5 skin fungi taxa – it also includes three controls. Standard curves for the assays were generated by running reference materials quantified by fluorescence (Quant-iT™ PicoGreen™ dsDNA Reagent, Thermo Fisher Scientific). The absolute quantification of each target (number of genomic copies per µL) was interpolated from the standard curves. Analysis of the results and generation of graphs were made in Excel or using the micriobiomeanalyst (MicrobiomeAnalyst)

Results.

1) Results from the skin measurements

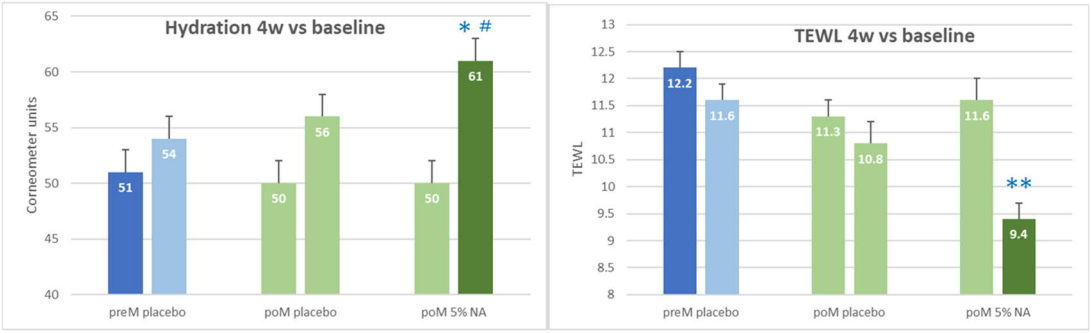


Figure 2 Skin measurements of the premenopausal group (preM) and postmenopausal groups (poM) before day0 (left column) and after treatment day28 (right column). NA =5% niacinamid formulation. Absolute measured values are shown for Corneometer and TEWL. ** p<0.0001 Anova both to placebo of poM and preM groups after treatment, * p<0.0001 to preM placebo day28, # p<0.001 to poM placebo day28

Skin measurements showed almost the same starting moisture level for the three groups at study start and moderate changes after the placebo treatment with plus 6% hydration on the premenopausal group (70% of subjects showed increase) and plus 11% for the postmenopausal group (88% of subjects positive) and clearly the outperformance with the niacinamide formula plus 23% (97% of subjects positive). For the TEWL measurement before the treatment we observed slightly reduced (not significant) values for the poM groups and again placebo showed moderate reductions (preM minus 5% reductions seen for 27% of the

volunteers and poM minus 4% for 19% of volunteers) whereas the clear benefit on TEWL reduction was again seen for the niacinamide formula minus 19% and 91% of volunteers showed reduction of TEWL.

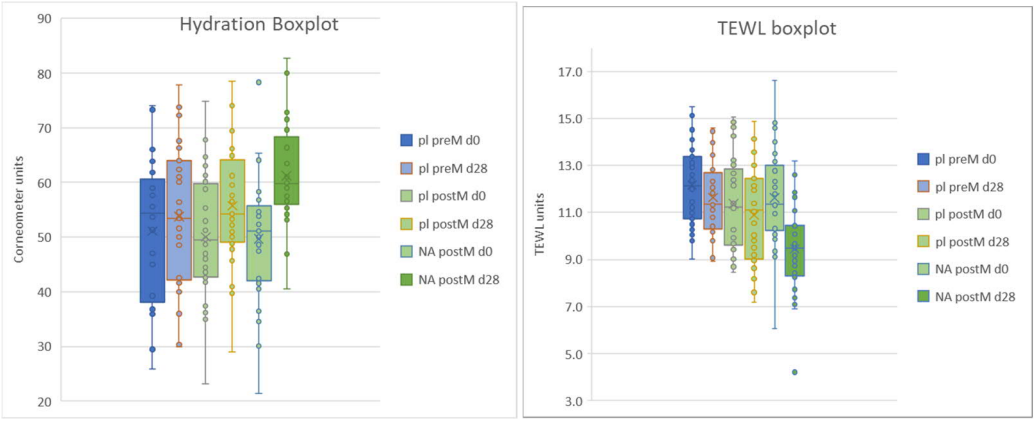


Figure 3 Boxplots of corneometer and TEWL values for the different groups before (d0) and after (d28) treatment

2) Metabolomic results taken from tape3 and 4

Metabolomics quality control: After data reduction, the total number of features detected in each of the three methods were summarized in Table 2. The internal standard variance calculated for Polar, FFA and Ceramide methods using methionine-d3, docosanoic acid-d43, and NS-d18:1-d7/16:1 were 34%, 37% and 15%, respectively, between pool and all samples. However, normalization with internal standards led to greater outliers and spread of pool in PCA score plots. Therefore, the metabolomic data were analyzed as is without any internal standard normalization. Despite the slight large variance in internal standards, the pooled samples showed a tight cluster for all three methods indicating good instrument reproducibility and reliable data.

Table 2 Metabolomic data summary

	Polar	FFA	Ceramides
Final metabolite features	5831	2363	1595

Correlation of skin measurements with metabolomic data: Consistent to the clinical trial conditions, niacinamide and its related metabolite derivatives were only detected at high levels at Day 28 for post-menopausal product treated group (Figure 4). A group of high responders were selected with TEWL reductions more than 2.5 units comparing day 28 to day 0 (subject V5, V8, V9, V11, V27, V49, V52, V55, V59 and V60). For the high performers in post-menopausal product group, a strong negative Pearson correlation of greater than 0.7 were noted between TEWL and niacinamide (and its derivative) levels detected in the skin strips (Table 3). Similarly, a moderate positive correlation was noted between measured hydration levels and niacinamide.

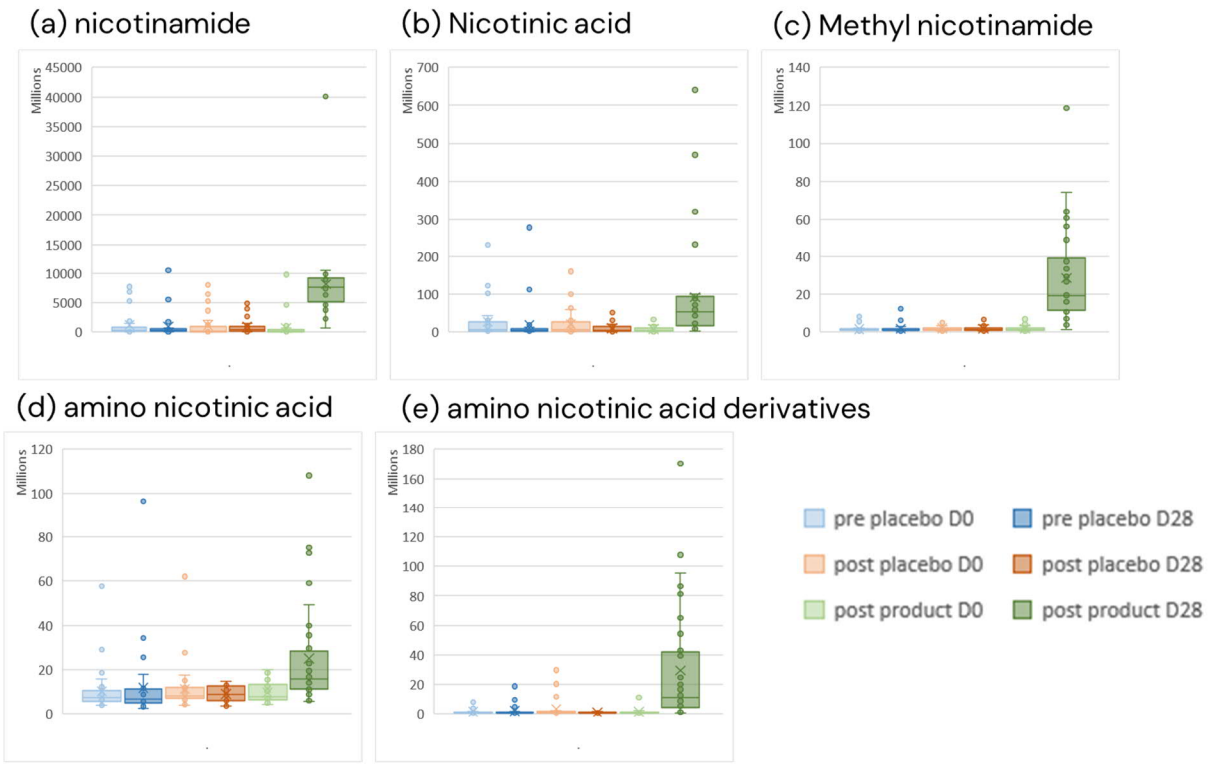


Figure 4 Nicotinamide (niacinamide) and its deverbatives detected in skin strip samples. (a) nicotinamide, (b) nicotinic acid, (c) methyl nicotinamide, (d) amino nicotinic acid, and (e) amino nicotinic acid derivatives.

