Supplemental Data to Quantitative Imaging Mass Spectrometry of Renal Sulfatides: Validation by classical mass spectrometric methods.

Authors

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Supplemental Methods

MALDI imaging mass spectrometry

Frozen mouse kidney was sliced into 10 µm sections using a Leica CM1950 cryostat (Leica Biosystems, Nussloch, Germany) at a chamber temperature of -15 °C and a specimen head temperature of -15 °C. Cryosections were mounted onto indium tin oxide (ITO)-coated conductive glass slides (Bruker Daltonics, Bremen, Germany) and dried for 30 min in vacuum. For washing studies one kidney section of wt- and of (Cst+Ugcg) $^{f/f\ Pax8Cre}$ mice was placed on the left hand side of the ITO-glass slide and the consecutive sections of each kidney were placed on the right hand side of the same ITO-glass slide. Washing was performed for only one side as previously described (1). In brief, on the left hand side adjacent sections from both wt and (Cst+Ugcg) $^{f/f\ Pax8Cre}$ were dipped 3 times for 15 sec in 50 mM ammonium formiate (pH 6.4; 4 °C) and dried afterwards for 15 min under vacuum. Afterwards, 9-AA (3.88 mg/mL in acetonitrile/water (80:20, v/v)) was deposited onto the complete slide (including the non-washed sections) using a SunCollect MALDI Spotter (SunChrom, Friedrichsdorf, Germany). Air pressure was 2.5 bar. The Matrix was deposited in nine layers, with increasing flow rate, i.e. 10 µL/min for the 1st layer, 15 µL/min for the 2nd layer, 20 µL/min for the 3rd layer, and 25 µL/min for all other layers.

Mass spectrometric measurements were performed using an Autoflex Speed MALDI TOF/ TOF instrument (Bruker Daltonics,) equipped with a smartbeam laser (2000 Hz) and controlled by flexControl 3.4 software (Bruker Daltonics). The extraction voltage was 19 kV, and gated matrix suppression (<650 Da) was applied to prevent saturation of the detector with matrix ions. Mass spectra were obtained in negative ion-reflector mode in the *m/z* range from 700 to 1100 Da using delayed extraction and a laser frequency of 1000 Hz. Images were acquired at a spatial resolution of 50 µm with 200 laser shots per position. Spectra were saved and the images constructed using flexImaging 3.0 software (Bruker Daltonics). Mass filters were chosen with a width of 0.2 Da. In all analyses blood–derived lipids where not separately taken into account.

Evaluation of Regions of Interest (ROIs) in MALDI IMS

Representative ROIs from each renal region, papillae, medulla or cortex in each section (unwashed, wt and (Cst+Ugcg) $^{f/f\ Pax8Cre}$ as well as washed wt and (Cst+Ugcg) $^{f/f\ Pax8Cre}$) containing 100-120 measurement points, where assigned in flexImaging 3.0 software (Bruker Daltonics, Bremen, Germany). Average spectra of each ROI where calculated using ClinProTools Version 3.0 (Bruker Daltonics, Bremen, Germany).

Statistics

All statistical calculations (mean, standard deviation and one-way ANOVA post-hocTukey tests) were performed with GraphPad Prism Version 5.04 software.

Supplemental References

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2. Hsu, F. F., and J. Turk. 2004. Studies on sulfatides by quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes that include an unusual internal galactose residue loss and the classical charge-remote fragmentation. *J Am Soc Mass Spectrom* 15: 536-546.

3. Sandhoff, R., S. T. Hepbildikler, R. Jennemann, R. Geyer, V. Gieselmann, R. L. Proia, H. Wiegandt, and H. J. Grone. 2002. Kidney sulfatides in mouse models of inherited glycosphingolipid disorders: determination by nano-electrospray ionization tandem mass spectrometry. *J Biol Chem* 277: 20386-20398.

