Kennedy Krieger Institute
Research. Treatment. Education.

November 2020

Much has changed in the world since the last fall update- and for those of you who have received our Spring COVID-19 update, this document will encompass those updates as well. We certainly hope that this report finds everyone well despite this new world we live in. The current COVID19 pandemic has had the most profound impact on everything we do as human beings and unfortunately will continue to affect all of us for the coming year. I would like to thank you for your trust and the amazing contributions you have made to help our LBSL research program. Despite the pandemic we have made major progress over the last year, and we now have a better understanding of the disease process and are gearing towards therapeutic targets for LBSL.

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

IMPACT OF THE PANDEMIC AND OUR RESPONSE

Around the third week of February 2020, Kennedy Krieger Institute's senior leadership put into action a COVID19 task force in anticipation of an upcoming surge and we started to project the impact of the pandemic on our patients and operations. On March 16th 2020, the Governor of Maryland closed public schools which resulted in an immediate impact on all of our activities, employees and patients. In response to this decision, Johns Hopkins University stopped all trainee activities and all research operations aside from critical research related to COVID19. Like most hospitals, we incurred massive financial loss due to the cancelation of elective clinical appointments. Within 5 days we were able to implement a telemedicine-based approach, and by the beginning of April, we had ramped up telehealth visits to over 5000 visits per week at Kennedy Krieger, with only 50-80 patients per week coming to the Institute. Meanwhile, we had to dramatically restrict access to our inpatient neurorehabilitation unit which houses very fragile children. As of today, I am blessed to report that we have not had a single patient who contracted COVID19 at Kennedy Krieger.

Regarding research, we had to shut down all lab operations aside from animal colony maintenance, maintenance of critical cell lines (like the LBSL stem cells) and equipment maintenance. We furloughed half of our laboratory technician staff utilizing a work share program, that allowed each technician to work half time for the essential functions mentioned above, and receive unemployment benefits for the other 50% of their time through the CARES Act. On June 15th, we returned to the research laboratories on a more limited basis with strict infection control and social distancing rules in place. Since then, our technicians have returned to full time though strict infection control and distancing are still in place.

I would like to thank my team members for their dedication and commitment to research despite the global pandemic. I am especially grateful for the efforts of **Dr. Christina Nemeth Mertz** and **Dr. Amena Smith Fine** who lead our basic research and clinical research efforts, respectively. They are both outstanding and highly talented young investigators supervising the other staff members.

PROGRESS AND CURRENT STATE OF RESEARCH

Overall, we have made significant progress within this one year in understanding disease mechanism and have started to explore a new avenue of targeted therapy using an Antisense Oligonucleotide (ASO) strategy. Please find a summary of our progress and upcoming plans.

1. Animal Studies

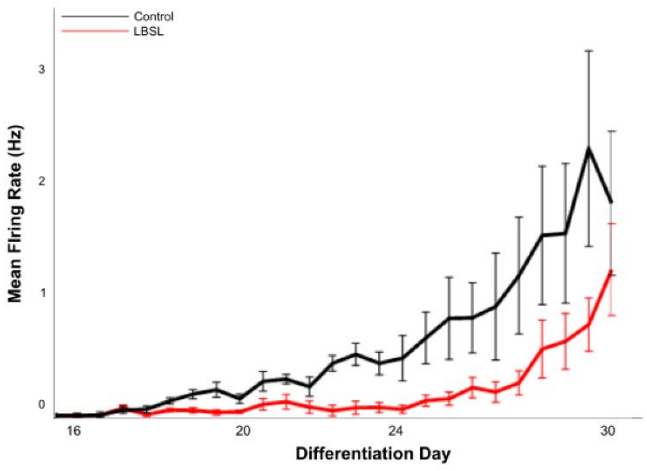
As a reminder, though a collaboration with a German group we developed a mouse model where neurons in the brain are missing the DARS2 gene (conditional neuronal knockout, referred to as "CamKII-DARS2 KO") and showed that these mice develop dramatic brain atrophy and severe behavioral abnormalities. This work was recently published in a prestigious journal called *Experimental Neurology*. We believe that the mouse animal model will be essential moving forward both for further understanding disease mechanism and also to test potential therapeutics before moving to human trials.