Table3 Pearson correlation between in-skin niacinamide and methyl nicotinamide to performance measurements in TEWL and corneometer units. The calculation was limited to the high performing subjects with TEWL difference less than -2.5 in post-menopausal product group for day 0 and 28.

Pearson correlation	**TEWL (high performer)**	**Hydration (high performer)**
Niacinamide	-0.76	0.59
methyl nicotinamide	-0.74	0.33

Significant metabolites showed difference between pre- and post-menopausal skin: Multivariant analysis showed no major metabolic differences between pre- and post-menopausal skins. OPLS-DA were performed for pre- and post-menopausal data for either Day 0 or Day 28 for each of the three analytical methods. Only FFA data at day 0 comparison shows a statistically significant between pre- and post-menopausal with Q2(cum) at 0.6 (two conditions are considered metabolically different if Q2(cum)>0.4). The remaining OPLS-DA all had Q2(cum) significantly below 0.4 and suggesting very little metabolic differences between pre- and post- menopausal skin (Table 4).

Table4 OPLS-DA results comparing pre- versus post-menopausal skin for either Day 0 or Day 28; Day 28 versus Day 0 product treatment in post-menopausal skin.

	Pre- vs. post-menopausal Day 0			**Pre- vs. post-menopausal Day 28**			**Post-menopausal Day 28 vs. Day 0 product treatment**		
	R^2X	**R^2Y**	**Q^2(cum)**	**R^2X**	**R^2Y**	**Q^2(cum)**	**R^2X**	**R^2Y**	**Q^2(cum)**
Polar method	0.162	0.147	0.0693	0.246	0.483	0.074	0.498	0.676	0.349
FFA method	0.461	0.964	**0.587**	0.276	0.697	0.168	0.486	0.876	**0.494**
CER method	0.129	0.164	-0.0292	0.268	0.394	-0.0281	0.498	0.749	0.368

Since multivariant analysis like OPLS-DA tends to bias towards metabolites with higher MS signal, univariant analyses were performed to interrogate individual metabolite differences between conditions. Subtle metabolic differences were observed for pre- and post-menopausal skin. Comparing pre- and post-menopausal skin, the post-menopausal subjects at Day 0 from placebo and product groups were combined and compared to the pre-menopausal subjects at Day 0 using moderate unpaired Student's T-test. For Day 28 comparison were only focused on comparing pre- versus post-menopausal subjects with placebo treatment. The criteria for significant metabolites were set to have p value less than 0.05 and fold change greater than 1.5. Only 0.2-1.6% of the total metabolites were considered statistically significant with Benjamini Hochberg FDR. To explore subtle metabolic differences, any metabolites that fell into the significant criteria without FDR correction were considered and analyzed for biological relevance (Table 5).

Table5 Number of significant metabolites detected between pre- versus post-menopausal skin using moderate unpaired Student's T test with $p<0.05$ and fold change greater than 1.5

	Day 0		Day 28	
	Up regulated in post-menopausal	Down regulated in post-menopausal	Up regulated in post-menopausal	Down regulated in post-menopausal
Polar method	294	111	481	171
FFA method	106	37	108	85
Ceramide method	10	27	0	123

NMF, amino acids and some dipeptides were found to be present at significantly higher levels in post-menopausal skin compared to pre-menopausal subjects (Figure 5). Significant decrease of cytosine and methylcytosine were also found in post-menopausal skin. For FFA, most unsaturated long chain fatty acids (LC-PUFA) were decreased significantly in the post-menopausal subjects, and lesser age effect were found to the saturated FFA (Figure 5, Table 6). Compared to pre-menopausal skin, overall up-regulation of the histidine catabolic pathway were observed in post-menopausal skin. More details on ceramides analysis are shown in the lipidomics section.

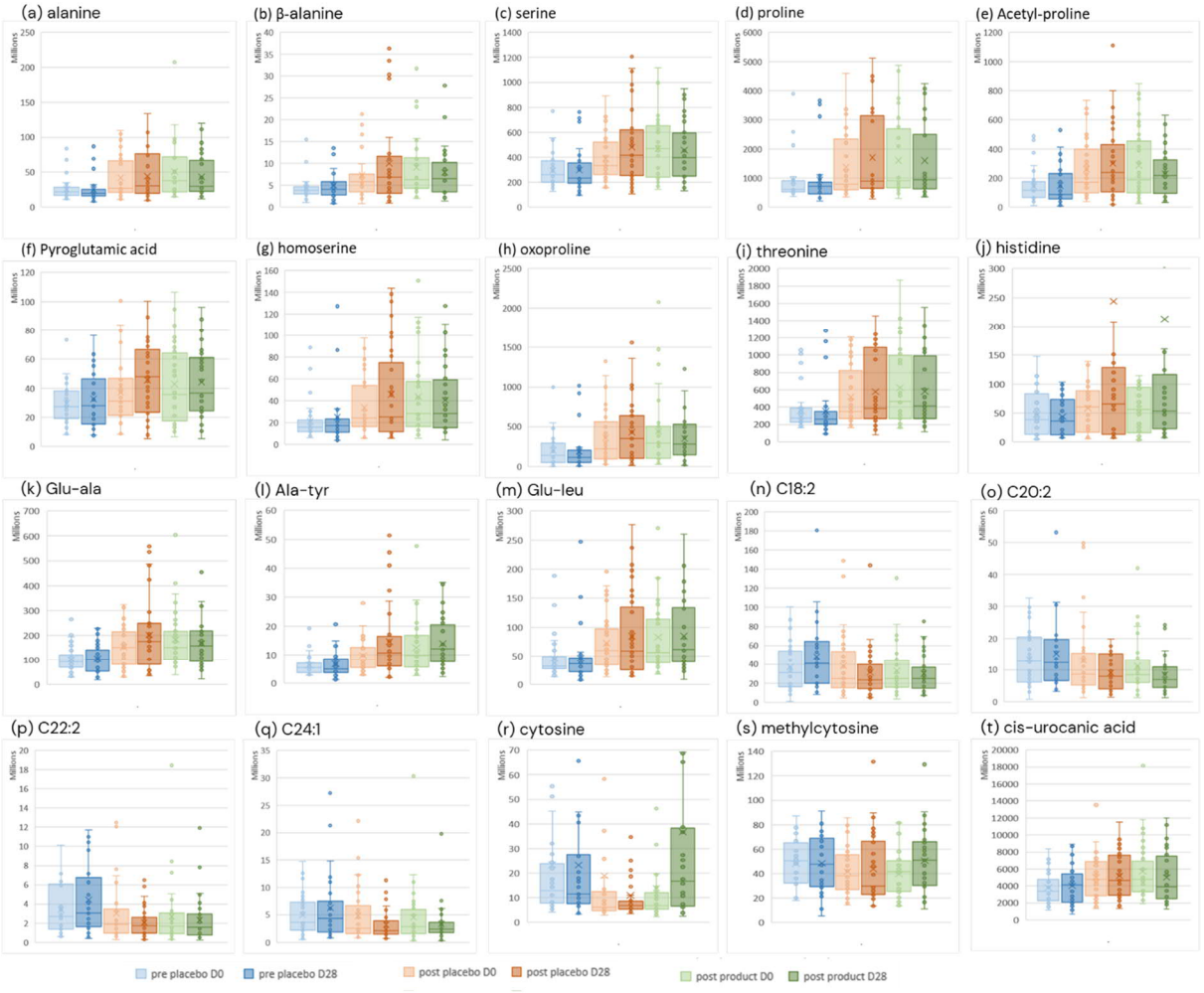


Figure5 Metabolites that show significant pre- versus post-menopausal differences. (a) alanine, (b) β-alanine, (c) serine, (d) proline, (e) acetylproline, (f) pyroglutamic acid, (g) homoserine, (h) oxoproline, (i) threonine, (j) histidine, (k) glu-ala, (l) ala-tyr, (m) glu-leu, (n) C18:2, (o) C20:2, (p) C22:2, (q) C24:1, (r) cytosine, (s) methylcytosine, and (t) cis-urocanic acid.

