Retinyl palmitate for high level efficacy when combined with 10-hydroxy stearic acid

Dominik Imfeld[1*], Mathias Gempeler[1], Stellla Xi[2], Baokai Pan[2], Zhenxiang Lin[2] and Anthony V Rawlings[3]

[1]*DSM Nutritional Products Ltd., Basel, Switzerland*

[2]*Shanghai Pechoin Daily Chemical Co., Ltd, Shanghai, China*

[3]*AVR Consulting, Northwich, UK*

*corresponding author, DSM Nutritional Products Ltd, Wurmisweg 576, 4303 Kaiseraugst, Switzerland, Dominik.Imfeld@dsm.com

Background: Retinol is recognized as a gold-standard skin care ingredient for anti-aging activity, but it has its limits for the use level and stability in formulation. Besides retinol various retinyl esters are also widely used and in certain settings retinyl esters tend to have a higher stability than retinol but are less active. The first aim of this work was to assess the effect of retinol and retinyl palmitate (RP) on their potential anti-aging efficacy and the second aim was to assess its potential synergistic effect when combined with 10-hydroxstearic acid (10-HSA).

Methods: We used *ex vivo* abdominal human skin (45- and 49-year old female donors) for the application of the test compounds (retinyl palmitate at 0.18% and 0.73%, retinol at 0.05% and 0.1%) to the skin surface. The biological activity of the compounds was then assessed after 6 days of treatment by quantification of collagen I or III in the dermis via histologic immunostaining.

Results: We obtained a dose dependent increase of collagen I induction to 144% compared to solvent treated control (100%) at 0.73% retinyl palmitate (RP). In another study the stimulation of 0.5% retinyl palmitate (RP) on collagen III production was at 248% and at this higher use level nevertheless slightly better than retinol at 0.1% with 213% response.

Finally, we showed that the additional boosting concept with 10-HSA as PPAR-alpha agonist also worked well in combination with retinyl-palmitate (RP). The combination reached almost an additive level of collagen III compared to the single ingredients (10-HSA +72%, RP +149%, combination +192% vs control = 0).

Keywords: (retinyl palmitate; retinol; 10-hydroxystearic acid; PPAR-alpha agonist;).

Introduction. Retinol and its derivatives are generally accepted as the gold standard active ingredient for anti-aging treatments and the effectiveness has already been proven many times with clearly visible results in various studies with up to several months treatment. However, retinol also has known drawbacks such as instability in formulation and can also cause issues with skin irritation although irritation issues are only relevant for a subgroup of sensitive people and correlates to the use of higher in concentrations. In certain applications the use of retinyl palmitate (Vitamin A palmitate) could be a preferred alternative because it is generally more stable than retinol and less irritation issues have been reported. In nature retinyl palmitate (RP) is widely present in living organisms. It is the palmitic ester form of retinol and as such an inactive precursor of retinol. It is also abundant in skin as storage form of the vitamin A. However once retinyl palmitate is applied topically on skin, an additional conversion step to retinol is required before it can be further processed to retinoic acid, the active form (figure 1). The efficiency and the mechanisms of conversion via microbial or skin derived enzymes is not yet clearly known and for this reason we wanted to test it on human skin to directly quantify the positive effects.

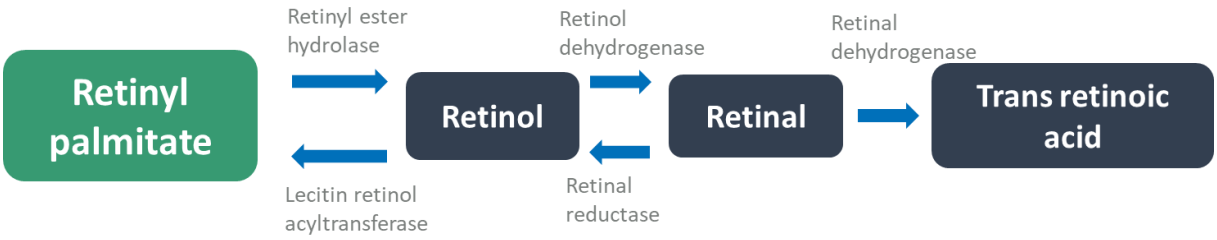

Figure 1: Retinyl palmitate and its enzymatic conversion steps to retinol and retinoic acid. Retinoic acid is the active form binding and activating the retinoic acid receptor (RAR). Retinoic acid occurs in both the trans- and the cis-form.