Supplemental Tables

Supplemental table 1: UPLC-MS^2 transitions and retention times of analyzed NS-SM4s sulfatides

NS-SM4s Species	Precursor (*m/z*)	Fragment (*m/z*)	Retention time (min)
d18:1,14:0 (Standard)	750.5	96.9	0.65
d34:1	778.5	96.9	0.76
d36:1	806.6	96.9	0.83
d18:1,19:0 (Standard)	820.6	96.9	0.94
d38:1	834.6	96.9	1.02
d40:1	862.6	96.9	1.21
d41:1	876.6	96.9	1.31
d42:1	890.6	96.9	1.43
d43:1	904.7	96.9	1.59
d44:1	918.7	96.9	1.76
d18:1,27:0 (Standard)	932.7	96.9	1.96
d42:2	888.6	96.9	1.21

Supplemental table 2: UPLC-MS^2 transitions and retention times of analyzed AS-SM4s sulfatides

AS-SM4s Species	Precursor (*m/z*)	Fragment (*m/z*)	Retention time (min)
d34:1	794.5	96.9	0.73
d36:1	822.6	96.9	0.80
d38:1	850.6	96.9	0.99
d40:1	878.6	96.9	1.17
d41:1	892.6	96.9	1.27
d42:1	906.6	96.9	1.39
d43:1	920.7	96.9	1.55
d44:1	934.7	96.9	1.69
d42:2	904.6	96.9	1.19

Supplemental table 3: UPLC-MS^2 transitions and retention times of analyzed NS-SM3 sulfatides

NS-SM3 Species	Precursor (*m/z*)	Fragment (*m/z*)	Retention time (min)
d18:1,14:0 (Standard)	912.7	96.9	0.68
d34:1	940.8	96.9	n.d.
d36:1	968.8	96.9	0.89
d18:1,19:0 (Standard)	982.8	96.9	0.97
d38:1	996.8	96.9	1.06
d40:1	1024.8	96.9	1.28
d41:1	1038.8	96.9	1.38
d42:1	1052.8	96.9	1.52
d43:1	1066.8	96.9	1.71
d44:1	1080.8	96.9	1.86
d18:1,27:0 (Standard)	1094.8	96.9	2.08
d42:2	1050.8	96.9	1.27

Supplemental table 4: UPLC-MS^2 transitions and retention times of analyzed NP-SM3 sulfatides

NP-SM3 Species	Precursor (*m/z*)	Fragment (*m/z*)	Retention time (min)
t34:0	958.8	96.9	n.d.
t36:0	986.8	96.9	n.d.
t38:0	1014.8	96.9	0.93
t40:0	1042.8	96.9	1.11
t41:0	1056.8	96.9	1.23
t42:0	1070.8	96.9	1.36
t43:0	1084.8	96.9	n.d.
t44:0	1098.8	96.9	n.d.
t42:1	1068.8	96.9	1.11

Supplemental table 5: Normalized values for correlation of the three mass spectrometric methods.

	MALDI IMS			**MALDI on target**			**UPLC-ESI-MS^2**		
	Papillae	Medulla	Cortex	Papillae	Medulla	Cortex	Papillae	Medulla	Cortex
NS-SM4s 34	5.76	2.99	0.72	4.31	3.68	0.26	3.29	3.84	0.46
NS-SM4s 36	0.89	0.30	0.44	1.22	1.37	0.00	1.42	0.89	0.30
NS-SM4s 38	6.21	2.30	0.72	5.07	5.04	0.25	5.26	5.41	0.88
NS-SM4s 40	18.80	6.59	1.16	15.72	13.08	0.66	12.22	9.66	0.46
NS-SM4s 42	40.04	6.16	0.91	40.78	17.52	1.43	31.68	10.27	0.34
NS-SM4s 44	28.30	0.57	0.31	32.89	5.82	1.55	46.13	6.08	0.05
summe	100*	18.91	4.26	100*	46.51	4.15	100*	36.15	2.49
AS-SM4s 34	0.60	0.33	0.59	0.29	0.41	0.09	0.30	0.32	0.02
AS-SM4s 36	0.26	0.37	0.22	0.02	0.20	0.00	0.02	0.82	0.02
AS-SM4s 38	2.83	15.17	1.70	3.16	12.12	0.99	1.18	20.17	1.22
AS-SM4s 40	12.34	53.80	7.53	8.80	52.53	5.42	5.30	50.50	3.10
AS-SM4s 42	6.33	30.33	3.63	3.69	34.75	3.10	3.07	27.58	1.58
AS-SM4s 44	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.61	0.00
summe	22.36	100*	13.67	15.96	100*	9.6	10.12	100*	5.94
NS-SM3 34	0.00	0.00	0.00	0.72	0.00	0.00	0.04	0.01	0.10
NS-SM3 36	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.03	0.06
NS-SM3 38	2.09	0.25	1.44	5.76	0.00	0.00	2.34	0.66	0.24
NS-SM3 40	14.30	0.61	6.19	14.33	2.66	4.23	11.58	3.02	1.30
NS-SM3 42	46.55	1.29	4.26	37.55	5.58	4.17	41.25	7.65	1.13
NS-SM3 44	37.06	1.00	1.06	41.64	5.72	3.25	44.56	8.70	0.03
summe	100*	3.15	12.95	100*	13.96	11.65	100*	20.07	2.86

