Jain Foundation 2024 Dysferlin Conference
All Program Summaries

Session I – Clinical Studies
This session focused on recent analyses of clinical data, primarily collected in the International Clinical Outcome Study for Dysferlinopathy (COS), as well as two studies to improve patient diagnosis.

Volker Straub (Newcastle University – volker.straub@ncl.ac.uk) gave a great overview of the history of clinical and genetic studies of dysferlinopathy as well as the goals and findings from the International Clinical Outcome Study for Dysferlinopathy (COS). COS is the first multi-center natural history study for dysferlinopathy and has been collecting longitudinal data from >300 individuals with dysferlinopathy, some of whom have been in the study for over 10 years. Volker highlighted some of the important findings of COS: determination of which outcome measures work best, development of a dysferlin specific outcome measure (NSAD), identification of a dysferlinopathy specific MRI signature, and elucidation of factors that influence rates of progression. This information increases clinical trial readiness and aids in disease management. There is more to be learned from continuing to mine the COS data and exploring additional clinical questions such as disease modifiers, exercise regimes, and evaluation of additional ethnic cohorts.

Meredith James (Newcastle University - meredith.james@ncl.ac.uk) discussed the development of standards of care for dysferlinopathy by a team of experts and how the COS data is being used to inform disease management and care guidelines. The resulting guidelines provide guidance for all stages of disease (ambulant, transitioning, and non-ambulant) across nine high level domains: diagnosis, neuromuscular, respiratory, cardiac, nutrition/bone/endocrine, pregnancy, surgical, vaccination, and psychosocial. Implementation of such standards, based on a multidisciplinary model of care, is needed to maximize function and quality of life, and facilitates clinical trials by standardizing care across trial sites.

Harmen Reyngoudt (Institute of Myology – h.reyngoudt@institut-myologie.org) presented data from the COS studies assessing longitudinal analysis of MRI scans as a way of monitoring disease progression and/or therapeutic interventions, as well as determining if it could be a predictor of decline. The MRI analyses, done in both upper and lower limbs, consisted of longitudinal assessments of fat fraction (FF) and water T2. A specific pattern of fatty replacement was noted in both upper and lower limbs, which significantly increased over time and strongly correlated with functional measures. The water T2 value in dysferlinopathy showed a significant increase relative to healthy controls. The T2 values stayed elevated over multiple years and represent a marker of active muscle damage. Another important finding from COS is that quantitative MRI measurements at different sites require qualification and standardization to generate comparable, analyzable, and consistent data.

Mary Neal (Newcastle University – mary.neal@ncl.ac.uk) presented the assessment of sodium dynamics in response to exercise using Na-MRI of the tibialis anterior (TA) in healthy volunteers versus individuals with dysferlinopathy. The exercise consisted of 25 reps of isometric dorsiflexion of the TA of the strongest leg at maximum effort. Water T2 and Na-MRI were measured at baseline pre-exercise and at several time points up to 30 minutes post exercise. The data showed significantly higher sodium concentrations and water T2 values at baseline in dysferlinopathy compared to healthy volunteers, likely reflecting the pathological state of the muscle. A significant increase in sodium was observed immediately post exercise in healthy volunteers, but the change in sodium following exercise in dysferlinopathy was highly variable likely due to the heterogeneity of disease states between participants, indicated by TA fat fractions ranging from 5-52%. Therefore, assessment of disease populations may need to be done by studying individuals longitudinally, rather than comparing individuals. Nevertheless, this data demonstrates the sensitivity of sodium fluctuations for detecting physiological and pathophysiological states.

Ana Topf (Newcastle University - ana.topf@ncl.ac.uk) described the formation of a new collaborative network, called Latin-Seq, that aims to provide genetic diagnosis to patients with neuromuscular disease (NMD) in Latin America. The aims of the study are to provide genetic diagnosis through exome sequencing,

learn about disease prevalence, identify geographic variants, identify new causative genes, and support local clinic development for diagnosis and management of NMDs. This is a highly collaborative effort, with the Latin-Seq consortium comprising more than 50 sites in 18 Latin American countries recruiting and counseling patients, staff from Newcastle managing the study and performing genetic analysis, and CNAG in Barcelona doing the exome sequencing. Patient recruitment and sample collection is ongoing and so far, 389 blood, saliva, or DNA samples have been collected from patients and family members. The first set of sequencing results are currently being analyzed.

Young Jae Moon (Children's National Research Institute – yjmoonos@jbnu.ac.kr) performed a longitudinal study on female BlaJ mice and a cross-sectional study on patients to investigate the involvement of spinal muscles in dysferlinopathy. For the mice, histological and MRI analysis was performed on both spinal and limb muscles at ages ranging from 6-22 months. Fatty infiltration was observed earlier in the spine than the quadriceps of BlaJ mice. The most affected spinal muscle in mice was the iliocostalis, with muscle fiber damage preceding adipogenic degeneration. MRI analysis in 6 individuals with dysferlinopathy showed that most patients with limb muscle degeneration also showed adipogenic degeneration of all the spinal muscles, with the iliocostalis muscle being the most severely affected, as was observed in the BlaJ mice. These results identify fatty degeneration of the iliocostalis spinal muscle as an early feature of dysferlinopathy, which could allow diagnosis of dysferlinopathy by non-invasive MRI even before the onset of limb muscle degeneration.

Session II Featured Topics
This session presented recent findings which the Jain Foundation thinks provide new insights into the function of dysferlin and the pathological mechanisms of dysferlinopathy.

