

Suivi du métabolisme des lipides dans la biologie avec l'HPTLC quantitative

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Application of HPTLC method for the assay of lipids

Use of HPTLC plates in qualitative and quantitative manner

- for physical detection with iodine

- for chemical detection with specific reagents ,

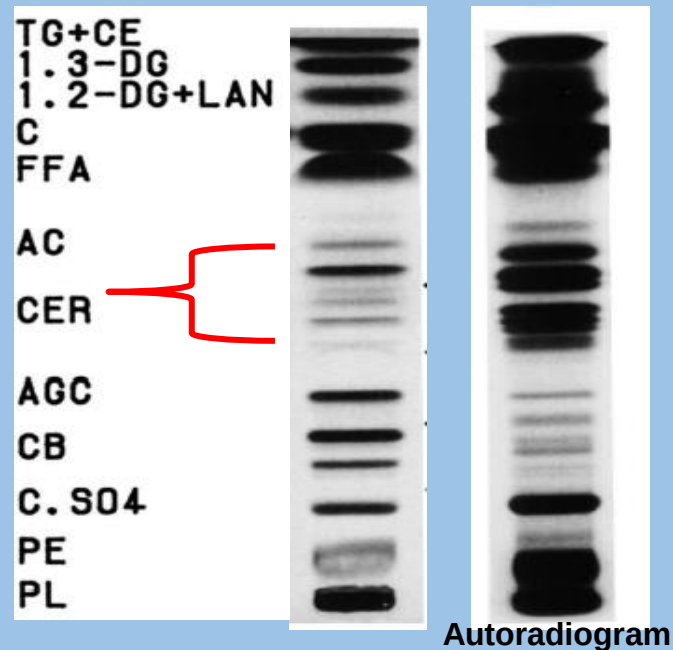
- for immunochemical detection with antibodies,

- for radiochemical detection of labeled spots (autoradiograms)

This method gives useful information about the nature of the lipids before going to more sophisticated analytical methods such as HPLC, mass spectrometry, gas chromatography.

Thin-layer analysis of total lipids versus isolated lipid fractions

Total lipids



One-dimensional TLC (10 cm height)
of total keratinocytes lipids

Sequential migration in :

- chloroform-acetone-methanol 76:4:20 (1 cm)
- chloroform-acetone-methanol 80:10:10 (7,5 cm)
- chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm)

First approach to lipid fractionation

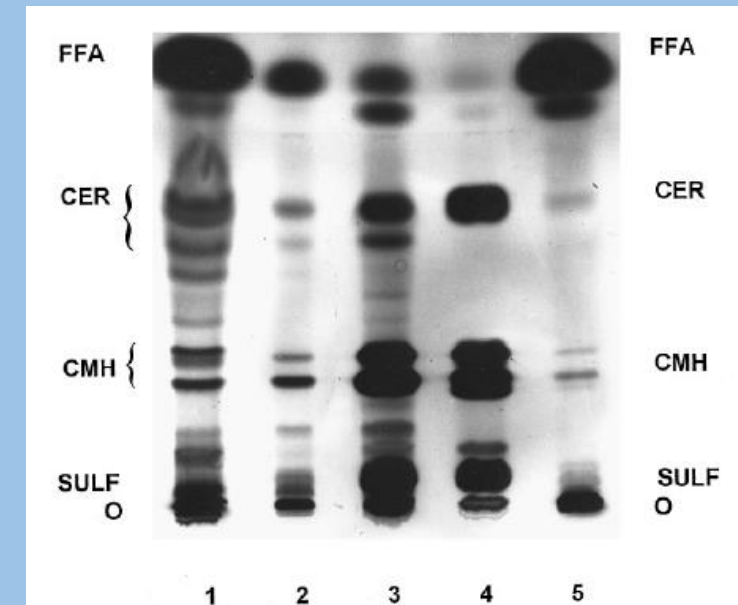


FIG. 7. HPTLC plate of NSL isolated from cultured rat RMG (lane 1), pig retina (lane 2), and rat brain (lane 3). About 3 μ g lipid P was spotted by lane. Standards were run on lanes 4 (CER, CMH, and SULF) and 5 (FFA and SM) (the SM remains at the level of the origin, O). Migration was performed in (1) C:M:H₂O (95:20:2, v/v/v) and (2) hexane:diethylether:Ac (80:20:2, v/v/v) as solvents. FFA, CER, and CMH were well separated. Abbreviations as in text.

Skin barrier defect and disease

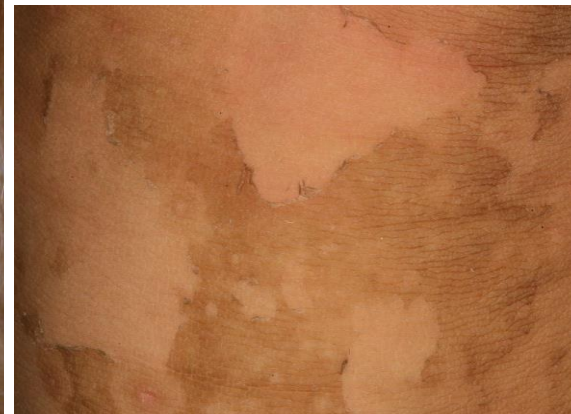
- Involved in several dermatoses (atopy, psoriasis, ichthyosis)
- Correlation between the depth of the SC and the composition of free and protein-bound lipid classes
 - biochemical lipids modifications and their phenotypic consequence due to the mutation various enzymes



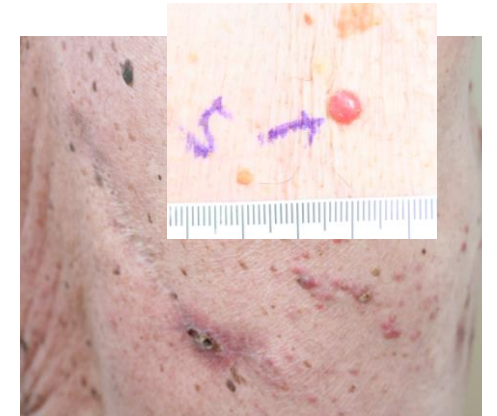
atopy



GR ichthyosis



Human ARCI
Dr E. BOURRAT



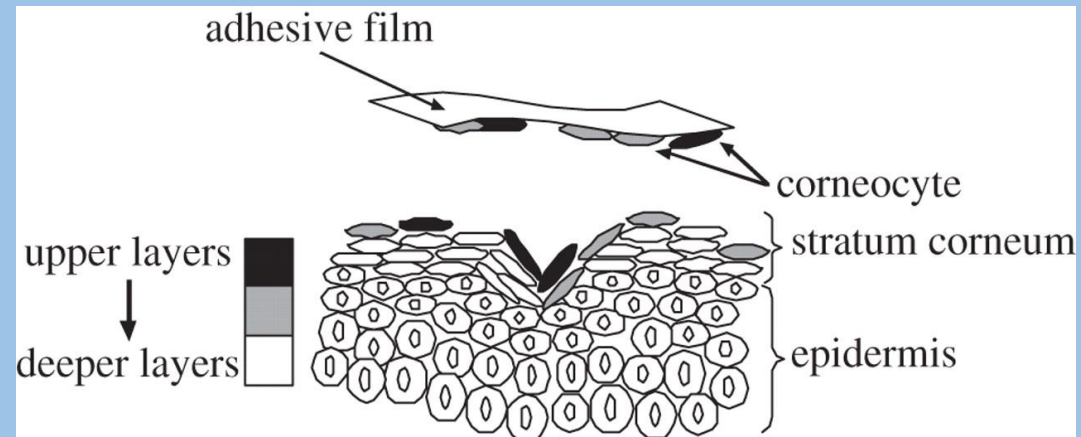
Tumeurs

Tape stripping methodology

- Removal of sequential layers of the SC using adhesive tapes and can be performed with minimum discomfort to subjects and relative ease *in vivo*.
- Quantify the SC thickness, total protein content of the SC depth profiles of various SC biomarkers.
- Method for studying kinetics and penetration depth of drugs
- Depth analysis study: determining the kinetics and penetration depth of different kind of permeants

Factors to be considered

- Number of tape strips
- Peeling force applied for removal
- Anatomical sites
- Skin hydration
- Skin cohesion



SC tape stripping, cells & tissue lipids extraction

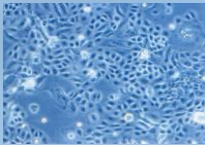
Lipid separation on LC-NH2 columns, HPTLC, ITLC, LC-MS/MS lipid identification

A. Extraction of whole lipids

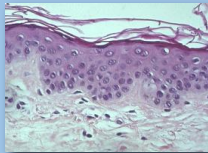
Tape Stripping
(D-Squam, scotch tape, surgical tapes)