	MALDI IMS			**MALDI on target**			**UPLC-ESI-MS^2**		
	Papillae	Medulla	Cortex	Papillae	Medulla	Cortex	Papillae	Medulla	Cortex
NP-SM3 34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NP-SM3 36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NP-SM3 38	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	2.46
NP-SM3 40	1.21	1.19	48.20	0.00	0.00	49.39	1.33	0.65	48.79
NP-SM3 42	1.40	1.11	51.80	0.00	0.00	50.61	1.34	0.28	48.70
NP-SM3 44	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.05
summe	2.61	2.3	100*	0	0	100*	3.23	0.93	100*

*: For each mass spectrometric method, the sum of intensities of the most abundant sulfatide subgroup within a renal region was set to 100 %, which were NS-sulfatides in papillae, AS-sulfatides in medulla, and NP-sulfatides in cortex.

Supplemental Figure Legends

Supplemental figure 1: Dependence of sulfatide retention times on type of head group and type and size of ceramide anchor on C18-column.

Regression with a third grade polynom leads to regression coefficienst in the range of $0.9984 \leq R^2 \leq 1$: NS SM4s, $y = 0.0003x^3 – 0.123x^2 + 0.2165x – 0.792$, $R^2 = 0.9999$; NS-SM3, $y = 0.0002x^3 - 0.0082x^2 + 0.133x - 0.2277$, $R^2 = 0.9994$; AS-SM4s, $y =-0.00003x^3 + 0.0078x^2 - 0.1883x + 1.8745$, $R^2 = 0.9984$; NP-SM3, $y= -0.0013x^3 + 0.913x^2- 2.0875x + 16.18$, $R^2 = 1$.

Supplemental figure 2: Major sulfatide SM4s fragments produced by collision induced dissociation using an ESI-triple quadrupole-tandem mass spectrometer.

The major fragments depicted correspond to those described earlier (2). The intensity of all these fragments together with the residual molecular ion peak was normalized to 100%. Sulfatide SM4s(d18:1;h24:1)(AS-SM4s) produces with a significant higher probability fragment ions in the range of $507 \leq m/z \leq 568$ than the corresponding sulfatide SM4s(d18:1;24:1) (NS-SM4s). Because all standards used were NS sulfatides, we corrected all AS-SM4s data obtained by ESI-(QqQ)-MS/MS analysis through the transition to HSO_4^- (*m/z* 97) with a factor of 1.114. (n=5; ***p<0.001).

Supplemental figure 3: Residual sulfatides SM4s in kidney of mice deficient in renal tubular epithelium sulfatides as determined by UPLC-ESI-MS^2.

Residual available extracts had been normalized to tissue dry weight (d.w.). Assuming 75% water content in kidneys, these values would correspond in total to about 35 pmol of sulfatides per mg tissue wet weight, which would correspond to less than 10% of wild type content (3). (n =3)

Supplemental figure 4: MALDI-IMS of renal sulfatides from a wild type, a renal tubular cell-specific CST-deficient and a systemic CST-deficient mouse.

Note the complete loss of AS-SM4s in the medulla (central region) of the systemic Cst-deficient kidney in contrast to residual AS-SM4s-signals in the kidney with the ephitilia cell-specific Cst-deficiency only.

Supplemental figure 5: Logarithmic correlation plot of MALDI IMS and UPLC-ESI-MS^2.

Plotted are the sulfatide species NS-SM4s (green; $R^2 = 0.806$), AS-SM4s (red; $R^2 = 0.967$), NS-SM3 (yellow; $R^2 = 0.927$) and NP-SM3 (blue; $R^2 = 0.997$). All dots are annotated with the renal region (P = papillae, M = medulla, C = cortex) and their amount of carbon atoms in the ceramide anchor. For visualization purposes, compounds with zero intensity in one method were plotted on the corresponding axis. (n = 3 for each, MALDI IMS and UPLC-ESI MS^2).

Supplemental figure 6: Logarithmic correlation plot of MALDI IMS and MALDI on target.

