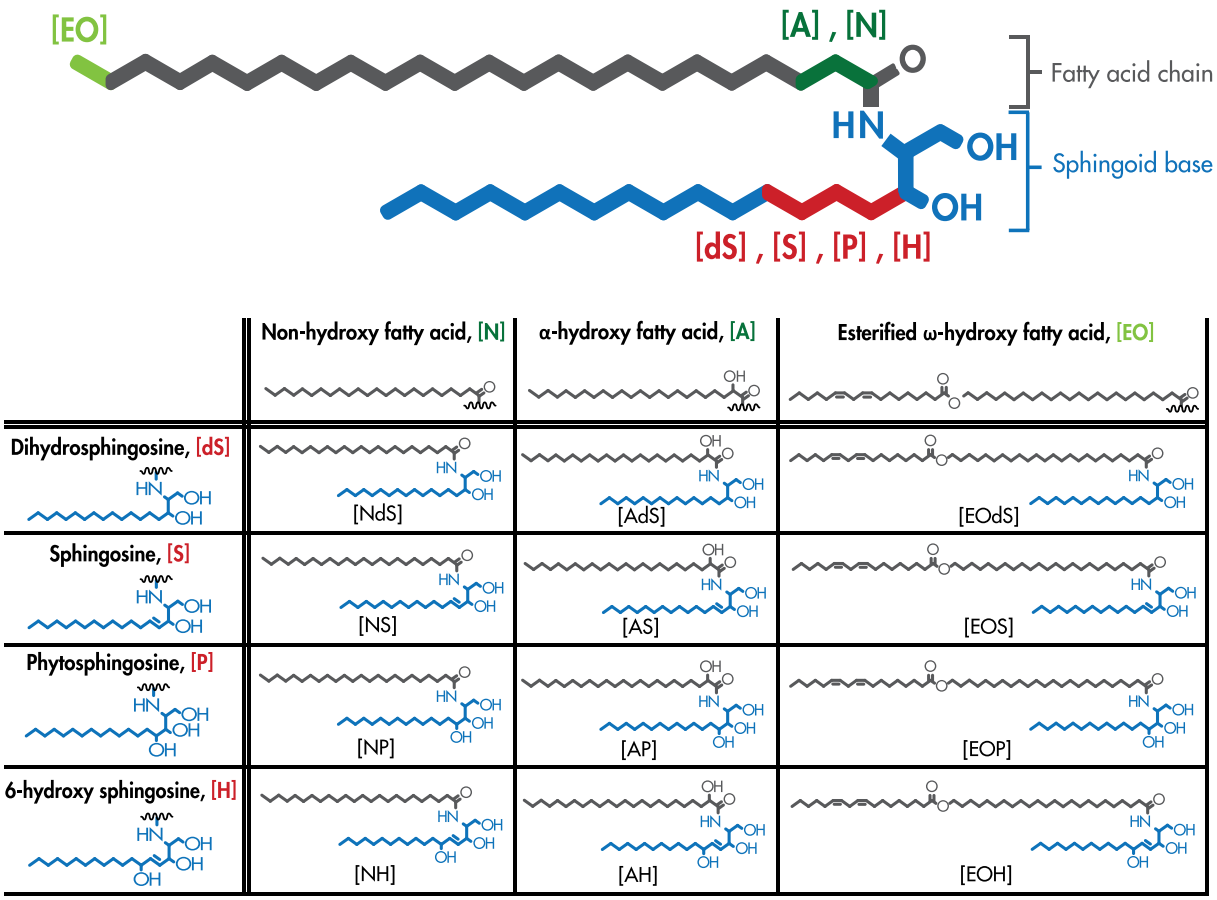
