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Batten Disease: Clinical Aspects, Molecular Mechanisms, Translational Science, and Future Directions

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Abstract

The neuronal ceroid lipofuscinoses, collectively the most common neurodegenerative disorders of childhood, are primarily caused by an autosomal recessive genetic mutation leading to a lysosomal storage disease. Clinically these diseases manifest at varying ages of onset, and associated symptoms include cognitive decline, movement disorders, seizures, and retinopathy. The underlying cell biology and biochemistry that cause the clinical phenotypes of neuronal ceroid lipofuscinoses are still being elaborated. The 2012 Neurobiology of Disease in Children Symposium, held in conjunction with the 41st Annual Meeting of the Child Neurology Society, aimed to (1) provide a survey of the currently accepted forms of neuronal ceroid lipofuscinoses and their associated genetic mutations and clinical phenotypes; (2) highlight the specific pathology of Batten disease; (3) discuss the contemporary understanding of the molecular mechanisms that lead to pathology; and (4) introduce strategies that are being translated from bench to bedside as potential therapeutics.

CLINICAL ASPECTS

Moderator: David Pearce, PhD, Sanford Children's Hospital, Sioux Falls, South Dakota

Natural Histories and Classification of the Neuronal Ceroid Lipofuscinoses

Jonathan Mink, MD, PhD, University of Rochester, Rochester, New York: Dr Mink provided an introduction of the neuronal ceroid lipofuscinoses and a survey of the 9 commonly accepted genetic loci mutations and their associated clinical phenotypes. The historical timeline associated with the discovery of the neuronal ceroid lipofuscinoses begins with Stengel in 1826, who recorded the first juvenile-onset form, although he did not describe a specific pathology. In 1903, Frederick Batten reported a juvenile-onset form with "cerebral degeneration with macular changes." By 1905 Vogt and Speilmeyer also reported a similar disorder. Jansky and Bielschowsky later found a similar late-infantile onset in 1913. Kufs described an adult-onset form with a characteristic feature of vision loss in 1925.

Lastly, Haltia and Santavuori described an infantile-onset form in 1973. The commonality among the diseases became increasingly apparent with each new discovery.

The neuronal ceroid lipofuscinoses represent a class of disorders that encompass a number of distinct biological entities that vary with age of onset, share the biochemical property of accumulating an autofluorescent lipopigment — ceroid lipofuscin — and ultimately cause cerebral degeneration. This waxy, fatty pigment-like storage material stains positively with Luxol fast blue, periodic acid-Schiff, and Sudan Black B. Other clinical features found in most of the neuronal ceroid lipofuscinoses include an associated movement disorder, progressive cognitive decline, epilepsy, and retinopathy. Some key differences among these diseases include clinical features, age at onset, cell biology, biochemistry, and the rate and characteristics of disease progression.

The neuronal ceroid lipofuscinoses encompass such a broad class of disorders classification of specific diseases has been a challenge. Historically the various neuronal ceroid lipofuscinoses were classified by age of onset: infantile (Haltia-Santavuori disease), late-infantile (Jansky-Bielschowsky disease), juvenile (Batten-Spielmeyer-Vogt disease), and adult (Kufs disease). Another classification scheme that developed was grouping based on ultrastructural abnormalities. Four main ultrastructural patterns are found in the neuronal ceroid lipofuscinoses: (1) granular osmiophilic deposits, (2) curvilinear profiles, (3) fingerprint patterns, and (4) rectilinear profiles. Conventional schemes attempt to differentiate the neuronal ceroid lipofuscinoses based on the presumed genetic locus that is mutated.

Unfortunately, each classification system has limitations — age of onset is not always a reliable predictor of the specific neuronal ceroid lipofuscinosis type, the ultrastructural patterns can vary, and genotype-phenotype associations may vary. There is also a rough association between age at onset and genotype, where more severe mutations are predicted to cause a more severe truncation or loss of protein and are more likely to lead to early onset, although this is not foolproof. Fortunately, these attributes can be used collectively to guide rational diagnostic testing.

An overview of the 9 major neuronal ceroid lipofuscinoses and the respective genetic locus mutation and clinical features were discussed. The congenital neuronal ceroid lipofuscinosis is the CLN10 disease. It is characterized by a deficiency in cathepsin D and leads to clinical features of microcephaly, lack of brain development, respiratory insufficiency, neonatal seizures, and absence of neonatal reflexes. Newborns bearing this mutation are rarely viable for more than a few days.

CLN1 disease represents the classic early infantile disease, but also has the most variation, having both juvenile and adult-onset forms. The ultrastructural appearance of the ceroid lipofuscin in CLN1 patients is in the form of granular osmiophilic deposits. The classic infantile-onset form presents between 6 and 24 months of age with rapid loss of motor and cognitive abilities. These patients typically show symptoms of seizures, myoclonus, ataxia, and, later, visual failure. The decline is quite rapid and leads to a vegetative state with spasticity late in the disease. By the age of 2, neurologic function is significantly impaired,

there are macular and retinal changes, but no abnormal pigment, and early extinction on electroretinography. In the early stages there is generalized cerebral atrophy, hypointensity on T2-weighted images of thalamus and basal ganglia, and generalized cerebral atrophy seen on imaging.

Other forms of CLN1 include a late-infantile variant that appears between 2 and 4 years of age. This form is associated with visual and cognitive decline followed by ataxia and myoclonus. The juvenile variant of CLN1 has characteristic onset between ages 5 and 10 and is associated with cognitive decline, seizures, motor decline, and vision loss. Vision loss occurs notably later in the course of the disease, around ages 10 to 14. Adult-onset CLN1 occurs after age 18 and has characteristic features of cognitive decline, depression, ataxia, Parkinsonism, and vision loss.

CLN2 disease has a typical age of onset between 1 and 4 years. It has the highest incidence of all the juvenile neuronal ceroid lipofuscinoses. CLN2 is marked by psychomotor regression that leads to the loss of abilities, typically by age 2. It is characterized by severe refractory epilepsy between ages 2 and 4, most frequently involving myoclonic seizures and tonic-clonic seizures and, to a lesser degree, tonic seizures and absence seizures. Other clinical features include severe myoclonus that can lead to spastic quadriparesis and ataxia in those with slower disease progression. Vision loss is a characteristic feature of CLN2 and is caused by prominent retinal degeneration in the macula without a cherry red spot.

The classic juvenile neuronal ceroid lipofuscinosis is the CLN3 disease, or Batten disease. Age of onset is typically between ages 4 and 7. This form of neuronal ceroid lipofuscinosis has a very characteristic pattern of progression, including gradual onset of blindness between years 4 and 6, progressive cognitive decline between years 7 and 10, behavioral problems (including angry outbursts, physical violence, and depression between years 8 and 10), generalized tonic-clonic seizures between ages 10 and 12, parkinsonism between ages 11 and 13, and severe dysarthria. Associated behavioral problems include anxiety, perseveration, irritability, and aggressive behaviors. There is a very characteristic dysarthria associated with CLN3 after age 10. It was recently discovered that children in their late teens and early 20s develop cardiac conduction abnormalities.

Dr Mink and colleagues have found through various natural history studies that there is a relatively normal distribution of the age of onset of symptoms such as vision loss, seizure, motor, cognitive decline, and behavioral changes in CLN3 patients. The earliest effected symptom is typically vision loss in both males and females, followed by cognitive decline. Notably, males experience behavioral changes earlier than females.

The variant late-infantile forms of the neuronal ceroid lipofuscinoses include CLN5, CLN6, CLN7, and CLN8. CLN5 has a highly variable age of onset, ranging from 4 to 17 years. Characteristic clinical features of this disease include psychomotor regression, ataxia, myoclonic epilepsy, and visual failure. This was once known as a Finnish variant, although it is not limited to this specific population. CLN6 age of onset ranges from 18 months to 8 years, but the disease has the potential to appear even later. Seizures occur in more than 60% of individuals under 5 years of age and there is early vision loss in 50% of cases, as well as

motor delay, dysarthria, and ataxia. This rapid deterioration leads to death between 5 and 12 years of age. There is an associated recessive form of Kufs disease with progressive myoclonic epilepsy with later development of dementia and ataxia but no vision loss.

CLN7, or the Turkish variant (although it is not limited to Turkish ancestry), starts between 2 and 7 years. It presents with seizures, progressive motor decline, myoclonus, cognitive changes, and vision loss. CLN8 has 2 forms: the Northern epilepsy and the variant late-infantile neuronal ceroid lipofuscinosis. The Northern epilepsy is characterized by progressive myoclonus epilepsy and seizures that worsen with age.

CLN4 disease is also referred to as the classic Kufs disease — an adult-onset neuronal ceroid lipofuscinosis. An associated mutation is the autosomal dominant form called Parry disease, which is associated with a mutation in the *DNAJC5* gene. Onset of CLN4 occurs after age 30 and has characteristic clinical features of ataxia, progressive dementia, seizures, and myoclonic jerks, but no visual loss.

The neuronal ceroid lipofuscinoses encompass a broad class of diseases with a number of biological similarities or functional interactions between the involved genes, which in turn leads to commonality in symptoms and some commonality in cellular pathology. Yet, there are distinct differences in biochemistry, age at onset, order of progression, and specific symptoms. The variability of these genetic neurodegenerative diseases begs the question about selective neuronal and cellular vulnerability. Why are these genes that are expressed throughout the body having preferential or differential involvement in the nervous system or in different neuronal populations? Ultimately, knowing the clinical presentations can help guide rational diagnostic testing.

Genetics of the Neuronal Ceroid Lipofuscinoses

Katherine Sims, MD, Massachusetts General Hospital, Boston, Massachusetts:

Associating the genetics of the neuronal ceroid lipofuscinoses with their clinical phenotype is important for conducting evaluations and selecting rational diagnostic testing. Identifying genes and specific genetic locus mutations can help in isolating the aberrant pathways and effective misplaced cellular biology in the disease. The ultimate goal is to use all of these factors to discover therapeutic interventions.

The general genetic inheritance for all but one of the neuronal ceroid lipofuscinoses is autosomal recessive. When the proband is carrying the mutant alleles, the incidence is quite penetrant and is estimated to be 1 in 12 500 live births within the Northern European Caucasian population. The hallmark pathology of these disorders is the lipofuscin storage material that accumulates in cytosomes. It is believed that the storage product is an important toxic component of the disease pathology but the specific details of the cell biology are still being elaborated.

To identify the disorders as a group, we have to characterize them by their age of onset, by the predominant or sequential clinical features they show, and by their pathologic inclusions, and identify their enzymatic deficiency in CLN1 or CLN2 or the molecular aberrations. As a clinician, think of the diagnosis in the context of a neurodevelopmental history and exam.

Use ancillary testing to elaborate the clinical phenotype but the basis of the diagnosis rests on enzymatic or primarily DNA molecular testing.

CLN10 is a severe congenital form of neuronal ceroid lipofuscinosis that features a mutation in the lysosomal aspartic protease cathepsin D gene. Many forms of the mutation have been described, including a stop mutation in the most severe form, a splice mutation (which is less severe in pathobiologic effect and results in a late-infantile form), and a missense mutation, which can cause a juvenile-onset form. The CLN10 subtype leads to rapid developmental failures, blindness, and seizures.

CLN1 is the classic late-infantile mutation, which is deficient in palmitoyl-protein thioesterase 1. The 2 subtypes of CLN1 highlighted in Dr Sims' talk were those prominent in the Finnish and US population. The Finnish variety is characterized by a homozygous point mutation, which results in the classic early onset form of the disease. The US population has several forms of *CLN1* genetic mutations that result in infantile, late-infantile, juvenile, and adult onset of the disease. The most common mutations in the US are stop mutations that lead to the infantile form of the disease, but point mutations that lead to juvenile and adult onset of the disease have also been documented.

CLN14 disease is characterized by mutations in the potassium channel tetramerization domain 7-protein (KCTD7). This mutation causes an infantile-onset disease that manifests with seizures, motor and developmental failure, and lipofuscin inclusions with fingerprint structures. KCTD7 may function in crosstalk between ubiquitin/proteasome system and the lysosomal autophagy pathway. Since this gene has been isolated relatively recently, more information is needed to isolate its mutation spectrum.

CLN2 is a classic infantile form of neuronal ceroid lipofuscinosis, with a deficiency in tripeptidyl peptidase 1 protein. Seventy-five percent of patients carry either a mutation in the intronic splice site in intron 5 or an exonic stop mutation on exon 6. Missense, nonsense, splice, and deletion mutations have also been identified in the disease.

There are 4 variant late-infantile forms: CLN5, CLN6, CLN7, and CLN8. As typically seen, most of these mutations have also been identified in juvenile and adult cases. *CLN5* produces a soluble lysosomal glycoprotein which, when mutated, leads to an aberrant protein that is stuck in the endoplasmic reticulum. This specific mutation is referred to as the Finnish variant but other mutations in *CLN5* exist and can be pan-ethnic in expression. Aberrant *CLN6* leads to a mutation of a transmembrane protein with unclear function. In addition to late-infantile expression, CLN6 can also be observed in the adult-onset progressive myoclonic epilepsy cohort. CLN7 is a major facilitator of superfamily transporter protein, which localizes to the lysosomal membrane. Though originally associated with the Turkish community, it is pan-ethnic in expression and seldomly seen in juvenile and adult-onset forms. Lastly, CLN8 was originally identified with the Finnish cohort and shows clinical features of severe epilepsy and cognitive decline without visual loss. It is currently believed that a late-infantile form also exists where added clinical features of myoclonus, visual loss, and cognitive decline also exist.

The juvenile form of *CLN3* mutation is known as Batten disease. *CLN3* encodes a lysosomal transmembrane protein. In over 75% of the patient population, when the *CLN3* mutation is present there is a 1.01 kb deletion. Clinical phenotypes in compound heterozygous cases are relatively similar to the classic homozygous patients described by Dr Mink.

The last group of mutations Dr Sims discussed involve the late-onset forms of the disease. Kufs disease is primarily a sporadic or autosomal recessive mutations in the *CLN4* gene. Classically the adult forms have been separated into 2 groups based on clinical phenotype. Significant seizure disorders, myoclonus, and early psychiatric features characterize Type A. Type B encompasses features such as deficits in mobility range and dementia; seizures are uncommon. The *DNAJC5* locus is the only autosomal dominant form of the neuronal ceroid lipofuscinoses to date. It is also called the Parry type and has a characteristic age of onset between the teen years and the 30s. Prominent clinical features include dementia, seizures, and myoclonus. This gene encodes a cysteine string protein alpha protein that seems to be neuronal-specific in expression. It may function in synapse function or synaptic degradation. The exact biologic function remains elusive.

The *CLN11* or progranulin mutation has been identified in 2 different situations. In a single-family report, *CLN11* mutations were shown to appear after an autosomal recessive mutation.¹ Another study showed a *CLN11* mutation that resulted from a heterozygous mutation in FTLTDP.² A single-nucleotide polymorphism variant was identified in the *TMEM106B* gene whose specific function is unknown but has expression changes that both increase the risk of frontotemporal dementia and also change the risk of disease penetrance in the face of progranulin mutations. *CLN11* mutations highlight the potential relationship between common neurodegenerative diseases and rare lysosomal diseases and the pleiotropic effect of heterozygous and homozygous mutations.

ATP13A2 is a P-type ATPase protein mutation designated as CLN12. Though the exact biologic function of this protein remains elusive, it seems to transport inorganic cations and may play a role in balancing lysosomal pH. This protein has been associated with the PARK9 Kufor-Rakeb syndrome, a juvenile parkinsonism, through its involvement in the lysosomal autophagy pathway as well as the ubiquitin/proteasome system with alpha-synuclein storage. When mutated in neuronal ceroid lipofuscinosis, CLN12 has an age of onset in the second decade with clinical symptoms of seizures, cognitive failure, and movement disorder. It also displays characteristic neuronal ceroid lipofuscinosis-like inclusions.

Clinicians can use characteristic phenotypes augmented by neuroradiology or electrophysiology capacities and classic pathologic findings to support a neuronal ceroid lipofuscinosis diagnosis. If the neuronal ceroid lipofuscinosis is believed to be caused by a CLN1 or CLN2 mutation than enzymatic deficiencies can be tested to confirm this diagnosis. All other NCL mutations would have to undergo molecular testing. In the future it is possible NCL mutations will become a part of newborn screening as an attempt to identify the disease before neurologic phenotypes present.