Plotted are the sulfatide species NS-SM4s (green; $R^2 = 0.913$), AS-SM4s (red; $R^2 = 0.985$), NS-SM3 (yellow; $R^2 = 0.948$) and NP-SM3 (blue; $R^2 = 0.998$). All dots are annotated with the renal region (P = papillae, M = medulla, C = cortex) and their amount of carbon atoms in the ceramide anchor. For visualization purposes, compounds with zero intensity in one method were plotted on the corresponding axis (n = 3 for each, MALDI IMS and MALDI on target).

Supplemental figure 7: Logarithmic correlation plot of UPLC-ESI-MS^2 and MALDI on target.

Plotted are the sulfatide species NS-SM4s (green; $R^2 = 0.870$), AS-SM4s (red; $R^2 = 0.960$), NS-SM3 (yellow; $R^2 = 0.979$) and NP-SM3 (blue; $R^2 = 0.998$). All dots are annotated with the renal region (P = papillae, M = medulla, C = cortex) and their amount of carbon atoms in the ceramide anchor. For visualization purposes, compounds with zero intensity in one method were plotted on the corresponding axis (n = 3 for each, UPLC-ESI MS^2 and MALDI on target).

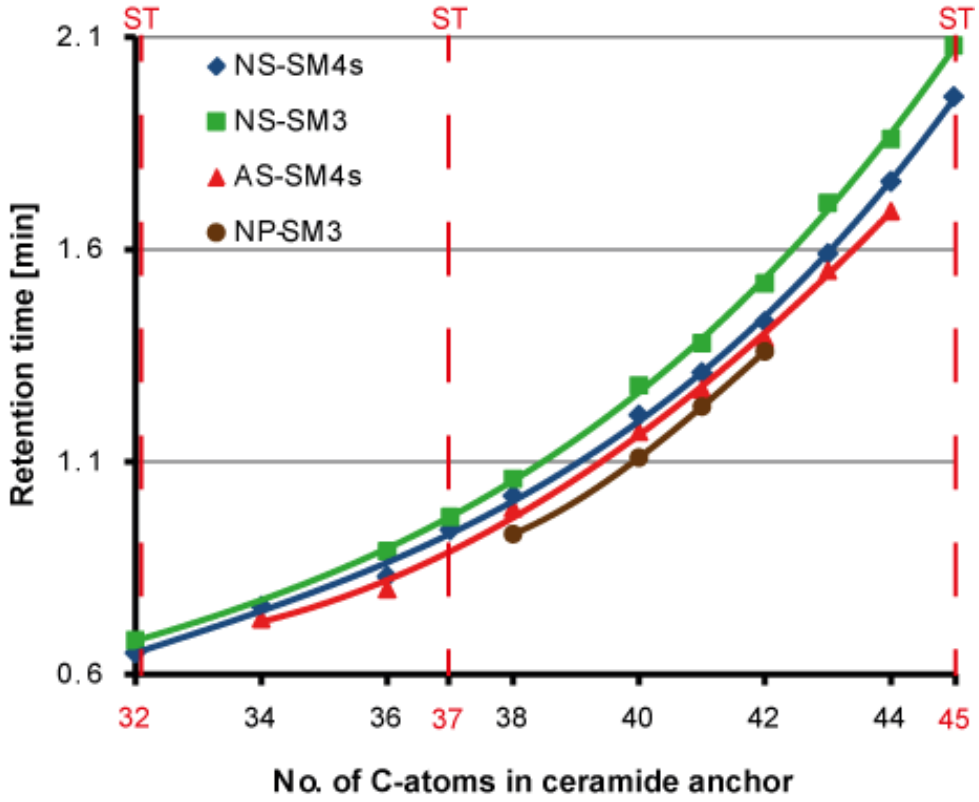
Supplemental figure 8: MALDI IMS signal of Phosphatidylinositol(38:4) with and without prior washing of kidneys sections from wild type mice and mice with a renal tubular cell-specific Cst-deficiency.

