Study Title:Evaluating mitochondrial dysfunction in patients with neurofibromatosis type 1

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Background and Rationale

Neurofibromatosis 1 (NF1) is the most common type of neurofibromatosis with a broad spectrum of clinical manifestations in multiple organs of the body, including benign and malignant tumors (1–3). This autosomal dominant disease arises from several thousand genetic variants in the chromosome 17, but about 50% of the mutations are de novo (25). Moreover, mitochondrial DNA mutations in the loop region of the genome were seen in 37% of cutaneous neurofibromas and in 50% of plexiform neurofibromas, as also in non-tumor cells like non-affected skin cells (28). Although it is unclear if those mitochondrial mutations have functional effects, other evidence implicates that the impairment of neurofibromin causes mitochondrial dysfunction (MD).

The relation between neurofibromin and mitochondria is structural and functional. Neurofibromin can be co-localized within and extracted from mitochondria (26). Losing neurofibromin, guanosine-5'-triphosphate (GTP) hydrolysis decreases, activating RAS, a protein complex that stimulates mitochondrial degradation (27). Neurofibromin might also modulate mitochondrial respiration. Animal models showed mitochondrial respiration inhibition characterized by increased superoxide anion production and decreased adenosine triphosphate (ATP) generation that was reverted with transgene expression of neurofibromin (9). Loss of neurofibromin also activates extracellular signal-regulated-1/2 (ERK 1/2), affecting its interaction with mitochondrial chaperone tumor necrosis factor receptor associated protein 1 (TRAP1), decreasing succinate coenzyme Q reductase(10,27). Although NF1 animal models show mitochondrial dysfunction, literature evaluating mitochondrial function in patients with NF1 is scarce. Another factor possibly impacting mitochondrial function is the use of MEK inhibitors. These drugs are the only FDA-approved treatment for plexiform neurofibroma, a type of tumor that grows in the nerves of NF1 patients. MEK inhibitors can cause an increase in the oxygen consumption rate, and they can alter the mitochondrial metabolism affecting complex I and the electron transport chain (24,29). **We hypothesize that NF1 patients exhibit a range of MD that contributes to adverse health outcomes**. Validation of this hypothesis will establish a bioenergetic phenotype among NF1 patients to better stratify them than current clinical descriptions. This information will then be used for identifying relationships between patient symptoms and mitochondrial function to demonstrate its value in the clinic (Aim 1). Further, patient mitochondrial function will serve as an important mechanistic readout on the consequences of neurofibromin mutations and impacts of other factors including lifestyle, diet, and drugs (like MEK inhibitors) that can improve or harm mitochondrial function (Aim 2). These findings would expand beyond the current limitations of results from model neurofibromin knockout studies to more relevant complexities observed with patients. Taken together, information gained from patient mitochondrial function assessments would create opportunities to develop more personalized, targeted therapeutic interventions for improving patient outcomes.

The mitochondrial function in patients with NF1 and how it correlates with clinical symptoms will be investigated for the first time in this proposed study. This novel experimental design will generate more clinically relevant findings in humans than current in vitro and in vivo animal studies. Moreover, the strategy leverages a minimally invasive blood test using peripheral blood mononuclear cells (PBMCs) to assess mitochondrial function. Studies with PBMCs detected metabolic stress in patients and acted as biomarkers for MD in diseases including diabetes (22), neurodegenerative disease (23), cardiovascular disease (21), as well as many cancers (14–16). The proposed study will be the first demonstration of PBMCs as biomarkers for MD for NF1 patients. This new technique will expand the phenotyping toolbox for patients to include

bioenergetic demands due to the disease as well as in response to interventions like the growing field of MEK inhibitors for NF1 treatment (1,4,24). This pilot project's candidate biomarker approach on mitochondrial function focuses on clinically relevant questions such as fatigue, pain, cardiac, and renal function. However, our results could be correlated with other important NF1 phenotypes and even data from future systemic unbiased multi-omics approaches to interpret their meaning within patient bioenergetics.