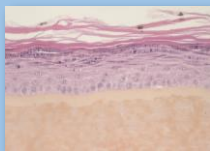
Cell culture



Skin sample

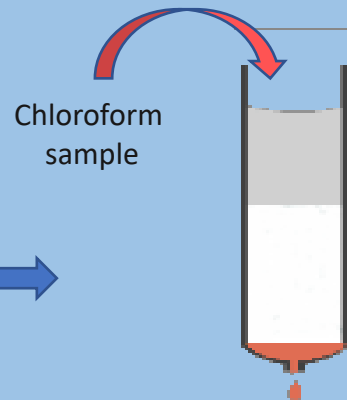


Reconstructed skin



organs, tissues, tumors

B. Elution of lipid fraction (FFA, ceramides)

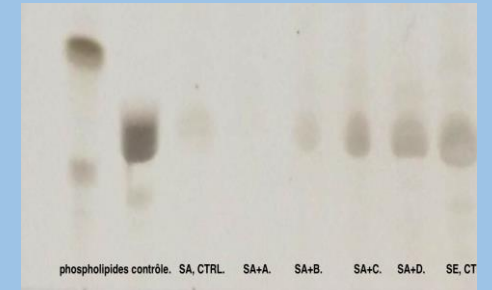


Fractions
Ex: FFA, ceramides

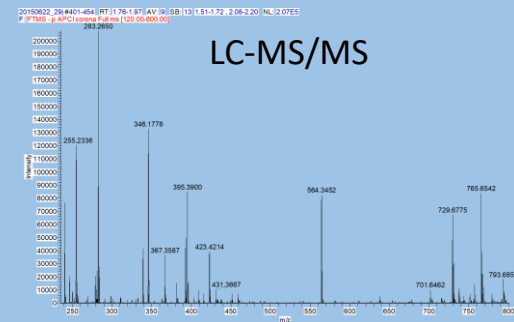
Confocal microscopy
Electron microscopy

C. HPTLC, LC-MS/MS, ITLC

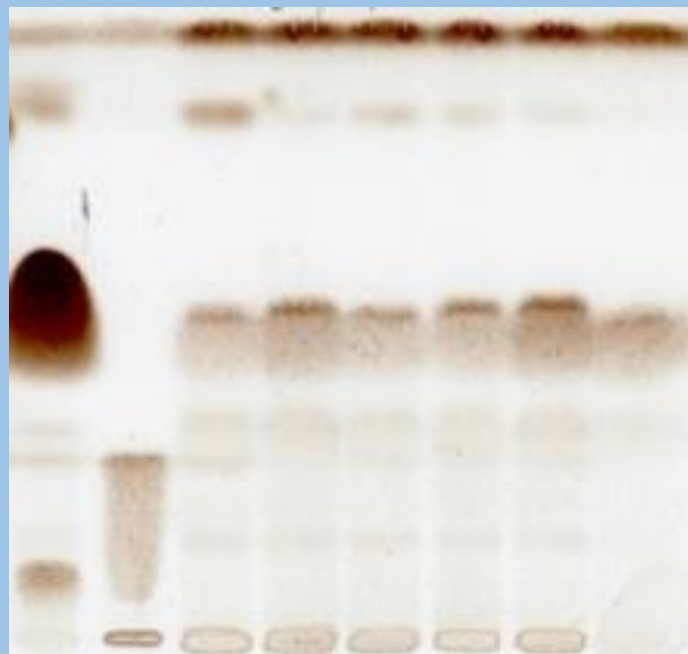
HPTLC-Chemical Identification on HPTLC plate



ITLC-antigen Identification on HPTLC plate



Lipides neutres et acides gras de la fraction de lipides libres du SC d'une peau humaine normale



TG

Acides oleique

Acide linoleique
(alpha. hydroxy)

Acide linoleique
(omega hydroxy)

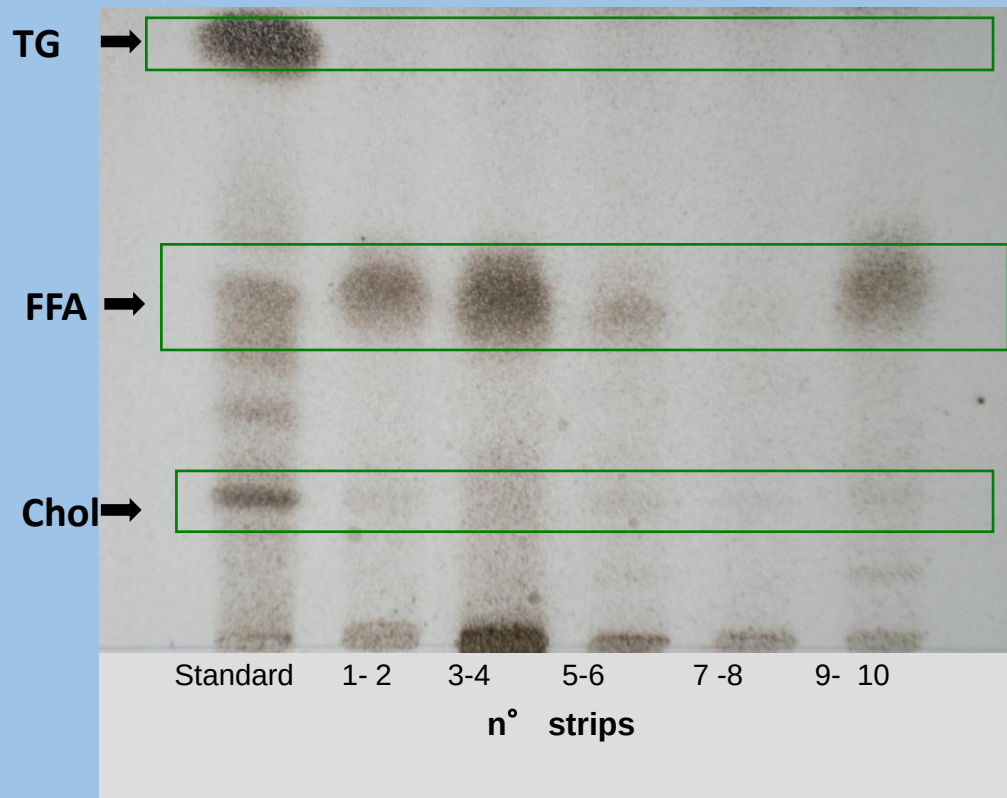
ST1. ST2. Ech.1 2. 3. 4. 5. 6.

strips SC de l'extérieur vers intérieur (peau humaine)

HTLPC, Migration- Hexane/ diethylether/acide acétique 70:30:1 (vol/vol), Visualisations- acétate de cuivre

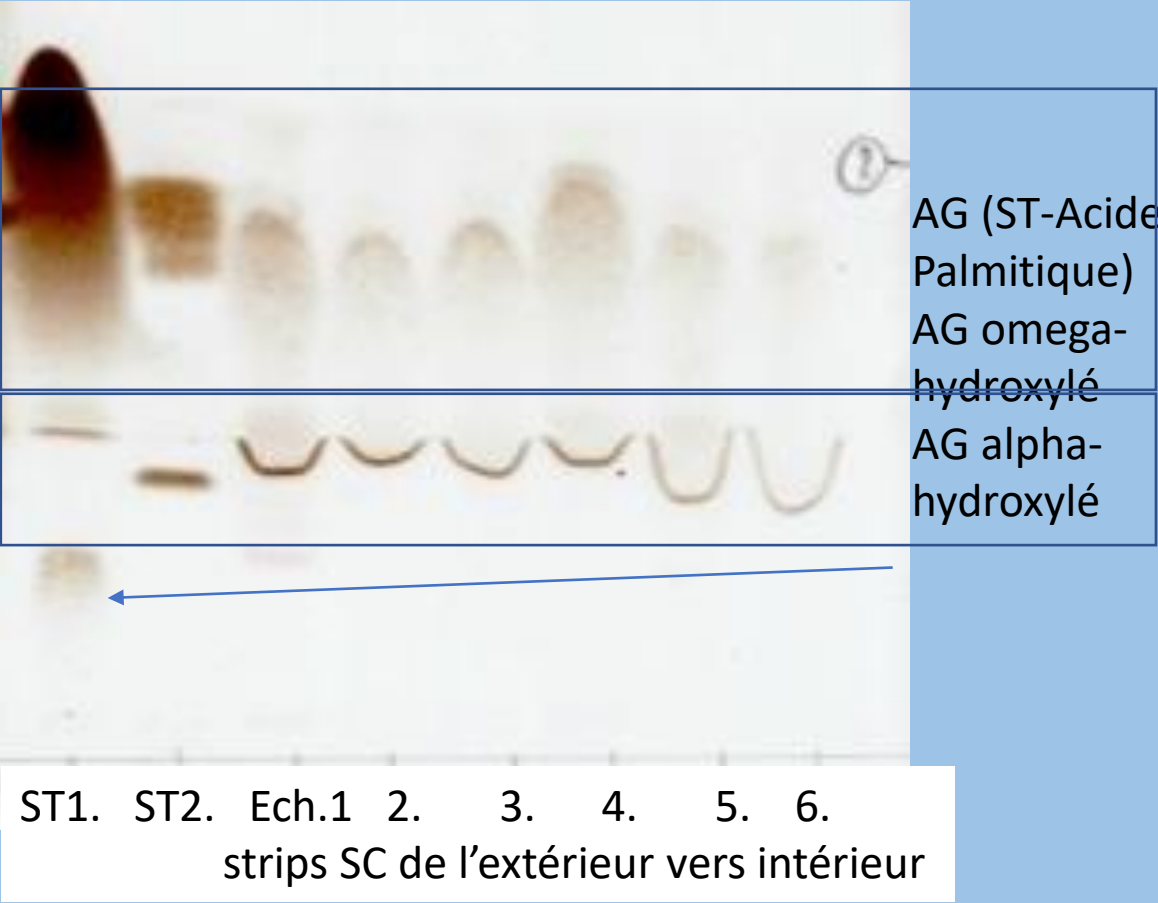
Lipides neutres et acides gras de la fraction de lipides libres du SC d'une peau ichtyosique

Présence d'une certaine hétérogénéité dans le contenu des acides gras libres et du cholestérol et une absence des triglycérides chez le chien ichtyosique, résultant en un SC irrégulier et une fonction barrière cutanée altérée



Détection des Acides Gras dans des échantillons biologiques par HPTLC

Quantification d'acide linoléique dans les acides gras du SC, de peau de chien avant et après diète

