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Study Title: Frataxin mRNA in Biofluids

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1. Project title: Measurement of Frataxin mRNA in NHP and human biofluids

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3. Abstract: Friedreich ataxia (FRDA) is the most common form of inherited ataxia. It is recessively inherited and is caused by a nucleotide repeat expansion in the FXN gene that leads to reduction in the amount of frataxin protein. Frataxin deficiency leads to mitochondrial dysfunction, impaired energy production and excess oxidative stress. Novel strategies are being developed to enhance frataxin levels in these patients as a therapeutic strategy including gene replacement, CRISPR based interventions and synthetic transcription factors. There is urgent need for biomarkers that can reflect the pathophysiology of the disease and document target engagement and reversal the pathophysiology with such treatments. In this study, we plan to identify and quantitate frataxin mRNA in CSF and plasma derived exosomes in human patients with FRDA. These human studies will be preceded by similar studies to quantitate human FXN mRNA and cynomolgus FXN mRNA from plasma- and CSF-derived exosomes and other tissues from cynomolgus previously administered human FXN by AAV9 vector by intravenous and intrathecal routes. We will perform transcriptome profiling of mRNAs derived from these biofluids to provide baseline information needed to subsequently develop targeted assays that measure FXN mRNA levels relative to reference mRNAs.

4. Background

   Friedreich ataxia (FRDA) is a rare, autosomal recessive disorder which causes progressive imbalance, coordination problems, speech difficulties and eventual need for assistive devices for mobility and often, early mortality. The disorder typically has onset in early teen years with progression to a wheelchair bound status in the 3rd or 4th decade but some will have onset later in life. A substantial proportion of patients have a hypertrophic cardiomyopathy that can lead to heart failure and arrhythmias. The disease is caused by an unstable, biallelic expansion of a repetitive GAA tract in the *FXN* gene, though rarely, some patients have an expansion in one allele and a conventional mutation in the other. Over the last 20 years, we have documented that the protein, frataxin, has a mitochondrial function and the loss of this leads to impaired ATP synthesis and generation of oxidative stress that lead to the manifestations of the disease. Age at onset, frataxin levels and likely disease progression rate are partly related to the size of the GAA expansion.

   Several therapies are in development for Friedreich's Ataxia[1]. Many of these, including gene therapy, seek to restore physiologically relevant levels of the Frataxin (FXN) gene product or protein, using strategies to either re-activate the endogenous gene or supply Frataxin by exogenous [e.g. viral] sources[2,3]. Ultimately, demonstrable improvements in function will be needed for drug approval, but biomarkers will likely play an important role to help determine optimal dosing paradigms and provide early signs of potential efficacy. While several efforts are underway to measure FXN protein[4], metabolic markers, and

other imaging/behavioral outcomes, assays to measure FXN mRNA levels in various biofluids such as cerebrospinal fluid (CSF) have not been established.

Exosomes are vesicular secreted structures that transport a variety of biological materials including DNA, RNA, proteins and lipids and are being investigated as possible sources of a variety of biomarkers in disease states. We have recently developed a method to isolate exosomes, harvest their RNA cargoes, and deep sequence the polyA+ population, yielding full-length, spliced and processed mRNAs for the study of CNS disorders. We noticed that FXN mRNA was robustly expressed in our samples, suggesting that development of a potential biomarker for FXN levels using CSF-derived exosomes might be feasible

Here, building on our experience with CSF exosome-derived mRNAs from other contexts, we propose to develop methods to measure FXN mRNA in exosomes derived from CSF and plasma. We will use biofluids and tissues from gene therapy-dosed non-human primates as a guide and then, determine baseline levels of FXN mRNA expression in CSF and plasma of human FRDA subjects. We will perform transcriptome profiling of mRNAs derived from these biofluids to provide baseline information needed to subsequently develop targeted assays that measure FXN mRNA levels relative to reference mRNAs.

5. Specific aim: to identify and quantitate human FXN mRNA from plasma- and CSF-derived exosomes from FRDA subjects and healthy controls; levels will be correlated to previously determined GAA repeat lengths and related functional metrics. We hypothesize that levels of FXN mRNA will be lower in CSF- and plasma-derived exosomes from FRDA subjects as compared to similar biofluids from control subjects.

6. Research Plan

   This is an exploratory pilot study and no sample size estimate has been performed. We plan to screen 14 FRDA patients to allow for any screen fails or withdrawals. This will allow us to recruit 10 adult FRDA patients from a large cohort of such patients followed by Drs Subramony and Corti, either in the clinic or for other research studies, such as natural history and imaging studies. All screened participants will have already had genetic mutation testing done as part of their previous clinical care. We will include subjects with a range of GAA repeat size so as to assess if estimated FXN mRNA values bear a relationship to the repeat size.

