Published in final edited form as:

Exp Neurol. 2015 January ; 263: 102–112. doi:10.1016/j.expneurol.2014.09.020.

Biomarkers for disease progression and AAV therapeutic efficacy in feline Sandhoff disease

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Abstract

The GM2 gangliosidoses, Tay-Sachs disease (TSD) and Sandhoff disease (SD), are progressive neurodegenerative disorders that are caused by a mutation in the enzyme β -N-acetylhexosaminidase (Hex). Due to the recent emergence of novel experimental treatments, biomarker development has become particularly relevant in GM2 gangliosidosis as an objective means to measure therapeutic efficacy. Here we describe blood, cerebrospinal fluid (CSF), magnetic resonance imaging (MRI), and electrodiagnostic methods for evaluating disease progression in the feline SD model and application of these approaches to assess AAV-mediated gene therapy. SD cats were treated by intracranial injections of the thalami combined with either the deep cerebellar nuclei or a single lateral ventricle using AAVrh8 vectors encoding feline Hex.

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Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Significantly altered in untreated SD cats, blood and CSF based biomarkers were normalized after AAV gene therapy. Also reduced after treatment were expansion of the lysosomal compartment in peripheral blood mononuclear cells and elevated activity of secondary lysosomal enzymes. MRI changes characteristic of the gangliosidoses were documented in SD cats and normalized after AAV gene therapy. The minimally invasive biomarkers reported herein should be useful to assess disease progression of untreated GM2 patients and those in future clinical trials.

Keywords

Lysosomal storage disorder; neurodegenerative disease; gene therapy; biomarkers; hexosaminidase; GM2 ganglioside

Introduction

The GM2 gangliosidoses are lysosomal storage diseases caused by inherited deficiency in a hydrolytic enzyme, β -N-acetylhexosaminidase (Hex; EC 3.2.1.52), and clinically characterized by progressive neurological impairment resulting in premature death. Initially, failure to attain developmental milestones or loss of acquired skills signals the onset of clinical disease, which ultimately progresses to loss of all body faculties, a semi-vegetative state, and death by the age of five years. The GM2 gangliosidoses include both Tay-Sachs disease (TSD) and Sandhoff disease (SD), which are almost indistinguishable clinically. Hex functions in the ganglioside degradation pathway by cleaving the terminal N-acetylgalactosamine residue from GM2 ganglioside. Structurally, Hex is comprised of two subunits, α and β , which dimerize to form distinct isozymes. A mutation in *HEXA*, encoding the α subunit, leads to a deficiency of the HexA isozyme (α/β) and results in TSD. A mutation in *HEXB*, encoding the β subunit, leads to a deficiency in both HexA (α/β) and HexB (β/β) and causes SD.

Although mouse models are invaluable in biomedical research, their brain size is >1000 times smaller than an infant [1-3] and their ~2-year lifespan precludes longer-term studies. Feline models are a logical intermediate between mice and humans because of similarities in brain organization and neuropathology [4-7]. With a brain size 50× larger and more complex than mice, cats are also an important model for therapeutic evaluation [4-8].¹

A feline model of SD, first identified in the 1970s [9], has been well-characterized [10, 11] and presents an unparalleled opportunity to test therapeutic options for translation to humans. Human TSD and SD are caused by a variety of mutations with varying levels of endogenous protein [12], in contrast to knockout mouse models that express no residual protein [13]. Feline SD is caused by a 25-base-pair inversion in the terminal exon of *HEXB*, resulting in ~15% of normal protein levels and < 3% of normal enzyme activity, allowing

¹Abbreviations: adeno-associated virus (AAV); α -mannosidase (Mann); aspartate aminotransferase (AST); β -galactosidase (β gal); β -N-acetylhexosaminidase (Hex); blood urea nitrogen (BUN); brainstem auditory evoked response (BAER); central nervous system (CNS); cerebrospinal fluid (CSF); deep cerebellar nuclei (DCN); electroretinogram (ERG); intracerebroventricular (ICV); lactate dehydrogenase (LDH); Niemann-Pick C (NPC); peripheral blood mononuclear cell (PBMC); Sandhoff disease (SD); Tay-Sachs disease (TSD); thalamus (Thal);.

for therapeutic assessment of a severe phenotype in the presence of minimally functional endogenous enzyme [10].

Adeno-associated virus (AAV) gene therapy has shown great promise in both murine and feline models of SD. With an untreated life span of ~18 weeks, SD mice treated with bilateral injections of the striatum and deep cerebellar nuclei (DCN) with monocistronic vectors encoding human Hex α - and β -subunits survived up to 2 years, the maximum age permitted by institutional animal welfare mandates [14,15]. SD cats treated with the same vectors with bilateral injection to the thalamus alone lived to 7.0 and 8.2 months of age, compared with an untreated life span of 4.5 ± 0.5 months ($n = 11$). Due to a marked humoral immune response to human Hex, SD cats were subsequently treated with feline-specific vectors and lived to 10.4 ± 3.7 months of age ($n = 3$), or 2.3 times as long as untreated cats [16]. While injection to the thalamus significantly increased lifespan, this injection route failed to treat the cerebellum. Additional routes of delivery to treat the cerebellum in SD cats are under investigation. When SD cats were treated by bilateral injection of the thalamus and DCN, Hex activity reached supranormal levels throughout the brain (2.7- to 45-fold normal) and spinal cord (4.2- to 14-fold normal), with reduction of GM2 ganglioside storage by 72 – 100 % [73]. When direct DCN injections were replaced with intracerebroventricular (ICV) delivery via the lateral ventricle, results were similar and have been reported in preliminary form (McCurdy, 2013). Results in both murine and feline studies support the therapeutic potential of AAV vectors for SD.