Another possibility to circumvent the stability and irritation issues of retinol was to combine retinol with a boosting active to get the expected benefits of retinol or its derivatives at reduced dosage. We saw the opportunity that this may be achieved by combining with PPAR agonists. Like other nuclear receptors, PPAR's bind to DNA of the peroxisome proliferator response element within the promoter region of the target gene in the form of homo or also heterodimers. Heterodimerization is known with the ubiquitous retinoid-X receptor (RXR) and thus amplify retinoic derived gene expression response [1]. From this we could expect a synergy on anti-aging benefits and may offer routes to reduced skin irritation from retinoids. Co-treatment of retinol with PPAR)-alpha agonist has been reported to reduce skin irritation issues [2]. We discovered previously the PPAR agonist 10-HSA and showed on various in vitro, ex vivo and in vivo study also anti-aging benefits on skin for example stimulation of collagen synthesis,

reducing the size of pores on facial skin and reducing the visibility of age spots [3]. Furthermore, we demonstrated the boosting effect on the efficacy of retinol when combined with 10-HSA as (PPAR)-alpha agonist [4]. A strong synergistic response on collagen III synthesis was observed. The level of collagen III gain was more than doubling the sum of the individual ingredients. However, we haven't shown so far that this concept could also be applied to other retinol derivatives such as retinyl-esters.

1) The first objective of the study was to measure ex vivo on human skin the efficacy of retinyl palmitate on boosting collagen I and III and fibrillin-1 level in the dermis.
2) The second objective was based on our retinol boosting concept to measure ex vivo on human skin the efficacy on collagen III synthesis by the combined treatment of retinyl-palmitate with 10-HSA and compare to the effect of the single ingredients.

Materials and Methods.

Ex vivo skin preparation, treatment, sampling and Type I collagen quantification:

Human skin from abdominal plastic surgery was obtained from healthy Caucasian 45year old woman (reference: P2329-AB45) with a phototype II, circular skin explants of 11 ± 1 mm in diameter were prepared. The explants were kept for survival in BEM culture medium (BIO-EC's Explants Medium) at 37°C in a humid, 5 % CO2 atmosphere.

The study was performed on human skin tissue, obtained from surgical residues of donor in full respect with the Declaration of Helsinki and the article L.1243-4 of the French Public Health Code. The latter does not require any prior authorization by an ethics committee for sampling and using surgical wastes.

A separate study was performed for collagen III testing with a different donor, a 49-year-old woman (reference: P2348-AB49) with phototype II-III.

Product preparations:

The vehicle V of all tested products was 30% propylene glycol (Sigma ref. W294004, batch SHBL1904)/ 70% ethanol (VWR, ref. 20821.467, batch 20E064025). The stock solutions of the products retinol and retinyl palmitate were prepared in the vehicle V on day 0. They were then stored in aliquots in the dark at -20°C. (a new aliquot was used each day of treatment).

Product application:

On day 0 (D0), D1 and D4, the vehicle V and the products were applied topically on the basis of 2 µl per explant (2mg/cm2) and spread using a small spatula. The control explants did not

receive any treatment expect the renewal of the culture medium. The culture medium was half renewed (1 ml/well) on day 1 and day 4.

Sampling:

For each condition 3 explants were treated. On day 0, 3 explants from the batch T0 were collected and cut in two parts. Half was fixed in buffered formalin solution and half was frozen at -80°C. On day 6, 3 explants from concerned batches were collected and processed in the same way than on day 0.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Skin analysis	x						x
Product application	x	x	(x)*		x		

Table 1: study schedule, *for the collagen III study the product was applied on day 2 instead of day1

Histological processing

After fixation for 24 hours in buffered formalin, the samples were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automat. The samples were embedded using a Leica EG 1160 embedding station. 5-µm-thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were mounted on Superfrost® histological glass slides. The frozen samples were cut at 7-µm thickness with a Leica CM 3050 cryostat. The sections were then mounted on silanized glass slides Superfrost® Plus. The microscopical observations were realized using a Leica DMLB or Olympus BX43 or BX63 microscope. Pictures were digitized with a numeric DP72 or DP74 Olympus camera with cellSens storing software.

