Kennedy Krieger Institute
Research. Treatment. Education.

November 2022

Greetings to our LBSL family and friends. We hope this update finds you and your loved ones safe, wherever you may be in the world. I would again like to thank you for your trust and for the amazing contributions you have made to help make our LBSL research program what it is. We were very excited to see many of you this summer at our third LBSL Patient Conference. In attendance, either in person or virtually, were 120 individuals representing six countries around the world. We are humbled by your support and will continue the momentum from this summer into 2023. Every year we have a better understanding of the disease process and towards therapeutic targets for LBSL.

I am taking this opportunity to provide you with an update about our LBSL research program and the Kennedy Krieger Institute overall.

PROGRESS AND CURRENT STATE OF RESEARCH

Over the past year, we have made great progress in research. Our pre-clinical research continues to be led by Dr. Christina Nemeth Mertz and the clinical research program is led by Dr. Amena Smith Fine. Most importantly, we have made significant progress with developing two different approaches for gene targeted therapy for LBSL as elaborated below.

PRE-CLINICAL RESEARCH

Cell Studies:

For those who may not be familiar, a few years ago we collaborated with a stem cell expert here at Kennedy Krieger, Dr. Mingyao Ying, to turn blood cells from LBSL patients into induced pluripotent stem cells (iPSCs). These iPSC were then treated with different factors to become nerve cells that we can grow and manipulate in a dish. We have established a nerve cell culture model for several LBSL patients and have compared those to several control cells. Over the last few years, we have been optimizing our techniques and determining the conditions necessary to best handle these fragile and valuable cells.

A major achievement this year is that we have obtained **"isogenic control" cells** for several of our patient lines. These cells are patient cells that have had the LBSL mutations corrected through gene editing in a lab. We then take these corrected cells and run them alongside unedited patient cells in all experiments. This technique controls all other genetic variability that exists in patients, ensuring that any abnormalities exhibited by patient cells is a result of the LBSL mutation. This type of control is considered the highest standard for scientific experiments as it completely eliminates variabilities that may be present between individuals that may be related to their ancestral background or other genetic variables. These isogenics controls are very expensive to generate. We were able to create these cells this year, which will help the validity of our work when under scientific review.

With your help, we were also able to secure the purchase of a **high-content screener**. This is an imaging system capable of housing cells (so that cells can be examined for extended periods of time and still be able to breathe) and imaging them at an extremely high resolution. This type of imager can image thick specimens, including cerebral organoids or "mini brains." Importantly, this machine also simultaneously analyzes samples, ensuring that data analysis is consistent and reproducible. It will enable us to acquire data more quickly increasing our output.

Single Cell RNA-sequencing to understand disease process in mini-brains:
Last year we updated you on the progress of growing LBSL cerebral organoids or "mini brains." These mini brains grow for several months in a dish and can grow several different cell types like human brains. Shiqi Guang, a former post-doc in the lab, ran these studies which are now under review for publication. In short, after reaching a state of maturity, each mini brain was separated into single cells and sequenced to be able to study the gene expression and composition of every different cell type within these structures. What we found was interesting, and very surprising to us. Full length "normal" DARS2 is necessary for normal protein function. We observed that certain cell types, most notably mature nerve cells, almost always fail to produce the full-length form. Other cells are still capable of producing full-length form, but also produce smaller versions which do not eventually produce protein; these small versions are usually degraded by the cell. The process by which these transcripts make these different forms is called "splicing" (for a great review of splicing, please watch this video on YouTube https://www.youtube.com/watch?v=CCGX05JKtSU). Errors in splicing tend to occur more frequently in LBSL patient cells, and these errors are not just in DARS2 but also in other genes/ proteins. We also observed that depending on the type of mutation a patient has, different cell processes may be activated. Though most LBSL patients present with a similar clinical phenotype, the way the cells respond to the lack of DARS2 may be different and may inform us on the exact nature of this dysfunction. These data are especially important to the big picture, and more on this below. Additionally, these data make sense based on our understanding from others' work, that not all cell types respond the same to the patient mutation, and we believe this is one particularly important reason to continue to study nerve cells, specifically. Dr. Christina Nemeth Mertz presented these data at Kennedy Krieger's Pediatric Neuroscience Consortium meeting in February, and at this year's United Leukodystrophy Foundation meeting in Itasca, IL in June 2022.