Interpretation of therapeutic efficacy in animal models and human patients would benefit from the establishment of minimally invasive, highly sensitive, reproducible methods of tracking disease progression. Such biomarkers should correlate with results of routine neurological exams and other clinical evaluations, yet provide easy and objective measures of clinical disease. To date, a few prospective biomarkers have been identified in murine models and human SD patients, but as they are technically difficult and time consuming, they have not been adopted into clinical practice [17].

Lysosomal storage diseases (LSD) share many pathophysiologic features (e.g. neurodegeneration), which make biomarkers developed and validated in one LSD potentially useful for others. Elevations of aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) have been noted in serum and CSF of patients with Tay-Sachs disease [18-20] and canine GM1 gangliosidosis [21]. After development in a mouse model of Niemann-Pick C (NPC) disease [22], a fluorescent, acidotropic probe (LysoTracker) has recently been validated as a biomarker in more than 100 NPC patients [23]. LysoTracker fluorescence increased with expansion of acidic compartments such as lysosomes, correlated with age and disease progression in untreated patients, and decreased in response to substrate reduction therapy.

To date, limited research has been conducted on biomarkers of SD and no results have been reported in a large animal model. In this study we evaluate biomarkers for feline SD and validate these biomarkers after efficacious intracranial AAV gene therapy. To our knowledge, this is the first effort to investigate the validity of a number of minimally

invasive measures to track disease progression and therapeutic response after AAV gene therapy in a large animal model of SD.

Materials and Methods

Animals and Surgery

All animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Bilateral injection of the thalamus and DCN were performed according to a previously published protocol [16]. ICV injection was performed under ultrasound guidance to confirm the correct placement of the injection needle. After visualization of the left lateral ventricle with an 8-5 MHz Philips HDI 5000 ultrasound probe (Philips Healthcare, Andover, MA), a single entry site was made through the skull with a 20G spinal needle. Vector was then delivered using a Hamilton syringe (Harvard Apparatus, Holliston, MA) fitted with a 25G non-coring needle (Harvard Apparatus). A total of 200uL was delivered in 10-15 uL aliquots at a rate of 3-5 uL/second with approximately 1 minute between aliquots. Cats were treated at 4-7 weeks of age, prior to symptom onset.

At humane or a predetermined endpoint animals were euthanized by pentobarbital overdose (100 mg/kg) and transcardially perfused with cold, heparinized saline. The brain was divided into coronal blocks of approximately 0.6 cm from the frontal pole through the cerebellum. The right hemisphere of the brain was preserved in optimal cutting temperature (OCT) medium for determination of enzyme activity. The left hemisphere of the brain and all other tissues were formalin fixed or flash-frozen in liquid nitrogen and preserved at -80 °C.

AAV Vectors

The feline HEXA/B cDNAs were cloned into an AAV backbone as previously described [16]. Feline HEX transgene expression was driven by the hybrid CBA promoter including the CMV immediate-early enhancer fused to the chicken β -actin promoter [24]. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was included for enhancement of gene expression. AAVrh8 vector stocks encoding feline Hex subunits were produced as previously described [25].

Blood and cerebrospinal fluid analysis

Blood was collected from the jugular vein at predetermined time points after tranquilization with dexmedetomidine (0.4mg/kg IM). Blood for complete blood count was put into EDTA tubes and analyzed using ADVIA 120 Hematology System (Seimens Medical Solutions, Malvern PA). Blood for serology was placed in heparinized tubes and analyzed using a Cobas C311 chemistry analyzer (Roche Hitachi, Basel, Switzerland, Tokyo, Japan). For quantification of echinocytes, Diff-Quick stained blood smears were scanned on an Aperio ScanScope CS2 (Vista, CA) and 3 40 $\times$ magnification fields were counted.

CSF collection was performed while under general anesthesia. Cats were anesthetized using dexmedetomidine (0.4mcg/kg IM) and ketamine (10mg/kg IM), underwent tracheal intubation and anesthesia was maintained using isoflurane (0.5-2%). CSF was collected

from the cerebellomedullary cistern as previously described [26]. CSF samples were collected and aliquots were analyzed for cytology and total protein. Samples were then immediately centrifuged at $100\times g$ for 5 minutes to remove any blood contamination. Samples then underwent one freeze thaw cycle at -80°C and were analyzed using Cobas C311 chemistry analyzer (Roche Hitachi, Basel, Switzerland & Tokyo, Japan).

LysoTracker® staining and quantification of PBMCs

PBMCs were isolated from whole blood with Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Isolated PBMCs were incubated with 200 nM of LysoTracker® Green (Molecular Probes, Grand Island, NY) for 20 minutes in the dark, centrifuged for 5 minutes at $200\times g$ in an IEC HN-SII centrifuge (Damon/IEC, Needham Heights, MA), and resuspended in 250 μL of MACS buffer. Samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences, San Jose, California) and data collected using CFlow software. Further sorting of select samples was conducted using MoFlow XDP cell sorter (Beckman Coulter, Brea, CA) and differential cell counts were performed on prepared cytopins.