Cell viability control

The cell viability of the epidermal and dermal structures was controlled on formalin-fixed paraffin-embedded (FFPE) skin sections after to Masson's trichrome staining, Goldner variant. Viability was assessed by microscopy. Only viable samples were further analyzed.

Collagen I immunostaining

Collagen I immunostaining was performed on frozen skin sections with a polyclonal anti-collagen I antibody (Monosan, ref. PS047), diluted at 1:50 in PBS-BSA 0.3% and incubated for 1 hour at room temperature. The staining was revealed by AlexaFluor 488 (Lifetechnologies, ref. A11008). The nuclei were post-stained using prodidium iodide. The staining was realized manually and assessed by microscopical observation.

Collagen III immunostaining

Collagen III immunostaining was realized on frozen skin sections with a polyclonal anti-collagen III antibody (SBA, ref. 1330-01), diluted at 1:200 in PBS-BSA 0.3% and incubated

for 1 hour at room temperature with a biotin/ streptavidin amplifying system and revealed with VIP (Vector laboratories, Ref. SK-4600), a substrate of peroxidase giving a violet staining once oxidized. The staining was realized manually and assessed by microscopical observation.

Image analysis for both collagen I and III quantification were performed on n = 9 images per test condition according to a standardized procedure using CellSens software.

Fibrillin-1 immunostaining

Fibrillin-1 immunostaining was performed on frozen sections with a monoclonal anti-fibrillin 1 antibody (Novus biological, ref. NB110-8146, clone 11C1.3) diluted at 1:500 in PBS, BSA 0.3% and Tween 20 at 0.05% for 1h at room temperature, with a biotin/ streptavidin amplifying system and revealed with FITC (Invitrogen, ref. SA 1001). The nuclei were counterstained with propidium iodide. The immunostaining was assessed by microscopical observation and image analysis.

Results.

Retinyl palmitate triggered stimulation of collagen I production:

The raw data from collagen immuno-staining are shown in Table 2, the normalized data to control = 100% are shown in figure 2.

	Vehicle day6	0.05% Retinol day6	0.18% RP day6	0.73% RP day6
Mean	55.6	64.4	74.6	79.9
SD	7.6	17.3	11.1	8.1

Table 2: Values represent % collagen 1 immuno-stained area from total surface in the papillary dermis (RP = retinyl palmitate) determined by image analysis

