Research Paper

Increased Sphingomyelin and Free Sialic Acid in Cerebrospinal Fluid of Kearns-Sayre Syndrome: New Findings Using Untargeted Metabolomics

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ABSTRACT

Background: Kearns-Sayre syndrome (KSS) is caused by duplications and/or deletions of mitochondrial DNA (mtDNA) and is typically diagnosed based on a classic triad of symptoms with chronic progressive external ophthalmoplegia (CPEO), retinitis pigmentosa, and onset before age 20 years. The present study aimed to diagnose two patients, on suspicion of KSS.

Methods: One of the patients went through a diagnostic odyssey, with normal results from several mtDNA analyses, both in blood and muscle, before the diagnosis was confirmed genetically.

Results: Two patients presented increased tau protein and low 5-methyltetrahydrofolate (5-MTHF) levels in the cerebrospinal fluid (CSF). Untargeted metabolomics on CSF samples also showed an increase in the levels of free sialic acid and sphingomyelin C16:0 (d18:1/C16:0), compared with four control groups (patients with mitochondrial disorders, nonmitochondrial disorders, low 5-MTHF, or increased tau proteins).

Conclusions: It is the first time that elevated sphingomyelin C16:0 (d18:1/C16:0) and tau protein in KSS are reported. Using an untargeted metabolomics approach and standard laboratory methods, the study could shed new light on metabolism in KSS to better understand its complexity. In addition, the findings may suggest the combination of elevated free sialic acid, sphingomyelin C16:0 (d18:1/C16:0), and tau protein as well as low 5-MTHF as new biomarkers in the diagnostics of KSS.

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Introduction

Neurodegenerative disorders can be broadly classified according to the clinical features, most commonly movement disorders and cognitive or behavioral problems. The presentation of a pure symptom among patients is rare, whereas combined manifestations are dominant. This dominance signifies the coexistence of several common fundamental processes associated with

progressive neuronal dysfunction/death in neurodegenerative disorders.[1] In this regard, mitochondrial dysfunction is a unifying feature for neurodegenerative mitochondriopathies, a group of disorders that include mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes syndrome; the myoclonic epilepsy with ragged red fibers syndrome; maternally inherited Leigh syndrome; the neuropathy, ataxia, retinitis pigmentosa syndrome; and the Kearns-Sayre syndrome (KSS).[2]

KSS, a rare mitochondrial disorder,[3] is caused by duplications and/or deletions of mitochondrial DNA (mtDNA). The large-scale deletion range is 1.1 to 10 kb,[4,5] and the most common is a 4977-bp deletion.[5] The resultant mtDNA rearrangements affect respiratory chain and the energy metabolism of several tissues, in particular high-energy consumers like the brain and muscles.[6] KSS is clinically characterized by a classic triad of symptoms with chronic progressive external ophthalmoplegia (CPEO), retinitis pigmentosa, and onset before age 20 years. The patients may also have other symptoms such as cardiac disease (heart block), cerebrospinal fluid (CSF) protein content of >0.1 g/L, muscle weakness, neurological abnormalities (ataxia, neural deafness, intellectual disability, dementia, convulsions and neuropathy), and several endocrine disorders (diabetes mellitus, thyroiditis, hypoparathyroidism, hypogonadism, and short stature).[6-8]

Cerebellar ataxia can be seen in several neurodegenerative and nondegenerative disorders, making the final diagnosis in these patients challenging.[9] For example, the presentation of a 14-year-old African American boy with spinocerebellar ataxia type 7 mimicked KSS.[10] Another study reporting a disease called "cerebellar ataxia with elevated cerebrospinal free sialic acid" (CAFSA) could identify free sialic acid as the common metabotype between recruited patients, both with POLG mutations and without genetic diagnosis, and a confirmed case of KSS.[11] The level of 5-methyltetrahydrofolate (5-MTHF) was decreased in the CSF of patients too. The authors concluded that CAFSA is related to mitochondrial alterations through either POLG mutations or a mechanism similar to KSS. The first CAFSA case was described in 2009, as a new clinicobiological entity.[12]

The present study aimed to diagnose two patients, on suspicion of KSS. Therefore, a stepwise diagnostic evaluation of KSS along with quantifying candidate molecules such as free sialic acid, 5-MTHF, and tau protein was performed using routine analysis. Further investigation was carried out using untargeted metabolomics, which identified a strikingly changed metabolite profile. Untargeted metabolomics is used for testing as well as generation of hypothesis.

Materials and Methods

Chemicals (liquid chromatography-mass spectrometry analysis)

Water used was of type 1 (>18 MΩ cm), obtained from MilliQ ultrapure water purification system (Merck Millipore, Darmstadt, Germany). Methanol was purchased from Rathburn Chemicals (Walkerburn, Scotland). Formic acid (98%), *N*-acetylneuraminic acid, and sphingomyelin 16:0 (d18:1/16:0) were from Merck.

Subjects

Patient I (#1) is a 19-year-old woman, from nonconsanguineous parents. The pregnancy was normal, gestational age 35 weeks, birth weight 2.5 kg, and height 48 cm. Development was normal during the first year; however, a slightly progressive delayed motor development was observed after age one year. Table 1 shows the symptoms at different ages. Ophthalmoplegia was recognized at age 14 years, involving lateral rectus muscle, immobile or limited

eye movement, bilateral lateral rectus paresis, droopy eyelids, mild ptosis, and weakness of orbicularis oculi. Chest X-ray and echocardiogram were normal. Cerebral magnetic resonance imaging at age six, 10, and 11 years was normal; however, T2-high signal in the occipital, thalamus, and brainstem regions, with no lactate on spectroscopy, was found at age 13 to 14 years.

Patient II (#2) is an eight-year-old boy presenting with motor and verbal developmental delay, afebrile seizures, stiff gait, and poor balance at age 1 year and 7 months. Seizures were initially short generalized clonic seizures and focal seizures with autonomic symptoms and reduced consciousness. Initially the patient was perceived as mildly ataxic; however, one also suspected features of dystonia and chorea. At age 3.8 years, he had episodes of paresis of the right foot and leg, but electromyography and neurography were normal. No signs of affection of eye motility or retinal pigmentation were observed. From age seven years, his motor and language abilities have regressed. At the same time, his epilepsy deteriorated markedly, leading to a prolonged nonconvulsive status epilepticus. Cerebral magnetic resonance imaging at age three years revealed a diffusely increased T2/fluid-attenuated inversion recovery signal, which gave the suspicion of a metabolic condition at the level of the cerebral peduncles and involving the substantia nigra. Electroencephalogram progressed from not revealing definite epileptic discharges to near-continuous multifocal discharges on an encephalopathic background activity. Lately runs of rapid spikes followed by depression have occurred during sleep, which is in accordance with Lennox-Gastaut syndrome. The patient is treated with multiple antiepileptic drugs with very limited success. He receives calcium folinate supplements with no obvious clinical effect.

