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Meeting report

# 6th Dysferlin Conference, 3–6 April 2013, Arlington, Virginia, USA

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**Abstract**

The 2013 Dysferlin Conference, sponsored and organized by the Jain Foundation, was held from April 3–6, 2013 in Arlington, VA. Participants included 34 researcher speakers, 5 dysferlinopathy patients and all 8 members of the Jain Foundation team. Dysferlinopathy is a rare disease that typically robs patients of mobility during their second or third decade of life. The goals of these Dysferlin Conferences are to bring experts in the field together so that they will collaborate with one another, to quicken the pace of understanding the biology of the disease and to build effective platforms to ameliorate disease. This is important because the function of dysferlin and how to compensate for its absence is still not well understood, in spite of the fact that the dysferlin gene was identified more than a decade ago. The objective of this conference, therefore, was to share and discuss the newest unpublished research defining the role of dysferlin in skeletal muscle, why its absence causes muscular dystrophy and possible therapies for dysferlin-deficient muscular dystrophy patients.


*Keywords:* Dysferlin; Muscular dystrophy; Limb girdle muscular dystrophy; LGMD

## Session 1: Exploring the function of dysferlin

This session explored the potential functions of the dysferlin protein.

**Robert Bloch (USA)** used immunoflorescent labeling and a pH sensitive GFP moiety linked to the C-terminus of dysferlin to show that dysferlin is localized to the transverse tubules both *in vivo* and *in vitro*. This localization of dysferlin to t-tubules suggests a role for dysferlin in maintaining t-tubule integrity. To test this hypothesis, t-tubules in both dysferlin deficient A/J and wild-type muscle were subjected to injury *in vivo* and *in vitro* and outcomes were assessed. In the first injury model, the hindlimb muscles *in vivo* were subjected to large strain eccentric injury (LSI) and torque loss and recovery, t-tubule disruption, necrosis, and inflammation were measured. While the initial loss of contractile strength was similar in both wild-type and A/J muscles, the t-tubule structure was maintained in wild-type but highly disrupted in the dysferlin null muscle soon after LSI, as observed by the level of DHPR disruption. In a separate injury experiment, FDB myofibers were subjected to osmotic shock (OSI) in culture. Measurements of t-tubule disruption, changes in Ca2+ transients and baseline, and changes in the efflux of the impermeant dye sulforhodamine B (SulfB) from the t-tubule lumen, were then performed. OSI rapidly caused dysregulation of Ca2+ homeostasis, disruption of DHPR organization and a slowing of SulfB efflux from the t-tubules of dysferlin-null myofibers, consistent with a disruption of the t-tubule structure. Expression of full-length dysferlin protected against the changes seen after OSI, indicating that these effects are dysferlin dependent. Taken together, these results show that dysferlin plays an important role in stabilizing the structure and function of t-tubules in myofibers both *in vivo* and *in vitro* and that these injury assays could be used in evaluating potential therapies for dysferlinopathy.

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**Aurelia Defour (USA)** used several different murine and human cell lines to characterize the subcellular defects present when dysferlin is absent. The dysferlin deficient muscle cell lines evaluated included C2C12 shRNA dysferlin, H2 K-A/J myoblasts derived from a cross between the dysferlin deficient A/J line and the immortomouse, and an immortalized human dysferlin deficient cell line generated from the myoblasts of a dysferlinopathy patient. Evaluation of these lines showed that while lack of dysferlin did not affect the ability of these cells to proliferate or differentiate, it did affect their repair capability using two independent methods for cell membrane injury and repair [1]. In addition, because lysosomal exocytosis is associated with repair of injured cell membrane, she went on to test if dysferlin deficiency affects lysosomal exocytosis. Using several assays for lysosomal exocytosis such as cell membrane LAMP1 staining, live lysosomal trafficking and docking with TIRF, she and her colleagues showed that there are fewer lysosomes at cell membranes and that there is a delay and reduction in injury-triggered lysosome exocytosis. These findings demonstrate a role of dysferlin in cell membrane repair and the docking and calcium triggered fusion of lysosomes. These effects likely contribute to poor healing of dysferlin deficient muscle cells and could help explain some of the underlying pathophysiology of dysferlinopathy.

**Angela Lek (Australia)** presented data around the role of dysferlin in repair of membrane injury. Using ballistics induced damage of human skeletal myotubes, she showed that endogenously expressed dysferlin is rapidly mobilized and enriched at sites of injury along with MG53 in a Ca2+ dependent manner. However, only a portion of dysferlin appears to be present at the sites of injury as only the C-terminal antibody (Hamlet-1) and not the other more N-terminally located dysferlin antibodies can detect dysferlin at sites of injury. This data is supported by Western blots that show the presence of a 72 k-Da "mini-dysferlin" Hamlet 1 positive band only in myotubes damaged in the presence of calcium. Further, the levels of this 72 k-Da band correlate with the levels of full-length dysferlin, and this "mini-dysferlin" is not produced in myotubes derived from dysferlinopathy patients. Given the size of the band and that it contains the Hamlet-1 epitope, the 72 k-Da band is proposed to contain C2E, C2F, and the transmembrane domain. This makes the fragment look a lot like synaptotagmin. In addition, the formation of the 72 k-Da mini-dysferlin is sensitive to calpeptin, which suggests that this fragment of dysferlin is produced by calpain cleavage. This data suggests that dysferlin could act as a modular protein produced through enzymatic cleavage and that the various functions attributed to dysferlin could be performed by different portions of the protein.

