スフィンゴ脂質のトランスレーショナルバイオマーカーとしての活用

Utilization of sphingolipids as translational biomarkers

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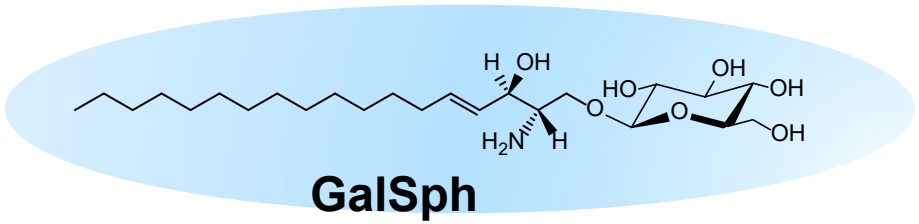
2023.03.02 JBF

Better Health, Brighter Future

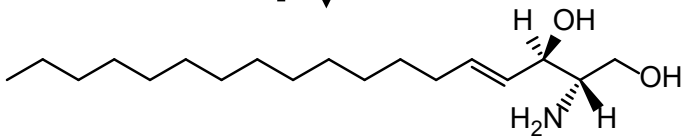
Physiological and pathological roles of sphingolipids



GalSph

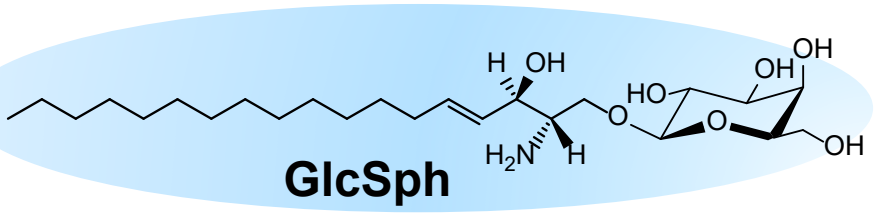
Galactosyl-sphingosine transferase

GalCase **Krabbe disease**



Sph

GCase **Gaucher disease**



GlcSph

- Galactocerebrosidase (GalCase) hydrolyses galactose residues from various substrates including GalCer and GalSph.

- Glucocerebrosidase (GCase) is involved in lysosomal degradation of GlcCer and GlcSph to Cer and Sph, respectively.

Krabbe disease

GalSph is significantly accumulated in brain. GalSph is a cytotoxic lipid, capable of inducing cell death. KD patients with infantile type usually die at an average age of 13 months. Individuals with juvenile type survive longer. Adult KD patients usually die in 2-7 years after diagnosis.

To date, at least 147 mutations in *GALC* have been documented, with 80 of them considered to be “severe” due to their impact on GalCase activity (Mol Genet Metab., 111, 2014).

Pathogenic mutations in *GALC* are associated with a dramatic increase in levels of GalSph within the CNS and peripheral nervous system (PNS). GalSph levels are a better biomarker for clinical diagnosis compared with GalCase activity (Front Med., 8, 2021).

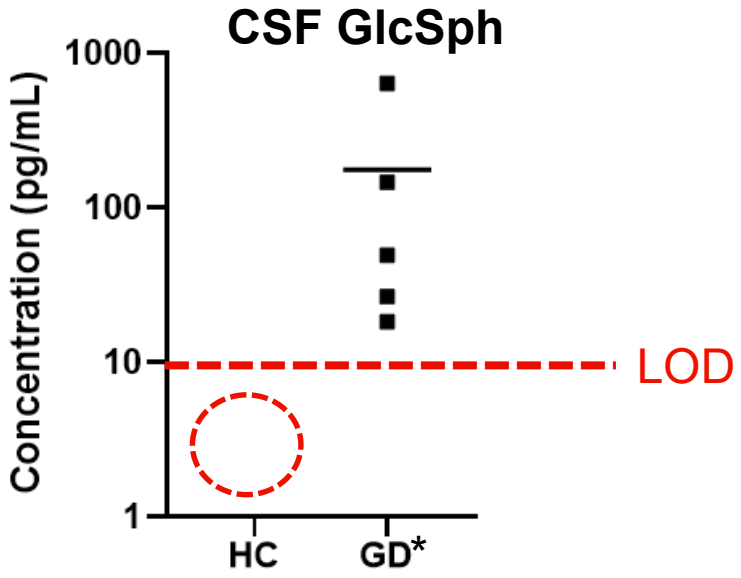
GalSph concentrations in dried blood spots (DBS) are well established. However, GalSph concentrations in the cerebrospinal fluid (CSF) of normal healthy humans have not been fully assessed yet.

Gaucher disease

Gaucher disease (GD): Accumulation of GlcSph, a cytotoxic lipid, in some cell types including neurons is associated with severity of disease (Int J Mol Sci., 21, 2020; bioRxiv, 2022).

GlcSph was detected in human CSF for GD patients (39 pg/mL), while LLOQ (5 pg/mL) in GBA-PD patient and healthy control (Brain, 146, 2023).

GlcSph was detected in human CSF for GD patients as below, while <LOD (10 pg/mL) in healthy control (Ann Clin Transl Neurol., 3, 2016).



Objective

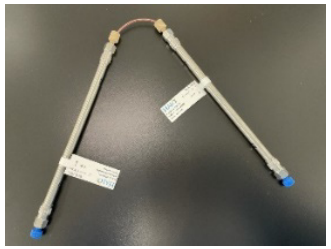
To have all samples quantifiable!
→quantify effect of medicine.
→quantitatively separate health and disease.

*The GlcSph concentrations in GD CSF were referred from Ann Clin Transl Neurol., 2016.