Metabolomics

Sample collection, preparation, and analysis

Plasma and CSF samples from patients (#1 and #2) and four control groups (patients with mitochondrial disease, patients with low 5-MTHF in CSF without KSS, adult patients with increased tau protein in CSF, and other/nonmitochondrial patients) were collected. The three mitochondrial disease control patients had the following diagnoses: ACAD9 deficiency, MT-ATP6 mitochondrial disease, and DNA polymerase subunit gamma (POLG)-related disorder. The four patients with low 5-MTHF in CSF without KSS had either methylenetetrahydrofolate reductase deficiency or secondary cerebral folate deficiency, with 5-MTHF levels ranging from 11 to 25 nmol/L. The eight tau controls were adult patients with delirium with increased tau protein in CSF.[13] The five nonmitochondrial patients were without diagnosis and not suspected to have inborn errors of metabolism. The samples were stored in −20°C (and −80°C for 5-MTHF measurement) before analysis (Table 2). All CSF and plasma samples were centrifuged for 10 min at 3600 rpm (4°C). The CSF samples were transferred directly to a liquid chromatography (LC) vial, whereas plasma samples were prepared by protein precipitation mixing plasma with MeOH (1:3 v/v), whirlmixed, centrifuged at 4°C using Fresco 21 Microcentrifuge (Thermo Scientific; Waltham, MA, USA) for 10 min at 14,000 rpm, and transferred to an LC vial. To ensure high-quality data and to correct for analytical drift during the analysis, a pooled quality control (PQC) was made by mixing an aliquot of all samples (CSF and plasma, respectively), and this PQC was analyzed repeatedly throughout the sequence.

Analysis of CSF and plasma samples from patients and controls was performed on an in-house developed and validated metabolomics platform covering a broad range of metabolites. In brief, the platform is based on reversed-phase liquid chromatography coupled to a high-resolution mass spectrometer, using electrospray

TABLE 1.
Disease Trajectory of Patient 1

Onset (Age)	Manifestation	Therapy
Intrauterine	Premature birth (5 weeks earlier)	None
Birth	Caesarean section due to premature rupture of the membranes	None
Infancy	Normal postnatal period	None
Childhood, 5 y	Frequent upper respiratory tract infections	Adenectomy and middle ear drainage
Childhood, 6 y	Hypermetropia and astigmatism	Followed by eye doctor
Childhood, 6 y	Downslowing, poor weight gain	Referred pediatric ward first time, normal examination
Childhood, 9 y	Chronic frontal headache with vomiting and abdominal pain	Normal MRI at this time, EEG with slow waves, no EPI activity. Treated with migraine medications without effect
13 y	Stomach pain, clinical reflux	Impedance pH monitoring confirmed GERD. Treated with PP
13 y	Social withdrawing, decreased working memory capacity and apraxia, both apraxia of speech and orofacial apraxia	New EEG taken, now normal
13 y	Language difficulties	None
13 y	Thoracolumbar scoliosis & prominent kyphosis	None
14 y	Ophthalmoparesis/CPEO (first recognized) and imbalance	MR spectroscopy: high signal in the occipital, thalamus, and brainstem regions. Elevated protein, cells, and IgG in CSF
14 y	Retinitis pigmentosa	None
14 y	Increasing hypomimia, bradykinesia, and slow movements	None
15 y	Very low level of 5-MTHF and high level of tau protein in CSF	Started with folinic acid
15 y	Diabetes mellitus	Antidiabetics

Abbreviations:
5-MTHF = 5-Methyltetrahydrofolate
CPEO = Chronic progressive external ophthalmoplegia
CSF = Cerebrospinal fluid
EEG = Electroencephalogram
GERD = Gastroesophageal reflux disease
MR = Magnetic resonance
MRI = Magnetic resonance imaging
PPI = Proton pump inhibitor

ionization operated in both negative and positive ionization modes.[14] Data for all detectable metabolites in each sample were collected, giving rise to each sample's unique metabolome.

After data collection, further processing of the metabolomes was performed in two different ways. First, the signal of *N*-acetylneuraminic acid (Neu5Ac), a molecule of interest due to the clinical presentation of the patients, was filtered out from the dataset. Neu5Ac was found by extracting the deprotonated molecular mass of $[M-H]^- = 308.0987 \pm 5$ ppm from the metabolomes and confirming its identity (level of confidence 1, Table 3).

After filtering out exact molecular masses to look for known metabolites, different data processing approaches were used on the untargeted metabolomes to look for new altered metabolites in CSF samples from patients and the different control groups (Table 2). PQC was analyzed using ddMS2 top5 method, that is, the five most abundant ions entering the mass spectrometer at any given time will be fragmented producing a fragmentation spectrum that can in turn be used for identification. A PQC from all samples was analyzed between every fifth sample to ensure good-quality data and enable signal correction. The overall quality of the metabolomics data was evaluated by principal component analysis (PCA). Clustering of the PQC certifies the proceeding use of the untargeted data in further statistical analysis. Significant metabolites providing specific metabolic signatures for each group were found using volcano plot, that is, comparison of molecular features between groups with criteria *P* value <0.05 and log2 fold change >1.

Data processing and statistics

The metabolome contains the metabolic profile of each sample, with all detected metabolites characterized by mass to charge ratio, retention time, and relative abundance. Compound Discoverer 3.1 (Thermo Scientific) was used for data processing and statistical analysis. Concentrations, in arbitrary units, were expressed using trend charts of areas of chromatographic peaks. Box-and-whisker plots were used to display the variation between the groups.

General metabolic screening

The general metabolic screening of urine, plasma, and CSF was performed at the Department of Medical Biochemistry, Section for Inborn Errors of Metabolism, Oslo University Hospital, Norway. The laboratory participates in several external quality programs: LabQuality and European Research Network for evaluation and improvement of screening, diagnosis and treatment of Inherited Metabolic Disorders.