   Inclusion criteria

   a. Patients with symptomatic FRDA (men and women) related to documented biallelic GAA expansion in the FXN gene
   b. Age over 18 years
   c. No contraindication to spinal tap including any coagulopathy or local infections
   d. Competent to provide informed consent

   Exclusion criteria

   a. Unable or unwilling to provide informed consent

b. Any uncontrolled medical illness that may increase the risk of spinal tap such as ongoing infection as determined by the investigators
c. FRDA related to an expansion in one allele and a conventional mutation on the other
d. Positive pregnancy screening

Research methods

a) Subject evaluation. The study involves two visits. Subjects are known to the PI through standard of care clinic. All subjects prior to participation will have had genetic testing performed to confirm biallelic GAA expansion in the FXN gene. To provide potential subjects opportunity to review the study, a consent will either be provided during a clinic visit or remotely by Zoom (visit 1); for the latter option the consent will be emailed via DocuSign to participant for review prior to the visit. Subjects who agree to the study during an in-person visit will sign the consent on the same day and a copy will be provided to them. Subjects who consent remotely will return a signed consent to the investigators. All the study procedures will be done during the second visit which will be scheduled within a reasonable time.
b) Participating subjects will have labs drawn for estimation of INR, PTT and platelet counts for review before visit 2 to ensure no evidence for a coagulopathy
c) During visit 2, the designated coordinator or primary investigator will obtain basic demographic data such as age, gender, and age at onset of FRDA along with vitals (blood pressure, heart rate, temperature, respiratory rate, and weight). Female subjects of childbearing potential will be asked to submit a urine pregnancy test before continuing the visit. A positive pregnancy test will be an exclusion of participation and the visit will be halted. The primary investigator (SHS) will perform a physical exam to include FRDA related medical and neurological history and examination. Severity of FRDA will be assessed using the modified Friedreich ataxia rating scale (mFARS), FRDA activities of daily living scale (FARS ADL) and disease stage determination as has been done in prior studies. Also, 5 to 10 mLs of blood will be collected during this time.
d) Spinal fluid collection. This will be done at the interventional radiology suite under fluoroscopic guidance by experienced personnel. The procedure will be done using standard aseptic precautions and using local anesthesia. The primary investigator (SHS) will be present at these sessions together with any lab personnel and coordinators that will be involved in the study who will help in collecting the sample in appropriate tubes. Approximately 15 mLs of CSF will be collected.
e) Measurement of FXN mRNA. We have developed methods to profile exosomes from CSF-derived exosomes, which in our experience has been the most difficult biofluid from which to harvest exosomes due to low quantity[8,9]. Plasma-derived exosomes present a lower degree of difficulty relative to CSF. To generate these transcriptomes, we first centrifuge CSF samples (up to 2 ml) at 3000 g for 10 min at 4° C. We then isolate exosomes by precipitation using miRCURY (Qiagen) following the manufacturer's protocol, but we incubate overnight at 4° C. We extract total exosomal RNA (exRNA) using the miRNeasy Serum/Plasma kit (Qiagen). After exRNA extraction, samples are DNase treated in-solution and cleaned up by SPRI beads. To prepare a transcriptome library from the extremely low input of exRNA, we

use the SMART-Seq mRNA LP kit (Takara). Library quality control is performed using a Fragment Analyzer (Agilent). Paired end sequencing is the performed on an Illumina NextSeq 2000, using a depth of greater than 50 million reads per sample. Reads are mapped by STAR, and gene expression is quantitated by Kallisto. We have already performed such studies in a set of CSF samples (from subjects with no FRDA) that were assessed in another study and these reads clearly show enrichment within exonic regions of *FXN* gene, and none from introns and intergenic regions. These samples, which will be used as controls for the current study, were obtained by the Wang lab from Stanford University under an MTA]. The transcripts per million (TPM) values are surprisingly abundant, with medians of ~50 TPM in control and DM1 samples. We propose to perform similar procedures to identify and quantitate human FXN mRNA in biofluids from our human subjects. We have parallel studies to evaluate this methodology in tissues and fluids from cynomolgus previously administered human FXN by AAV9 vector by intravenous and intrathecal routes.

f)

Statistical analysis. We will obtain transcript per million (TPM) FXN mRNA levels as determined by Kallisto and determine whether this value correlates with mFARS, FARS ADL, and disease stage determination. Spearman and Pearson correlations will be performed.