**Kevin Sonnemann (USA)** utilized recombinant dysferlin proteins to understand how dysferlin modulates membrane fusion events and is recruited to sites of damage. He presented *in vitro* data that demonstrated that dysferlin is able to bind lipids, aggregate vesicles, and stimulate fusion between muscle v-SNARE and muscle t-SNARE containing vesicles in a dose dependent and calcium dependent manner. Using purified recombinant GFP-dysferlin mixed with fluorescent (protein-free) vesicles, he also showed that dysferlin localizes to areas of membrane tubulation upon addition of calcium, suggesting that dysferlin directly modulates membrane lipids by inducing or stabilizing extreme membrane curvature. Lastly, using frog oocytes he showed that dysferlin is recruited approximately 30 s after the wound, that this recruitment requires the presence of the transmembrane domain, and that dysferlin recruitment is prevented when DAG expression is inhibited by injection of SPK609. DAG is enriched at the site of membrane damage and in the absence of DAG recruitment, the membrane fails to repair. Together, these data indicate that dysferlin may perform a primary role in membrane fusion and offers mechanistic insight into the membrane repair deficiency in dysferlinopathies.

**Paul S. Blank (USA)** presented techniques to evaluate the repair potential of dysferlin deficient skeletal muscle fibers and myotubes using fluorescence microscopy to measure indicator uptake, leakage, and calcium activity. The uptake assay involves membrane wounding and a measurement of the increase in cellular fluorescence. The increase in fluorescence correlates with membrane repair. Paul pointed out that FM-143, a reagent commonly used for this type of assay, may not be appropriate because baseline membrane labeling is affected by the amount of time the cells or fibers are in culture. He demonstrated that the nuclei-staining indicator Sytox Green does not have these problems. When cells containing the indicator calcein red–orange are wounded, a "puff" of indicator is released at the wound site. The leakage amount and time course correlates with membrane resealing. The last technique measured changes in intracellular calcium using a combination of the calcium indicator dyes fluo-4 and Fura Red. Using this assay, an increase in calcium levels is observed at a wound site that returns to the pre-wound level following successful resealing. However, in the absence of resealing, the calcium levels remain high and spread throughout the injured cell. In all three assays, dysferlin deficient muscle was able to reseal in physiological Ca2+, but differed in the developed calcium load and wounding threshold, supporting a role for dysferlin in the membrane wounding and repair process.

**Jyoti Jaiswal (USA)** discussed how the actin cytoskeleton regulates membrane remodeling during cell membrane repair and how dysferlin trafficking is associated with this process. By comparing the cell surface proteome of injured and uninjured cells, he and his colleagues identified various organelles and processes, such as mitochondria, actin polymerization and distribution, and microtubule growth that changed in

response to membrane injury. Mitochondria accumulate at injured sarcolemma and are required for myofiber repair [2]. Using real time imaging of the cytoskeleton during cell membrane repair, he went on to illustrate that injury causes loss of microtubules at the site of injury and depolymerizing microtubules does not affect cell membrane repair. This approach also demonstrated the importance of actin in the membrane repair process by showing that injury dynamically regulates local polymerization and depolymerization of cortical F-actin. Remodeling of actin, but not microtubule cytoskeleton increased fusion of dysferlin vesicles in response to injury. These findings suggest a local control of actin remodeling and that membrane trafficking regulates membrane repair.

**Pascal Bernatchez (Canada)** showed that dysferlin and myoferlin are expressed in human and animal cancer cells and tumors. However, the role these two proteins play in these cells appear to differ dramatically. While knocking down myoferlin using siRNA reduced proliferation, as indicated by reduced tumor size and cell numbers, knocking down dysferlin had no effect. This data indicates that cancer epithelial cell lines could prove to be an important tool for studying the apparent heterogeneous role that ferlins play in the regulation of proliferation.

## Session 2: Impact of dysferlin's absence

This session addressed the consequences of the absence of dysferlin.

**Jeffery Molkentin (USA)** described the contribution of calcium to the disease process that occurs in muscular dystrophies and how the Na+/Ca2+ exchanger might contribute to the process. An interesting twist to the story is that the disruption of sodium homeostasis may contribute to calcium overload in the dystrophic muscle because a sodium gradient of sufficient magnitude is needed for the Na+/Ca2+ exchanger to move Ca2+ out of the muscle cell. If the Na+ level inside the cell becomes too high, the exchanger may be driven in the opposite direction (reverse mode), allowing Ca2+ to leak into the cell. Overexpression of sodium calcium exchanger 1 (NCX1) in mouse models of both dysferlin deficiency and sarcoglycan deficiency exacerbates muscle pathology, which was tracked by histology and serum creatine kinase levels. Furthermore, knocking out the NCX1 gene in mouse models of muscular dystrophy resulted in an improvement in the disease. Dr. Molkentin suggested the mechanism for this exacerbation in muscular dystrophy was due to the endoplasmic reticulum damage that occurs when calcium is overly abundant in the cytosol. These results suggest that inhibitors of the NCX1 Na+/Ca2+ exchanger may have therapeutic potential in the muscular dystrophies.

**Elizabeth McNally (USA)** presented her work in myoferlin/dysferlin doubly deficient (FER) mice. Compared to singly deficient mice, creatine kinase in the blood of dysferlin deficient mice is increased in FER mice, and the t-tubule network in the myofibers is abnormal. Muscle fibers from FER mice fail to swell like wild-type fibers following osmotic shock, suggesting a loss of action potential across the membrane. She also showed that the lipid content in dysferlin deficient muscle fibers is high and reported that the muscle cells are greasy to touch. She showed a striking image of lipid droplets blebbing from the surface of a FER muscle fiber, which supports the data presented in this session by Miranda Grounds.