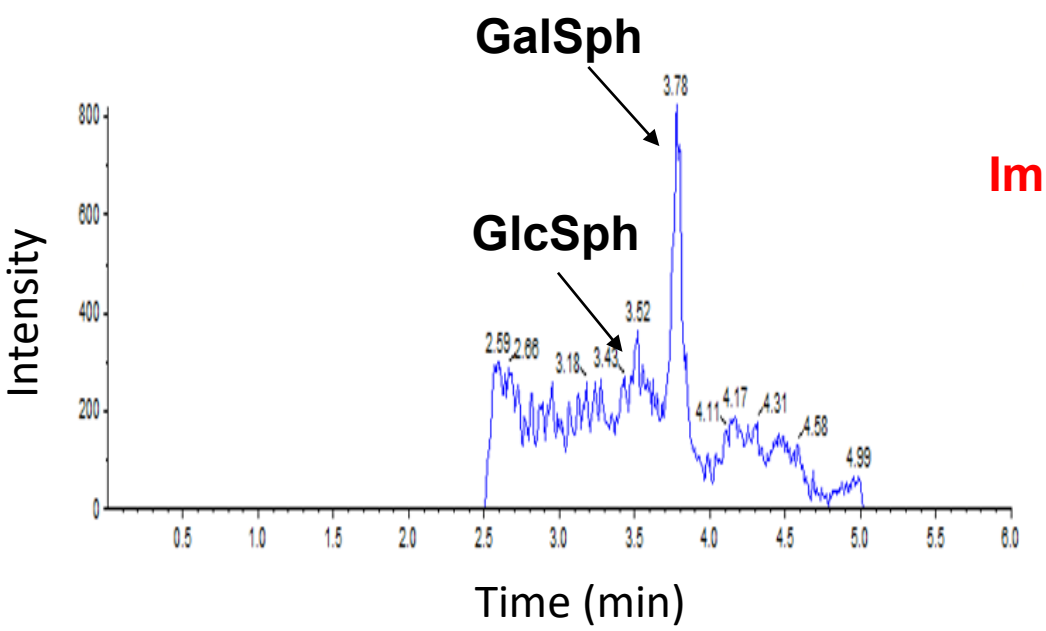
Improvement of sensitivity

-PP
-Gradient
-HILIC column
-API5000

-SPE
-Isocratic
-Double HILIC column
-Mobile phase
-QT6500

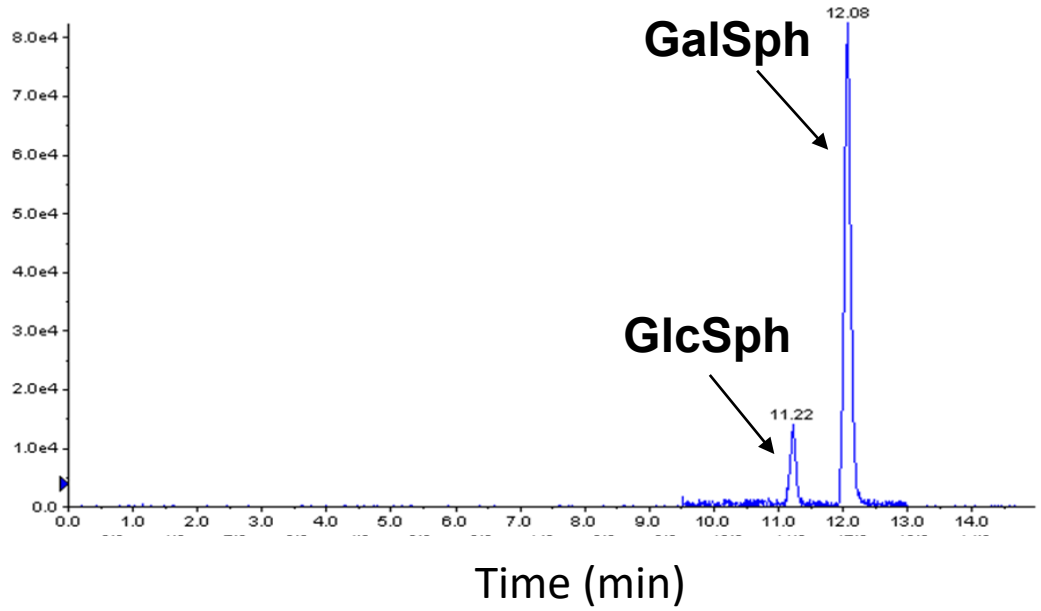


Human CSF



Improved sensitivity
>50~100 fold

Human CSF



Matsumoto SI, et al., J Pharm Biomed Anal. 217 2022

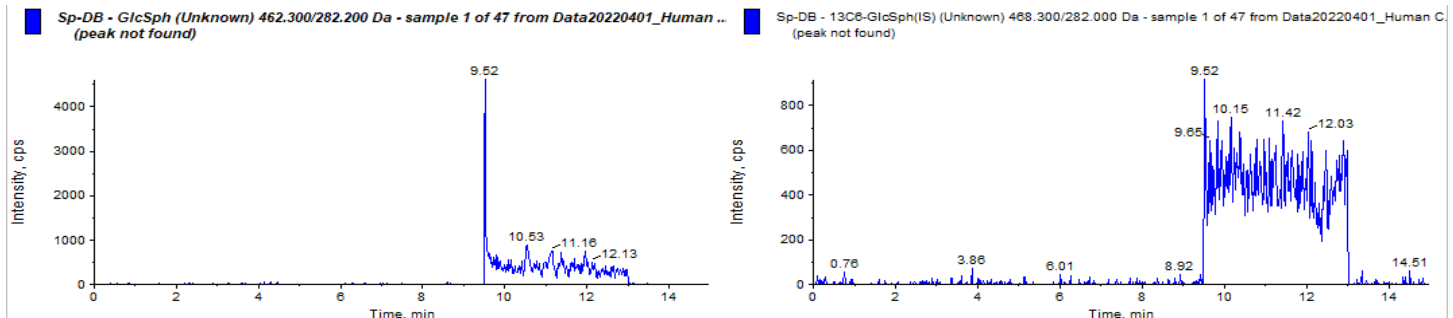
Chromatograms of GlcSph and IS in Surrogate matrix

Surrogate matrix; 0.1% BSA

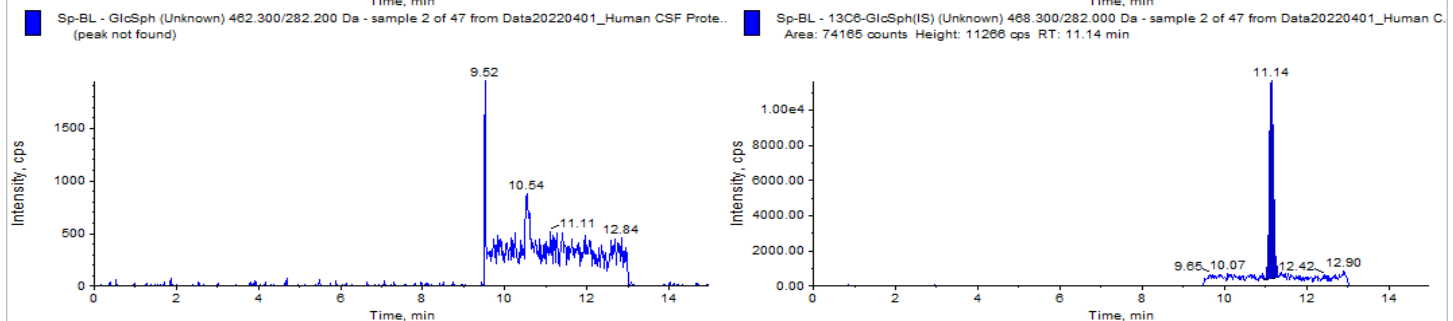
Glc-Sph

IS (Glc-Sph-$^{13}C_6$)