Results

Metabolic screening

Patient #1

General metabolic screening in urine did not show any specific findings, including normal pattern of organic acids and levels of purines/pyrimidines. There were normal levels of copper in urine. Plasma lactate had near-normal values. In addition, we observed normal concentrations of amino acids, very-long-chain fatty acids, homocysteine, thiamine, and folic acid in plasma, and normal results from carbohydrate-deficient transferrin (CDT) analysis.

Analyses of CSF have been performed three times (2019/2017/2016). Main findings were increased leukocytes $14/10/8 \times 10^6$/L (reference interval [ref.] 0 to 4), glucose 5.8/12.9/4.4 mmol/L, lactic acid N.D/3.3/2.0 mmol/L (ref. 0.8 to 2.8), and increased protein 1.7/1.61/1.84 g/L (ref. 0.15 to 0.45). Albumin index, at age 14 years, was 24 (ref. <9), indicating moderate barrier failure, with no oligoclonal bands detected. Amino acids in CSF showed unspecific small changes from reference values and normal concentration of alanine. Total tau protein was grossly elevated, 3550 ng/L (ref. <250) (Sahlgrenska University Hospital, Sweden). Neurotransmitter analyses showed very low levels of 5-MTHF, <2 nmol/L (ref. 30 to 116), but normal levels of biogenic amines and pterins (Heidelberg Hospital, Germany). Free sialic acid was elevated, 113 μmol/L (ref. 2 to 24.4) (Radboud UMC, Nijmegen, Netherlands).

TABLE 2.
Overview of the Samples (Patient #1 and Patient #2), Controls (High Tau, Mito, Low 5-MTHF, and Non-mito), Materials (CSF and Plasma), and Year of Sampling for Samples Included in the Metabolomics Study

Sample	Material	Year of Sampling					
		2009-2012	2016	2018	2019	2020	2021
P #1	CSF		X				
P #2	CSF			X	X	XXX	
P #2	Plasma			X	X	XXX	
High tau	CSF	XXXXXXX					
Mito	CSF				X	XX	
Mito	Plasma				X	XX	
Low 5-MTHF	CSF				X		XXX
Low 5-MTHF	Plasma				X		X
"Non-mito"	CSF						XXXXX
"Non-mito"	Plasma						XXXXX

Abbreviations:
5-MTHF = 5-Methyltetrahydrofolate
CSF = Cerebrospinal fluid
P = Patient
The CSF samples were used both for the targeted approach and the untargeted comparison. The plasma samples were used for targeted approach only. Each X represents samples taken at different time within the specific year.

TABLE 3.
Metabolite Identification Confidence Level

	Level of Confidence	Requirements
↑ Increasing confidence	Level 1: Validation identification	Reference standard match (MS-MS spectrum and retention time match)
	Level 2: Putative identification	MS-MS spectrum match, using online databases
	Level 3: Tentative structure	MS1 database search, supported by additional information
	Level 4: Proposed molecular formula	Match of mass spectrum, isotopes abundance distribution
	Level 5: Unique feature	No identification. Exact molecular mass only; ±5 ppm

For biological interpretation of data, highest level of metabolite identification is a necessity. The levels of confidence identification range from 1 to 5, where 1 is the highest level. Using our in-house library, containing exact mass of molecular ion, fragmentation spectrum, and retention time of reference compounds analyzed on our platform, enables level 1 identification. For level 2 and below, Compound Discoverer uses Chemspider for proposal of molecular formula based of accurate mass, and mzCloud for online fragmentation pattern, increasing the confidence in proposed molecular identity.
Adapted from Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA (2016) Untargeted metabolomics strategies—challenges and emerging directions. J Am Soc Mass Spectrom. 27:1897-1905.

Genetic analyses were performed both in blood and muscle. The results from exome sequencing in blood (intellectual disability gene panel and mitochondrial gene panel [nuclear DNA]) were normal, including normal POLG sequencing. mtDNA sequencing in muscle (Nijmegen, Netherlands) was normal, except for a rare sequence variant, heteroplasmic (27%) mtRNR2 in muscle m.3173 G>A of unknown significance. Polymerase chain reaction (PCR) analyses of MT-TL1, MT-TK, MT-ATP6, and common deletion (MT-DNA8470-13466del) were normal in blood and muscle. Also, the analysis of the respiratory chain with oxygraphy and spectrophotometric enzyme assay in muscle did not show any pathology. However, cytochrome c oxidase (COX)-negative fibers detected in muscle could point to mitochondrial dysfunction.

Finally, by using long PCR/walking PCR on muscle sample (Haukeland University Hospital, Bergen, Norway), the diagnosis was found: long PCR revealed an mtDNA deletion of approximately 7 kb with breakpoint 6683 to 13659. Figure 1 summarizes the laboratory results in urine, plasma, blood, and CSF.

Patient #2
General metabolic screening in urine did not show any specific findings, including normal pattern of organic acids and levels of purines/pyrimidines. The results from basic plasma evaluation

including blood gases and lactate/glucoses were normal during the disease course. Plasma amino acid profile, acylcarnitines, very-long-chain fatty acids, homocysteine, and carbohydrate-deficient transferrin analyses were normal.

Analyses of CSF demonstrated very low value of 5-MTHF, <5 nmol/L (ref. 31 to 130), and markedly increased tau protein, 7050 ng/L, indicating severe cortical/axonal decay. Total protein in CSF was 1.6 g/L (ref. 0.15 to 0.45) and albumin index 24 (ref. <9), indicating moderate barrier failure. Lactate values and amino acid profile were normal. Neopterin in CSF was slightly elevated; 43 nmol/L (ref. 7 to 32) (Heidelberg Hospital, Germany), but Aicardi-Goutières syndrome was excluded on genetic analyses.

A muscle biopsy was analyzed and showed a generally too low diameter of type 2 fibers on microscopy. There were no "ragged red fibers" and a normal staining for COX, succinate dehydrogenase, and NADH. Oxygraphy of the muscle showed an intact respiratory chain in the mitochondria in the muscle; however, spectrophotometry revealed a slight reduction of the activity of complex II. Activity of the respiratory chain enzymes in fibroblasts was also analyzed in Nijmegen, the Netherlands, with normal results.

