# Supplemental

## Supplemental material and methods

### Mass spectrometric analysis by direct infusion of lipid extracts and standards.

For initial survey analysis, lipid extracts corresponding to 0.1 mg dry weight of epidermis were dissolved in 1 mL of methanol containing 5 mM ammonium acetate and were injected directly into the ESI-source with a flow rate between 5 and 10 µL/min.

Quantification of free glucosylceramides and ceramides.

Free glucosylceramides and ceramides from epidermal lipid raw extracts were quantified by UPLC-ESI-(QqQ)tandem mass spectrometry according to (1).

# Supplemental Tables

## Supplemental Table 1: UPLC-gradient elution of 1-O-acylceramides for tandem-mass spectrometric detection.

| Time [min] | Flow Rate [ml/min] | Solvent A [%] | Solvent B [%] | Slope |
|---|---|---|---|---|
| Initial | 0.45 | 100 | 0 | Initial |
| 0.10 | 0.45 | 100 | 0 | Linear |
| 0.20 | 0.45 | 92 | 8 | Linear |
| 5.00 | 0.45 | 10 | 90 | Concave ($X^2$) |
| 5.25 | 0.45 | 10 | 90 | Linear |
| 5.5 | 0.45 | 100 | 100 | Linear |
| 6.5 | 0.45 | 100 | 100 | Linear |