**Tatiana Cohen (USA)** hypothesizes that inflammation inhibits myogenesis, and that the dysferlin deficient muscle is in a constant inflammatory state. Using *in vitro* experiments, she showed that incubating myoblasts with pro-inflammatory macrophages (M1) inhibits myoblast fusion while incubating them with wound-healing (M2) macrophages supported myoblast fusion. Further, genes involved in differentiation were down-regulated in myoblasts incubated with M1 macrophages and up-regulated in myoblasts incubated with M2 macrophages as compared to myoblasts incubated with neutral (unactivated/M0) macrophages. Tatiana showed that dysferlin deficient myoblasts are more susceptible to the effects of M1 macrophage associated cytokines than wild-type myoblasts, and that blocking inflammatory cytokines improved myoblast fusion. These findings suggest that drugs that regulate inflammation may be beneficial in dysferlin deficiency.

**Joseph A. Roche (USA)** presented data on the response of dysferlin-null muscle and control muscle to a single bout of large-strain eccentric (lengthening) contractions performed *in vivo*. Over a 72 h period following eccentric contractions, the number of damaged fibers is minimal in control muscle (<5%); but, in dysferlin-null muscle, myofiber damage progressively increases over 10 min, 3, 6, 12, 24, 48 and 72 h, with a sharp increase occurring beyond 12 h. Prior to widespread myofiber damage in dysferlin-null muscle, there is elevated endoplasmic reticulum stress, disruption of the ryanodine receptors, disruption of the transverse tubules and Z-disks, release of cytochrome C from the mitochondrial network and an increase in TUNEL+ myonuclei. The data suggest that for the same level of mechanical loading through a single bout of eccentrically-biased exercise, dysferlin-null muscle responds adversely and undergoes changes leading to delayed myofiber death, whereas control muscle shows minimal signs of myofiber damage.

**Miranda Grounds (Australia)** discussed her finding that fat accumulates in the muscle cells of dysferlin deficient mice. In collaboration with Simone Spuler, she showed that the fat accumulation observed in mice was also present in dysferlin deficient human muscle biopsies. Dr. Grounds hypothesized that lipid accumulation in dysferlin deficient muscle cells could be due to either a change in the nuclear program from muscle cell to

adipocyte, or accumulation of so many lipid droplets in the muscle cells so as to cause them to be overwhelmed with fat. Electron microscopy evidence shown supported accumulation of lipid droplets in muscle cells near mitochondria.

**Terence Partridge (USA)** explained the method with which the "stable isotope labeling amino acids in mouse" (SILAM) mice were made and their utility in discovering biomarkers by comparing heavy isotopes in SILAM mice to normal mice using mass spectrometry. He showed that after 3 generations (F2), SILAM mice on a C57BL/6 background had stably incorporated $^{13}C_6$ Lysine. In BLAJ mice, scaffolding protein expression is increased, suggesting that there is an issue with structure of the muscle cell in absence of dysferlin. This mouse will be used for future comparisons to identify other proteins that are over or under expressed in muscular dystrophy mouse models.

**Volker Middel (Germany)** presented the work generated in his laboratory showing that dysferlin is required for membrane repair in zebrafish. They found that dysferlin appears rapidly at the site of wounding and that its accumulation is independent of the size of the wound or how much dysferlin is present in the cell. They also showed that the C-terminus is required for trafficking of dysferlin to the wound site, and that dysferlin recruitment is sufficient for resealing the membrane hole.

## Session 3: Animal models of dysferlinopathy

This session focused on animal models that were made for studying dysferlinopathy, highlighting the benefits and challenges of several systems that were recently developed.

**Matthew Hirsch (USA)** discussed his characterization of the BlaJ (Dysf−/−) dysferlin deficient mouse model that was generated by Dr. Isabelle Richard (France). This work is essential because full characterization of the model is necessary before therapeutic studies can be performed. Hirsch analyzed a cohort of BlaJ and wild-type C57 black 6 mice for 1 year and 7 months, studying serum creatine kinase levels, behavioral phenotypes, physical manifestations and live imaging of muscle degeneration. Compared to wild-type, BlaJ mice had elevated creatine kinase levels at every time point tested. In behavioral analyses, 66 week old BlaJ mice showed significant deficiencies compared to wild-type in rotor-rod, marble-bury, grip strength, and swimming speed challenges. Overall exploration and rearing frequency differences were observed during extended monitoring of BlaJ mice as early as 15 weeks of age. Histological analyses showed that BlaJ mice had more centralized nuclei and a higher uptake of Evans blue dye after injury. BlaJ mice also develop large fat deposits in the hip region that are often accompanied by tumors but there were no significant differences in life expectancy. BlaJ mice also appear to have an increase in brain weight but the significance of this was unclear.

**Peter Currie (Australia)** described previous analyses showing that dystrophin-deficient zebrafish have many aspects similar to the pathology seen in humans, indicating that zebrafish is a good potential model for studying muscular dystrophies. He is now using zinc finger (ZFN) technology (Sigma–Aldrich) to create a number of zebrafish lines with mutations in genes associated with muscular dystrophies including dysferlin, myoferlin, otoferlin, FKRP and AHNAK. He found that ZFNs are very efficient in generating mutations in the zebrafish genome. The first target was dysferlin and he generated 12 independent mutations (both insertions and deletions) in less than 9 months with a single ZFN. He is currently evaluating the phenotype of the dysferlin-null zebrafish.

**Louis Kunkel (USA)** discussed his creation of a dysferlin-mutant zebrafish line. Kunkel used TALEN technology to create 2 stable deletions in exon 1 of dysferlin. Transcripts containing exon 1A have high homology to human dysferlin variants 8–15 and this variant is more highly expressed in brain, eye, heart and skeletal muscle than the other tissues. Western blot analysis using Hamlet showed that dysferlin expression is reduced in mutant fish but RNA levels are normal. Full phenotypic analysis has not yet been completed and the fish are available for other labs to use.

