Kennedy Krieger Institute
Research. Treatment. Education.

10/13/2023

To: Beth & Mike McGinn
CureLBSL Foundation
3520 S. Wakefield Street
Arlington, MD 22206

Re: LBSL RESEARCH PROGRESS REPORT
(Period: 4/1/2023-10/1/2023)

Dear Beth,

We are delighted to provide you our twelve-month progress report on the Moser Center's LBSL Research Program. It has been a busy six months and I am pleased to say that we are well on track despite a few hiccups. As with most things, science does not always go as planned and we have encountered some difficulty with cells that we will discuss below. In spite of this, we have made exciting progress beyond what was anticipated and have gotten some extremely encouraging results from our gene therapy and ASO experiments.

In summary, 1) we show that AAV9 injected into the brain ventricles improves behavioral function of our previously reported mouse model; 2) we have developed a new spinal cord mouse model in addition and are testing the therapy in this new model as well; 2) we show that ASO therapy improves neuronal function in the dish and is taken up by neurons in the brain when injected into the nervous system, 3) we have patented these technologies and are exploring with Johns Hopkins Technology transfer several options of finding a commercial path towards clinical development of these therapeutics. Meanwhile, we continue to make significant progress on our clinical research collaborations to obtain data needed to design the right clinical trials in LBSL. I am extremely grateful for our amazing research team's hard work and your continuous support.

Please find below a list of our Milestones for the 6-to-12 month period:

MILESTONE 1: GENE THERAPY PROJECT

a. *Compete efficacy studies in 3 LBSL and 3 isogenic cell lines assessing the effect of AAV9 on DARS2 gene expression, dendrite growth.*

We continue to get promising results using the *DARS2*-AAV9 on our patient neurons, and proper gene transcripts are produced and **function (neurite outgrowth) is improved** (Figure 1).

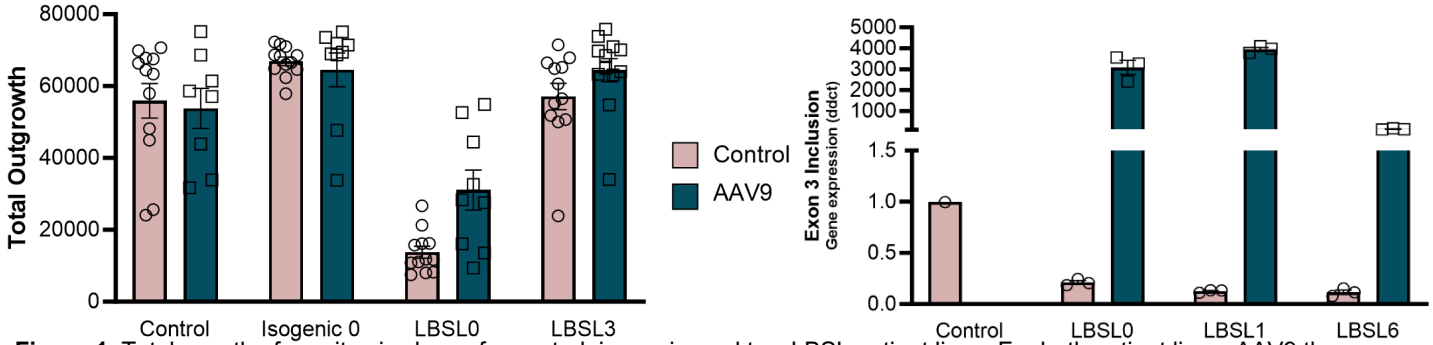


Figure 1. Total growth of neurites is shown for control, isogenic, and two LBSL patient lines. For both patient lines, AAV9 therapy improves growth by ten of treatment (data shown here). Similarly, mature LBSL neurons show less inclusion of exon 3 within DARS2 transcripts compared to control. Treatment with AAV9 significantly increases the number of transcripts including exon 3, relative to control.

Challenge with isogenic cell lines: For full transparency, I would like to discuss this issue first. The isogenic controls are our patient lines with the LBSL mutations corrected using CRISPR. This work was completed by Applied Stem Cell, a reputable company in the field. We and others have noted our isogenic controls to at times have an odd cellular morphology and to not behave (in terms of gene expression or outgrowth) as healthy controls. In assays, these cells have more closely resembled patient lines. We are in the process of sequencing these cells, and have contacted the company who grew new frozen stocks of our cells to test for growth, free of charge. Isogenic controls are pivotal to demonstrate reliability of our research and so repairing these cells is also pivotal. While this has caused some delays, we are still highly encouraged that LBSL lines clearly grown more neurites with gene therapy when compared to the healthy control and compared to untreated cells. When this work is complete, we will be able to more reliably measure patient lines compared to isogenic controls across several assays. Because of this issue, the experiments below used healthy control cells, or compare treated to untreated patient cells.

b. Complete pilot biodistribution studies for AAV9 in mice.

CamKII-*Dars2* knock-out mice that exhibit a severe neuronal phenotype were given *DARS2*-AAV9* at 8 weeks of age, through an intracerebroventricular injection (injection through the skull, into the brain ventricle where the AAV9 can be circulated by cerebrospinal fluid). As you know, AAV9 is used as a vehicle to deliver full-length DARS2 to cells within the central nervous system. We have started preliminary analysis of the biodistribution of *DARS2*-AAV9, and while this work is ongoing, after 8 months, we observe cells that express a green fluorescent protein (indicating *DARS2* integration) in multiple areas of the brain. Importantly, we only injected the AAV9 into one side of the brain, and are able to observe expression on both sides, and densely within the cortex (Figure 2). Based on the morphology of these cells, we believe them to be neurons, however future analyses will verify cell types. In Figure 2, green indicates AAV9 expression.