## Supplemental Table 2: Multiple SRM transitions (MRM) used for detection and quantification of 1-O-acylceramides.

| 1-O-Acylceramide | | | Transitions | | 1-O-Acylceramide | | | Transitions | |
|---|---|---|---|---|---|---|---|---|---|
| 1-O-Acyl | So | N-Acyl | $[M+H]^+$ & $[(M-H_2O)+H]^+$ | Fragment **c** | 1-O-Acyl | So | N-Acyl | $[M+H]^+$ & $[(M-H_2O)+H]^+$ | Fragment **c** |
| HO-groups, C-atoms : double bonds | | | | [m/z] | HO-groups, C-atoms : double bonds | | | | [m/z] |
| 14:0 | d17:1 | 16:0 | 734.7 & 716.7 | 488.5 | 14:0 | d18:1 | h16:0 | 764.7 & 746.7 | 518.5 |
| 16:0 | | | 762.7 & 744.7 | | 16:0 | | | 804.8 & 786.8 | |
| 18:0 | | | 790.8 & 772.8 | | 18:0 | | | 832.8 & 814.8 | |
| 20:0 | | | 818.8 & 800.8 | | 20:0 | | | 860.8 & 842.8 | |
| 22:0 | | | 846.8 & 828.8 | | 22:0 | | | 888.9 & 870.9 | |
| 24:0 | | | 874.9 & 856.8 | | 23:0 | | | 902.9 & 884.9 | |
| 26:0 | | | 902.9 & 884.9 | | 24:0 | | | 916.9 & 898.9 | |
| 14:0 | d17:1 | h16:0 | 750.7 & 732.7 | 504.5 | 25:0 | | | 930.9 & 912.9 | |
| 16:0 | | | 790.8 & 772.8 | | 26:0 | | | 944.9 & 926.9 | |
| 18:0 | | | 818.8 & 800.8 | | 14:0 | d18:1 | 18:0 | 776.7 & 758.7 | 530.5 |
| 20:0 | | | 846.8 & 828.8 | | 15:0 | | | 790.8 & 772.8 | |
| 22:0 | | | 874.9 & 856.8 | | 16:0 | | | 804.8 & 786.8 | |
| 24:0 | | | 902.9 & 884.9 | | 18:0 | | | 832.8 & 814.8 | |
| 26:0 | | | 930.9 & 912.9 | | 20:0 | | | 860.8 & 842.8 | |
| 14:0 | d17:1 | 18:0 | 762.7 & 744.7 | 516.5 | 22:0 | | | 888.9 & 870.9 | |
| 16:0 | | | 790.8 & 772.8 | | 23:0 | | | 902.9 & 884.9 | |
| 18:0 | | | 818.8 & 800.8 | | 24:0 | | | 916.9 & 898.9 | |
| 20:0 | | | 846.8 & 828.8 | | 26:0 | | | 944.9 & 926.9 | |
| 22:0 | | | 874.9 & 856.8 | | 14:0 | d18:1 | 20:0 | 804.8 & 786.8 | 558.6 |
| 24:0 | | | 902.9 & 884.9 | | 15:0 | | | 818.8 & 800.8 | |
| 26:0 | | | 930.9 & 912.9 | | 16:0 | | | 832.8 & 814.8 | |
| 14:0 | d17:1 | 20:0 | 790.8 & 772.8 | 544.5 | 18:0 | | | 860.8 & 842.8 | |
| 16:0 | | | 818.8 & 800.8 | | 20:0 | | | 888.9 & 870.9 | |