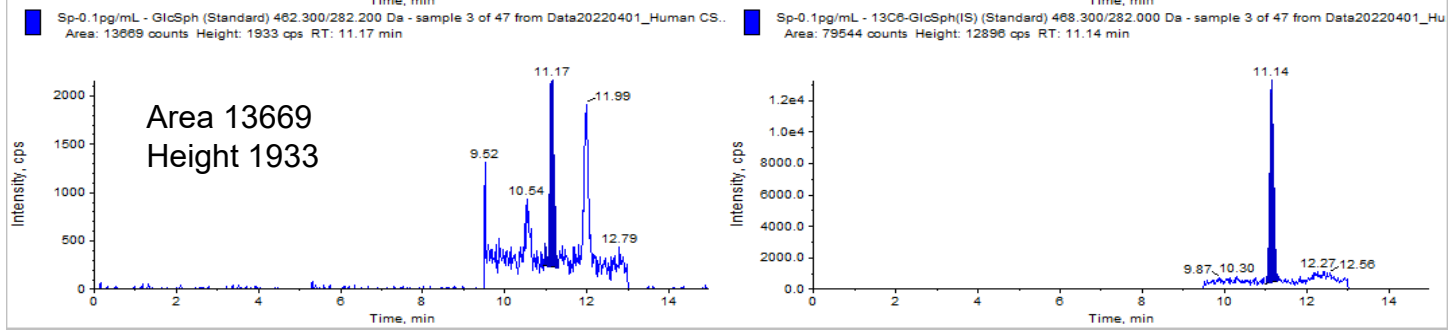
Double Blank



Blank

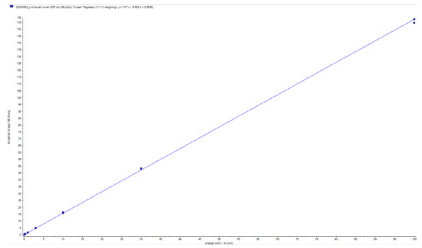


0.1pg/mL



Standard curve

	Accuracy (%)	
Std(pg/mL)	1	2
0.1	97.3	91.7
0.3	98.7	100.9
1	99.3	99.7
3	103.7	102.9
10	100.3	103.2
30	102.3	101.8
100	98.4	99.8



QC (spiked in surrogate)

	Accuracy (%)				
	1	2	3	Ave.	SD
QC_L	75.4	87.1	76.8	79.8	6.4
QC_M	77.4	81.8	86.7	82.0	4.7
QC_H	82.7	82.0	80.7	81.8	1.0

QC (spiked in human CSF)

	Accuracy (%)				
	1	2	3	Ave.	SD
QC_L	90.1	91.4	86.8	89.4	2.4

Tends to show low accuracy in surrogate matrix.

LLQC,LQC,MQC,HQC=0.3, 1, 10, 80 pg/mL

Intra- and inter-day assay precision and accuracy for GlcSph and GalSph in CSF.

Surrogate matrix; 0.1% BSA with 3% heparin

Analyte	Matrix		Added concentration (pg/mL)	Intra-day (n=5)						Inter-day (n=15)	
				Batch-1		Batch-2		Batch-3			
				CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)	CV (%)	RE (%)
GlcSph	Surrogate	LLQC	0.1	5.7	7.2	10.1	-5.5	9.3	6.3	9.8	2.6
		L1QC	0.3	8.1	4.3	9.7	-1.8	5.4	3.3	7.8	1.9
		L2QC	1	4.8	12.4	6.0	10.0	6.0	-3.1	8.4	6.4
	CSF	L2QC	1	3.6	7.3	4.7	-1.8	5.5	2.3	5.7	2.6
		MQC	10	7.4	4.4	5.1	-1.2	3.6	2.3	5.7	1.8
		HQC	240	4.4	-2.3	2.3	-10.8	4.9	-10.6	5.8	-7.9
GalSph	Surrogate	LLQC	0.1	9.4	-1.1	5.7	1.9	8.1	-4.1	7.7	-1.1
		L1QC	0.3	8.4	0.9	7.4	4.1	11.0	1.9	8.4	2.3
		L2QC	1	6.9	11.0	4.1	3.1	5.8	-3.7	8.0	3.5
	CSF	L2QC	1	7.0	2.2	0.9	-4.2	12.7	4.0	8.8	0.6
		MQC	10	2.8	4.2	7.5	-2.5	3.7	11.9	7.4	4.5
		HQC	240	2.2	2.2	2.6	-10.9	2.2	-10.1	7.0	-6.3

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QC precision and accuracy in all assays met the acceptance criteria (CV, RE <20%).

Stability of GlcSph and GalSph in matrices under various conditions

	Temperature	Period		Analyte conc.	CSF % remaining	Plasma % remaining	Analyte conc.	Brain % remaining
GlcSph	On ice	4 h		L2QC	112	87.6	MQC	110
	10℃ (Autosampler)			L2QC	100 (54 h)	101 (136 h)	MQC	104 (136 h)
	-65℃	1 month		L2QC	107	88.4	MQC	104
	Freeze/thaw	3 cycles		L2QC	102	85.3	MQC	101
GalSph	On ice	4 h		L2QC	110	80.2	MQC	107
	10℃ (Autosampler)			L2QC	99.5 (54 h)	102 (136 h)	MQC	93.0 (136 h)
	-65℃	1 month		L2QC	103	85.0	MQC	107
	Freeze/thaw	3 cycles		L2QC	96.9	85.9	MQC	97.0