Nenad Bursac (Duke University-- nbursac@duke.edu) reported findings from an in vitro model of dysferlinopathy using engineered muscle tissues, or "myobundles," created from induced pluripotent stem cells (hiPSCs). Compared to myobundles cultured from healthy control iPSCs, dysferlin-deficient myobundles have normal development and structure, but display several phenotypic differences. These include: (i) decreased contractile force due to reduced calcium transient amplitude; (ii) impaired recovery from osmotic shock injury; and iii) abnormal response to fatty acid supplementation, including mitochondrial abnormalities and accumulation of lipid droplets inside myotubes. RNAseq revealed transcriptomic signatures of deficits in calcium handling, oxidative metabolism, and mitochondrial function in LGMD2B myobundles. Dantrolene and Vamorolone were able to restore some, but not all, of the abnormalities in dysferlin-deficient myobundles. Inducing an RyR1 Ca2+ leak in healthy myobundles recapitulated development of the contractile and metabolic LGMD2B disease phenotypes, but not impaired recovery from osmotic shock. This suggests that calcium leak alone is insufficient to drive the dysferlin-deficient phenotype. In another set of experiments, the authors investigated the cell-autonomous effect of dysferlin deficiency on the function and polarization of macrophages differentiated from LGMD2B iPSCs (iMPs). Comparison of dysferlin-positive and dysferlin-negative iMPs revealed no significant differences in phagocytosis or structural changes in response to standard pro- and anti-inflammatory factors. However, dysferlin-deficient macrophages exhibited reduced endocytosis (of AcLDL) and clearance of myotube debris.

R. Bryan Sutton (Texas Tech University Health Sciences Center - roger.b.sutton@ttuhsc.edu) Dr. R. Bryan Sutton has made significant contributions to our understanding of ferlin protein structure. Ferlins are challenging to analyze due to their large size and complex structure. Initially, researchers believed that ferlins consist of multiple synaptotagmin-like C2 domains arranged as "beads on a string". This model of dysferlin structure included a considerable amount of linker, making it difficult to interpret pathogenic mutations. However, the development of new computational tools like AlphaFold and RoseTTAFold has been critical in defining dysferlin's structural domains, although experimental confirmation is still necessary to validate the predictions. Dr. Sutton's recent research has focused on membrane binding and the impact of known pathogenic mutations on the dysferlin C2G domain, which is crucial for dysferlin's adherence to the membrane and its role in membrane bending and fusion. Several pathogenic mutations associated with this domain affect Ca2+ binding, altering its ability to bind to phospholipids. These findings significantly contribute to our knowledge of ferlin structure and function.

Chiara Nicoletti (Sanford Burnham Prebys Medical Discovery Institute-- cnicoletti@sbpdiscovery.org)
Karim Ismat (Children's National Research Institute-- khussainis@childrensnational.org)
Both Chiara and Karin presented results from a joint project between the two research groups to catalog and analyze gene expression patterns at the single cell level in dysferlin-deficient and control mice of various ages.

Chiara presented a longitudinal analysis of gene expression changes at the single-cell level and targets for intervention. The team assayed the transcriptional profile of mononuclear cells from Bla/J and wild-type mice by single cell RNA-Seq from quads (symptomatic) and TA (largely asymptomatic) at ages of four months (presymptomatic), seven months (early symptomatic) and eleven months (symptomatic). Some key observations from these single cell experiments were: 1) There are specific FAPs and myeloid cell subpopulations that show differential enrichment in different muscles/time points; 2) Dysferlin deficiency causes a gradual increase in pro-adipogenic FAPs in quad over time, but this happens only at late stage in TA; 3) FAPs from quad show an accumulation over time of a pathogenic subpopulation expressing Timp1, Thbs4, and Postn; 4) DYSF KO cells show enrichment of a subpopulation expressing markers typically associated to M1, M2 macrophages, and an upregulation of genes linked to inflammation, endocytosis and mitochondrial activity; 5) DYSF KO quads at 4 and 7 months have the highest level of ligand-receptor interactions between

FAPs and macrophages. An interactive dysferlinopathy single-cell atlas of this dataset is being created and will be shared with the community.

Karim focused on the identification of macrophage populations that drive adipogenesis in dysferlinopathy. Analysis of the dysferlinopathy single cell atlas pinpointed high levels of TIMP1 and galectin-3 (Gal-3) expression associated with disease specific FAP and macrophage populations, respectively. A co-culture system was used to assess how signaling between macrophages and FAPs may drive the fates of both the cell populations. These assays showed that dysferlinopathic macrophages rich in Gal-3 increased spontaneous adipogenesis of FAPs. Furthermore, exogenous Gal-3 given to wild-type mice in connection with injury recapitulated the increased adipogenesis observed in dysferlinopathy. Thus, aberrant interactions via Gal-3 can drive muscle adipogenesis, and Gal-3 emerges as a potential therapeutic target.

Session III: Genetic Therapies

This session focused on approaches to treating dysferlinopathy that involve restoring the muscle's ability to produce the dysferlin protein. There were four presentations in the session: two on gene transfer, one on gene editing, and one analyzing the amount of dysferlin needed to rescue the phenotype.

For gene transfer therapy, a challenge is that the dysferlin cDNA is too big to fit in a single AAV, the viral vector used in almost all current gene therapies. An approach to circumvent this hurdle is to deliver the gene in two pieces (dual vector). Recombination can occur at the DNA level, at the RNA level, or at the protein level; all these approaches are being investigated for genes beyond the AAV size limit.

Douglas Anderson (University of Rochester – Doug_anderson@urmc.rochester.edu) talked about the development of an alternate approach to dual vector gene therapy based on recombination of RNA, referred to as StitchR. Dr. Anderson's presentation walked us through the development of the StitchR platform which uses ribozyme-activated RNA trans-ligation to "stitch" together the pieces of a gene at the RNA level and how his lab has begun applying the StitchR approach to delivering full-length dysferlin to mice. Early indications are that StitchR has the potential to achieve high levels of dysferlin expression in skeletal and cardiac muscle, which can be further enhanced using muscle specific AAV capsids.

Oliver Rogers (Sarepta Therapeutics orogers@sarepta.com) discussed the dual vector SRP-6004 dysferlin gene therapy being developed by Sarepta Therapeutics, which uses the DNA reconstitution approach to produce the full-length *dysferlin* protein. The therapy is currently in clinical trials using intravenous delivery. Dr. Rogers' presentation focused on pre-clinical work in dysferlin-deficient mice that was done to inform the clinical trials and characterize the PK/PD properties of the therapy including the degree of vector transduction and reconstitution in tissues, levels of dysferlin protein expression in target tissues, functional restoration and safety.