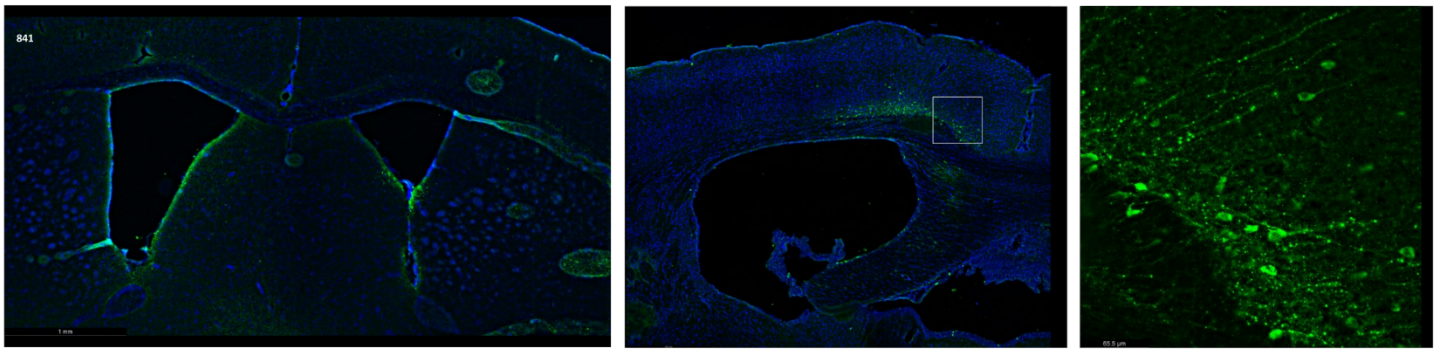


Figure 2. Anterior regions of the brain show green signal in both ventricles, indicating circulation of the virus (left). In more posterior sections, green signal can be observed within the cortex, an area in which neurons are lost within this model (middle image). A higher magnification image in this region (right) reveals green signal to be within neurons.

The next steps for this project include analysis of cortical thickness, to determine if gene therapy was sufficient to reduce neuronal death in the cortex. Secondly, we have designed probes to identify where *DARS2* transcripts exist in the brain, and which originated from mouse and which originated from the delivered (carrying human *DARS2*) AAV9. These results will help us understand how much *DARS2* transcript needs to be present in order for a functional change to occur.

**Dars2* refers to the mouse gene, while capital, *DARS2* refers to the human gene, which was delivered via AAV9.

c. Com*plete pilot studies on effect of AAV9 on animal behavior in mice*

As published in 2020, CamKII-*Dars2* knock-out mice show a behavioral change at around five months of age. When given AAV9 carrying human *DARS2*, we show that behavior is improved from untreated mice (Figure 3). The dose given was based on literature values and was administered as a one-time-only treatment. While this therapy did not reverse the behavioral phenotype completely, **these results show clearly that AAV9 treated**

animals are less severely affected and we will validate these results in future experiments and may achieve full recovery using different drug dosing/route.

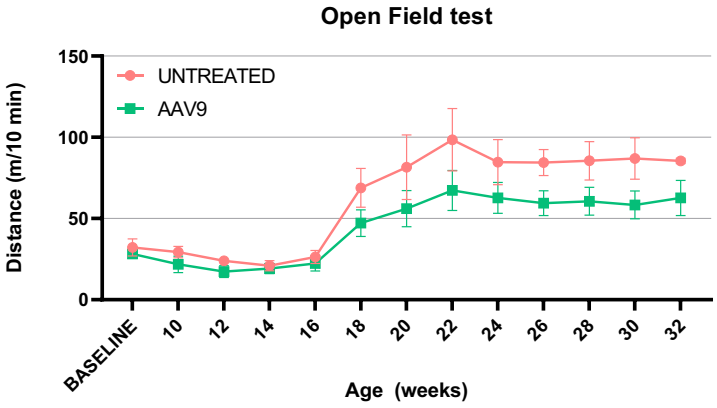


Figure 3. All mice included in this experiment were knock-out animals. Some received treatment (*DARS2*-AAV9 to the brain) and some received saline. Activity was measured over a 10-minute period every other week for six months. A clear difference can be seen between animals receiving treatment and those that did not. The red, untreated, line in Figure 3 very much resembles the degree of behavioral change we observed with these mice in previous experiments (published 2020).

A NEW LBSL MOUSE MODEL

During this time we have also made significant progress on a new LBSL mouse model. This model has reduced *Dars2* within the dorsal root ganglia, or the populations of sensory neurons that are located just beyond the spinal cord. These mice, as discussed in the previous update, show a significant motor phenotype, characterized by hind limb dysfunction and smaller size. When the distance between their front or hind legs is measured, they show a wider stance (referred to as base of support, BOS) compared to wild-type mice (see Figure 4).

We administered *DARS2*-AAV9 to these mice, intrathecally, as a single injection, when they were either 5 or 7 days old. At one month of age, we tested the gait of these mice and found their BOS to be less variable (Figure 4), and more similar to control than untreated mice. Similarly, their weight loss was less significant, and again, more closely resembled control than untreated. These data again suggest that replacement with full-length, healthy *DARS2* can rescue the phenotype caused by constitutive *Dars2* deficiency. We have harvested tissue from these mice, and analysis is ongoing.