Enzyme Assay

The activity of lysosomal enzymes was determined using a previously published protocol [11]. The fluorescence of 4-methylumbelliferyl (4-MU) substrates (β -gal, 0.5 mmol/l 4-MU- β -D-galactoside, pH 3.8; HexA, 1 mmol/l 4-methylumbelliferyl 6-sulfo-2-acetamido-2-deoxy- β -D-glucopyranoside (MUGS), pH 4.2; total Hex, 1 mmol/l 4-MU-N-acetyl- β -D-glucosaminide (MUG), pH 4.3; and α -mannosidase, 2 mM 4-MU- α -D-manopyranoside, pH 4.2) was measured at an excitation/emission of 360 nm/450 nm on a BioTek Synergy HT plate reader (BioTek, Winooski, VT). Specific activity was expressed as nmol 4-MU cleaved/mg protein/hour.

1.5T MRI Quantification

MRIs were performed in SD cats at humane endpoint and in AAV-treated cats at 16 weeks post treatment. Cats were anesthetized with dexmedetomidine (0.4mcg/kg) and ketamine (10mg/kg), and maintained on 0.5-2% isoflurane after tracheal intubation. All MRI scans were performed on an Infinion 1.5 Tesla MRI scanner (Phillips Healthcare, Andover, MA) using a lower extremity quadrature coil (Phillips Healthcare, Andover, MA). Scan parameters were as follows: 2D transverse T2 weighted turbo spin echo (TSE) images with 5mm^3 resolution, TR/TE of 4072/141.7 ms, echo train length of 16, BW of 20.8 KHz/pix, and 3 averages.

MRI based cortical and cerebellar changes were quantified using pixel intensity measurement. Pixel intensities were measured in quadruplicate at the individual area of interest. The average pixel values were then normalized to the intensity of CSF, also measured in quadruplicate, on that particular slice. All measurements were performed using the probe tool included in EFilm 3.2 software (Merge Healthcare, Chicago, IL, USA).

BAER

BAER assessments were performed in SD cats at 8-12 weeks and at humane endpoint (approximately 16-23 weeks). Cats were sedated with 0.4mcg/kg dexmedetomidine and BAER was performed using a Nicolet VikingQuest electrodiagnostic instrument (CareFusion, San Diego, CA). BAER was performed using earplug and hollow tubing auditory transmission apparatus, a click stimulus of 11.4 Hz at 75dB, contralateral noise at 40dB. Electrode placement was as previously described [27] and is as follows: mastoid reference (negative electrode), vertex reference (positive electrode) and ground placed on midline at the level of the occipital condyles. Response to click stimulus was acquired over 1.5 ms sweep with an amplitude of 0.2 μ V and 1000 averages. Measurements were repeated 3 times per side per time point. Peak identification was performed as previously described [28, 29].

ERG

ERGs were performed at 12 weeks and humane endpoint in SD cats. Ophthalmologic examination was performed and eyes were dilated using 1-2 drops tropicamide (1% solution). Cats were then sedated using dexmedetomidine (0.4mcg/kg) intramuscularly. Cats were dark adapted for 15 minutes prior to the procedure. Scotopic ERGs were recorded using a BPM-100 instrument (Retiongraphics, Norwalk, CT) with an active corneal contact lens electrode (Bausch & Lomb, Rochester, NY). The reference and ground electrodes were placed subcutaneously at the lateral canthus and skull vertex. This procedure was performed twice per eye per time point.

Results

Biomarker analysis was performed on untreated SD and normal control cats from birth to 23 weeks of age, the approximate humane endpoint for SD cats. Seven additional SD cats were treated with a 1:1 formulation of monocistronic AAVrh8 vectors encoding feline Hex α and β subunits (AAVrh8-fHEXA/B). SD cats were divided into two treatment cohorts based on injection route: bilateral thalamic and DCN injection (Thal+DCN, n=3) or bilataraal thalamic and ICV injection (Thal+ICV, n=4). For most analyses, animals were segregated into 3 age groups reflective of disease severity: 0-5 weeks, presymptomatic; 8-12 weeks, mild disease; 16-23 weeks, moderate to severe disease. All samples in the 0-5 week age group were from untreated animals, even for cats ultimately assigned to treatment cohorts.

Cerebrospinal fluid analysis

Routine CSF analysis revealed significant elevation of AST activity in SD cats. Elevated AST activity in CSF was detected as early as 0-5 weeks of age compared to normal controls ($p < 0.01$) and continued to increase with disease progression. Complete normalization of CSF AST was documented in the Thal+DCN treatment group by 16-23 weeks. In the Thal+ICV treatment group there was a large decrease in CSF AST levels ($p < 0.01$ compared to untreated) without complete normalization ($p < 0.05$ compared to normal) (Fig. 1a). The increase in CSF LDH activity in SD cats became statistically significant at 8-12 weeks of age ($p < 0.01$). LDH levels in CSF were normal in both AAV treatment groups at 16-23 weeks of age (Fig. 1b). CSF cytology, creatine kinase activity, total protein, and total

calcium were also tested in SD and normal cats, but differences were not noted. Normal AST and LDH levels were established using cats of all ages, and levels remain constant across all age ranges.