Camille Bouchard (Université de Laval – camille.bouchard@crchudequebec.ulaval.ca) presented work on editing dysferlin point mutations by Prime Editing, a technique developed by Dr. David R. Liu using a form of CRISPR-Cas9. This technique permits modification of a single nucleotide in the DYSF gene and thus to precisely correct point mutations. Thus far the team has corrected patient-derived cell lines with four different mutations, and plan to begin testing their prime editing platform in a mouse model carrying the mouse equivalent to the human R1905X mutation. This mouse model was recently created by the Jain Foundation. One important finding regarding gene editing of dysferlin and other muscle genes is that editing efficiency is much better in muscle cells, where these genes are expressed, than in fibroblasts, where these muscle genes are not expressed.

Joe Yasa (University of Sydney – joe.yasa@sydney.edu.au) shared findings on three mouse lines which are knock-outs for Exon 40a but still expresses reduced amounts (10-90%) of dysferlin isoforms not containing Exon 40A. All the mice expressing reduced amount of dysferlin were generally free of pathological features of dysferlinopathy even when expressing only 10-20% of normal dysferlin levels. There were, however, multivesicular tubular aggregates seen in the muscles of the mice with the lowest dysferlin expression, in spite of the muscles displaying normal gross histology. The timing and significance of these tubular aggregates has yet to be determined.

Session IV: Lipids/Cholesterol

Growing evidence is pointing to the involvement of loss of homeostasis of lipids and cholesterol in the pathogenesis of dysferlinopathy. The speakers in this session presented a variety of observations in in vitro myobundles, in mice, and in patient samples documenting how muscles' management of lipids is altered in the absence of dysferlin.

Pascal Bernatchez (University of British Columbia–pascal.bernatchez@ubc.ca) discussed abnormalities in cholesterol handling in dysferlin-deficient mice, and findings in blood samples of human dysferlinopathy patients. The connection between dysferlin and cholesterol was first hinted at when Dr. Bernatchez's lab crossed ApoE knockout mice (a humanized mouse model of high cholesterol) with dysferlin-deficient mice. The ApoE mice have no muscle pathology themselves, but unexpectedly, knocking out the ApoE gene made the muscle pathology of the dysferlin-deficient mice much worse. Also, it was found that high dietary cholesterol made the dysferlin/ApoE double knockout (DKO) phenotype even worse, while the drug Ezetimibe, which blocks dietary cholesterol absorption and bile-excreted cholesterol reabsorption in the intestine, improved the DKO phenotype. Similar effects were seen when crossing the ApoE with mdx mice (DMD model), indicating that loss of cholesterol homeostasis may be a common feature in muscular dystrophies. In blood samples from Duchenne and Becker patients, levels of total cholesterol, HDL, LDL, and triglycerides all tended to be elevated. In blood samples from the COS study, dysferlinopathy patients were also determined to be dyslipidemic, although in contrast to Duchenne, HDL levels tended to be lower than normal. In addition, levels of triglycerides and ratios of total cholesterol/HDL tended to be elevated in those with dysferlinopathy. In dysferlin-deficient mice, it was found that muscles accumulate excess levels of free cholesterol, which is likely toxic to cells. There is also an upregulation of the HMGCR enzyme (which catalyzes the rate limiting step in cholesterol synthesis and is the target of statins) in muscle but not in liver. Thus, the circulating lipid abnormalities seen in dysferlinopathy may originate in muscles.

Alastair Khodabukus (Duke University – aik12@duke.edu) presented work on dysferlin's regulation of cholesterol homeostasis using an hiPSC-derived skeletal muscle "myobundle" model of LGMD2B. The dysferlin deficient myobundles show decreased force generation, increased susceptibility to osmotic shock injury (OSI), and lipid droplet accumulation, which mimic the functional and metabolic impairments that occur in dysferlin-deficient skeletal muscle. In addition, the following key observations were reported: 1) Metabolomics analysis showed a significant increase in free cholesterol and cholesterol esters in LGMD2B myobundles compared to WT in a cholesterol-free medium; 2) Decreasing plasma membrane cholesterol with methyl beta cyclodextrin (MBCD—reversibly binds cholesterol) impaired membrane repair in WT, but had no effect on LGMD2B myobundles. In contrast, increasing plasma membrane cholesterol levels with MBCD-bound cholesterol improved membrane repair in LGMD2B myobundles, but had no effect on wild type. 3) In WT myobundles, increasing cholesterol accumulation by inhibiting lysosomal cholesterol export with U18666A (inhibitor of NPC1) did not impact force generation, but significantly decreased membrane repair capacity and increased lipid droplet formation. Increasing plasma membrane cholesterol partially compensated for these effects of U18666A; 4) Pharmacological inhibition of cholesterol biosynthesis with pravastatin or cholesterol esterification with CI-976 in LGMD2B myobundles increased force generation and membrane repair capacity, decreased lipid droplet accumulation, and enhanced osmotic OSI functional recovery; 5) LGMD2B myobundles display increased statin resistance (i.e. compensatory HMGCR increase) compared to healthy myobundles and the use of a HMGCR degrader, SR12813, increases force and promotes OSI recovery in LGMD2B myobundles. Taken together these findings suggest that loss of dysferlin alters cholesterol homeostasis which impairs membrane repair and metabolism. In future work, the authors propose to a) assess the abundance of mevalonate pathway intermediates, b) study LDL cholesterol uptake, c) evaluate HMGCR degradation and d) validate these findings in mouse models.