Neurobehavioral Aspects of CLN3

Heather Adams, PhD, University of Rochester Medical Center, Rochester, New York:

Dr Adams characterized various aspects of neurobehavioral features of Batten disease and discussed how these data were obtained. She first reviewed the development and current use of the Unified Batten Disease Rating Scale. This clinical rating instrument is used to assess physical, behavioral, and functional capability in juvenile neuronal ceroid lipofuscinosis. The Unified Batten Disease Rating Scale includes a physical assessment done by a trained neurologist, as well as a clinician's global assessment of how well the child is doing at that point in time across different neurobehavioral domains. This global assessment was validated against a number of standardized measures of cognitive and behavioral evaluations. It was found that there are significant relationships between each of these measures and the global assessment of the Unified Batten Disease Rating Scale. The use of the Unified Batten Disease Rating Scale has allowed Dr Adams and her colleagues to evaluate the neurobehavioral aspects of Batten disease with much greater efficiency.

Before the development of the Unified Batten Disease Rating Scale, there were a number of challenges in the assessment of Batten disease progression. In terms of changes in cognitive function, there were variable rates of IQ decline across children, as well as variability in the types of measures that were used to acquire such data. Also, common symptoms of Batten disease include blindness and the development of a characteristic speech pattern that hinders their ability to communicate verbally. This makes obtaining a comprehensive cognitive assessment rather difficult. In the past, researchers were presented with a dual challenge of evaluating children who must rely upon verbal communication because of their vision loss, yet are limited in their ability to do so.

Because of these challenges, one of the ways the Unified Batten Disease Rating Scale evaluates cognition is to look at the point at which a child is no longer able to perform a task. To assess attention and memory, clinicians use a task called digit span. It has 2 components, digit span forward and backward. Digit span forward provides an indication of just simple attention. The clinician reads a series of numbers and the child is asked to repeat them back. Digit span backwards is a more complex task of working memory, which has higher cognitive processing demands. The clinician reads a series of numbers and the child is asked to repeat them in the reverse order.

The results of the digit span forward tasks have shown that simple attention for many children is relatively well-conserved until the middle teen years. The percentage of children who are still able to complete the simplest version of this task is around 90%. The results of the digit span backward tasks have shown that even at the youngest ages, working memory in 40% to 50% of children is diminishing. It was expected that working memory would improve as children got older, but actually the opposite is the case. Dr Adams pointed out that despite the general decline in ability over time for task, there was considerable variability from year to year.

Another task used to evaluate cognitive function is verbal fluency. It also has 2 components, one that assesses semantic information and one for phonemic information. The results have shown a clear pattern of decline over time in the percentage of children who can no longer

complete the simplest form of the task. However, there is no significant difference between the semantic and phonemic components of the task. There is also variability in this cognitive assessment. Until about age 12 or 13, there are still a few children who are able to perform in the average range of this test of verbal ability. Nonetheless, even at the youngest age of 6, there are children who struggle at the lowest possible level of the task. It is not yet currently understood why there are vast differences from age to age.

Dr Adams and her colleagues also conducted a behavioral study that involved about 30 children. A standardized assessment of behavior, the Child Behavior Checklist, was used in this study. Measures of this assessment include anxious/depressed mood, withdrawn/depressed mood, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. Although there were certainly some areas where there were more clinically significant difficulties, such as attention problems or aggression, the results have shown that children were having difficulties in all areas measured across the board.

Dr Adams went on to discuss the possibility of whether there are sex differences in the disease. She alluded to a behavioral study in Finland that examined this same question. The researchers in Finland measured children with Batten disease using the Child Behavior Checklist. The results showed that females fared worse in certain areas. Dr Adams and her colleagues replicated this study with a similar measure, but the results did not display the same patterns in behavior. They did, nevertheless, revisit the idea in terms of cognitive performance. The cognitive tasks assessed were digit span forward, digit span backwards, and vocabulary. Although the differences in the distributions between males and females were not statistically significant, there was a definite trend toward loss of cognitive skill over time in females, but an increase in development of these skills in males.

Another way the neurobehavioral data has been used is to understand the impact of the disease on the family at large. Dr Adams and her colleagues used a parent assessment to measure family function in terms of how well they are doing as a result of their child having Batten disease. After comparing this assessment to the standard child behavior measure, there was an association between greater child behavioral problems and lower family and parent functioning. Additionally, the illness' influence on unaffected siblings was also assessed. Behavior problems in affected siblings were shown to be associated with higher levels of sad and anxious moods, as well as social withdrawal in unaffected siblings. This certainly points toward the value of providing more comprehensive support to families as a whole.

There also has been some discussion of genotype-phenotype relationships in Batten disease. In juvenile CLN3, as an autosomal recessively inherited form of the disease, there has always been a question of whether individuals who are compound heterozygotes may have a more attenuated disease course. To answer this question, dimensions from the Child Behavior Checklist were compared with the genotyping in children confirmed to have CLN3. The results, however, showed no difference between homozygotes and heterozygotes.

Despite significant advances in studying Batten disease, Dr Adams pointed out a major challenge in rare disease research. Because there are so few children to evaluate, it takes a tremendous amount of time to accumulate a body of knowledge and actually see patterns emerge. A small sample size also means that even just one outlier can push or pull distributions. With this in mind, it is important to try to develop different, less traditional approaches to evaluating data. This particular field certainly requires flexibility and creativity to balance having a thorough assessment with understanding the unique challenges the disease presents.

Neuropathology of the Neuronal Ceroid Lipofuscinoses

Jonathan Cooper, PhD, Kings College London, United Kingdom: Dr Jon Cooper presented an overview of what is currently understood in terms of the neuropathology of the neuronal ceroid lipofuscinoses. He focused on the pathogenesis of the different forms of Batten disease, and how mutations in particular genes lead to the effects noted in the brains of affected individuals.

Researchers aim to understand the sequence of events of the disease, identify landmarks of how particular organisms are affected by the disease (which may be pathological or behavioral), and use this information to intervene and influence disease progression. While human disease is traditionally the gold standard, there is a limit to information that can be acquired because researchers can only study the end-stage impact upon the brain in Batten disease. Therefore, a large number of animal models are used, ranging in complexity across many different species. The foundation of the work with animals is a series of naturally occurring or genetically modified mouse models, which include knockout and knock-in mice. Knockout mice are mice that do not have the specific protein of interest at all, and knock-in mice are mice that have had the disease-causing mutation introduced into their genome.

To understand how the biology of these mouse models are compromised, pathologists take the mice brains and look at them in different ways. They are interested in how gene or protein expression changes in parts of the brain at various stages of the disease. The first step is to cut sections of the brain and stain them for an assortment of makers. Then a number of different counting methods are used to extract data. These data are used to test hypotheses about how the disease has impacted the brain or whether different therapies have actually had an effect on them. Dr Cooper then mentioned an issue of using mouse models and stressed the importance of studying larger animals. Because mice brains are so small and simple, it is relatively easy to fix them. However, treating a larger and more complex brain is much more difficult. This is where having genetically accurate organisms is useful, especially when looking at the delivery and biodistribution of therapies within the brain. Also, from a pathological standpoint, large animal models actually display pathology that is closer to the human disease in terms of effects seen in the brain.

Using these animal models, pathologists have been investigating the staging of these diseases, as well as which cell types and parts of the brain are affected. For the enzyme-deficient infantile form of Batten disease, it was initially assumed that neuron loss occurred to very similar extents throughout the neuronal ceroid lipofuscinosis brain. However,

although neuron loss is indeed widespread at the end stages of disease, there is notable selectivity in its earlier stages. This indicates there are certain portions of the brain that are much more resistant to the effects of the diseases than others. To investigate this trend, researchers focused on the defined pathways from the thalamus to the cortex, particular areas that are affected by the disease, rather than looking at the brain as a whole. A number of important events that influence this type of cell activity have been discovered.

One of these key events involves synapse activity. It is known there is an impairment of axonal transport, and it is speculated there is involvement of astrocytes at the synaptic compartment. The first thing seen is synapse reorganization at the presynaptic compartment of the proteins, which is accompanied by electrophysiological changes. This leads to synaptic loss, which invariably occurs before the loss of neuron populations. To determine how this occurs, researchers have been directing their attention to the axon initial segment. Because the axon initial segment is the first portion of the axon that is important in terms of transportation down the axon, it may very well be related to those presynaptic defects. Additionally, from a seizure point of view, it also has important functional roles in terms of whether cells actually fire or initiate an action potential. Dr Cooper and his colleagues have been gathering data about the axon initial segment in at least one form of the neuronal ceroid lipofuscinoses. Another significant event is related to the activation of different populations of glia. In these disorders, as well as other lysosomal diseases, researchers have noticed an activation of glial cell populations occurring before neuron loss. Because of this, glial activation has become the best predictor of where neurons are subsequently going to die within the brain.

Dr Cooper then switched over to the transmembrane protein-deficient juvenile form of Batten disease. In this form of the disease, a similar series of events is seen, but it occurs in a different order. While neuron loss occurs as the disease progresses, there is a quite unusual activation of both astrocytes and microglia early on in juvenile neuronal ceroid lipofuscinosis. The synaptic pathology in this variant is also similar to the infantile form, but there is evidence of excitotoxicity. Additionally, there appears to be an autoimmune response, an event that is specific to the juvenile form. The key question of all these events is what is their contribution to the pathogenesis of this particular form of the disease?

When compared with the robust activation of astrocytes and microglia seen in the infantile form of the disease, this response is somewhat attenuated in the CLN3 mouse model of juvenile neuronal ceroid lipofuscinosis. This trend is also seen in the human form of the disease. In terms of the synaptic pathology of juvenile neuronal ceroid lipofuscinosis, it has been shown that there is an imbalance between excitation and inhibition in the brain. Metabolic studies have shown that the amount of glutamate, the brain's primary excitatory transmitter, was elevated. Not only was this accumulating presynaptically, but also different populations of neurons were selectively vulnerable to this excess of glutamate. Dr Cooper and his colleagues are currently using different classes of glutamate receptor antagonists to see if this will have an influence upon the disease phenotype of mice.

Regarding the autoimmune response, studies have shown that autoantibodies appear to be present in both mice and humans affected with juvenile neuronal ceroid lipofuscinosis.

These autoantibodies enter the brain via a breach within the blood-brain barrier. To determine whether this has any effect on disease pathology, researchers tested a genetic blockade and immunosuppressant therapy on mice with juvenile neuronal ceroid lipofuscinosis. This treatment strategy has shown to have positive effects on the brain pathology of those mice, and effectively reverse the characteristic motor defects caused by the disease. This preclinical data has led to the Phase 1 trial that will be taking place at the University of Rochester in the near future.

In regard to different cell types, Dr Cooper and his colleagues have been exploring the interactions between neurons, astrocytes, and microglia in both types of disorders. In particular, they have recently been attempting to determine which cells are the main contributors to the disease, whether the biology of these cells themselves is compromised, and which cells might be amenable to treatment. Data so far have shown that the biology of astrocytes and microglia in particular certainly differs in these diseases. These cells have been shown to exhibit altered secretion of substances that may be considered neuroprotective, that may be involved in the crosstalk between cells, or that may influence microglia migration. In co-culture systems, data suggests there may be a direct impact of dysfunctional glia on neurons, and treating those glia actually reverse many of the deleterious effects of the disease.

Dr Cooper concluded his talk by pointing out the overlooked sites of pathology of Batten disease. Much of the current work is focused on the cortex, thalamus, and cerebellum, but there are clearly other parts of the brain and body that need to be targeted, too. It is important to remember that these neuronal ceroid lipofuscinoses are not just disorders of the brain, and that to be successful therapeutically, the other places in the body affected by the disease must also be considered.

Questions and Answers—Dr Maria: This question is to Kathy Sims, and perhaps to others on the panel. We heard from Dr Mink and many of you about the extreme variability in presentations and the wide array of genes that produce the disorder. How are we going to work with genomic sequencing in newborn screening? Because as that makes its way into sort of a routine for screening, what are your thoughts on the clinical implications, both in the way of prognosis and treatment by identifying one of these genes at birth?

Dr Sims: I think this is an important question, and I'm not sure I have the answer to it, but I could highlight some of the—maybe the concerns, both the positive effects and maybe some of the risks of that kind of screening. Even in non-newborn screening we're faced with the same problems sometimes in interpreting the potential pathologic consequences of a mutation. Even in the childhood or adult patient that same question, not infrequently, comes up. The databases are good and they will continue to get better so that we can try to make pathologic assessment of the molecular changes. But there are cases in which it is not clear, and we don't have good biological function assays that can immediately confirm or contest the diagnosis.

It's obviously much more critical if you're going broad-based population screening or newborn screening, because you have to know and you have to be able to interpret the

results. I think even now it is difficult to absolutely rely on second gen sequencing. Its fidelity is not as good as regular Sanger sequencing. I think the technologies will improve so that the data will be good, but the bioinformatics assessment is going to continue over the next years in the foreseeable future to challenge us. If we had functional assays that were reliable, and the components, and certainly that's possible in CLN1 or 2, but the others, yet, remain problematic. So we have to use known databases and try to make reasonable phenotypic correlations, but I don't think it's going to be straightforward for quite a while.

Dr Pearce: Just as an addendum to that, and a pertinent point, I just attended a rare diseases clinical trials meeting. A well-known company who develops diagnostics said they took 150 children that they had not identified a diagnostic test for. They did not know what the gene was. They did whole genome sequencing on 150 children and they actually only identified pathogenic mutations in 20% of those. I think that alludes very much to bioinformatics issues and just really knowing what is a pathogenic mutation right now.

Audience member 1: I have 2 questions. The first question is for Dr Mink. Is myoclonus necessary for the diagnosis of Batten disease? I've always thought of it as the hallmark, but if you have someone with many of the other characteristics, should you still consider Batten disease?

Dr Mink: Myoclonus is not a hallmark of Batten disease. It is a very common feature, particularly in CLN2 diseases. On the other hand, as you may have seen in the video I showed, that was a child who had predominantly chorea, and so there may be some misclassification of the individual movement disorders. We had hoped at one point that each form might have a unique pattern of movement disorder that might help us in directing our diagnostic assessment. And what we're coming to recognize is that there is a fair amount of variability. Often in the textbook chapters, myoclonus is pointed to as one of the major features of NCLs generally, but we almost never see it in CLN3 disease, and it may or may not be present in CLN1. It's really CLN2 where it's very prominent. Then in some of the others, CLN8, for example, it's myoclonic epilepsy, which is different in some important ways from subcortical myoclonus, but not all is easy to sort out clinically.

Audience member 1: Thank you. Then just a little further clarification from Dr Sims. So is there, in fact, a panel now that's available or is that something that's in the future?

Dr Sims: It's close. It's coming.

Audience member 2: Given a definite genetic diagnosis of NCL, how many families, who are counseled, actually don't have further kids? Do you have a sense of that? We don't have a sense of that. I think some of these decisions are going to be based on the diagnosis that we're talking about. For CLN3, parents may have had all of the kids they're going to have by the time their first child is diagnosed, because their first child may not be diagnosed until age 6 or 7, and so that may become a non-issue for those families. I think that, and I'm sure the others on the panel can talk to some of the options that are available to families for family planning if they are considering growing their family further and know that they have genetic risk for this disease.

Dr Sims: I might just add that prenatal testing is available, so that if the diagnosis is made—and I think that's a critical issue if it's made quickly rather than over a long series of months and years, then that molecular information is immediately available for family planning if the family chooses that. I, myself, can't speak to the population experience, but, hopefully, we have the tools to make a change in that if the family wishes.

Audience member 3: I was wondering in juvenile Batten if we have any idea in terms of the aggression that is seen in these children. Is there any evidence that that's triggered by seizure activity and some of these bouts of aggression?

Dr Adams: The seizures, in at least the juvenile form, seem to be, for many children, not a prominent feature, and when they do occur can be reasonably well-controlled with the first AED. So we don't really see postictal aggression as a major problem for these children. We see the aggression occurring before seizures ever develop, and as more belonging to the overall dementia syndrome that these children experience.

Dr Mink: We did look a little bit at some of our natural history data, and there doesn't seem to be any correlation at any time, and these are cross-sectional studies, but there doesn't seem to be any correlation between severity of seizures at the time of assessment and severity of behavioral problems.

Dr Adams: For many children, behavioral problems are a nonspecific symptom early in the course of the disease. So children who present with behavioral problems, there's some attentional difficulties in school, may be considered just to have garden variety ADHD, for example, but then when other symptoms start to come on line, such as vision loss, then the picture pulls together. But those things will happen a number of years — maybe even before this first seizure is ever noticed.