### *Jain Foundation initiatives: preparing for pre-clinical trials*

Mouse models of dysferlinopathy are critical for monitoring disease progression in response to potential interventions. However, the mouse models of dysferlinopathy don't show a robust overt phenotype, and the disease progression can only be followed using tedious histological evaluations of the muscle only apparent at a late age and require that the animal be sacrificed. The late onset and tedious methods necessary to evaluate dysferlinopathy in mice are a barrier to therapy development, dramatically increasing the time and cost of evaluating any particular intervention. This problem is magnified further with the desire to test dozens of possible interventions or make comparisons between interventions. **Doug Albrecht** described the Jain Foundation's efforts to use new technologies to increase our ability to track the changes that occur with disease progression in mice, with a goal of establishing preclinical testing platforms that will help evaluate and compare interventions.

The first platform that was tested to evaluate the dysferlin deficient "BlaJ" animals takes advantage of cutting edge video image analysis to identify changes in the behavior of the mice. The idea is that the behavior of the mice will change as they compensate for weakness or muscle soreness. The Jain Foundation in partnership with a contract research organization,

PsychoGenics, compared dysferlin null and normal animals in two different platforms that either assessed natural cage activity and the response to a variety of physical and psychological challenges, or that sensitively monitors the gait of the animals. Both of these platforms were able to discriminate between normal and dysferlin deficient animals at the earliest time point tested (3 months old), as well as at later time points (6 and 9 months). This first study using these methods suggests that they are sensitive enough to detect behavioral changes that stem from the dysferlin deficiency earlier than can be detected histologically. During the conference, several attendees expressed concern that the platforms may be picking up small differences between normal and dysferlin deficient mice related to small differences in their genetic background rather than the disease process, and suggested that future studies evaluate littermates to help control for this. The Jain Foundation plans to use littermates to validate the initial findings in future studies.

PsychoGenics also conducted a hindlimb fatigue challenge on the animals. In this test, the animals are forced to stand on a narrow ledge with all of their body weight on their hindlimbs and their spontaneous activity is measured before and after. This challenge is difficult enough that the normal animals show a marked decrease in spontaneous activity after the challenge, and the preliminary results suggest that the dysferlin deficient animals are slightly more sedentary, suggesting that it takes them longer to recover.

The Jain Foundation also conducted studies at Charles River Research Discovery Services (CRL) in Finland to monitor the volume of the TA, Gastrocnemius, Psoas, and Gluteus muscles in BlaJ and normal C57Bl6 by MRI and measured composition of the Gastrocnemius and Gluteus by 1H MRS. CRL used a high power (11T), small bore MRI magnet to achieve good spatial resolution in rodents, and was able to detect an initial drop in the muscle volume of Gluteus muscle at 6 months of age, followed by a statistically significant drop in volume by 9 months. The composition of the Gluteus also began to change at 6 months with an increase in the lipid content that became pronounced by 9 months. The mice will be imaged again at 12 months, and we expect that the differences will become even more pronounced in the Gluteus, and may become apparent in other muscles such as the TA and Gastrocnemius. Overall, these studies look very encouraging, as MRI and MRS are a non-invasive method for monitoring changes in the volume and composition of the Gluteus muscle in response to interventions. The ability to follow individual animals overtime and monitor their response to an intervention should increase our ability to detect subtle improvements, and reduce the numbers of animals required for an effective study. In addition, MRI and MRS are monitoring changes in the muscle that correlates directly to the disease process. Since MRI and MRS are commonly available clinical tools, any findings that are made in mice should easily translate to the patients.

CRL also monitored the Basal Metabolic Rate (BMR) of the animals in response to cold stress. Because muscle is the site of most energy consumption in the body, we hoped to see some perturbation in the amount of energy consumed with the progression of the disease, which might be exacerbated by cold stress, forcing the animals to use their muscles to shiver to maintain body temperature. Animals were tested at 3, 6 and 9 months of age, with a 12 month time point pending completion. These studies found no difference in the dysferlin deficient mice room temperature at any age tested so far, but did find some subtle gender specific differences at the 6 and 9 month time points. It may be that the muscle deterioration in the animals is not sufficient at 9 months to result in a measurable difference in BMR, but perhaps at 12 months there will be larger differences.

Finally, the Jain Foundation's efforts to characterize a dysferlin/dystrophin double deficient mouse line (BLAJ/mdx) as a model to help increase the pace of genetic therapy exploration were described. The concept is based on the premise that dystrophin helps stabilize muscle membranes, and dysferlin helps repair them, so if both are missing it should be catastrophic for the muscle. If this is true, then we might be able to assess the effectiveness of genetic interventions that restore dysferlin earlier and with better sensitivity than in BlaJ/mdx mice, but only if there is a synergistic effect that dramatically worsens the disease. Initial publications from the Campbell and Han laboratories suggest this is the case. BlaJ/mdx and mdx mice were tested in a variety of behavioral and functional tests, including downhill treadmill running, grip strength, rotorod (a coordination test), and serum CK levels at 1, 3 and 6 months of age. At the time of the conference, the 1 and 3 month time points were complete, with the 6 month time point pending. Following the 6 month time point, the animals will be sacrificed, and their muscles analyzed for total weight, numbers of centrally nucleated fibers, and fiber diameter. In the first two time points the BLAJ/mdx and mdx mice performed equally well in the all of the tests, with no clear exacerbation of the disease.

## Session 4: Genetic therapies for dysferlinopathy

This session discussed genetic therapies for dysferlin deficiency including adeno-associated virus replacement of the dysferlin gene, compensation for the absence of dysferlin by increasing expression of another muscle protein and expression of a partial dysferlin gene by exon skipping.