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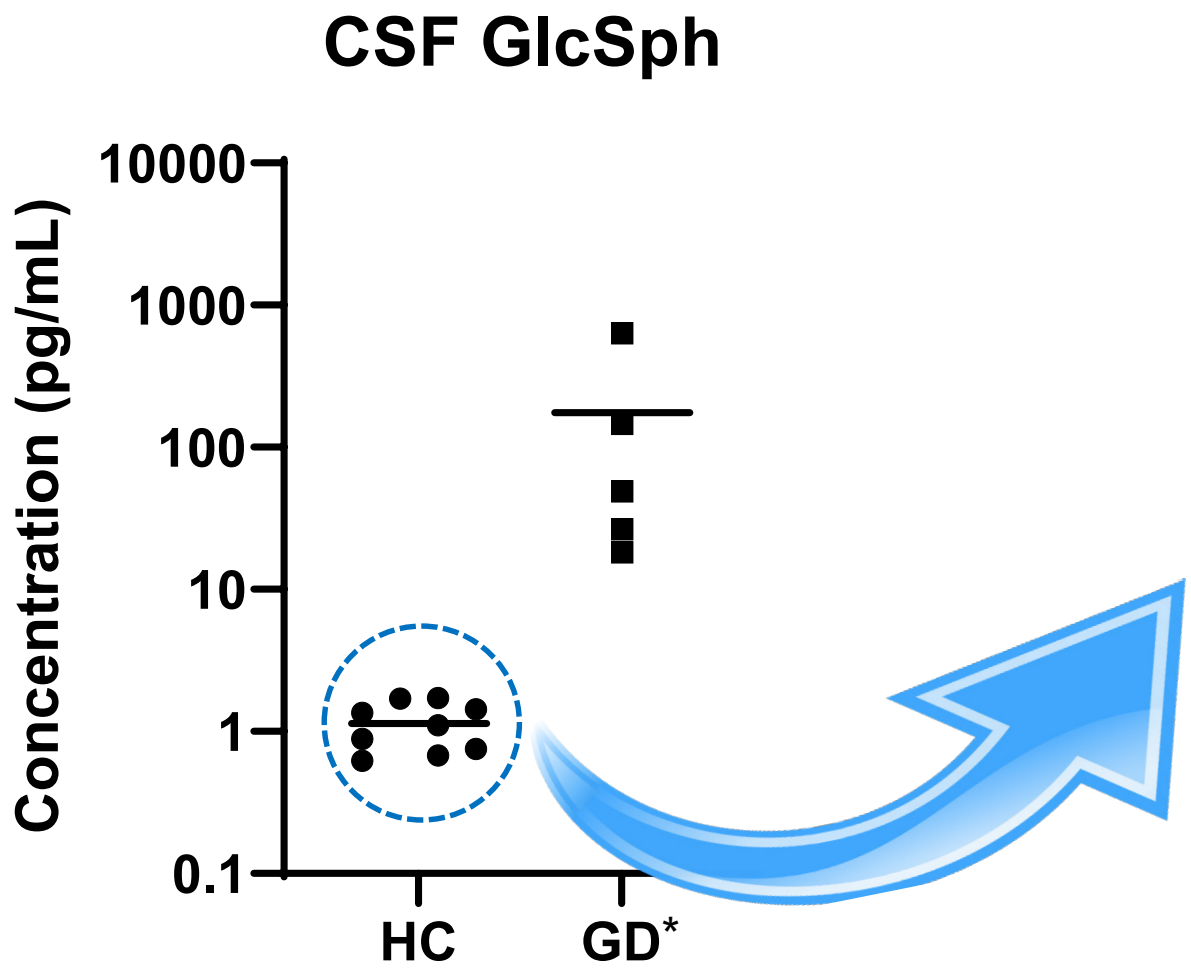
GlcSph and GalSph were stable in CSF, plasma, and brain with more than 85% remaining.

Method comparison for GlcSph

Method	Brain 146(2), 2023	Ann Clin Transl Neurol. 3(3) 2016	Takeda
Sample requirement			CSF: 200 µL
Pretreatment			SPE Strata-X
Recovery			>80%
Internal standard	Not disclose	Not disclose	10 pg/mL, 20 µL
Column			HALO HILIC 4.6x150mm x2
Pump Run time			Isocratic 15 min/run
Mobile phase			0.1% HCOOH, 5mM $HCOONH_4$
Injection volume			50 µL
Equipment		QTRAP5500	QTRAP6500
LLOQ	**5 pg/mL**	**10 pg/mL**	**0.1 pg/mL**

This established method for CSF delivered 50-100-fold improvement in the LLOQ compared to that reported for the CSF of a GD patient.

Concentrations of GlcSph in individual human CSF



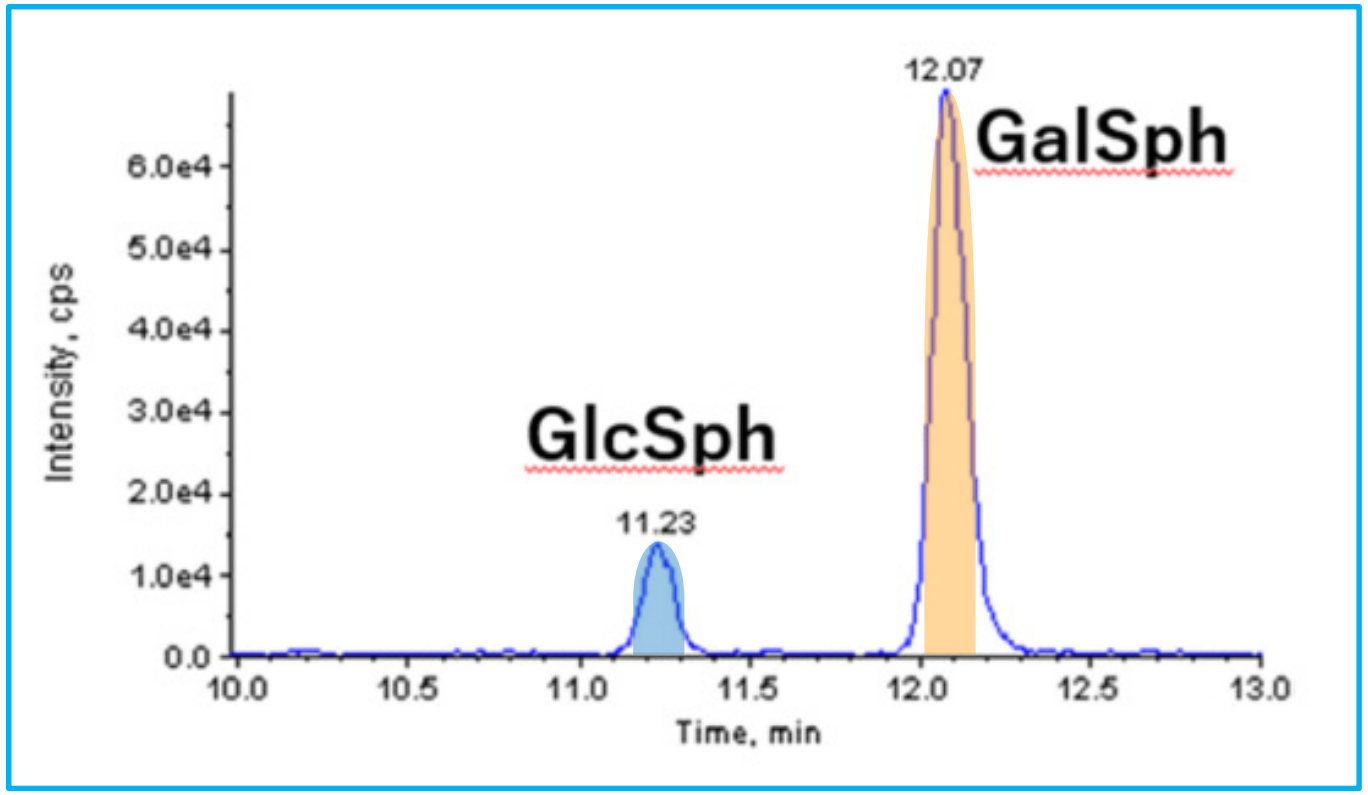
Sample Name	Calculated Concentration (pg/mL)
8223	0.68
8231	0.62
8232	1.35
CSF17	0.75
CSF18	1.71
8244	1.11
8255	1.43
8256	0.89
8290	1.72
Ave.	1.14
SD	0.43

Succeeded to detect Glc-Sph in all CSF samples!