| 18:0 | | | 846.8 & 828.8 | | 22:0 | | | 916.9 & 898.9 | |
| 20:0 | | | 874.9 & 856.8 | | 23:0 | | | 930.9 & 912.9 | |
| 22:0 | | | 902.9 & 884.9 | | 24:0 | | | 944.9 & 926.9 | |
| 24:0 | | | 930.9 & 912.9 | | 26:0 | | | 973.0 & 955.0 | |
| 26:0 | | | 959.0 & 940.9 | | 14:0 | d18:1 | 22:0 | 832.8 & 814.8 | 586.6 |
| 14:0 | d17:1 | 22:0 | 818.8 & 800.8 | 572.6 | 16:0 | | | 860.8 & 842.8 | |
| 16:0 | | | 846.8 & 828.8 | | 18:0 | | | 888.9 & 870.9 | |
| 18:0 | | | 874.9 & 856.8 | | 20:0 | | | 916.9 & 898.9 | |
| 20:0 | | | 902.9 & 884.9 | | 22:0 | | | 944.9 & 926.9 | |
| 22:0 | | | 930.9 & 912.9 | | 24:0 | | | 973.0 & 955.0 | |
| 24:0 | | | 959.0 & 940.9 | | 25:0 | | | 987.0 & 969.0 | |
| 26:0 | | | 987.0 & 969.0 | | 26:0 | | | 1001.0 & 983.0 | |
| 14:0 | d17:1 | 24:0 | 846.8 & 828.8 | 600.6 | 14:0 | d18:1 | 24:0 | 860.8 & 842.8 | 614.6 |
| 16:0 | | | 874.9 & 856.8 | | 15:0 | | | 874.9 & 856.8 | |
| 18:0 | | | 902.9 & 884.9 | | 16:0 | | | 888.9 & 870.9 | |
| 20:0 | | | 930.9 & 912.9 | | 17:0 | | | 902.9 & 884.9 | |
| 22:0 | | | 959.0 & 940.9 | | 18:0 | | | 916.9 & 898.9 | |
| 24:0 | | | 987.0 & 969.0 | | 20:0 | | | 944.9 & 926.9 | |
| 26:0 | | | 1015.0 & 997.0 | | 22:0 | | | 973.0 & 955.0 | |
| 14:0 | d18:1 | 16:0 | 748.7 & 730.7 | 502.5 | 23:0 | | | 987.0 & 969.0 | |
| 16:0 | | | 776.7 & 758.7 | | 24:0 | | | 1001.0 & 983.0 | |
| 18:0 | | | 804.8 & 786.8 | | 25:0 | | | 1015.0 & 997.0 | |
| 20:0 | | | 832.8 & 814.8 | | 26:0 | | | 1029.0 & 1011 | |
| 22:0 | | | 860.8 & 842.8 | | | | | | |
| 23:0 | | | 874.9 & 856.8 | | | | | | |
| 24:0 | | | 888.9 & 870.9 | | | | | | |
| 25:0 | | | 902.9 & 884.9 | | | | | | |
| 26:0 | | | 916.9 & 898.9 | | | | | | |

## Supplemental References

1. Amen, N., D. Mathow, M. Rabionet, R. Sandhoff, L. Langbein, N. Gretz, C. Jackel, H. J. Grone, and R. Jennemann. 2013. Differentiation of epidermal keratinocytes is dependent on glucosylceramide:ceramide processing. *Hum Mol Genet*.