**Louise Rodino-Klapac (USA)** began the session by introducing AAV5-MHCK7.dysferlin, an adeno-associated viral (AAV) vector that allows transfer of "full-length" dysferlin into recipients, despite the large size of dysferlin

relative to the packaging capacity of the vector. The system they have developed allows expression of dysferlin to persist at least a year *in vivo* with only full-length, not partial fragments of dysferlin detectible in the muscle. Dysferlin expression following AAV delivery improved pathology, increased specific force and resistance to fatigue in the diaphragm of treated mice, and restored membrane repair. The Rodino-Klapac laboratory intends to moving this virus forward through pre-clinical development, with the intention to initiate Phase I clinical trials in humans by early 2015. Strategies for treating human recipients, such as isolated limb vascular delivery were also discussed as a solution to get dysferlin into all muscles necessary during treatment. Finally, Dr. Rodino-Klapac also described a second AAV vector system to deliver dysferlin being developed as either a second generation or a backup.

**Isabelle Richard (France)** discussed the challenges of inserting the dysferlin gene into an adeno associated viral (AAV) vector, since the gene is larger than its packaging capacity. She compared the "full-length" packaging strategy (also called fragmented genome reassembly) to split and overlapping AAV vectors that recombine to yield one full-length dysferlin gene *in vivo*. She found that dysferlin could be expressed using all vectors, but was still in the process of completing these experiments at the time of this talk and could not yet draw conclusions about the comparative efficacy of the strategies.

**Matthew Hirsch (USA)** showed that 65% of wild-type expression of dysferlin following adeno-associated viral vector (AAV) delivery improved muscle integrity in dysferlin deficient mice 3 weeks post-transduction. Indirect evidence suggested that even mild overexpression of dysferlin was detrimental to skeletal muscle in wild-type mice. These findings highlight the necessity of finding an optimal and safe dose for dysferlin gene delivery using a gene addition strategy. He also investigated the amount of AAV vector transduction of muscle fibers following intramuscular injection and presented evidence that not all muscle fibers express the transgene equally following delivery. It was indicated that the distribution of the vector transgene product varies with respect to the AAV capsid following intramuscular injection. Importantly, this observation, in addition to evidence of dysferlin toxicity during overproduction, suggests that care must be taken to achieve a uniform dysferlin distribution following a gene addition strategy as AAV vectors move into the clinic for the treatment of dysferlinopathy.

**Marc Bartoli (France)** and co-investigator Martin Krahn, have designed an anti-sense oligonucleotide (AON) that skips exon 32 of dysferlin to allow cells to produce a functional truncated form of the protein. In particular, Dr. Bartoli presented data that showed that the resulting protein protected the AON-treated dysferlin deficient human cell from membrane lesion and osmotic shock. The laboratory designed a mouse model of dysferlinopathy that has a nonsense mutation in exon 32. Characterization studies of these mice are still underway, but they plan to assess whether muscular dystrophy can be prevented using this AON treatment.

**Karine Charton (France)** presented data that Ano5 is expressed less abundantly in dysferlin deficient BLAJ mice compared to control muscle. This suggested that increase of Ano5 expression could compensate for the absence of dysferlin. Ano5 is relatively small and does not present the same challenges in packaging into an adenoviral vector as dysferlin. The full-length Ano5 dysferlin was successfully overexpressed by adenoviral delivery, but there was no improvement in the muscle pathology in dysferlin deficient mice. This work suggests that Ano5 overexpression may not be a useful therapeutic to pursue for the treatment of dysferlinopathy. Their findings were published shortly after the conference [3].

### *Jain Foundation initiative: Online LGMD Diagnostic Tool*

The Jain Foundation LGMD Diagnosis Tool is a computer-based diagnostic decision support tool whose purpose is to recommend which LGMD genes are the most promising candidates for sequencing to determine a patient's subtype, based on the clinical presentation. It uses a Bayesian algorithm, which multiplies the probabilities that a given symptom will, or will not, be observed in a particular disease. The probabilities for different diseases are then calculated based on how closely the patient's phenotype matches what is typical for each disease. Currently, the tool includes autosomal dominant LGMDs 1A-1G, autosomal recessive LGMDs 2A-2O, as well as Nonaka Distal MD/Hereditary Inclusion Body Myopathy. The tool is available on the Jain Foundation website with free registration (www.jain-foundation.org/lgmd-subtyping-tool/).

The Bayesian probability scores are taken from the medical literature. For every disease listed in the tool, we have based an initial algorithm of the phenotype based on the medical literature. The tool was refined by testing approximately 50 published cases from the medical literature covering most forms of LGMD. The tool was able to pick the correct diagnosis as the most likely choice in over 70% of the tested cases, and as one of the top three choices in over 90% of cases. Currently, the Jain Foundation uses the Tool to determine if sequencing of the dysferlin gene is warranted for registered patients. Of those patients for whom the tool determined that LGMD2B was the most likely diagnosis and for whom genetic sequencing has been completed, about 90% were found to have dysferlin mutations.

## Session 5: Dysferlin structure

This session focused on the folding structure of dysferlin. The most noteworthy structural feature of dysferlin is the presence of seven C2 domains. C2

domains in other proteins are known to bind Ca2+ and/or phospholipids, and it is thought that these domains play key roles in dysferlin's cellular function. Dysferlin also contains a DysF domain of unknown function, which is unique to dysferlin and related proteins such as myoferlin.