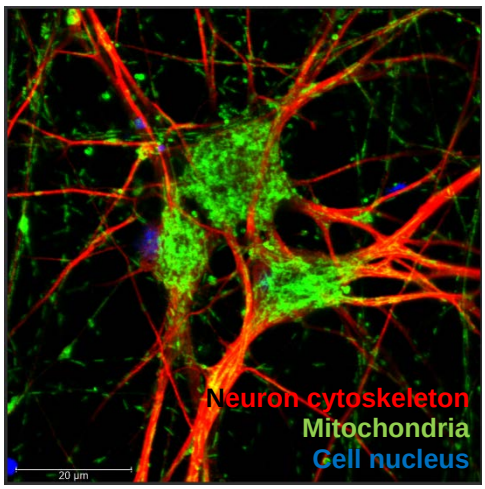
We have so far tested this model to assess the effect of two different novel drugs. One drug was a potent antioxidant delivered selectively using a nanodendrimer platform and it did not have any clear effect on the animals. The second drug, a so called ISR inhibitor (detailed in last fall's update), was also unsuccessful. In fact, we halted the second drug study as many of the treated mice appeared worse when getting treated. Understanding why the ISR inhibitor made the animals worse will likely give us new clues as to how we can reverse the deficits seen in the model. We plan to do more studies investigating the different molecular pathways involved to understand these mechanisms not only in mice but also in patient derived cells.

In summary, we believe that the animal model is very helpful for testing other therapeutics and also as a platform to test gene therapy. While both drug trials have failed so far, we continue to breed these animals and manage a large colony of mice and are planning to test other new therapeutics.

2. Cell Studies

As a reminder, we collaborated with a stem cell expert, Dr. Mingyao Ying at Kennedy Krieger, 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 continue to maintain in the petri dish (pictured below). In summary, we established a nerve cell culture model for several LBSL patients and are comparing those to control cells. Last year, we had done work on a single LBSL cell line and one control cell line. We have made significant progress along the study of these cells and have shifted a majority of our efforts towards these cell culture studies.





Left: The average firing rate of neurons shows LBSL cells to have a lower rate at each time point.
Right: Stained neurons derived from patient and control iPSCs.

We are now happy to report that we have 6 patient iPSC lines and 4 control cell lines. We can measure certain aspects of cell development, growth, and function in these cells and worked through the COVID shutdown to develop a Python based Artificial Intelligence (AI) computer code capable of analyzing some of these complex cell metrics. This work was enabled through a collaboration with Dr.

Mathias Unberath, a machine learning expert at Johns Hopkins School of Engineering. We use this AI tool to track individual cells, of which there are a few hundred thousand in each petri dish, and over multiple days' worth of photos to gain valuable information about their health and activity. We also use a different system to observe the effects of patient-specific mutations on neuronal cell firing. The ability and frequency of neuronal firing dictates their entire ability to function and this machine enables us to look at the similarities and differences of our patient mutations on functions. While this work has been done on our initial cell lines, we must now transform our newly received lines and run them through these same tests. These experiments will take some time, but we have established the methods and the expertise to complete the work. We are excited as these platforms are allowing us to understand what the consequence of the gene mutation is on nerve cell function, and are perfectly suited to test therapeutics in the dish.