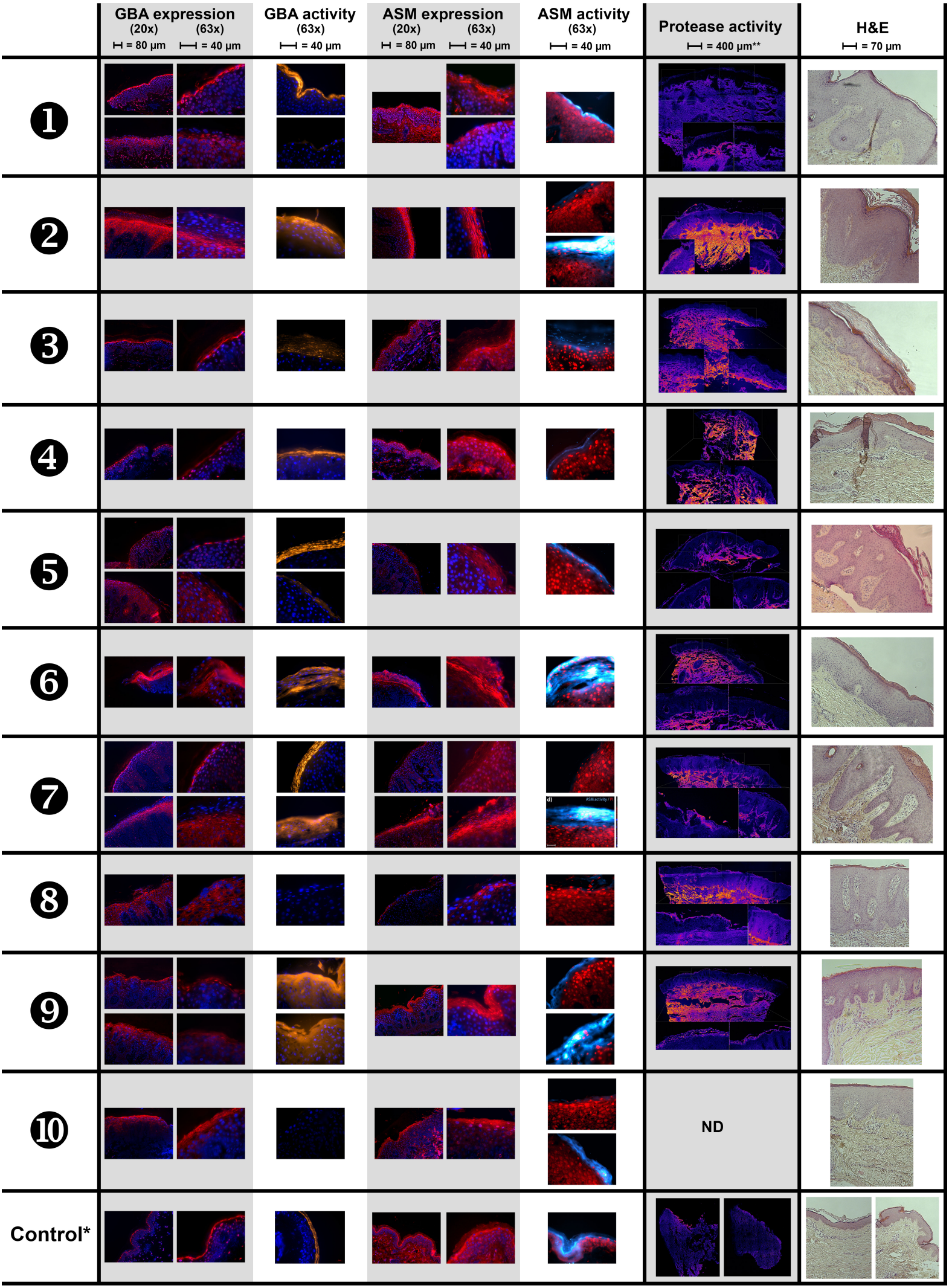
Supplement