Audience member 4: I had a question regarding diagnostic workup. In the sense that as everyone has alluded to that there is a lot of genotypic-phenotype variability, if we have in front of us a young girl, 8 years of age that we are thinking of juvenile NCL, we send for CLN3 and it's negative. When in the other case, we have a 6-month-old baby boy, who is sent for the most common ones, and it become negative. Is it worth maybe until we have a whole genome or exome sequencing to send for skin biopsy, which maybe can give us a sense if this is at least in the NCL category, and then we can give some advice to the family, and then continue to try to find if this NCL3, 5, 6, 7? Because I think at one point it becomes very costly to begin sending and sending more and more genes instead of giving a skin biopsy. So I just wondered if we have presented the case and the first pass is negative, should we just first go then to skin biopsy?

Dr Mink: If I could comment first. My recommendation is use the clinical history to help guide you. If you suspect an NCL in an 8-year-old, who has relatively preserved vision, it's probably not CLN3, for example. And so there are some things that can help guide you to make it a little bit more efficient. Skin biopsy, ultrastructural diagnosis can be particularly fingerprint bodies can be relatively predictive of what type it is, but the other ultrastructural findings—you're basically it's age at onset, it's relative incidence or prevalence of the disorder that help guide you in what order to go, and then maybe the ultrastructure will help

you or maybe it won't. I don't know. Kathy or Jon, do you want to comment further? It is difficult, but I think you need to use all of that information together.

Dr Sims: I would agree that the skin biopsy is one piece of the diagnostic assessment. And until we have the really reliable capacity to screen molecularly, we're still back sorting it out from the clinical presentation, age of onset, the characteristic features, and the pathology, it it's helpful. If it's GRODs, it's going to direct your testing for sure. So there are certain flags that will really clarify things, but I think the real problem is that the negative biopsy or the atypical presentation is a real phenomenon and it leaves you still needing a full screen, and that is expensive sequentially. It's still reasonable to do that. But when we can just say we think this is in this category of disorders based on the clinical presentation, and the path, and the electrophysiology, or the MRI, then you just will screen for all known CLs and candidates.

I wish I could say that was available today, but it won't be but months away. I think that will give us all a better tool. There will be negative cases and ones that are difficult to interpret, but I think we'll make far faster headway in identifying these children, and this will be important in therapy to try to mitigate the disease process early rather than later, and do genetic counseling. I would do the biopsy. Always.

Audience member 5: I had a question about the—a couple of questions about the neuronal loss and whether it's the excitatory cells or the inhibitory cells. And then how that relates to the onset of the epilepsy. And if different types of anticonvulsants are perhaps more helpful than others and if that could help us understand the pathophysiology.

Dr Cooper: I'll address the neuron loss part and then pass over to someone else to talk about seizure medications. When I started doing this, we thought, well, it would be obvious to look at the inter-neuron populations and they clearly are affected, and they are the first thing that we actually picked up on. But when you start looking at the excitatory cells, they're also clearly affected, and it becomes more a case of where those cells are, and which brain regions they're in, and which pathways they're in that determines their cell activity rather than whether they're inhibitory or excitatory. That's more important. It's intriguing why neurons in particular pathways should be affected and why, in particular, we see sensory pathways being so clearly affected more than motor pathways, although they are also involved to certain extents. We don't understand that properly. We do see the pathology spreads within those pathways, which we think is important, and we do think that it's that interaction between the neurons and glia that is a key part of it. But, we're still trying to pick apart why should it be that certain populations are affected. Hopefully we'll tell you that in years to come.

Dr Mink: In terms of seizure—antiepileptic or anti-seizure medicine effect, there has not been any systemic study. We've collected a lot of data that Erika Augustine's looking at. What we have right now is fancy anecdotes. We can tell you what the majority of children with—the type we study is CLN3—are on, and those who have had good benefit versus not so good benefit, but nothing systematic. There does seem to be overall, my general impression is, a relationship to the type of seizure and the type of medication that is

prescribed, but that doesn't necessarily mean specific efficacy. So those who have generalized tonic-clonic seizures with CLN3 disease are more likely to be on valproic acid or lamotrigine, and are likely to have pretty good control. Those who have myoclonic seizures with CLN2 disease tend to be on a lot of medicines, and they don't have very good control at all, but that's the nature of myoclonic epilepsy.

Dr Pearce: Just as an addendum to that, there were a couple of more recently published studies Jon alluded to in his talks with the mouse models. The mice models for the CLN3 or juvenile do not get seizures, but there are some anticonvulsants that seem to alleviate the disease. So we need to dig a lot deeper in that before that can be actually translated to the patients.

Audience member 6: Not a question, but more of a general comment. There have been several questions about there's not a next-gen sequencing panel yet available for NCL. But I think all of us as clinicians need to be mindful that there have been a lot of next-gen panels that have been developed just over the last couple years, and many of these are symptom-specific. So, for example, if we look at the epilepsy panel that GeneDx labs offers, they cover 8 of the known NCL genes as part of their testing. So even though you may not be testing for epilepsy specifically, if you are worried about NL, this is something that's available. I think this instance is true for many other diseases, but it will be nice when Dr Sims has her pan available.

Dr Sims: I agree. I should have said and highlighted that. What I don't have is any personal experience in the quality control that's coming out of that kind of testing. Again, I know how difficult it is to do the interpretations of the data, even off the plan Sanger sequencing. So I think it all remains yet to be seen whether we can move forward, but I don't think it's far in the future. Thank you for highlighting that.

Audience member 7: I just noticed that a couple of you mentioned parkinsonism features in some of your patients. Given that people have noticed a parkinsonism link with Gaucher disease, is there any evidence of alpha-synuclein buildup in Batten's autopsy brains or mice model brains?

Dr Cooper: Very good question and that's one we're actively looking at. We've been trying to understand the basis of parkinsonism and, naturally, went looking in the substantia nigra. It turns out not to be the dopaminergic cells that go there, it's actually the inhibitory cells. So whether it's classically like that or it just ends up at the same way, we don't know. But we're not actively hunting and looking at things like alpha-synuclein distribution of inclusions and doing this across multiple forms of the disease to try and figure out if there is something different. For example, we're also keen and also actively looking at what might be links to other more common neurodegenerative diseases like tauopathies, etc. So I think that's an area that we will be looking at and we'll be reporting back about in more detail in the years to come.

Dr Mink: And if I can just comment for a minute on the parkinsonism. So that seems to be the most common movement disorder in JNCL. There are data going back 15 years or so now showing with fluorodopa PET scans that there is nigrostriatal dopamine or striatal

dopamine deficiency in those individuals, and there is loss of the nigral dopamine neurons in a couple of small postmortem—well, a couple of postmortem cases that have been described. There's other pathology and it's widespread, but it does seem to be that form, maybe some predilection the teenage years for the nigrostriatal dopamine system. And those patients do respond moderately to supplemental — at least in terms of posture on stability. It doesn't provide them dramatic improvement of their function, but it does have some modest benefit.

MOLECULAR MECHANISMS

Moderator: Jonathan Mink, MD, PhD, University of Rochester, Rochester, New York

Neuroinflammation and Microglial Activation in Juvenile Neuronal Ceroid Lipofuscinosis

Tammy Kielian, PhD, University of Nebraska, Omaha, Nebraska: Dr Kielian's talk focused on juvenile Batten disease, the most commonly inherited progressive neurodegenerative disease of childhood, affecting an estimated 1:100 000 live births. It is caused by an autosomal recessive mutation in the *CLN3* gene and has clinical features that include impaired cognitive function, motor abnormalities, behavioral issues, seizures, blindness, and premature death. In addition to the classic accumulation of autofluorescent ceroid lipopigments, there is an association of cortical atrophy and ventricular enlargement as the disease progresses.

The molecular function of the CLN3 protein is largely unknown, but it is believed to primarily localized to the lysosomal membrane and takes part in endocytosis and intracellular protein trafficking, lysosomal homeostasis, autophagy, mitochondrial function, amino acid transport, oxidative stress, neuronal excitatory, and cell cycle regulation. There is an increasing understanding of CLN3 mutation due to mouse models. Sue Cotman at Harvard developed the CLN3^{ex7/8}, which recapitulates the 1 kb mutation deletion present in nearly 85% of patients who have juvenile neuronal ceroid lipofuscinosis.³ Magnetic resonance spectroscopy of juvenile neuronal ceroid lipofuscinosis brain and ex vivo analysis of brain extracts from CLN3 knockout mice indicates there is an increase in glutamate concentration and decrease in gamma-aminobutyric acid. Neuron loss is believed to result from increased excitatory and decreased inhibitory inputs.

Dr Kielian and colleagues' recent work on identifying inflammatory paradigms in juvenile Batten disease hypothesizes that inappropriately primed microglia secrete elevated levels of inflammatory cytokines and glutamate that eventually contribute to neuron death in juvenile neuronal ceroid lipofuscinosis. The functional roles of microglia are varied as they play a trophic role modulating neural repair, scavenge for apoptotic debris, and participate in synaptic remodeling. When chronically perturbed, microglia can produce a wide array of pro-inflammatory molecules and free radicals such as superoxide and nitric oxide. Initially when developing this study, Dr Kielian and colleagues focused on interleukin-1 beta because of this cytokine's involvement in neuroinflammation and neurodegeneration of neurons. Dr Jon Cooper also has shared his findings that interleukin-1β is elevated in the juvenile neuronal ceroid lipofuscinosis brain.

Before presenting her research, Dr Kielian reviewed the mechanism by which interleukin-1 β is activated and released from the cell. Two necessary signals trigger the production of interleukin-1 β and subsequently allow its release from the cell. In terms of juvenile Batten disease, Signal 1 is often neuronal debris, which binds to toll-like receptors on the surface of microglia and causes downstream signaling that leads to transcription of the pro-form of the cytokine. In Dr Kielian's study, she uses freeze-thaw extracts from primary neurons, embryonic day 16, to simulate the neuronal debris of the condition. Signal 2 is needed to activate the proteolytic complex in the cytoplasm called the inflammasome. Once the inflammasome is activated, caspase-1, the downstream effector, is activated and cleaves the inactive pro-interleukin-1 to its active form, which is then secreted. Rosemary Bustami's study indicated that ceramides are elevated in juvenile neuronal ceroid lipofuscinosis patients' brains, so ceramides were used as Signal 2.⁴

In Dr Kielian's study, primary microglia were harvested from neonatal CLN3^{ex7/8} or C57BL/6 wild-type mice at 1 day to 2 days of age. These cells were treated in vitro with Signal 1 (neuronal debris) and Signal 2 (ceramide). The 2 primary experiments were to determine caspase-1 activity using the FLICA reagent and to analyze interleukin-1 β and other pro-inflammatory mediators by microbead arrays. The first experiment showed that when Signal 1 and 2 were present there was a significant increase in caspase-1 activity in CLN3^{ex7/8} when compared with the C57BL/6 wild-type. This suggests these CLN3 mutant microglia may be unusually primed to respond to these stimuli, which are elevated in the brains of patients with juvenile neuronal ceroid lipofuscinosis.

Dr Kielian also studied the amount of interleukin-1 β secreted from these microglia. Ceramide and neuron lysates induced robust interleukin-1 β production by the CLN3^{ex7/8} microglia. To support that caspase-1 regulated the release of interleukin-1 β , Dr Kielian presented studies that showed a dose-dependent reduction of interleukin-1 β cytokine in the presence of caspase-1 inhibitor. Interestingly, tumor necrosis factor- α expression also displayed a dose-dependent reduction of expression as caspase-1 inhibitor was increased. This suggests there is a potential autocrine-paracrine role of interleukin-1 β in further triggering microglia to produce other proinflammatory cytokines causing an amplification effect in CLN3^{ex7/8} mice. Other cytokines found to have a significantly higher expression in the CLN3 mutant mice included interleukin-15. Interleukin-15 is a B cell activating cytokine that leads to antibody release. The correlation between interleukin-15 and juvenile neuronal ceroid lipofuscinosis is not yet understood, but the finding was interesting.

Dr Kielian also observed that caspase-1 played a role in modulating the concentration of extracellular glutamate. When caspase-1 inhibitor was added in the absence of neuronal lysate or ceramide stimuli a heightened release of glutamate occurred when compared to unstimulated cells. This causes interest in the potential link between caspase-1 regulating inflammation as well as its role in glutamate balance, which is known to be elevated in juvenile neuronal ceroid lipofuscinosis brains.

Propentofylline is a phosphodiesterase-4 inhibitor that has been shown to inhibit microglia and astrocyte inflammation in spinal cord injury and neuropathic pain models. It is primarily shown to attenuate inflammatory cytokine release by microglia and augment glutamate

transporter expression in astrocytes. The current question is whether propentofylline can modulate aberrant responses in *CLN3*^{ex7/8} glia. Studies have shown that adding increasing concentrations of propentofylline is able to significantly inhibit interleukin-1 β as well as tumor necrosis factor-alpha release from the *CLN3*^{ex7/8} mutant microglia. Follow-up studies are currently being conducted to further inquire about the benefits of this compound.

The last topic covered in Dr Kielian's presentation was her work on astrocytes and gap junctions. Gap junctions are important for the homeostatic environment of the brain. They play a role in diluting out toxic molecules such as glutamate from the microenvironment. This is a feature of specific importance in juvenile neuronal ceroid lipofuscinosis brain, which has elevated concentrations of glutamate. Joining of 2 hemichannels when 2 neighboring cells are in close proximity forms conduits in which toxic molecules are diluted out. However, during pathology these hemichannels open and allow bidirectional trafficking of molecules between the extracellular and intracellular milieus, causing a disruption of the metabolic gradients in the central nervous system.

Dr Kielian is curious to see whether the disruption of the metabolic gradients in the central nervous system may contribute to neurotoxicity later in disease. In a study where acute brain slices from *CLN3* mutant and wild-type animals were monitored for rate of ethidium bromide uptake as a test of ease of hemichannel access within somatosensory barrelfield 1 cortex, thalamus, visual cortex, and hippocampus, a heightened expression of hemichannel activity was noted to be higher for the *CLN3* mutant than the wild-type. This suggests that in these areas the balance has been tipped to a potentially unfavorable way of opening the hemichannels. This could disrupt the metabolic gradients and be one potential mechanism for neuronal loss.

CLN3: What Does It Do?

David Pearce, PhD, Sanford Children's Hospital, Sioux Falls, South Dakota: Though the details of the *CLN3* protein remain elusive, Dr Pearce provides details on several studies that are attempting to elucidate the biological role of this protein. *CLN3* protein is a transmembrane protein believed to be associated with every subcellular compartment, though commonly associated with lysosomes and endosomes. The loss of function *CLN3* mutations associated with juvenile Batten disease is believed to result from a truncation of the protein, the most common mutation being the 1-kilo base deletion of the *CLN3* gene.

Saccharomyces cerevisiae is an optimal model for studying *CLN3* mutations because the primordial function of the protein is conserved in this model. Btn1p is 59% similar and 39% identical to human *CLN3* protein.⁵ Using this model can allow researchers to discover the function of *CLN3* in simple cells and apply these discoveries to more complicated systems. Pearce and colleagues have found that yeast that lack Btn1p had a lower lysosomal pH and pumped more protons into the media than their wild-type counterparts. The vacuole ATPase (VATPase) is aberrantly functioning in these mutant models, causing an unbalanced vacuolar pH and acidic extracellular environment. Although this finding was interesting, the link between aberrant Btn1p activity to VATPase activity produced an overwhelming negative association.

Localization of the CLN3 protein is an interesting topic. CLN3 protein associates with structures that are inherent to the lysosome, late endosome, early endosome, golgi, nucleus, and mitochondria. The working hypothesis of Pearce and colleagues is that there is a differential localization of the CLN3 protein depending on the environmental condition. Within the yeast model, Pearce and colleagues studied the colocalization of Btn1p and the vacuole under a stressful environmental condition at pH 6 and a normal environmental condition at pH4. The study showed that under stress the Btn1p co-localized with the vacuolar membrane. Under normal conditions Btn1p was no longer co-localizing with the vacuolar membrane. This supports the idea that Btn1p is primarily a glycosylated lysosomal or vacuolar protein at elevated pH and at low pH it is an unglycosylated protein found outside of the vacuole in yeast.