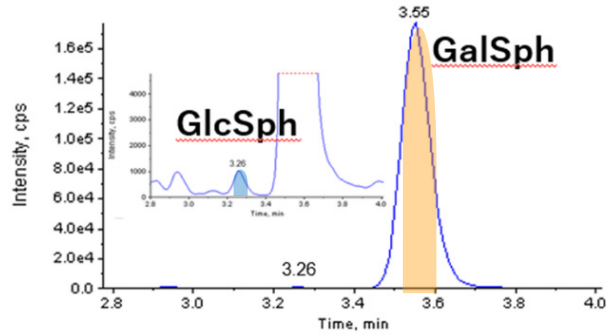
*The GlcSph concentrations in GD CSF were referred from Ann Clin Transl Neurol., 2016.

Chromatograms of GlcSph and GalSph in human CSF, plasma and brain

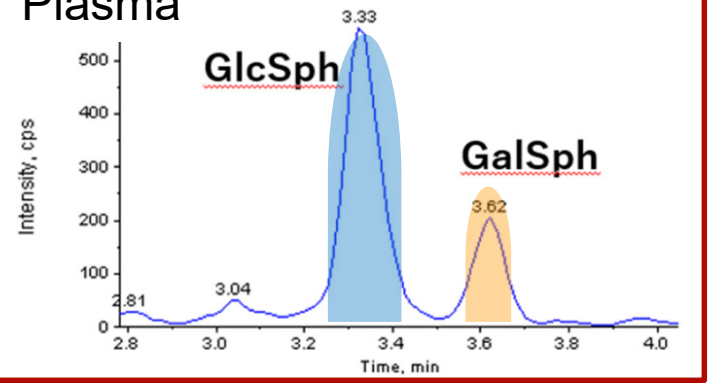
CSF



Brain



Plasma



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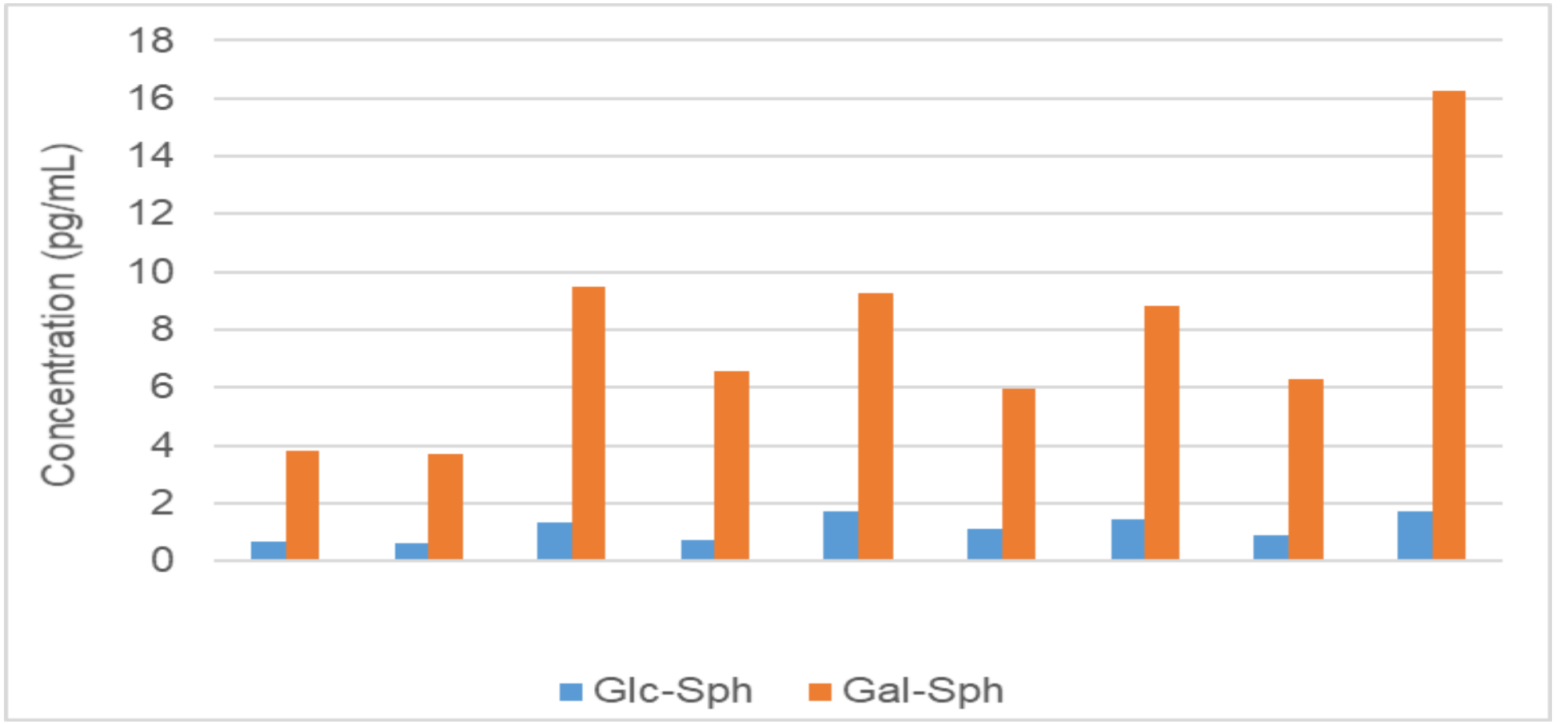
The pattern of GlcSph/GalSph ratio is different between CSF and plasma, suggesting CSF conc. would be good surrogate for GlcSph change in brain.

Concentrations of GlcSph and GalSph in individual human CSF



GalSph levels are higher than GlcSph in all healthy control samples.