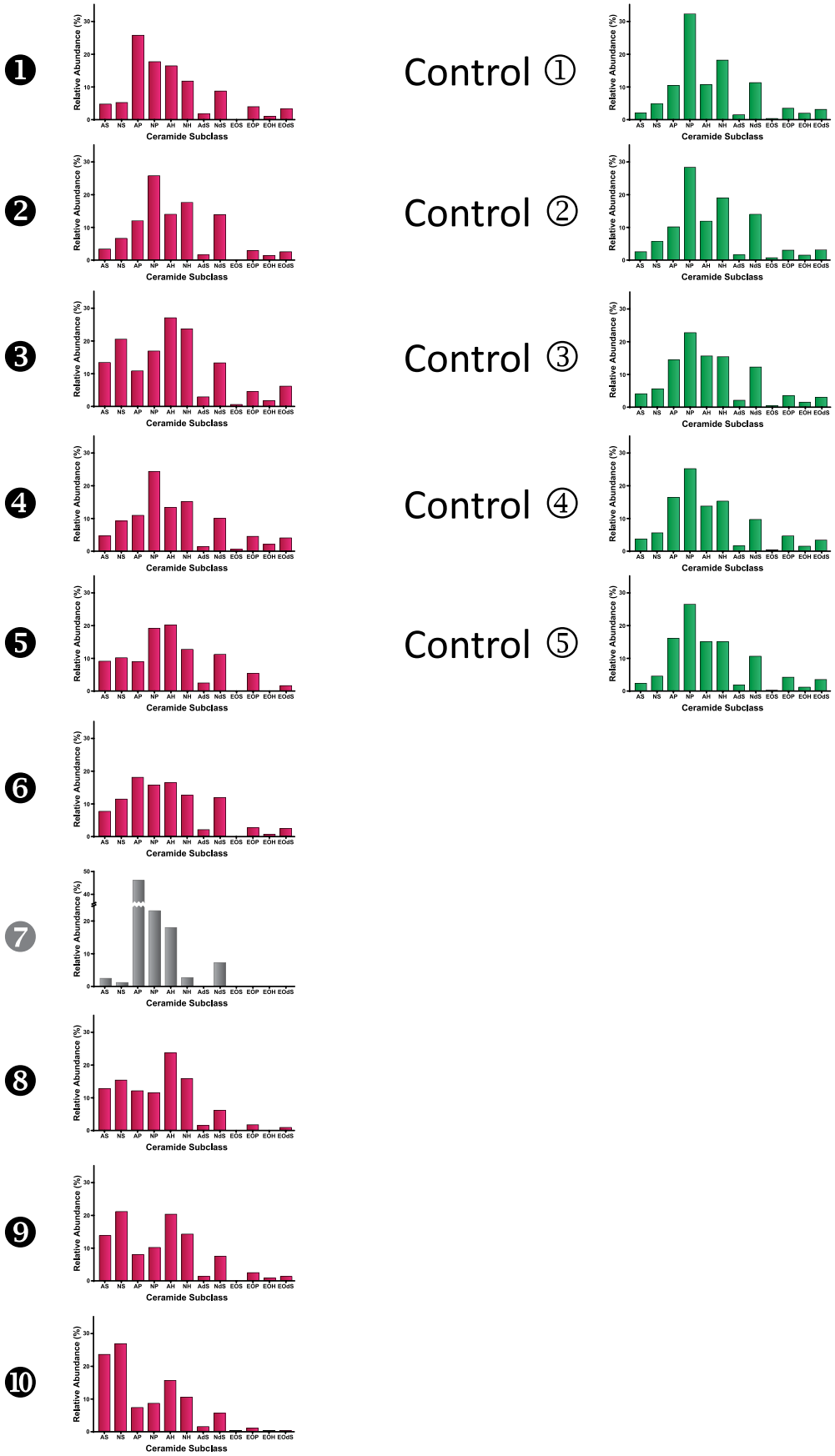
SUPPLEMENTAL FIGURES



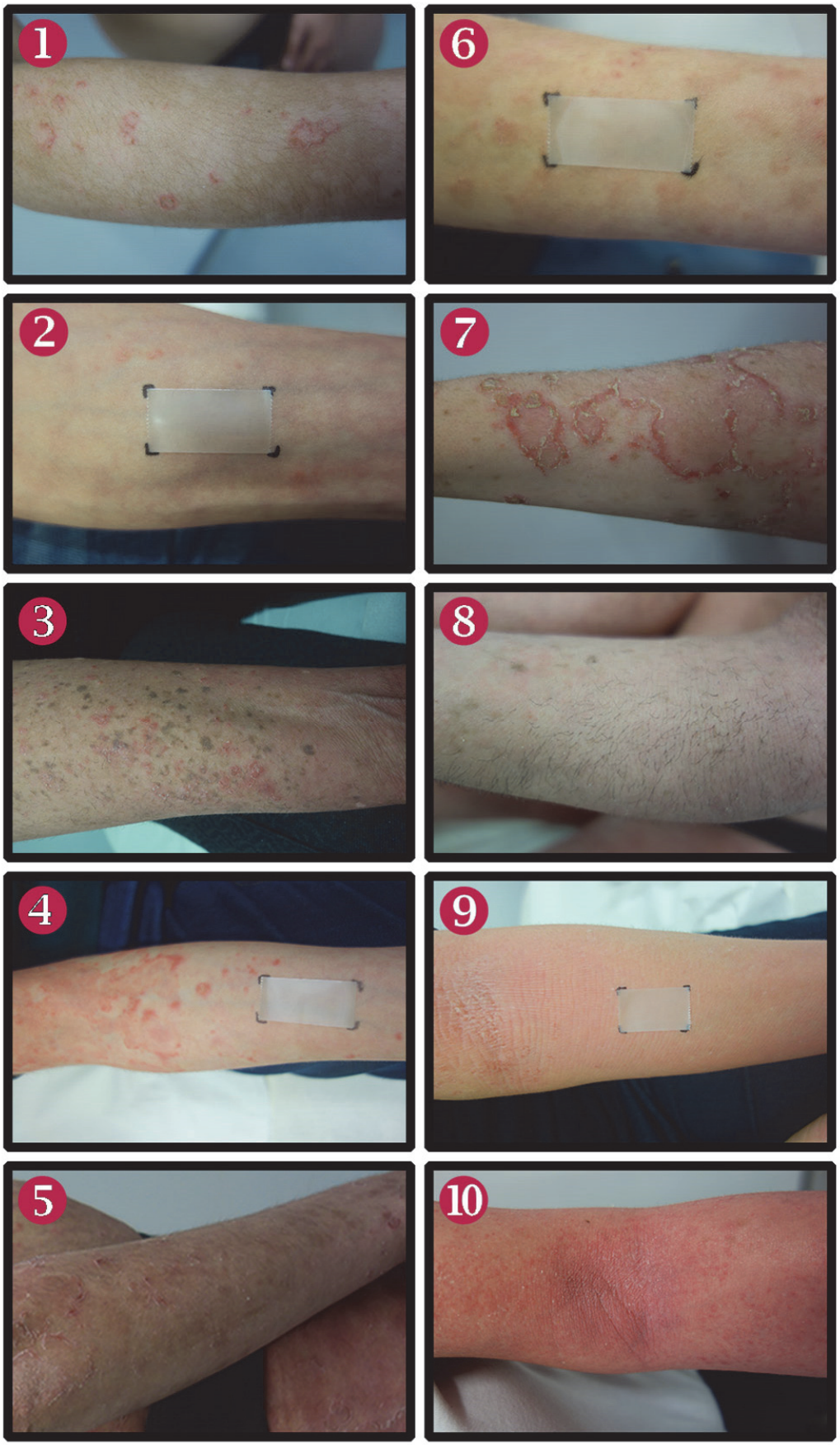
Supplemental Figure S1: ceramide subclass nomenclature, based on the molecular architecture. Ceramides bear two chains: one fatty acid chain and one sphingoid base. In human skin, both chains can vary in their structure at specific locations (red/green). The nomenclature is based on the abbreviation for the fatty acid chain (Esterified Omega-hydroxy fatty acid [EO], Non-hydroxy fatty acid [N], Alpha-hydroxy fatty acid [A]), combined with the 1 or 2 letter abbreviation for the sphingoid base (dihydroSphingosine [dS], Sphingosine [S], Phytosphingosine [P], 6-Hydroxy sphingosine [H]). Together, this results in the presence of the 12 most common subclasses: [AS], [NS], [AP], [NP], [AH], [NH], [AdS], [NdS], [EOS], [EOP], [EOH], [EOdS] (Motta et al., 1993, van Smeden et al., 2011).



Supplemental Figure S2: Overview of all stainings in each individual NTS subject and representative examples for the healthy control group. *: all healthy subjects demonstrated comparable photographs for the different stainings, therefore only 1 representative image is shown. **: This scale bar represents the main microscope picture, not the zoomed/boxed highlighted areas.