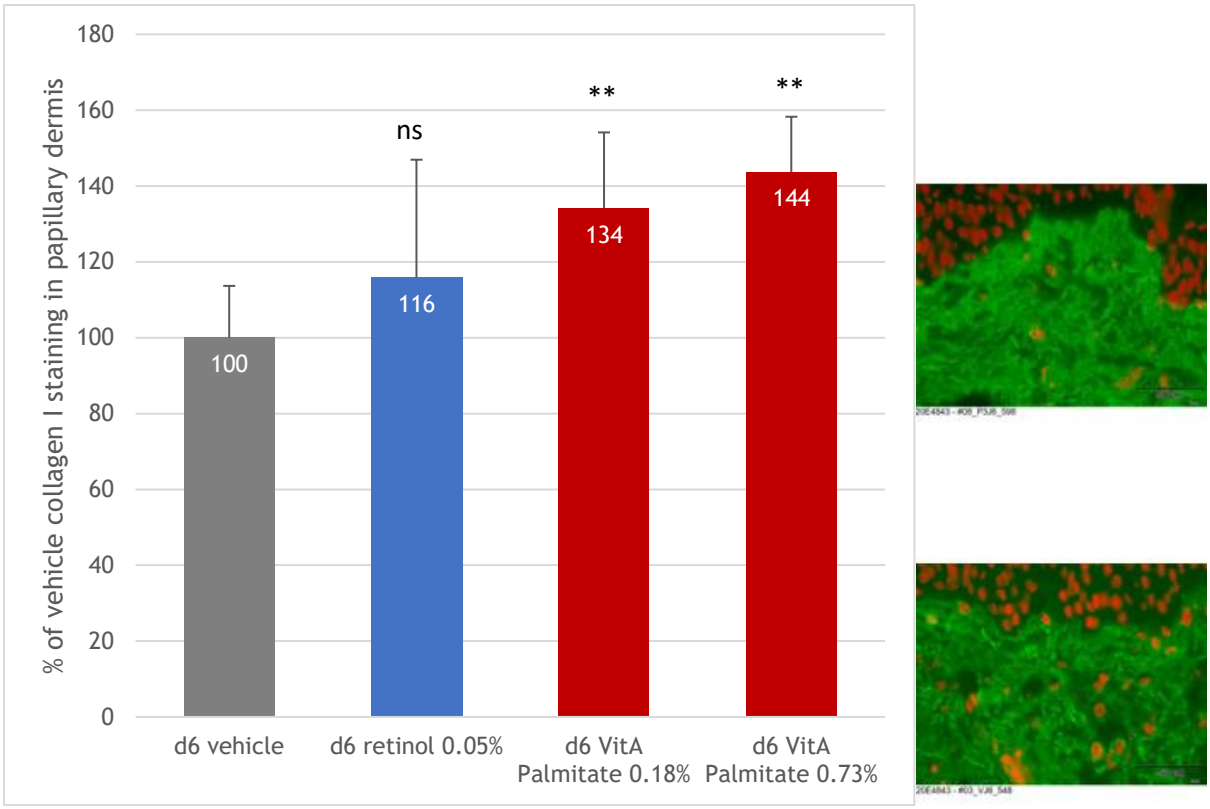

Figure 2: Results of collagen I immunostaining on *ex vivo* human skin from a 45years old female donor. Values represent % stained area in the papillary dermis normalized to the control (100%). d6 = stained after 6 days of treatment, solvent was 30% propylenglycol/70% ethanol, n=9 replicates analysed per condition and error bars represent standard deviations. P-value by t test. Images on right side represent examples of histologic preparations showing green fluorescence in dermal part from collagen I immunostaining with lower image from vehicle and upper image at 0.73% retinyl palmitate. Cell nuclei are visible in red.

<u>Retinyl palmitate triggered stimulation of collagen III production</u>

An additional study using skin from 49 years old women the potential stimulation of retinyl palmitate on collagen III production was assessed and compared to retinol. After 6 days of incubation retinyl palmitate at 0.5% strongly stimulated the level of collagen III to 248% compared to control (100%) and was slightly better than retinol at 0.1% with 213%. The values are shown in Table3. It is known that collagen III reacts stronger to stimuli therefore the higher values for collagen III stimulation (>200%) compared to collagen I (< 150%) were not surprising.

	Vehicle day6	0.1% retinol day6	0.5% RP day6
Mean score	27 (**100%**)	57.4 (**212.6%**)	67.1 (**248.5%**)
SD	6.8 (25.2)	4.7 (17.4)	8.3 (30.7)
p-value	-	< 0.01	< 0.01

Table 3: Results of collagen III immunostaining on *ex vivo* human skin from a 49years old female donor. Values represent % stained area in the papillary dermis, values in brackets in % normalized to the control (100%). RP = retinyl-palmitate, d6 = stained after 6 days of treatment, solvent was 30% propylenglycol/70% ethanol, n=9 replicates per condition and error bars represent standard deviations (SD). P-value by t test.

Retinyl palmitate stimulation of fibrillin-1 production along the dermal epidermal junction

Fibrillin-1 staining along the dermal epidermal junction was performed. Surprisingly the retinol alone did not show an increase although an increase can be expected. For 0.18% retinyl palmitate a significant increase of +19% was obtained ($p<0.05$) compared to vehicle treated skin.

	Vehicle day6	0.05% Retinol day6	0.18% RP day6	0.73% RP day6
Mean	26.3	24.7 (n.s.)	31.4 ($p<0.05$)	30.4 ($p<0.05$)
SD	2.6	5.5	5.8	4.9

Table 4: Results of fibrillin-1 immunostaining on *ex vivo* human skin from a 49years old female donor. Values represent % stained area in the papillary dermis. RP = retinyl-palmitate, d6 = stained after 6 days of treatment, solvent was 30% propylenglycol/70% ethanol, n=9 replicates per condition and error bars represent standard deviations (SD). P-value by t test vs vehicle d6.