**Altin Sula (UK)** presented the determination of the inner portion of the DysF domain of dysferlin. The DysF domain is a nested domain common to all the Type I ferlins (dysferlin, myoferlin, and Fer1L5). At the 2008 Dysferlin Conference, Nicholas Keep, the head of the laboratory, presented a structure determination of the inner DysF domain of myoferlin by NMR. The present work determines the analogous structure of dysferlin by X-ray crystallography. This domain consists of a long two-stranded beta sheet which is held together by arginine and tryptophan residues. A number of pathological mutations have been found in this region of the protein; in most cases, an arginine or tryptophan is changed to a different amino acid, which is predicted to disrupt the beta-strand binding.

**Bryan Sutton (USA)** presented work on the C2A domain of dysferlin. This domain, which comprises the N-terminal portion of the protein, has two different forms due to two alternate start exons of dysferlin, which comprise about 40% of the amino acid sequence of C2A. Dr. Sutton's laboratory and collaborators determined the 3D structure of both versions of C2A by X-ray diffraction, and measured Ca2+ and phospholipid binding by isothermal titration calorimetry and differential scanning calorimetry. The alternate version of this domain, C2Av1, which is encoded by the alternate start exon 1a, is more basic that the canonical C2A as it contains more arginine and lysine residues. These positively charged amino acids bind to the site, which in canonical C2A binds calcium ions. Unlike C2A, which functions as a Ca2+-activated phospholipid binding domain, C2Av1 does not exhibit Ca2+-dependent phospholipid binding. It is noteworthy that the Ca2+ binding site of C2A has a very weak binding affinity for Ca2+, so it will only bind Ca2+ at higher than physiological intracellular Ca2+ concentrations. Also, both domains have very marginal thermodynamic stability and unfold at slightly higher than physiological temperatures.

*C2 domains workshop*

**Bryan Sutton (USA)** led a tutorial on C2 domains of dysferlin. C2 domains are named by analogy to one of the domains of Protein Kinase C. C2 domains are present in many proteins and occur in two types. Each C2 domain is composed of four pairs of beta sheets. The two types of C2 domains differ in the order in which the paired beta sheets occur in the primary amino acid sequence. Synaptotagmins have Type 1 C2 domains; in contrast, five of the seven C2 domains in full-length dysferlin and myoferlin are thought to be Type 2. In the primary amino acid sequence, beta sheet domains can often be recognized by alternating hydrophobic and hydrophilic amino acids, which are oriented to the core and the periphery, respectively, of the beta-sheet when folded.

In addition to the C2 domains and the central DysF domain, it appears that dysferlin also contains an alpha helical domain in exons 21–24, just after the C2C domain.

**Session 6: Pursing interventions**

This session focused on therapeutic strategies that could reduce the symptoms of dysferlinopathy using non-genetic methods of intervention.

**Joshua Zimmerberg (USA)** discussed how dietary interventions using exogenous lipids might slow progression of dysferlinopathy. Previously, he used a laser to wound whole muscle fibers and utilized a calcein red–orange leakage assay to show that DHA can strengthen cell membranes. Consequently, he has begun testing new classes of lipids and is characterizing the lipid that had the largest effect on membrane stability. When ingested, the lipid is partially digested and partially incorporated into phospholipids. Dr. Zimmerberg is currently evaluating the toxicity of the lipid in mice coupled with lipidomics to determine how much of it is incorporated into cell membranes and the effects the lipid has on other membrane lipids. He is also developing array tomography to determine whether these lipids reverse muscle disorganization in A/J mice and has initiated a study to look for a biomarker that can be used to track disease progression.

**Se-Jin Lee (USA)** described his ongoing work on the myostatin pathway. Mutations in myostatin have been shown to dramatically increase muscle mass. Since this discovery, a number of pharmaceutical companies have been interested in developing drugs that affect the myostatin pathway to treat muscular dystrophies. Although it would not stop disease, inhibiting the myostatin pathway might tip the balance enough to alleviate patient symptoms. One potential problem of inhibiting the myostatin pathway could be the premature exhaustion of satellite cells. Additionally, inducing muscle hypertrophy in the context of muscular dystrophy may cause supplementary myofiber damage and Dr. Lee's results suggest that this is a valid concern. By crossing dysferlin-null mice with follistatin knock-out mice (F66), Lee has shown that myostatin inhibition seems to increase muscle damage and leads to elevated CK levels. Thus, the beneficial effects of myostatin inhibition in enhancing muscle mass and strength should be weighed against the potential detrimental effects of increased myofiber damage.

**Kanneboyina Nagaraju (USA)** described his work on a glucocorticoid analog called VBP-15. Glucocorticoids reduce inflammation and work in multiple inflammatory diseases but their chronic use causes severe side effects. He showed that it is possible to dissociate the

glucocorticoid beneficial effects from side effects. Dr. Nagaraju showed that VPB-15 treatment reduces the effects of cell injury and helps reseal damaged membranes, suggesting that VBP15 is a potential therapeutic for dysferlinopathy. Dysferlinopathy patients generally do not respond to glucocorticoid treatment. Pre-necrotic stage mdx mice that were treated for 6 weeks with VBP-15 showed significant improvement in grip strength and EDL muscle force. VBP-15 also reduced muscle inflammation and unlike prednisone, it does not stunt growth and has no side effects on bone in mice.