Genetic analysis (gene panels) of the mitochondrial and nuclear DNA from blood and muscle was done. Analysis of common deletions (mtDNA8470-13466del) was negative in both leukocytes and muscle. Whole mtDNA sequencing from the laboratory in Nijmegen was also normal. There were no deletions on long PCR. A separate sequencing of genes relevant for cerebral folate deficiency

and POLG was also normal. The patient does not have a final diagnosis yet.

Metabolomics

Targeted approach

By extracting the signal of the exact molecular mass from the metabolome, in combination with the use of our in-house library, Neu5Ac was detected (level 1) in the CSF in all samples from Patients #1 and #2. The levels of Neu5Au were clearly elevated in both patients compared with the four different control groups (Fig 2). In plasma, low signals for this metabolite and no group differences were found (data not shown).

Untargeted metabolic comparison

To verify low analytical drift and ensure data of high quality, PCA was performed on two sample types (i.e., CSF and plasma) and two ionization modes (i.e., negative and positive). As shown in Fig 3, the clustered data points for Patients #1 and #2 are separated from the rest of the metabolomes, indicating unique metabolic similarities between the two patients compared with the controls.

Following PCA, volcano plot, comparing patients and controls on a group level, revealed significant group-specific metabolic signatures. Among identified molecules, those at confidence level 5 were not investigated further due to resource-demanding/time-consuming annotation processes. Significant findings with their
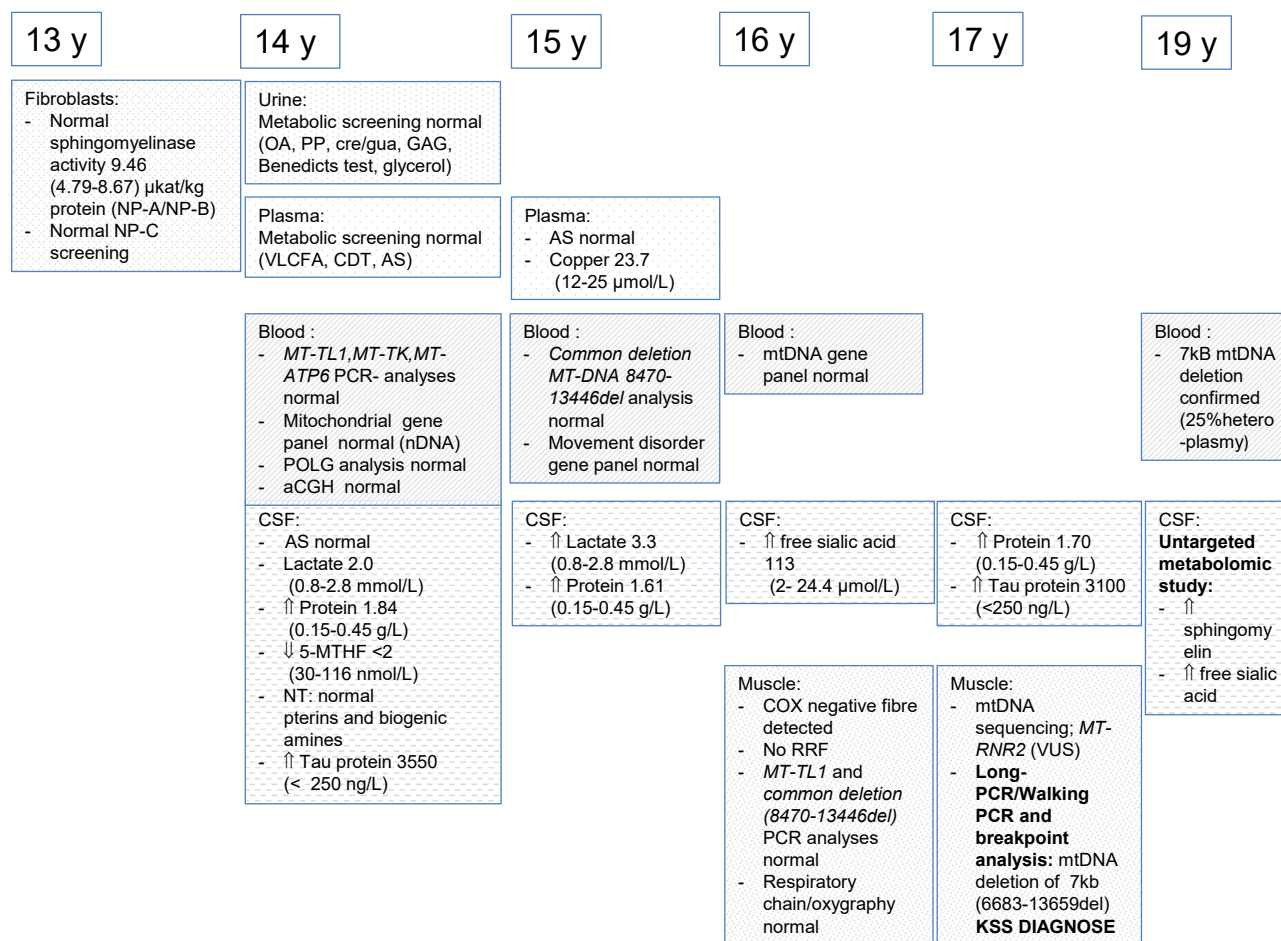
FIGURE 1. Summary of the laboratory results in patient #1. NP, Niemann-Pick; OA, organic acids; PP, purines and pyrimidines; cre/gua, creatine and guanidinoacetate; GAG, glycosaminoglycans; VLCFA, very-long-chain fatty acids; CDT, carbohydrate-deficient transferrin; AS, amino acids; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; aCGH, array comparative genomic hybridization; CSF, cerebrospinal fluid; 5-MTHF, 5-methyltetrahydrofolate; NT, neurotransmitters; RRF, ragged red fibers; VUS, variant of uncertain significance. The color version of this figure is available in the online edition.

corresponding level of confidence are presented in Table 4. For example, sphingomyelin C16:0 (d18:1/C16:0) was clearly elevated in the patient samples (Fig 4 and Table 4). Other molecules including 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, gluconic acid, glutamic acid, glyceric acid, and proline were also identified at level 1.

Discussion

Using a stepwise diagnostic evaluation for KSS, the present study could diagnose one case (KSS patient), and another similar, but undiagnosed patient with ataxia shared abnormal levels of four CSF molecules with the patient with KSS: 5-MTHF, free sialic acid, sphingomyelin C16:0 (d18:1/C16:0), and tau protein. Of these, a reduction in level was observed only for 5-MTHF, whereas the levels of the other three were increased.