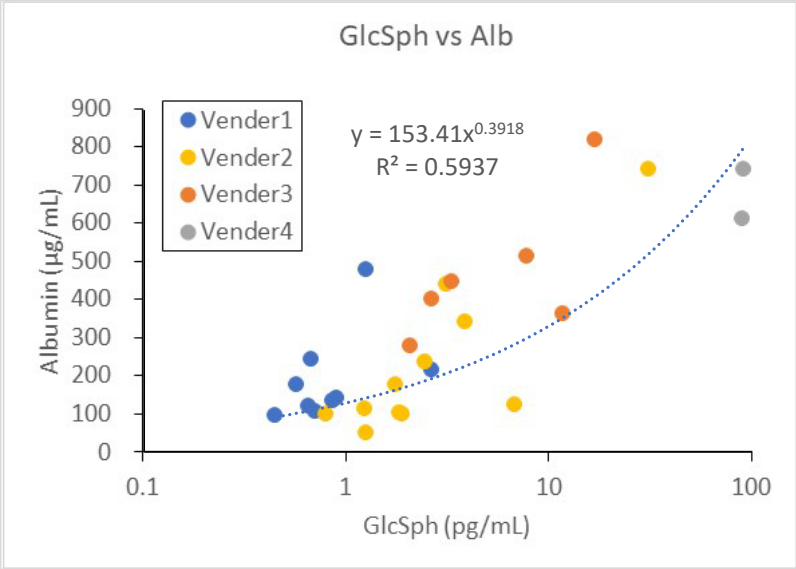
GlcSph and GalSph concentration in normal human CSF (vender difference)

	GlcSph (pg/mL)		GalSph (pg/mL)
Vender1, 9 donors (mean)	1.1	<	7.79
Vender2, pool donors	0.51	<	3.04
Vender3, pool donors	13.3	>	9.11
Vender4, pool donors	157	>	26

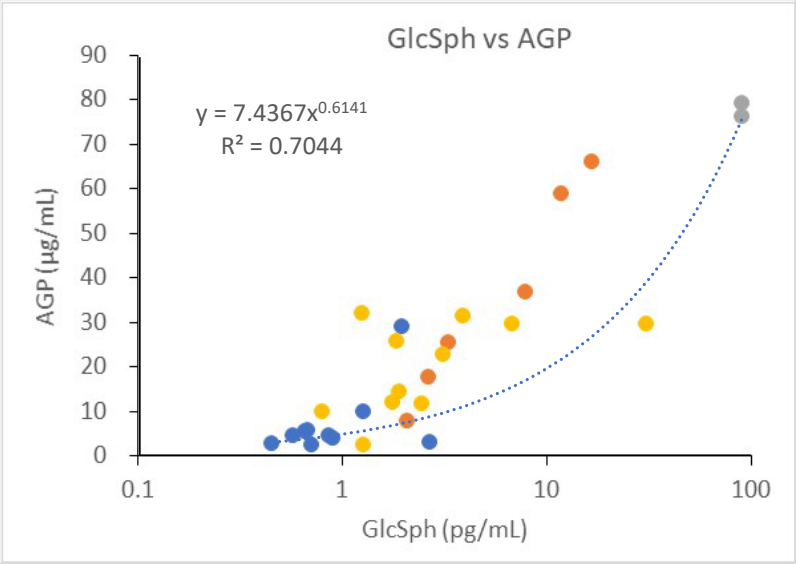
Suspected plasma contamination in CSF samples

Blood/plasma contamination markers in CSF

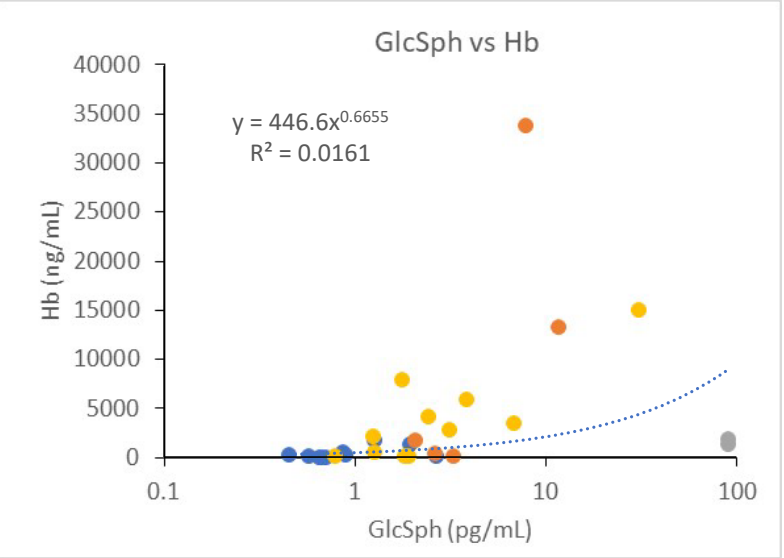
Albumin



AGP



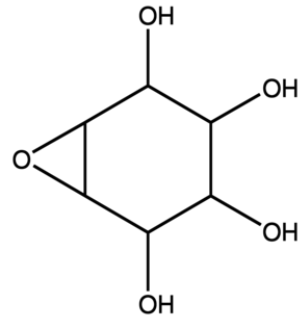
Hemoglobin



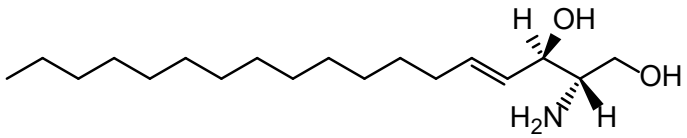
Albumin and AGP are good contamination marker of plasma in CSF.

Concept and study design for Gaucher disease model

CBE (conduritol-β-epoxide)