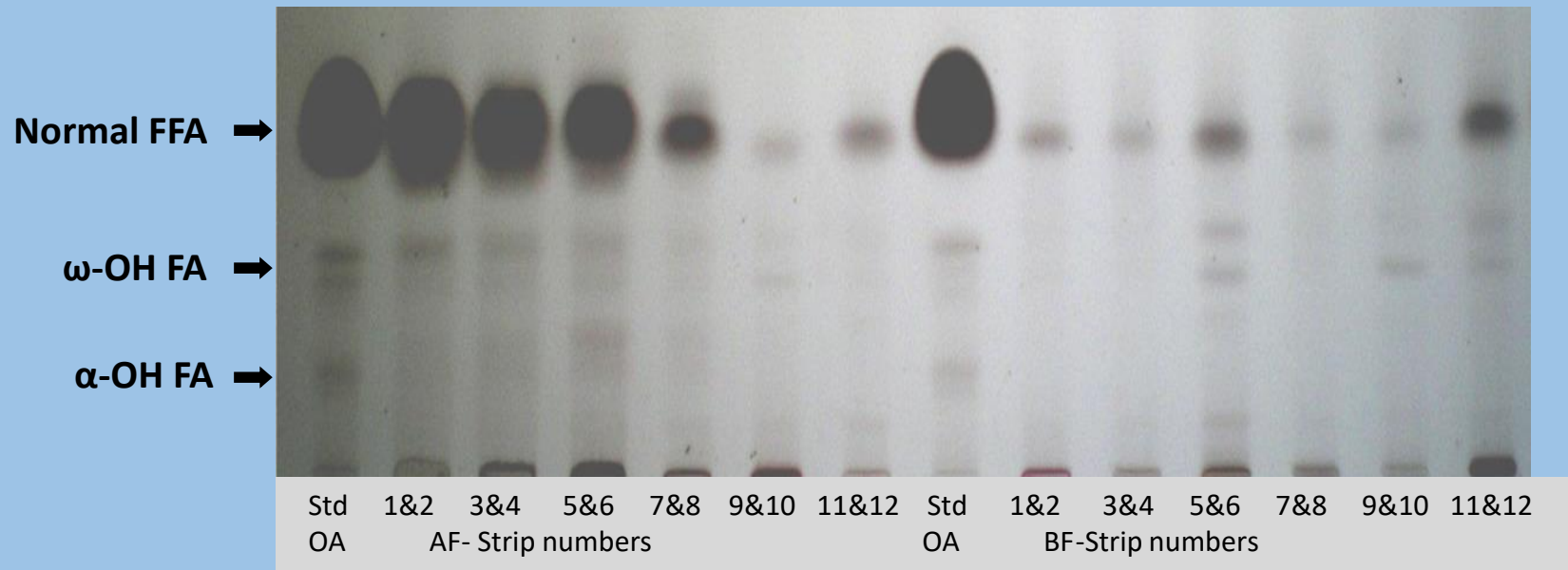


C18:2 in free lipids of 10 strips (quantification en LC-MS)					
prefed 12 weeks (n=5)			after 24 weeks (n=5)		
Total (µg/mg protein)	% as free C18:2	% as acylacid	Total (µg/mg protein)	% as free C18:2	% as acylacid
32.5	11.4 %	88.6%	31.4	12.1 %	87.9 %
14.6	10.7 %	89.3 %	23.4	9.6 %	90.4 %
43.9	7.9 %	92.1 %	39.5	11.5 %	88.5 %
23.1	9.8 %	91.2 %	30.2	10.7 %	89.3 %
14.4	10.3 %	89.7 %	21.4	11.2 %	88.8 %
25.7 ± 13.0			29.2 ± 6.8		

***4g/Mcal pendant 12 semaines**

HTLPC, Migration- Chloroforme/ méthanol/acide acétique 95:4,5:50 (vol/vol), Visualisations- acétate de cuivre

Acides gras de la fraction de lipids libres des strips de SC de la peau de chiens atopiques

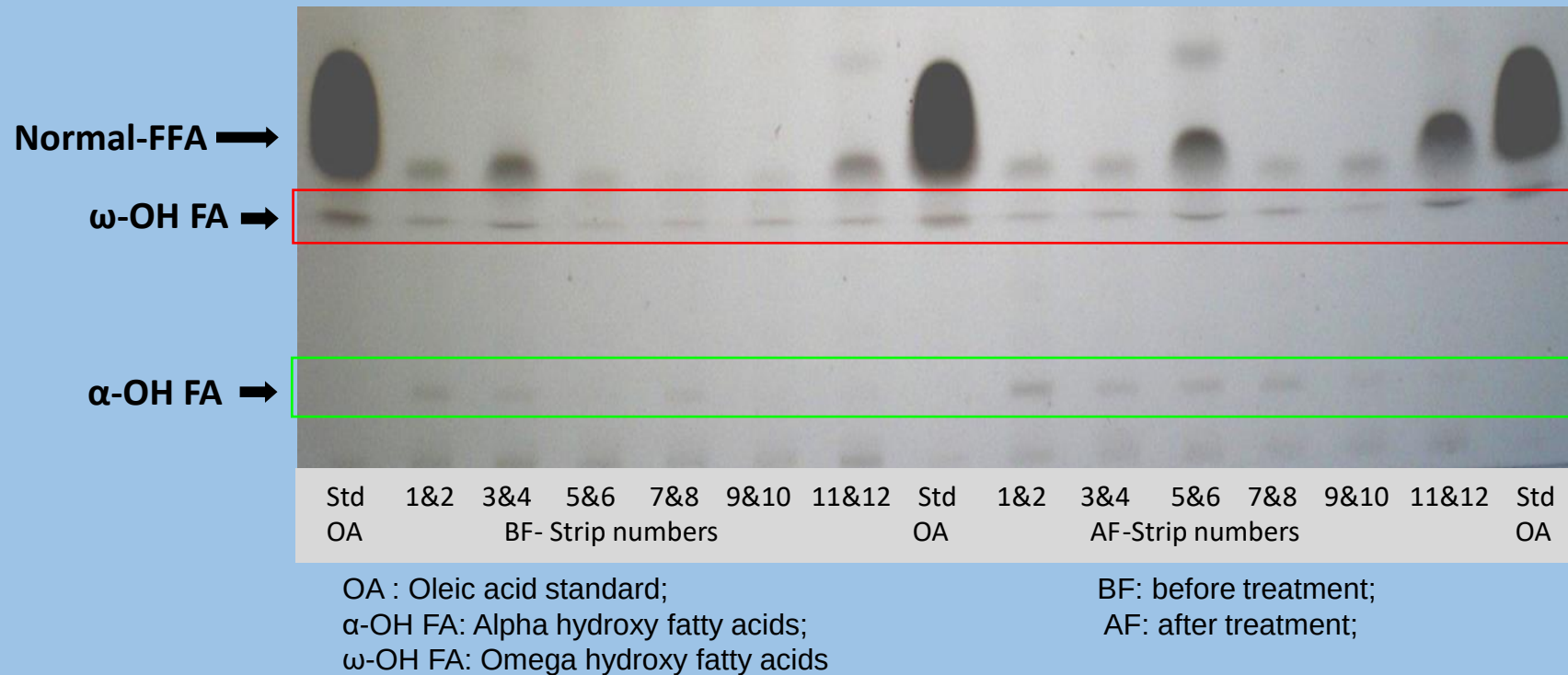


OA: Oleic acid standard;
 α -OH FA: Alpha hydroxy fatty acids;
 ω -OH FA: Omega hydroxy fatty acids.

BF: before treatment;
AF: after treatment;

Observation : Strong increase of normal fatty acids in the upper layers after treatment with SLC

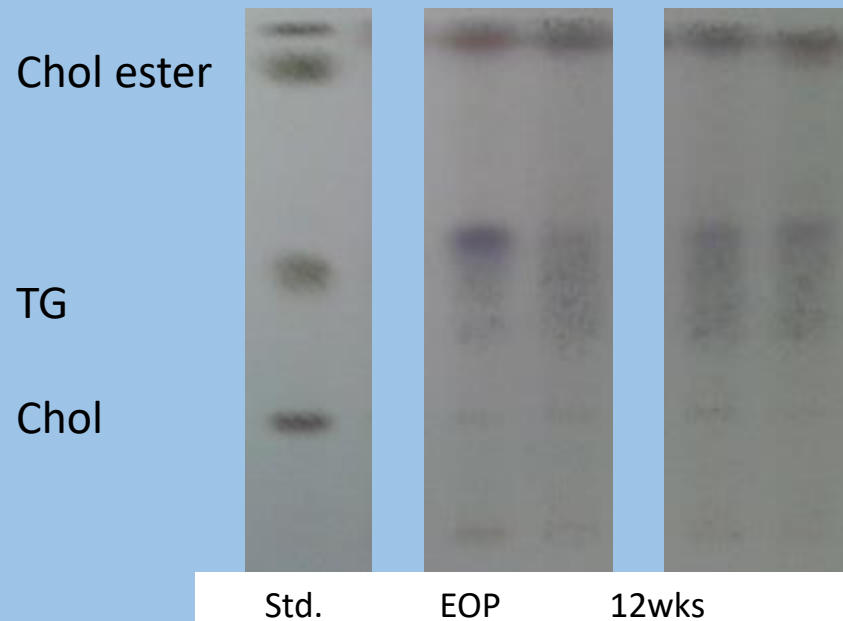
Acides gras de lipidides liés aux proteins des strips de SC de la peau de chiens atopiques



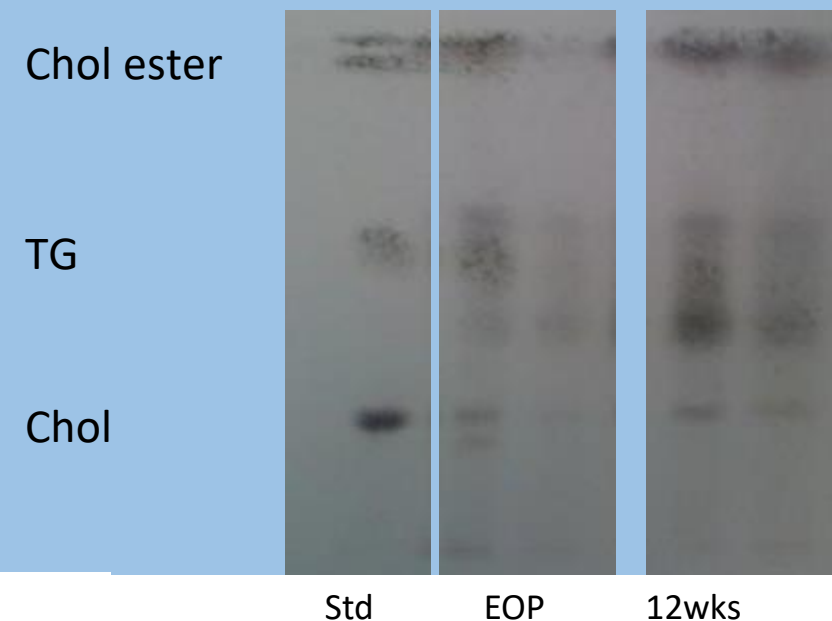
Observation : Elevation in protein-bound alpha-hydroxy and omega-hydroxy fatty acids after treatment with Megaderm®