Values obtained for PI(38:4) in papillae and medulla were normalized to corresponding values obtained in the cortical region, in which, due to low sulfatide levels, the lowest total ion intensities were obtained. Therefore we assumed the lowest ion suppression effects in cortex. Normalized values of phosphatidylinositol, PI(38:4), obtained after washing are in light purple and those without prior washing are in dark purple color. In analogy to the main figure 5D, bars for wild type values are

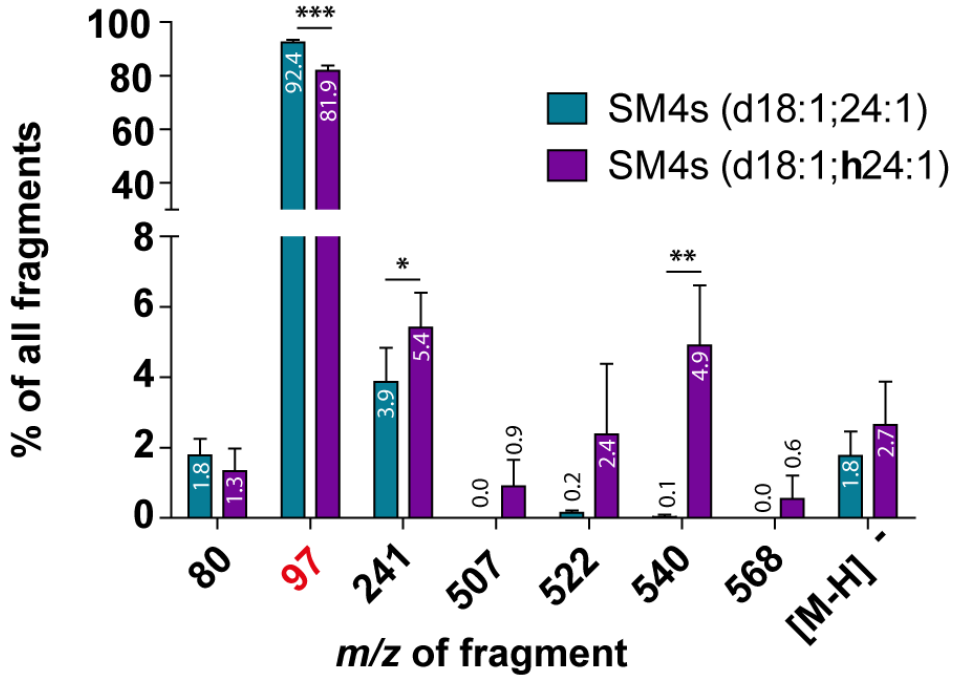
without and bars for mutant values are with black diagonal stripes. Note, as compared to wild type, the higher relative PI intensities in medulla and papillae of mutant kidneys remained after washing salts and other water soluble compounds away, i.e. after eliminating suppression effects caused by these solutes. Three independent experiments were performed (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Supplemental Figures