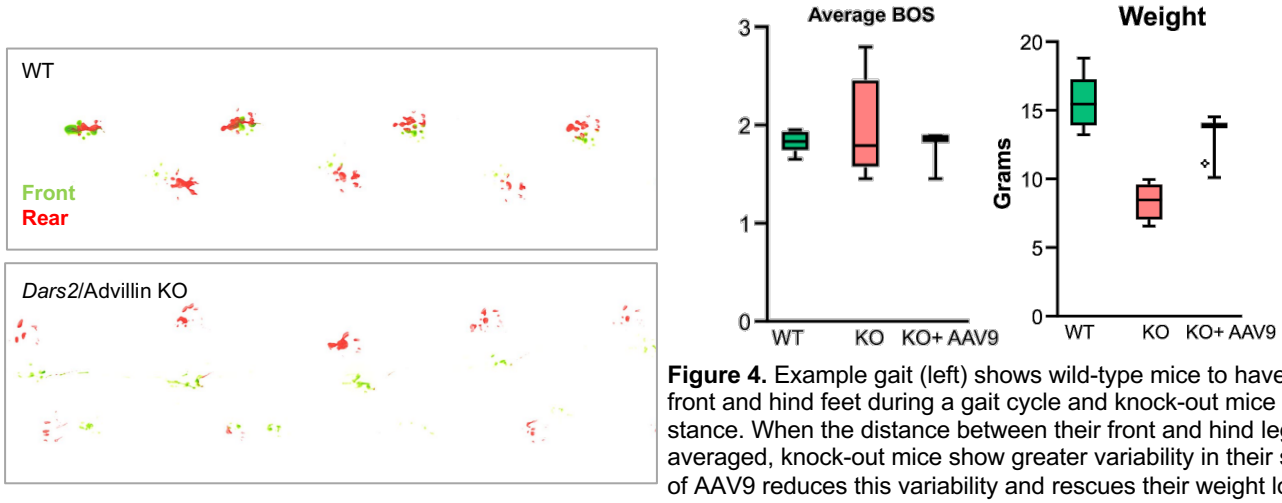


Figure 4. Example gait (left) shows wild-type mice to have overlapping front and hind feet during a gait cycle and knock-out mice have a wider stance. When the distance between their front and hind legs is averaged, knock-out mice show greater variability in their step. Delivery of AAV9 reduces this variability and rescues their weight loss.

MILESTONE 2: ASO PROJECT

a. *Complete efficacy studies in assessing the effect of ASO on patient neurons in several LBSL cell lines assessing gene expression, dendrite growth and mitochondrial activity.*

We have previously demonstrated the effects of ASO therapy on gene expression, specifically showing that ASO can increase in exon 3 inclusion within DARS2 transcripts. **New data show functional improvements in neurons as a result of ASO therapy in terms of mitochondrial function as measured by the Seahorse (Figure 5), and by neurite outgrowth (Figure 6). Neurite outgrowth data were collected using the new**

high content screener funded by you. Additionally, we show successful uptake of ASO into neurons when plated in a dish (Figure 7).

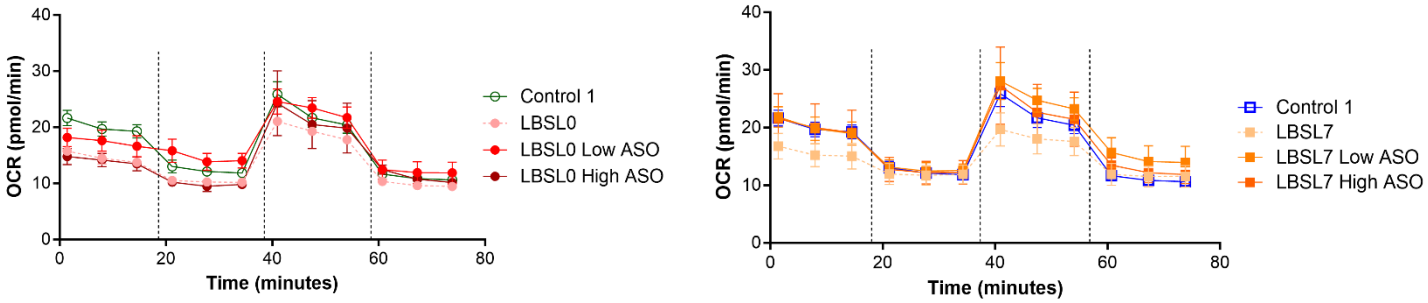


Figure 5. The oxygen consumption rate (OCR) was measured in several stages during a 'mitochondrial stress test'. Data from time zero to 20 minutes indicate baseline respiration of cells. In both cell lines shown above, the dotted patient lines show lower baseline mitochondrial activity compared to ASO treated and control. From minutes 40-60, the cells are pushed to their maximum respiratory capacity and again, treated patient cells show higher levels of respiration compared to untreated cells.

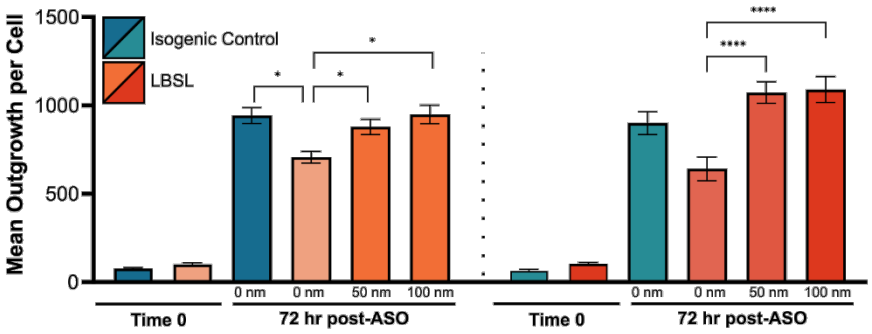


Figure 6. The average outgrowth (neurites, or processes from neurons that help their growth and functioning) is shown over a period of several days. Time zero demonstrates that early after cells are placed into dishes, their arborization or morphology is similar. After 5 days in culture, and 72 hours after ASO treatment, it is shown that untreated (0 nm ASO) patient cells have the fewest or shortest outgrowth and treated patient lines resemble isogenic control levels.