Bradley Launikonis (University of Queensland–b.launikonis@uq.edu.au) talked about cholesterol and its role in membrane function in LGMD2B. The aim of the project is to understand if the amount of membrane cholesterol is different in dysferlin positive and negative muscles by varying the levels of cholesterol using cholesterol complexing agents, methyl-β-cyclodextrin and saponin. One of the early observations from this

project is that BlaJ TA muscle shows a greater dependence on membrane cholesterol than WT TA to resist sarcoplasmic reticulum Ca2+ leak. The group is keen to see what the effect of depleting t-system membrane cholesterol will have on the heavily dystrophic psoas muscle.

Stacey Keenan (University of Melbourne–stacey.keenan@unimelb.edu.au) presented on the heterogenous lipidome of dysferlin deficient mice, examining sex, age and muscle type. It is well known that a defining feature of dysferlinopathies is the dramatic changes in lipid composition, which manifests postgrowth and increases in severity with age. In this study, we investigated the muscle lipidome from female and male mice aged 3, 10 and 26 months of age, across four different muscle groups (quad, gastroc, EDL, and soleus) in BLA/J mice. We found distinct age and sex differences with signal intensity across 738 lipid species and 36 lipid classes and found significant sex and age differences in nine lipid classes. Compared to their younger counterparts, aged males determined the largest lipidome composition changes. Furthermore, soleus and EDL had the greatest lipid content while quadricep and gastroc displayed the largest remodelling changes. This coincided with dramatic changes in lipid metabolism gene expression markers involved in lipid synthesis, uptake and esterification. In total, this lipidomic analysis provides new insights into the changes accompanying dysferlin deficiency and paves the way for targeted subcellular and temporal lipidomics.

Zeren Sun (University of British Columbia–zerensun@student.ubc.ca) analyzed whether dietary approaches capable of modulating circulating lipoprotein-associated cholesterol and/or triglycerides (TG) can exacerbate muscle wasting of severe dysferlin/ApoE double knockout (DKO) mice. It has been previously reported that the DKO mice fed a fat-based “western diet” containing 0.2% cholesterol can show humanized levels of circulating lipids with a 6-fold exacerbation of fibro-fatty infiltration along with ambulatory dysfunction by 11 months of age. Since the western diet contains both cholesterol and TG, it was unclear which – cholesterol or TG – is responsible for disease exacerbation. The aim of this project was to differentiate the roles of dietary cholesterol and TG in the progression of dysferlinopathy. To do this DKO mice were fed four different diets from 2-5 months of age 1) a control diet (low in fat, 0% cholesterol), 2) a TG diet (60% fat, 0% cholesterol), 3) a cholesterol-rich diet (10% fat, 2% cholesterol), and 4) a cholesterol/TG diet (60% fat, 2% cholesterol). Muscle lesions were evaluated by Masson’s trichrome stain. Intramuscular cholesterol accumulation was characterized by filipin stain, Amplex Red, and Western blotting of two key cholesterol-regulating enzymes, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and low-density lipoprotein receptor (LDLR). The cholesterol-rich diet raised circulating cholesterol and further accelerated ambulatory dysfunction and appearance of muscle lesions at an earlier age (4-5 months). While the TG-rich diet had little effects on muscle lesions, the cholesterol/TG diet increased both circulating cholesterol and TG and unexpectedly prevented the appearance of severe muscle lesions typically observed with the cholesterol-rich diet. The cholesterol/TG-rich diet also normalized the 2-fold increase in intramuscular and intramyofiber free cholesterol accumulation seen in hindlimb muscles of the DKO mouse, as well as the abnormal upregulation of HMGCR and LDLR. In conclusion, high circulating cholesterol exacerbates the phenotype of dysferlinopathy, but dietary triglycerides may be protective, suggesting modification of the intramuscular cholesterol abnormalities and TG-rich diets as therapeutic interventions.

Session V: Potential Therapies 1
This session discussed potential interventions to reduce the effects of dysferlin's absence. The talks focused on the contributions of energy storage, metabolism, and membrane repair, to the muscle pathology that occurs in dysferlinopathy and how we might be able to slow or stop disease progression.

Cesar Cardenas (Universidad Mayor, Chile– julio.cardenas@umayor.cl) presented research on the impact of a ketogenic diet on dysferlin knockout mice. Mice consistently fed a ketogenic diet before the onset of the disease showed enhanced motor balance and strength, but intermittent use of a ketogenic diet, or beginning the ketogenic diet after onset of muscle pathology, did not improve muscle performance. Targeted proteomics on mitochondrial genes was conducted. Western blots of BlaJ mouse muscle on a ketogenic diet show higher PGC-1 alpha expression, a key regulator of mitochondrial biogenesis, compared to WT levels on standard chow. Upon further investigation, mitochondrial mass did not change on the ketogenic diet, although an increase in mitochondrial function was observed, suggesting that a ketogenic diet induces changes in mitochondria. No histological improvements were seen in various muscle groups from BLAJ mice on a ketogenic diet compared to those fed normal chow. With some positive trends to some readouts, but also some with no change, it is difficult to tell if the ketogenic diet is having a benefit in dysferlinopathic mice.

Regula Furrer (University of Basel– regula.furrer@unibas.ch) discussed metabolic dysregulation in dysferlinopathy. Increased glycogen accumulation in dysferlin deficient muscles is observed by histological staining, EM, and biochemical analysis. Patterns of protein expression indicate that glycogen accumulation is due to increased glucose uptake, increased conversion of glucose to glycogen, and reduced glycolysis. Increased glycogen is observed at an early stage of the disease (7 months), before significant muscle atrophy has occurred in Dysf-/- mice. Increasing glycogen content by overexpressing PGC-1α in dysferlin deficient mice accelerates disease progression, which is opposite of the benefits that have been reported in mdx mice. Promoting the dynamics of glycogen synthesis and breakdown through exercise ameliorated muscle atrophy and improved endurance performance, but increased muscle damage in Dysf-/- mice. This study demonstrates that glucose metabolism is altered in dysferlinopathy and that the pathology the mice experience is not only the result of a membrane repair defect. The authors propose that it may be beneficial to combine membrane repair therapies with interventions modulating glucose/glycogen metabolism and a non-damaging exercise regimen.