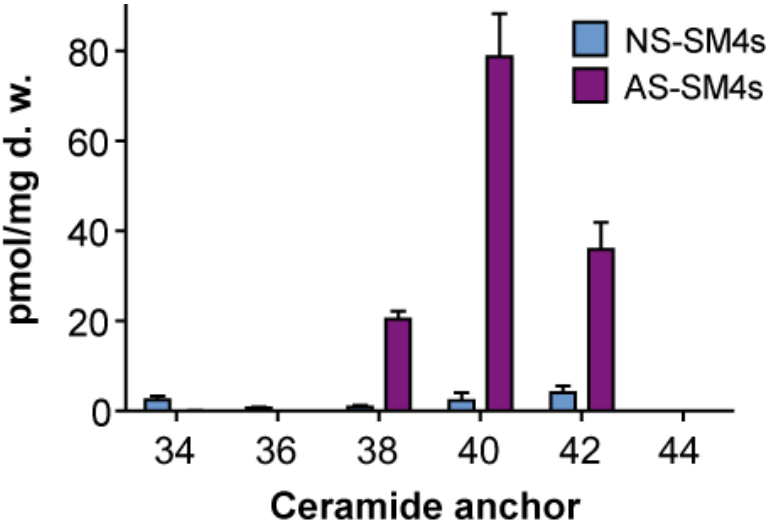
Supplemental Figure 1



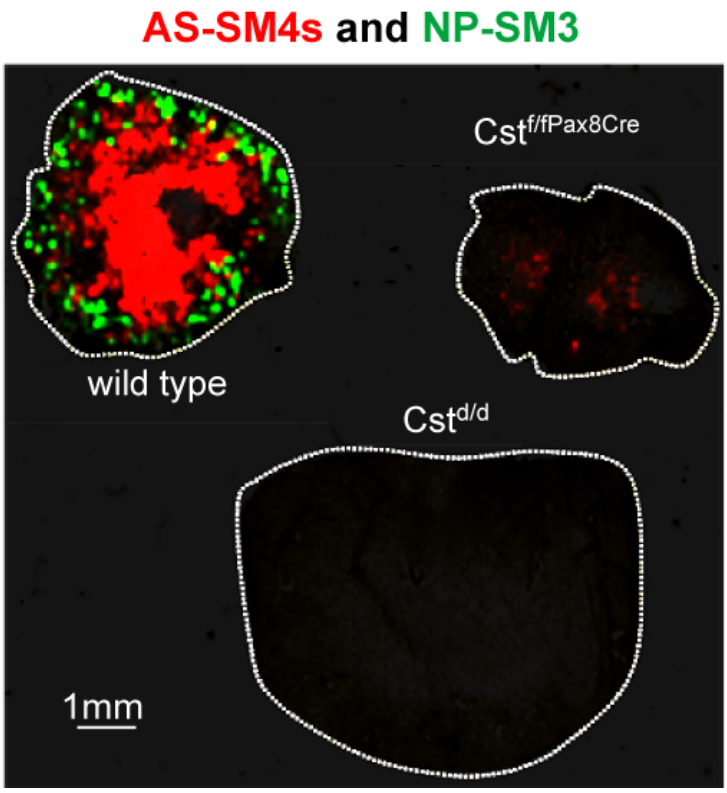
Supplemental Figure 2