Hypothesis and/or **Specific Aims** or **Objectives**

Neurofibromatosis 1 (NF1) is one of the most common hereditary tumor predisposition syndromes afflicting ~1 in 2750 people worldwide (1,2). Hallmark traits include peripheral and central nervous system tumors along with skin and eye lesions, cognitive deficiencies, musculoskeletal abnormalities, and multiple types of cancer (2,3). The clinical phenotype arises from point mutations (90%) and deletions of the NF1 tumor suppressor gene encoding neurofibromin (4). A well-characterized neurofibromin function is stimulation of Ras GTPase activity leading to Ras inactivation and suppression of its signals through multiple effectors, including mTOR, MEK, ERK, and potentially cAMP/PKA indirectly (5–7). Emerging evidence suggests that neurofibromin regulates cellular and organismal metabolism. The decreased respiratory quotients (ratio of eliminated CO_2 to consumed O_2) reported for NF1 patients implicate neurofibromin defects as a source of altered basal metabolic rates (8). Similarly, animal knockout models have reduced respiratory quotients and increased energy expenditures (9–11). Further analysis of isolated cells lacking neurofibromin show lower basal and coupled respiration, lowered maximal and spare respiratory capacities, and elevated reactive oxygen species, underscoring the importance of neurofibromin on mitochondrial function (10). Supplementation with L-carnitine, a mitochondrial nutrient important for fatty acid oxidation and gluconeogenesis, improved weakness and fatigue in NF1 patients (12,13). Due to a diverse array of genetic lesions, **we hypothesize that NF1 patients exhibit a range of mitochondrial dysfunction (MD) as contributing factors to adverse health outcomes**. We will test the hypothesis by isolating peripheral blood mononuclear cells (PBMCs) from NF1 patients recruited through the new Adult UAMS Neurofibromatosis Clinic at the Winthrop P. Rockefeller Cancer Institute and measure mitochondrial function for correlative studies with clinical phenotypes and symptoms. While novel for NF1 patient studies, the use of PBMCs for mitochondrial studies has precedence in assessing function with a variety of cancers and other diseases (14–23). The effort for this NF1 pilot study involves expertise in pathogenesis and treatment of NF1 (Dr. Erika Santos-Horta), mitochondrial biology (Dr. Nukhet Aykin-Burns), and metabolism (Dr. Grover P. Miller) to achieve the following aims:

Aim 1: Demonstrate mitochondrial respiration efficiency of PBMCs inversely correlates with clinical symptoms of NF1 patients.

We will use an Agilent Seahorse XF Pro Analyzer to measure the cellular and mitochondrial bioenergetics of PBMC isolated from NF1 patients. We predict patients with milder symptoms of fatigue, pain, liver and cardiac function, creatine kinase (CK), and body mass index (BMI) will demonstrate better mitochondrial function in their circulating cells.

Aim 2: Assess whether therapeutic interventions impact mitochondrial function and metabolic plasticity of circulating cells of NF1 patients.

We hypothesize that MD among NF1 patients sensitizes them to therapeutic interventions targeting mitochondria. We will assess the impact of vitamin D treatment to potentially improve mitochondrial function and explore if MEK inhibitors like Koselugo (KOS, Selumetinib, FDA-approved treatment of plexiform neurofibromas in NF1) harm mitochondrial function in patients with NF1, due to their impact in the RAS pathway (24). We predict that the impact of treatments on mitochondrial function will lead to insights on mechanisms of action for NF1 patients and possibly serve as diagnostic and/or prognostic biomarkers for patient outcomes.

Preliminary findings will demonstrate a powerful, novel approach for assessing and understanding the differences in bioenergetic status among NF1 patients and open new research venues. The next steps will study the degree of MD and its correlation with 1) different NF1 mutations (deletions, truncations), 2) other NF1 complications like cognition outcome and quality of life, and 3) prevalence and prognosis of cancers.

