ANTIOXIDANT THERAPY WITH N-ACETYLCYSTEINE FOR LEARNING AND MOTOR BEHAVIOR IN CHILDREN WITH NEUROFIBROMATOSIS TYPE I

NCT04481035

Note: As funding was obtained for a more definitive study, the present piilot study was closed and only descriptive analysis of primary outcome was performed.

CINCINNATI CHILDREN'S HOSPITAL MEDICAL CENTER

STUDY TITLE:
Antioxidant therapy with N-acetylcysteine for learning and motor behavior in children with neurofibromatosis type 1

SPONSOR NAME: CCHMC - ARC RASOPATHIES PROGRAM

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ABSTRACT:
Children with neurofibromatosis type 1 (NF1) commonly suffer from the effects of cognitive, behavioral, and motor impairments. At present there is no specific treatment for this NF1 complication. However, data from rodent models of NF1 along with uncontrolled clinical observations in children with NF1 suggest that the anti-oxidant, glutamate modulating compound N-Acetyl Cysteine (NAC) may reduce these impairments. Of particular interest is a murine study analyzing the central nervous system manifestations of NF1 at our institution. That study revealed a role for myelin-forming oligodendrocytes in the control of nitric oxide synthases (NOS) and their product, nitric oxide, in maintenance of brain structure and function, including regulation of behavior and motor control. Treating these mice with NAC corrected cellular and behavioral abnormalities. N-Acetyl Cysteine is available over the counter and has been used by thousands of individuals; moreover, it has shown some promise in clinical trials for psychiatric disorders.

In order to better understand treatment mechanisms, and possibly predict long-term outcomes, we propose concurrently to explore Specific Aim 1 (1.1, 1.2, and 1.3) exploratory potential disease biomarkers as outlined below. The primary outcome of this study is motor function rated with the Physical and Neurological Examination for Subtle Signs (PANESS), a validated scale that consistently demonstrates significant impairments in children with ADHD, and which our preliminary data suggest may demonstrate more extreme problems in children with NF1. The first exploratory biomarker is motor system inhibitory physiology, measured using Transcranial Magnetic Stimulation (TMS). Preliminary measures in our NF1 population also show abnormalities similar to established findings in ADHD. The second exploratory biomarker is metabolomics profiling for the biomarker of oligodendrocyte dysfunction in NF1 participants: autotaxin. Preliminary data in our NF1 population showed specific signal abnormalities in the NF1 population compared to healthy controls. Therefore, we propose to perform a double-blind placebo controlled, prospective, Phase IIa study to explore safety, tolerability, and efficacy of NAC on learning and motor behavior in children with NF1 aged 8 through 16 years old.

PURPOSE OF STUDY:

The aims of this application are to gain information in children with NF1 about possible clinical benefit of anti-oxidant treatment and to develop and evaluate quantitative brain-based and blood biomarkers relating to presence of NF1, symptom severity, and response to antioxidant therapy. Clinically, 50% of children with NF1 are underperforming or failing at school [1]. This frequently leads to decreased educational attainment and fewer opportunities as adults. An important first step was our preliminary work using the PANESS scale and Transcranial Magnetic Stimulation (TMS)-evoked Short Interval Cortical Inhibition (rSICI) in children with NF1. We propose to develop and extend our understanding of NF1-related motor and learning behavior in response to antioxidant therapy with NAC. The purpose of the present study is to 1) evaluate tolerability, safety, and clinical benefit of NAC in this double-blind placebo controlled study; 2) to evaluate motor function (PANESS) and physiology (TMS) biomarkers at baseline and after treatment; and 3) to quantify metabolomics profiles at baseline and after treatment. We propose to study 20 children with NF1, ages 8-16 years, at baseline and after completion of 8 weeks of treatment with NAC.

NAC therapy, if successful, is expected improve these parameters. Our trial endpoints are: Does behavior improve? Does motor function improve? Are there TMS biomarkers that reflect the presence of NF1 and the response to NAC treatment? Are there metabolomics measures that reflect the presence of NF1 and the response to treatment? We hypothesize that we can identify predictive measures as a foundation for an application for funding for a larger, more definitive, placebo-controlled trial involving biomarkers and clinical outcomes. We believe this work has the potential to lay groundwork for future use of relevant biomarkers for treatment and outcomes research for NF1 as well as other biologically similar conditions, collectively designated the "RASopathies" (due to involvement of the RAS family of proteins) and ultimately to guide development of more effective treatments based on disease pathophysiology.