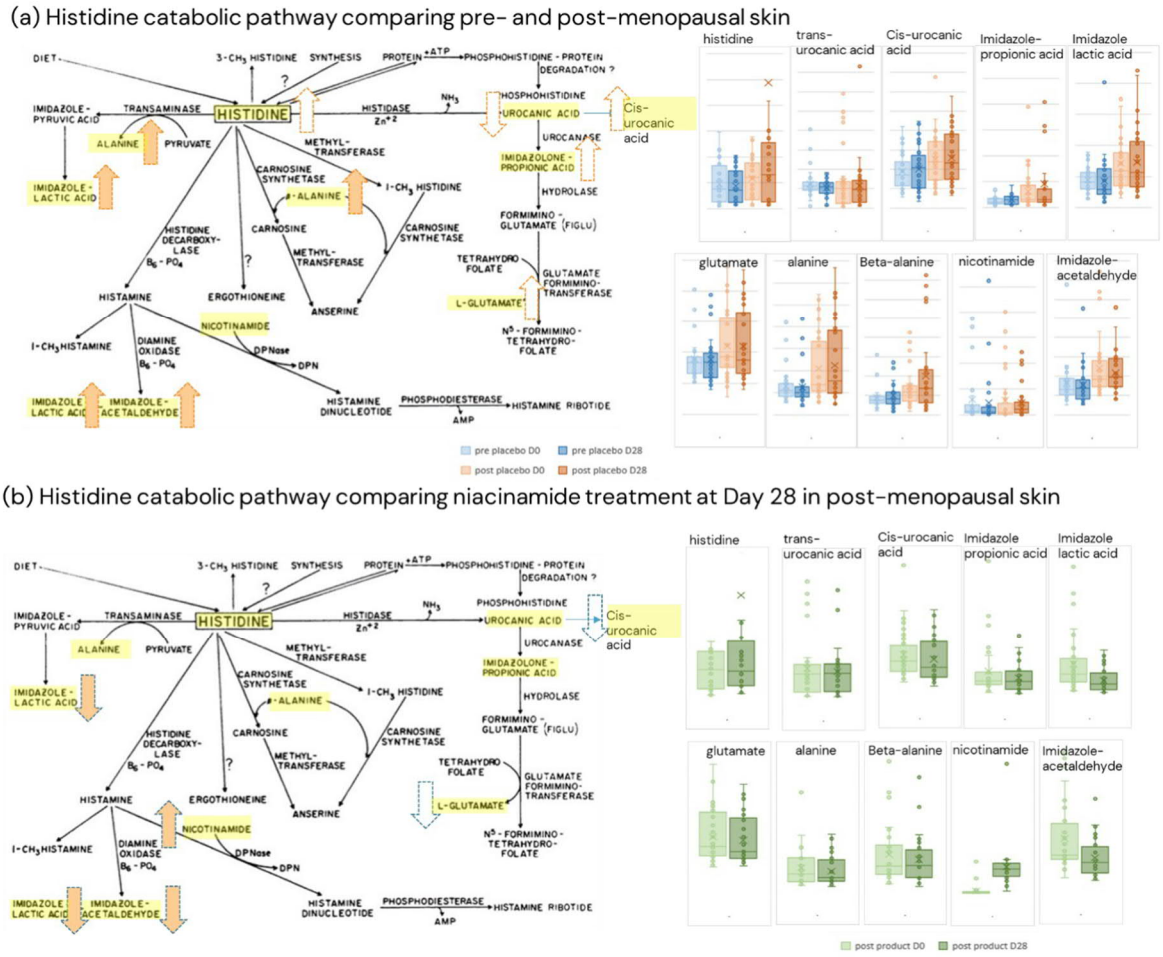
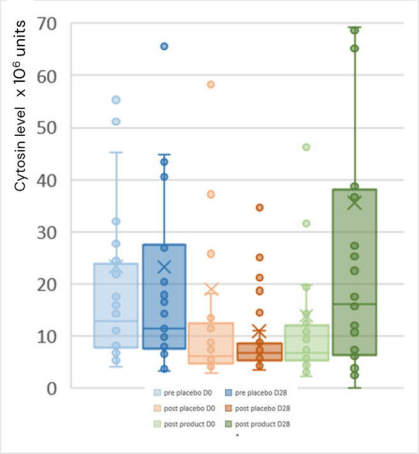


Figure6 Histidine catabolic pathway and its intermediates showing (a) differences between pre- versus post-menopausal skin, and (b) effect of niacinamide treatment. The detected metabolites were highlighted in yellow, metabolites with statistical significance were annotated with a solid arrow indicating up or down regulated in (a) aged skin or (b) Day 28 of niacinamide treatment; white arrow indicate trends of up or down regulated in (a) aged skin or (b) Day 28 of niacinamide treatment.

Table6 Detectable FFA that either significantly down-regulated in post-menopausal skin compared to pre-menopausal subjects, or unaffected with age.

FFA significantly decreased in post-menopausal skin compared to pre-menopausal	C12:0, C16:1, C18:2, C20:1, C20:2, C22:1, C22:3, C24:1, C24:2, C24:4, C26:1, C32:2, C32:3, C34:3, C34:4
FFA unchanged between pre- and post-menopausal skin	C6:0, C8:0, C9:0, C10:0, C14:0, C15:0, C16:0, C18:0, C18:1, C18:3, C20:0, C20:3, C22:0, C20:3, C22:0, C22:2, C22:4, C24:0, C24:3, C26:0, C28:0, C28:1, C30:0, C30:1, C30:2, C32:0, C32:1, C34:1, C36:1, C36:3, C36:4

Significant metabolites showed niacinamide effects in post-menopausal skin: Niacinamide was found to induce subtle metabolic changes in post-menopausal skin. From OPLS-DA studies, slight metabolic differences were observed for post-menopausal skins before and after product treatment with OPLS-DA analysis with Q2(cum) around 0.4 (Table 5). Univariant analysis using pairwise student's t test were also applied to find significantly changed metabolites at Day 28 that were uniquely associated to the niacinamide treatment (Figure 6).



We could show large increase in NMF and amino acid levels in aged skin. Niacinamide partially showed a tendency of moderate reduction on their levels (Figure 5). For example proline, acetylproline and oxoproline were lower after niacinamide treatment. We could not observe an influence of niacinamide on FFA levels in post-menopausal skin. However, niacinamide was able to significantly improve cytosine and methylcytosine levels in post-menopausal skin, reducing cis-urocanic acid levels and imidazole acids levels in histidine catabolic pathway (Figure 5-6).

Figure 7 Cytosin levels detected in the different groups at day0 and day28 with strong recovery of cytosin levels in the niacinamide treated group

3) Results of the lipid analysis from tape6

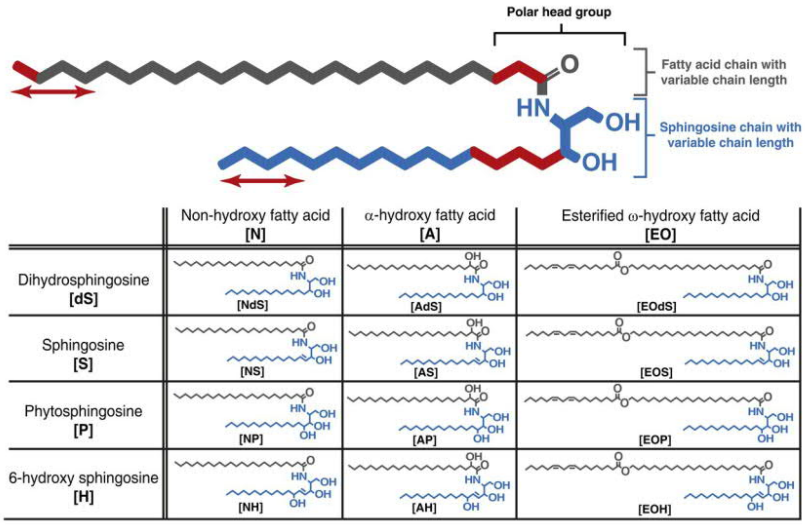


Figure 8: Structure and Nomenclature of Ceramides. Taken from [8]

With our analytical process and considering only lipids occurring in at least 70% of all the samples we could detect 297 ceramide lipid species to be compared. Due to the large dataset, we focused on the most obvious findings in this publication. At day0 the two post-menopausal cohorts PoM_d0B3 and PoM-d0pl could be considered as independent but equivalent groups and had as expected very similar total lipid content 106 vs 108 pmol/μg. The premenopausal cohort showed slightly but not significantly less lipid amount (90 pmol/μg) at day0. However, all the cohorts showed significant reductions of lipids after the treatment at day28 compared to day0. Apparently the B3 product could partially compensate this effect as the lipid reduction was less for the B3 cohort (-38%) than for the PoM placebo (-52%) or the preM placebo (-51%) and in direct comparison at day28 the B3 group showed significantly more lipid content (+27%) than the placebo group.

Table7 total detected lipid content in tape strip no 6 per cohort and normalized by protein content (PoM = postmenopause, PreM = premenopause, d=day, pl=placebo, B3= niacinamide treated. Units are ratio lipid/protein [pmol/mg], detected lipids excluding sebum lipids (Table15). P-values are based on an unpaired Mann-Whitney U test.