Hence, this proposal will generate future projects that will aid the team in achieving the long-term goal of determining mechanisms driving NF1 clinical phenotypes and symptoms to stratify and treat patients. To that end, this pilot study will foster a collaborative team across disciplines and departments at UAMS to leverage the first and only neurofibromatosis clinic in Arkansas at UAMS for improving adult NF1 patient care, and also expand the spectrum of research done by the Drs. Miller and Aykin-Burns, who have not researched populations with genetic diseases so far, and Dr. Santos Horta, who has not researched mitochondrial function.

Study Design and Procedures (sometimes called "Methods")

Although all NF1 patients have genetic changes in the same gene, the clinical presentations of the disease are widely diverse. Thus, it is important to determine non-invasive biomarkers that would help stratifying NF1 patients, who present with highly variable and unpredictable clinical manifestations. Some of the comorbidities seen in NF1 are also shared with patients with mitochondrial diseases (e.g., short stature, fatigue, pain). Thus, our main goal will be to demonstrate a co-association of clinical symptoms and MD that exists in these patients.

Aim 1: Demonstrate that mitochondrial respiration efficiency of PBMCs inversely correlates with clinical symptoms of NF1 patients.

Parameters associated with MD of PBMCs will be assessed as potentially shared cellular pathology in NF1 patients. We hypothesize that MD based on parameters (e.g., coupling efficiency and respiratory capacity) positively correlates with more adverse outcomes as reflected in the symptoms of fatigue, pain, liver, kidney, and cardiac function. Patients over 18 years old, previously diagnosed with NF1, will be eligible for this project. A subject will be registered in the study only after obtaining a signed copy of the Informed Consent Form, completing all pretreatment evaluations, meeting all eligibility criteria, and presenting no exclusion criteria. The neurofibromatosis clinic at UAMS currently cares for 100 patients a year, and it is in a phase of expansion. It is expected that the clinic will see 100 to 140 patients in the next year. We will also recruit 10 to 15 healthy individuals as the control group. Mitochondrial function and cellular bioenergetics will be measured in PBMCs and platelets isolated from blood collected at consent and every 3-4 months for one year. Freshly isolated cells will be plated in coated XF96-Pro well plates using assay media, and the plate will be centrifuged. After the

attachment is confirmed, the cells will be then incubated in a non-CO2 incubator for 20 min at 37°C. The cellular bioenergetics of PBMCs or platelets will be determined by using a standard mitochondrial stress test as we previously published via consecutively injecting oligomycin, an uncoupler (FCCP), and the mitochondrial electron transport chain blockers antimycin A and rotenone into the wells as we previously published (30–37). Clinical repercussions of MD in NF1 will be measured through serial scores of fatigue and pain on the same days that PBMC and platelets are collected. Fatigue will be assessed through the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F). FACIT-F is the only fatigue scale that has been validated in dermatological and painful disease, as it is commonly used in neurological disorders (38). Pain will be assessed by the NRS-11 scale, as recommended by the patient-reported outcome group of the Response Evaluation in Neurofibromatosis and Schwannomatosis (REIS) (39). Other biochemical and metabolic functions to be followed will be CK, cardiac function through echocardiogram, body mass index, liver, and kidney function.

Aim 2: Assess whether therapeutic interventions impact mitochondrial function and metabolic plasticity of circulating cells of NF1 patients. We hypothesize that NF1 patients' inherent mitochondrial dysfunction makes them more responsive to therapeutic interventions resulting in further mitochondrial injury (e.g., by MEK inhibitors, including KOS) or improved function (e.g., by vitamin D). Most patients are vitamin D deficient so the standard of care involves supplementation that could improve mitochondrial function given its essential role in many related processes. Also, we will carry out an exploratory study on the potential adverse effects of KOS on adult NF1 patients who are beginning therapy during the study window. While promising in treatment, the use of this drug has a high incidence of significant adverse side effects like cardiotoxicity (30%). The cause for toxicity is unknown yet may involve further compromised mitochondrial function as reported for other similar tyrosine kinase inhibitors (40). The combination of these studies will provide a critical preliminary assessment of the sensitivity of the mitochondrial measures to patient interventions as well as the possible capacity to correlate with favorable or harmful outcomes.