Blood chemistry and complete blood count

Analysis of 18 routine blood chemistry analytes revealed several values that were significantly altered in untreated SD cats. AST activity steadily rose in SD cats and was significantly different from normal control cats at 16-23 weeks of age ($p < 0.01$). After treatment with AAVrh8-fHEXA/B, SD cats showed complete normalization of AST at 16-23 weeks regardless of injection route (Fig. 2a). Unlike CSF, blood LDH levels were not different between normal and SD cats at any time point (data not shown). Mild but significant reductions were seen in albumin concentration by 8-12 weeks of age in SD cats compared to normal controls ($p < 0.01$), and by 16-23 weeks of age both AAV treatment groups showed normal albumin levels (Fig. 2b). Similarly, calcium was significantly decreased by 8-12 weeks of age in SD cats when compared to normal controls ($p < 0.05$), but was normalized by 16-23 weeks subsequent to treatment in the Thal+DCN cohort (Fig. 2c). The earliest difference was a reduction in serum cholesterol levels, which reached a significance of $p < 0.01$ in SD cats 0-5 weeks of age, compared to normal controls, and continually decreased with disease progression. In the Thal+DCN treatment group there was no improvement in cholesterol levels at any time point, while there was partial normalization by 16-23 weeks of age in the Thal+ICV treatment group (Fig. 2d). Blood Urea Nitrogen (BUN) concentration was elevated in untreated SD cats at humane endpoint ($p < 0.01$; data not shown). Mean platelet volume was increased in SD cats at 16-23 weeks and was restored to normal levels in the Thal+DCN treatment group ($p < 0.05$) but not in the Thal+ICV treatment group (data not shown).

Complete blood count data showed mild to moderate anemia with significant reduction in red blood cell count in SD cats by humane endpoint ($p < 0.01$; Fig. 2e), without significant changes in the hemoglobin content or red blood cell size (data not shown). Also there was a slight increase in neutrophils in untreated SD cats by 5 weeks of age compared to normal cats of the same age ($p < 0.05$) (Fig. 2f). Neutrophil counts continued to increase with disease progression, reaching greater significance ($p < 0.01$) by 8-12 weeks of age, and levels stabilized until the humane endpoint. At the last data point analyzed (16-23 weeks of age), neutrophil counts were normal in the Thal+ICV treatment group, but were significantly higher than normal in the Thal+DCN treatment group ($p < 0.05$).

LysoTracker analysis of PBMCs

To determine whether expansion of the lysosomal compartment itself could serve as a marker of disease progression, we measured relative LysoTracker fluorescence in peripheral blood mononuclear cells (PBMCs) beginning at 8 weeks of age and successively in four week intervals until SD cats reached the humane endpoint. LysoTracker is a fluorescent, acidotropic probe used to selectively label acidic organelles in live cells to examine the biosynthesis or pathogenesis of lysosomes. At 8 weeks of age, the average percent of LysoTracker positive PBMCs in SD cats was 25.6%, progressively increasing to 47.9% at humane endpoint ($n=5$, 20.6 ± 1.3 weeks) (Fig. 3a). In contrast, normal control littermates

maintained between 6.5% and 10.3% of LysoTracker positive PBMCs for the duration of the experiment. Histological analysis of sorted, LysoTracker positive cells revealed that > 65% were granulocytes (Fig. 3c), a common contaminant in PBMC populations isolated by density gradient centrifugation. However, there was no difference in the percentage of granulocytes in PBMC populations from normal ($2.87\% \pm 0.03$) and SD cats ($2.68\% \pm 0.05$), indicating that increased numbers of LysoTracker positive cells were not due to an increased proportion of granulocytes in SD samples after density gradient isolation. In the Thal+ICV treatment group there was partial normalization of the lysosomal compartment, averaging 37.5% LysoTracker positive cells ($n=3$, 22.3 ± 1.0 weeks) at sixteen weeks post-injection (Fig. 3b). However, this value was significantly higher than normal control cats ($n=5$, $p < 0.05$) and not significantly lower than SD cats ($p > 0.05$). Statistical analysis was not conducted for the Thal+DCN treatment group due to low sample numbers for this assay ($n=2$).

Echinocytosis

Analysis of blood smears of SD cats revealed an abnormally large population of echinocytes, or spiculated red blood cells. In SD cats 0-5 weeks of age, echinocytes comprised 25.3% of all red blood cells (Fig. 4a). At 8-12 weeks of age, the percentage of echinocytes increased to > 50% of all red blood cells and this was maintained until SD cats reached the humane endpoint. In contrast, echinocytes comprised 4% of the red blood cell population in normal control cats. At humane endpoint, the percentage of echinocytes in SD cats was significantly greater ($p < 0.05$, $n=4$) than normal control cats (Fig. 4c, d). Thal +DCN injection of AAV failed to attenuate echinocyte formation, while Thal+ICV injection showed partial normalization and large variability within the cohort (range 2.1% - 54.0%) 16 weeks post treatment (Fig. 4b, e, f). Statistics were not conducted on the treatment groups due to few data points available ($n=2$ each).

Peripheral lysosomal enzyme activity and histopathology

In the absence of β -N-acetylhexosaminidase, other lysosomal enzymes are upregulated by an incompletely defined mechanism. Specifically, β -galactosidase (β gal) and α -mannosidase (Mann) activities are elevated in numerous tissues of SD cats. Enzyme activity measured in PBMCs from SD cats showed a 2-fold increase in β gal and a 2.5-fold increase in Mann when compared to normal controls. Sixteen weeks after AAV-mediated gene therapy, activities of both enzymes were decreased, but to varying degrees. At 16 weeks after treatment, the degree of reduction of β gal and Mann in PBMCs appeared to correlate with the level of Hex restoration in the brain and liver (Fig. 5a). Specifically, cat 7-972 attained the highest Hex activity at the thalamic injection site, reaching 92-fold normal, and also had complete normalization of Hex activity in the liver. In PBMCs, β gal activity was completely normalized and Mann was reduced more than 75% compared to untreated SD cats. In contrast to 7-972, a cat with ~one-half as much Hex activity at the thalamic injection site (7-957) and only 57% of normal Hex activity in the liver did not have any reduction of β gal and Mann levels in PBMCs.