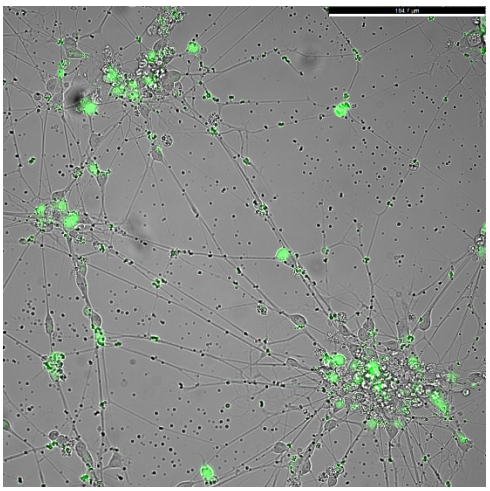


Figure 7. We imaged neurons that were treated with a fluorescently-tagged ASO. This ASO was also tested in functional studies to ensure that the tag does not interfere with normal function of the ASO. Still, the tag is for uptake studies only. This image was taken hours after treatment, and uptake is rapid. Future testing will examine ASO within neurons after the neuronal medium (liquid in which they live) has been refreshed.

b. Complete pilot biodistribution studies for ASO in mice

In adult wild-type mice, we administered ASO either to the brain (intracerebroventricular), or to the spinal cord (intrathecal) and observed distribution after 6 days. From these mice, we collected liver, kidney, heart, brain, spinal cord, and blood. Preliminary data show that the ASO is taken up by neurons within the brain when the ASO is delivered to the brain (Figure 8, top). When delivered to the spinal cord, we see some signal within the brain and expect that repeated dosing would result in greater observation of the ASO signal within this tissue (Figure 8, bottom). Higher magnification imaging of the ASO within regions of the brain of intracerebroventricular injected mice show that the ASO (green) overlaps with the neuron (red) signal (Figure 9). To check, we also identified astrocytes, an abundant and important cell within the brain, however we did not

observe much overlap in signal (pink) with ASO. **We were pleased to observe the neuronal uptake, as we believe that neurons in LBSL are the most susceptible to DARS2 deficiency.**

Spinal cords are in the process of being 'cleared' or turned translucent and stained with antibodies to identify cell types. This technique allows us to keep the spinal cord whole and use a special microscope called a light-sheet microscope to see where the ASO has deposited in 3D without artifacts of tissue sectioning. This process is lengthy, so these data will be available for the next update. Importantly, no signal is observed within the kidney or liver (see Figure 10 and 11, respectively), blood serum analysis came back negative, and analysis of heart tissue distribution and toxicity is ongoing.

c. *Engage GMP manufacturer for large scale clinical grade ASO production for human trials*

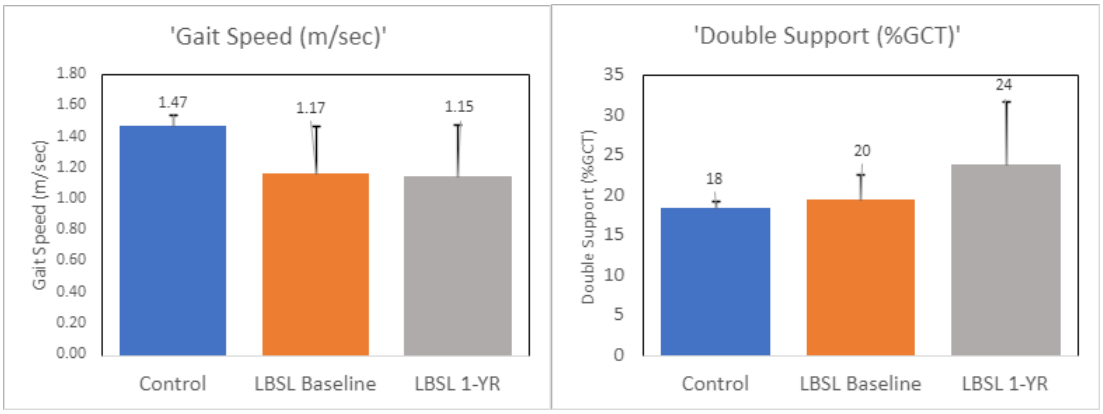
We have engaged a GMP manufacturer who has provided a cost quote for synthesizing GMP manufactured ASO product for us. This will be necessary once we complete the first round of toxicity studies of our ASO. The FDA will likely require us to test the GMP manufactured products in two species before giving it to patients. We hope that these efforts can be conducted on the commercial side as we have patented our products and are currently searching for a company to license the technology and move forward towards clinical development.

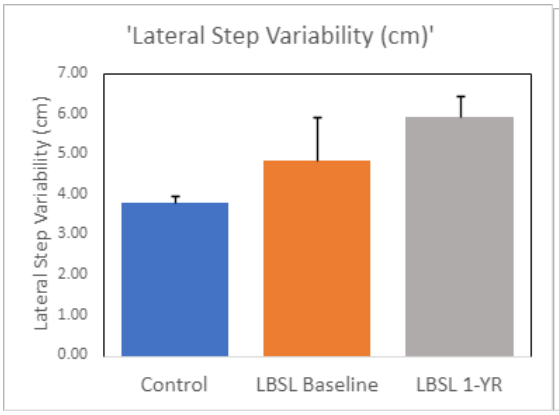
MILESTONE 3: CLINICAL RESEARCH

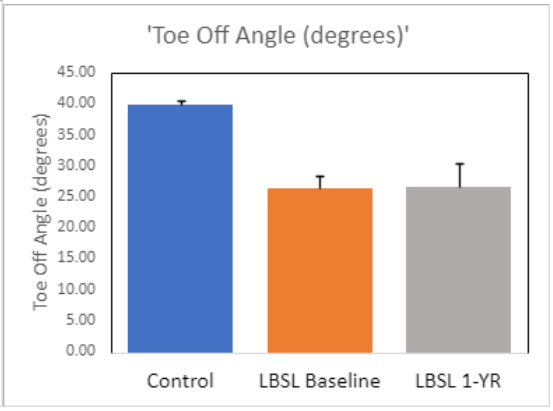
a. *Complete interim analysis of follow up data from US and Netherlands (AMC)*

1 year follow-up data from a subset of LBSL patients in the Netherlands has been transferred and analysis is in process. The Netherlands group has also collected a large dataset of healthy control data for comparison of performance on the walking and balance tests. The series of graphs below represent control and patient performance and on the 6MWT (6-minute walk test, fast-as-possible pace), the Timed Up and Go Test, and 4 standing balance tests (Feet Apart Eyes Open, Feet Apart Eyes Closed, Feet Together, Eyes Open, Feet Together Eyes Closed).