Cholesterol esters in protein-bound lipids of the *Stratum Corneum*

SC des Chiens nourris avec diète **4g/Mcal**
pendant **12 semaines**



SC des Chiens nourris avec diète **10g/mcal** pendant **12 semaines**



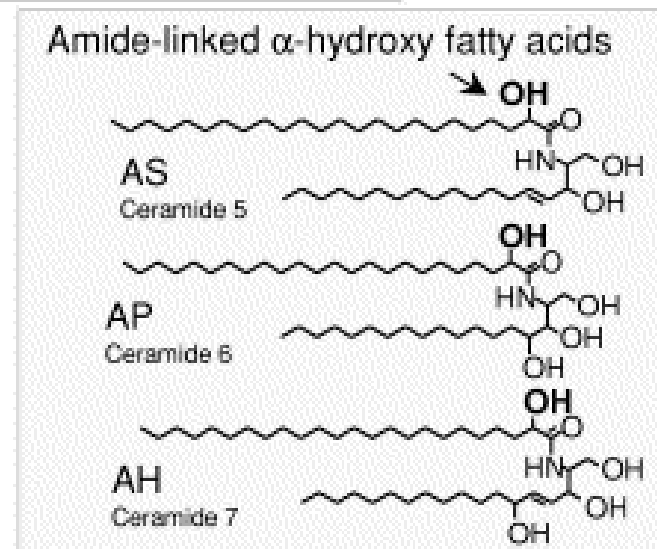
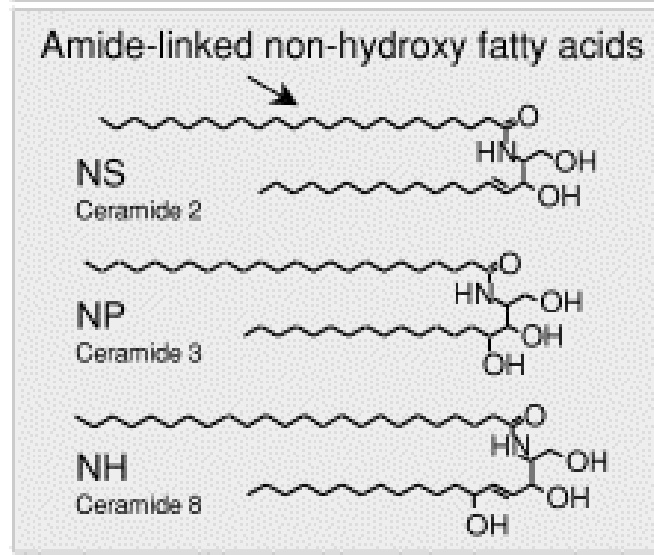
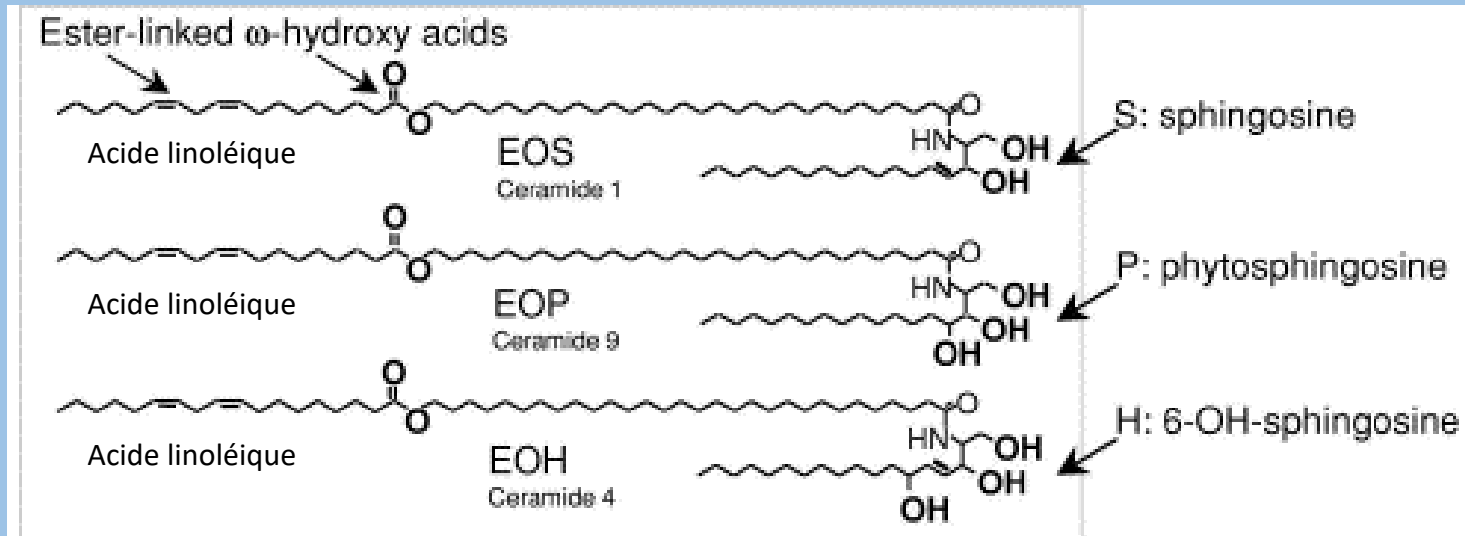
Observation: la concentration de cholestérol ester dans la fraction des lipides liés aux protéines ne change pas significative pendant la diète enrichie en acide linoléique

Composition en céramides libres et couplés aux protéines dans le Stratum Corneum du chien et de l'homme.

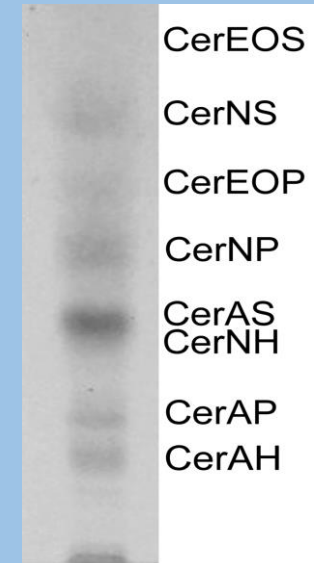
	Type de céramide	Chien	Homme
Céramides libres	Cer 1 [EOS]	3%	8%
	Cer 2 [NS]	19%	20%
	Cer 3 [NP]	28%	18%
	Cer 4 [EOH]	traces	4%
	Cer 5 [AS]	33%	17%
	Cer 6 [AP]	10%	9%
	Cer 7 [AH]	6%	12%
	Cer 8 [NH]	traces	9%
	Cer 9 [EOP]	4%	2%
Céramides couplés aux protéines	Cer [OS]	62%	59%
	Cer [OP]	8%	33%
	Cer [OH]	30%	8%

Poniec , Weerheim,
Lankhorst, Wertz ,
Hill ,....

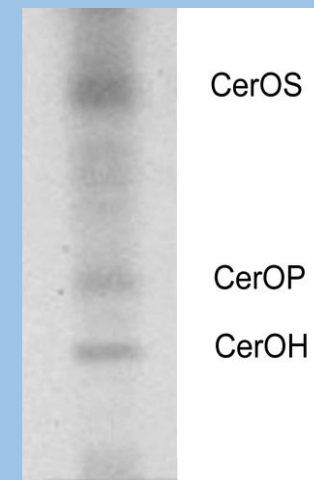
Human Stratum Corneum Ceramides



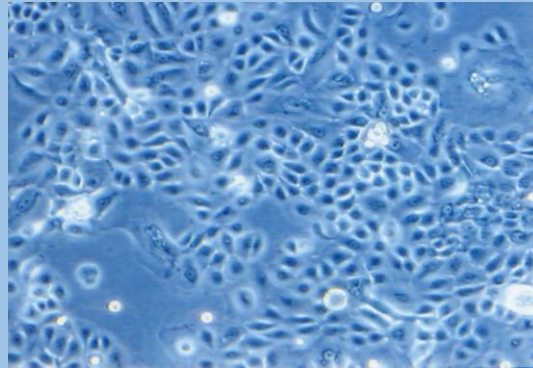
Free SC Ceramides



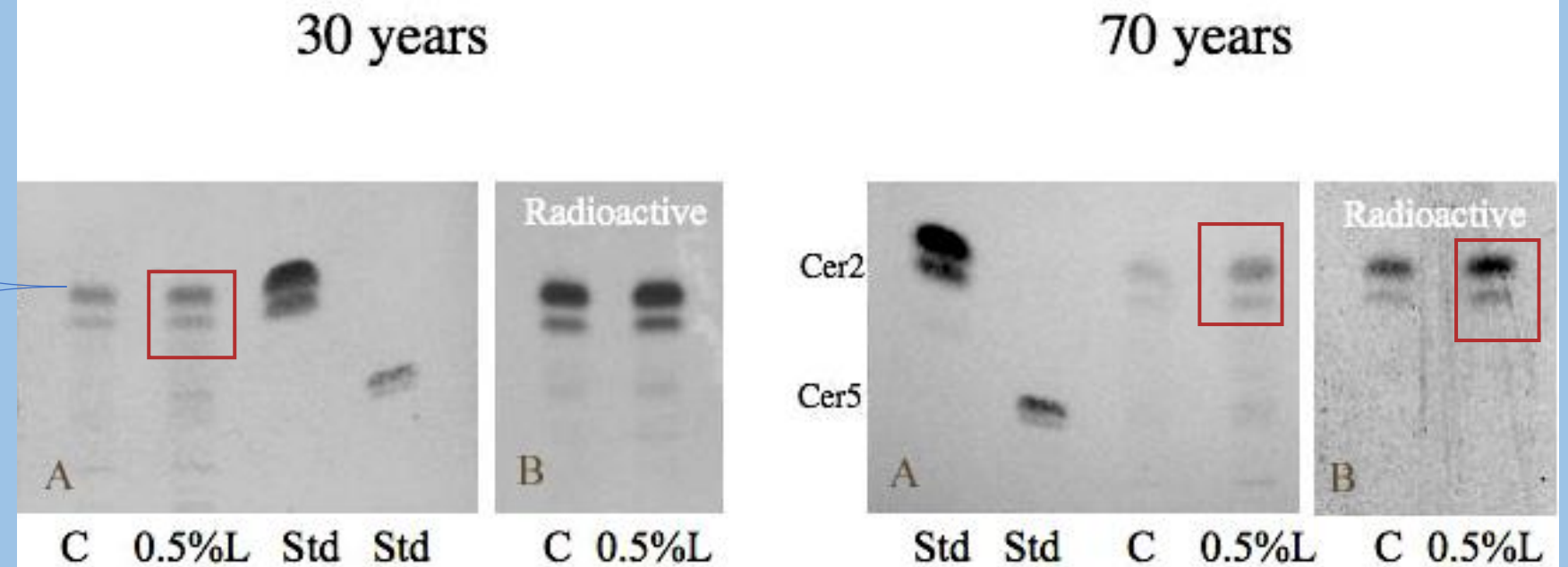
Protein-bound SC Ceramides



Alpha-hydroxy-ceramides biosynthesis is stimulated by the peptides active in keratinocytes from old donors