NIH funding:
This year, we are extremely happy to announce we have secured NIH funding for an LBSL study. As alluded to above, this study is to form an understanding of how DARS2 interacts with other proteins and RNA molecules in a cell. We know that LBSL results in complex changes within a cell, and the studies proposed in this grant are to specifically identify DARS2 partners, which may reveal unique roles for DARS2 in the nerve cell and reveal how different mutations may result in differential patterns of gene expression. Importantly, the findings from these studies may be meaningful for the understanding of other disorders of mitochondrial tRNA synthetases, and we are delighted that the NIH recognized the significance of this work.

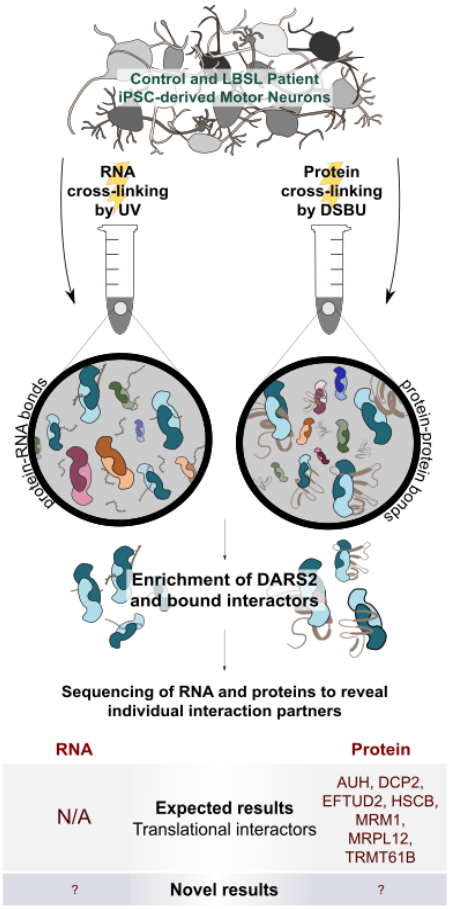

***Figure 1**. Schematic depiction of the new NIH funded project. LBSL neurons will be grown and DARS2 within these cells will be permanently linked to other interacting molecules, either RNA or protein. We will then identify these RNAs and proteins to determine what they do in the cell, and how LBSL may affect their function.*

Gene Targeted Therapies for LBSL

At this year's patient conference, we showed preliminary data for two different types of gene targeted therapy:

- AAV9 based gene therapy: AAV9 stands for adeno-associated virus type 9, which is a type of virus that is commonly used for treating genetic neurological disorders. In a nutshell, these viruses are able to infect brain cells but do not replicate and do not cause any inflammation, and we have genetically modified the virus so that it serves as a vehicle to deliver the normal DARS2 gene into the nervous system.
- Antisense Oligonucleotide (ASO) therapy: ASOs are short pieces of RNA that can be used to "hide" the DARS2 mutation in cells resulting in cells making the correct version of the gene.

These type of approaches have been used in many different conditions and several therapeutics have received FDA approval for other neurological conditions. We have chosen to pursue both of these avenues, since each approach may have different degree of efficacy and come with distinct patterns of side effect profiles.

Our studies using AAV9 vectors are in collaboration with talented scientists led by Dr. Piotr Walczak at the University of Maryland School of Medicine. They have vast experience creating and validating such viruses and have made small quantities for us to test in our LBSL patient cells. Our findings so far, from experiments run by our research technician **Adam Ratajczak**, show that we can bring the normal DARS2 gene into the cells and are currently studying whether this strategy can improve the function of LBSL cells. For example, Figure 2, illustrates how with this therapy we can improve the growth of LBSL nerve cell wires.