Human CLN3 has been found in association with multiple proteins, including calcein, fodrin, SBDS, and myosin IIB. None of these proteins are associated with lysosomal function. This supports the working hypothesis that CLN3 protein may be localized to other areas outside of the lysosome. To translate the findings of the yeast study to mammalian CLN3, Pearce and colleagues selected osmotic stress (increased sucrose and salt in the media) as the manipulated environmental variable. pH manipulations were not suitable, as they caused cell death. The study created a cell line with controlled expression of CLN3 under the ubiquitin promoter. The results showed that increased osmolarity upregulated myc-CLN protein and mRNA. Additionally, when assessing localization osmotic stress decreased golgi localization of the myc-CLN3 and increased lysosomal localization of myc-CLN3. Intermediate localization of CLN3 is still possible, as it has been noted to be in the early endosomes.

Neuronal Ceroid Lipofuscin: Marker or Cause of Disease?

David Palmer, PhD, Lincoln University, New Zealand: The role of the fluorescent storage material that is ubiquitously expressed in patients affected with neuronal ceroid lipofuscin is complex. Is it a result of the genetic mutation or does the accumulation cause the disease? It is indeed a storage material that stains positively with Luxol fast blue and periodic Schiff base. There is a peroxidation theory that states ceroid lipofuscin is created through an oxidation reaction of polyunsaturated fatty acids followed by a reaction with an amine of a protein or phospholipids resulting in a fluorescent Schiff's base. Dr Palmer and colleagues find that when analyzing phospholipids associated with the storage bodies, there was significant retention of linoleic acids, linolenic acids, and additional phospholipids. This weakens the peroxidation theory, because if polyunsaturated fatty acids are undergoing a significant amount of peroxidation there should be a low concentration of the reactants.

Upon analyzing the actual biochemical composition of the core storage body material, Dr Palmer and colleagues were able to sequence the protein make-up of the storage material. Fifty percent of the storage body mass was identified as the complete and functional c-subunit of the mitochondrial ATP synthase. This is a very unusual and highly insoluble protein, which supports its histological property of having a fatty waxy surface property. Subsequent experiments have also shown c-subunit to be the dominant stored product in the storage material in CLN2, 3, 5, 6, 7, and 8 forms. CLN1 and 10 were the notable exceptions,

and both had different proteins making up the storage material that were associated with the characteristic granular osmiophilic deposits ultrastructure.

The c-subunit is one of 16 different protein components that comprise the mitochondrial adenosine triphosphate synthase. Its functional role is to sit in the mitochondrial membrane drive the rotor around as protons go through the protein. This allows for subsequent conformational changes to occur and allows the catalytic component of the protein to generate adenosine triphosphate from adenosine diphosphate and inorganic phosphate. This may lead one to think that *CLN* mutations could be associated with energetic discrepancies, but at this time no arguments are sustainable enough to support this theory.

If the ceroid lipofuscin storage material is predominantly composed of c-subunit of adenosine triphosphate synthase, where is the fluorescence coming from? Palmer and colleagues have found that upon dissolving the storage bodies fluorescence vanishes. When the c-subunit is reconstituted with normal membrane phospholipids, fluorescence appears again. This shows that fluorescence is a physical artifact of the way the proteins proximal lipids are packed. The distance between the layers is sufficient for quantum dot fluorescence from diffractive source.

The discovery of this c-subunit accumulation begs the question how is the genetic mutation related to the storage body accumulation and subsequent neurodegeneration? In 2-year-old New Hampshire sheep, when comparing *CLN6* to wild-type brains there was significant cortical atrophy noted in the affected brain. Further analysis showed there is also global accumulation of storage material throughout the brain layers, although some regionality is noted where the storage material accumulation is light and small like the motor cortex.

Dr Palmer introduces certain physiological dimensions to consider when thinking about neuronal ceroid lipofuscinoses. The first posits that location and connectivity determine survival and non-survival more than cell type does. This stresses the importance of cellular communication and where these neuronal cells are positioned. Extending this idea, Dr Palmer studied the effect of intercellular communication by studying chimeric sheep that had a combination of normal and partly affected cells. Sheep that had 75% affected cells in their brains were seen to live like normal sheep.

Through Dr Palmer's studies it is apparent that perhaps ceroid lipofuscin is a misnomer. Though they do exhibit histological manifestations, it is the c-subunit of adenosine triphosphate synthase that appears to be accumulating. The reason or function behind this accumulation in the storage bodies remains unclear. It does appear that neuronal ceroid lipofuscins are not a cause of disease.

Questions and Answers—Dr Mink: Well, let me ask a question then. Let me ask 2 questions. First of all, the evidence presented for *CLN3* for a strong inflammatory component was quite clear. Do you think that is a feature of all of these NCLs or do you think that it's—Jon Cooper presented some data on relative timing of when there seems to microglial activation in different forms. But do you think that that's a common feature of this?

Dr Pearce: We routinely use the other mouse models with CLN1 and CLN2 and 6 as controls for a lot of the studies that we do for CLN3 or for JNCL. We do see that there clearly are neuroinflammatory responses, but as I think Jon articulated, the timing of them can be very different. What makes juvenile exquisitely different is the fact that they have these circulating auto-antibodies, a number of which we've characterized. I think we'll hear a little bit more about that this afternoon. And there is no autoimmune response, as I understand it, that's been demonstrated in the NCLs. It'll be curious to see with all of these new forms coming along if CLN3 stays that way.

Dr Palmer: For me—sorry

Dr Kielian: That's OK. Go ahead.

Dr Palmer: That's certainly in the sheep. It begins prenatal and regionally, the astrogliosis and the microglial activation a little bit prenatally, perinatal. So yeah, there's no doubt that it's involved and very much involved, really, and that's certainly true for the CLN6 and CLN5 forms.

Dr Pearce: Tammy?

Dr Kielian: Yeah. So I'd just like to add some recent things that we've done. We've taken different brain regions and have looked for just kind of homogenizing tissues, and mediators. In those early ages, we looked at 1, 2, and 3 month postnatally. We can see glial activation. We can see these subtle perturbations, but we don't see overt proinflammatory mediator expression at those earlier stages. That differs a lot, I think, with what Mark Sands' group has shown in infantile NCL, where they see very early increases, usually in proinflammatory mediators. So I think it may also depend on the kinetics of the disease process. Right now, we're trying to look later in disease to see if we can see more global evidence of inflammation. But I think what we're kind of thinking is that earlier there are more subtle changes, and then they just build to crescendo later.

Dr Mink: What's your question?

Audience member: I hope this is a fair question of this panel. Given that there are a certain number of normal, if you will, skin or white blood cell biopsies in affected patients, we see that this ubiquitous storage material is not necessarily present in every single cell of a certain tissue type. I'm just wondering what the percentage is of negative, if you will, biopsies that we might be performing, and if you have any thought about why it would be negative in an affected patient.

Dr Palmer: Well, the big problem is you don't see them in fibroblasts unless you biopsy some nervous tissue. I think that's a sampling issue.

Dr Maria: Are there any disorders other than NCLs where we find this subunit C collection?

Dr Palmer: There are minor amounts reported in some cells in some of the mucopolysaccharidosis 4, I think, and I think in parts of the brain, so you don't have this ubiquitous accumulation. And it's very much an NCL phenomenon.

Dr Mink: Question?

Audience member: Is MR spectroscopy helpful in evaluation of these patients?

Dr Kielian: I think so.

Dr Mink: I guess the question is, is there any specificity to it and how sensitive is it. We saw some data that were presented, but I think on a clinical basis whether changes you might see in MR spectroscopy—certainly, the things that are looked for routinely on a clinical basis don't include glutamate or GABA, and you certainly—I'm trying to remember what the references are, but certainly do see a progressive loss of so-called neuronal markers. But whether that's specific for Batten or whether even looking for glutamate GABA differences in this disorder versus other neurodegenerative diseases... I think we don't know.

Dr Kielian: Yeah, I would agree with that. I think that it could be used as a way to track therapeutics to try to normalize those levels, but, also, acetylaspartate is the metabolite that's used to track neuronal integrity. And that's changed a lot in other neurodegenerative diseases as well. So I think as a primary diagnostic it wouldn't work probably very well because you probably couldn't rule out a lot of other things. But I think in certain instances our plan is to use it as a therapeutic indicator. I think there it would be helpful to see if any kinds of targeting therapies can bring down that glutamate and try to bring up that GABA.

TRANSLATIONAL SCIENCE AND CLINICAL FRONTEIRS

Moderator: Marc Patterson, MD, Mayo Clinic, Rochester, Minnesota

Gene Therapy and Enzyme Replacement

Dr Beverly Davidson, PhD, University of Iowa, Iowa City, Iowa: Dr Davidson opened this session discussing gene therapy and enzyme replacement strategies for late-infantile neuronal ceroid lipofuscinosis. She began her talk by highlighting the major issue of using enzyme replacement therapies for lysosomal storage diseases of the brain. Due to ineffective passage of enzymes across the blood-brain barrier, intravenous injection of recombinant tripeptidyl peptidase 1 is not likely to have any therapeutic significance for late-infantile neuronal ceroid lipofuscinosis. While it is possible to permeabilize the blood-brain barrier experimentally, this is not a feasible option in a clinical setting where repeated enzyme administration may be required.

An alternative approach to delivery is to bypass the blood-brain barrier completely with direct injections to the ventricular space of the brain. Dr Davidson and her colleagues hypothesized that if enzyme could be distributed to the cerebrospinal fluid, it could possibly provide widespread access to all part of the brain. To test their hypothesis, they performed a 14-day intraventricular infusion of tripeptidyl peptidase 1 enzyme on CLN2 mice, and analyzed the mice after 25 days. Results of tripeptidyl peptidase 1 activity assays show that intraventricular recombinant enzyme infusion resulted in enzyme distribution to numerous central nervous system regions, as well as increased tripeptidyl peptidase 1 activity throughout the brain beyond heterozygous levels. Glial fibrillary acidic protein immunoreactivity staining was used to examine the progression of gliosis in the primary

motor cortex. Late-infantile neuronal ceroid lipofuscinosis mice that received enzyme replacement therapy showed a significant diminution of gliotic response. Enzyme replacement therapy-treated late-infantile neuronal ceroid lipofuscinosis mice also exhibited attenuation of resting tremor phenotype, decreased autofluorescence in primary motor cortex, and partial recovery of neurons within the deep cerebellar nuclei.

In terms of gene therapy, Dr Davidson and her colleagues have been moving forward with a tripeptidyl peptidase 1-deficient dog model. Previous studies have shown that although intraventricular tripeptidyl peptidase 1 infusion in this dog model improved clinical phenotypes and increased life span, it led to unstable levels of recombinant protein within the brain. To achieve steady state levels of the protein, Dr Davidson and her colleagues looked for a vector that could transduce ependymal cells, cells that line the ventricles, for long-term secretion. They turned to adeno-associated viruses. These small, nonpathogenic viruses can be completely gutted. Because of this, these viral vectors can be manipulated to not replicate and only express a certain protein of interest. Adeno-associated viruses, or AAVs, also provide for persistent long-term expression without integration into genomes. They are currently being used in trials for many genetic diseases.

In a former study, Dr Davidson and her colleagues discovered a particular AAV that did transduce the ependyma. The study also showed that it could reverse lysosomal storage disease in another animal model, the MPS7 mouse. To determine which AAV strain would work best for the dog ependymal, they screened a number of different AAVs, and eventually found that the standard serotype AAV2/8 transduced the best. To assess the effect of this gene therapy strategy on ventricular enlargement, Dr Davidson and her colleagues injected AAVs expressing tripeptidyl peptidase 1 into a ventricle in dog models. Results showed a significant reduction in ventricular enlargement in treated tripeptidyl peptidase 1-deficient dogs. Immunoreactivity staining indicated that expressed human protein was broadly distributed in the central nervous system. Additionally, autofluorescent analysis displayed a substantial decline in the levels of fluorescent material in brains of treated dogs. One of the clinical phenotypes that was tested was visual-evoked potential. While affected dogs have a decline in this readout, treated dogs were able to retain that visual-evoked potential.

Despite the broad distribution of enzyme, the same extension in life span that was shown in the enzyme replacement therapy studies was not seen. Dr Davidson and her colleagues speculated the cause to be an animal immune response to the human enzyme. To investigate this further, a number of short-term experiments that followed recombinant protein levels over time were conducted in additional dogs. Results showed there were high levels of enzyme activity early after transduction of ependymal cells, but virtually no detectable levels in the cerebrospinal fluid at later points in time. Additionally, there were varying levels of neutralizing antihuman tripeptidyl peptidase 1 antibodies in both normal and diseased treated dogs. Dogs that had the lowest levels of neutralizing antibody titers had the highest levels of enzyme activity in the cerebrospinal fluid. This indicates that treated dogs do in fact have an immune response to the therapy, and that response is what causes the drop in the level of enzyme activity. This certainly raises caution for the treatment of individuals that are null.

Whether the dog study is predictive of human response to gene therapy is another question Dr Davidson and her colleagues seek to answer. To determine this, a series of parallel studies in nonhuman primates is currently being performed. Data so far has shown that AAV2 does transduce the ependymal well in nonhuman primate brains. Also, an in vivo assessment of intraventricular delivery of tripeptidyl peptidase using AAV in primates showed considerable distribution of enzyme, with above normal levels of activity in the ependymal regions, cortical regions, caudate, thalamus, and hindbrain.

Dr Davidson finally discussed another strategy to achieve global enzyme delivery, which involves taking advantage of the vasculature. Dr Davidson and her colleagues hypothesized that transduction of vascular endothelial cells would allow for apical and basolateral secretion of recombinant enzyme. The idea is that the endothelia would be used as a reservoir for enzyme secretion, so the endothelia themselves would express the enzyme and secrete it. When delivered basolaterally, some of that enzyme would transduce pericytes, as well as the underlying astrocytes and neurons of the neuropil. To achieve this, Dr Davidson and her colleagues developed and tested vectors modified for brain endothelia targeting work after systemic delivery. Recombinant bacteria phages were used to identify peptide epitopes that might bind to the endothelia. Phages were injected into the brains of diseased mice, and these brains were harvested 30 minutes later. Phages that were bound to the brain endothelia were recovered, and those sequences were extracted, purified, and put back into new mice. This process was repeated several times until just a couple of sequences that bound to brain endothelia were left. These sequences were then cloned into the capsid of AAV.

Thus, there were 2 types of AAV involved in this study, wild-type AAV and an engineered AAV that travels to the endothelia. Diseased mice were given tail vein injections of one type of AAV or the other. Wild-type AAV delivered via the tail vein in mice travels to the liver. Results indicated that in these mice, only the enzyme deficiency in the liver was corrected. Mice injected with the modified AAV had an increased level of enzyme activity in all regions of the brain tested. There was also a significant reduction of both the tremor and gliotic response in mice injected with modified AAV. These phenotypes in mice injected with wild-type AAV did not change. These results suggest this promising treatment approach may be an alternative to repeated delivery of enzyme or neurosurgical delivery of AAVs.

Combinational Therapies

Dr Mark Sands, PhD, Washington University School of Medicine, St. Louis, Missouri:

Dr Sands presented an overview of a series of experiments that investigated the effects of combinational treatments for infantile neuronal ceroid lipofuscinosis. With respect to treatment for infantile neuronal ceroid lipofuscinosis, a number of preclinical experiments have demonstrated partial improvements in disease parameters. These include intracranial gene therapy, small molecule-based drugs, stem cell-based therapies, and bone marrow transplant. The clinical responses to these single treatment regimens have been relatively minimal and modest.

Given the limited effectiveness of these therapies, it was hypothesized that therapies used in combination would better target the complexity of disease. To test this, Dr Sands and his colleagues combined central nervous system-directed gene therapy with various strategies that target some of the secondary consequences of the disease. Because of its transduction efficiency and high distribution levels in the central nervous system, researchers used the adeno-associated second-generation viral vector, AAV2/5, for gene therapy. Dr Sands also briefly discussed the palmitoyl protein thioesterase-1 activity deficient mouse model that was used in these experiments. These palmitoyl protein thioesterase-1-deficient mice suffer from the same pathological changes and functional deficits as patients with infantile neuronal ceroid lipofuscinosis. On the basis of these similarities, the palmitoyl protein thioesterase-1-deficient mouse became an excellent tool for investigating potential therapies for the treatment of infantile neuronal ceroid lipofuscinosis.