**Robert Bloch (USA)** has shown that dysferlin is present in t-tubules where it stabilizes t-tubule structure and function. T-tubules are extensively damaged in muscles lacking dysferlin and this damage requires extracellular Ca2+ *in vitro*. Therefore, a calcium channel blocker like diltiazem, currently used to treat cardiovascular diseases, may prevent muscle damage in dysferlinopathy patients. Dr. Bloch showed that diltiazem protects t-tubules from disruption, promotes recovery of torque and reduces both necrosis and inflammation after LSI *in vivo*. Diltiazem also protects against damage to t-tubules (measured with an impermeant dye, Sulforhodamine B), against disruption of the organization of dihydropyridine receptors (DHPRs) and against dysfunctional calcium signaling after OSI to muscle fibers *in vitro*. In muscle cells lacking dysferlin subjected to injury, t-tubules are disrupted and myoplasmic calcium levels increase, leading to the muscle being irreversibly damaged. Diltiazem stabilizes t-tubule structure and function, probably by blocking DHPR and preventing calcium influx, thereby reducing muscle damage in dysferlin deficient cells. These results suggest that diltiazem may be a potential therapeutic strategy for dysferlinopathy.

**Eduard Gallardo (Spain)** presented his work on bone marrow transplantation (BMT) of dysferlin deficient mice. Dysferlin is highly expressed in the blood and since the lack of dysferlin is associated with inflammation, he asked if restoring dysferlin in blood monocytes could restore muscle function without actually adding dysferlin back directly to skeletal muscle. Dr. Gallardo found that BMT in the A/J Dysf$^{prmd}$ mouse model restored the expression of dysferlin in monocytes and macrophages but not in skeletal muscle. Animals who were treated with BMT showed mild functional improvement even if the transplant did not contain dysferlin although the effect was moderately better when dysferlin was added. He hypothesizes that the improvements seen might be due to the mobilization of muscle stem cells or the release of growth factors that favor muscle regeneration. Further experiments are necessary to determine the cause of improvement in mice receiving BMT and to test if it persists 6 months post-treatment. Dr. Gallardo also described a strain of A/J mice with the same genetic background as the A/J Dysf$^{prmd}$ mouse model but without a mutation in Dysf that can be used as a control in future studies [4].

**François-Jérôme Authier (France)** described his work on polyvalent immunoglobulin (po-Igs), which could potentially alleviate muscle pathology by decreasing the widespread complement activation seen in dysferlinopathic muscle. Dysferlin deficient SJL/J mice were given 5 consecutive weekly infusions of human po-Igs. The results showed that although treatment with po-Igs led to a significant increase in forelimb strength and hind limb strength, spontaneous activity and muscle pathology remained unchanged. These results suggest that po-Igs could potentially be used as a therapeutic strategy, but more sensitive mouse phenotyping assays are necessary to fully characterize the effects of po-Igs in mice.

### *Jain Foundation initiative: assay development for drug screening*

**Doug Albrecht** described the Jain Foundation's efforts to translate some of our understanding of dysferlin's role in a cell into assays that might help identify chemical entities that modulate or replace dysferlin's function. The Jain Foundation's efforts are ongoing and are being conducted by Contract Research Organizations (CROs) that routinely work with pharmaceutical companies on assay development and compound library screening. The initial steps of this effort are focused on determining whether published observations that relate to dysferlin's function in cells can be turned into simple assays that can be used to rapidly screen thousands of chemicals for their ability to revert dysferlin null cells back toward normal behaviors. The major hurdles in these studies are practical, and the experiments described are not meant to comment on the underlying biology of each assay, but rather are focused on overcoming a variety of difficulties related to accurately monitoring complicated cellular processes in a way that can be done thousands of times in a relatively short time. Dr. Albrecht described the current state of 5 assays that are being considered.

(1) Transferrin-488 accumulation.

The assay that is the farthest along and is looking most promising is based on the observation of Demonbreun et al. [5] that dysferlin-null myoblasts take up much more transferrin (labeled with a fluorescent dye) than normal cells. The uptake and monitoring of labeled transferrin levels in cells has been optimized over a wide range of cell culture conditions to produce a robust difference between dysferlin positive and negative mouse cell lines. The final validation step for this assay is to reintroduce dysferlin in the null cell line to confirm that the effect is dysferlin dependent. Once this is complete, the assay will be ready to move into the next phase of development, which is to miniaturize the assay and conduct a preliminary screen on a small library of compounds to assess its performance.

(2) IL-1β secretion by ATP/LPS stimulated dysferlin deficient myotubes.

The methods appropriate to monitor IL-1 β secretion in a high throughput assay were not sensitive enough to detect the secretion of this cytokine from the cell lines used in the assay. Therefore, this assay was abandoned as it is not thought to be suitable for screening compound libraries.

(3) Absent ATP secretion from wounded or depolarized dysferlin deficient myotubes.

This assay attempts to monitor ATP levels in the media surrounding the cells following depolarization. Initial results were encouraging, but background levels of ATP in the media made it difficult to detect robust differences in cultures of normal and dysferlin deficient cells. While this assay may still have some potential, further development has been put on hold while other options are pursued.

(4) Differentiation of dysferlin deficient myoblasts to myotubes.

Several labs have reported that dysferlin deficient myoblasts isolated from patients and mice do not form myotubes as well as normal myotubes. This assay uses cutting edge "high content" microscopy to monitor the size and length of myotubes. Initial experiments suggest a 3-fold difference at defined time points between normal and dysferlin deficient cells. Additional optimization and validation work is in progress.

(5) IGF1-R expression on the surface of dysferlin deficient myotubes.

In the same paper that showed an increase in transferrin accumulation in dysferlin deficient cells, Demonbreun et al. [5] also demonstrated that dysferlin deficient muscle cells are insensitive to growth factor IGF-1 and that the IGF-1 receptor is not making it to the surface of the muscle cells. High content imaging was used to determine if the difference in surface IGF-1R could be distinguished between normal and dysferlin deficient cells. Because this difference is not related to the absolute amount of IGF-1R but rather its position within the cell or on its surface, the automated microscopy system was unable to reliably distinguish this subtle difference and this assay is no longer being pursued.