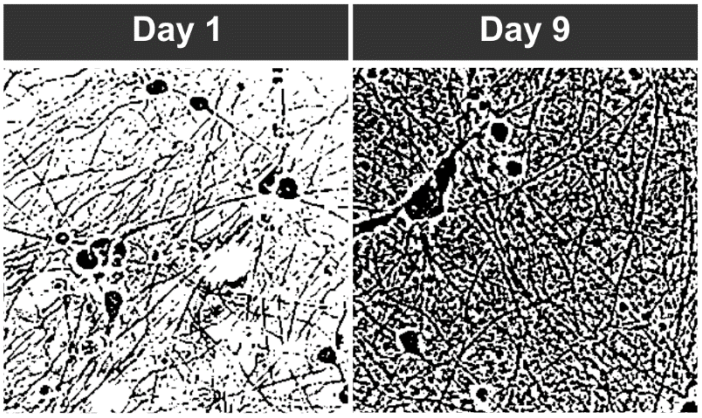

Figure 2. *Overlay images of LBSL neurons on Day 1 and Day 9 after treatment with AAV9 carrying DARS2. The cell neurites, or "wires" that allow communication between cells, are increased by Day 9 in AAV9 treated cells compared to LBSL neurons that received no treatment.*

ASO therapy (depicted in Figure 3) in LBSL patient cells also shows promise. We have tested a few different ASO constructs over the last two years and even tested the ability of nanoparticles to enhance delivery of ASOs to the brain. While nanoparticle experiments were not successful in delivering ASOs to mature neurons in a dish, we have since modified the ASOs to be more stable and suitable for this type of delivery. Parallel studies are being conducted as described above to answer the question of how this treatment may affect cell function, and how dosing can be optimized to ensure a long-lasting effect. Our talented postdoctoral fellow, **Dr. Manou Amanat**, has been hard at work growing cells, testing ASOs, and validating his findings.

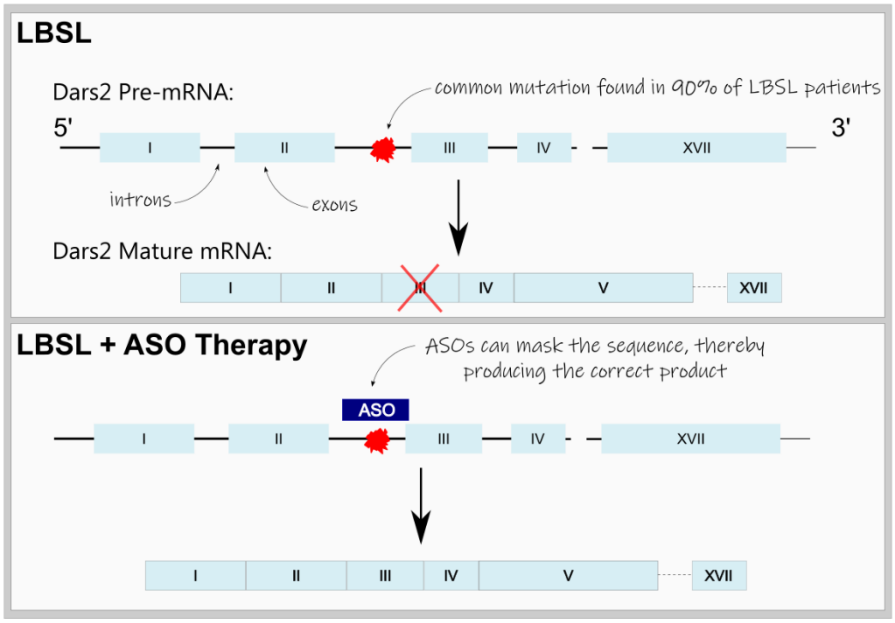

Figure 3. *Schematic of ASO therapy in LBSL. The most common mutation in LBSL affects a cell's ability to make a functional protein. An ASO is a short sequence that is designed to bind to the mutation area, masking the mutation, and tricking the cells into producing a healthy product.*

Plan for the upcoming year in the lab:
For both AAV9 and ASO studies, we will incorporate the mouse model to determine if, how, and when each of these constructs travels to the brain. We will also need to determine other tissues that may accumulate these constructs and if that has any effect. Finally, it will be important to determine if any toxicity is apparent. We are well set up in our mouse studies to be able to conduct these types of experiments soon. Importantly, in studies led by our technician, **Inés Garofolo**, we are now testing two additional mice that lack DARS2 in specified cells. One mouse lacks the gene in early white matter cells, and the other in a specific neuronal population of the spinal cord. We look forward to understanding how the gene deletion affects these cells, and these mice.