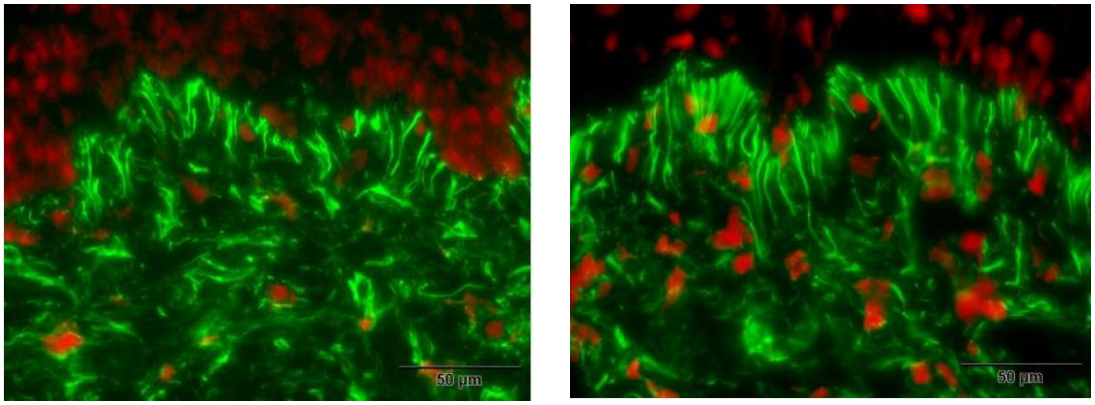

Figure 3: Representative images with green fluorescence of fibrillin-1 immunostaining on *ex vivo* human skin from a 49years old female donor. Image on the left side is the vehicle treated skin after 6 days (vehicle solvent was 30% propylenglycol/70% ethanol) and image on right side is treated with 0.73% retinyl palmitate in same solvent.

10-HSA boosted the efficacy of retinyl-palmitate

As we showed in the past that 10-HSA and retinol together resulted in great synergistic boost of collagen synthesis the aim of this study was to find out if such a boosting effect could still be obtained in the combination of 10-HSA and retinyl palmitate. In fact, the combination of retinyl-palmitate with 10-HSA also worked out very well and reached the highest level of efficacy. In our study the combination effect reached almost the additive level from the single ingredients but not reaching out to a synergistic output (10-HSA +72%, RP +149%, combination +192%, see Fig4). However, this doesn't exclude that retinyl-palmitate and 10-HSA together may reach synergism when used at different ratio and concentrations.