The first experiment combined central nervous system-delivered AAV-mediated gene therapy with the small molecule drug phosphocysteamine. The AAV-mediated gene therapy was used to supply persistent localized regions of high-level expression in the central nervous system, and phosphocysteamine was used to cleave thioester linkages in palmitoylated proteins in regions with low palmitoyl protein thioesterase-1 activity. The rationale behind this experiment was that continuous delivery of phosphocysteamine would complement the high distribution of enzyme activity that arose from the gene therapy, thereby reducing the storage burden and increasing efficacy. To evaluate the effect of the combinational therapy, palmitoyl protein thioesterase-1-deficient mice were given direct intraparenchymal injections of AAV2/5 on postnatal day 2. On postnatal day 28, intraperitoneal delivery of phosphocysteamine was initiated, and continued on a daily basis for the rest of the animal's life.

The effects of the different treatment strategies on life span were examined. Untreated palmitoyl protein thioesterase-1-deficient mice had a shorter life span when compared with normal mice. Mice treated with phosphocysteamine alone had no effect on life span compared with untreated mice. Treatment with either AAV only or AAV and phosphocysteamine significantly increased the life span of untreated mice. The combination of AAV and phosphocysteamine displayed a slight trend toward increased life span compared with AAV only, but the difference in longevity was not statistically significant. To investigate the effects on treatment on motor function, the mice were tested on a rocking rotorod paradigm. Untreated palmitoyl protein thioesterase-1-deficient mice and mice treated with phosphocysteamine alone had equally declining motor function. Treatment with AAV only showed a significant improvement in motor function. AAV+phosphocysteamine treatment showed an even greater statistically significant improvement in motor function when compared to untreated mice.

The second experiment combined AAV-mediated gene therapy with Minoxidil, a potent anti-inflammatory drug that targets glial cells in the brain. The rationale behind this experiment was that the AAV would supply persistent levels of palmitoyl protein thioesterase-1 activity, while Minoxidil would target the inflammatory response. The experimental design was identical to the first experiment, replacing phosphocysteamine for Minoxidil. The results of the experiment showed that when compared with those receiving AAV treatment alone,

mice treated with both AAV and Minoxac showed greater improvement in both life span and motor function. Mice treated with combined therapy lived 2 to 3 weeks longer and had a larger statistically significant improvement in motor function than mice treated with AAV alone. Mice treated with Minoxac alone showed no difference in life span or motor function when compared with untreated mice.

The final experiment combined AAV-mediated gene therapy with bone marrow transplantation. It was expected that AAV therapy would supply persistent levels of palmitoyl protein thioesterase-1 activity while the bone marrow transplant would either supply additional enzyme to the central nervous system through migration of donor-derived cells, or have an immunodulatory effect. In this experiment, mice were identified at birth, genotyped at postnatal day 2, and were given intracranial injections of the gene therapy vector at postnatal day 3. At postnatal day 4, the mice were subjected to a normal bone marrow transplant under myeloreductive conditions. They were exposed to 400 rads of radiation and injected with palmitoyl protein thioesterase-1-positive bone marrow cells. The life span and motor function were analyzed as the mice aged.

As expected, bone marrow transplant alone had no effect on life span when compared with the life span untreated mice. Untreated mice generally have a life span of about 8 months. Mice receiving AAV alone lived for 12 months, showing the typical significant increase. Mice treated with both AAV and bone marrow transplantation lived for 18 to 18.5 months, which is the largest improvement in life span with any of the single or combination therapies that have been tested so far. In terms of motor function, results were similar. Mice treated with AAV alone showed significant improvements. Mice treated with combined therapy displayed even greater improvements in motor function. For mice that received bone marrow transplant alone, motor function actually declined significantly.

In summary, the results of the experiments show that combinational therapies are associated with varying levels of improvement, whereas secondary treatments alone essentially have no benefit. The combination therapy of AAV and phosphocysteamine minimally improved motor function but had virtually no effect on life span when compared with AAV treatment alone. The combination of AAV and Minoxac significantly improved both life span and motor function. AAV combined with bone marrow transplant dramatically increases life span and improves motor function.

Clinical Trials: Challenges and Opportunities

Dr Erika Augustine, MD, University of Rochester Medical Center, Rochester, New

York: Dr Augustine's talk centered on the challenges and opportunities in rare disease clinical trials. She began her discussion by reviewing the current state of treatment in the neuronal ceroid lipofuscinoses. Since Stengel first described patients with neuronal ceroid lipofuscinosis 200 years ago, there is still lack of an FDA-approved disease-modifying therapy for any neuronal ceroid lipofuscinosis. Although researchers have been able to target specific symptoms such as seizures and behavioral problems, strategies for treatment are fairly independent of the actual underlying neuronal ceroid lipofuscinosis diagnosis. Promisingly though, there have been several clinical trials in the neuronal ceroid lipofuscinoses in recent years.

Dr Augustine then stressed the importance of understanding the goal of developing cures. Since the treatment goal can be to stabilize, improve, or reverse symptoms, establishing a specific objective is a crucial step in every aspect of therapeutic development. Dr Augustine then considered neuronal ceroid lipofuscinoses in the context of rare disease challenges. The neuronal ceroid lipofuscinoses are rare diseases, which the Orphan Drug Act defines as disorders affecting fewer than 200,000 individuals in the US. While the focus on the neuronal ceroid lipofuscinoses is an extremely small population, more than 8,000 recognized rare diseases affect almost 30 million individuals collectively. Because many of the concepts addressed are common to rare diseases, Dr Augustine emphasized that researchers must take advantage of the opportunity to learn from others who face similar obstacles.

Dr Augustine went on to discuss challenges in creating therapies for rare disease, which can be organized into 2 categories. One category relates to proof-of-concept, which encompasses preclinical factors such as the discovery of target agents and the establishment of potential therapies. In terms of rare disease pathophysiology, many questions remain unanswered. Researchers face the challenge of having to develop a therapeutic target and assess whether the target is appropriate in a setting of unclear pathophysiology. Specific to neurodegenerative disorders, there is the additional concern of central nervous system delivery and crossing of the blood-brain barrier.

Another issue Dr Augustine touched on was the animal model. There has always been a question of how well preclinical animal models predict human disease and drug activity. How accurate are these models for the target in question, mode of delivery, and estimation of anticipated clinical benefits? Target engagement can be assessed postmortem in animal models, but for some targets, it might not be possible to achieve that in vivo in humans. Also, significant components of human disease such as behavioral and cognitive characteristics are quite difficult to evaluate in animal models.

As rare disease researchers facing these preclinical challenges, it might not be economically feasible to develop all that is necessary across the translational spectrum at a single academic center, or set of centers. With that in mind, Dr Augustine stressed the importance of using existing resources, and highlighted the National Center for Advancing Translational Sciences Therapeutics for Rare and Neglected Diseases program. This program aims to stimulate drug discovery and speed development of new and repurposed drugs. A unique aspect of this program is its access to preclinical study resources. This is definitely a good opportunity for both bench and clinical researchers to come together and work to accelerate development of new interventions for neuronal ceroid lipofuscinosis.

The other category of rare disease challenges relates to trial execution. This involves developing studies that will prove whether treatment options are effective. Dr Augustine pointed out that although these challenges are not unique to rare diseases, they are certainly amplified by the limitations that studying a small population bring. The first of these challenges relates to clinical trialists. The field of rare disease research needs individuals with interest to design trials for rare disorders and who understand the rigors of experimental therapeutics. Unfortunately, the number of clinician scientists to conduct these trials is declining. The best way to combat this issue is to encourage young people to learn about and

partake in the science of clinical trials itself. Dr Augustine highlighted a number of clinical trial training opportunities, including experimental therapeutics fellowships, National Institute of Neurological Diseases and Stroke Clinical Trial Methods Course, ASCENT Training for Neurotherapeutics Discovery, and NeuroNEXT.

The next issue involves finding the funds, knowledge, and resources to complete the study and take on the many regulatory issues that may arise. On the matter of drug development, common diseases tend to have a larger economic impact, and since rare diseases affect a smaller market, they have a lower turn on investment. A solution to this problem is the Orphan Drug Act, which has been developing incentives to stimulate therapeutic development for rare disease. These incentives include protocol assistance from the FDA, tax credits, and longer market exclusivity. Although this program has been reasonably successful, it is important to think about other kinds of opportunities in funding for academic-initiated trials, academic-industry partnerships, the use of the FDA Orphan Product Grant Program, and federal funding mandates for rare disease. Dr Augustine did mention that the consensus, however, is not a funding issue, but rather a resource issue.

Another significant obstacle is being able to select the right sample and recruit participants in an ethical manner. To recruit a sufficient sample, and to do so in a timely manner, is certainly a challenge given the small number of affected, eligible individuals. Other problems related to the recruitment of small populations deal with early diagnosis, geographic dispersion, placebos, therapeutic misconception, consent, risks, and benefits. Dr Augustine went on to discuss the various opportunities to help advance new therapies for limited populations. These include partnering with patient organizations, developing registries of individuals interested in clinical trial participation, and taking advantage of technologies like telemedicine in assessments.

The final and most important challenge in this category involves the study design, measures, and outcomes themselves. Dr Augustine first brought up the double-blind, randomized controlled trial. This design minimizes selection bias, distributes confounders equally between study groups, and facilitates blinding, which improves participant compliance and retention, and limits investigator bias in outcomes assessment. Although this design is considered the gold standard for establishing cause and effect in a therapeutic setting, it presents a number of hurdles in rare disease research. Blinded randomized controlled trials usually look for small effects, which can be very costly and time-consuming, as well as require a large number of patients. None these aspects are feasible for rare diseases, however, especially not in ones rare as the neuronal ceroid lipofuscinoses.

Trial design is certainly strengthened through use of a comparator, but in a fatal degenerative disorder, ethical questions about use of placebo may arise. This also becomes an issue of sample size. Randomization does not apply in a single-group study, so other means are needed to minimize bias. Also, as it relates to analysis, there is a dual disadvantage. By necessity, these are small studies, but there is also individual variability in clinical course, both of which diminish a study's power. Designing statistical techniques that maximize data from small numbers of subjects is vital. It is ideal to avoid missing an effect

due to study design and sample size, and have a sense that there is a true lack of biological effect.

When considering how to address these concerns, better understanding natural history is imperative. Historical controls present their own challenges, but a registry of specific, prospective data may introduce efficiency into future studies. Additionally, existing resources for improving trial design are certainly available, which includes the Office of Orphan Products Development Science of Small Clinical Trials annual course. Direct interaction with the FDA itself regarding study design and outcomes is encouraged. Also, Dr Augustine mentioned the National Institute of Neurological Diseases and Stroke Network for Excellence in Neuroscience Clinical Trials, where protocol-working groups are formed for each study being considered for execution through the network. Lastly, researchers should take advantage of the opportunity to use alternative designs. It is important to think beyond traditional parallel group or open-label studies to add design features that will potentially increase knowledge gain from a single study.

Outcome measures are another significant challenge in terms of trial execution. In rare diseases, validated measures of disease activity and progression generally are lacking. There are some clinical measures, but they are in need of refinement. Also, there is a role for biomarker development that can help support proof-of-concept or biological plausibility of interventions in early phase studies. This is especially needed in juvenile neuronal ceroid lipofuscinosis, where enzyme assay is not relevant and the course of disease is slow. Additionally, researchers may not yet fully be aware of what outcomes are important to patients and their families. With these issues in mind, it is important to think about the ways to refine clinical rating scales to better quantify these multifaceted diseases, to increase their precision related to small changes, and to address ceiling and floor effects. This is surely the time to engage families about outcomes meaningful to them, and establish consensus regarding common use of outcomes, both preclinical and clinical.

Dr Augustine concluded her talk with a review of certain clinical trials whose design features address many of the rare disease challenges that were discussed. A current study is examining the effectiveness of the drug Cystagon in treating infantile neuronal ceroid lipofuscinosis. To develop a homogeneous group for entry, this open-label, single-group study set restrictions based on both gene mutation and age. Also, the study was centrally conducted at the National Institutes of Health. Thus, patients were required to travel to a single center. Dr Augustine also pointed out a series of Stem Cell Inc. trials. One specific study was unable to continue its second phase because of a lack of eligible patients identified within a reasonable timeframe. This trial was an example of how there can still be challenges in trial execution because of small cohort size, even when finances and resources are stable. The last study discussed was a juvenile neuronal ceroid lipofuscinosis mycophenolate study, which had an alternative crossover design. Each participant served as his or her own control, which allowed researchers to maximize the information gained from a single sample. The clinical trialists worked with the Batten Disease Support & Research Association and used a contact registry to initiate recruitment of subjects, which was tremendously helpful.

Questions and Answers—Audience member: I was just curious. The 2 studies with the drugs, you did the mice, you did the gene therapy at day 2, and then you waited 28 days for the drugs. For the bone marrow, it was done the day after the gene therapy. Is there a chance that some of that increased effect was because you intervened earlier? If you had done the Minozac at day 4 and continued it, would you have seen a similar effect?

Dr Sands: Well first of all, I don't know the answer to that question. I think it's certainly a possibility. Working with neonatal mice is tricky. Giving a neonatal mouse an injection every single day—it's tough enough giving adult animals injections like that. With newborn animals, you have the added problem that you to give it back to mom, and oftentimes, if the mice are handled too much when they're young, the mothers will either ignore them or kill them. So I think it's possible, certainly, that that window that we didn't give the small molecule drugs, we might have gotten increased efficacy. I think it's possible. But I don't think we would've gotten the same response that we did with the bone marrow transplant, but that's just a guess on my part.

Audience member: I think one of the things that is interesting and certainly was discussed in all of the talks today that before we contemplate further for clinical trials, it would be really interesting to understand and maybe you'd know more that hasn't been shared but understanding where the therapeutic window is for these patients specifically. And it sounds like, obviously, in the different animals models it's different, but that has clear implications, again, in thinking about these are rare diseases, we have a limited number of patients, and making sure that we design the trials, as was discussed in the last talk, so that we are fairly certain, or at least we have our best guess to actually have the potential to see an outcome measure change. I think that that speaks to this therapeutic window that we really need to understand, I think, before we recruit further patients for these trials.

Dr Davidson: I completely agree. I think the last thing we want to do is to perform a trial in an individual in late-stage disease and sort of freeze them in that state. Again, you aren't able to assess whether you're having any therapeutic benefit or not or if it's something with the natural history of that disease or that particular mutation that allows for prolonged survival. We can learn only so much from animal models. I think it will be until we have more interventions in children before we learn how these may modulate the disease course. And it's, obviously, going to be different for INCL and LINCL and JNCL.

Dr Sands: It's a complicated situation because what you would like to do is treat these children as early as possible when they're presymptomatic to get the best outcome. For most of these Phase 1 trials, treating a newborn child is not an option. In fact, most of the kids, of course, aren't picked up until they're symptomatic. In many of these diseases that's too late. In fact, in INCL, Sandy Hoffman just published a paper with enzyme replacement therapy, and she showed that if you started the enzyme replacement when the mice were 8 weeks of age, which they're still presymptomatic at that state, there was no clinical benefit whatsoever. However, if she treated the mice starting at birth, there was a significant benefit.

Dr Davidson: I think this comes full circle back to this morning's presentations and from Dr Mink and colleagues where we need to continue to do natural history trials in these diseases.

We need to keep enrolling as many children as possible. From the neuropsych side, from Heather's data, to standard neurological phenotypes. The more we know, the more informed we can be as we move forward.

Dr Augustine: And I might add to that. Not so much thinking about how far does the window extend, but thinking in the other direction of how early we can go. When we have therapies to offer, that really will change thinking as it relates to asymptomatic or presymptomatic diagnosis for siblings. I think when we have therapies on the table, then we really have to reconsider what our standard has been for asymptomatic testing.

Dr Mink: It's a question for the panel and maybe one that could be extended into the later panel discussion, too. As Erika pointed out in her talk, experimental therapeutics has really become a science of its own, particularly moving into some of these areas like therapeutics for rare disease, and adaptive trial design, et cetera. Is there (1) an effort, and (2) perhaps the first question, a therapeutic development sense, too? So as I heard your talk, Bev, your talk, Mark, and wondering, OK, how do you make that decision; how do we make that decision; OK, we're ready to go. Let's ignore the FDA role in approving it, let's ignore the money role in approving it. Given that there are more potential targets than potential patients, perhaps, how do you know, as the preclinical investigators, to make that decision to say this is something that really should go forward given, again, all the multiple permutations that are possible?