Keratinocytes culture + active



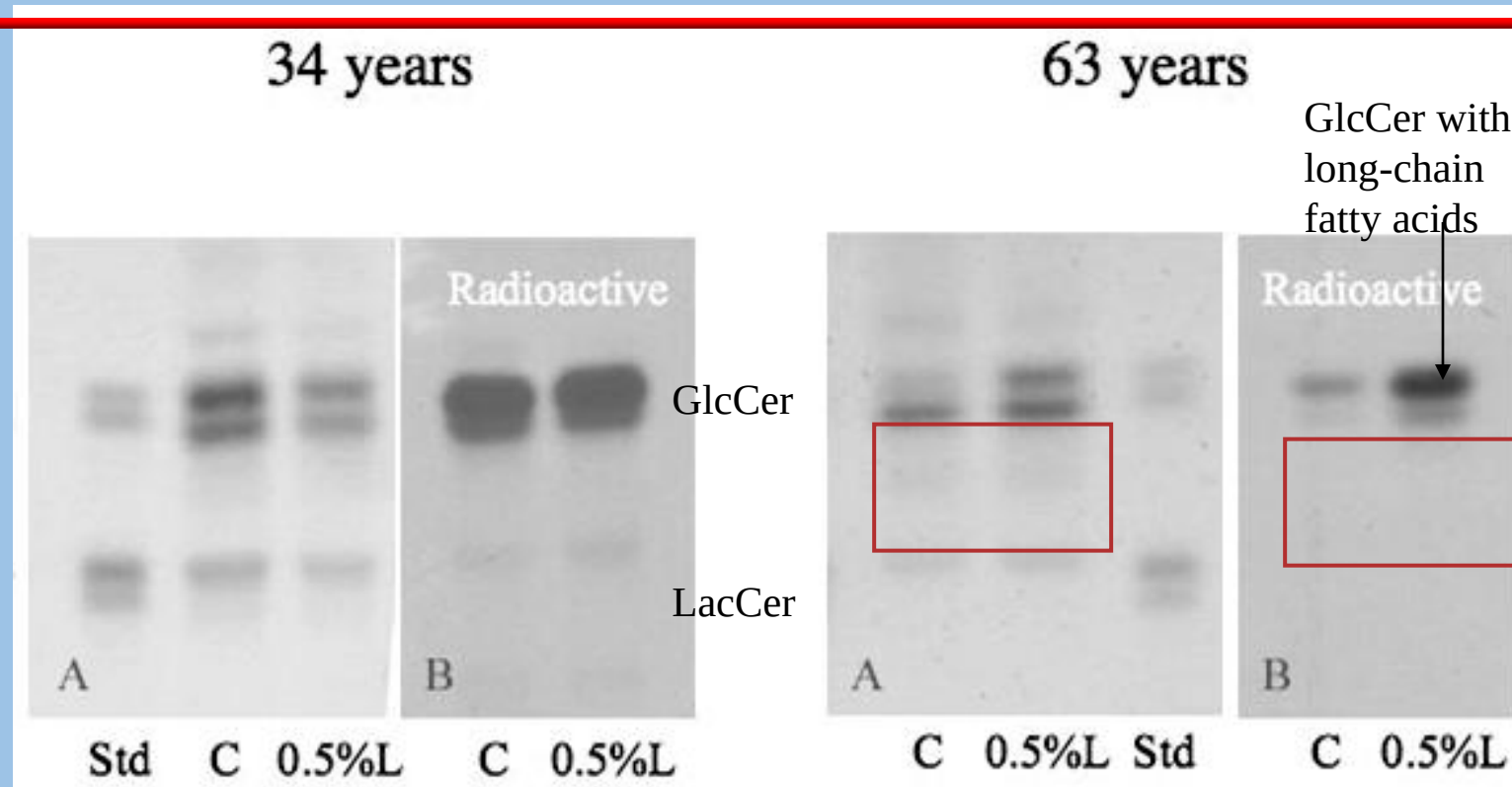
The peptides active boost the enzymatic activity up to the rates seen in young donors

*Lipids were extracted, fractionated into specific classes and analyzed by HPTLC

*Radioactive lipids were visualized by autoradiography.

Popa et al, Int. J. Cosmet Sci. 2010, 32(3): 225-32.

Increase of neutral glycolipids biosynthesis in basal keratinocytes isolated from aged donors versus young donors after treatment with peptides (0.5%)

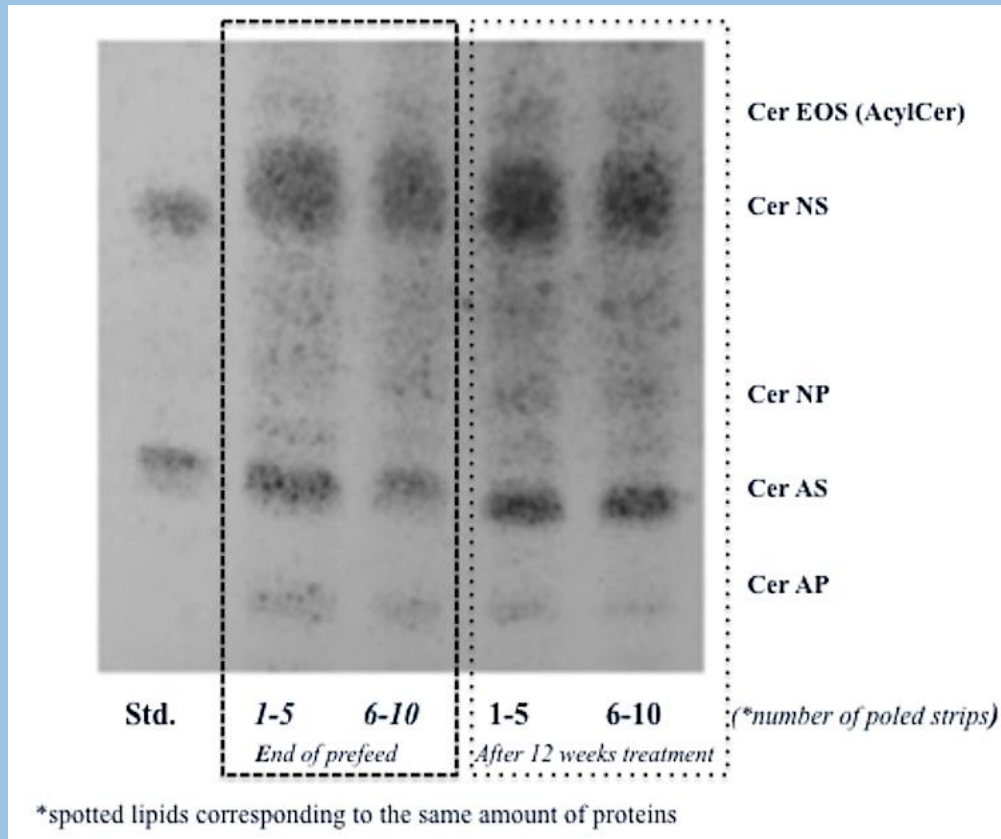


Observation 1: Lower amount of glucosylceramides in the aged donor.

Observation 2: The peptides of the potato extract normalizes the amount of the glucosylceramide in the aged donor and especially the turnover of the species with long-chain fatty acids

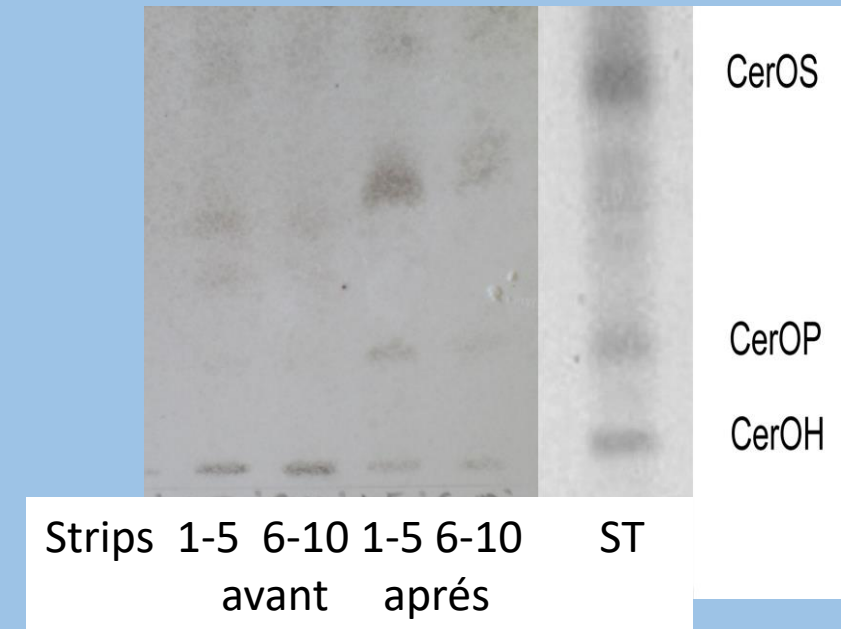
Détection des céramides dans des échantillons biologiques par HPTLC