Supplemental Figure S3: SC ceramide subclass profile of each individual NTS subject (and healthy control group). For each ceramide subclass, the relative abundance (% of peak areas) is plotted. Note that the amount of lipids obtained from NTS⑦ was very limited, making the outcomes less reliable compared to all other subjects.



Supplemental Figure S4: Photographs of the forearm of all 10 NTS patients. NTS ②,④,⑥,⑨ demonstrate the tape strip on the ventral forearm.

SUPPLEMENTAL TABLES

Supplemental Table S1: Pearson correlation coefficients of all 12 analyzed ceramide subclasses

[NdS]	[NS]	[NP]	[NH]	[AdS]	[AS]	[AP]	[AH]	[EOdS]	[EOS]	[EOP]	[EOH]	Ceramide subclass
1	-0.56*	0.67*	0.54*	0.43	-0.67*	-0.21	-0.59*	0.46	0.50	0.57*	0.56*	**[NdS]**
	1	-0.84*	0.01	0.11	**0.96***	-0.55*	0.45	-0.26	-0.30	-0.39	-0.39	**[NS]**
		1	0.27	-0.11	-0.86*	0.19	-0.77*	0.49	0.37	0.63*	0.43	**[NP]**
			1	0.54*	-0.17	-0.78*	-0.32	0.54*	0.52*	0.65*	0.63*	**[NH]**
				1	0.07	-0.62*	0.020	0.17	0.69*	0.16	0.51	**[AdS]**
					1	-0.40	0.50	-0.37	-0.38	-0.56*	-0.54*	**[AS]**
						1	0.05	-0.32	-0.41	-0.29	-0.30	**[AP]**
							1	-0.56*	-0.26	-0.73*	-0.47	**[AH]**
								1	0.33	0.74*	0.66*	**[EOdS]**
									1	0.52*	0.72*	**[EOS]**
										1	0.82*	**[EOP]**
											1	**[EOH]**

Note the high correlation between [AS] and [NS] (R=0.96), indicating that an increased level of [AS] will be accompanied by an increased level of [NS] in this cohort of NTS subjects. * indicates a significant correlation ($p<0.05$).

Supplemental Table S2: Characteristics of patients with Netherton syndrome included in the study

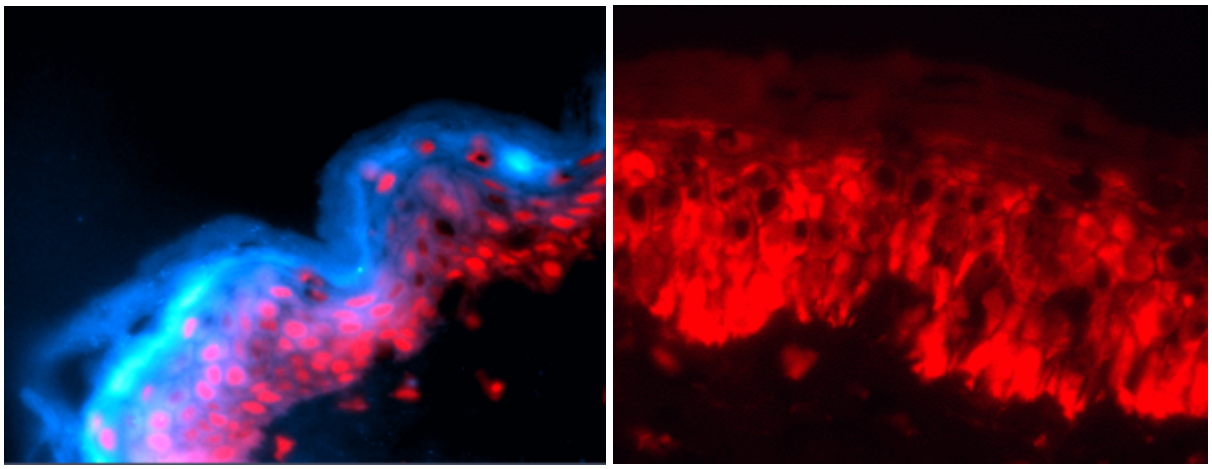
NTS	Age (y/o)	Sex	Flare	Acanthosis	Scaly Erythroderma	ILC
①	21	F	-	3	0	1
②	16	M	+	2	0	1
③	57	F	-	2	0	2
④	12	M	+	1	0	3
⑤	26	F	+	2	0	3
⑥	18	F	+	3	0	3
⑦	13	F	+	3	1	3
⑧	41	M	-	3	2	0
⑨	34	F	-	2	2	0
⑩	17	F	-	3	3	0