Cohort 1	Mean Cohort 1	n	Cohort 2	Mean Cohort 2	n	p-value	Sign
PoM_d0B3	106 ± 45	25	PoM_d0pl	108 ± 48	26	0.99	N.S.
PoM_d0B3	106 ± 45	25	PoM_d28B3	66 ± 22	25	0.000016	****
PoM_d0B3	106 ± 45	25	PreM_d0pl	90 ± 47	24	0.032	*
PoM_d0pl	108 ± 48	26	PoM_d28pl	52 ± 13	26	0.000000005	<****
PoM_d0pl	108 ± 48	26	PreM_d0pl	90 ± 47	24	0.11	N.S.
PoM_d28B3	66 ± 22	25	PoM_d28pl	52 ± 13	26	0.019	*
PoM_d28B3	66 ± 22	25	PreM_d28pl	44 ± 17	24	0.0004	***
PoM_d28pl	52 ± 13	26	PreM_d28pl	44 ± 17	24	0.11	N.S.
PreM_d0pl	90 ± 47	24	PreM_d28pl	44 ± 17	24	0.00000098	<****

Figure9 PCA Score plot of all samples colored by day0 versus day28 with increased clustering after treatment at day28

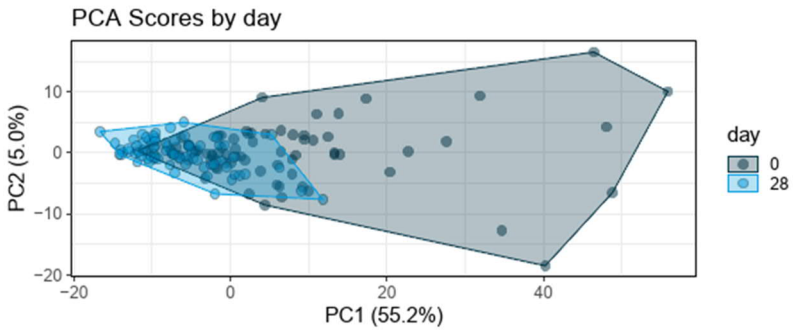


Table8 molar amount of lipid per protein in each lipid class of PreM group compared to PoM group after treatment.

	PreM d28		PoM d28					
name	Mean± SD	n	Mean± SD	n	PoM/PreM	p-value	BH	
AP	2.1 ± 1	24	3.1 ± 0.99	26	1.46	0.0017	0.01	**
AdS	2.3 ± 1.1	24	3.2 ± 0.8	26	1.36	0.0022	0.01	**
NP	3.3 ± 1.6	24	4.6 ± 1.2	26	1.38	0.0025	0.01	**
AH	5.3 ± 2	24	6.7 ± 2.3	26	1.26	0.0250	0.076	N.S.
EOdS	0.24 ± 0.33	18	0.12 ± 0.1	22	0.516	0.0580	0.12	N.S.
NdS	3.8 ± 1.4	24	4.5 ± 1.1	26	1.19	0.0610	0.12	N.S.
NH	5.9 ± 2.3	24	7 ± 2.1	26	1.2	0.0970	0.17	N.S.
AS	3.6 ± 1.5	24	4.1 ± 1.2	26	1.13	0.2400	0.34	N.S.
EOS	5.7 ± 2.9	24	6.2 ± 1.8	26	1.09	0.2600	0.34	N.S.
EOH	3.6 ± 1.6	24	4 ± 1.3	26	1.09	0.3800	0.45	N.S.
EOP	0.22 ± 0.11	18	0.19 ± 0.1	24	0.851	0.4100	0.45	N.S.
NS	8.1 ± 3.7	24	8.5 ± 2.7	26	1.05	0.5700	0.57	N.S.

After the treatment PoM showed more ceramides in all classes except for EOdS. As seen in Table8 the content of AP, AdS and NP was significantly elevated.

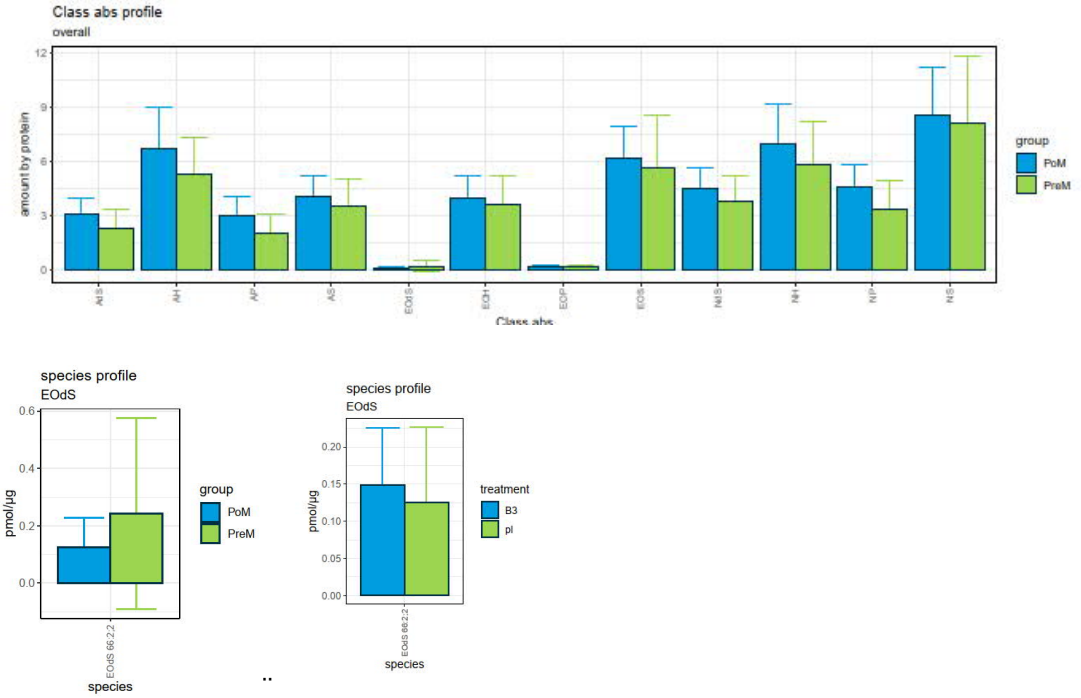


Figure10 molar amount of lipid per protein in each lipid class of PreM group compared to PoM group after treatment for all ceramides and EOdS in separate graph and also compared with niacinamide (B3) treatement

EOdS was the only ceramide with non-significant upregulated trend (day28) in the premenopausal cohort. In the B3 treated cohort the EOdS again was slightly higher than in the postmenopausal, however the data were not statistically significant.

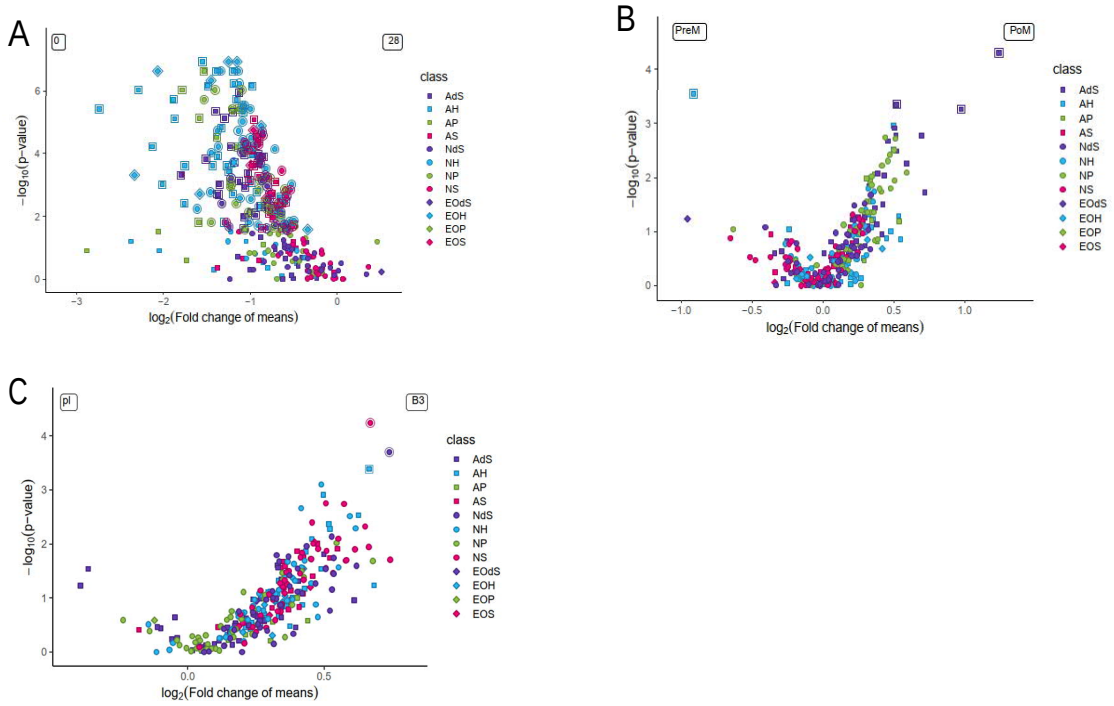


Figure11 Volcano plots of species comparing A PreM day0 and day28, B at day28 PreM and PoM and C at day28 PoMpl and PoMB3 groups. Logarithmic p-values of the Mann-Whitney-U Test without correction for multiple testing are displayed on the y-axis, Logarithmic fold changes of means are shown on the x-axis. Labels at the top indicate the cohort in which the values show a higher mean according to the fold change. Points with additional outlines (e.g. ◎ vs. ○) show significant hits after Benjamini-Hochberg correction for multiple testing.