CLINICAL RESEARCH
While we are hopeful that we will identify a working therapy in animals and cells, it is critical that we better characterize the progression of LBSL clinically. In order to test any drug in a patient, we first need to understand at what pace the disease worsens and how variable this disease is. Importantly, this is not just a scientific question but a vital issue that needs to be fully addressed before the Food and Drug Administration (FDA) permits human trials.

Accordingly, with the help of our LBSL patient community, we have made great progress in collecting outcome variables and studying LBSL. We have recruited several new subjects since the last update and have been receiving more contacts from international patients who want to participate in research at our site and we have a strong ongoing international collaboration with several institutions across the US and Europe. We are anticipating a boost to recruitment this year, as Cure LBSL has launched the LBSL patient contact registry this month.

In April 2022, we published a manuscript about the feasibility of using the wearable sensors for home assessment of walking and balance impairment in LBSL in the journal Annals of Clinical and Translational Neurology. Our key findings were that the Lateral step variability (LSV) and step height during walking are increased for LBSL patients during brief walking tests compared with healthy volunteers (Figure 4). These are both markers of unsteady walking/ataxia that have been reported as important in other neurologic disorders with ataxia as well.

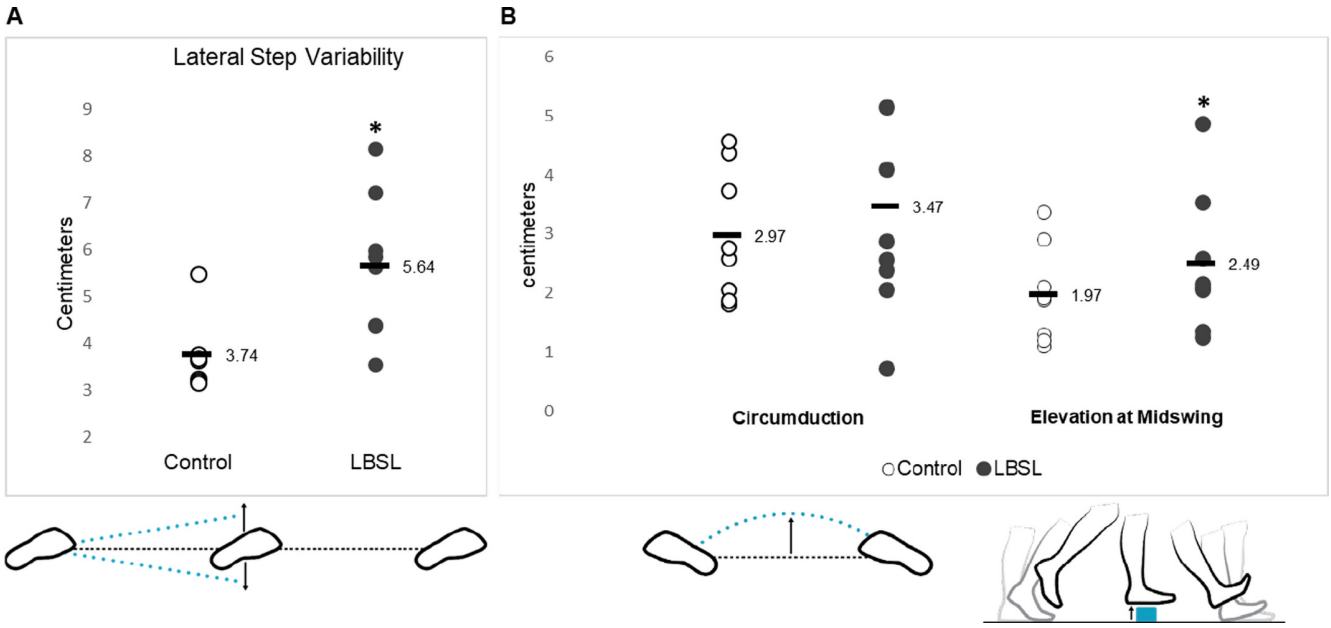