In SD cats, approximately 95% of hepatocytes contained large, discrete, intracytoplasmic vacuoles which displaced the nucleus of the affected cell peripherally (Fig. 5b). Additionally

the stroma of the portal triads was mildly edematous and rare Kupffer cells adjacent to the sinusoids were swollen and contained fine, clear cytoplasmic vacuoles. In contrast, normal cat hepatocytes lacked vacuolation and had centrally located nuclei (Fig. 5c). Sixteen weeks after intracranial AAV therapy, cat 7-972 had normal levels of Hex activity in the liver and architecture was largely normalized, with some hepatocytes containing multiple, small intracytoplasmic vacuoles (Fig. 5d). In comparison, vacuolated hepatocytes were more prevalent in cat 7-957 (Fig. 5e), which had reduced Hex activity in both the brain and liver.

MRI

Alterations in brain architecture were evaluated non-invasively using MRI. T2 weighted MRI of SD cats imaged at humane endpoint (4.5 ± 0.5 months) showed profound brain atrophy as well as indistinguishable or inverted gray matter and white matter intensities (Fig. 6b). Changes of signal intensity ($p < 0.05$) were noted within the olfactory bulb, precuneate gray matter, caudate nucleus (Fig. 6e), caudal colliculus, white matter of the temporal lobe and occipital cortex at the level of the midbrain (Fig. 6f), corpus callosum, suprasplenial gyrus gray matter and white matter at the level of the thalamus (Fig. 6g), DCN and ventral brainstem (Fig. 6h). Sixteen weeks after AAV gene therapy, brain architecture was normalized as measured by preservation of gray matter and white matter intensities and prevention of cortical atrophy. Signal intensities were restored to normal in the Thal+DCN group in the majority of brain areas, with partial normalization in the DCN. Statistical analysis was performed only for the Thal+DCN cohort ($n=3$), since only 1 animal from the Thal+ICV cohort was imaged (Fig. 6c-d).

BAER and ERG

To characterize auditory pathway abnormalities in feline SD, brainstem auditory evoked response (BAER) was measured at 8-12 weeks and 16-23 weeks (humane endpoint) of age. In untreated SD cats, increased latencies were observed in waves II, III and V at 16-23 weeks and in waves II and V at 8-12 weeks, correlating with neurologic disease progression (Fig. 7a). Interwave latencies of I - III were significantly increased in SD cats at 16-23 weeks and interwave latencies for III-V and I-V were increased at both 8-12 and 16-23 weeks of age (Fig. 7b). BAER was not measured in AAV-treated SD cats.

Scotopic electroretinograms (ERG) revealed no significant difference ($p > 0.05$) between the amplitude or implicit times of the A or B waves in SD cats when compared to normal. The implicit time of the A wave was 17.1 ± 2.5 ms, 16.5 ± 1.9 ms, and 15.3 ± 0.5 ms for SD cats at 8-12 weeks, SD cats at 16-23 weeks, and normal kittens of similar age, respectively. The implicit times for the B waves were 58.0 ± 7.8 ms, 58.6 ± 3.7 , and 56.1 ± 3.0 ms for SD cats at 8-12 weeks, SD cats at 16-23 weeks, and normal kittens of similar age, respectively. The amplitude for the A wave was -30.4 ± 38.3 μV, -26.4 ± 47.3 μV and -20.6 ± 12.5 μV for SD cats at 8-12 weeks, SD cats at 16-23 weeks, and normal kittens of similar age, respectively. The amplitude of the B wave was 159.2 ± 47.1 μV, 119.8 ± 39.2 μV, and 145.2 ± 56.6 μV for 8-12 week old SD cats, SD cats at 16-23 weeks, and normal cats, respectively.

Discussion

In lysosomal storage diseases, patient evaluation historically has been restricted to disease etiology and severity. With the development of new therapeutics, like enzyme replacement, chaperones, substrate reduction and gene therapy, biomarker discovery is of increasing importance for objective assessment of treatments. Biomarkers are particularly relevant to TSD and SD, where only small cohorts of patients are available to participate in clinical trials. In these cases, effective biomarkers could be used to compensate for limited patient numbers by providing physicians with validated methods for evaluation [30]. Here we described multimodal biomarkers in a feline model of SD and applied these biomarkers in a therapeutic setting.

The natural history of neurologic disease progression in SD cats consists of a profound whole body tremor, spasticity and progressive neurologic decline [9]. Sixteen weeks after pre-symptomatic gene therapy, SD cats did not exhibit profound neurologic deficits and were almost indistinguishable from normal controls. AAV-treated cats developed mild hind limb weakness, but subsequently disease progression in treated cats was stabilized. At the study endpoint of 16 weeks post-injection, subtle tremors of the head and tail were present in all AAV-treated cats; however, none developed overt whole body tremors, which are evident in untreated SD cats at an early age [73]. Biomarkers described herein reflect changes in the neurologic state of untreated SD cats as well as amelioration of disease after AAV gene therapy.