Volcano plots in Figure11 A, B, C show the global view on the changes on ceramide species over time for premenopausal group (A) and in the direct comparison premenopausal vs postmenopausal (B), finally the positive effect on increased ceramides after B3 treatment (C)

Table9 molar amount of lipid per protein in each lipid class of PoM placebo group compared to B3 treated group

	pl		B3				
name	Mean ± SD	n	Mean ± SD	n	B3/pl	p-value	BH
NS	8.5 ± 2.7	26	11.7 ± 4.3	25	1.38	0.0054	0.037
NdS	4.5 ± 1.1	26	6 ± 2	25	1.34	0.0061	0.037
AS	4.1 ± 1.2	26	5.3 ± 1.9	25	1.30	0.0120	0.049
EOS	6.2 ± 1.8	26	8.1 ± 3.2	25	1.31	0.0300	0.084
NH	7 ± 2.1	26	8.7 ± 2.9	25	1.24	0.0350	0.084
AH	6.7 ± 2.3	26	8.5 ± 3.1	25	1.27	0.0510	0.100
EOH	4 ± 1.3	26	5.1 ± 2.1	25	1.28	0.0920	0.160
EOdS	0.12 ± 0.1	22	0.15 ± 0.077	22	1.19	0.1100	0.170
AP	3.1 ± 0.99	26	3.8 ± 1.7	25	1.26	0.2000	0.260
NP	4.6 ± 1.2	26	5.1 ± 1.7	25	1.11	0.3000	0.360

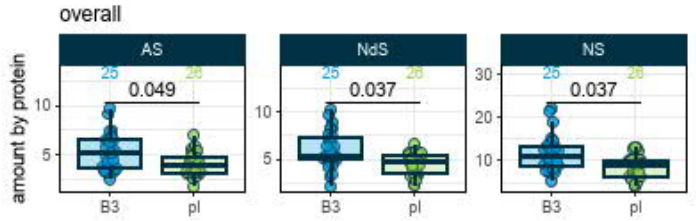


Figure12 After the B3 treatement the ceramides were again generally higher however the most significant elevations were measured for NS, NdS and AS (Table9).

Also, this is seen in the time course of the treatement with B3 from day 0 to day28 against the trend of significant reduction of majority of ceramide species the EOP, NdS, NS and EOdS had the least and non-significant reductions (Table10).

Table10 molar amount of lipid per protein of PoM group before and after B3 treatment

name	Mean (d0)	sd(d0)	n(d0)	B3 mean (d28)	sd(28)	n(d28)	fold change of means (d28/0)	p.value	BH	Signif
AH	18.1	7.2	25	8.5	3.1	25	0.471	0.00000006	0.00000072	<****
AP	9.2	4.3	25	3.8	1.7	25	0.418	0.0000003	0.0000018	<****
EOH	9.9	4.7	25	5.1	2.1	25	0.512	0.0000042	0.000017	****
EOS	13.1	7.3	25	8.1	3.2	25	0.617	0.00043	0.0013	**
NH	13.1	6	25	8.7	2.9	25	0.664	0.0015	0.0035	**
AdS	5.5	3.2	25	3.5	1.2	25	0.636	0.0056	0.011	*
AS	7.4	3	25	5.3	1.9	25	0.715	0.0067	0.011	*
NP	7.6	4.2	25	5.1	1.7	25	0.67	0.0074	0.011	*
EOP	0.29	0.15	21	0.19	0.12	22	0.64	0.03	0.04	*
NdS	7.7	3.5	25	6	2	25	0.787	0.15	0.18	N.S.
NS	13.7	5.9	25	11.7	4.3	25	0.857	0.37	0.4	N.S.
EOdS	0.14	0.092	17	0.15	0.077	22	1.06	0.46	0.46	N.S.

Some significant changes were seen in the fatty acid profiles mostly in the long-chain bases of the dihydrosphingosine and phytosphingosine type: 17:0;2, 17:0;3, 18:0;3 and 16:0;2 and

interestingly for 16:0;2 this PoM related change was significantly reversed by the B3 treatment. See Table 11 and 12.

Table11 significant changes on long-chain bases between preM and PoM group after the 28-day treatment

	PreM		PoM				
name	Mean ± SD	n	Mean ± SD	n	PoM/PreM	p-value	BH
L-17:0;3	0.66 ± 0.31	24	1 ± 0.33	26	1.58	0.0002	0.0097
L-17:0;2	0.73 ± 0.32	24	1.1 ± 0.33	26	1.49	0.0003	0.0097
L-16:0;2	0.23 ± 0.13	24	0.36 ± 0.13	26	1.56	0.0008	0.0180
L-18:0;3	1.7 ± 0.75	24	2.4 ± 0.62	26	1.41	0.0011	0.0180

Table12 significant changes on fatty acid chains between PoM placebo and B3 treated group after the 28-day treatment

	pl		B3				
name	Mean ± SD	n	Mean ± SD	n	B3/pl	p-value	BH
L-16:0;2	0.39 ± 0.15	26	0.26 ± 0.11	25	0.674	0.0009	0.040
17:0;0	0.1 ± 0.054	25	0.16 ± 0.067	25	1.590	0.0011	0.040
18:0;0	1.3 ± 0.41	26	1.6 ± 0.18	25	1.200	0.0028	0.068

Finally, some Pearson correlations of TEWL and hydration to lipid components were done (Table13 and 14)

Table13 high correlations of ceramides with hydration level in the preM group at day0

name	r	p.value	BH adj. p.value	correlating with
EOdS	0.6867	0.0023	0.0142	hydration
AH	0.5847	0.0027	0.0142	hydration
NH	0.5675	0.0038	0.0142	hydration
NS	0.5563	0.0048	0.0142	hydration
AS	0.5448	0.0059	0.0142	hydration
NdS	0.5304	0.0077	0.0153	hydration
EOH	0.4804	0.0175	0.0260	hydration
EOP	0.5197	0.0189	0.0260	hydration
EOS	0.4733	0.0195	0.0260	hydration
NP	0.4487	0.0279	0.0334	hydration

In general, higher amounts of ceramide species had positive Pearson correlation to hydration at day0 for the premenopausal group, and EOdS was on the top of the list. In the postmenopausal group at day0 these correlations less evident, however EOdS had again the strongest correlation (Table14).

Table14 correlations of ceramides with hydration level in the PoM group at day0

name	r	p.value	BH adj. p.value	correlating with
EOdS	0.6437	0.0016	0.0197	hydration
NH	0.4665	0.0163	0.0774	hydration
NS	0.4556	0.0193	0.0774	hydration
AH	0.4216	0.0319	0.0775	hydration
EOP	0.4381	0.0323	0.0775	hydration
AS	0.4071	0.0390	0.0781	hydration
NP	0.3459	0.0834	0.1292	hydration
NdS	0.3431	0.0862	0.1292	hydration
AdS	0.3321	0.0974	0.1299	hydration
AP	0.2941	0.1447	0.1737	hydration

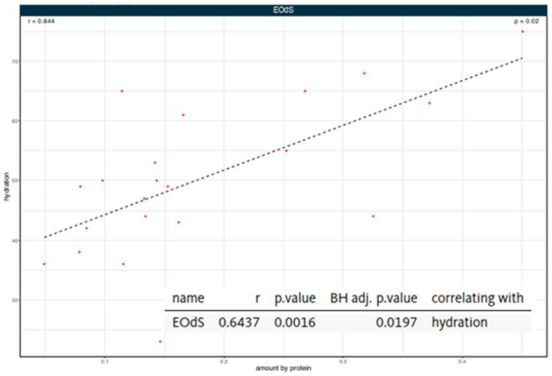
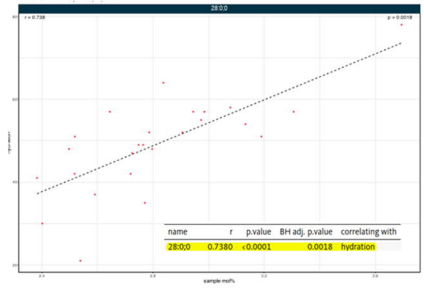


Figure13 EOdS with positive correlation to hydration units in the postmenopausal group at day0

In several comparisons the EOdS showed good and positive correlation with hydration (Figure13) and negative correlation with TEWL. One of the strongest correlations to hydration was seen for the fatty acid chain 28:0;0 (Figure14).



	pl		B3				
name	Mean ± SD	n	Mean ± SD	n	B3/pl	p-value	BH
L-30:0;2	0.019 ± 0.01	24	0.031 ± 0.015	25	1.67	0.0002	0.014
17:0;0	0.096 ± 0.057	25	0.2 ± 0.11	25	2.07	0.0004	0.016
L-19:1;2	0.51 ± 0.17	26	0.76 ± 0.31	25	1.48	0.0011	0.026
L-20:1;2	1.9 ± 0.68	26	2.7 ± 1	25	1.44	0.0016	0.030
28:0;0	2.8 ± 0.88	26	3.8 ± 1.3	25	1.38	0.0025	0.036

Figure14 This FA chain was also significantly upregulated by B3 treatment compared to the placebo group (poM)

Several more correlations at moderate to higher degree of significance for lipid species to TEWL and hydration were identified but not further shown in this publication.