Noah Weisleder (Ohio State University– noah.weisleder@osumc.edu) described efforts to improve sarcolemmal membrane repair. The TRIM72/MG53 protein contributes to sarcolemmal membrane repair in part through its interaction with dysferlin. Previous studies showed recombinant human MG53 (rhMG53) delivered exogenously by injection showed promising results in improving sarcolemmal repair in both dysferlinopathy and Duchenne Muscular Dystrophy. However, there were off-target effects of MG53 protein on metabolism mediated by its E3 ligase activity. This led to engineering improved versions of rhMG53 by deletion analysis of protein domains. An improved construct named MyoTRIM was developed that contains amino acid replacements which eliminates metabolic effects and improves solubility while still maintaining membrane repair efficiency. MyoTRIM improves membrane repair in BLAJ muscle ex vivo, and is more efficacious than the original rhMG53 in BlaJ mouse cardiac muscle.

Matthew Watt (University of Melbourne– matt.watt@unimelb.edu.au) discussed inhibition of the enzyme diacylglycerol acyltransferase 2 (DGAT2) enzyme as an approach to inhibit the accumulation of triglycerides in dysferlin-deficient muscle. Previous work in Dr. Watt's lab showed that fat accumulation coincides with a dramatic increase in DGAT2, which catalyzes the final reaction in the synthesis of triglycerides. DGAT2 upregulation in dysferlinopathy occurs independently of DGAT1, which shows no change. DGAT2 inhibition was accomplished by treating mice with AAV carrying a construct that expresses a shRNA against DGAT2, which was effective in 70% of the animals and achieved an average of 35% downregulation of DGAT2. The animals with DGAT2 inhibition had increased energy expenditure and more browning of body fat, but no change in body weight or the weights of individual muscles. The treated mice had moderately improved performance on the balance beam but did not reach the level of wild type mice, and still had a greatly

increased lipid content in muscle. No significant histological changes were observed in the psoas muscle with DGAT2 inhibition. It was concluded that DGAT2 inhibition is unlikely to be an efficacious treatment for dysferlinopathy.

Session VI Potential Therapeutics II
This session continued the discussions on identifying downstream changes that occur in dysferlin's absence and different ways to intervene in the disease process in muscle.

Mohan Viswanathan (MIT –mohanv@mit.edu) presented his findings using small molecules to stabilize *DYSF* patient missense mutations, which can prevent their degradation and restore their localization and function. The ability of multiple compounds to restore plasma membrane localization of 64 *DYSF* pathogenic missense mutations was assessed. 4-PBA was selected from the screening assay for further experiments. 4-PBA acts as a chemical chaperone, helping protein trafficking and folding and is a clinically approved drug used for urea cycle disorders. Further tests of 4-PBA were conducted on the *DYSF* L1341P mutation. HEK and dysferlin-deficient GREG cells were transfected with dysferlin containing the missense mutation *DYSF* L1341P. Transfected cells treated with 4-PBA showed improved membrane repair, suggesting that the L1341P containing dysferlin protein is getting to the plasma membrane and is functional. 4-PBA was then tested in a 12 month study in MMex38 mice, which are homozygous for the mouse equivalent of the L1341P human mutation ($mDYSF^{L1360P}$). 4-PBA was given in drinking water starting at 2-months of age MMex38 were evaluated using the balance beam assay at 3-month intervals and at 14 months of age, the treated and untreated were sacrificed and the EDL, TA, Quad, Gluteus, Psoas, and Gastrocnemius were analyzed by histology. The authors report 1) 4-PBA increases muscle weight in female mice, but not males, 2) 4-PBA shows improvement in psoas histology and 3) 4-PBA-treated female mice show improved balance beam performance. Key outstanding questions from the study include: Do the differences in benefit correlate with the amount of dysferlin expressed? Are the sex differences in disease progression masking 4-PBA benefits in males? Do male and female Mmex38 mice metabolize the drug differently? Would different dosing reveal benefits in males?

Pam Van Ry (Brigham Young University – pvanry@chem.byu.edu) presented her group's efforts on "Evaluating Therapeutic Activity of Galectin-1 in LGMD2B/R2". Previous research has shown multiple potential protective roles of Galectin-1 in muscular dystrophy. Using this data, the authors sought to test the efficacy of recombinant human galectin-1 (rHsGal-1) as a novel therapeutic option for dysferlinopathy using *in vitro* and *in vivo* models of LGMD2B/R2. Results showed that rHsGal-1 increases membrane repair capacity in dysferlin null myotubes and myofibers. Experiments to understand the mechanism of rHsGal-1 treatment showed that the increase in membrane repair in $Dysf^{-/-}$ myotubes is dependent on Galectin-1's carbohydrate recognition domain (CRD) and *in-vitro* rHsGal-1 treatment modulates the inflammatory response through the NFĸ-B pathway and cytokines. An *in vivo* one month treatment of Bla/J mice showed benefits in membrane repair and upregulation of anti-inflammatory cytokine expression; confirming the beneficial effects of rHsGal-1 treatment in inflammation and membrane repair. Functional improvements of a 6-month rHsGal-1 treatment include enhanced exploratory ambulation and balance beam performance. Histology showed that rHsGal-1 treatment increased myofiber diameters and decreased central nuclei in the psoas muscle. These results reveal that Gal-1 can reduce signs of disease through changes in integral myogenic protein expression as well as membrane stabilization. The authors hope to perform lipidomic, proteomic and immunomodulation studies in future.