Immunohistochemistry and genetic testing (nuclear DNA, mtDNA sequencing, and long-range PCR) were applied for the two patients. Immunostaining showed COX-negative fibers in the muscle sample from the first patient, implying mitochondrial dysfunction. COX-negative fibers have previously been shown in patients with KSS.[15-17] However, the patient faced a diagnostic odyssey, with normal results from several mtDNA analyses, both in blood and muscle. It is therefore of utmost importance to follow the clinical symptoms and biochemical results, even if the genetic testing does not give a diagnosis. Finally, long-range PCR confirmed an mtDNA deletion. In addition to the clinical features,[18] muscle pathology and molecular genetic analysis are considered reliable methods for KSS identification.[19]

The reported histopathological, neuropathological, and molecular findings (e.g.,[15,20-23]) in KSS have progressively increased since the first description of the disease in 1958.[3] Nevertheless, it is still unclear what triggers the onset of the symptoms and how the cells respond to mtDNA deletions. In a unique study investigating the pathophysiologic mechanism, the untargeted gene expression profile of nuclear-encoded genes in 6 cell types containing mtDNA deletions from patients with KSS and CPEO was examined.[24] The aberration was shown to increase protein damage, inhibit the ubiquitin-proteasome system, decrease amino acid salvage, and activate autophagy.

The second patient did not (yet) present CPEO, one of the classical symptoms of KSS.[3,18] Likewise, genetic analysis failed to identify mtDNA deletions. However, routine CSF analysis indicated a decreased level of 5-MTHF and increased level of tau protein, the same pattern as observed in the patient with KSS. Untargeted metabolomics approach further detected increased free sialic acid

and increased levels of sphingomyelin C16:0 (d18:1/C16:0) in both patients. In line with our findings, an untargeted metabolomics study using nuclear magnetic resonance spectroscopy revealed increased free sialic acid in a new complex unknown neurological disease, called CAFSA.[11,12] Regarding tau protein, its rise in CSF has been found in several neurodegenerative diseases,[25] especially Alzheimer and Creutzfeldt-Jakob diseases.[26] However, high CSF tau has never been reported in patients with KSS. Similarly, it is the first time that the sphingomyelin C16:0 (d18:1/C16:0) level has been found to be elevated in patients with KSS.

It is difficult to explain the above-mentioned results and determine associations among the variables, but the findings might be related to lipid raft. Neu5Ac, the major member of sialic acid family in humans,[27] was shown to act as a signaling molecule through activation of Rho/Rho-associated coiled-coil-containing protein kinase signaling pathway.[28] Active Rho proteins present on lipid rafts (membrane microdomains), where they involve in, for example, insulin and insulinlike growth factor-1 signaling.[29,30] Rho family of GTPases (Rho GTPases) are important regulatory molecules that link surface receptors to organization of the actin-microtubule cytoskeletons and mediate many diverse critical cellular processes. In neurons, they play an essential role in regulating morphology, particularly regulation of Rho GTPase dendritic arborization, spine morphogenesis, axon guidance, and neuronal survival-death.[31]

Lipid rafts are defined as small (10 to 200 nm), heterogeneous, highly dynamic, and (chole) sterol-sphingolipid-enriched domains that compartmentalize cellular processes.[32] Importantly, the segregation of proteins is rendered on the raft by selective inclusion or exclusion of these molecules.[33] Sphingomyelin, the major component of lipid rafts, along with its metabolites plays a significant role as second messengers in signal transduction events. Besides being necessary for the activity of some receptors, membrane proteins (e.g., amyloid precursor protein, gp120, and PrP) associated with some diseases share the same sphingomyelin recognition site, implying the contribution of lipid rafts in diseases like Alzheimer.[34]

Most information about tau protein belongs to research on Alzheimer disease. Tau is a family member of microtubule-associated proteins[35] with susceptibility of being modified post-translationally (e.g., hyperphosphorylation, glycosylation, and cleavage)[36,37] causing disease initiation and progression.[38] Tau actively takes part in the regulation of lipid metabolism and alongside other Alzheimer-related proteins cross talks with membrane lipids, particularly lipid raft.[39] The link between Rho/Rho-associated kinase signaling and (hyper) phosphorylation of tau
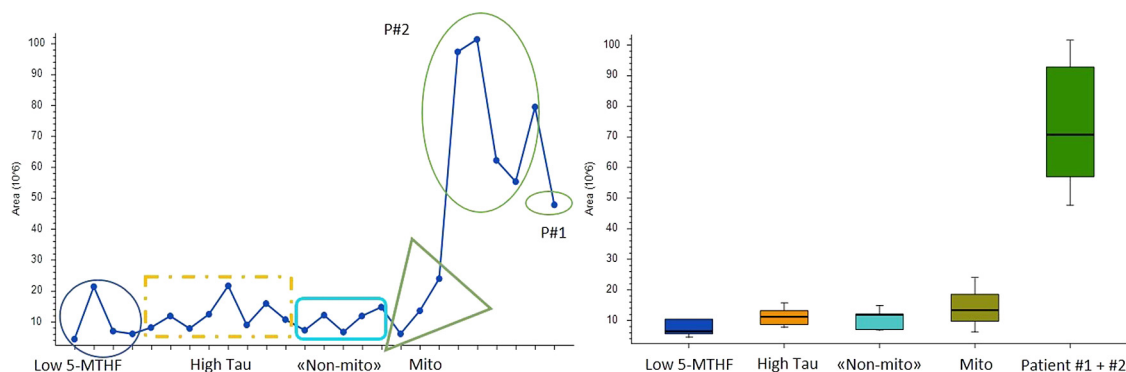