Fraction céramides libres du SC



Strips. SC. de la peau de chien en bonne santé avant et après une diète de 12 semaines enrichie en acide linoléique
Total Céramides libres: **132.6** $\pm$ 20.4 avant et **212.2** $\pm$ 23.5 ($\mu\text{g}/\text{mg}$ protéines $\pm$ SD) (sur 5 échantillons)

Fraction céramides liés aux protéines du SC



Strips. SC. de la peau de chien en bonne santé après une diète de 12 semaines enrichie en acide linoléique
Céramides liés aux protéines **33** $\pm$ 10.8 avant et **38** $\pm$ 9.9 après ($\mu\text{g}/\text{mg}$ protéines $\pm$ SD) (sur 5 échantillons)

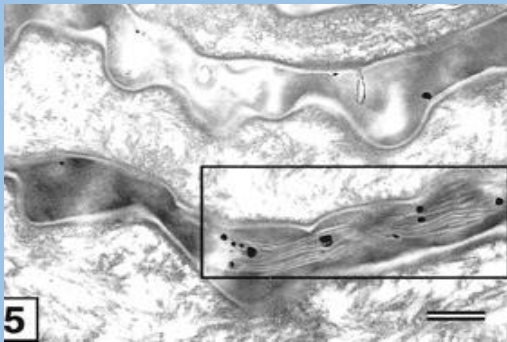
Increase of ceramides in stratum corneum of group 2 dogs fed 24 and 45 weeks with LA-enriched diet. Average values were obtained after scan densitometry of five ceramides HPTLC plates processed from the strips of five dogs from group 2. The ceramide quantities were normalized against the protein content of the strips

	Group 2 dogs prefed 12 weeks	Group 2 dogs fed 24 weeks
Free ceramides ($\mu\text{g}/\text{mg}$ proteins $\pm$ SD)	132.6 $\pm$ 20.4 (n= 5)	212.2 $\pm$ 23.5 (n= 5)
Protein-bound ceramides ($\mu\text{g}/\text{mg}$ proteins $\pm$ SD)	33 $\pm$ 10.8 (n= 5)	38 $\pm$ 9.9 (n= 5)

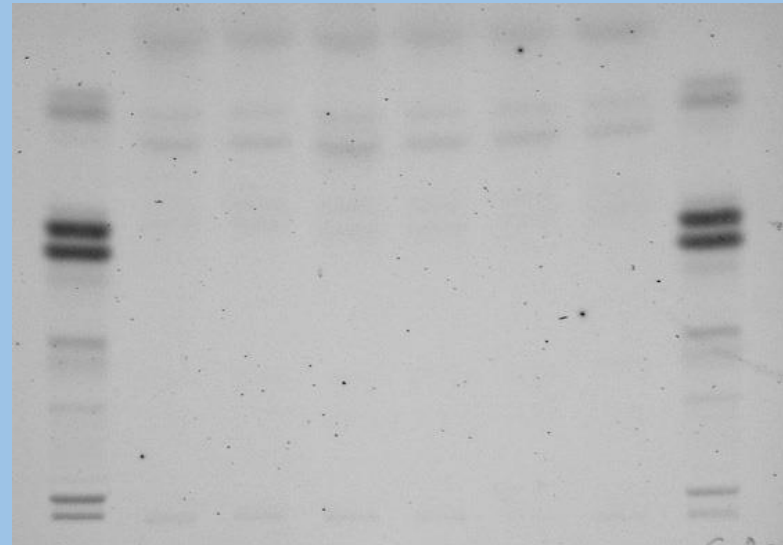
Free glucosylceramides of canine atopic stratum corneum

Non-lesional area

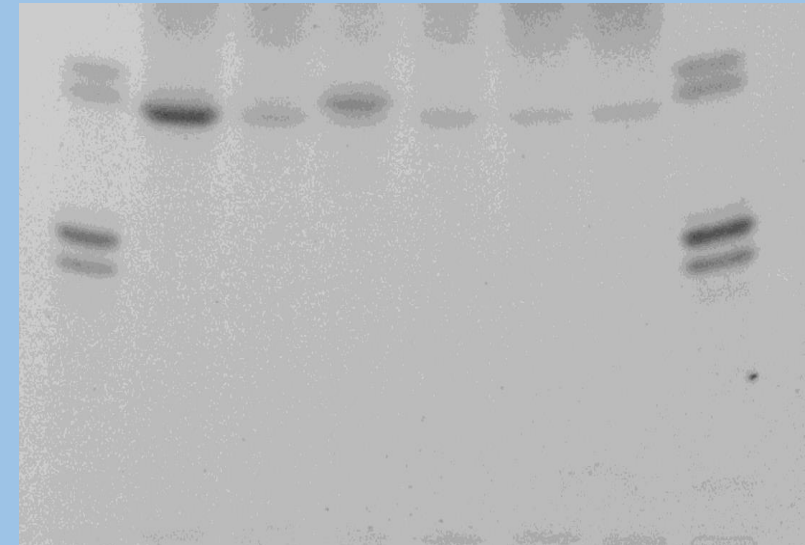
Lesional area



Stratum
Corneum (SC)



Std 1+2 3+4 5+6 7+8 9+10 11+12 Std
Strip number

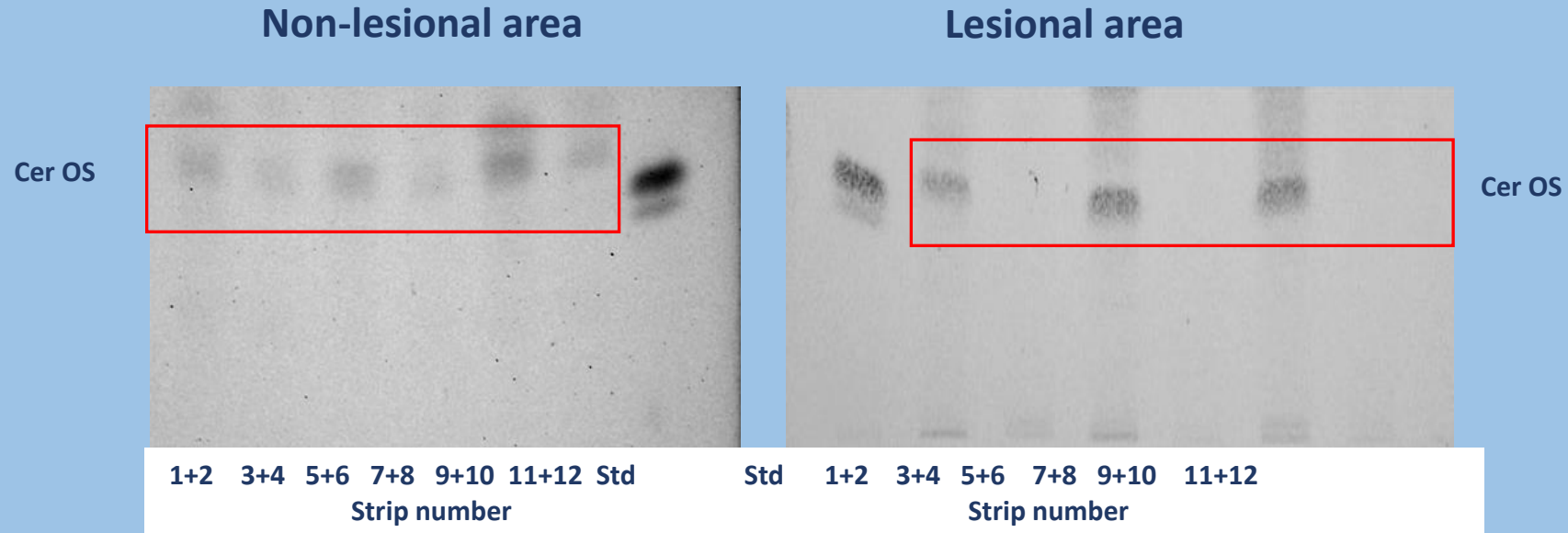


Std 1+2 3+4 5+6 7+8 9+10 11+12 Std
Strip number

Although in the normal stratum corneum, glucosylceramides are fully hydrolyzed into ceramides and are therefore absent, some molecular species are still present in the canine atopic stratum corneum, in lesional as well as non-lesional areas

Popa et al, Arch Dermatol Res, 2011 b

Stratum Corneum of atopic dog with an heterogeneous distribution of the protein-bound ceramides

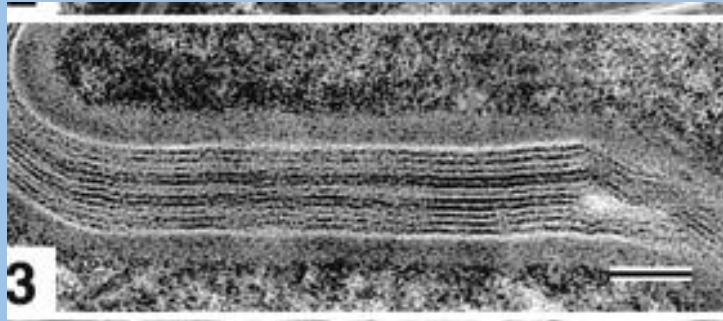


- ◆ Protein-bound ceramides in the atopic stratum corneum are heterogeneously distributed among the layers, and this feature is even more prominent in the lesional areas.
- ◆ The CerOP species present in the normal dog is absent.