Dr Davidson: I guess I'd like to ignore the FDA, but we don't. No. I'm just kidding if the FDA is here.

Dr Sands: You're safe.

Dr Davidson: Yeah, I'm safe. When we start to have these positive effects in rodent models, it's very exciting. We're getting to the point where we can do this repeatedly with a number of different interventions now, and then it's moving onto larger animal models. And even just safety testing in nonhuman primates to see whatever it is—the proof of the principle of what we're investigating holds true in a larger brain. You heard a lot about sheep models and dog models, et cetera. I think if there's an inkling that our hypotheses may be correct, then it's really time to engage the clinical investigators and the FDA. I've found them very receptive to just kicking around ideas on what do they think are the necessary experiments to file an IND to move these experiments into humans. And the more investigations we can have in this field, the more clinical neurologists that are engaged at all levels, yourself, Rob Steiner, Erika, et cetera, the more we can learn from you all.

Dr Sands: Right. Exactly. So it's a very good question, Jonathan. I don't know what the answer is except part of my thinking is these are invariably fatal diseases. There is nothing out there for them. That doesn't mean we become cavalier and just throw any old thing at it. There's got to be good science. There's got to be a good rationale. But, again, there's nothing out there. And given how rare they are, it's not like we can get a large pharmaceutical company to invest half a billion dollars into going through every permutation.

There has to be a different paradigm, I think, for us to move these things forward effectively, especially when we think about the stuff we're doing with the combination. Are they going to force me to do each individual arm, some of which have no efficacy whatsoever, and then combine them? Well, we'll run out of patients. And, again, like with bone marrow transplant, it would be unethical to do a bone marrow transplant in an INCL child. It's been done. It doesn't work. How do we do that? Like I said, I think there needs to be a whole new mindset here to move these things forward. I don't know what it is, but, anyway. To simply answer your question, if I've got good solid mouse data, it's a rational experiment, I can repeat it, I say we start thinking about it.

Dr Maria: My question was on the theme of treating early and doing that in a presymptomatic way. If genomic sequencing is to be part of neonatal screening going forward, as the costs have fallen for sequencing, there's every expectation that that will become our new screener at birth, have you given any thought to functional genomic aspects? In other words, my understanding is that the placenta is roughly two-thirds the genome of the child. So could one be looking at placental cells and if genomic sequence identified NCL3, could one be using those cells in some functional assay to look at therapies or combinations of therapies to sort of add some specificity to what would need to be done in a presymptomatic way? It seems to me we may have an opportunity to develop in the dish some assays that might have some predictive validity. Is that wild and crazy or does that make sense?

Dr Davidson: I don't think it's wild and crazy. I think it does make sense. Maybe placenta because there's such a huge maternal component to the placenta if you're not careful where you isolate it. But other tissues that you could harvest and do functional genomics is not a bad idea. A cheek swab would be probably the easiest or blood from the baby when they poke the heel for some of the more common disorders.

Dr Maria: What would those assays be?

Dr Davidson: Maybe it would be in response to some small molecule therapy. For example, some mutations may be more responsive to some therapies. Another, you would be able to implement that therapy. It's more in line with personalized medicine as we think about going forward. Or it may be that this particular mutation will only respond to a full enzyme replacement, for example, or a full gene replacement. But I think there certainly are things that you could think about down the road in doing it in an individualized manner.

Dr Sands: I know there are certainly people developing the high throughput assays, obviously, in cell-based systems, and those might be able to be applied, for example, if there's some meaningful readout.

Dr Davidson: What impressed me this morning, too, was the general notion that for many of these diseases there's some very highly prevalent mutations. And particularly for JNCL where the phenotype is pretty tight. I think we may have a better opportunity to say that a particular therapy is working because the natural history is a little tighter. For some of the other diseases, I think we just need more and more patients to begin to develop the kind of things that you're suggesting.

Dr Palmer: Have you tried either a blood transfusion rather than a bone marrow transplant in your combined therapy or have you tried just the radiation without the bone marrow transplant?

Dr Sands: That's a good question. So we have not tried just the transfusion in this model. Now, what I will tell you is in NPS7 we've done experiments where we've taken fully differentiated cells. We've done experiments where we've done macrophage infusions. We've done separate experiments where we've done lymphocyte infusions to see if there's any benefit, and there was, but they were transient in nature. And, unfortunately, we didn't look to see if there were any immunodulatory effects in those experiments. But I think if you did that, if there was any response at all, it would probably be transient. To your second question, because we thought that experiment was going to be a busy, we didn't do all the right controls. We have mice on the shelf right now that receive the gene therapy and radiation alone, and they're now about 10 months of age. So I still can't interpret the data. But we did that experiment in the mouse model of Kreb A disease, so the twitcher mouse, where we saw the same kind of synergy between the gene therapy and the bone marrow transplant. We gave them radiation alone, the gene therapy, and there was absolutely no benefit whatsoever to the animals that got radiation and gene therapy, even though we did see higher levels of expression, just like what I showed in INCL. So I don't think, although I don't have any proof of this, I don't think it's simply increased expression. I think it's more complicated than that. But I don't have the data in INCL yet. Ask me in a couple of months.

EXECUTIVE SUMMARY OF THE DAY

Dr David Pearce and Dr Jonathan Mink

Session I covered the clinical aspects of Batten disease. Dr Mink reviewed the natural histories and classification of Batten disease, concentrating mainly on comparing and contrasting the clinical presentation of each subtype. While age of onset and the order of symptom presentation are key components in Batten disease classification, there is a great deal of variability, even within specific types of neuronal ceroid lipofuscinosis. Thus, these measures cannot reliably predict Batten disease subtype. Dr Sims gave a comprehensive overview of Batten disease genetics. She covered the most common mutations in the genes leading to the disease and how they affect protein function. Although the different forms of neuronal ceroid lipofuscinosis have certain facets in common, each individual disease has a different biochemistry and cell biology. Nevertheless, there may be very important interactions between the proteins involved in the different forms, as well as other types of neurodegenerative diseases.

Dr Adams discussed the neurobehavioral aspects of CLN3. She focused primarily on the Unified Batten Disease Rating Scale and the clinician global impression of cognitive function and behavior. Because of the disease's rapid progression, early onset of the different forms of neuronal ceroid lipofuscinosis is more difficult to study. Using careful, quantitative approaches to neuropsychological and behavioral features, it is possible to characterize the phenotypes of these disorders and see how they change over time. Dr Cooper gave the final talk in this session over the neuropathology and neurobiology of Batten disease. He emphasized the role of synapse, as well as the activation of supporting

cells in the brain, such as astrocytes and microglial. The dysfunction of these cells may be a crucial event in Batten disease pathology.

Session II covered the molecular mechanisms of Batten disease. Dr Kielian discussed the role of inflammation and the immune system in Batten disease. She highlighted the importance of microglial cells in Batten disease pathology and how these cells may become vital therapeutic targets. Dr Pearce talked about the current knowledge of CLN3 protein. Although this transmembrane protein has been profoundly studied, the function of CLN3 is still not understood. Nevertheless, studies are beginning to suggest that the CLN3 protein may work in lysosomal acidification, in vesicular transport, or as an osmolarity regulator. Dr Palmer closed this session reviewing Batten disease storage material. He explained that this storage material is principally comprised of subunit C of the mitochondrial adenosine triphosphate-synthase complex. However, this material may not be correlative to the disease because of its heterogeneity, as well as the differential accumulations seen in various models and cell types.

Session III covered the translational science of Batten disease. Dr Davidson opened this session discussing gene therapy and enzyme replacement strategies for late-infantile neuronal ceroid lipofuscinosis. Enzyme replacement can be used to directly infuse recombinant tripeptidyl peptidase 1 into the brain. Gene therapy for late-infantile neuronal ceroid lipofuscinosis involves using adeno-associated viruses to insert the normal *CLN2* gene into brain cells of mouse and dog models. Dr Sands discussed combinational gene therapies in infantile neuronal ceroid lipofuscinosis. Studies have shown that bone marrow transplantation along with AAV treatment leads to significant benefits over bone marrow transplantation or AAV treatments alone in mice. Finally, Dr Augustine pointed out the challenges and opportunities of clinical trials in Batten disease. Because of a lower number of researchers, a smaller sample size, and fewer available funds, clinical trial issues are magnified across all rare diseases.

FUTURE DIRECTIONS PANEL DISCUSSION

Dr Mink: Let me introduce who's coming up. I will stand in for Story Landis, who was unable to get out of DC because of Hurricane Sandy. Our panelists include Marc Patterson, who moderated the previous session, who is an expert on metabolic disorders of childhood, and who is at the Mayo Clinic. Beverly Davidson, who you also heard from in the last sessions, talked about gene therapy, from the University of Iowa. Bob Steiner, who is a medical geneticist/pediatrician from Oregon Health & Science University, and was involved in the human stem cell trial in CLN1 and CLN2 disease. Gary Clark, who is at Texas Children's Hospital and Baylor College of Medicine, and is the director of the Batten Disease Center of Excellence there. And because I don't believe that Ann made it from Washington, we have a special guest joining our panel, Dr Angela Schulz, who is from Hamburg, Germany, and is leading a cooperative multinational European consortium for studying different forms of Batten disease.

Dr Maria: We've had a wonderful review of where we are in the way of the science and the clinical aspects. The purpose of the panel is really to advise on future directions. What are the priorities going forward across the spectrum that we covered earlier today?

Dr Patterson: So for people who talked about using whole exome and whole genome sequencing, Kathy Sims gave a beautiful presentation, and we've talked about newborn screening. But I'd like to be a little bit more productive and suggest there's one lysosomal disease where it's a great genetic success story, and that's Tay-Sachs disease. That's not because of newborn screening. It's because of carrier screening. That hasn't been a practical proposition for any of these diseases until now. And it's perhaps not quite there, but I think with the prospect of whole exome sequencing, which is in clinical practice and I'm sure there are many people in this room, I certainly have used it many times, and I think many people already have, that we will have the prospect of doing very broad carrier screening. Now, I understand that there are a lot of technical and ethical aspects to that, which are worth discussing, and in no way diminishes the fantastic work that colleagues here and who are not here have done in similar diseases; to me, this is really the way to defeat these diseases. It's to let people have this information before they can see the child. Because, as I say, you can hold up the example of Tay-Sachs disease, for which there is still no disease-modifying therapy 33 years after the enzyme deficiency was described. Forty-three years I should say. So I'd like to open that to my colleagues for their discussion and comments, and members of the audience.

Dr Davidson. I think that's a fantastic thing to bring up. I just wonder if 23andMe, given the prevalence of these mutations, for example, the common deletion in JNCL, if that's even on the panel for 23andMe, which a lot of us in the room have probably sent our cheek swabs to. But I don't know the answer to that. Do you?

Dr Patterson: I don't know if that's covered. But I think one of the things that was emphasized in previous talks, and Erika had mentioned as well, is education. There's an enormous need for education, not just within the profession—we have a group of people here who are very interested in this topic and I'm sure keep up with this subject—but for the population in general. I think there's a great deal of misunderstanding about this. I can tell you in the state I come from, we can't keep dried blood spots for more than 2 years because people are worried that somehow the government is going to manipulate this information about their genomes. Which makes me wonder which planet these people live on. The government could barely deliver mail, so I don't see how we can run huge conspiracies, but I think it really is a way forward.

Dr Schulz: I can comment a little bit on the dried blood spot screening where you can measure the enzymes for CLN1 and CLN2 disease. Of course, Germany is where I'm from. It's much, much smaller than the US, so it's easier to try to contact most of the child neurologists and to bring the word out there what is NCL disease, when do we have to think about NCL disease. Since we have been trying to do this, starting at our center in Hamburg, we have the number of dried blood spot samples that we get for diagnosis over the last 5 years has more than tripled in a year. Initially, we thought well, now, everybody is just sending dried blood spots when they have something weird in their clinic, because in

Germany it costs like 50 euro and then you can exclude CLN1 or CLN2, which is rather cheap.

But, still, we found now when we looked at our statistics, the number of patients that we have diagnosed, especially for CLN2, has doubled over the last 5 years. So there is, I think, a benefit from really bringing the word out there, and, especially, I think, together with also testing siblings and other relatives to see whether they are carriers—which sometimes is a hard thing to find the health insurance to cover the costs for this testing, even in Germany, and we have a more social health system than you have here in the U.S. But, still, there the health insurance is very careful to do that. But the enzyme testing is really cheap, I would say, and so this way at least you might be able to find more patients. And the more patients you know, the better genetic counseling you are able to do. And then, again, the sibling testing or relative testing would kick in. So I think this is a good way to start from.

Dr Mink: So it's a prevention model based on information.

Dr Schulz: Yeah.

Dr Mink: Let me throw a monkey wrench in this. There was actually a very nice series of articles in Time magazine this past week or 2 on genetic information, genetic testing in children. It's one matter if you can say aha, you have this mutation and, therefore, we can say this is a classic Mendelian recessive condition, and if you have children with someone else who has this mutation that there's a 25% chance you'll have a child with that. But one of the things we've learned from more extensive and more modern sequencing is there are all these modifiers, too, and we don't really have a good handle on that.

One of the things we learned from Hurricane Sandy is that people who lived in Hoboken, New Jersey, said "oh, I've been through these before, and even if you say there's a 50% chance that I'm going to be flooded out of my house, I've never experienced it. I'm going to take that chance." What do we do from the educational standpoint or how do we deal with this uncertainty where we say OK, well, this confers a 13.2% risk of you having a child with this disorder, that's a number that to a lot of people means absolutely nothing unless they've already had a child with this disorder, and they say that there's no acceptable risk? What do we do with that?

Dr Patterson: I think you're conflating some different things here. As you point out, at the moment, we're talking about autosomal recessive disease. And it's true there must be modifiers for those because there's enough variability in most of the phenotypes. So I think that's the low-hanging fruit, if you will, in this situation.

I think when you're talking about risk factors that is a difficult issue. There's no question about it. The example you provide, I think, is a good one. I can tell you when I was at NIH and we were doing the enzyme replacement studies for Gaucher disease, we had a physician, who was very prominent in the area, who had had 3 children after the first 2 were diagnosed. She knew very well the odds. So I think we have to differentiate between our providing information to the people, and the way they act on it. We can't control stupidity. We're about to have an election and I hope it doesn't manifest itself, but I'm worried about that,

and you could take that any way you like. But I think we mustn't confuse those things. We must educate people as best we can. We'll have to make sure we have appropriate counseling. That's going to take a lot of resources, and there are not enough board-certified geneticists and genetic counselors to do the work. I think that's a huge issue. I'm not minimizing this. But it's happening.

As you point out, 23andMe, you can send your DNA off already. I'm sure in your practice, as I have, I've seen parents who brought me results to interpret. So I think we have to be very proactive about this. I think we should recognize the challenges, but also the opportunity, because I do think that we have an opportunity to really eliminate a lot of these diseases. It's not going to happen overnight. But I think in the long term, that's an achievable outcome. In the meantime, I think understanding these diseases is better. I think having patients involved in registries and developing therapies is entirely appropriate. But I don't think we should forget that long-term goal.

Dr Clark: I was just going to try and see if we could maybe move in a little bit different direction.

Dr Mink: Absolutely.

Dr Clark: I would argue that if you think about HIV infections, we could identify all HIV. We haven't eliminated the disease, but we have turned it into a chronic disease with successful treatment. So I'd like to turn the conversation a little bit to trying to identify how we could do a clinical trial. And much like Dr Augustine talked about, talk about some of the paradigms that we need to consider.

I know that there are a number of targets. We've talked about a number of potential disease-modifying therapies that could come down the pike. How do we do a clinical study? I think one of the things that Jonathan has done so well, and he hasn't talk about it today, was by developing this clinical rating scale, and using it prospectively, he has acquired data that we can now go back and look retrospectively, and, therefore, test hypotheses. We've talked about a hypothesis recently that he can data mine this wonderful clinical material for us, and perhaps we can test hypotheses almost prospectively, but retrospectively in a way. So Jonathan, if you could talk a little bit about that and the power of that, and your published study on flupirtine where you showed it didn't have efficacy.

Dr Mink: This all started—it's actually kind of an interesting history about how research gets done that I will spare you. But it came about from a relatively chance encounter between a biochemist and geriatric neurologist, who were sharing notes about what each other did. Fred Marshall, who was the instigator of this from a clinical standpoint, asked Dave Pearce, "So you want to cure Batten disease, how will you know when to find the cure?" His point was we have no outcome measures, we have no good biomarkers, we really have nothing other than these kids die after many years. That's what initiated the development of this rating scale.