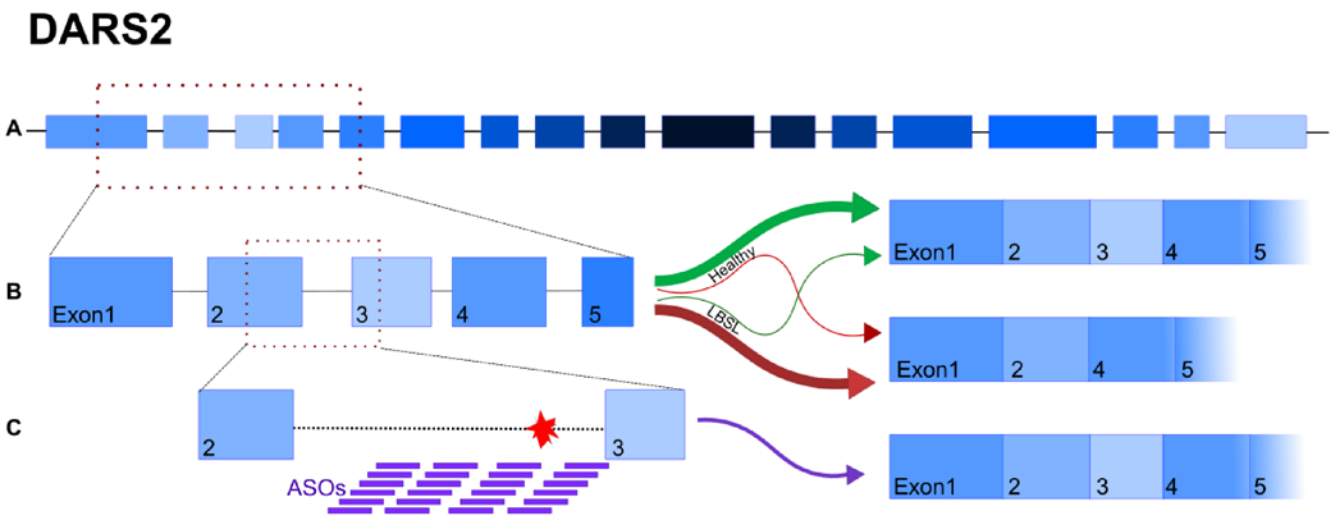
More recently, through another collaboration with a Johns Hopkins School of Medicine investigator, Dr. Anne Hamacher Brady, who studies mitochondria and mitochondrial signaling using advanced imaging techniques, we are hoping to highlight novel functional differences related to mitochondrial activity in these cells. This technique will also be useful for determining which cell processes are most affected by therapeutics.

Analysis of mitochondrial proteome: With the help of **Dr. Stephen Fried**, a quantum biologist at Johns Hopkins who is also a distinguished NIH new innovator award recipient, we conducted a full analysis of the mitochondrial proteome (exhaustive analysis of proteins within the cells' mitochondria) in one cell line and we will prepare the other cells lines for this same analysis. To our great surprise, in the examined cell line, there was no misspelling in the amino acid sequence of mitochondrially encoded proteins, this means that despite the DARS2 mutation which in theory affects the availability of Aspartate-tRNA, there is still sufficient tRNA to allow the addition of aspartate during protein synthesis. However, we do see that nearly 100 mitochondrial proteins are altered in LBSL. We must now repeat this experiment with our other cell lines. If these findings hold with repeated experimentation, it would suggest that we may be able to target abnormalities in the mitochondria further downstream from the actual mutated gene.

Cerebral Organoids (minibrains): With the help of Dr. Mingyao Ying's group here at Kennedy Krieger, we have also established our own in-house cerebral organoid protocol. Combining multiple other protocols, our talented student, Shiqi Guang, has developed a new protocol for generating brain organoids that contain neurons, myelin producing cells, as well as inflammatory cells. We plan to do a high-throughput analysis of these organoids to determine a cell-specificity of dysfunction. Because organoids can take several months to grow, we deemed these experiments too essential to halt during COVID. Although still too preliminary, there may be some histological differences visible in LBSL organoids. These organoids are important as they contain multiple cell types which will give us insight into the cell type-specific activities and/or dysfunctions in LBSL.