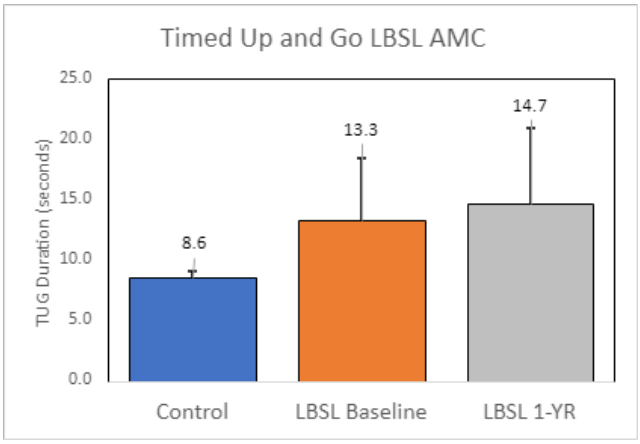
6-minute Walk Test. AMC LBSL patient gait speed was decreased by a large, clinically meaningful margin (0.3m/s) at baseline compared to age- and sex-matched healthy controls. However, after 1 year, LBSL gait speed did not decline further relative to baseline. Double Support (the percentage of time spent during the gait cycle with both feet on the ground) was increased in LBSL vs. Controls at baseline, and further increased after 1 year, indicating a decline in gait stability. Similarly, the lateral step variability, a proxy of gait ataxia/variable stepping pattern, further increased during the interim. The toe off angle is the angle of the foot during step off as a stride is initiated, and a smaller angle can be seen in association with leg spasticity, weakness or impaired proprioception. The toe off angle was significantly decreased in the LBSL cohort by 15%, and this change was stable after 1 year.



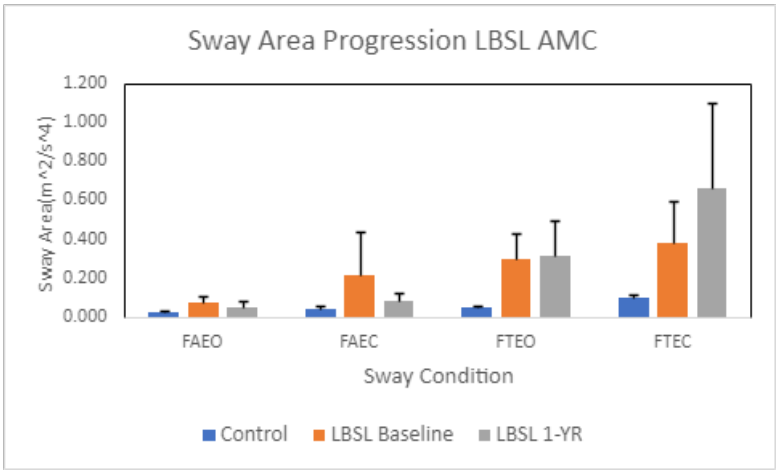




Timed Up and Go Test. In this fast-as-possible 3 meter walk test, participants rise from a chair, walk, turn and sit down. The TUG duration (seconds) was significantly increased for LBSL participants, with a further minimal increase after 1 year.



Sway Tests. Similar to our findings in the KKI LBSL cohort, the sway area during a 30-second standing balance test was greatest in the feet together, eyes closed (FTEC) condition. Although not statistically significant, there is a trend of further increased sway in the FTEC condition after 1 year, and the mean sway area is 6-fold larger than the age and sex-matched control sway area. **Importantly, these results bear promise that sway with feet together, eyes closed maybe an early indicator of disease progression and could be used as an outcome measure in a trial.**



b. Start obtaining data from Finland

Our research collaborators at the University of Helsinki (PI Dr. Emil Ylikallio) have been enrolling patients since Spring 2023, 4 subjects have been enrolled and tested so far, with continued interest generated through social

media and their clinical practice. Our data transfer agreement is pending, and we anticipate sharing interim analysis results as part of the Spring 2024-6 month report.

Our newest research collaboration at the Hospital Pequeno Principe in Curitiba, Brazil (PI Dr. Josiane Souza) is in progress. Final site IRB edits were made in October 2023. 7 interested patients have been engaged through clinicians working with Dr. Souza or by CureLBSL social media and contact registry efforts. 2 OPALs kits have already been purchased and are being stored in the U.S. until we determine a vendor to facilitate the most secure, cost-effective means to deliver the equipment to our partners, anticipated by the end of October 2023.

c. *Initial engagement with FDA regarding a Patient-Focused Drug Development (PFDD) vs listening session*

We are excited to announce that a very successful FDA patient listening session was held on 5/31/2023. This is thanks to the excellent efforts of the CureLBSL Team, and Melody Kisor in particular for hard work to engage the patients and families and capture their voices in such a compelling format. Dr. Amena Smith Fine provided a clinical overview and has advised Melody on the post-meeting FDA executive summary report. The full known spectrum of LBSL was represented (infantile, juvenile, and adult onset), the signs/symptoms, and the impacts on patients/families through a series of interviews, brief videos and educational slides. The concluding question was "What is the primary symptom you're looking to address?" (Answers: Balance, stability, seizures, breathing, weight management). Attendance was high, and FDA participants included representatives from the Office of the Commissioner, Center for Biologics Evaluation and Research, Center for Devices and Radiological Health, Center for Drug Evaluation and Research, and many non-FDA attendees from foundations including the NIH/NCATS (National Center for Advancing Translational Sciences). We are meeting with CureLBSL 10/20/23 to start planning engagement for Critical Path Innovation (CPIM) and PFDD meetings in summer or fall 2024.

d. *Identify regulatory consultant for FDA IND filing*

We are considering a short list of consultants and determining an appropriate budget for these next steps. Current plans for the PFDD are that this meeting would be spearheaded by Cure LBSL (perhaps jointly with NORD), with invited clinical speakers from Kennedy Krieger and Amsterdam. NORD is preparing a quote for PFDD planning services and communications. The Critical Path Innovation meeting will be led by our research team at KKI with support from Cure LBSL. We are gathering information on consultants who are experienced in working rare disease patient advocacy groups. Next meeting with CureLBSL 10/20/23.