Subjects who present to the clinic and match the inclusion/exclusion criteria for either the NF1 or control group will be provided a copy of the consent for review. A thorough discussion of the consent will be provided by study staff. If the subject agrees to participate, the following will be completed at each of the 3 total visits required by the study. Each visit will occur at 14 weeks (+/- 2 weeks):

Visit 1

- The subject's medical history will be gathered. This will include information regarding a subject's height, weight, medications used, vitamin D levels, kidney, liver, and heart function.
    - For control subjects, the PI will confirm that the subjects do not have NF1 by family history and physical exam.
- Questionnaires regarding pain and fatigue will be provided for the subject to review and answer.
- Standard-of-care lab draws will continue to occur.
- An additional 10 mL of blood will then be drawn for mitochondrial testing purposes.

Visit 2

- Questionnaires regarding pain and fatigue will be provided for the subject to review and answer
- Standard-of-care lab draws will continue to occur. An additional 10 mL of blood will then be drawn for mitochondrial testing purposes.

Visit 3

- Questionnaires regarding pain and fatigue will be provided for the subject to review and answer
- Standard-of-care lab draws will continue to occur. An additional 10 mL of blood will then be drawn for mitochondrial testing purposes.

	Control Group	NF1 Group		
	At time of Consent	Baseline/Visit 1	Visit 2 (14 weeks +/- 2 weeks)	Visit 3 (14 weeks +/- 2 weeks)
Medical History		X		
FACIT-F QoL		X	X	X
Numeric Pain Rating QoL		X	X	X
Research Lab – Blood	X	X	X	X
SOC labs		X	X	X

Study Population

This study will look to enroll 40 to 45 adults over 18 years old diagnosed with NF1 as well as 10 to 15 adults over 18 years old without NF1 (the control group).

Inclusion Criteria

NF1 group

- Over 18 years old
- Diagnosed with NF1
- Agreeable to participating in the study

Control group

- Over 18 years old
- Agreeable to participating in the study
- Not the first degree relative (biological parent, sibling, or child) of the NF1 patient who is in the NF1 group

Exclusion Criteria

- NF1 group
  - Under 18 years old
  - Not agreeable to participating in the study
- Control group
  - Under 18 years old
  - Diagnosed with NF1
  - The first degree relative (biological parent, sibling, or child) of an NF1 patient who is participating in the study

Examples of recruitment methods include, but are not limited to the following:

- Patients with NF1 at the UAMS Adult NF1 clinic will be invited to participate in the study during their regular clinic appointments. If the patient is interested in participating in the study, a Cancer Clinical Trial Office nurse will be called to consent the patient.
- Spouses, friends, and non-relatives of NF1 patients who come to the UAMS Adult NF1 Clinic will be invited to participate in the control arm of the study at the time of the patient appointment. If the non-patient is interested in participating in the study, a Cancer Clinical Trial Office nurse will be called to consent the control group volunteer. They will then receive a single blood draw at the time of consent.

Risks and Benefits

Risk is minimal as this is not an intervention study and the only procedure is collection of 10 mLs of blood. A blood draw can lead to discomfort, redness, bruising, or infection at the blood collection site.

The questions being asked by the FACIT-F and Pain Scales may cause patients to become sad or upset.

A risk to study subjects is the potential for loss of confidentiality of study data.

Measures to protect the confidentiality of study data will be implemented as described in the Data Handling and Recordkeeping section below.

There will be no direct benefits to the study participants; however, knowledge gained from the study could potentially benefit patients in the future.

Data Handling and Recordkeeping

The Principal Investigator will carefully monitor study procedures to protect the safety of research subjects, the quality of the data, and the integrity of the study.