Sebum lipids from tapes were also analyzed. For cholesterol esters (CE), diacylglycerols (DAG) and triacylglcerols (TAG) there was a tendency for higher sebum lipids in the premenopausal cohort but the differences were not significant. Whereas the effect of niacinamide treatment was negligible on the sebum lipids (Table15).

Tabel15 sebum lipids in PreM, PoM and niacinamided treated groups

name	sd(PreM)	mean(PreM)	n(PreM)	sd(PoM)	mean(PoM)	n(PoM)	fold change of means (PoM/PreM)	p.value	BH	Signif
DAG	70.1	57.7	24	31.1	29	25	0.502	0.058	0.13	N.S.
TAG	523.8	389.1	24	237.9	220	26	0.565	0.082	0.15	N.S.
CE	51.3	79.6	23	46.7	65.6	26	0.824	0.34	0.43	N.S.

name	sd(pl)	mean(pl)	n(pl)	sd(B3)	mean(B3)	n(B3)	fold change of means (B3/pl)	p.value	BH	Signif
TAG	237.9	220	26	188.3	237.1	25	1.08	0.38	0.52	N.S.
DAG	31.1	29	25	24.4	28.6	24	0.988	0.76	0.84	N.S.
CE	46.7	65.6	26	69.9	78.3	25	1.19	0.79	0.84	N.S.

4) Results from microbiome analysis results using tape no1

The microbiome analysis was limited to a set of 50 different species. However, qPCR analysis was performed, and we obtained quantitative results on the abundance of the bacteria/fungi per sample.

As a result, the number of genomic copy's per each individual could be visualized in Figure 15 and demonstrate the high inter-individual variability for taxa such as *Cutibacterium acnes*, *Corynebacterium kroppenstedtii* and others. In Figure16 the changes with the treatment from day0 to day28 are visualized with most obvious strong increase in *C. acnes* load for the premenopausal group after 28 days. Data on taxa and % change are also shown in Table 16. The apparently strong % differences presented are however mostly not confirmed by statistical significance due to the large individual variability.

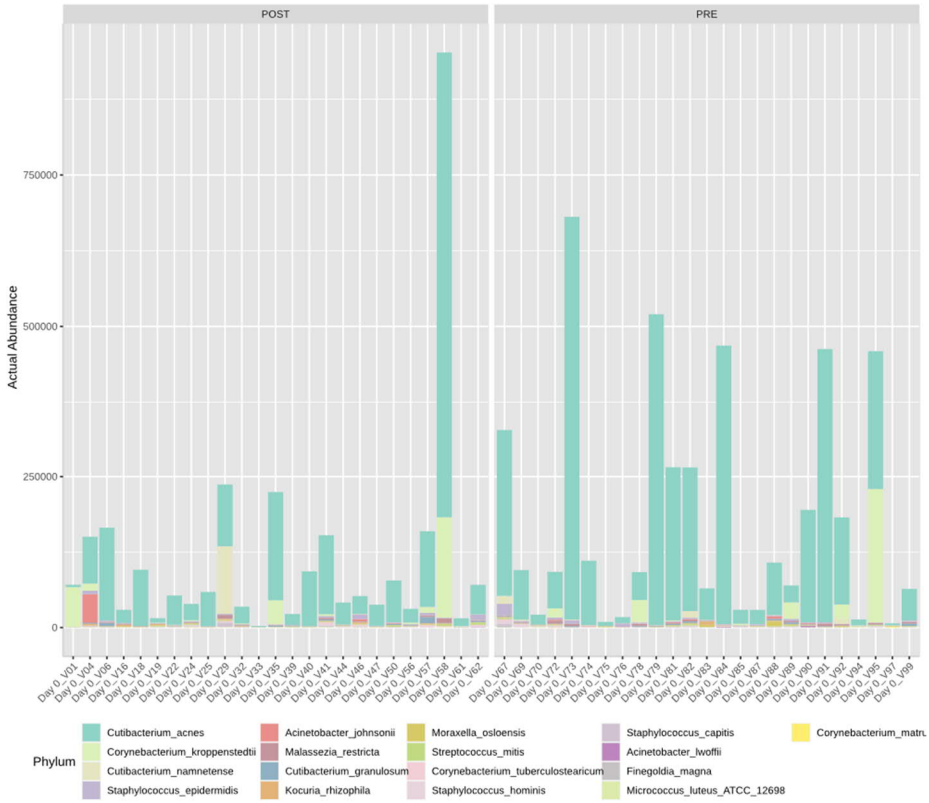


Figure15 Taxa abundance per individual at day 0 on the pre- and postmenopausal group

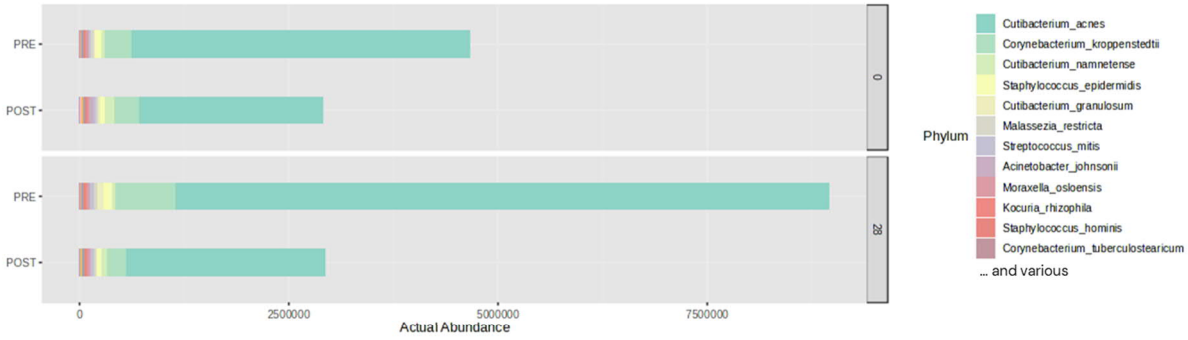


Figure16 total abundance of taxa per group pre- vs postmenopausal and at day0 and day28

The absolute quantifications (taxa genomic copy numbers) are shown in Table 16 for the 17 most abundant species. Most of the changes are not statistically significant. One remarkable result was the lower *Moraxella osloensis* counts in poM to 60% at day0 and 28 compared to PreM which was increased by niacinamide treatment by more than 600%.

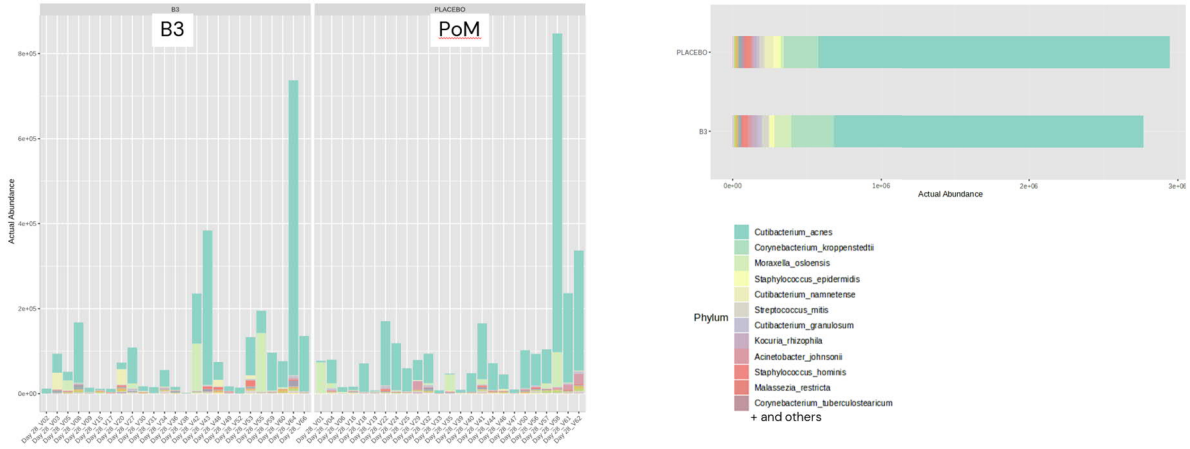


Figure17 the comparison of microbial counts after treatment with placebo and NA (B3) formulation of postmenopausal volunteers