2. Jennemann, R., R. Sandhoff, L. Langbein, S. Kaden, U. Rothermel, H. Gallala, K. Sandhoff, H. Wiegandt, and H. J. Grone. 2007. Integrity and barrier function of the epidermis critically depend on glucosylceramide synthesis. *J. Biol. Chem.* **282**: 3083-3094.

## Supplemental figure legends

### Supplemental Figure 1: Validation of the UPLC-ESI-MS/MS-method for quantification of 1-O-acylceramides .

A) Dilution series of 1-O-palmitoyl ceramide(d18:1;16:0) versus 7.65 pmol of internal standard, 1-O-oleoyl ceramide(d18:1;17:0) per mL corresponding to 76.5 fmol of injected internal standard. For each dilution 3 different samples were measured. B) Dilution series of lipid extracts from wild type (WT) and from glucosylceramide-deficient (KO) epidermis versus 7.65 pmol of internal standard. Samples from three different mice were recorded for each series. C) Dilution series of 1-O-palmitoyl ceramide(d18:1;16:0) versus 7.65 pmol of internal standard in the absence or presence of adhesive tape extracts; n = 3. Without tape extract a linear curve with a slope of 1.063 ± 0.021 (95% confidence interval: 1.016 to 1.110) and with tape extract a linear curve with a slope of 1.259 ± 0.02622 (95% confidence interval: 1.200 to 1.319) were obtained.

## Supplemental Figure 2: Tandem-mass spectrometry precursor ion spectra of 1-O-acylceramides.

Epidermal extracts from CerS3-deficient mice were analyzed by direct infusion. Acylceramides (precursor ions) leading to the fragment 264 (in red) contain a C18-sphingosine (d18:1). Signals of this +264 precursor ion scan represent the sum of all potential 1-O-acylceramides containing this sphingoid base. Subgroups of these acylceramides (blue capital letters) also lead to the formation of fragments (in red) representing ceramide-backbones containing N-linked saturated C16- (d18:1;16:0, m/z 502, series 16), hydroxy-C16- (d18:1;h16:0, m/z 518, series h16), C22- (d18:1;22:0, m/z 586, series 22), or C24-acyl chains (d18:1;24:0, m/z 614, series 24). Peaks annotated with the same blue capital letter contain the same O-linked acyl chain. Peaks annotated in grey are derived from in source water loss of the acylceramides and differ by a mass of 18 u.

## Suppl. Fig. 3: Comparison of the retention times of 1-O-acylceramides and EOS-ceramides on RP 18-column.

A), B), and C) correspond to 3 subsequent injections of epidermal lipid extract from CerS3-deficient mice spiked with the internal standard (IS), Cer(18:1;d18:1;17:0), on reversed phase 18 column. Eluting compounds were recorded by tandem mass spectrometry in multiple SRM-mode as described in the *Material and Methods* section. A different lot of solvent systems A and B had been used, which resulted in slightly shifted retention times as compared to Fig. 3. A) Total ion chromatogram (TIC) of 1-O-acylceramides with a C18-sphingosine base and an N-linked hydroxy palmitoyl residue. The TIC includes also the internal standard. B) TIC of 1-O-acylceramides with a C18-sphingosine base and an N-linked lignoceroyl residue. The TIC includes also the internal standard. $C_1$) SRM of the internal standard within the run C. C2) TIC of EOS-ceramides (linoleic acid esterified ω-hydroxy ceramides) with a C18-sphingosine base and a N-linked saturated ultra-long ω-hydroxy acyl chain from run C. Note, 1-O-acylceramides with an N-linked lignoceroyl residue elute similar to or even later than EOS-ceramides.

## Suppl. Fig. 4: Quantification of epidermal 1-O-acylceramide levels from $LPLA_2$-, keratinocyte-specific GlcCer-S-, nGlcCerase-deficient, and control mice.