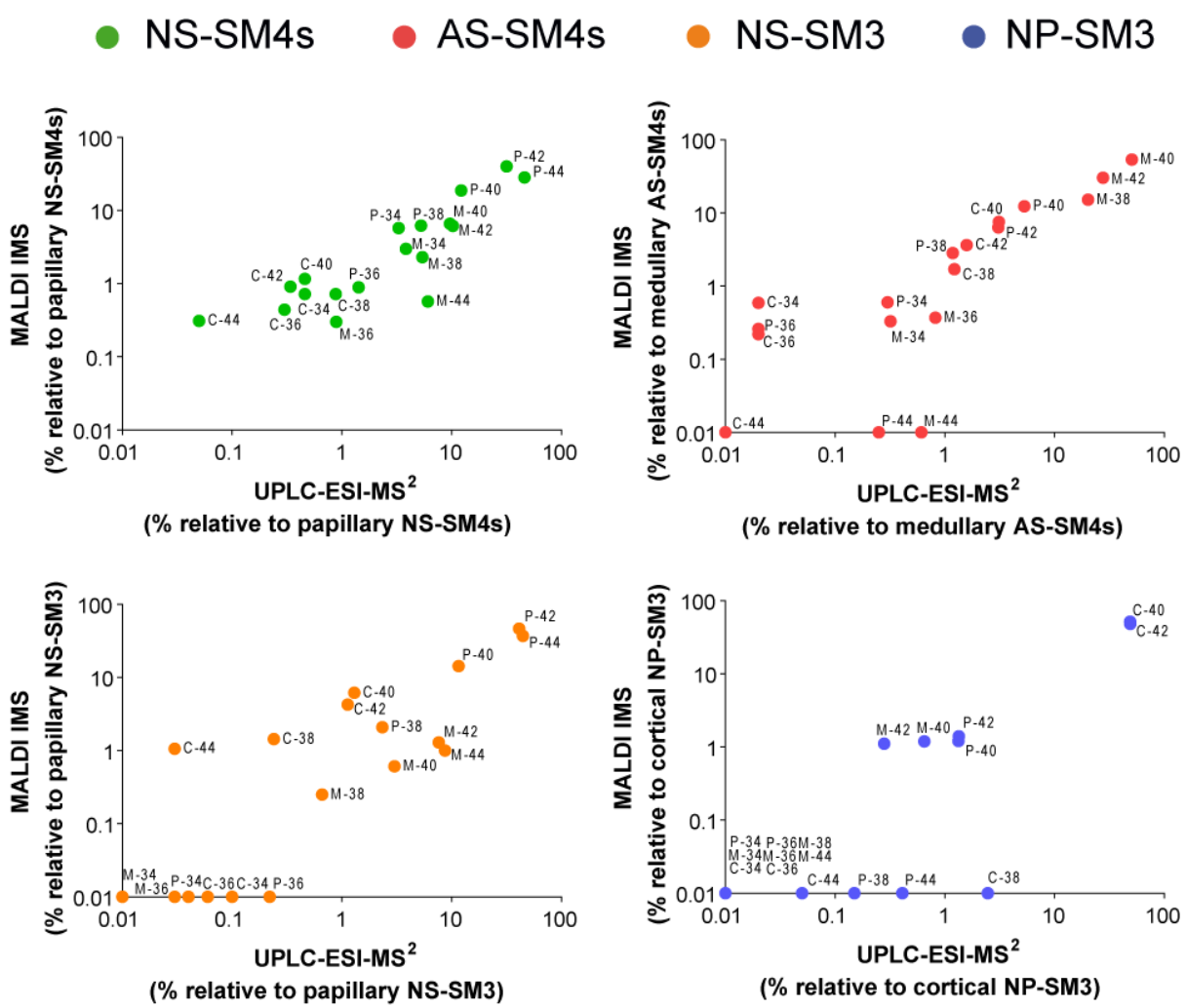
Supplemental Figure 3



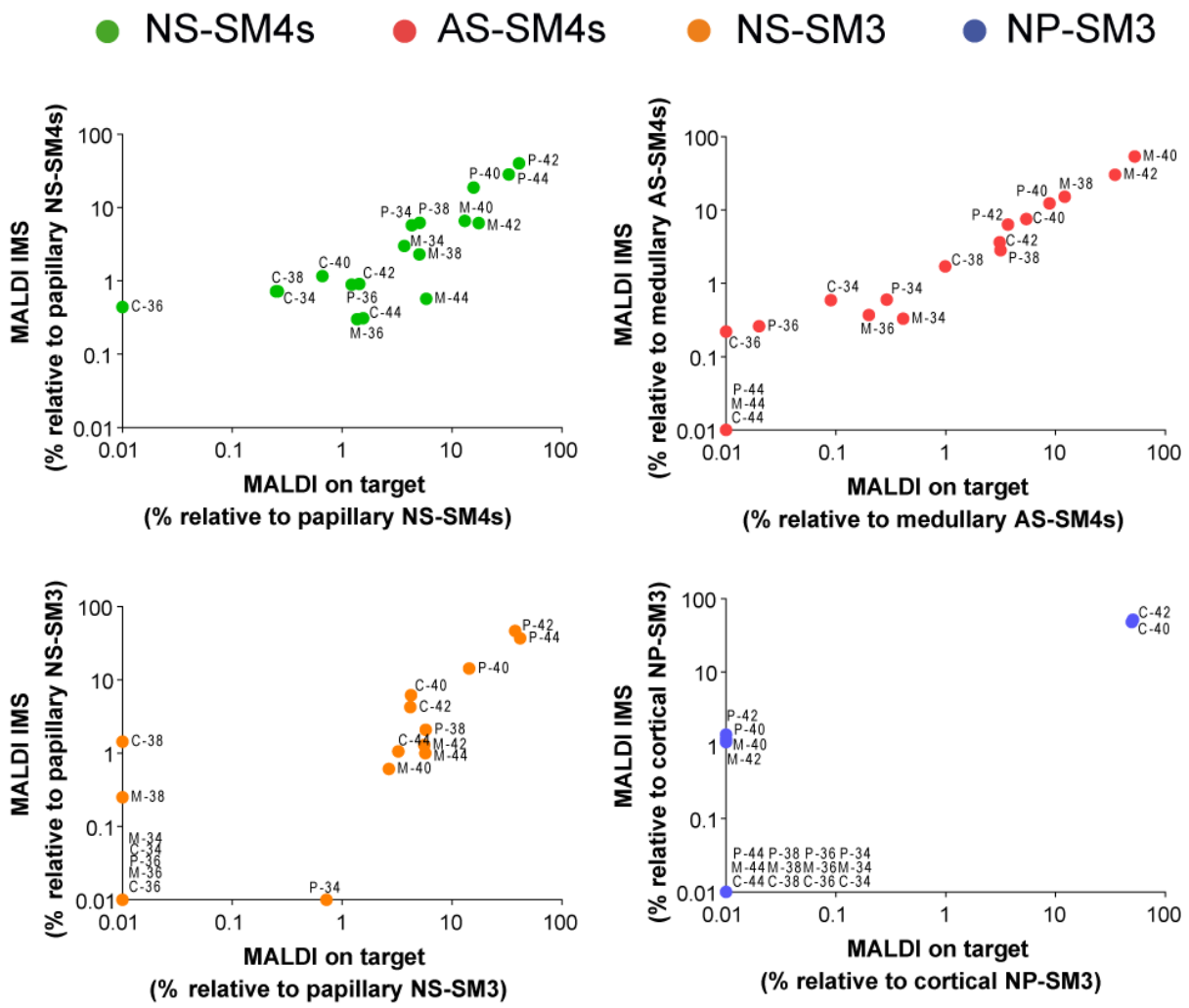