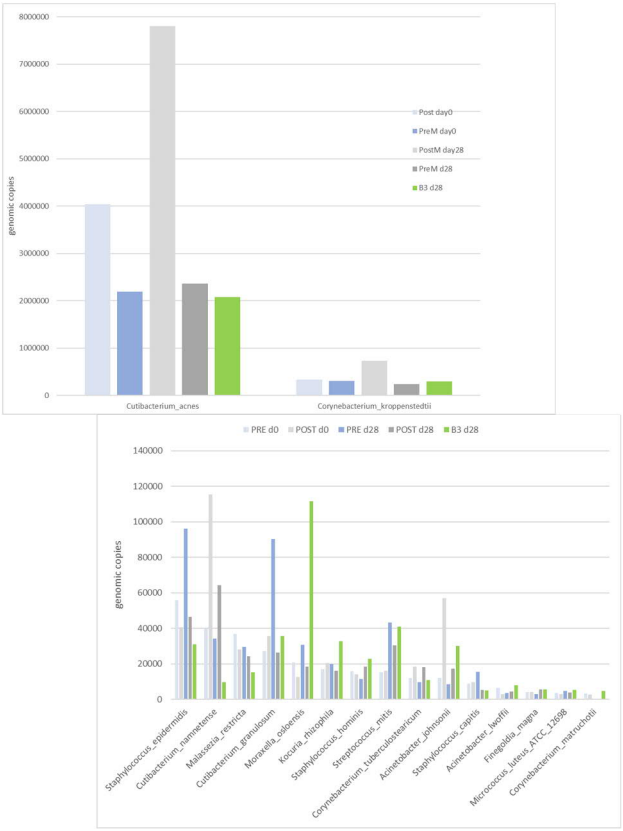
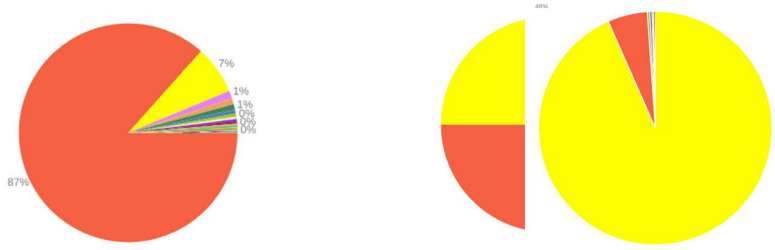


Figure18 visualization of microbe abundance in pre and postmenopausal panel and after niacinamide treatment (B3) for simplification without error bars

Table16 Taxa data on the premenopausal and postmenopausal groups at day0 and after treatment with placebo or niacinamide (B3) at day28, also the % of preM and of PoM are shown.

	taxa name	PRE d0	POST d0	% of PRE	PRE d28	POST d28	% of PRE	B3 d28	% of POST
1	Cutibacterium_acnes	4036313	2193163	54.3	7804994	2365474	30.3	2080925	88
2	Corynebacterium_kroppenstedtii	332623	307017.1	92.3	734450.1	238532	32.5	291432	122.2
3	Staphylococcus_epidermidis	55864.7	40788.8	73	95978.6	46353.2	48.3	30945.4	66.8
4	Cutibacterium_namnetense	39909.8	115490.7	289.4	34352.7	64237.5	187	9752.2	15.2
5	Malassezia_restricta	36873.6	28249.9	76.6	29611	24255.5	81.9	15390.2	63.5
6	Cutibacterium_granulosum	27370.3	35616.7	130.1	90393.9	26338.6	29.1	35778.1	135.8
7	Moraxella_osloensis	20765.2	12515.3	60.3	30597.8	18431.4	60.2	111459.5	604.7
8	Kocuria_rhizophila	17121.2	20624.4	120.5	19903.8	16275	81.8	32737.7	201.2
9	Staphylococcus_hominis	15728	14073.6	89.5	11361.1	18434.6	162.3	22964.4	124.6
10	Streptococcus_mitis	15243.2	16161.4	106	43166.4	30329.2	70.3	40889.7	134.8
11	Corynebacterium_tuberculostearicum	12005.3	18471.2	153.9	9862.7	18061.3	183.1	10963.2	60.7
12	Acinetobacter_johnsonii	11990.2	57087.8	476.1	8431.9	17417.4	206.6	30101.1	172.8
13	Staphylococcus_capitis	8850.7	9784.8	110.6	15561.4	5419.5	34.8	4936.5	91.1
14	Acinetobacter_lwoffii	6442.9	3093	48	3553.7	4480.6	126.1	7874	175.7
15	Finegoldia_magna	4148.5	4283.3	103.2	3148.1	5542.4	176.1	5737.3	103.5
16	Micrococcus_luteus_ATCC_12698	3546.2	3094.9	87.3	4895.6	3875.3	79.2	5344.8	137.9
17	Corynebacterium_matruchotii	3298.9	2788.4	84.5				4770.3	115.4

From the individual dataset it becomes obvious that each group includes some individuals with extremely high abundances e.g. for *C. acnes*, *C. kroppenstedtii*, *C. namnetense* with strong influence on the average group result. For example, by exclusion of one individual (no95) with high load of *C. kroppenstedtii* from the premenopausal group the average of *C. kroppenstedtii* reduces from 332623 (108% of poM) to 111203 genomic copies (36% of poM). In Figure 19 two individuals are specifically shown with higher load of *C. kroppenstedtii* as compared to the average of the group.



Average group result at day0 of premenopause	Volunteer no 95 from preM group at day0, normal skin	Volunteer no1 from poM group at day0, dry skin
C. acnes 87%, C. Kropp 7% TEWL 12.2, Corneometer 50CU, protein on tape 9.6U	*C. acnes* 50%, C. Kropp 50% TEWL 10.1 Corneometer 51CU, protein on tape 13.9U reduced cohesion	*C. acnes* <10%, C. Kropp >90% TEWL 8.7, Corneometer 36CU, protein on tape 13.9U reduced cohesion

Figure19 Left to right pie diagram of microbial composition with *C. acnes* in red and *C. kroppenstedtii* in yellow

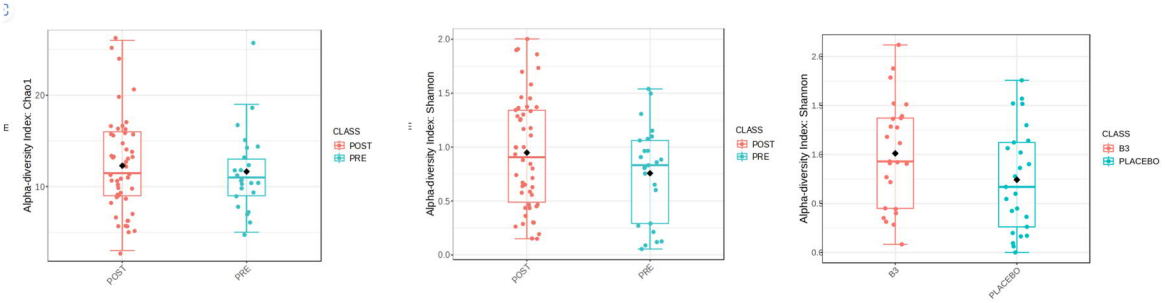


Figure20 Alpha diversity indices at day 0 with minor differences and with slightly higher Shannon index for PoM placebo when compared to niacinamide (B3) group.

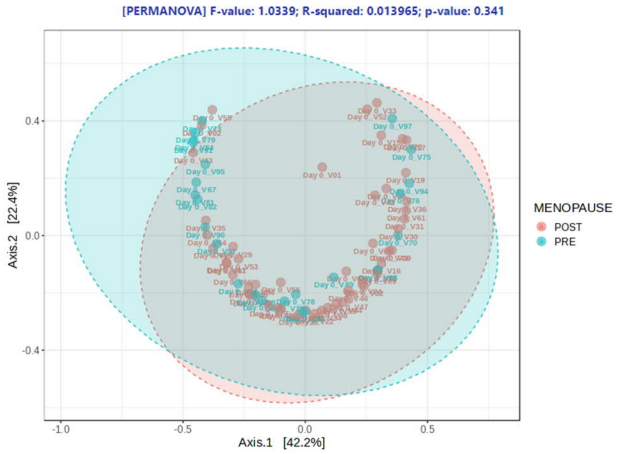


Figure21 Beta diversity at day0, highly matching beta diversity of preM vs PoM pl group with high (Microbiomeanalyst: Distance method, Bray curtis index, stat. method Permanova), a similar picture was obtained comparing niacinamide group with placebo group after treatment (not shown)

By performing t test/annova analysis comparing individual species abundances at day0 none of the species showed significant differences. *C. acnes* had the lowest p-value at 0.07 due to the higher abundance observed in the preM group. The statistics at day28 didn't show any significance either and for *C. acnes* it was again at p = 0.07. The same result without significances was obtained for the niacinamide treated versus placebo group. Was saw that the abundance for *M. osloensis* was for the niacinamide group at 600% compared to the PoM placebo group but not statistical significance was not confirmed (p-value = 0.09). In the violin plots for *M. osloensis* of these two groups obviously at least two outliers can be seen in Figure23.