Data from Fig. 5 are dissected according to the type of acyl chain esterified to the first hydroxyl group of the ceramide. 1-O-Acylceramides containing saturated 1-O-acyl chains from C14 to C26 and parent ceramide backbones with either a C17-sphingosine (d17:1) or a C18-sphingosine (d18:1) combined with the N-linked acyl chains hC16:0, C16:0, C18:0, C20:0, C22:0, or C24:0 were quantified by UPLC-MS/MS. 1-O-acylceramide levels were compared between control (**c**) and the different knockout mice (**k.o.**) at birth (**P0**) and 4 days postnatal (**P4**). The full list of compounds is listed in supplemental table 2. $LPLA_2$: group XV lysosomal phospholipase $A_2$, GlcCer-S: glucosylceramide synthase, nGlcCerase: neutral glucosylceramidase; n = 3 for $LPLA_2$ (P0 and P4), and GlcCer-S control and knockout (k.o.) groups, n=6 for nGlcCerase controls and n=4 for nGlcCerase-knockout.

## Suppl. Fig. 5: Comparison of alkaline sensitive ceramide (alk. sensitive Cer) levels with the amount of 1-O-acylceramides (1-O-AcylCer) in both, control (contr.) and GlcCer-S deficient (k.o.) epidermis at postnatal day 4.

Ceramides containing C18-sphingosine had been quantified before from epidermal lipid extracts by precursor ion (m/z +264) scanning before and after mild alkaline treatment (saponification)(2). Previously however, only the data obtained after mild alkaline treatment had been published (2). Data obtained before saponification correspond to free ceramide levels (free Cer). Subtracting the amount of free ceramides from the quantities of total ceramides obtained after saponification yields the amount of alkaline sensitive ceramide derivatives (alk. sensitive Cer). These data are compared with the amount of C18-sphingosine containing 1-O-acylceramides (1-O-AcylCer) determined now from epidermal extracts before saponification. The total amount (∑) refers to the sum of (1-O-acyl)ceramides with a N-acyl chain length of 16 to 24 C-atoms. The total amount of parent ceramides significantly increases after saponification in both, controls and GlcCer-S deficient epidermis. Note, the amount of alkaline sensitive ceramide derivatives roughly equals the amounts of 1-O-acylceramides determined in both, controls as well as mutants; n = 3.

## Suppl. Fig. 6: Levels of free glucosylceramides and ceramides with the structural backbone found in 1-O-acylceramides in Gba2-mice.

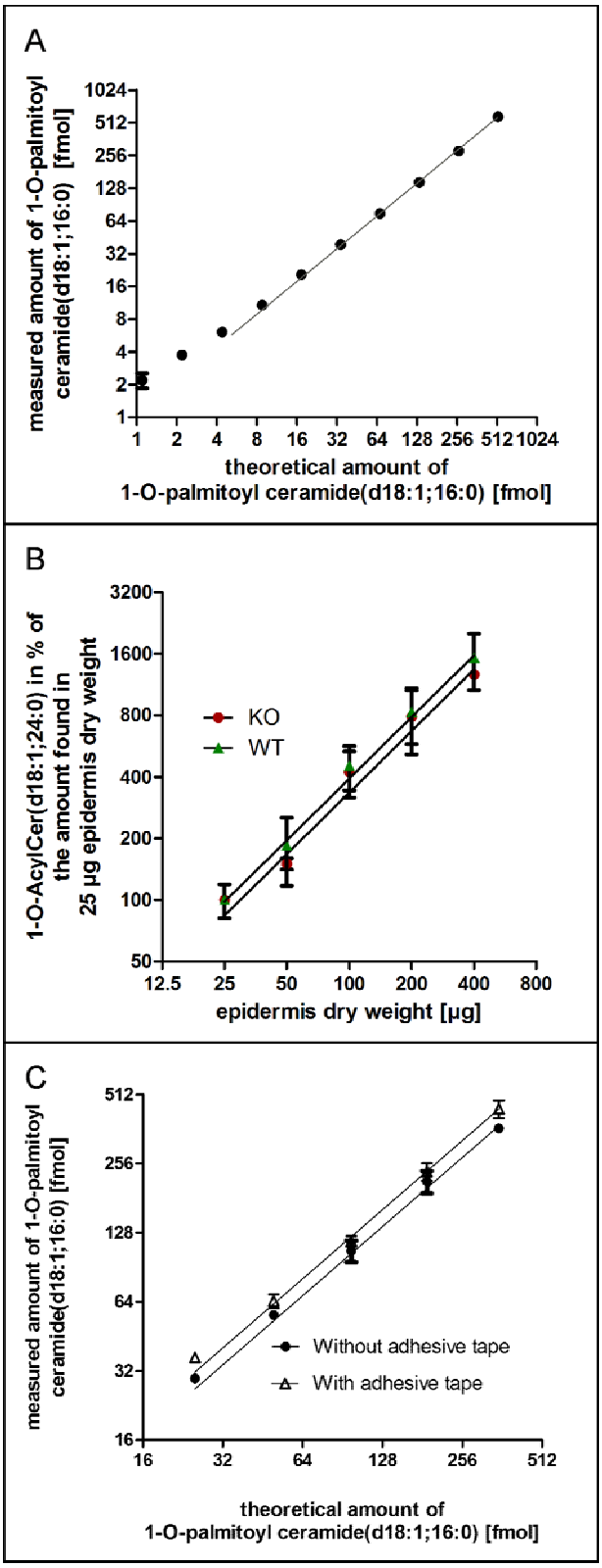
Glucosylceramide and ceramide levels were determined from lipid raw extracts. Note, the sum of the plotted GlcCers increases significantly (2.7fold), whereas the sum of the plotted ceramides does not change. However, long chain ceramides significantly decrease.