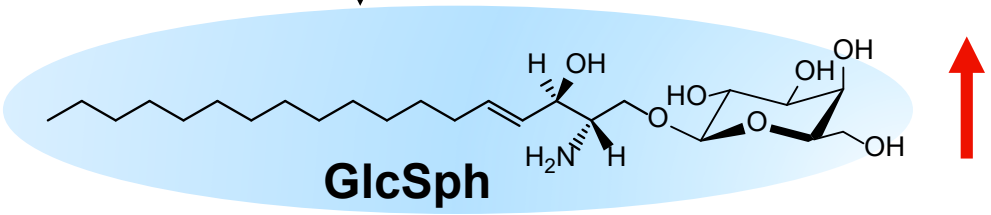


Irreversible competitive inhibitor of GCase



Sph

GCase — **CBE**



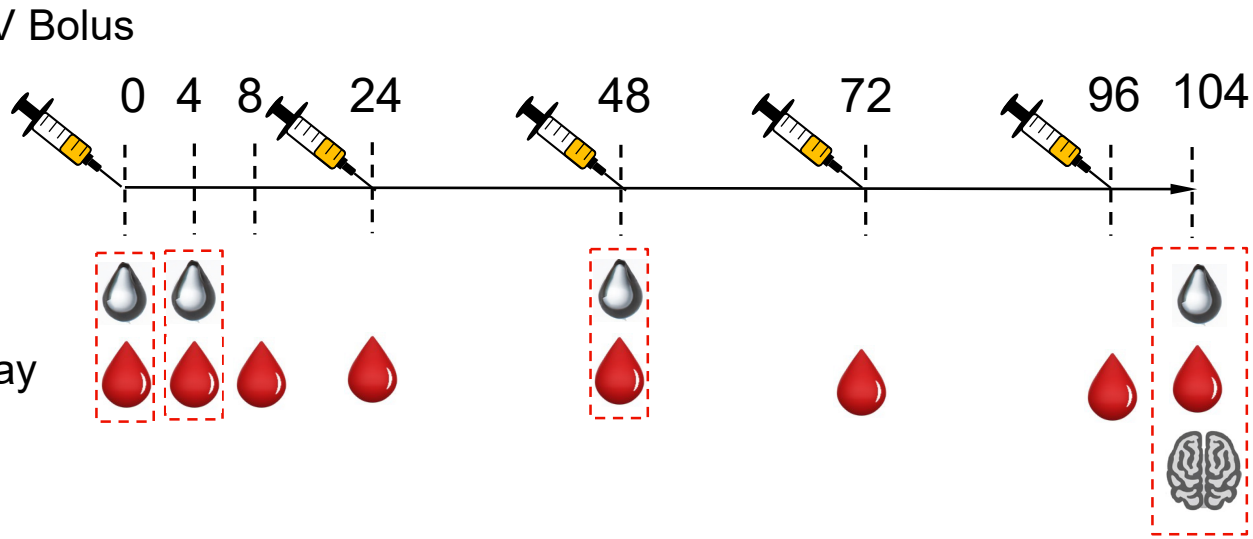
GlcSph

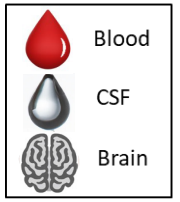
Group 1 (n=3)

Vehicle (saline)

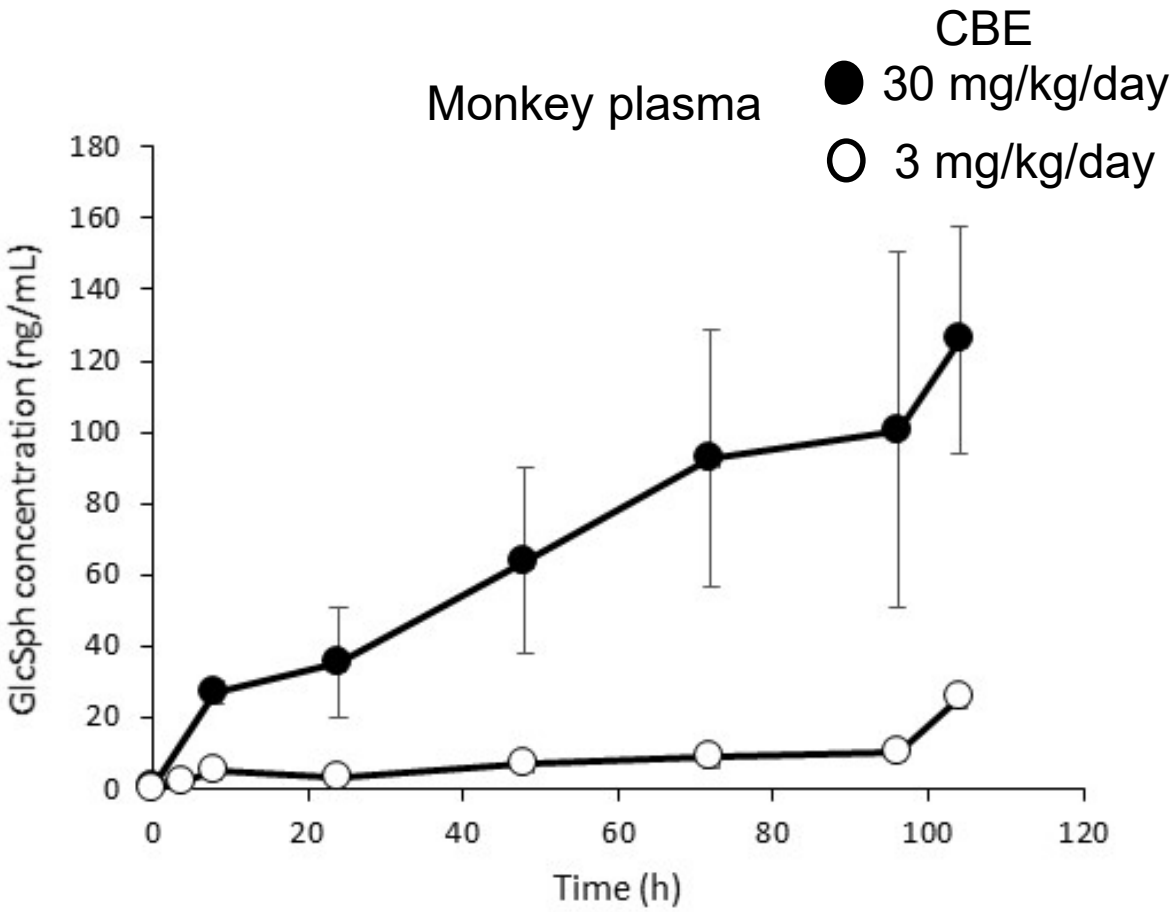
Group 2 (n=3)

CBE 0.3-30 mpk/day





Monkey model for Gaucher disease

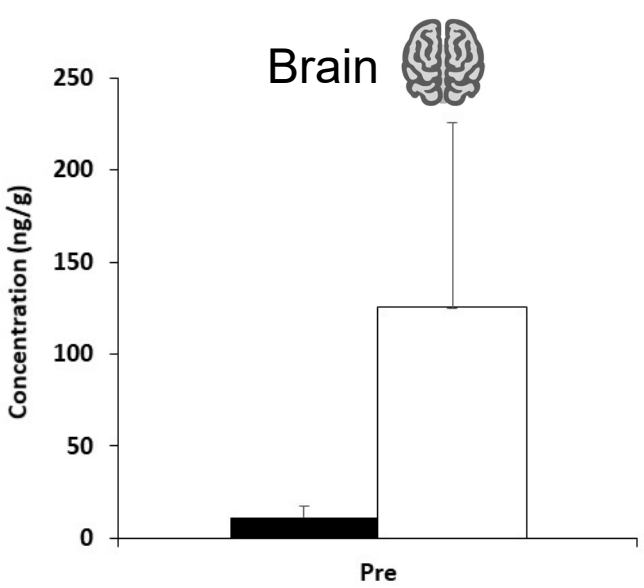


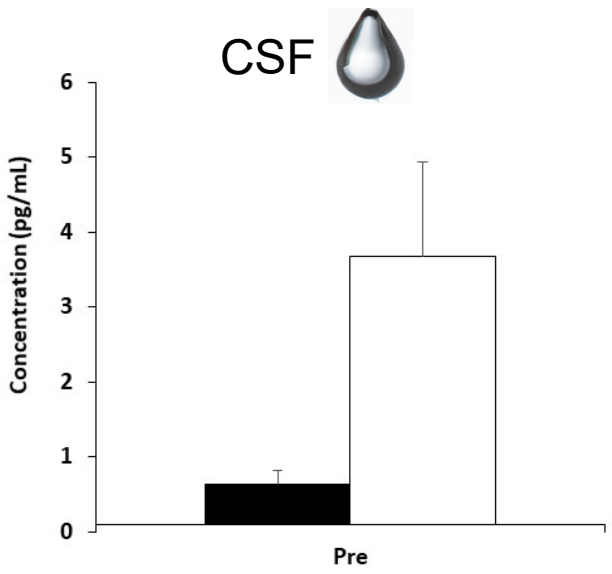
Human	Plasma/Serum
HV	0.48 ng/mL[a]
GBA-PD	0.82 ng/mL[a]
GD (type3)	19.3 ng/mL[b]