Requested Funding for 4/1/23-9/30/23 period:
$636,000 Salaries and Supplies (for 6 months)
Total: $636,000 direct costs

In order to continue our research we request that you please donate the above requested amount to our program.

Payment Instructions:
Checks should be made payable to: **Kennedy Krieger Foundation** and sent to the attention of:

Leslie A. Marsiglia
Office of Philanthropy
707 North Broadway
Baltimore, Maryland 21205
Phone: 443-923-2750, email: fatemi@kennedykrieger.org

Thank you again for all your support and your trust in our work. We are eager to continue our important work and are inspired by your dedication and commitment to find a cure for LBSL.

Sincerely,

Ali Fatemi, MD, MBA

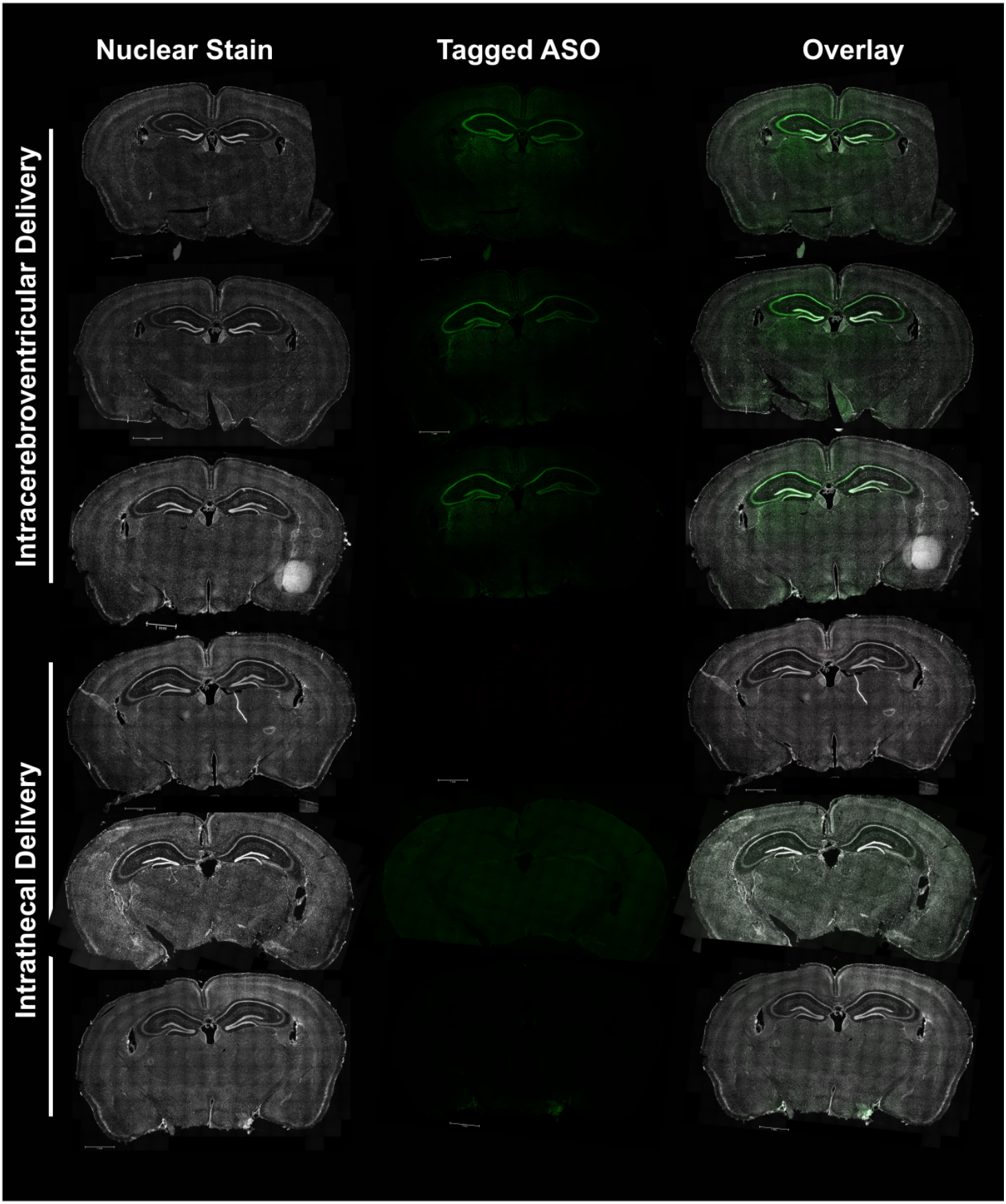


Figure 8. Brain sections from six wild-type mice are shown, three with brain injections (top) and three with spinal cord injections (bottom). Images on the left show brain structures (level of the hippocampus) using a stain for the cell nucleus. The middle column shows the green signal of ASO, and the third column is the overlay.