Supplemental Figure 4



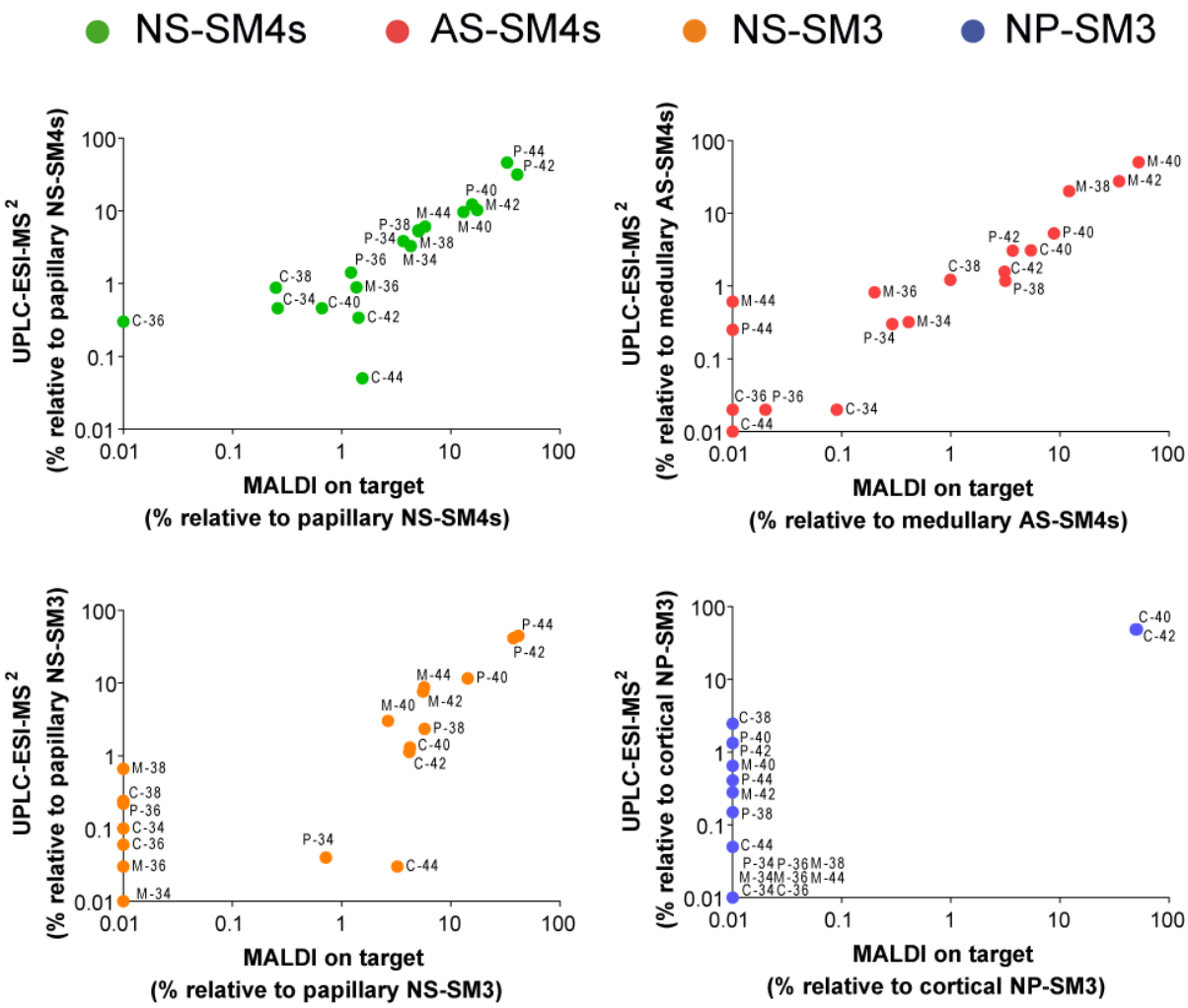
Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8