## Suppl. Fig. 7: 1-O-acylceramides in the human stratum corneum sorted according to the acyl moiety linked in 1-O-linkage.

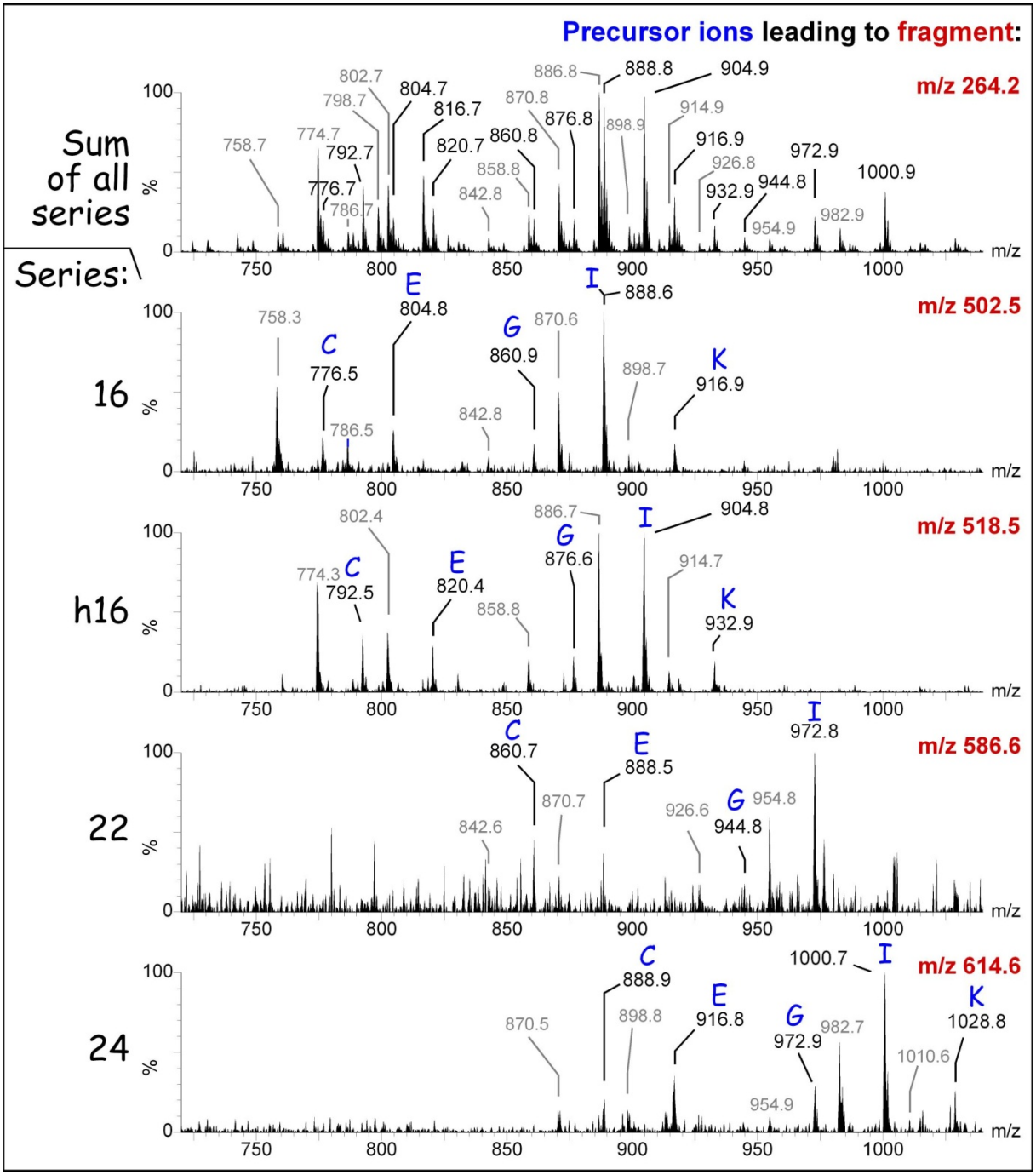
1-O-acylceramides presented by the sum of all parent ceramides (d18:1;(h)14-26:0) found in human stratum corneum. Data of figure 6 were grouped to obtain supplemental figure 4.