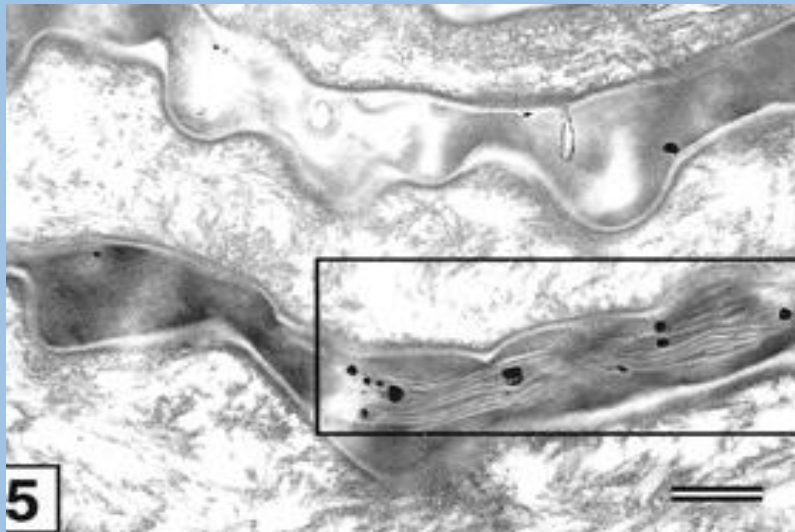
Popa et al, Arch Dermatol Res, 2010

Observation of the metabolic changes in lipids of Stratum Corneum by HPLTC/tape stripping (D-Squame in metabolic illness and during a topical or oral treatment)

Stratum Corneum of a healthy skin

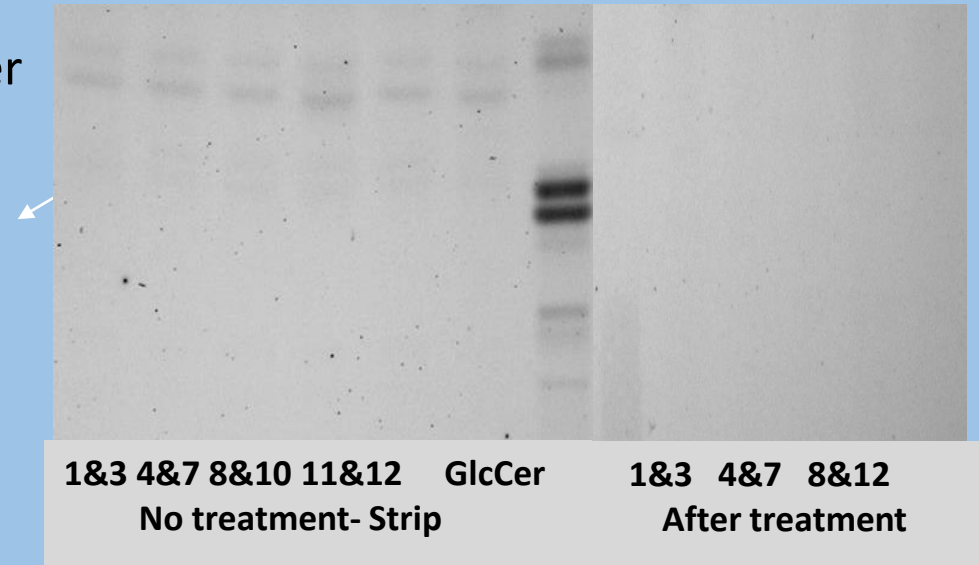


Stratum Corneum (SC of atopic dogs)



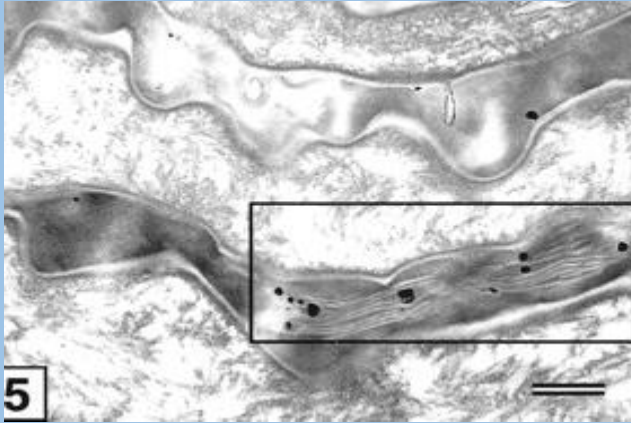
Protein-bound glucosylceramides of the atopic Stratum Corneum disappear following topical Sphingolipid cream (SLC) treatment

GlcCer



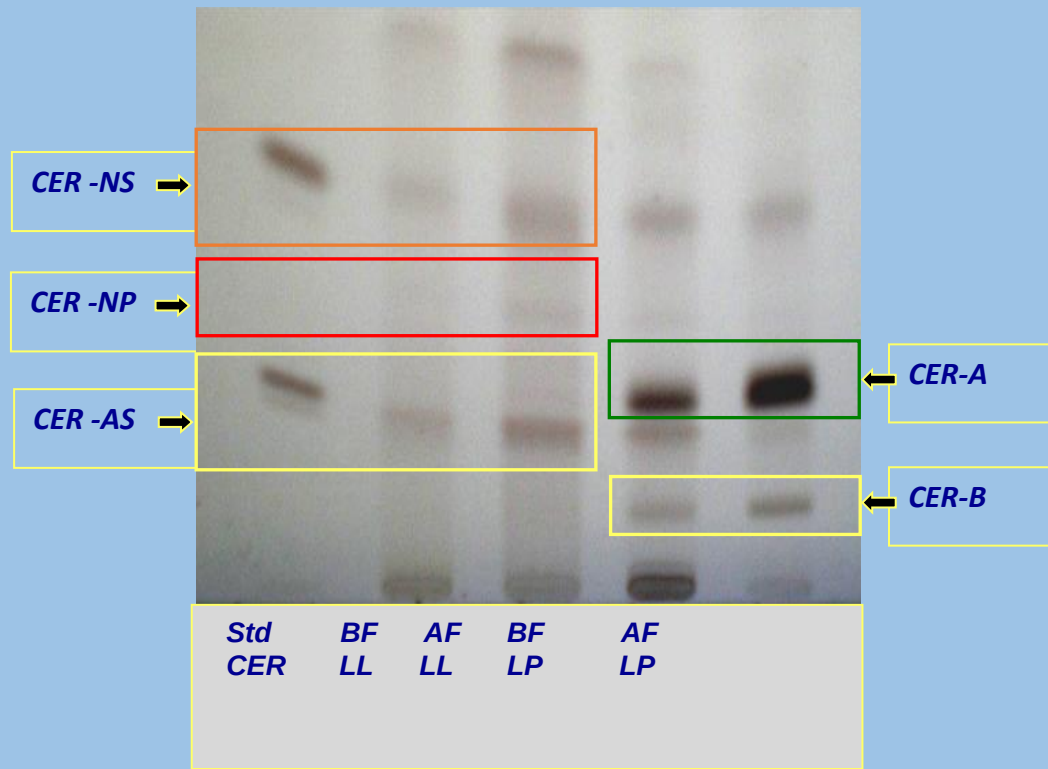
Popa et al, Clin Exp Dermatol., 2011

Objectivation des changements métaboliques des céramides par l'analyse tape stripping (D-Squame)/HPLTC sur des SC avant et après un traitement

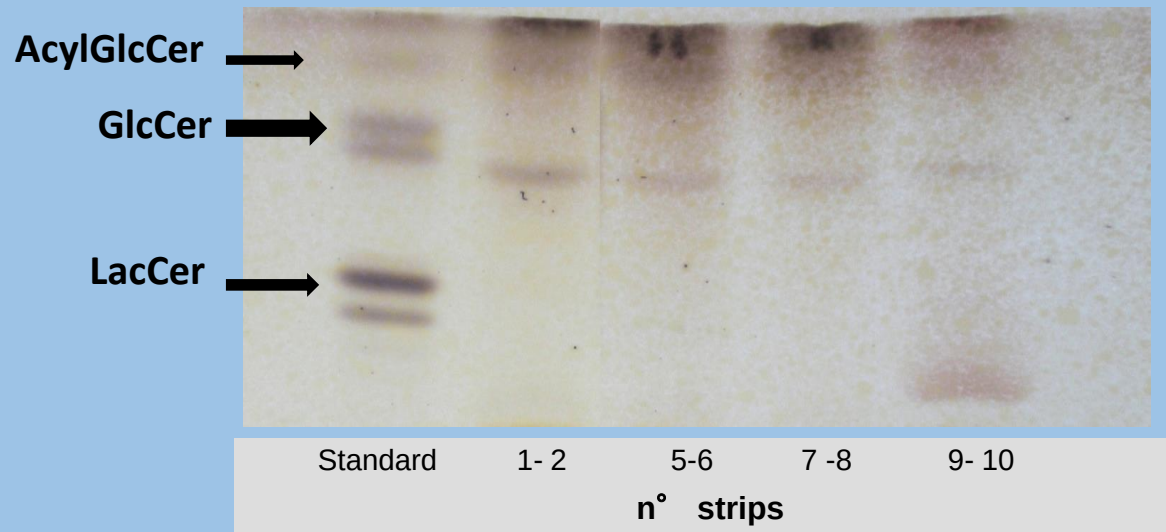


Stratum Corneum (SC)

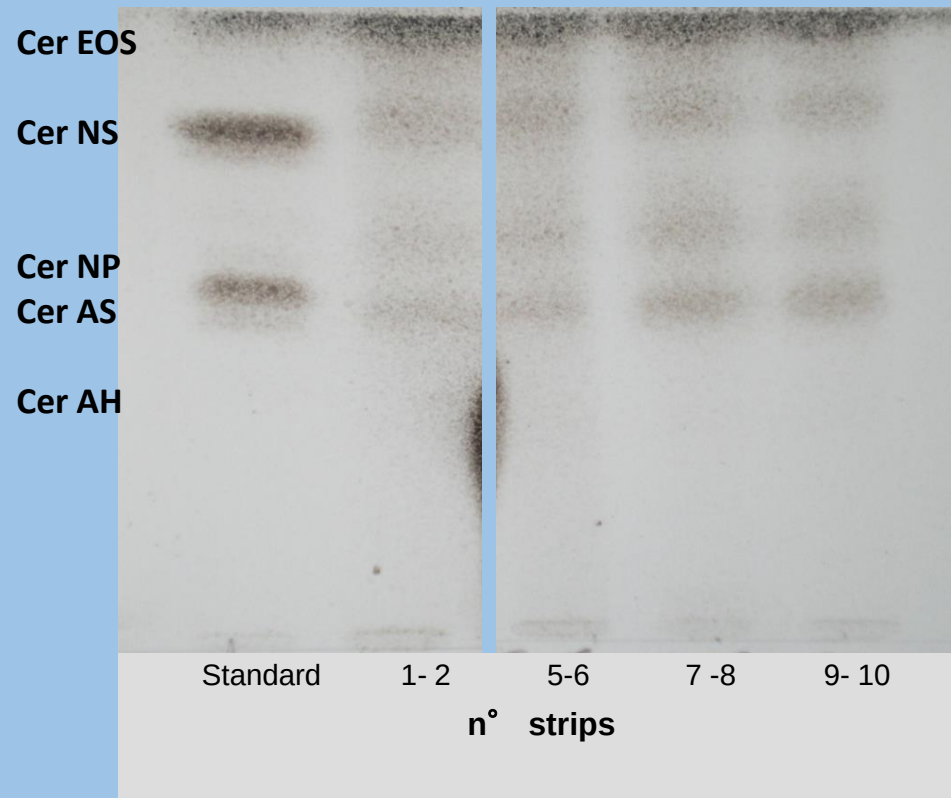
Céramides libres (LL) et liés aux protéines (LP) de SC
d'une peau de chien atopique avant (BF) et après
(AF) un traitement oral avec Megaderm®