We now have, in some individuals, longitudinal data for 11 years, and for a lot of individuals 1 year worth of data. But let me give you 2 examples. There was this idea that

emerged from some in vitro work that a compound that's used as a nonsteroidal anti-inflammatory in Europe, flupirtine, might prevent apoptosis. So based on this and based on the fact that there was nothing else really available as disease-modifying therapy, many families obtained flupirtine from Europe, had their children take flupirtine, and thought maybe their children weren't doing as poorly as they might have done without flupirtine.

We were able to then take our data and see if there was a difference between children who had been exposed to flupirtine and those who had not been exposed to flupirtine. Then we also asked the families a lot of questions in a survey. We were able to determine that although parents felt that their children might be doing better if they had gotten the flupirtine than those who hadn't, actually, we had no objective data to support that. That, again, is an opportunity to look at something that was in pretty widespread use in this narrow population, and to see if there was at least a big effect or a small or no effect.

Another one, and this is, I think, very important following up on what we heard at lunch today. So we have these data and a mother of one of the recently deceased children with juvenile NCL came to chat with our research group at one of the BDSRA meetings. She said I noticed that of all the families I met when my daughter was first diagnosed, those whose children were boys with JNCL are doing much better than the girls. I thought OK, maybe. That's just an observation, but there might be something there. Let's go back and look at our data. What we found was, on average, boys had onset of blindness a year earlier than the girls, but, on average, the girls died a year earlier than the boys, and they lost functional independence, on average, a year earlier than the boys. So later onset, earlier death, earlier loss of independence, suggesting it may be a more rapidly progressive course in the females, even though this is an autosomal recessive condition. Again, whether that will lead to any important work that will guide future therapy or not, we don't know, but it is a wonderful opportunity to test those hypotheses on already existing data.

Dr Clark: I was just going to say that, so as we look and we can test some hypotheses with his existing data, we can also begin to think about bringing some things to clinical trials. We do have a Batten disease program at Baylor, and we do have some potential targets for therapy. As we've thought about clinical trials, much as Dr Augustine talked about, we thought maybe we should be developing some biomarkers. There is some literature to support use of some biomarkers in JNCL or CLN3 disease. Our thoughts were our ideal biomarker would be one that would be disease-specific, could be measured in a noninvasive fashion, and was reproducible and quantifiable. Other than hearing today about glutamate elevation in MR spectroscopy, I can't think of anything that really fits the bill.

What we want to do and what I have IRB approval for is measurement of CSF proteins that have been shown to be elevated in Batten, specifically neuron-specific amylase. We also proposed to measure 14-3-3 epsilon that is elevated in Jakob-Creutzfeldt and differentiates that disease from Alzheimer's disease. We proposed to measure tau. Then in a clinical trial we might have efficacy, as we heard earlier, that there are widespread effects in other tissues. So perhaps when we contemplate a trial, we may actually get peripheral effects and not hit our target. Therefore, we may need to modify the delivery of the drug, or whatever the compound is, or whatever we're doing, gene therapy, etc.

We also proposed to, and we are working to develop a quantifiable immunoflow cytometry. So we can measure lymphocytes autofluorescence. And then urine dolichols have been shown to be elevated in the disease as well. So we'd like to measure all those things and at least begin by showing that there is a difference in those biomarkers between disease patients and controls that we will acquire in our hospital. But the real use in a trial means that we have to have a therapy that works so that we can show that these biomarkers are modified in the therapy. Unfortunately, we don't have that at this point in time.

Dr Davidson: I think one of the issues with biomarkers, particularly for rare diseases, we're kind of riding on the heels of Alzheimer's disease and Parkinson's disease that are much more prevalent, and even they're doing the proteomics on CSP, and exosomes and CSF, and exosomes and blood, and cells has not yet picked up one single biomarker. But I think that the machinery is getting to this stage where it's going to happen pretty soon. I think it would be fantastic to engage experts in those arenas in profiling biomarkers in CSF exosomes, for example, as well as peripheral tissues.

Dr Pearce: One of the wonderful things about working in this and working with John is he talked about over the years they've done this natural history study of all the children, and we've been collecting the blood samples over the years, and, obviously, John knows this. The way this has gone so quickly or so slowly is it takes many years to look for those biomarkers, and we're at the point where we have the biomarkers. The paper is literally just going to be submitted, and it's going to be applied to John's clinical trial. So we will actually have that. They are a little bit less invasive than taking CSP. It's obviously blood-borne biomarkers.

Angela has published a paper as well where she did some micro ACE biomarkers, and some of our data matches with that. I think we're close to having the biomarkers. I think more importantly, actually applied into a clinical trial. Whether, of course, they become validated in that clinical trial is going to be the really big question. I think now that we should be proud as a field that we're at that point, because I don't believe there are too many diseases there.

My question is we have a very esteemed panel here, and we have a lot of junior investigators here as well. So on their behalf, I'd like to ask what should they be doing for their careers right now, whether they want to become clinical faculty, or whether they want to become physician scientists. So just a little bit of a short sort of snapshot on what the advice and its current environment would be I think would be a good idea.

Dr Schulz: Maybe I could answer this as I'm, I think, the most junior person here at the table. When I started to work in this field, I had a wonderful and I still have a wonderful mentor in Germany, and he's really like a very old, traditional child neurologist. He taught me from the very beginning. He said the only way you can do good clinical science, good clinical research is if you not only study the patient, but if you care for the patient. You're a young person so you listen to this and you're like yeah, of course that's what I do. But over the years, I really learned that this is a huge effort. And we have also—and I'm happy that we're collaborating on these natural history studies with Jonathan Mink, who has the rating

scale, we have similar rating scales for juvenile NCL, also for late-infantile NCL in Europe. I'm happy to coordinate now a European collaborative effort focused on natural history studies to put all those in an NCL patient database. But we would not be able to do this without the help of the parents. We have the families who come to our NCL clinic every 6 months. I know similar in Rochester. This is for all the families a huge effort. For the patient, this is really troublesome. For an environment this can be painful if you take samples, especially CSF, I think, is the sample that's very precious and tells us a lot, but for the children it's not always simple to get through this.

So we have the experience. The more you really try to care for these patients, the easier it is for the families to come back again and again. This is the way you can collect prospective natural history data. We can do a lot retrospectively, but I think for all these clinical trials that are about to start sooner or later, prospective data collection will also be the key issue because only this way we can actually really collect very sensitive scoring data that we're not able to just gain from patient charts. We need to see the patients on a regular basis, I think. This is the only way to evaluate these studies that are coming.

Dr Steiner: I'd like to add 2 points about junior investigators and people just starting out. One is, I would recommend you follow your passion. Find something that you're really interested in that really excited you and follow that. Then maybe related to that, find good mentorship. If you can align both of those, you're in great shape. Find a mentor who does work in the area that fascinates you. I think that's the best advice I can give.

Dr Patterson: I would just amplify my colleagues' comments and emphasize the care for patients. It's something I think you can do very effectively in partnership with the patient groups. That's certainly been my experience over nearly 25 years from now. You regularly participate in their meetings. You're there as a resource. I always point out to families I always learn more from them at the meetings than I could possibly teach them. It's the sort of thing that I can become international. Personally, I visit Germany, and Poland, and England, and other countries every year at the request of these groups. So it's very fulfilling, and I think it's a great way to help families and to help the field advance.

Dr Clark: Could I also add that for junior people I say, I tell them— and many of them approach me and say what if I don't get the grant, what if I don't get this paper, it's rejected this time—that's part of business. What I tell them is I make the comparison to fishing. I say they call it fishing, they don't call it catching. You have to enjoy that process of going through the review process—of the rigor of the review process, and in some ways, these kind of meetings are preparing you for that because you get to hear us sort of criticize each other about the approaches that we're having toward a clinical problem or a research problem. That's part of the review process that you will undergo as you move forward in the field. But enjoy that process. Learn from that process. It's hard to take rejection sometimes, but some of us thrive on it. If you don't give up you will be successful. If you do give up you won't be successful.

Dr Maria: One of our colleagues in the audience asked a question. First, I should say that we have a session dedicated to stem cells tomorrow morning, in which we hope to amplify

many of the great points that you've made. One of our colleagues in the audience raised a question of why haven't we talked more about imaging. Why have we talked about the modalities? We heard about the visual evokes, and then ERGs. Can you discuss a little bit kind of what the state of the art is with structural and metabolic imaging, and a review of that? And can you say something about sensitivity and specificity of imaging, but also of some of the neurophysiologic assessment tools? I think that would be helpful.

Dr Steiner: There is a brand new article that's published, I think it's AJNR on imaging, specifically in one of the forms of Batten disease. It's the late-infantile NCL from the Cornell Group who are doing a clinical trial. And it's a very nice article. I think one of the things it illustrates is the difficulty here in applying these techniques to rare disorders with heterogeneity. But they managed to be successful, I would say, in this endeavor. Basically, they applied a number of different techniques, structural imaging, volumetrics. I don't remember whether that article talks about spectroscopy, but I think it's mainly structural and volumetrics.

Dr Mink: Yeah, DTI.

Dr Steiner: So they basically collected all the data, looked at the variables, and then did the statistical method where you can put all the variables—

Dr Davidson: Combinational.

Dr Steiner: Combinational analysis. There's another word for it as well.

Dr Davidson: Principle component.

Dr Steiner: Principle component analysis, exactly. And so I think that's a first way to start developing biomarkers by way of imaging. But, again, this is work that's still in its early stages.

Dr Mink: I think one of the things they did was they showed using DTI in particular that there clearly was a change that correlated with disease progression, and using a modification of Alfreda Kohlshuder's Hamburg scale that they call the modified Hamburg scale, that there is a correlation between these DTI measurements and deterioration using a relatively simple clinical rating scale. But what it doesn't do is allow you to send your patient for an MRI scan and have them do DTI, and MR spectroscopy, and whatever, and come away with a diagnosis this must be CLN2. It's not clear. We don't know whether it's true or not. But at this point, we don't know whether there's a specific change or nonspecific.

Likewise, we heard earlier from Tammy about the glutamate GABA spectroscopy in the animals. Again, is that something we might see in multiple forms of grey matter disease, if you will, or multiple forms of NCL, or is it specific, and are there specific ratio patterns on CTs, and are they developmentally specific, or disease-stage specific? I think we're not there. Likewise, I think the finding on something, like for diagnostic studies, there's not a Batten EEG pattern. Although ERG is pretty sensitive in at least in some forms, there are other things that cause retinal abnormalities as well.

So it comes back to what do we train for? We clinicians, we train as clinicians, and we train to be able to put together the picture based on some data here, some data here, some data here. Right now, aside from enzymatic testing and genetic testing, there is not a test where you send your patient off and then come back an hour later and you have the answer, like there might be for a glioblastoma, for example. Other thoughts?

Dr Patterson: Maybe just a comment about biomarkers and surrogate markers of disease progression. I think you have to be careful in distinguishing those. There are a lot of diseases where we can find various analytes in different tissues, which will be high or low in the disease state, as compared to controls. But if you're going to use those as markers of disease progression and response to interventions, I think it's very important that you have that longitudinal data, which is exactly what you're getting. And that you show that normalizing or near normalizing the level of that analyte corresponds to clinical improvement. There's a best example I think we have that is very long chain fatty acids in X-linked ALD, where in patients, in boys who are symptomatic normalizing that level with Lorenzo's oil does not correspond to clinical improvement. I think it's an extremely important endeavor, but that's just something I think we all have to keep in the back of our minds. That improving a lab test does not necessarily mean you've cured the condition. Or maybe it's too late. The burden of disease is excessive.

Dr Clark: Right, but in this disorder, you may not expect improvement. You might expect a plateau.

Dr Patterson: Sure. Stabilization

Dr Clark: So I think you have to use both the clinical rating scales and some biomarker. But if we don't use biomarkers and we only use clinical rating scales, and other things such as neuropsychological testing with lots of variability, as we saw earlier today, I think you're not going to get an answer as to whether or not a treatment is efficacious for generations maybe.

Dr Patterson: No, no. I agree. I agree. But I'm just saying you have to just have that note of caution. And some things do improve. In Niemann-Pick C, the level of calbindin in the CSF improved. Amazingly enough, even a disease, which has an average of 5 years' delay in diagnosis, which NPC does, you can actually see improvements in some of these markers, which otherwise increase over time. So, no, I agree with you completely, Gary, but I just say there's always just that littler reservation to keep in mind.

Dr Clark: Sure.

Dr Mink: Heather.

Dr Adams: I wanted to ask the panel to consider the challenges and opportunities in Phase 3 trials, clinical trials, for rare diseases, such as Batten disease. But certainly this question might apply to the 8,000-plus other rare diseases that we encounter—the challenges and opportunities for increasing our sample size, increasing our opportunity to demonstrate efficacy with international collaboration on these trials. Because I think that's something I

know Angela has been tackling with an international collaborative registry in Europe. I think that is setting the framework for the work to be done. But I see that with these rare diseases, with our small sample sizes, within each community, and within each country, it really becomes incumbent upon us to look at the entire population available for study, and help the entire population when the trial becomes available.

Dr Schulz: Yeah, I think maybe I can comment on this a little bit. We're very happy in Europe that we got funding from the European Commission to transfer our German patient database to more on a European level, but we're also now working on to get it on a more international level to include other countries, such as Turkey, South American countries, and, of course, we're very much interested also in even more collaboration here with the U.S. We are in close contact with the Rochester group, which I very much appreciate. So I think we can only study these rare diseases in a collaborative international effort, because we've been talking a lot about infantile, late-infantile, and juvenile NCL diseases here today. But the first 2 talks this morning state that now we are talking about 13 to 14 different NCL diseases, and these are all clearly different disease because they are all caused by different genetic defects. So we have many more patients out there. For CLN5, CLN6, CLN7 to do a natural history study, you can't do this without an international collaboration. This is impossible.

I think there are really 2 things that will be important. One is to have international collaborative efforts, to have good rating scales also for those diseases including adult NCL disease types. We have no rating scale so far, and I think there's a lot of work that needs to be done, so from adult neurologist's point because I'm not very experienced with adult NCL patients. Maybe others are. And, also, the early diagnosis, I think, is very important. Because this will, one, increase the number of patients that we have for natural history study, and it's important that we have young patients at early disease stages also for experimental therapy studies. Because it's a big challenge not only the small number of patients per se, but the even smaller number of young patients early in the disease that might be eligible for those treatment studies.

Dr Mink: I'm going to do something very provocative. We heard at lunch today about the first patient to undergo a human neural stem cell transplant, and we have one of the key investigators in that study here, but we haven't had anybody else talk about cell-based therapy. To quote my dean, who said, "Well, this is all very interesting and important research, but until we can cure type 1 diabetes mellitus with a stem cell transplant, I'm not willing to talk about human therapy using neuronal stem cell transplant." So what do you think about the future of cell-based therapy, particularly thinking about those diseases where it's an enzyme-bound protein or, I'm sorry, a membrane-bound protein as opposed to a soluble enzyme? Do you think that there is—I don't want to talk about the value of doing research—but do you think there is an important future for human therapy using cell-based therapy?

Dr Steiner: I think that the companies involved in this type of research picked the low-hanging fruit. I think what they picked makes a lot of sense. So they, obviously, picked disorders caused by enzyme deficiency. Lysosomal enzyme deficiencies where if you can

get a very small amount of soluble enzyme circulating in the brain, you can potentially ameliorate these conditions. So I do think this is a reasonable approach, and I do think there's a future for this type of therapy, at least for the lysosomal storage diseases that are caused by enzyme deficiencies. In those disorders, you don't need to fix a large proportion of cells. You only need to transplant cells so that they secrete enough enzyme to ameliorate the disease. For other types of disorders, like CLN3 membrane associated proteins, I think it's much more difficult, because I think the number of cells you need to correct or replace with normal cells is much higher, and that's a more difficult target.

Dr Clark: I would just say that I think it depends. I think it's basically saying what you're saying, but in a very succinct way, is it a cell autologous or is it a cell exogenous effect? If it is a cell autologous, I guess the stem cell approach probably won't work, right, because you're going to have to replace every neuron in the nervous system or whatever cell that's being affected by the cell autologous process. So it has to be a different strategy. I was intrigued by Dr Sands' work with the adeno-associated virus that does have neuronal predilection. But what is the efficiency of the delivery of the viruses? I know that the new-generation delivery systems are getting better and better, but if we have to fix every single neuron in the nervous system, that might be a difficult approach too. I don't know. But those sorts of approaches are going to have to be used to get something into a neuron.