CSF and blood based biomarkers

Clinicopathologic parameters measured here are already regularly used in medicine. AST (EC 2.6.1.1) is a ubiquitously expressed enzyme that catalyzes the interconversion of aspartate and α -ketoglutarate to oxaloacetate and glutamate and, in the CNS, functions to manage levels of excitatory neurotransmitters. Elevations of AST in the central nervous system previously have been reported in human GM2 gangliosidosis, Alzheimer's disease, canine GM1 gangliosidosis, brain insult and stroke and are thought to be due to leakage from neurons and other CNS cells [18,19,21,31,32]. Similarly, LDH (EC 1.1.1.27 & EC1.1.1.28), which catalyzes the interconversion of pyruvate and lactate, is ubiquitously released from all cells after damage to the plasma membrane. LDH is thought to be a biochemical index of neuronal cytotoxicity [33] with elevations in a wide array of neurological disorders including meningitis [34], prion disease [35], leukemia [36], trauma [37], infantile spasms [38], stroke [31] and canine GM1 gangliosidosis [21]. LDH has been used to distinguish various neurological disorders [37,39]. In our feline SD model, levels of AST and LDH in CSF correlate with disease progression and therapeutic impact after gene therapy. Plasma AST levels also reflect neurologic status in the SD cat, but plasma LDH was not informative.

Albumin, the main protein component of plasma, is reduced in human GM2 gangliosidosis [19] and in SD cats by 8-12 weeks of age. Intracranial delivery of AAV-gene therapy resulted in restoration of plasma albumin levels. Calcium levels trended similarly, with reduced levels in the SD cat and restoration after AAV gene therapy, but its changes are likely a direct consequence of reduced albumin-calcium binding in circulation.

Unfortunately, ionized calcium was not assessed. Altered calcium homeostasis is a common feature of lysosomal storage diseases [40, 41], and elevated cytosolic calcium has been shown in SD mice and attributed to an endoplasmic reticulum defect [42]. Hepatic disease, as suggested in Figure 5, is present in both human [43] and feline [6] SD, and is a potential cause of albumin reduction. The precise role of albumin remains unknown, but interestingly human serum albumin has been suggested to promote neuronal survival through production of glutamate [44], to prevent formation of amyloid β self-association in Alzheimer's disease [45], and to improve outcomes in models of focal cerebral ischemia [46-48].

Circulating cholesterol was significantly decreased in the presymptomatic SD cat (< 5 weeks of age), and reduction continued with onset and advancement of neurologic signs. Restoration was observed at 16-23 weeks of age in the Thal+ICV cohort, implicating it as a potential biomarker of SD. Cholesterol is a secondary storage product in many LSDs, including GM2 gangliosidosis, and is thought to be sequestered in the cell due to its affinity for sphingolipids [49]. Niemann-Pick type C cultured fibroblasts exhibit a marked reduction in exogenous cholesterol, resulting from increased intracellular storage [50]. These reports suggest that cholesterol may be taken up and sequestered intracellularly in the SD cat, resulting in low plasma levels. Future experiments will evaluate intracellular cholesterol sequestration.

The red blood cell membrane of the TSD patient contains many alterations, including changes in lipids, glycoprotein and protein levels [51-55], and altered erythrocyte membrane composition has been reported to increase susceptibility to oxidative stress and echinocytosis [56, 57]. This change in RBC morphology is associated with apoptosis and phagocytosis by macrophages and is a potential etiology for anemia [58, 59]. Anemia is a prominent finding in several lysosomal storage disorders, including Gaucher disease [60] and Niemann-Pick type C [50], and is used as a powerful biomarker to score severity and need for enzyme replacement therapy [61]. Our findings suggest that this may also be an applicable clinical test in SD, though anemia may have numerous causes in storage diseases and its response to gene therapy in the current study is ambiguous.

Complete blood counts revealed nearly a threefold increase of neutrophils in the blood of SD cats at humane endpoint. Neutrophilia is also observed in the Gaucher mouse, with a hypothesized etiology of intracellular storage in antigen presenting cells and release of granulocyte colony stimulating factors to mobilize hematopoietic progenitor cells. Additionally, neutrophil recruiting chemokines CXCL-1 and -2 were also detected in sera from Gaucher mice [62]. Levels of CXCL and granulocyte colony stimulating factors have not been measured in SD cats but could further elucidate the cause of neutrophilia.

Of the standard parameters evaluated in the current study, AST and LDH levels in CSF are likely the most robust biomarkers, but the invasiveness of this test may reduce its use in clinical practice. Serum AST mimics CSF levels and, although elevations of serum AST can be non-disease specific, its use is supported by similar increases in human GM2 patients [19]. Serum cholesterol is among the most sensitive blood-based biomarker that we report, with significant reductions by 5 weeks of age and continued decline with disease severity.

As a known storage product in gangliosidosis, cholesterol may prove to be a sensitive indicator of lysosomal dysfunction.

A unique and potentially powerful indicator of lysosomal pathology, LysoTracker recently has been validated as a biomarker in the mouse model of NPC and more than 100 NPC patients. te Vruchte et al demonstrated elevation of the acidic compartment of circulating CD19+ B cells, which correlated with age and disease progression [23]. Marked expansion of LysoTracker positive cells to nearly fivefold normal was demonstrated in PBMCs of SD cats as well. Sixteen weeks after AAV gene therapy, LysoTracker staining was only marginally reduced, indicating failure to affect storage in peripheral blood cells. This finding was in agreement with background levels of Hex in PBMCs after AAV therapy, 1.69% of normal (n=3), which is comparable to untreated controls (1.84% of normal, n=5). In contrast, substrate reduction therapy with miglustat substantially reduced LysoTracker levels in NPC [23], which may not be surprising since miglustat was administered systemically and gene therapy in the current study was administered intracranially.

Partial correction of the periphery after intracranial delivery of gene therapy has been described in mice with mucopolysaccharidosis IIIA [63] and neuronal ceroid lipofuscinosis [64], and distribution of AAV to peripheral tissues has been described in nonhuman primates after intraparenchymal brain injection [65]. Similarly, our data suggest that intracranial injection of AAV can lead to correction of pathology in peripheral organs, particularly liver, but variability of results in the current study will require optimization of the delivery method in future experiments. In addition, graded reduction of secondary lysosomal enzyme activity (β gal, Mann) in PBMCs that reflects the degree of clinical benefit in the liver and/or CNS is enticing, but larger numbers of treated animals are required to validate these potential biomarkers.