Gianni Giarrano (Ohio State University – giarrano.1@buckeyemail.osu.edu) reported results from experiments on improving sarcolemmal membrane repair by modulating the PI3K/Akt1 pathway in dysferlin deficient skeletal muscle. Endocytosis and exocytosis are involved in membrane repair and are regulated by the PI3K/Akt1 pathway, which is reduced in dysferlinopathy patient derived cells. The team reports that upregulating the PI3K/Akt1 pathway *in-vivo* increases plasma membrane repair. In addition, the PI3K/Akt1 pathway is activated upon ballistic injury to cells and when either PI3K or Akt1 is constitutively activated, endocytosis and exocytosis activity is upregulated. Given this data, the group sought to evaluate the therapeutic potential of upregulating PI3K/Akt1 signaling in the absence of dysferlin. The authors report that 1) Electroporation of constitutively active PI3K or subcutaneous injection of the Akt1 agonist SC79 in dysferlin-null mouse skeletal muscle increases plasma membrane repair capacity and 2) SC79 treatment reduces CK values and increases body weight of Bla/J mice.

Session VII: Calcium Handling

This session discussed a variety of observations on changes in calcium handling in dysferlin-deficient muscle. Muscle contraction is triggered by a large release of calcium from the sarcoplasmic reticulum, but calcium in large amounts is toxic, and cells in general usually have very low calcium concentrations. Muscles have developed an elaborate calcium handling system to deal with these opposing requirements. There are various lines of evidence that dysferlin is part of calcium management, and that calcium handling doesn't work normally in dysferlin's absence.

Robert Bloch (University of Maryland rbloch@umaryland.edu) presented results of experiments studying the contribution of different C2 domains of dysferlin and other proteins to T-tubule localization, membrane repair, and proper calcium handling. The C2A domain of dysferlin was shown to be sufficient to restore normal calcium signaling and membrane repair but could not by itself localize efficiently at the triad junction. When the C2A domain of myoferlin, or the C2 domain of PKCα was substituted for dysferlin C2A, regulation of calcium signaling wasn't restored. A combination of dysferlin C2A and PKCα C2, however, properly localized to the triad junction and also stabilized calcium signaling as well as supported membrane repair, demonstrating that a combination of these two domains may be an approach to achieving a "minigene" capable of restoring two of dysferlin's important functions.

Aldo Meizoso Huesca (University of Queensland—a.meizosohuesca@uq.edu.au) discussed measurements of sarcoplasmic reticulum calcium leak rates in various muscles of wild type and dysferlin deficient mice at various ages. Findings from these experiments include the following: 1) the psoas muscle, which is highly affected in dysferlinopathy, has a greater intrinsic leak rate in wild type mice than does the much less affected TA; 2) the greater calcium leak rate in psoas is correlated with greater RyR1 phosphorylation, which is accompanied with higher sympathetic input, increased basal PKA-dependent signaling and may be related to differing amounts of thermogenesis between muscles; 3) in BlaJ mice, only psoas has greater calcium leak rate than the corresponding wild type muscles, and the leak increases with the animal's age; 4) in psoas of older BlaJ animals, the mitochondria contain a large fraction of the intracellular calcium, implying impairment of their function. These observations may provide an explanation as to why some muscles—those with greater intrinsic calcium leak rates—are more susceptible to damage in dysferlinopathy.

Callum Quinn (University of Manchester—callum.quinn@manchester.ac.uk) discussed calcium handling in dysferlin-deficient cardiac muscle. Cardiac muscle also expresses dysferlin but isn't affected by its absence as badly as skeletal muscle. In part this may be due to cardiac muscle contraction being initiated by the muscle's own calcium release, rather than by membrane depolarization induced by nerve signals. Nevertheless, the following changes were observed in dysferlin-deficient cardiac mouse muscle: changes in the gross organization of ventricular transverse-axial-tubules (TATs), changes in TAT ultrastructure and a narrowing of the dyadic cleft. In addition, dysferlin-deficient cardiac muscle was found to be more susceptible to damage from osmotic shock, and to induced arrhythmia.

Session VIII Dysferlin and Immune function

There is significant cross talk between the muscle and the immune system, especially after muscle injury and during muscle regeneration. Studies conducted both *in vitro* and *in vivo* in dysferlin deficient mice and humans have demonstrated that the immune system responds abnormally in the absence of dysferlin. This session covered insights into the immune system in the context of dysferlin deficiency.

Noah Weisleder (Ohio State University -- noah.weisleder@osumc.edu) presented findings on how antibodies to certain proteins can compromise sarcolemmal membrane repair. TRIM72/MG53 rapidly translocates to plasma membrane disruption sites upon injury. Previously, the authors showed that antibodies against TRIM72 impair membrane resealing, and that autoantibodies against TRIM72 are present in the sera of some patients with Idiopathic Inflammatory Myopathies (IIM). Furthermore, repair is compromised in the presence of sera with elevated anti-TRIM72 titers, and similar effects were observed in multiple patient sera samples regardless of myositis subtype. Dysferlinopathy has similar pathological hallmarks to IIM, so the authors investigated whether autoantibody responses are also present in dysferlinopathy. The authors found that serum from dysferlinopathy patients compromised membrane repair. However, in certain patients this was observed regardless of whether serum levels of TRIM72/MG53 antibodies were high or low, indicating that antibodies to other proteins might also be interfering with repair. Antibodies to Annexin A6, but not Annexin A5, were found to also compromise membrane repair and are present in dysferlinopathy patients' serum. FDB muscle treated ex vivo with commercial Annexin A6 antibodies showed a significant repair defect. From these data the authors suggest that increasing membrane integrity with agents like poloxamer or reducing the amount of autoantibodies present could potentially compensate for or reduce the membrane repair defects seen in dysferlinopathy. Future studies will test if depleting Annexin A6 autoantibodies from sera samples improves repair.