*Figure 4. Individual values of control and LBSL participants are plotted for the (A) lateral step variability (when considering three consecutive foot placements made by the same foot, this describes the variability of perpendicular deviations of the middle foot placement from the line connecting the first and the third, with positive values indicating movement to the outside), (B) circumduction (the maximum amount that the foot travels perpendicular to forward movement during an individual stride, with positive values indicating movement to the outside) and (C) elevation at midswing (the height of the foot sensor measured at midswing, relative to its start position while standing) during the 2-min walking test. The horizontal black bar is labeled with the cohort mean value. *p<0.01.*

During standing with the eyes closed, LBSL participants show rapid changes of body movement (known as jerk) covering a large sway area (Figure 5). This difficulty with maintaining balance during standing without aid of visual input indicates problems with the spinal cord white matter tracts that receive sensory information from the body. We are now tracking how these variables change during assessments every 6 months at participants' homes.

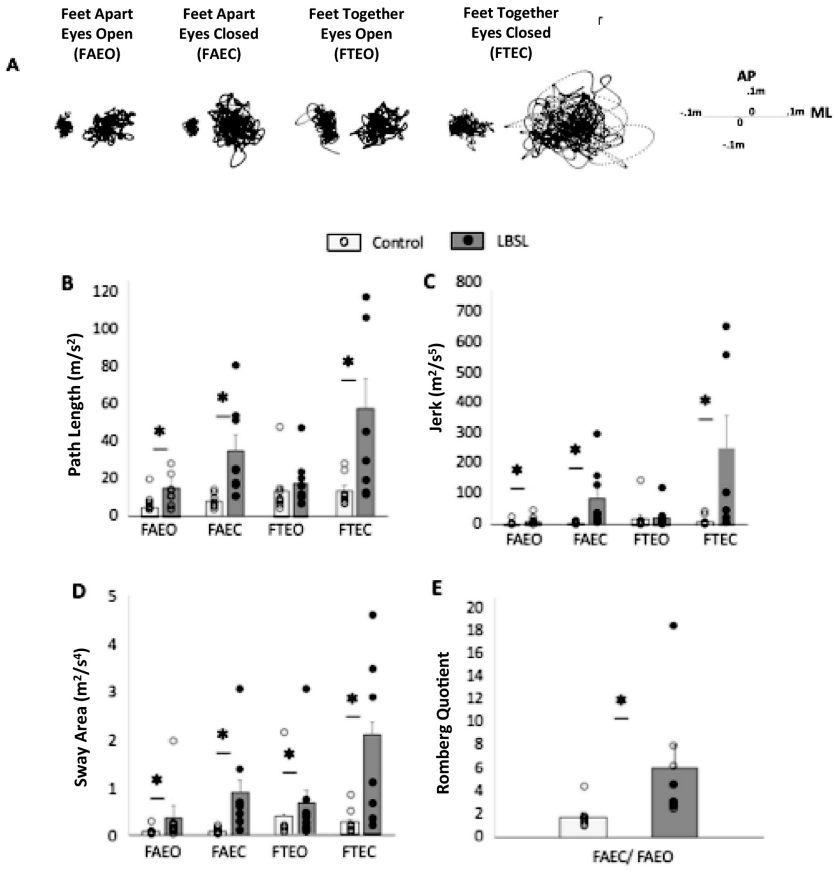

*Figure 5. (A) The mean sway paths of the control and LBSL cohorts are plotted across four postural sway conditions, feet apart eyes open (FAEO), feet apart eyes closed (FAEC), feet together eyes open (FTEO), and feet together eyes closed (FTEC). Trial duration is 30 sec. (B–D) Graphs represent the quantified mean path length (total length of the sway path in the anterior/posterior direction), jerk (smoothness of sway from the time derivative of the sway path in the anterior/posterior direction), and sway area (the area of an ellipse covering 95% of the sway angle in the coronal and sagittal planes) of the control and LBSL cohorts. (E) A Romberg Quotient was calculated for each individual as the sway area with the feet apart eyes closed divided by the sway area with the feet apart and eyes open. The graph represents the mean Romberg Quotient for the control and LBSL groups. *p < 0.01 control versus LBSL.*