Dr Davidson: Well, with Mark's stuff he's introducing the vector very early and so, essentially, putting the enzyme, the gene, and a large proportion of the brain. I think the other intriguing data that was raised today was David Palmer's data where he essentially created a chimeric animal that had normal cells and mutant cells, and was able to essentially ameliorate disease in those animals. I think more studies like that need to be done in larger animal models to begin to get a sense of whether it's a cell autonomous problem going on or not. If there is some non-cell autonomous benefits to therapy for these membrane proteins, I think it would be very exciting. I think another thing that we don't know for some of these disorders—again, lacking numbers of patients and natural history trials where we have samples available early in disease as opposed to late-stage diseases—where is the inciting event? Is the entire brain affected all at once or are there very regional foci where the onset occurs? You don't see it clinically until something manifest to much more of the brain. And maybe you only need to correct smaller regions focally that will lead to a long-term benefit. It's all speculation. It's a lot of things we don't know.

Dr Maria: Dr Mink has invited provocative questions, so I'll try to follow with one. We've heard from John that there's been tremendous excitement in events over the last 5 or 6 years or so. So for each of you, there's probably a long list of unanswered questions, but one of the things that I'd love to hear you articulate is what you see as being within your respective interests, in field, what you consider to be your key unanswered question at this point?

Dr Steiner: Maybe we'll go in order down the table. Mine's easy. I'd like to see a next-phase trial go on with neural stem cells in infantile, late-infantile NCL to see if the therapy works. You saw there were problems with recruitment, but there are ways around that. So the first clinical trial, the results of which will be published shortly, the patients were so far advanced in their disease, like Daniel that you heard about today, that it was very unlikely to have

efficacy, and that was really a safety study. So I'd like to know whether, sort of back to John's question, whether purified neural human stem cells are an effective therapy for infantile and late-infantile NCL.

Dr Davidson: Well, I work in both JNCL and LINCL, so I have 2 different things. But since I talked on LINCL, I'll focus on that today. I think I would like to really pin down how much enzyme and where we need for full clinical benefit. The animal models still get sick. They still succumb to the disease. We can prolong onset of disease symptoms, but they're not cured. I think it's important to know what's the level of enzyme replacement that's required. Is it 5% in this part of the brain and 30% in another part of the brain? And to devise experiments to begin to address that.

Dr Clark: There are a lot of things I'd like to know. But my interests right now are in the juvenile form. So what does CLN3 do is my major burning question. And by knowing what I think you probably would be able to design some therapies. But I don't know and we've talked about this today. I think we probably don't want to wait for that. We probably do want to trigger a clinical trial sooner than perhaps when we get that understanding, even though it may be forthcoming. And so what is that point? I think we talked about that earlier. I think that's probably our burning question right now.

Dr Schulz: Yeah, likewise. There are many questions that are out there that would interest me, but I think there's one thing that has not so much been talked about today. One of my most burning questions, are we actually just talking about a neurodegenerative disease or might there also be on a longer run if someday we would be able to stabilize these patients with some sort of experimental therapy? We would run into completely different disease and see that other organ systems are affected by this disease as well. We find the storage material in many, many tissues of the body. As we learned, we don't know whether the storage material is the toxic cause of the disease, but still this shows that there is also something going on in all the other tissues. And we have seen and others have seen that in CLN1, CLN2, and CLN3 patients they are more prone to cardiac problems. They have heart rhythm abnormalities. We see in our patients that other parts of the central nervous system are affected. And I think in all the natural history studies we should start to think about these other organ systems, how they are affected, if they are affected. Because if we stabilize the neurological part of the disease, we need to know where are we running into; what might be the new problems of those patients, and would we need to treat the rest of the body as well?

Dr Patterson: Like my colleagues, I think there are many questions. I'd like for all of these types of disease to know what the point of no return is. In other words, where have you reached a stage with burden of disease where no intervention can be effective? I don't know how to answer the question. I don't know when patients are symptomatic how much of their symptomology reflects loss of neurons, which, effectively, are not replaceable at the moment, and how much reflects dysfunctional sick neurons which might be rescuable. I think perhaps developing techniques to answer that question initially in animals, and maybe subsequently in humans could be a very valuable guide to studies. Because I think in all of these diseases, one of the big challenges we have in clinical trials is that we often recruit patients simply because of the delay in diagnosis, who already have a very significant

burden of disease. And actually just reflect that burden of disease rather than the fact that the intervention is not rational or efficacious.

Dr Mink: As the moderator I'm supposed to stay quiet, but I'm going to answer that question anyway with 2 answers. One is I'm really increasingly interested in what the role of inflammation is. Is this just a bystander? Is it a parallel process when you have dying neurons you have to have changes that involve this complex proinflammatory system? Or is this fundamental to the pathobiology of these disorders, even though it's not the primary defect, if you will? If you were to completely eliminate the inflammatory response, would that be protective in itself or would that slow progression? I don't think we know. It's enticing because so many diseases, including Alzheimer's disease, have a significant inflammatory component. I think it's increasingly important to pay attention to those. But I'd like to know that. The other thing I'd like to know is related to you answer Marc, and to a question from the audience earlier about this idea of temporal therapeutic window. We know from a lot of degenerative diseases that by the time someone manifests symptoms, they've already lost an awful lot of cells. So the other question is, what's the earliest we can detect whether with biomarkers or something else what is the most effective way to screen, and what criteria do we use to identify what is the latest possible intervention to have a meaningful impact? As our lunchtime speaker pointed out, and as many of the families we've interacted have raised is, if your child is immobile, non-communicative, and completely dependent on others, do they really want a therapy that's going to arrest the disease in that state and prolong their child's life by 15 more years with minimal quality. And again, different people have different answers to that, but it raises, I think, a very important question.

Dr Patterson: Maybe one comment, John. Obviously, I don't know for NCL, but certainly in the other lipid storage disease and the lysosomal disorders, in animal models you can get 10% maybe 15% increase in life span by suppressing inflammation. So it certainly seems to be a contributor. It's certainly not a major one, and it seems to be downstream of the major defect, at least in that family of diseases where it's being studied. And Sandhoff is probably the best model that's been studied for that.

Dr Mink: Though, if we're 15% of the way away from a truly disease-altering therapy, is it a reasonable strategy to pursue this?

Dr Patterson: Sure.

Dr Mink: Again, I don't have the answer.

Dr Patterson: Well, it raises another interesting question maybe Bev, as an experimenter, could answer this. Because one of the things that troubles me, maybe perhaps from ignorance, but everyone's aware of the story with the SOD mouse model of ALS and how many rather small effects were reported in small studies. Basically, when large numbers of mice were studied, it turns out it was noise. So, again, I know there are very careful and thoughtful researchers. So the question is well, how many mice do you need to study to get a result? Because you may get a quote, "statistically significant result," but if you're using small numbers what does that mean?

Dr Davidson: Yeah, the group sizes I use, Mark uses, and David uses are generally quite large, and they're generally repeated more than one time. So it's not you do an N of 12 or 15 you get a result. You repeat that and see that again and again. I think for mice, I think we've cured them a lot of different ways. Given that their brains are this big, you can get that.

Dr Patterson: I think the size of the effect is very impressive in those cases. It's these small effects that you worry about.

Dr Davidson: Right. I think that what you're talking about with the G551D model is that in my lab, and your lab, and your lab, we're going to have different age of onset and different age of progression, and different mean death. So it's a little bit touchier animal model. I think some of these animal models of lysosomal surges, these are fairly robust and fairly repeatable amongst the different labs, as long as the chows are the same, and things like that.

Dr Mink: Other questions from the audience? Other things? I want to bring up one more provocative question. This is for the animal model people. Those of you in the audience please feel free to chime in, too. It's been speculated that mice are wonderful genetic models, but they don't live that long. And that in some disorders, like the more slowly progressive NCL disorders, like Huntington disease, like some of the other slowly progressive degenerative diseases, that it's not the proportion of the life span or the species, it's the number of years of accumulated toxicity. So that you may have to have 6, 7, 8 years of this abnormal function before you start to have enough impact to cause what we see as the human disease. Certainly, there are mouse models that manifest robustly, but to have a true phenocopy of what we see in human disease in a mouse is actually quite uncommon.

Dr Davidson: Well, I'll comment on that. I think it's very difficult and didn't know that you truly get sort of at the biomarker or surrogate marker. It's an analogy to that. Until you know that your animal model is a phenocopy or a good animal model of disease, you cure the animal model and the same therapy cures the humans. And, well, I guess it was a good animal model, but there's not many instances where that's the case. But on the other hand, it's all we have. I think it's certainly a step forward from cell-based models, in many cases, where you would have the variability of not having a tissue of multiple cell types as opposed to a cell line. And as you move up in animal models into larger mammals, maybe those phenotypes will be more relevant. For example, the dog has seizures. The dog model of LINCL has seizures the mouse model does not. I don't know if there's a larger animal model for INCL. I guess not. And maybe the sheep models, David can comment on those. And, again, until we can cure them, we don't know how good those models are.

Dr Palmer: Sorry. I missed a bit of what I was meant to comment on, Bev.

Dr Davidson: What? Oh. How good are the animal models?

Dr Palmer: In the analogy with the human diseases? The sheep are very good. There's a lack of seizure activity, so some of those properties are different. There's an argument in the veterinary world that sheep are a low-seizure activity animal anyway. They're amazingly stoic ruminants. Well, they come back to be bled quite happily, which is really amazing, really, when it does them no good whatsoever. Otherwise, though, no, it seems a very good

power level model. And there's an advantage with those populations. I think you don't want to underestimate it in the mice, too. What you've got over the human genetic variability, both in the background and in the animals. So these biomarkers of disease progression and so on and the animal populations they're far less spread out than they are in the human disease. Just to comment on something earlier. You run into a problem when your need begins to develop velocity of disease studies, and what you're up to, because you can measure the height of a million people and get an average height of humans to an extraordinary precision. But it doesn't help you at all to make the height of the next person that comes in the room. And it's exactly the same problem you're going to have in trying to take your single or few subjects up against that database. So that's just an aside. What I wanted to ask, though, is earlier in the day there was kind of a ghost raised really that I think was about in terms of early screening and genetic screening for predictive course of disease. In all the genetics, human genetic studies, has anyone found genuine atypical or nonpathological cases of what you would genetically describe as cases of Batten disease?

Dr Davidson: You mean a patient with a mutation that didn't manifest?

Dr Palmer: Yeah.

Dr Mink: We've seen a couple in our work of patients who clearly, on a clinical ground, don't have Batten disease. They have a neurologic disorder, but it's not Batten disease. In fact, it's a non-progressive, but they happen to have a point mutation on one allele of one of the NCL genes or another. And they have concluded from that well, probably there's a mutation on the other allele and it just wasn't discovered because my child has a neurologic disorder, and a point mutation. I don't know the answer to that except I can tell you that these are individuals that very clearly had a static non-progressive, probably birth-related insult to their central nervous system, as opposed to a progressive genetic disorder. So that's the closest I've seen to someone who might fit into that category, though it's not exactly what you posed.

Dr Palmer: No, but in a sense that's rather cheering news. In terms of in the future people going into therapeutic regimens or treatment regimens before the development of clinical symptoms, and some confidence that they are avoiding something that might—that will happen rather than trying to avoid something that might not have happened, if you follow me.

Dr Mink: However, before having seen several dozen children with various forms of Batten disease, I don't know that I would have been able to say that child didn't have Batten disease.

Dr Palmer: It's a long time ago now, I think it was 1999 or something, I was at a conference in Cambridge. There's an atypical form of the gene for Fabry's disease that causes heart attacks and with people in their 30s. I think there was a guy, Grabowski I think his name was, at Buffalo. I don't remember all the details, but it's kind of a cautionary tale.

Dr Patterson: Well, there is a cardiac-only variant of Fabry disease. You're absolutely right. The other interesting thing about Fabry is that heterozygotes do manifest, but they tend to

manifest later and less severely than the hemizygotes. So we don't know what the modifiers are that are responsible for that variability.

Dr Palmer: Well, my memory of this talk was that this was purely by accident. He was just doing a survey of the general population to find out how many people are carriers for Fabry's disease, and stumbled across this.

Dr Mink: I think we learned an important lesson recently about Kufs disease that this was thought to be a completely separate genetic entity, although no gene had been described. And, now, the so-called type A, which may or may not be a relevant distinction of Kufs disease, is actually not CLN4 disease, it's CLN6 disease. So we've learned that kind of going in the back door instead of looking at the phenotypic variability of a mutation; we've found that there's a completely discrete phenotype that is related to a mutation of a gene that had been implicated in a late-infantile variant of the disease. So again, I think that gets to your question too, that it's never simple.

Dr Schulz: And one more challenge in the future might be now we're clinicians, so when we first see a child with symptoms and due to the symptoms we do genetic testing. As you said, if you wouldn't have known the JNCL phenotype very well in this one child, you might not have been able to tell. What if we do genetic testing in asymptomatic children and we come back with a kind of, let's say, SNP in one of the NCL genes, and we have no symptoms to look at? And this is not a mutation that has been described before. It will be difficult to tell in some cases whether this child needs treatment or not. In the enzyme-affected diseases, we could measure the enzyme activity. But in all the other remaining 8 or 9 forms, this might be difficult. I think it will be challenging.

Audience member: So I'm just wondering if there is an NCL-type disorder that presents in a patient at what point do you consider it not to be an NCL disorder if there has been no genetic component of that disease found? And if there's enough of the neurobiology aspect that has been found, is there some sort of threshold and you say this is not NCL or this is NCL?

Dr Schulz: In our European consortium, we have agreed upon a diagnostic algorithm. One important part of this algorithm, even though it might be a little bit old-fashioned, is a skin biopsy, and looking with an electron microscope at whether you see the lysosomal inclusion with the classic ultrastructure. I think there's still a consensus among all the NCL researchers that if you do not see these typical lysosomal inclusions, you should be careful to talk about an NCL disease. So I can do the enzyme measurements, I can look for vacuoles and lymphocytes, and a blood smear if I have suspicion of a CLN3 disease, and then do the genetic testing. But if all this fails and clinically I have no clear idea of what kind of all these different NCLs these could be or whether this is an NCL disease at all, I would go for a skin biopsy first, do electron microscopic analysis. And if I see these inclusions, then I would go ahead and check genetically. But if you don't see those, you should be rather careful, I think. Or if an experienced pathologist doesn't see them, because I think it's tricky to do this, and not everybody has seen them before, and it takes a while to really look for them, I think.

Dr Mink: And as was pointed out earlier, you can't just look at fibroblasts.

Dr Schulz: Yeah. I need to have a skin biopsy where you have clean sweat glands where you can see the specific neurons, endothelial cells, so that's important.

Dr Patterson: I don't recommend it as a first test, but if you have a muscle biopsy and you stain it with titrate-resistant acid phosphatase, you'll often see evidence of lysosomal storage there. I admit this. I stumbled onto a case in this fashion once. And then we found curvilinear bodies and fingerprint inclusions on the skin biopsy.

Dr Clark: Now, if you had a patient who was 5 years old, who had a retinal degeneration and 2 mutations, loss of exon 7 and 8, and CLN3 would you still do a biopsy?

Dr Patterson: No. No. No. No. I think Gary, if you've got a typical clinical phenotype, you do the minimal testing you need to sustain a diagnosis.

Dr Clark: Right. But I thought in your—

Dr Patterson: But if you didn't have one of the mutations, but you were still concerned clinically, then I would do that.

Dr Clark: But what if they had a stop and then an exon 7/8 would you still...

Dr Schulz: Similar. I wouldn't go for it. Well, scientifically, of course, it's always nice to have a skin biopsy and then you can do fibroblast cultures, and use those fibroblasts for future research. But, usually, if you have a child at school age, with visual failures, with some kind of early signs of dementia, or something, you usually do the blood smear. If we see the lymphocyte vacuoles, we go for CLN3 genetics and usually this comes out positive, and then the diagnosis is made. But if I don't find anything in the CLN3 gene, then I go for a skin biopsy.

Dr Mink: Any last comments, words of wisdom? Any last questions from the audience? Well, thanks for your attendance. Thanks to Bernie for organizing this; another very successful symposium, I think. And thank you to everybody.

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