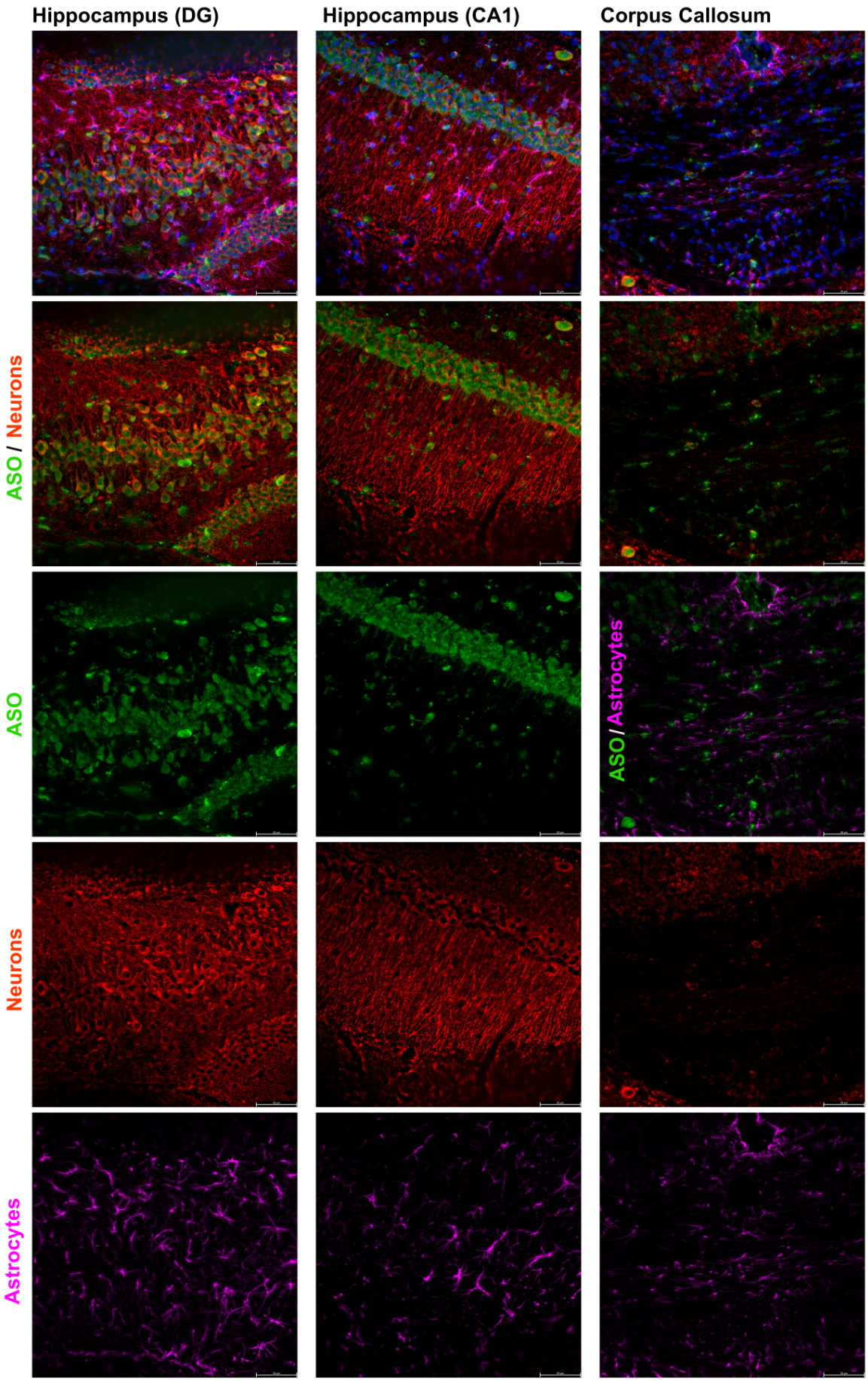


Figure 9. Higher magnification images of different brain regions show ASO (green) to overlap with neurons (red).