# Supplemental figures

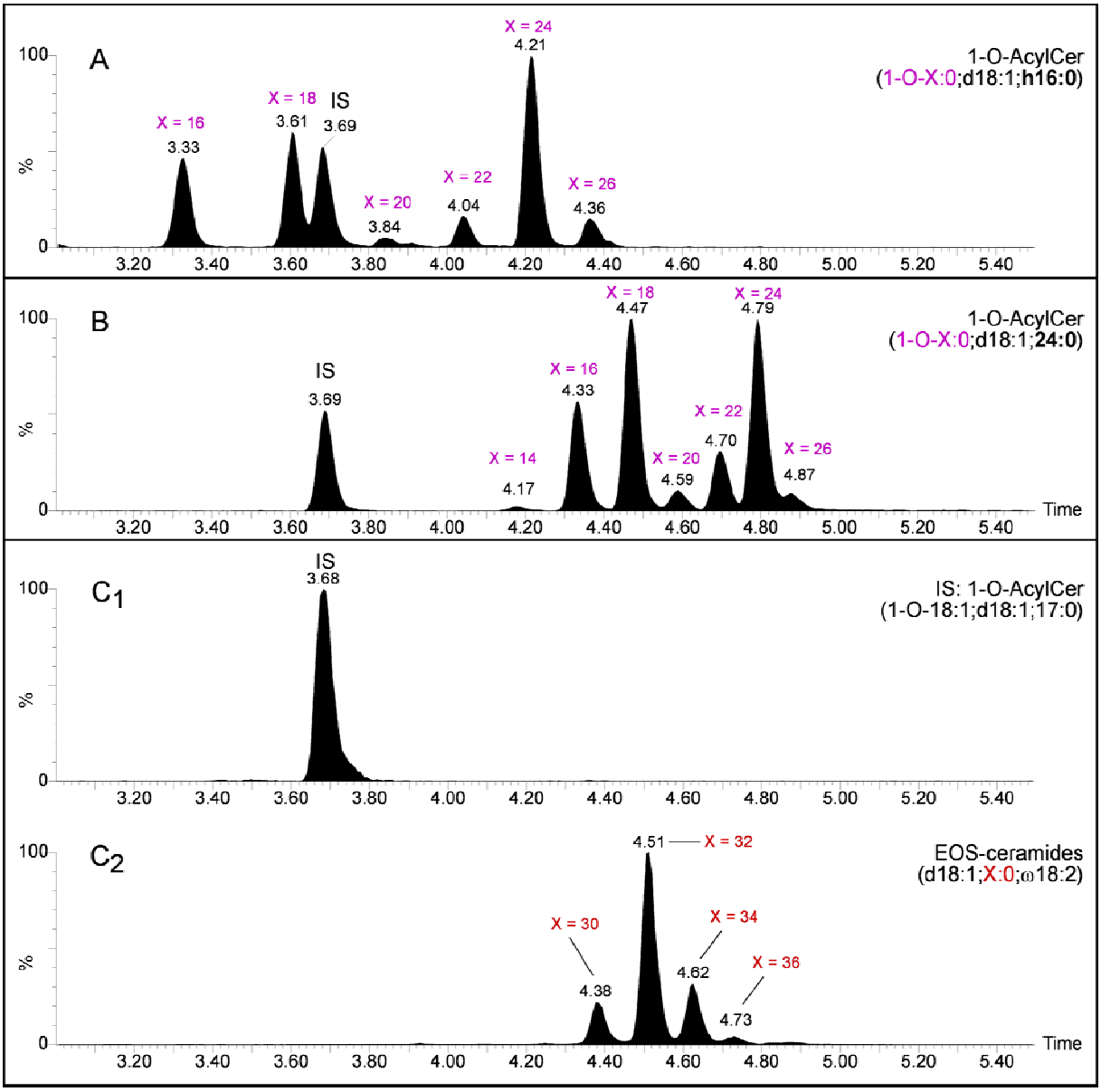
## Supplemental figure 1



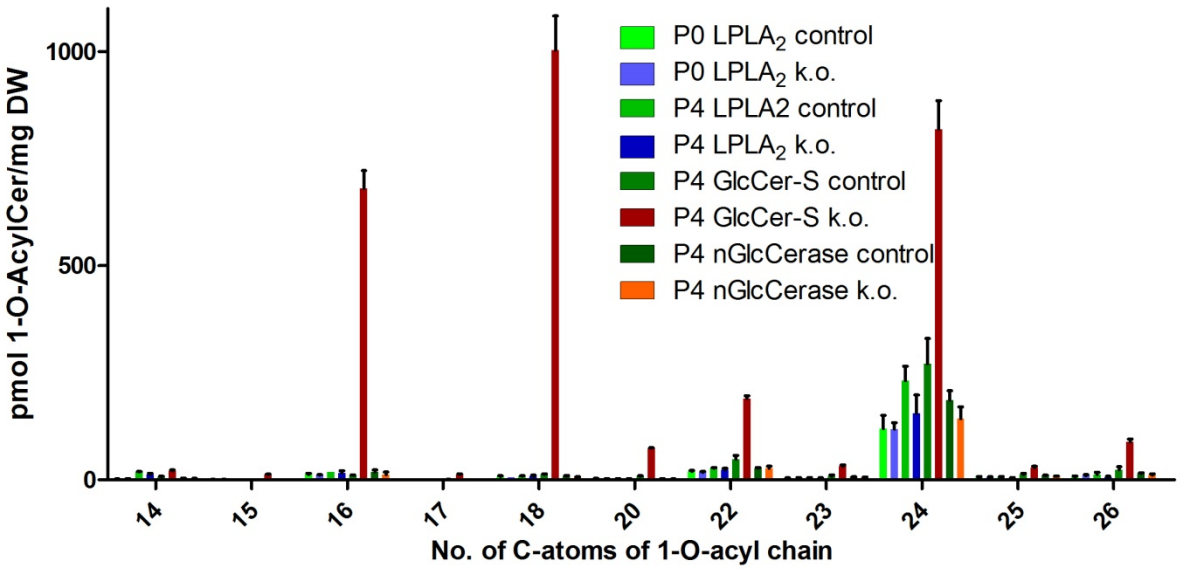
**Supplemental figure 2**



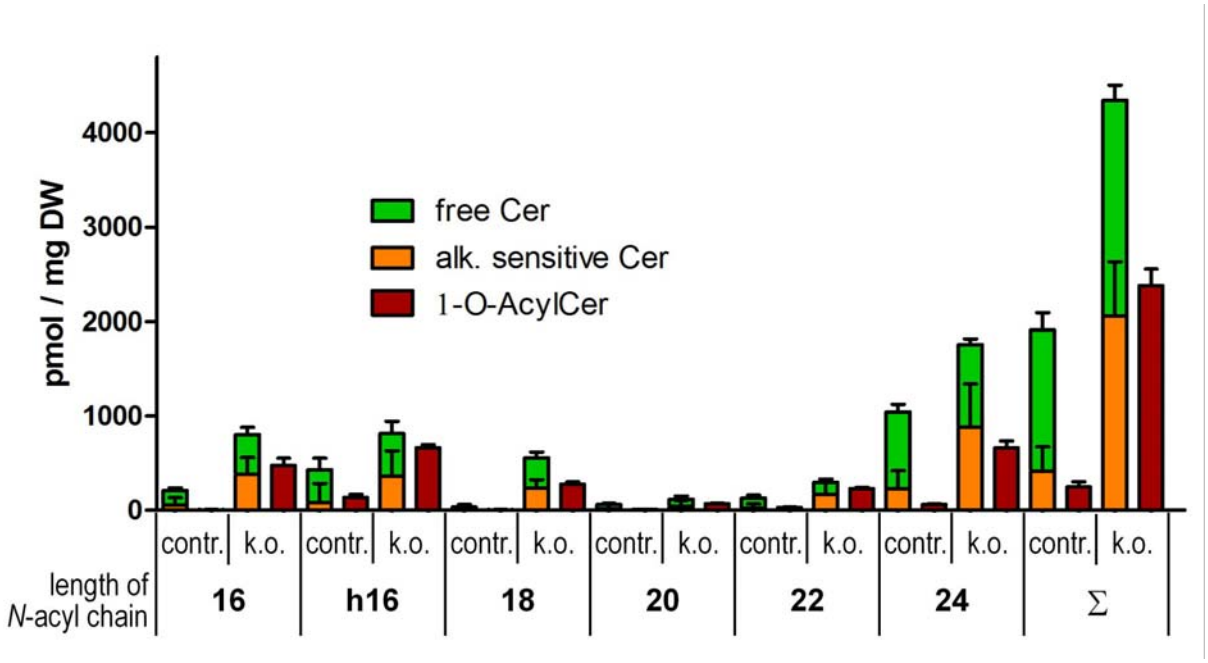