FIGURE 2. Trendline chart (left) and box-and-whisker plot (right) showing the peak areas and its distribution, respectively, for *N*-acetylneuraminic acid in the CSF of the samples from the patients (#1 and #2) and controls (low 5-MTHF, high tau, "non-mito" and mito). The data are generated using the mass spectrometry-based metabolomics platform. CSF, cerebrospinal fluid.
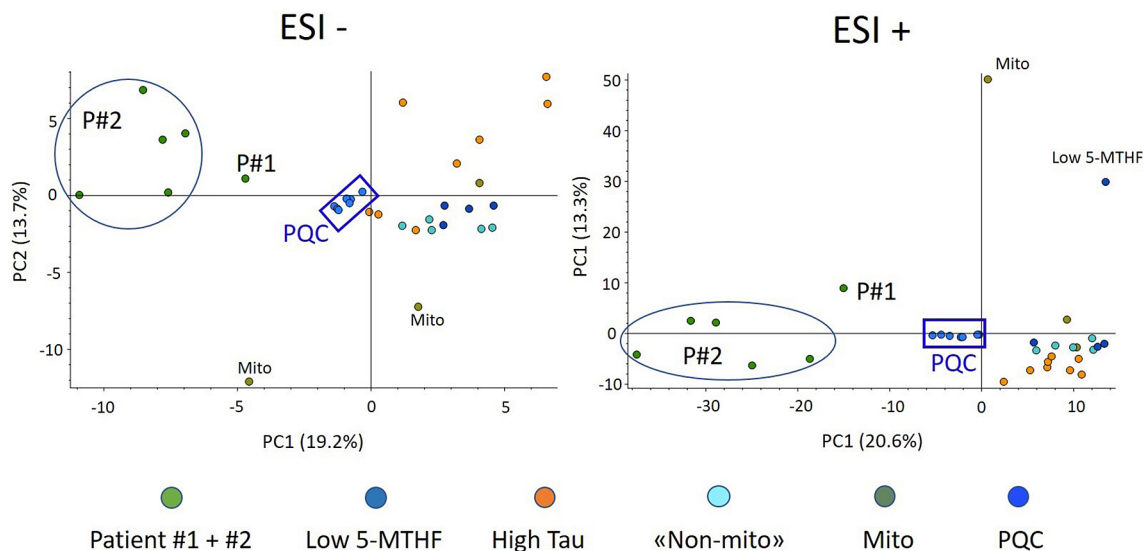
FIGURE 3. PCA plot of CSF metabolites in the samples from the patients (#1 and #2) and controls (low 5-MTHF, high tau, "non-mito" and mito). The circles show the distribution of the samples; Patients #1 and #2 correspond better than the rest of the samples on a holistic level, indicating more similarity in detected metabolomes. The data are generated using mass spectrometry-based metabolomics platform. PQC, pooled quality control; ESI–, negative electrospray ionization; ESI+, positive electrospray ionization; PCA, principal component analysis; CSF, cerebrospinal fluid; 5-MTHF, 5-methyltetrahydrofolate.

has been displayed using cholesterol-lowering medication.[40,41] Moreover, it has been shown that lipid rafts can be modified by the accumulation of tau and amyloid precursor protein.[42]

5-MTHF is one of the key sources of methyl groups in the brain. The decline in its levels disturbs methylation and/or redox potentials, resulting in consequences such as amyloid and tau protein accumulation and neuronal death.[43,44] Sialic acid also plays an important role in the alteration of tau through N-linked glycosylation.[45] Such hypersialylation leads to the accumulation of phosphorylated tau in Alzheimer and other tauopathies.[46] Growing evidence points out the impact of pathologic forms of tau, essentially hyperphosphorylated or cleaved/truncated form, on mitochondrial function through, for example, the generation of reactive oxygen species (for review see Ref.[38]).

A question that remains to be answered is why the free sialic acid level is increased in these patients. In the CAFSA cases, the authors left the question open as they could not find/suggest any link between sialic acid and mitochondrial metabolism.[11] However, one study has discovered reactive oxygen species' scavenging role, a new function, for sialic acid in skeletal muscles.[47] Apart from this, any alterations in the activity of enzymes participating in the biosynthesis of sialic acid (e.g., UDP-N-acetylglucosamine 2-epimerase) can lead to its overproduction.[48] Furthermore, it may be linked to the subcellular localization and enzymatic properties of different types of sialidase in the degradation of glyco-conjugates.[49] To shed light on the mechanisms of sialic acid elevation and its association with other variables, further work needs to be undertaken.

In addition to the above-mentioned molecules, the untargeted metabolomics approach revealed elevation in the levels of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, gluconic acid, glutamic acid, glyceric acid, and proline in the CSF of the two patients compared

TABLE 4.
Untargeted Metabolomics Comparison of CSF Metabolites Elevated in Patients (#1 and #2) Compared With the Control Group: Delirium, Mito, Low 5-MTHF, and Non-mito (see Table 2)

Compound	Level of Confidence	Patients/Controls Ratio	ESI
N-Acetylneuraminic acid	1	↑ (6.3)	-
2-Deoxy-2,3-dehydro-*N*-acetyl-neuraminic acid	1	↑↑ (11.4)	-
Sphingomyelin C16:0 (d18:1/C16:0)	1	↑↑ (22.0)	+
Gluconic acid	1	↑ (3.1)	-
Glutamic acid	1	↑↑ (24.5)	-
Glyceric acid	1	↑ (5.0)	-
Proline	1	↑ (4.6)	+
Glutarylcarnitine	2	↑↑ (15.0)	+
Saccharopine	2	↑↑ (17.5)	+
N-Acetylaspartylglutamic acid	2	↑↑ (15.6)	+
5-aminoimidazole-4-carboxamide ribonucleoside-riboside	2	↑↑ (18.5)	+
Succinyladenosine	2	↑ (3.9)	+
Xylitol	2	↑ (3.9)	+
Xylonic acid	4	↑ (4.0)	-
238.01478*	5	↑ (9.6)	-
250.11631*	5	↑ (7.9)	+