Dr. Amena Smith Fine continues to tap into institutional resources that we can utilize for this project, including the expertise in the Motion Analysis Laboratory at Kennedy Krieger headed by our Chief Science Officer, Dr. Amy Bastian, the neuropsychology core services, the highly advanced FM Kirby Research Center for Functional Brain MRI, and our clinical trials unit. Finally, Dr. Fine applied for an NIH Career Development Award (for 2023) in June 2022, which would support her salary as a leukodystrophies researcher and some research expenses for the next 5 years. Dr. Fine had a strong percentile score and will receive feedback and an award decision by early 2023.

Dr. Fine and Dr. Mertz joined with Beth McGinn and Melody Kisor to share proceedings of a special workgroup focused on defining our current preparedness for advancing clinical trials in LBSL. They worked with a team of scientists and clinicians in the US and internationally, including Dr. Marjo van der Knaap, to mark progress and set new goals. They presented this work at the GLIA-CTN Annual Investigator's Meeting at Children's Hospital of Philadelphia in October 2022. It was suggested that the workgroup develop guidelines for clinical care management soon.

Importantly, we have partnered with Professor Marjo van der Knaap and Dr. Marc Engelen at UMC Amsterdam to begin a parallel study to evaluate sensory motor outcome measures in LBSL using the wearable OPAL system that Dr. Fine has been using. Our collaboration began in summer 2019, and their evaluation of the European LBSL cohort is ongoing. Eight patients have had MRI scans and accelerometry testing in their lab so far and are undergoing recurring visits. We have also partnered with researchers at the University of Helsinki to be another natural history site for pediatric patients in

Europe. They have received OPALs kits and are undergoing training with our team, to start enrollment in late 2022.

We are progressing in our neuroimaging research at the Institute using the recently developed advanced MRI protocols of the brain and spinal cord, which is critical to advance our neuromotor outcomes research. We have performed scans of six healthy volunteers and six LBSL patients to date. This provided important data and allowed us to optimize the protocol and decrease the scan time duration, which is ideal for our youngest participants to complete their studies.

New Clinical Exercise study:
With our research physical therapist, **Jennifer Keller, MS, PT,** we have developed an exercise intervention program to address balance and strength in LBSL, which will involve in-person PT training while participants are visiting us for their imaging study and a home exercise component using the wearables afterward. We now have IRB approval and are enrolling as of November 2022.

Machine learning:
In addition to the wearable technology, we have developed new MRI techniques that we would like to apply to LBSL. We have spent much time developing machine learning tools to generate a neural network that can automatically analyze imaging data from the spinal cord. This has been in collaboration with Dr. Unberath, in the Department of Computer Science at Johns Hopkins University. Dr. Unberath has trained our postdoc, **Dr. Bela Turk**, in machine learning and Python, and has also provided us with a free master's degree student this year to help with the effort. Dr. Turk has also trained Dr. Fine and research coordinator **Dan Amos** in these machine learning methods, to improve the efficiency of our data analysis.

Plans for the Upcoming Year
We are planning to approach the Food and Drug Administration for a Critical Path Innovation Meeting now that our manuscript about feasibility of using wearables for home assessment in LBSL has been published (Annals of Clinical and Translational Neurology, April 2022). We hope that this meeting can be conducted within the next 2 years. We hope that the new contact registry will allow us to enroll more patients at our site and at our European collaborator sites. We are also hoping to engage in a partnership with our colleagues in Brazil.

Summary:
We have made several new discoveries and believe that we are on the right path towards developing therapeutics that we can then push forwards towards clinical trials. Meanwhile, we need to continue our human studies to identify the right set of outcome measures for the conduction of clinical trials. We have established a network of collaborators including national and international partners now conducting both basic and clinical work in LBSL. While there is still a long way to go, we are optimistic that our work will lead to fruition of new therapies and clinical trials for LBSL.

Thank you again for supporting us and entrusting us with this noble work. Please contact Leslie Marsiglia for details or questions (Marsiglia@kennedykrieger.org).