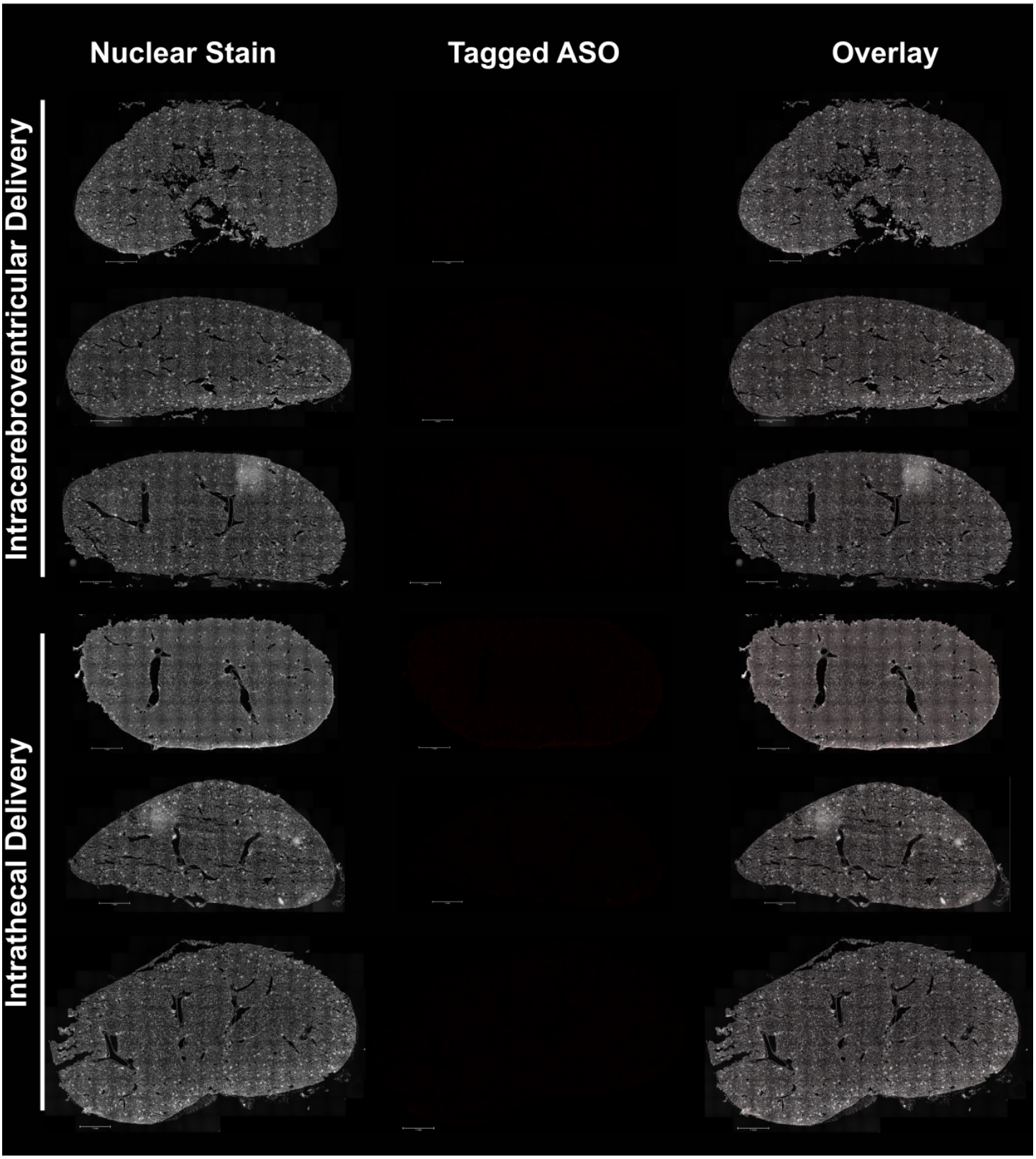


Figure 10. Kidneys from the same six ASO injected animals are shown and no green (ASO) signal is observed within these tissues. General morphology of the kidney looks normal. Blood tests were sent to test for kidney function and were returned normal; however validation of these data are necessary.

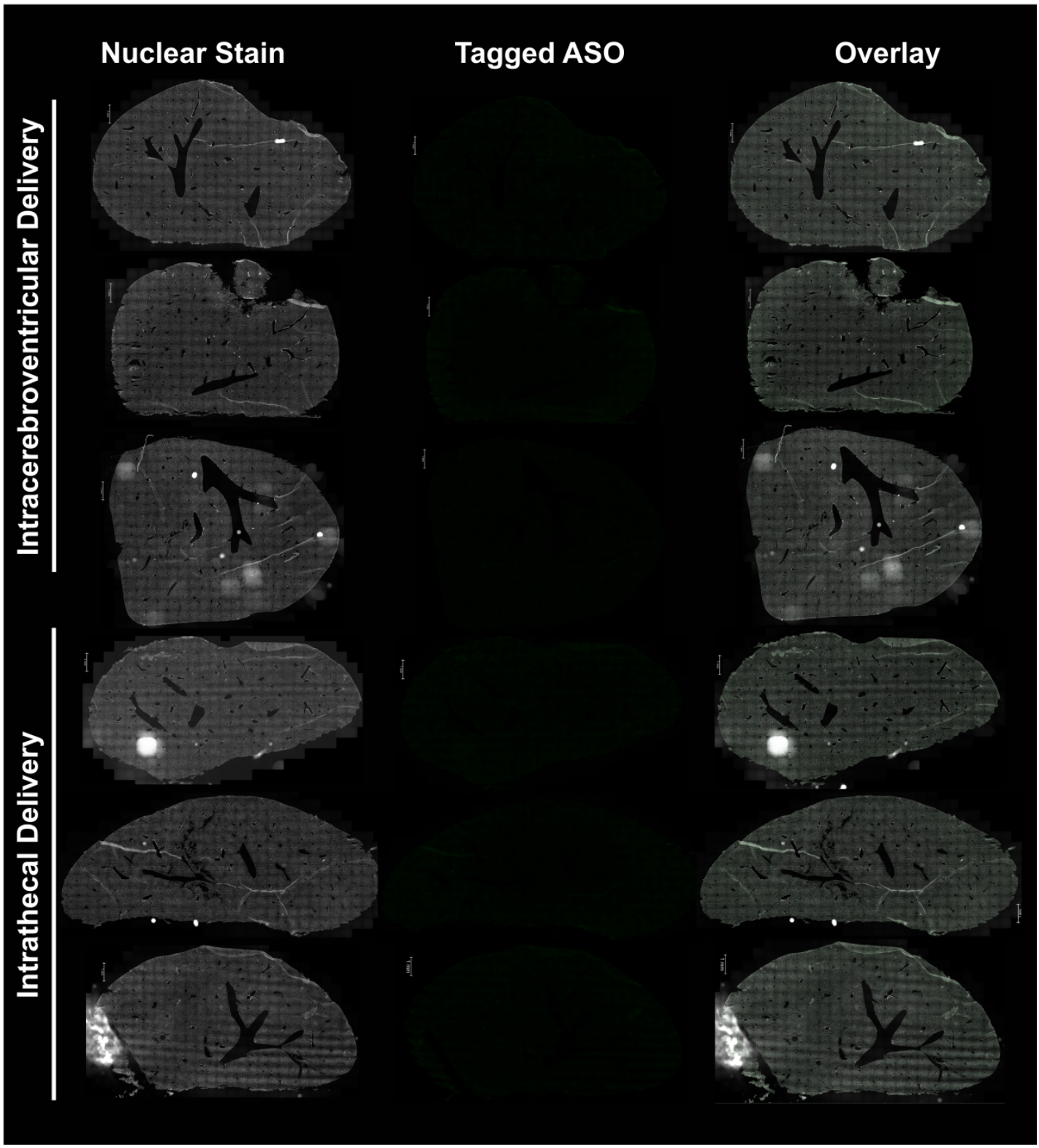


Figure 11. Livers from the same six ASO injected animals are shown and no green (ASO) signal is observed within these tissues. General morphology of the liver looks normal.