Abbreviations:
↑↑ = Ratio >10
↑ = Ratio 2—9.9
ESI = Electrospray ionization in either positive (+) or negative (−) ionization mode.
* Features that could not be identified are named by their molecular mass ±5 ppm.
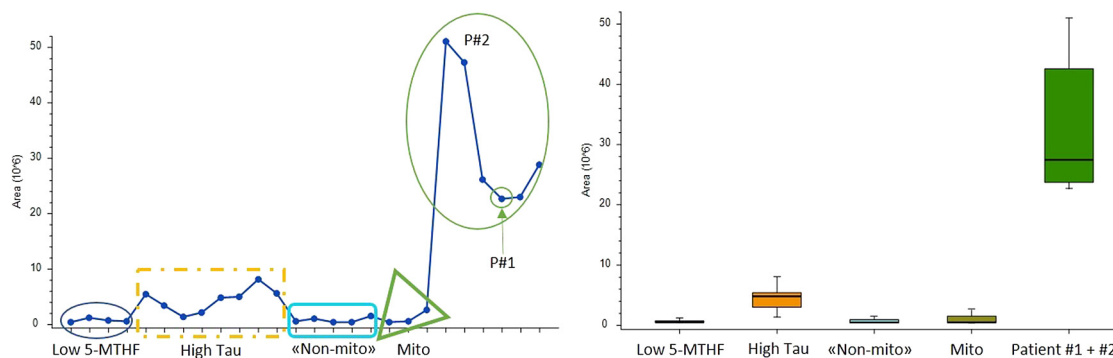
FIGURE 4. Trendline chart (left) and box-and-whisker plot (right) showing the peak areas and its distribution, respectively, for sphingomyelin C16:0 (d18:1/C16:0) in the CSF of the samples from the patients (#1 and #2) and controls (low 5-MTHF, high tau, "non-mito" and mito). The data are generated using the mass spectrometry-based metabolomics platform. CSF, cerebrospinal fluid; 5-MTHF, 5-methyltetrahydrofolate.

with the control groups. The underlying biochemical explanation for these biochemical alternations is not obvious. The observed increased in 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, in addition to sialic acid, could be attributed to the transition state analogue of sialic acid.[50] Regarding glutamic acid, an important neurotransmitter and key metabolite, we could speculate that the molecule is elevated secondary to epilepsy, migraine, or due to an accumulation in the brain secondary to disease.[51,52] For proline, this L-glutamate derivative is known to be elevated in the plasma or urine of several inborn errors of metabolism including mitochondrial diseases. However, the proline level quantified by the general metabolic screening in both patients was normal, also in CSF. A future study on a larger cohort of KSS is therefore suggested.

One limitation of this work was the low number of patients, and that the second patient is without final diagnosis, although our diagnostic follow-up and multiple test strategy could provide valuable information. For metabolomics, the lack of accurate quantitation and identification along with the complexity of data interpretation are generally considered the major drawbacks compared with standard laboratory methods. However, the approach is strengthened by including a pooled quality control, correcting for analytical drift and therefore increasing the quantitative accuracy. The use of an in-house-library also ensures the highest possible level of confidence in identification of the signals identified as significantly important in the study.

Conclusion

Increased tau protein and low 5-MTHF in the CSF of the patient with KSS was found concomitant with the elevation of sphingomyelin C16:0 (d18:1/C16:0) and free sialic acid. The last two molecules were detected using an untargeted metabolomics approach. Our study may suggest the combination of elevated free sialic acid, sphingomyelin C16:0 (d18:1/C16:0), and tau protein with low 5-MTHF as potential new biomarkers for KSS. Together with genetic analysis, these findings may take us one step closer to early diagnosis of the disease. Further untargeted metabolomics studies on patients with KSS are strongly recommended.

Acknowledgments

References

1. Dugger BN, Dickson DW. Pathology of neurodegenerative diseases. Cold Spring Harb Perspect Biol. 2017;9, a028035.
2. Swerdlow RH. The neurodegenerative mitochondriopathies. J Alzheimer's Dis. 2009;17:737—751.
3. Kearns TP, Sayre GP. Retinitis pigmentosa, external ophthalmophegia, and complete heart block: unusual syndrome with histologic study in one of two cases. AMA Arch Ophthalmol. 1958;60:280—289.
4. Ryzhkova AI, Sazonova MA, Sinyov VV, et al. Mitochondrial diseases caused by mtDNA mutations: a mini-review. Ther Clin Risk Manag. 2018;14:1933.
5. Shemesh A, Margolin E. Kearns Sayre Syndrome. StatPearls. Treasure. Island FL: StatPearls Publishing; 2021.
6. Maceluch JA, Niedziela M. The clinical diagnosis and molecular genetics of kearns-sayre syndrome: a complex mitochondrial encephalomyopathy. Pediatr Endocrinol Rev. 2006;4:117—137.
7. Berenbaum F, Cote D, Pradat P, Rancurel G. Kearns-Sayre syndrome. Neurology. 1990;40:193.
8. Harvey J, Barnett D. Endocrine dysfunction in kearns-sayre syndrome. Clin Endocrinol. 1992;37:97—104.
9. Pavone P, Praticò AD, Pavone V, et al. Ataxia in children: early recognition and clinical evaluation. Ital J Pediatr. 2017;43:1—9.
10. Gupta SN, Marks HG. Spinocerebellar ataxia type 7 mimicking Kearns—Sayre syndrome: a clinical diagnosis is desirable. J Neurol Sci. 2008;264:173—176.
11. del Mar Amador M, Colsch B, Lamari F, et al. Targeted versus untargeted omics—the CAFSA story. J Inherit Metab Dis. 2018;41:447—456.
12. Mochel F, Sedel F, Vanderver A, et al. Cerebellar ataxia with elevated cerebrospinal free sialic acid (CAFSA). Brain. 2009;132:801—809.
13. Idland A-V, Wyller TB, Støen R, et al. Preclinical amyloid-β and axonal degeneration pathology in delirium. J Alzheimers Dis. 2017;55:371—379.
14. Skogvold HB, Sandås EM, Østeby A, et al. Bridging the polar and hydrophobic metabolome in single-run untargeted liquid chromatography-mass spectrometry dried blood spot metabolomics for clinical purposes. J Proteome Res. 2021;20:4010—4021.
15. Moraes CT, Ricci E, Petruzzella V, et al. Molecular analysis of the muscle pathology associated with mitochondrial DNA deletions. Nat Genet. 1992;1:359—367.
16. Finsterer J, Winklehner M, Stöllberger C, Hummel T. Unusual phenotype and disease trajectory in kearns—sayre syndrome. Case Rep Neurol Med. 2020;2020, 7368527.
17. Diniz AG, Saritaş T, Aktaş S, et al. A case of kearns-sayre syndrome (the importance of enzyme staining for the diagnosis of Childhood mitochondrial diseases). Turk J Pathol. 2009;25:053—056.
18. Khambatta S, Nguyen DL, Beckman TJ, Wittich CM. Kearns—Sayre syndrome: a case series of 35 adults and children. Int J Gen Med. 2014;7:325.
19. Yu M, Yu L, Wang Z-X. Diagnosis and management of Kearns-Sayre syndrome rely on comprehensive clinical evaluation. Chin Med J (Engl). 2016;129:2519—2520.
20. Szalardy L, Fort Molnár M, Török R, et al. Histopathological comparison of Kearns-Sayre syndrome and PGC-1α-deficient mice suggests a novel concept for vacuole formation in mitochondrial encephalopathy. Folia Neuropathol. 2016;54:9—22.