## Supplemental figure 3



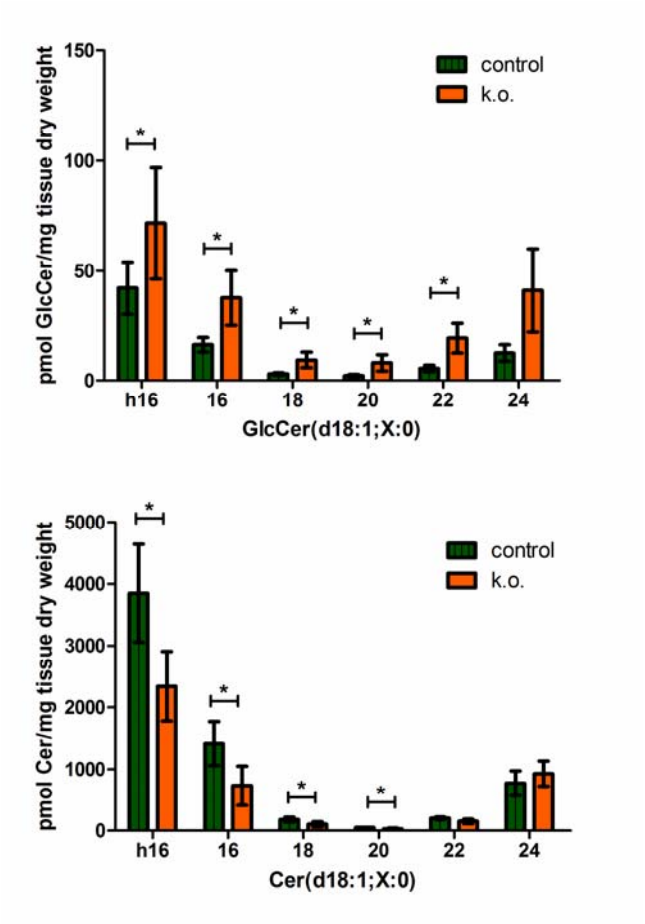
**Supplemental figure 4**



**Supplemental figure 5**



## Supplemental figure 6



**Supplemental figure 7**