## Session 7: The clinical perspective

This session presented data from several clinical projects that focused on better understanding dysferlinopathy.

**Susan Sparks (USA)** presented her clinical findings from 11 genetically confirmed dysferlinopathy patients who were part of a larger LGMD Natural History Study. The evaluations included strength and function tests, 6 min walk, timed tests, pulmonary and cardiac function tests, questionnaires to assess their quality of life and activity limitations, and blood tests to evaluate serum creatine kinase, growth factors, and cytokine levels. Her analysis showed that all muscle groups tested where significantly weaker than controls, but function in the upper extremity appeared to be more preserved than in the lower extremity. Also, as reported previously, she found that clinical presentation is highly variable as indicated by a wide range in values for both timed tests and muscle strength and that there is little to no cardiac or respiratory involvement.

**Anna Pastoret Caldero (Spain)** is trying to define the dysferlinopathy carrier frequency in the general population. To do this, she used the blood monocyte assay to evaluate the dysferlin protein levels in 147 normal volunteers. It is known that individuals that carry only one pathogenic dysferlin mutation express between 23% and 75% of the dysferlin protein. The goal was to use this assay to assess the carrier frequency for dysferlin mutations and extrapolate to determine the true disease frequency. The results showed that 16/147 (11%) normal volunteers had dysferlin expression in the carrier range, which is higher than expected. Three of the 16 predicted carriers had their dysferlin gene sequenced and 2/3 showed the presence of at least one predicted pathogenic mutation. Complete genetic analysis is needed on all 16 samples in order to arrive at conclusions regarding the carrier frequency.

**Jorge Bevilacqua (Chile)** presented the clinical and genetic characterization of dysferlinopathy in the Chilean population. The evaluations done consisted of a clinical assessment including motor function measure tests (MFM), electrodiagnostic tests, whole body MRI, echocardiogram, respiratory evaluation, as well as molecular evaluation of CPK levels, dysferlin protein expression in the muscle biopsy, and dysferlin genetic analysis. Of the 41 individuals, 31 belonging to 24 different Chilean families were shown to harbor point mutations in dysferlin. Eight mutations were consistently found in the cohort and 4 out of these accounted for 77.4% of the total. This suggests the presence of a founder effect for these mutations in the Chilean population. The clinical findings of initial weakness in the lower legs that progresses later to the upper limbs, normal NCV, major impairment of motor function observed through MRI and MFM analysis, and normal respiratory and cardiac function are similar to what has been previously reported in other dysferlinopathy cohorts.

### *Jain Foundation initiative: clinical outcome study (COS)*

**Laura Rufibach** of the Jain Foundation provided a status update for the Jain Foundation's dysferlinopathy clinical outcome study (COS). Oversight and funding for this study is provided by the Jain Foundation and the lead principal investigator is Dr. Kate Bushby from Newcastle University.

COS is an opportunity for patients with dysferlinopathies to participate in a clinical research study that aims to define outcome measures by measuring how these conditions develop and progress. This is the first study of its kind. The outcome measures identified are essential to the development and success of future clinical trials. In addition, the information collected will provide a better

understanding of dysferlinopathies, ensuring that optimal care and treatment is being provided for people with these conditions, and will hopefully lead to new and better therapies.

The specific aims of the study are: (1) To define the natural history of dysferlinopathy and occurrence of complications, (2) To determine the utility of specific outcome measures across a spectrum of severity, (3) To identify a large group of genetically confirmed and clinically characterized patients, (4) To build a resource of biomaterial with good clinical correlates to aid in the identification of biomarkers, (5) To build an international network of dysferlinopathy trial ready sites.

The study aims to recruit a minimum of 150 dysferlinopathy patients from 14 international study sites in the United States, United Kingdom, France, Germany, Italy, Spain, Japan, and Australia. The main eligibility requirement is that the individual be genetically confirmed by the identification of two pathogenic mutations in the dysferlin gene or one pathogenic dysferlin mutation plus $< 20\%$ normal dysferlin protein expression based on Western blot analysis of muscle or blood. Both ambulant and non-ambulant individuals are eligible, but for statistical reasons, a 2:1 ambulant/non-ambulant ratio must be maintained. As of March 2013, 7/14 sites were recruiting and 49 individuals, with a 2.26:1 ratio, had already been recruited and screened.

Medical, physiotherapy and MRI/MRS assessments are being performed, according to the patient's level of ability, on 6 occasions over 3 years (screening, baseline, 6 months, 1 year, 2 year, and 3 year). These assessments include: (1) Heart, lung and neurological examinations - all visits, (2) Measurements of muscle strength/functionality - all visits, (3) Echocardiography and electrocardiography - 2 visits, (4) Magnetic Resonance (MRI/MRS) investigations - 4 visits, (5) Questionnaires to evaluate activity/quality of life - 4 visits, and (6) Standard laboratory tests on blood samples - 4 visits.

Additional blood samples are also being requested for "biobanking." The extra blood for biobanking will be taken once a year. Serum, plasma, DNA and RNA is being extracted from the blood and stored in the EuroBioBank (www.eurobiobank.org) for anonymous use in approved research. In addition, a skin sample for biobanking is also being requested. Analysis of these biobanked samples is crucial for the identification of disease markers that will allow for monitoring of these conditions non-invasively during clinical trials. As of March 2013, 95% of participants had given biobank blood samples and 63% had consented to give skin fibroblasts.

For more information about the dysferlinopathy clinical outcome study, go to the study website at www.dysferlinoutcomestudy.org or email contact@dysferlinoutcomestudy.org.

*Note:* Please visit the Jain Foundation website (www.jain-foundation.org) for more details on the 6th Dysferlin Conference proceedings.

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*For their privacy, patient names have been omitted from this list.*

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