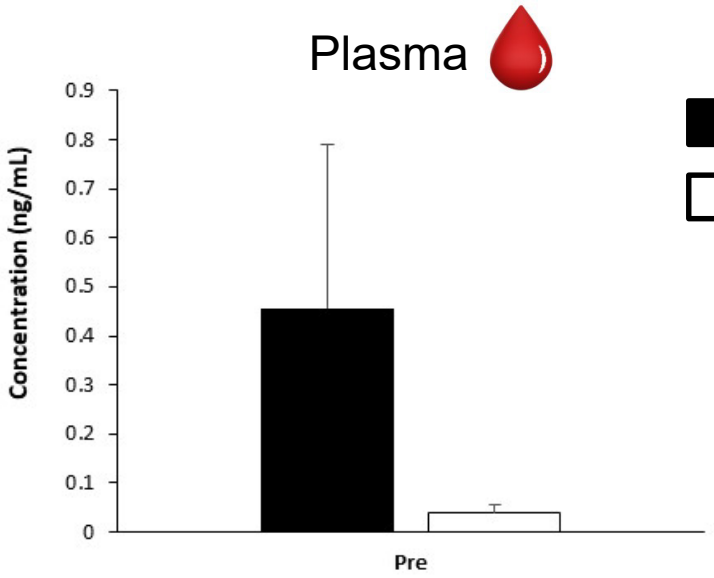
[a] Mov Disord., 37, 2022
[b] Brain, 146, 2023

Plasma GlcSph in monkey reached to GD patient's level by treatment of CBE (3 mg/kg/day, 5 days).

Endogenous GlcSph and GalSph conc. in monkey



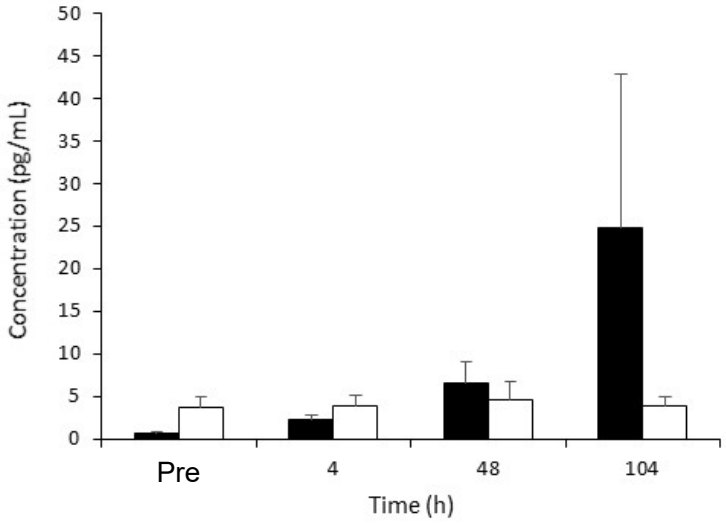




As same as human, the pattern of GlcSph/GalSph ratio in monkey is different between CSF and plasma.

Endogenous GlcSph and GalSph conc. in monkey

CSF

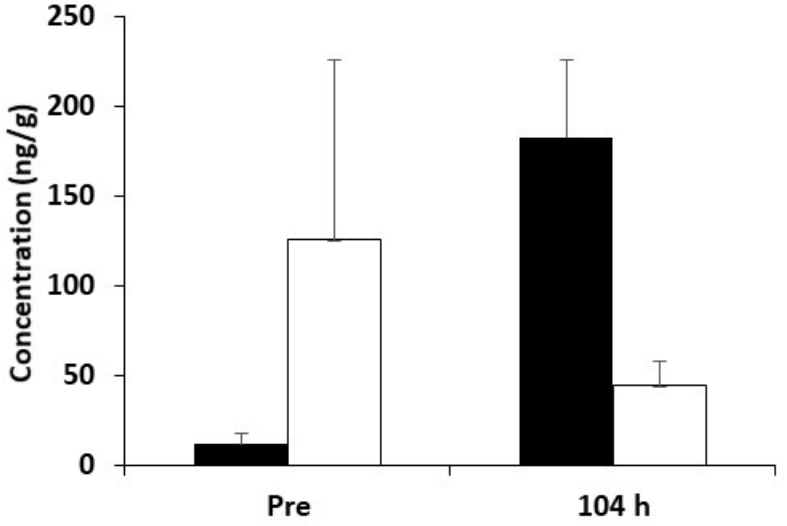


3 mg/kg/day CBE

Brain

GlcSph

GalSph



3 mg/kg/day CBE

Elevation of GlcSph in brain by CBE showed same trend with CSF, suggesting CSF conc. would be good surrogate for GlcSph change in brain.

Translation of GlcSph concentration among species

	Plasma	CSF	Brain
Human HV	0.348 ng/mL	1.07 pg/mL	4.67 ng/g
Human GD (type3)	19.3 ng/mL[a]	43.2 pg/mL[a]	138-2770 ng/g[b]
Monkey	0.110 ng/mL	0.635 pg/mL	11.3 ng/g
Monkey (CBE 3mg/kg, 5d)	25.8 ng/mL	24.8 pg/mL	184 ng/g
Rat	0.061 ng/mL	0.148 pg/mL	1.47 ng/g
Mouse	0.113 ng/mL	Unknown	3.66 ng/g
Mouse (CBE 100mg/kg, 4w)	Unknown	Unknown	>10000 ng/g[c]
GD mouse (L444P)	Unknown	Unknown	>200 ng/g[c]

[a] Brain, 146, 2023
[b] Acta Neuropathol., 65, 1984
[c] Anal Chem., 89, 2017

- GlcSph elevation in the monkey treated with CBE is well consistent with human GD patient.
- Rodents can also be used by modifying a dose of CBE.

Summary

- Highly-sensitive simultaneous quantitation method of glucosylsphingosine and galactosylsphingosine was established.
- The balance of GlcSph and GalSph in CSF would be a good surrogate of concentration change in the brain by targeted therapies.
- The monkey treated with CBE can be useful for the compound evaluation for GBA-related diseases as translational animal model.

Acknowledge

Shin-ichi Matsumoto
Sho Sato
Kentaro Otake
Hiroaki Shida
Kazumi Ohuchi
Kazuko Watanabe
Ayumi Kawamura
Misato Mori
Hiroshi Watanabe
Hideki Hirabayashi