7. Recruitment and retention. We currently have about 50 FRDA patients of all ages that participate in various research studies an ongoing basis. In addition, Dr Subramony follows a number of more advanced adult patients with FRDA in his weekly ataxia clinic. Despite the fact that FRDA has usually a childhood onset, the majority of patients in these studies are adults because of the prolonged course of the illness and we anticipate no problems with recruiting the required number of subjects. In addition, Dr Subramony also works closely with the national patient support organization (FARA) who are always ready to help with recruitment.
8. Payment for participation. We will compensate each subject $200.00 for completing the study (Visit 1 and Visit 2). In addition, at the PI's discretion, the study will reimburse hotel expenses (up to $300) for overnight stays for participants that the PI deems necessary.
9. Research patient costs. There is no cost to research patients to participate in this research study.
10. Inclusion of Women and Minorities. FRDA has a prevalence rate of approximately 5/100,000. There are no expected gender differences. Both men and women will be recruited to the study. FRDA is confined to Indo-Caucasian populations, presumably from the timing of the origin of the mutation in relation to human movement over the millennia. We expect our patients to primarily have Indo-Caucasian background. Children will be excluded because of the invasive nature of the spinal tap and also, the aims of the study can be achieved with adults only.
11. Data monitoring. Data management will be performed by the investigators as a team. Subject folders with source documents including informed consent documents will be kept in secured areas within the Fixel Center for Neurological Disorders, accessible only to authorized study personnel. Clinical and demographic data will be then entered into excel spreadsheets with subject name replaced by a study ID number; biofluid

containers will also be identified by study ID number. These data will be linked to findings from the biomarker analyses for further analyses. Hard copies of the informed consent forms and data records will be kept for a minimum of 5 years following completion of the study after which time the Principal Investigator will cross-shred and dispose of the documents.

12. Potential risks. Clinical data collection for this study is minimal and poses little risk. The risks include the discomfort of going through the necessary history, physical exam and neurological exam and possibility of falls during the examination.

    Spinal tap will be done under fluoroscopic guidance which can minimize risks and discomfort. The LP needle may still cause some discomfort and pain in the low back or shooting pain down the legs despite the local anesthesia. There is some risk for a post-spinal tap headache which may require bed rest or occasionally pharmacotherapy. There is remote risk of infections in the spinal fluid or a hematoma formation; these are potentially serious events. Allergic reactions may occur to the local anesthetic but the investigators will inquire into such allergies before the study.

    The risk of blood drawing from a vein includes discomfort at the site of puncture, possible bruising and swelling around the puncture site; rarely an infection; and uncommonly, faintness from the procedure.

    An investigator or study coordinator will obtain informed consent after details of the study and its requirements have been presented, and there has been an opportunity for the participant to ask questions. Consent will be obtained only after complete information has been provided to the participant about the purpose of the study, procedures, risks, benefits, contact persons, compensation, care for injury, and voluntary participation. The participant and study personnel obtaining consent will sign the institutional review board-approved consent form. The participant will receive a copy of the consent form after it has been signed. The original will be retained in the files of the Investigator. Subjects will be carefully screened to minimize any such risk such as being sure that there are no coagulation defects or ongoing systemic or local infections.

    Safety Monitoring:

    Spinal tap is planned to be done with fluoroscopic guidance and likely to be associated with modest discomfort. Subjects will have some pain in the back related local anesthesia injection. There is a slight risk of infection because the needle breaks the skin’s surface, providing a possible portal of entry for bacteria. A temporary numbness to the legs or lower back pain may be experienced. There is a small risk of bleeding in the spinal canal. Subjects will be monitored for 2 hours after the spinal tap and instructed on post-LP care to minimize the risk of post-LP headache. Should the participant experience an adverse effect from LP, severity will be determined by the PI. If the event is deemed serious, the subject may be routed to the UF Health Shands ED, under the discretion of the PI. They will have contact information for the investigators to reach out if needed after the study visit is completed.

This is a pilot observational study and it is planned that Dr. Subramony will oversee the safety of research subjects. He will be responsible for identifying any adverse events and their management if needed. No formal safety monitoring board will be created. Site investigator will assess all adverse events and report any serious adverse events within 5 business days of discovery to the UF IRB01. All adverse experiences will be recorded on the adverse event log.

13. Possible Benefits. There are no direct benefits that patients may expect to receive as a result of participation in this study. The potential benefit of this study is the advancement of scientific knowledge. The information gained from the assessments planned may provide a valuable biomarker for future studies that directly elevate frataxin levels as a therapeutic intervention in FRDA.
14. Conflict of Interest: There is no conflict of interest with this study beyond the professional benefit from the academic publication or presentation of the results.