NTS represents the Netherton subject, ILC mean Ichthyosis Linearis Circumflexa. F and M indicate Female and Male, respectively. + and – correspond to present and absent. Score: 0 = Absent, 1 = Minimal, 2 = Moderate, 3 = Severe.

SUPPLEMENTAL MATERIAL & METHODS

Developing and optimizing an *in-situ* zymography method for localization of active ASM

To visualize active ASM in human epidermis, we developed and optimized an *in situ* zymography method using 6-HMU-PC (6-hexadecanoyl-4-methylumbelliferyl-phosphorylcholine), a specific ASM substrate that results in fluorogenic product 6-HMU(van Diggelen et al., 2005). The following parameters were optimized (detailed results in supplement): *i) Concentration of substrate 6-HMU-PC:* a concentration around 0.5 mM provided best results. Lower concentrations of substrate led to less signal and therefore suboptimal contrast. Higher concentrations increased the risk for insoluble or precipitated substrate clusters. *ii) Incubation period:* Incubation at 37°C successfully converted 6-HMU-PC into fluorescent 6-HMU, which could easily be observed after one hour. When incubating for ~4 hours or longer, diffusion became significant, interfering with the results by reducing the contrast. *iii) pH Incubation buffer:* fluorescent product was optimal at pH 5.2, and clearly reduced at pH≥5.4. *iv) Sodium Taurocholate as buffer additive:* addition of 0.2% (w/v) sodium taurocholate resulted in a more consistent signal along the whole skin section. Sodium Taurocholate catalyzes the enzyme activity (Takagi 1999). *v) Washing procedure:* Washing the skin sections after incubation with 6-HMU-PC was necessary to remove non-specific signal. Washing with Milli-Q proved more efficient in addition of 1% tween, resulting in clearer images with increased contrast. This method revealed active ASM at the SC layers of human skin (Figures below), particularly at the SG/SC interface. Negative control (human skin with incubation of buffer instead of 6-HMU-PC solution) did not demonstrate active ASM.



In-situ zymography of ASM acivity, showing in blue/cyan active ASM (left figure). Negative control did not show ASM activity (right figure).

Scoring of lipid parameters

SC Ceramide scoring: Scoring all NTS subjects was based on the deviation from the control value. Subject 7 was excluded as the amount of lipids obtained was minimal, and therefore the obtained relative values for [AS] + [NS] and [EO] less reliable. For the relative abundance of [AS] + [NS] (%), a mild increase is indicated in yellow (values between 50-75th percentile), whereas a more drastic increase is categorized as red (values above the 75th percentile). For [EO], a mediocre or strong decrease in relative abundance would lead to respectively a 'yellow' or 'red' label (values between 25-50th percentile, and values below 25th percentile, respectively).

	SC Ceramides	
Subject	[AS] + [NS] (%)	[EO] (%)
①	9.8	8.2
②	9.8	6.5
③	24.0	9.2
④	13.9	11.2
⑤	19.0	6.9
⑥	18.7	5.5
⑦	3.5 *	0.0 *
⑧	27.7	2.4
⑨	34.7	4.5
⑩	50.0	1.3
Control	8.0 ± 1.2	8.7 ± 0.5

GBA+ASM scoring: Scoring of the lipid biosynthesis enzymes and protease activity was based on all microscopy images from all sections of each individual subject. For control skin, variation between parameters was very low compared to NTS patients. The localization of expressed and active protein was first described and compared to healthy (see Table S3 on next page). For each parameter (GBA expression, ASM expression, GBA activity, and ASM activity), this written description was translated into:

- Equal to healthy skin
- Minor Differences: Partially equal, partially different from healthy skin
- Major Differences: (Almost) Completely different from healthy skin