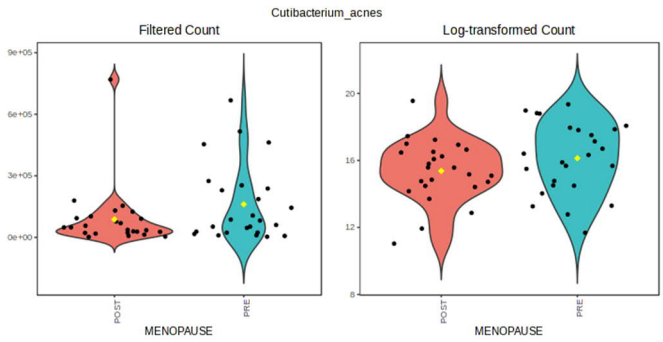


Figure22 Violin plots showing the distribution of *C. acnes* abundances within preM and poM at day0

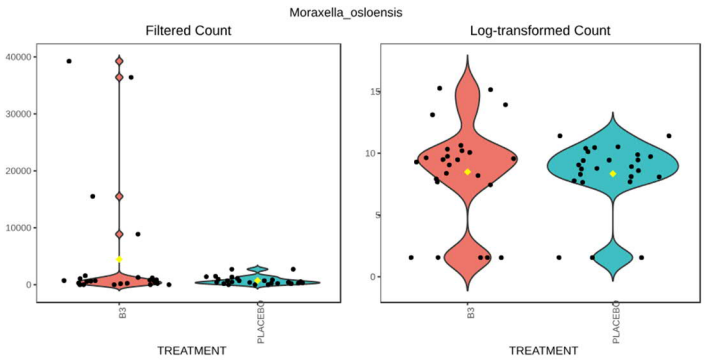


Figure23 Concerning *M. osloensis* abundance it seems at least two high outliers make the difference for the NA treatment (see plot on the left side)

Discussion

In our study we could not measure significant differences on the corneometer values (hydration) and TEWL values (skin barrier) between the pre- and postmenopausal groups. Rather there were a slightly lower TEWL values for the older groups. However, we confirmed that niacinamide can prevent epithelial water loss and improve hydration in post-menopausal facial skin. Niacinamide treatment in post-menopausal skin significant improved hydration and decreased in TEWL, despite the little changes without significance noted in NMF and amino acid levels. The high levels of niacinamide and its derivatives detected in the skin strip suggests the topical nicotinamide application to facial skin can be absorbed and metabolized. The in-skin niacinamide levels have a strong negative Pearson correlation with TEWL and a moderate positive Pearson correlation with corneometer units (hydration) suggest that high niacinamide absorbance in skin prevents epidermal water loss and improves hydration. Urocanic acid provides protection against harmful ultraviolet (UV) rays on skin [9]. As the final product of histidine catabolism, trans-urocanic accumulates in the epidermal as natural sunscreen, and upon UV exposure converts to cis-urocanic acid. No significant differences in trans-urocanic acid were noticed between pre- and post-menopausal group, and niacinamide treatment also led to no change in trans-urocanic acid level (Figure 6A). However, slightly higher level of cis-urocanic acid were detected post-menopause, suggesting less UV protection provided by aging skin. With niacinamide treatment in post-menopausal skin, significant decrease in cis-urocanic level was noted at Day 28 compared to Day 0 or to placebo treatment (Figure 6B). This decrease in trans- to cis-urocanic acid isomerization suggest niacinamide provides biological protection from UV damage in post-menopausal skin. UV from sunlight can also induce DNA damage in skin [10]. DNA damage from UV exposure can induce C-T transition, such as deamination of cytosine to uracil or methylcytosine to thymine as it was reported in both in vitro and in vivo models [10]. C-T mutation are highly prevalent in sequenced skin melanoma genomes [11]. Although for unknown reasons uracil and thymine were not detected in this study very clearly reduced cytosine and methyl cytosine levels were observed in post-menopausal groups suggesting greater DNA damage experienced by the aged skin (Figure 6). The most obvious result for niacinamide treatment in post-menopausal group was the significant the reduction of cytosine and methylcytosine after 28 days (Figure 6 and 7) as a reversal of the UV induced effect. It is suggested that niacinamide can reduce DNA damage experienced by facial skin post menopause.

Maintaining skin homeostasis at low pH is critical for forming permeable skin barrier and integrity of SC [12], and skin barrier is critical for preventing epidermal water loss. Aging is also known to elevate in skin surface pH [13]. FFA and urocanic acid from histidine catabolic pathway has shown to impact the pH of SC [14]. At post-menopause, total FFAs were decreased, which led to imbalance in skin pH and homeostasis, reduce skin flexibility, barrier, and protection against external environment. As compensation mechanism to maintain skin homeostasis against dry skin [14] and slower cell turn over [15], filaggrin-histidine-urocanic acid pathway were up-regulated (Figure 6A). NMF, histidine and intermediates in histidine catabolic pathways were all elevated post-menopause. Such increase in NMF amino acids have already been reported previously [14, 16]. As components of the filaggrin proteolysis product, NMF helps preserving molecular mobility in the SC. Though niacinamide had little impact on post-menopausal NMF and FFA levels, it was able to balance the histidine catabolic pathway and reduce some of the intermediate levels (including cis-urocanic acids and imidazole acids). Also worth mentioning is the effect of proline and derivative which were upregulated post menopause as potential result of collagen degradation whereas after niacinamide treatment a tendency for reduced levels of proline was measured. Additional to FFA, cholesterol and ceramides also make up the lipid classes in SC at approximately equimolar ratio [17]. The lipids and their composition are important to maintain skin barrier. Though many papers had reported the change of ceramide profiles in aged skin and as indication of impaired skin barrier [13,18], little menopausal induced ceramide

changes were noted in this study. However, we did a specific lipidomic analysis to look more in detail at the ceramides (see chapter “lipidomics). Unlike this study, where age difference between pre- and post-menopausal women were negligible, many age-related SC studies had greater age gap (> 10 years) between groups. This may contribute to more extraneous differences between lipid profile than reported in this study. Changes in skin barrier at SC and epidermal homeostasis following menopause are the key drivers for skin health concerns like dryness and sensitivity. In this clinical trial, we see evidence of niacinamide providing UV protection, minimize DNA damage and contributes to skin homeostasis. Similar effects were also noted in many other studies [19]. Skin tumor development was reduced with topical niacinamide [20], 5% niacinamide could reduce UV exposure and UV-induced immunosuppression [21]. Misra et. al. also reported elevated unchanged trans-urocanic acid but elevated levels of cis-urocanic due to polycyclic aromatic hydrocarbons, carcinogenic pollutants, and skin dysfunction [22]. Like increase pollutant exposure, similar skin physiology and dysfunction was also observed in post-menopause skin aging in this study.

Concerning ceramide modulation, we saw not a significant difference post vs pre however the 28days treatment caused a significant decline in overall ceramide content. The study was performed between August until early November hence the individuals had different seasonal starting and ending conditions for their 4 weeks treatment period. We therefore assume that most probably the ceramide decline was due to the treatment itself.

Interestingly the most significant positive changes by the niacinamide treatment were seen for ceramides NS, NdS and AS compared to PoM and comparing PreM and PoM the NS, NdS and AS were against the general trend not significantly upregulated in PoM group. It potentially can mean these ceramides are more in favor of the preM group and niacinamide treatment could recover the depletion of PoM skin. This is apparently in line with the finding of [18] also reporting a decline of NS and NdS post menopause and significant in vivo recovery by females under hormone replacement therapy and in vitro on keratinocytes NS and NdS were increased by oestradiol treatment. More investigations will be necessary to find out the possible role of NS and NdS in age related changes.

Finally, we observed a reduced level of sebum lipids in the postmenopausal panel in line with less *C. acnes* and Malassezia. NA has been previously shown to reduce sebum production [23] however in our study the NA treatment rather demonstrated a sebum balancing effect as for the post-menopausal group NA did not further reduce the sebum level.

Concerning the microbial results in agreement with previous studies [24] the most obvious difference was visible on the higher absolute abundance of *C. acnes* on the pre-menopausal group compared to the postmenopausal, this difference was even more impressive after 28days treatment, but statistical significance was not confirmed. For the lipophilic *C. acnes* the higher load matches the increased sebum lipids that were detected in the PreM group and to the general expectation to age dependent and probably postmenopause dependent decrease in sebum production. We also saw this trend for lipophilic *Malassezia restricta* with higher abundance in the PreM group. The niacinamide treatment was rather neutral on the abundance of *C. acnes* and sebum lipids. The diversity indices did not significantly change from premenopausal to postmenopausal and same after niacinamide treatment. However, in agreement with previous studies [24] the Shannon index tended to be higher for postmenopausal and NA treated probably due to the reduced abundance in *C. acnes* and consequent reduced deflation of species richness in the samples.

Some interesting individual examples with exceptional microbial composition are shown in Figure19. Correlation to hydration and protein on tape (SC cohesion) could give some indication between high load of *C. kroppenstedtii* and dry skin and reduced corneocyte cohesion (high

protein on tape). However further studies with higher number of such individuals would be necessary to confirm such a connection.

Conclusion.

Skin homeostasis was imbalanced at post-menopause indicated by elevated levels of NMF, histidine catabolic intermediates, and decreased FFA. 5% Topical niacinamide to post-menopausal facial skin am others recovered cytosin levels as potential marker for less DNA damage, and contributes to skin homeostatic maintenance. Niacinamide helped to increase ceramide synthesis preferably the categories NS, NdS and AS which were downregulated post menopause. Quantitative microbiome analysis showed high individual variability with a trend for higher C. acnes in premenopausal skin however more studies are needed with large cohorts of subjects to better understand the menopausal skin changes on metabolomic and microbial level.

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Conflict of Interest Statement.

The study was sponsored by DSM-Firmenich. DI, MP, BJ and MG were employed by DSM-Firmenich.

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