Paper Consents and paper forms will be stored at the Cancer Clinical Trials Office. A digital database for patient data entry will be developed by the Cancer Clinical Trials Office. The database will be housed on a UAMS-maintained server. All data will be coded with a unique identifying number upon entry into the database. The key to the code will be kept separately and maintained electronically on a password-protected computer. Only the PI and the study staff will have access to the key linking a patient's name to their data.

At the conclusion of the study, the samples and data will be indefinitely stored in a repository for future use. The key linking the samples and data to a subject's name will be retained indefinitely at the time of study closure in order to de-identify the data/samples.

If any patient desires to have their blood sample destroyed at any time during or after conclusion of this study, patient data will be decoded, and the sample will be destroyed upon the subject's request for withdrawal.

Specimen Handling and Storage

Blood samples will be collected from patients and controls during routine appointments. Samples will be given a unique identifying number, and then transported to Dr. Nukhet Aykin-Burns' laboratory where they will be tested and stored. A key linking the coded sample to the data will be maintained separately on a UAMS password-protected server. Transport will be done by Drs Nukhet Burns laboratory staff.

If the patient sample volume is not completely used for this project, it will be retained for further study if patient indicates so in the consent. This sample might be used for future research to study the pathophysiology of neurofibromatosis. In the case that there is an opportunity to use the storage blood, we will decode data and enter in contact with patient to request their consent for the new project.

Data Analysis

XF Pro mito-stress test data will provide direct measurements of basal, nonmitochondrial, and maximum respiration, and we will use these data to calculate the mitochondrial basal respiration, ATP-linked respiration, reserve respiratory capacity, proton leakage, and coupling efficiency in PBMCs and platelets isolated from the control group once and from NF1 patients longitudinally (3 times). At the end of each run, we will also determine the cellular bioenergetics and metabolic plasticity (switch between oxidative phosphorylation and glycolysis) of the cells via plotting extracellular acidification rate versus oxygen consumption rate. The NF1 and healthy control groups' mitochondrial function and metabolic plasticity parameters will be compared in addition to longitudinal changes in these parameters for each NF1 patient. Linear regression analysis will be utilized to establish the relationship between coupling efficiency, respiratory capacity (ATP-linked, reserve capacity etc.) as well as metabolic plasticity and NF1 clinical phenotype and biochemical scores for fatigue, pain, CK, cardiac function, BMI, and liver function. Analysis of variance for repeated measures will also be used to analyze changes in these parameters over time by each group (NF1 patients before and after Vitamin D and/or KOS administration).

This pilot study was designed to test the feasibility of our recruitment and implementation procedures and operational strategies. Preliminary data obtained from this pilot project will also be used to assess the variability of outcomes for power analysis of future larger studies. A biostatistician will be consulted during pilot data analysis and included in the team for statistical power calculations for future studies.

Ethical Considerations

This study will be conducted in accordance with all applicable government regulations and University of Arkansas for Medical Sciences research policies and procedures. This protocol and any amendments will be submitted and approved by the IRB as required.

No compensation will be given for patients or controls.

The informed consent of each subject, using IRB-approved consent materials, will be obtained before that subject begins any study procedures. All subjects for this study will be provided a paper consent form describing this study in language understandable to the study population. Consent materials will provide sufficient information for subjects to make an informed decision about their participation in this study. The person obtaining consent will thoroughly explain what the subjects need to know about the study, including study requirements, study risks and benefits. The consent process will take place during their clinic visit in the exam room. The consent discussion will occur at the same day of the first blood sample. Participant privacy will be maintained and questions regarding participation will be answered. No coercion or undue influence will be used in the consent process. This consent form must be signed by the subject**,** and the person obtaining the consent. The participant will receive a paper copy of the signed consent form, and the informed consent process will be documented in the research record.

The consent process will be documented separately via a written note in the research or medical chart for each subject.

Dissemination of Data

Results of this project will be presented in Neurofibromatosis and Neuro-oncology symposium and Conferences, as published in neuro-oncology and metabolism journals.

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