"Different" means either expression or activity at a different location, or a substantial different intensity (e.g. completely absent). When constructing Table I located in the main text, the expression of the two enzymes GBA+ASM was scored in the following manner:

o	(Identical to) Healthy skin
+	Minor differences in one of the two enzymes
++	Major difference in one of the two enzymes; Minor differences in both enzymes
+++	Major differences in both enzymes

	GBA+ASM enzymes	
Subject	Expression Score	Activity Score
①	++	+
②	+++	++
③	o	++
④	o	++
⑤	+++	+
⑥	+ *	++
⑦	++	++
⑧	++	+++
⑨	+++	+++
⑩	++	+++
Control	o	o

Supplemental Table S3. Written description of each expression/acitivity staining in NTS individuals compared with control.

Subject	GBA expression	GBA activity	ASM expression	ASM activity	Zymography (Casein Substrate)
1	Heterogenous, either primarily at SG/SC interface, or intracellular, throughout the epidermis	Heterogenous, either absent or primarily at SG/SC interface	Heterogenous, not localized throughout SC or primarily SG/SC interface	Present, primarily in outer SC layers	Mild casein activity in the stratum corneum
2	Intracellular, throughout the epidermis	Present, primarily in outer SC layers	SG/SC interface and many SC layers	Heterogenous, absent or present at parakeratotic cells	Strong casein activity in the stratum corneum, Heterogenous
3	Primarily SG/SC interface	Primarily SG/SC interface and lower SC layers, speckled	Primarily SG/SC interface	Present, speckled pattern	Absent
4	Primarily SG/SC interface	Present, primarily in outer SC layers	Primarily SG/SC interface	Present in outer SC layers	Absent
5	Heterogenous, either primarily at SG/SC interface, or intracellular, throughout the epidermis	Heterogenous, either almost absent or primarily at SG/SC interface	Heterogenous, not localized throughout SC or primarily SG/SC interface	Primarily at the SG/SC interface and lower SC layers	Mild casein activity in the stratum corneum
6	Intracellular, throughout the epidermis**	Not localized, throughout SC	Heterogenous, not localized throughout SC or primarily SG/SC interface	Heterogenous, Present at parakeratotic cells	Mild casein activity in the stratum corneum
7	Heterogenous, either primarily at SG/SC interface, or intracellular, throughout the epidermis	Heterogenous, either throughout SC, or almost absent	Heterogenous, absent or primarily SG/SC interface	Heterogenous, absent or present at parakeratotic cells	Moderate casein activity in the stratum corneum, Heterogeneous
8	Intracellular, throughout the epidermis	Absent	Heterogenous, absent or primarily SG/SC interface	Absent	Strong casein activity in the stratum corneum
9	Heterogenous, either in parakeratotic cells, or intracellular, throughout the epidermis	Not localized, throughout SC	Primarily in parakeratotic cells	Heterogenous, in outer SC layers or present at parakeratotic cells	Strong casein activity in the stratum corneum
10	Primarily SG/SC interface, but also intracellular, throughout the epidermis	Absent	Heterogenous, not localized or outer SC layers	Heterogenous: Primarily absent, more present at parakeratotic cells	ND
Control	Primarily SG/SC interface	Primarily localized at SG/SC interface and lower SC layers	Primarily SG/SC interface	Primarily at the SG/SC interface and lower SC layers	Absent

ND means not determined

Supplemental References

Motta S, Monti M, Sesana S, Caputo R, Carelli S, Ghidoni R. Ceramide composition of the psoriatic scale. Biochim Biophys Acta 1993;1182(2):147-51.

van Diggelen OP, Voznyi YV, Keulemans JL, Schoonderwoerd K, Ledvinova J, Mengel E, et al. A new fluorimetric enzyme assay for the diagnosis of Niemann-Pick A/B, with specificity of natural sphingomyelinase substrate. J Inherit Metab Dis 2005;28(5):733-41.

van Smeden J, Hoppel L, van der Heijden R, Hankemeier T, Vreeken RJ, Bouwstra JA. LC/MS analysis of stratum corneum lipids: ceramide profiling and discovery. J Lipid Res 2011;52(6):1211-21.