Lelinh Duong (Curtin University – lelinh.duong@curtin.edu.au) reported findings on the role of dysferlin in neutrophils and the impact on neutrophil function when dysferlin is absent. Neutrophils isolated from blood samples of Bla/J and WT mice (both genders and various ages) were evaluated for a variety of functional markers. The results concluded 1) Markers associated with chemotaxis, migration, and inflammatory functions showed changes between 3-month old Bla/J and WT; 2) Neutrophils from 3-month old Bla/J mice showed increased lipid content and production of pro-inflammatory IL-12 following activation; 3) In the 10-month old Bla/J cohort, a reduction in L-selectin positive neutrophils were observed, suggesting neutrophils in peripheral blood are potentially migrating to dysferlin deficient tissues or are transmigrating to the bone marrow; and 4) Neutrophils from the 19-22 month old cohort showed similar functional patterns between Bla/J and WT, likely due to neutrophil function dysregulation caused by aging. Taken together, these data suggest that dysferlin deficiency may lead to dysregulation in neutrophil migration and function prior to disease onset, and that neutrophil migratory markers could be biomarkers and potential targets for therapeutic interventions.

Connie Jackaman (Curtin University – connie.jackaman@curtin.edu.au) continued the topic of immune dysregulation in dysferlin deficiency. This study investigated the role of inflammatory cells in dysferlinopathy by analyzing intracellular inflammatory cytokine expression, Bodipy lipid content, and metabolic activity in immune cells from various tissues in Bla/J and WT mice. Key conclusions include: 1) Analysis of sites involved in the generation of immune cells suggests that at early ages (3 & 10 months) immune responses are driven by proximal lymph nodes and bone marrow, while at later stages (24-26 months) these responses are driven from the spleen; 2) Early site-specific myeloid cell changes and cytokines are evident in blood and bone marrow prior to pathology; 3) Immune-related structural changes in the absence of dysferlin are observed in the lymphoid organs particularly the lymph nodes at 10 months, while dysregulation of T cells in the spleen are seen at later time points; and 4) Dysregulated inflammation and metabolism in myeloid cells is seen in 10 months old Bla/J mice. Many of the immune changes observed in Bla/J mice at 10 months of age are similar to those seen in older WT mice, suggesting an early aging of the immune system in dysferlinopathy.

Session IX – New Insights
This session covered a variety of observations giving new insights into the behavior and functions of dysferlin and possible pathogenic pathways in dysferlinopathy.

Robyn Murphy (La Trobe University—r.murphy@latrobe.edu.au) presented results of a survey of how dysferlin expression levels vary with a variety of factors: sex, age, muscle, and fiber type. A few different muscles were analyzed in rats and mice, as well as biopsies from the human vastus lateralis muscle. In the human samples, there was no clear trend observed in dysferlin expression as a function of sex or age. Differences were seen in dysferlin expression between fiber types, but these weren't consistent between species. In human skeletal muscle, more dysferlin was seen in Type IIA fibers compared to Type I. In rats, however, the opposite trend was observed, while in mice there wasn't a significant difference in dysferlin levels between fiber types.

Stefan Wette (La Trobe University— s.wette@latrobe.edu.au) discussed the localization of dysferlin within muscle fibers. Dysferlin was first characterized as a sarcolemmal protein based on transverse cross-sections of muscle, then in longitudinal cross sections, was found to be localized to the vicinity of t-tubules. By mechanically skinning rat EDL fibers, the amounts of dysferlin in the sarcolemma in the fiber periphery and in the fiber interior (including the t-tubules) were compared by western blotting. Dysferlin localization within fibers was compared with other proteins including caveolin-3 and DHPR, using confocal microscopy. Most dysferlin (>85%) was found to be located deeper in t-tubules. Unlike caveolin-3 which was found almost exclusively near the entrance of t-tubules (as well as in the sarcolemma), most t-tubule dysferlin is localized deep in the tubules, likely near triad junctions in proximity (~200 nm) to DHPR.

Miranda Grounds (University of Western Australia – miranda.grounds@uwa.edu.au) discussed a long-standing question regarding dysferlinopathy: why do corticosteroids, which are helpful and part of the standard of care in DMD, have a harmful effect on dysferlinopathy, at least when administered daily? Dr. Grounds reported results of two studies using the glucocorticoid Dexamethasone (Dex for 4-5 weeks) in male BlaJ and wild-type (WT) mice (aged 5 and 10 months). Functional analyses of slow-twitch soleus and fast-twitch EDL muscles, which are less affected in dysferlinopathy, were minimally impacted by Dex at 10 months, The investigations focused on rapid gene expression changes, and histopathology of quadriceps and psoas muscles that are typically impacted at 10 months of age. Complement-associated gene expression was high in BlaJ muscles compared with WT, and Dex decreased immune-associated gene expression in WT muscles, but not in BlaJ with a significant increase in inflammasome-related related genes. A novel observation was pronounced staining for glycogen in myofibres of BLAJ quadriceps, exacerbated by Dex. These myofibres were large, pale and contained vacuoles, suggesting that excess glycogen may be causing myofibre death by oncosis.

Hannah Bulgart (Ohio State University— hannah.bulgart@osumc.edu) presented studies investigating links between dysferlin and Alzheimer's disease. Neurons in Alzheimer's Disease (AD have defective membrane repair, and beta-amyloid (observed in some biopsies of dysferlin-deficient muscle and a prominent feature of AD) impairs membrane repair in healthy cells. Alzheimer's disease models tend to have reduced dysferlin expression in neural cells, and inducing overexpression of dysferlin in cells from AD models improves membrane repair. These observations indicate that dysferlin facilitates membrane repair in neural tissue (where it's known to be expressed), and that the repair deficit due to reduced dysferlin expression may play a role in AD.

Peter Fridy (Rockefeller University— pfridy@rockefeller.edu) shared results of raising and using nanobodies (camelid antibodies) for dysferlin, and of using cross-linking mass spectrometry to characterize dysferlin's interactions with itself and with other proteins. Two nanobodies raised were able to specifically isolate dysferlin with efficiency comparable to a control monoclonal antibody. These nanobodies are listed in the Jain Foundation's Resources page. Mass spectrometric analysis of these isolations identified known binding partners of dysferlin as well as other potential interactors. Preliminary cross-linking mass spectrometry of

recombinant dysferlin indicated significant structural flexibility, with multiple binding geometries between domains.