21. Brockington M, Alsanjari N, Sweeney M, et al. Kearns-Sayre syndrome associated with mitochondrial DNA deletion or duplication: a molecular genetic and pathological study. J Neurol Sci. 1995;131:78–87.
22. Zeviani M, Moraes CT, DiMauro S, et al. Deletions of mitochondrial DNA in kearns-sayre syndrome. Neurology. 1988;38:1339.
23. Pitceathly RD, Fassone E, Taanman J-W, et al. Kearns–Sayre syndrome caused by defective R1/p53R2 assembly. J Med Genet. 2011;48:610–617.
24. Alemi M, Prigione A, Wong A, et al. Mitochondrial DNA deletions inhibit proteasomal activity and stimulate an autophagic transcript. Free Radic Biol Med. 2007;42:32–43.
25. Schraen-Maschke S, Sergeant N, Dhaenens C-M, et al. Tau as a biomarker of neurodegenerative diseases. Biomark Med. 2008;2:363–384.
26. Grangeon L, Paquet C, Bombois S, et al. Differential diagnosis of dementia with high levels of cerebrospinal fluid tau protein. J Alzheimer's Dis. 2016;51:905–913.
27. Kontou M, Weidemann W, Bork K, Horstkorte R. Beyond glycosylation: sialic acid precursors act as signaling molecules and are involved in cellular control of differentiation of PC12 cells. Biol Chem. 2009;390:575–579.
28. Zhang L, Wei T-T, Li Y, et al. Functional metabolomics characterizes a key role for N-acetylneuraminic acid in coronary artery diseases. Circulation. 2018;137:1374–1390.
29. Bickel PE. Lipid rafts and insulin signaling. Am J Physiol Endocrinol Metab. 2002;282:E1–E10.
30. Lacalle RA, Mira E, Gómez-Moutón C, et al. Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation. J Cell Biol. 2002;157:277–289.
31. Stankiewicz TR, Linseman DA. Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration. Front Cell Neurosci. 2014;8:314.
32. Pike LJ. Rafts defined: a report on the Keystone Symposium on lipid rafts and cell function. J Lipid Res. 2006;47:1597–1598.
33. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997;387:569–572.
34. Hicks DA, Nalivaeva NN, Turner AJ. Lipid rafts and Alzheimer's disease: protein-lipid interactions and perturbation of signaling. Front Physiol. 2012;3:189.
35. Tapia-Rojas C, Cabezas-Opazo F, Deaton CA, et al. It's all about tau. Prog Neurobiol. 2019;175:54–76.
36. Pritchard SM, Dolan PJ, Vitkus A, Johnson GV. The toxicity of tau in Alzheimer disease: turnover, targets and potential therapeutics. J Cell Mol Med. 2011;15:1621–1635.
37. Polanco JC, Li C, Bodea L-G, et al. Amyloid-β and tau complexity—towards improved biomarkers and targeted therapies. Nat Rev Neurol. 2018;14:22–39.
38. Quntanilla RA, Tapia-Monsalves C. The role of mitochondrial impairment in Alzheimer's disease neurodegeneration: the tau connection. Curr Neuropharmacol. 2020;18:1076–1091.
39. Walter J, van Echten-Deckert G. Cross-talk of membrane lipids and Alzheimer-related proteins. Mol Neurodegener. 2013;8:1–12.
40. Meske V, Albert F, Richter D, Schwarze J, Ohm T. Blockade of HMG-CoA reductase activity causes changes in microtubule-stabilizing protein tau via suppression of geranylgeranylpyrophosphate formation: implications for Alzheimer's disease. Eur J Neurosci. 2003;17:93–102.
41. Hamano T, Yen SH, Gendron T, Ko LW, Kuriyama M. Pitavastatin decreases tau levels via the inactivation of Rho/ROCK. Neurobiol Aging. 2012;33:2306–2320.
42. Ditiatkovski M, Mukhamedova N, Dragoljevic D, et al. Modification of lipid rafts by extracellular vesicles carrying HIV-1 protein Nef induces redistribution of amyloid precursor protein and Tau, causing neuronal dysfunction. J Biol Chem. 2020;295:13377–13392.
43. Obeid R, Kasoha M, Knapp J-P, et al. Folate and methylation status in relation to phosphorylated tau protein (181P) and β-amyloid (1–42) in cerebrospinal fluid. Clin Chem. 2007;53:1129–1136.
44. Obeid R, Herrmann W. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. FEBS Lett. 2006;580:2994–3005.
45. Liu F, Zaidi T, Iqbal K, et al. Role of glycosylation in hyperphosphorylation of tau in Alzheimer's disease. FEBS Lett. 2002;512:101–106.
46. Nagamine S, Yamazaki T, Makioka K, et al. Hypersialylation is a common feature of neurofibrillary tangles and granulovacuolar degenerations in Alzheimer's disease and tauopathy brains. Neuropathol. 2016;36:333–345.
47. Cho A, Christine M, Malicdan V, et al. Sialic acid deficiency is associated with oxidative stress leading to muscle atrophy and weakness in GNE myopathy. Hum Mol Genet. 2017;26:3081–3093.
48. Thomas GH, Reynolds LW, Miller CS. Overproduction of N-acetylneuraminic acid (sialic acid) by sialuria fibroblasts. Pediatr Res. 1985;19:451–455.
49. Yamaguchi K, Hata K, Koseki K, et al. Evidence for mitochondrial localization of a novel human sialidase (NEU4). Biochem J. 2005;390:85–93.
50. Kamei H, Shimazaki K, Nishi Y. Computational 3-D modeling and site-directed mutation of an antibody that binds Neu2en5Ac, a transition state analogue of a sialic acid. Proteins. 2001;45:285–296.
51. D'Andrea G, Granella F, Cataldini M, Verdelli F, Balbi T. GABA and glutamate in migraine. J Headache Pain. 2001;2:S57–S60.
52. Wishart DS, Tzur D, Knox C, et al. HMDB: the human metabolome database. Nucleic Acids Res. 2007;35:D521–D526.