Clinical biomarkers

Based on data reported herein, clinical tests such as MRI can be used to assess therapeutic efficacy of AAV mediated gene therapy in GM2 gangliosidosis. Human and feline GM2 gangliosidosis follow characteristic patterns of neurodegeneration, which on T2 weighted images show increased signal in the white matter and decreased signal in gray matter [66-68]. As glycosphingolipid storage increases in neuronal cell bodies of the gray matter and as the white matter undergoes demyelination [68], these two tissues become indistinguishable on MRI, or isointense. When glycosphingolipid accumulation in gray matter exceeds the lipid composition of white matter, an inversion of intensities is observed [66-68]. AAV gene therapy resulted in significant normalization of intensities in most brain areas regardless of injection route, suggesting that MRI will continue to be a valuable tool in evaluating success of novel therapeutics for GM2 gangliosidosis. Currently underway is evaluation of the SD cat at higher field strengths to provide more anatomical detail and advanced techniques like MR spectroscopy to map metabolites in the diseased brain.

Most SD and TSD patients develop deafness and blindness [69,70], so we tested hearing and sight in SD cats using BAER and ERG, respectively. In the cat, BAER peaks I, II, III and V are associated with the cochlear nerve, cochlear nucleus, nucleus of the trapezoid body, and caudal colliculus, respectively [28,71]. BAER tracings from SD cats suggest normal

function of the cochlear nerve but abnormalities in the cochlear nucleus, the nucleus of the trapezoid body and the caudal colliculus (Figure 7). These data correlate with findings in human patients [72], suggesting the cat is an appropriate model to study electrophysiology of the auditory pathway. Further electrodiagnostic similarities were found in the visual pathways of GM2 cats and human patients, in which ERGs were within normal limits [72]. Normal ERGs point to a non-photoreceptor etiology for decreased vision, and abnormalities may be detectable in future studies by using visually evoked potentials. Though BAER and ERG analyses were not performed on AAV-treated cats in the current study, a foundation was laid for future testing of the special senses after gene therapy, an important consideration for patients' quality of life.

Summary

Minimally invasive biomarkers are particularly relevant for orphan diseases, like GM2 gangliosidosis, with reduced numbers of patients. Regulatory procedures designed for more common diseases pose difficulty in small cohorts because of the time required to reach sufficient power. Therefore, development of novel therapeutics for rare diseases will benefit from more rigorous scrutiny using sensitive, validated biomarkers to monitor therapeutic response [30]. Here we describe clinically translatable biomarkers of feline GM2 gangliosidosis and describe their use in assessment of SD cats after AAV gene therapy. It is our hope that the biomarkers described herein will provide insight into the effects of AAV treatment on neurodegeneration and assist physicians in assessment of therapeutic efficacy in future clinical trials.

Acknowledgments

This work was funded by National Institutes of Health grant U01NS064096, the National Tay-Sachs and Allied Diseases Association, Horace W. Goldsmith Foundation, and the Scott-Ritchey Research Center. F.M.P. is a Royal Society Wolfson Research Merit Award holder.

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Highlights

- Clinically relevant biomarkers objectively evaluate feline Sandhoff disease.
- Blood, CSF, liver, MRI and electro-diagnostics are utilized to track disease.
- Intracranial AAV mediated gene therapy normalizes many of these objective measures.
- Minimally invasive biomarkers will inform future human clinical trials.

Fig. 1.

CSF levels of a) Aspartate aminotransferase (AST) and b) Lactate dehydrogenase (LDH) in CSF of normal, Sandhoff (SD), and SD cats 16 weeks after AAV gene therapy by Thal+DCN and Thal+ICV injection routes. Levels of AST and LDH significantly increase over time in the SD cat. AST and LDH are restored to normal levels after AAV gene therapy by Thal+DCN and Thal+ICV injection routes. Normal cats: n=14; SD cats: 0-5 weeks, n=9; 8-12 weeks, n=10; 16-23 weeks, n=9; Thal+DCN: 16-23 weeks, n=6; Thal+ICV: 16-23 weeks, n=6. (*denotes $p<0.05$ from normal, **denotes $p<0.01$ from normal, † denotes $p<0.05$ from SD, ‡ denotes $p<0.01$ from SD; error bars represent standard deviation)

Fig. 2.

Blood chemistry findings in normal, Sandhoff (SD), and SD cats 16 weeks after AAV gene therapy by Thal+DCN and Thal+ICV injection routes. a) Aspartate aminotransferase (AST) levels increase over time in SD cats when compared to normal age-matched controls and are normalized after AAV gene therapy by 16-23 weeks in both Thal+DCN and Thal+ICV cohorts. b) Albumin levels decrease with time in the SD cats and are normalized by 16-23 weeks of age in AAV-treated cats. c) Calcium levels also decrease over time, with normalization in the Thal+DCN group by 16-23 weeks of age. d) Blood cholesterol levels decrease over time and are only partially normalized in the Thal+ICV cohort at 16-23 weeks. e) Red blood cell number is decreased in SD cats. f) Neutrophils in SD cats were significantly increased, as compared to normal controls, at all time points. Treatment with AAV by Thal+ICV, but not Thal+DCN, returned the neutrophil count to normal levels. Normal cats: 0-5 weeks, n=10; 8-12 weeks, n=9; 16-23 weeks, n=8; SD cats: 0-5 weeks,

n=9, 8-12 weeks, n=11, 16-23 weeks, n=17 (except BUN n=14); Thal+DCN: 0-5 weeks, n=13; 8-12 weeks, n=9; 16-23 weeks, n=10; Thal+ICV: 0-5 weeks, n=6; 8-12 weeks, n=6; 16-23 weeks, n=8 (except AST n=7). (*denotes $p < 0.05$ from normal, **denotes $p < 0.01$ from normal, † denotes $p < 0.05$ from SD, ‡ denotes $p < 0.01$ from SD; error bars represent standard deviation)

Fig. 3.