Jain Foundation Initiatives I: Addressing Patient Identification, Clinical Care, Engagement
This session centered around the process of community engagement and how the Jain Foundation has been able to position the community for successful interventional trials through planned engagement. We conceptualize, design and execute key initiatives that are factors for success, such as: diagnostic innovation and access, swift patient recruitment, clinical education excellence and Dysferlin Registry growth. Most notably, the Jain Foundation has used engagement techniques to keep studies and trials on time.

Ecosystem Identification: Through 19 years of relationship building that fostered the patients and clinicians' willingness to share their experiences, the team has gained knowledge and insights about the interconnection of all stakeholders involved in our mission. Barriers and the reasons that exist are being addressed to drive change. Community stakeholders mentioned include patients, clinicians, laboratories, industry/drug developers, advocacy organizations, regulators, and researchers.

Past Initiatives: An overview of a hypothetical trial recruitment case study was shared that demonstrated how direct lines of communication with stakeholders and the Dysferlin Registry database enable the Jain Foundation to prepare to swiftly mobilize the community for trials. Also discussed were milestone initiatives and major learnings that have come from past initiatives such as:

- Free next genetic testing program from 2014-2017 in collaboration with LGMD Family Foundation Consortium, Genzyme/Sanofi, Emory Genetics, Dr. Madhuri Hegde and 2,000 patients.
- Multi-tiered diagnostic research which provided DNA, RNA and/or dysferlin protein expression analysis to over 400 individuals suspected of having dysferlinopathy.
- LGMD Patient days organized at clinics throughout the US.
- Dysferlin Registry member conferences in the US and India
- Various clinical studies such as the biomarker study and neutrophil assay validation study with the Indian patient community.
- Development of a custom, hybrid, private, application-based registry platform inclusive of genetically confirmed individuals.

Current and Ongoing Initiatives and Progress Associated with them:
- Dysferlin Registry: scaled to 1230 genetically confirmed dysferlinopathy individuals (as of June 2024) representing 55 countries.
- >10years of Global, Natural History Data Collection and Assessment Development (COS).
- Before Onset of Symptoms (BOS), a Study of Presymptomatic Individuals with Dysferlinopathy.
- US Centers of Excellence (COE) for Dysferlinopathy.
- Clinic Support Program (CSP).
- LGMD Advocacy Bundles-LAB (LGMD Awareness Foundation partnership).
- Latin-Seq and Neutrophil Validation Project (Diaz-Manera, Topf, Barresi)
- Indian Cohort Identification and Support (Dastur and Gaitonde, Mumbai).
- Communication Toolbox: NDEP, DR Newsletter, Quarterly Highlights.
- Targeted Registry Member Data Study – annual update on progression and support needs.

Challenges: The session concluded with reflection on the specific challenges the Jain Foundation faces relating to the needs of the community:
- Need for trial ready, functionally meaningful clinical assessments for non-ambulant patients.
- Patient Identification: Clinical and Laboratory pathways that automatically connect patients to our organization.
- Remote assessments, trial accessibility.
- Understanding why patients progress so differently.
- Discovering what protects a child's muscles from the absence of dysferlin before onset.
- Exploring and translating the changes in biomarkers over time (before onset, at onset, during progression).
- Laboratories accepting variant classification revisions and reissuing reports.

Jain Foundation Initiatives II: Program Summary coming soon

A Workshop for Connection: Real World Perspectives and Insights from Community Members

In this session, 9 patients who are members of the Dysferlin Registry, who live in the state of Texas, traveled to the conference venue to connect with one another and conference attendees who included dysferlin researchers, clinician researchers, and industry partners. Dysferlin Registry member attendees ranged in age from 16 to 77 years old and represented vastly different stages of the disease progression.

The session began with an introduction to Leon Shannon, a Dysferlin Registry member, who shared a documentary film he made in 2016. In the documentary, Leon's wife and friends from high school described moments when they began to notice changes in Leon's abilities and his gait. The film also shows Leon as he shared his routine, how he faces adversity, and the ways he is choosing to support his children. After the film, he gave a presentation to attendees to share how his life has changed since 2016 and what life is like now. You can view Leon's 2016 documentary here https://www.youtube.com/watch?v=oNaqFyKj33Y

Each of the 9 Dysferlin Registry members and their families then met in small groups for a 45-minute workshop with a Jain Foundation staff member and conference attendees. Often, a patient's experience is abstract to preclinical dysferlin researchers who may never meet a person with dysferlinopathy. This was a time to take turns sharing about themselves, challenges they encounter, and what their hopes are from future treatments. This fluid, reciprocal type of engagement activity in a conversational setting provided the opportunity to learn more about one another.

After the workshop, some researchers shared that the patients they met wanted to stay in touch and were willing to share with the entire lab in the future.

Some of the small group discussion takeaways from all community members included:

- The subject of time and being aware of how long each step of the discovery and drug development process is taking while patients continue to progress.
- Researchers encouraging young patients to continue to develop their minds and not give up on their intellectual passions.
- The trauma of becoming diagnosed, specifically relating to shock and a lack of prognosis.
- Considerations and gratitude for caregivers.
- The mental health challenges that come during all stages of disease progression.
- The need for treatment options and the willingness to participate in trials.
- The clinical challenges of not having materials or resources to offer people with dysferlinopathy.
- Fear of the progression into new parts of the body (especially the hands, feet) and not having enough knowledge to prepare ahead of time.
- Apprehension about going places for fear of falling, getting stuck or being unsafe.
- The need for an integration of natural history data and other research project data.
- The need for government funding for dysferlin research.
- The financial strain or burden on patients to afford the support they need to perform activities of daily living and remain as independent as possible.
- Stopping the progression would be enough reason to try a therapy for some patients.

This was the first time many of the researchers in attendance had ever met a person with dysferlinopathy. The session was a good start to building closer relationships among all community members. However, more time for the discussion to continue is warranted. Thank you to the Dysferlin Registry members and their families who were willing to join the conference for this special workshop session.