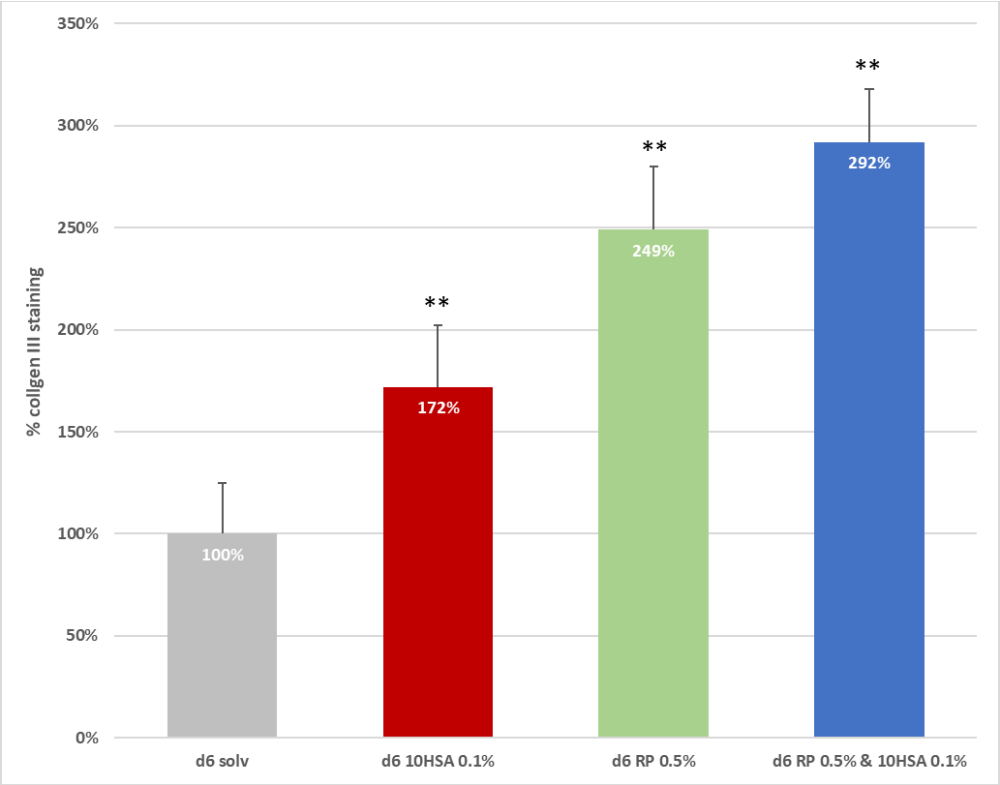

Figure 4: Results of collagen III immunostaining on ex vivo human skin from a 49years old female donor quantified as % stained area compared to the control (solvent only) normalized to 100%. RP = retinyl-palmitate, d6 = stained after 6 days of treatment, solvent was 30% propylenglycol/70% ethanol, n=9 replicates per condition and error bars represent standard deviations. ** Statistical significance versus control at $p<0.01$

Discussion. Retinyl palmitate (Vitamin A palmitate) can both stimulate collagen I and collagen III after topical application on human skin. From this we conclude that in skin retinyl palmitate is well converted to retinol and retinoic acid, the active form on skin, to exert the benefits. The performance of retinyl palmitate was similar to retinol and in our ex vivo studies the performance was even better than retinol at the given concentrations. As retinyl palmitate needs more conversion steps to the active form than retinol both the bioavailability and reaction time on skin expected to be lower than for retinol. For this reason and for a good performance we suggest the use level to be at least 3 to 5 times higher than for retinol. Finally, we showed that the additional boosting concept also worked well in combination with retinyl-palmitate. The combination reached almost an additive level compared to the single ingredients (10-HSA +72%, RP +149%, combination +192%).

Conclusion. In these studies, we now showed that retinyl palmitate is a direct booster of collagen I and III in human skin and the combination of retinyl palmitate (RP, 0.5%) with 10-HSA (0.1%) also worked out very well as collagen III synthesis was further increased.

Additional studies are needed to test the combination of 10-HSA and retinyl palmitate at different concentrations and ratios to eventually find synergistic effects.

In contrast to data reported by others [5], we showed retinyl palmitate to be a very valid candidate for antiaging treatments and higher level of performance could be achieved when combined with 10-HSA.

Acknowledgments. The ex-vivo experiments on human skin have been performed by Laboratoire Bio-EC Longjumeau, France. This research was financed by DSM Nutritional Products Ltd.

Conflict of Interest Statement. Dominik Imfeld and Mathias Gempeler are employees of DSM Nutritional Products Ltd., the sponsoring company of this research. Anthony V Rawlings is a consultant to DSM. Stellla Xi, Baokai Pan and Zhenxiang Lin are employees of Pechoin Co. Ltd.

References.

1. Rigano D, Sirignano C, and Taglialatela-Scafati O (2017) The potential of natural products for targeting PPARalpha. Acta Pharm Sin B. 7(4):427-38.

2. Kim B, Kim JE, Kim H, Lee JD, Choi KY, Lee SH (2012) Co-treatment with retinyl retinoate and a PPARalpha agonist reduces retinoid dermatitis. Int J Dermatol. 51(6), 733– 741

3. Schutz R, Rawlings AV, Wandeler E, Jackson E, Trevisan S, Monneuse JM, Bendik I, Massironi M, Imfeld D (2019) Bio-derived hydroxystearic acid ameliorates skin age spots and conspicuous pores. *Int J Cosmet Sci.* 41(3):240-56.

4. Rawlings AV, Wandeler E, Bendik I, Fuchs P, Monneuse JM, Imfeld D, Schutz R (2021) Effect of regioisomers of hydroxystearic acids as peroxisomal proliferator-activated receptor agonists to boost the anti-ageing potential of retinoids, *Int J Cosmet Sci.* 43(5):619-626

5. Bjerke, DL, Li R, Price JM, Dobson RLM, Rodrigues M, Tey C, Vires L, Adam RL, Sherrill JD, Styczynski PB, Goncalves K, Maltman V, Przyborski S, Oblon JE. (2021) The vitamin A ester retinyl propionate has a unique metabolic profile and higher retinoid-related bioactivity over retinol and retinyl palmitate in human skin models. Exp Dermatol. Feb;30(2):226-236