As a next step for both our cells and minibrains, we need to now start understanding mitochondrial function in LBSL at a more molecular level to truly determine what is happening pathophysiologically. I am happy to report that Dr. Joseph Scafidi, a renowned physician scientist, will be joining the Kennedy Krieger Institute at the end of 2020. Dr. Scafidi has been studying mitochondrial energy consumption using a system called the seahorse assay. This assay allows us to directly measure mitochondrial energetics and Dr. Scafidi is an expert in this area currently funded by the NIH to investigate mitochondria in other neurologic conditions. We intend to study all LBSL neurons and minibrains using this methodology next year once Dr. Scafidi has joined us. We should be able to complete these mitochondrial function studies by Fall of 2021.

Antisense Oligonucleotides: We have begun to closely examine antisense oligonucleotides (ASO) - short pieces of RNA that can be used to "hide" the DARS2 mutation in cells (see figure below). This technique is currently being used in Spinal Muscular Atrophy and is being tested for the rare Batten disease. In order to find a specific sequence of RNA that may have an effect, often hundreds of different ASOs need to be tested, and just before COVID, we began to test a handful of these sequences at a time. We are working with a Johns Hopkins RNA biologist, Dr. Shuying Sun, who is helping us to design and determine efficacy of these short RNAs in our cells. We will need to design hundreds of ASOs and test them to determine if any are effective in restoring the expression of the normal gene product.



A) DARS2 is made up of 17 coding regions (blue boxes). The most common mutation in LBSL is found right before the third coding region, or Exon 3. This mutation might contribute to disease symptoms. B) In healthy people, this site commonly produces an abnormal protein (red arrows) in addition to the normal full length protein (green arrows). In LBSL, a mutation here might further reduce the amount of healthy protein, and for patients without this mutation, mutations elsewhere in the gene likely still compromise the production of healthy protein thus making proper protein production at this junction more important. C) Antisense oligonucleotides (ASO, purple) can find this location, bind to the non-coding regions, and "hide" the mutation, allowing for protein machinery to read a corrected sequence and produce the full protein. We must test many ASOs to find one that works.

3. Human Studies

Human subject research has been hit very hard by COVID and onsite non-interventional human research was on hold until mid-Summer 2020. We have currently a petition under review to resume research in a safe manner and expect approval in 2 weeks. Meanwhile, we have been able to continue great aspects of our work through remote assessments.

Importantly, we were able to successfully retain Dr. Amena Smith Fine to stay at Kennedy Krieger as she has graduated from her Neurodevelopmental Disability Residency training. She has been promoted as an Assistant Professor of Neurology and will see patients at the Moser Center and spend the next few years focusing on LBSL research. The human studies need to continue. We currently have many 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. We have easy access to these resources and can utilize them for this project.

Importantly, we have partnered with Dr. Marjo van der Knaap and Dr. Marc Engelen at VUMC Amsterdam to begin a parallel study to evaluate sensory motor outcome measures in LBSL using the wearable OPAL system that Dr. Smith Fine has been using. Our collaboration began in summer 2019, and they now have an approved ethics committee protocol which replicates our study for subjects older than 16. We have completed remote training in our research methods with their group and have used the funding we have received here to purchase the wearable OPAL systems for them. We're excited to report that the evaluation of the European LBSL cohort has begun!

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 a lot of time developing machine learning tools to develop a neural network that can automatically analyze imaging data from the spinal cord. This has been in collaboration with Dr. Unberath. 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 for this year to help with the effort. Together, we have recruited a PhD student who just began her studies remotely, but is currently constrained in Colombia due to the pandemic but will hopefully join us in a few months.

We scanned our first LBSL patient and several healthy volunteers in February 2020 just before clinical research was suspended at the Institute due to COVID restrictions. When we are able to resume in-person research we will scan local patients and plan to scan more patients and controls as part of future LBSL family meetings. We are also planning to approach the Food and Drug Administration for a Critical Path Innovation Meeting once our manuscript is published.

Summary:
We have made several new discoveries and believe that we are on the right path towards identifying therapeutics that we can then push forward 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.

Thank you again for supporting us and entrusting us with this noble work. We look forward to seeing everyone during our Virtual Family Conference to be held November 14-16, 2020 where we will further discuss these projects. Please contact Leslie Marsiglia for details (Marsiglia@kennedykrieger.org).