LysoTracker quantification in peripheral blood mononuclear cells (PMBCs). a) Percent of positive cells increased with age in SD cats and was significantly higher than normal control cats by 12 weeks of age (8 weeks, n=5; 12 weeks, n=6; 16 weeks, n=6; endpoint, n=5). b) Thal+ICV gene therapy decreased the percentage of LysoTracker positive cells (n=3, 22.3 ± 1.0 weeks), yet values remained significantly higher ($p < 0.05$) than normal cats (n=5) at that time point. Results from the Thal+ICV cohort were not significantly different from SD cats ($p > 0.05$). Analysis was not conducted for the Thal+DCN treatment group due to low animal numbers (n=2). c) Cytopsin from sorted LysoTracker positive cells consisted primarily of neutrophils (scale bar = 50 μ M; inset scale bar = 10 μ M). (*denotes $p < 0.05$ from normal, **denotes $p < 0.01$ from normal, † denotes $p < 0.05$ from SD, ‡ denotes $p < 0.01$ from SD; error bars represent standard deviation)

Fig. 4. Echinocytes (spiculated red blood cells) in SD cats. a) In SD cats, echinocyte formation increases by 8-12 weeks of age (n=4) and b) remains elevated after AAV gene therapy (normal, n=4; SD, n=4; Thal+DCN, n=2; Thal+ICV, n=2). Representative photomicrographs show increased echinocytosis in the c) SD cat as compared to d) normal. Echinocyte levels appear elevated in the e) Thal+DCN and intermediate in the f) Thal+ICV groups. There was large variability in echinocytosis for the Thal+ICV cohort (range 2.1% - 54.0%). Nucleated cells in the field of view (neutrophils, lymphocytes and monocytes) are part of a typical feline blood smear. (*denotes $p < 0.05$ from normal; error bars represent standard deviation; black bar=10 μ M)

Fig. 5.

Brain and peripheral tissue enzyme restoration. a) Following Thal+ICV injection, levels of Hex A in the brain of cats reach >50-fold normal at the injection site and correspond directly to levels of Hex A achieved in the liver. Hex A in the brain and liver also directly correlate with normalization of Bgal and Mann in PBMCs. (Hex A was not elevated above untreated SD levels in PBMCs). Normal (n=4) and SD (n=5) values are provided for reference. Values are reported as specific activity expressed as nmol 4-MU cleaved/mg protein/hour. H&E stains of the liver show notable vacuolation of hepatocytes in b) SD cat at humane endpoint compared to c) a normal age-matched control cat. d) Sixteen weeks after intracranial AAV therapy, cat 7-972 had normal levels of Hex activity in the liver and architecture was largely normalized, with some hepatocytes containing multiple, small intracytoplasmic vacuoles. e) In comparison, vacuolated hepatocytes were more prevalent in cat 7-957, which had reduced Hex activity (black bar=10 μ M)

Fig. 6.

MRI changes in brain. Imaging was performed in untreated SD (n=6; humane endpoint), normal (n=3; age-matched), and SD cats 16 weeks after AAV gene therapy by Thal+DCN (n=3) and Thal+ICV (n=1) injection routes. White matter (WM) becomes isointense (loss of defined boundaries) or hyperintense (lighter than) to gray matter (GM) in the b) untreated SD cat compared to a) normal. Also, cortical atrophy in the untreated SD cat is apparent from widened sulci and enlarged ventricles. AAV gene therapy by c) Thal+DCN and d) Thal+ICV restored GM and WM intensities in most areas. Aberrant hyperintensities (arrow) are noted in the dorsal thalamus in both Thal+DCN and Thal+ICV groups at approximately the level of the injection site. MRI changes are quantified in the e) frontal cortex and striatum, f) occipital cortex and midbrain, g) thalamus, and h) cerebellum and brainstem. Significant changes in signal intensity were detectable in many brain regions of SD cats. Most importantly, many areas altered in SD cats were normalized in the Thal+DCN group. Interestingly, the deep cerebellar nuclei injection site had a signal intensity intermediate to untreated SD and normal. (*denotes $p < 0.05$ from normal, **denotes $p < 0.01$ from normal, † denotes $p < 0.05$ from SD, ‡ denotes $p < 0.01$ from SD; error bars represent standard deviation). Statistical analysis was not performed for the Thal+ICV cohort, since only one animal was imaged.

Fig. 7.

Brainstem auditory evoked responses in SD cats. a) In untreated SD cats, significant increases in latencies were noted in waves II and V at both 8-12 and 16-23 weeks of age. Wave III had increased latency at 16-23 weeks. b) Increased interwave latencies were present between waves III-V and I-V at 8-12 weeks, and between all waves at 16-23 weeks. Normal cats n=4, SD cats n=4 for all time points (*denotes $p < 0.05$ from normal, **denotes $p < 0.01$ from normal; error bars represent standard deviation).