Présence anormale de glucosylcéramides chez le chien ichtyosique montrant une dégradation incomplète en céramides nécessaires à une bonne barrière



Homogénéité dans le contenu des céramide libres
mais pas suffisante pour maintenir une barrière
cutanée étanche chez le chien ichtyosique



Identification des phospholipides dans le cas de l'ichtyose

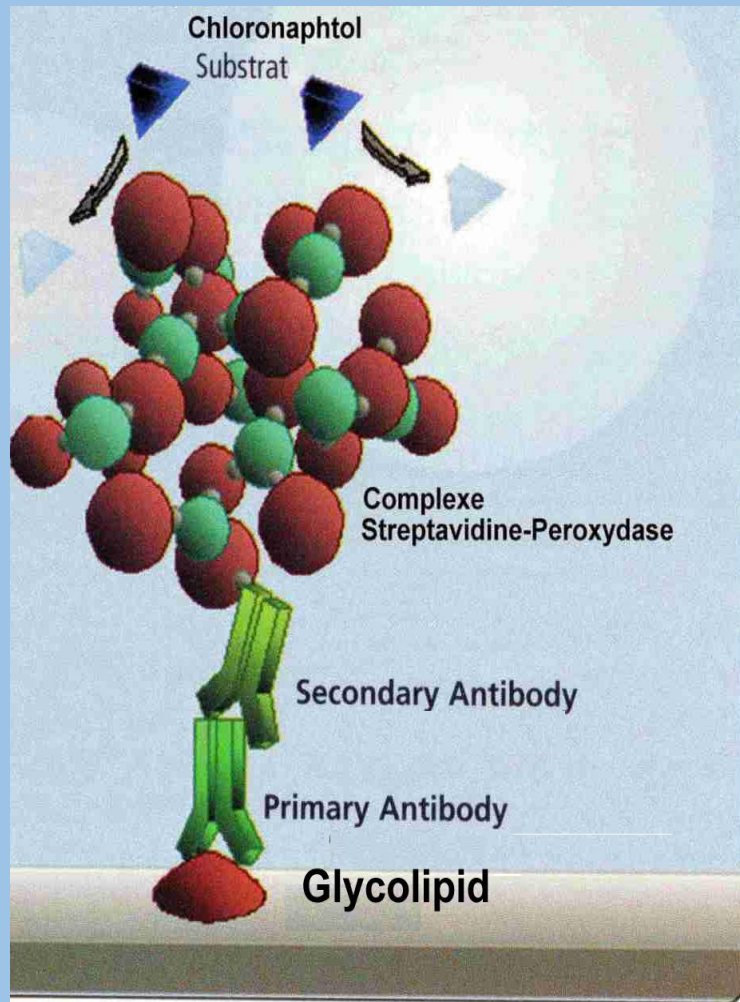
Présence anormale de phospholipides
neutres dans les lipides liés aux
protéines chez le chien ichtyosique

Phosphatidylcholine

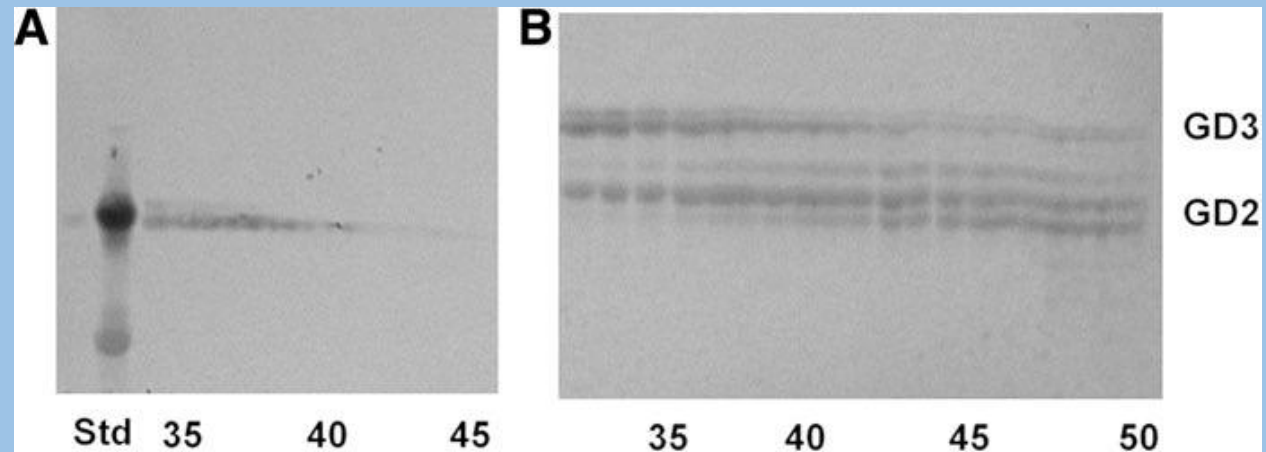
Métabolisme normalisé des phospholipides après le traitement chez le chien ichtyosique

ITLC – Immunostaining on TLC plates

Purification and structural characterization of de-N-acetylated form of GD3 ganglioside present in human melanoma tumors



I-TLC with specific antibody mAb SGR37 to de-N-AcetylGD3 compare to TLC
(A) immunostaining and (B) resorcinol-HCl.



Popa et al, Glycobiology, 17(4):367–373, 2007

HPTLC et ITLC -Detection de l' O-acetyl ganglioside

c acid is shown in Fig. 2.

Further examined by I

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ST GM3. GD3. O-acetyl-GD3 O-acetyl-GT3 GG. GG tumor tissue.
normal tissue



Tumeurs

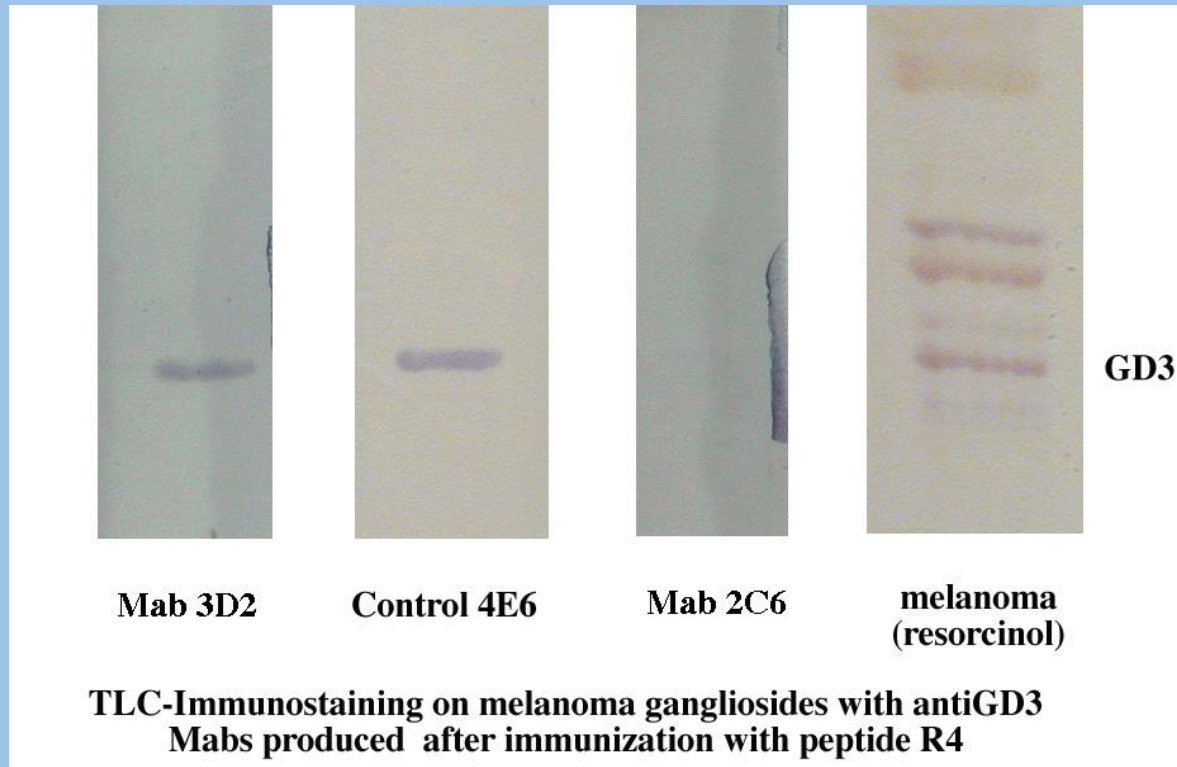
Susumu Ando

Gangliosides Expressed in Human Breast Cancer,
CANCER RESEARCH56, 5165-5171, 1996

*Autres cancers OAc-GD2

GD3-replica peptides selected from a phage peptide library induce a GD3 ganglioside **antibody** response

Immuno-HPTLC



Major Gangliosides of melanoma

GM3	Cer-Glc-Gal-NeuAc
GM2	Cer-Glc-Gal[NeuAc]-GalNAc
GD3	Cer-Glc-Gal-NeuAc-NeuAc

Conclusions....

- Understanding the sphingolipid rheostat in skin could be helpful in maintaining a certain homeostatic state (disease, aging, diet...)
- Methods such as HPTLC coupled with ITLC, LS-MS, GS , optical microscopy, confocal microscopy, electron microscopy are helpful in understanding lipids problems and metabolic lipids disorders.

Thank you for your attention