SPECIFIC AIMS

This study involves the following aims:

Specific Aim 1: Primary Outcome of Study

In children and adolescents with NF1, to characterize the behavioral and motor effects of 8 weeks of N-acetylcysteine (NAC) treatment in a cohort of 20 children and adolescents with NF1. We will evaluate tolerability, safety, and clinical benefit of NAC in this double-blind crossover placebo controlled study. **Aim 1.1**: Characterize effects of NAC treatment on motor function in kids with NF1 using the Physical and Neurological Examination for Subtle Signs (PANESS). This is a validated scale that consistently demonstrates significant impairments in children with ADHD, and which our preliminary data suggest may demonstrate more extreme problems in children with NF1 than age-matched healthy controls (unpublished data from CCHMC). We hypothesize that motor function scores rated with the PANESS scale will improve

after treatment with NAC. **Aim 1.2**: Characterize effects of NAC treatment on ADHD symptoms in children with NF1. We hypothesize that ADHD attention and hyperactive/impulsive symptoms, rated with the DuPaul DSM-5 based clinical rating scales, will improve after treatment with NAC.

Specific Aim 2: Experimental aim # 1
In the same cohort, we will identify potential novel biomarkers of neurodevelopmental burden in NF1. **Aim 2.1**: Describe the function and physiology of the motor system using Transcranial Magnetic Stimulation (TMS) as a possible disease biomarker of NF1. Preliminary measures in our NF1 population also show abnormalities similar to established findings in ADHD. We hypothesize that children with NF1 will have significantly less motor cortex inhibition using TMS measurements, and these measures will improve ("normalize") upon NAC treatment. We will compare to our internal age-matched healthy controls at Cincinnati Children's. **Aim 2.2**: We propose to evaluate autotaxin as a candidate biomarker of oligodendrocyte dysfunction in NF1 participants. Preliminary data from our biomarker discovery analysis of serum samples from healthy controls and NF1 patients showed lysophosphatidylcholine (LPC) depletion compared to healthy age/sex matched controls. In gene expression analysis autotaxin was elevated 4 times in neurofibroma Schwann cells compared to normal nerve Schwann cells. We will collect serum and plasma from participants to assess autotaxin/LPC axis prior and post-NAC therapy. We hypothesize that autotaxin axis abnormalities will be a biomarker of response to antioxidant therapy in our NF1 population.

Specific Aim 3: Experimental aim # 2
In the same cohort, to evaluate metabolomics profiles as a possible disease biomarker that is affected by NF1 and by treatment with NAC as per Aim 1. Hypothesis 4: We hypothesize that specific profiles will predict clinical response to antioxidant therapy compared to age-matched healthy control (unpublished data from CCHMC).

BACKGROUND:

Neurofibromatosis (NF1) – overview

Neurofibromatosis (NF1) is an autosomal dominant condition affecting many organs, resulting from mutations in the neurofibromin gene [2]. Characteristically, individuals with this condition have cutaneous manifestations, most prominently hyperpigmented macules termed "café-au-lait" spots. The neurofibromin gene functions in part as a tumor suppressor, and persons with NF1 are susceptible to malignant and benign tumors in multiple organs. What is less well studied is that individuals with NF1 commonly suffer from difficulties with behavioral and emotional regulation including ADHD symptoms, learning disabilities, and developmental delays in motor function [3]. These cause substantial morbidity in adulthood.

NF1 – challenges evaluating behavioral, emotional, and cognitive symptoms

Clinically, 50% of children with NF1 are underperforming or failing in school [1]. This frequently leads to decreased educational attainment and fewer opportunities as adults. The high prevalence of behavioral, emotional, and cognitive problems in persons with NF1 suggests that neurofibromin may play a role in structure and/or healthy, efficient function of the brain. Clarifying this is challenging because most likely behavioral, cognitive, and motor impairments in NF1 result, as in other genetic conditions, from multiple complex factors including environmental factors and genetic loading distinct from the major effect of mutations in neurofibromin [4]. Moreover, cognitive and behavioral symptoms are difficult to quantify. Rating scales capture impressions of parents and teachers in the important domains of home and school, but they are subjective ratings, not brain-based measures [5]. Moreover, if treatments during a clinical trial are beneficial, there may be biological changes that precede clinical ones. Measuring these changes may be important for validating treatment mechanisms and effects.

NF1 – neurobiological basis for behavioral, emotional, and cognitive symptoms and role for physiological biomarkers

It has long been known that children with NF1 have enlarged heads – macrocrania. More recent reseach has shown that children and adults with NF1 often have enlarged myelin-rich white matter tracts [6]. These findings have been associated with behavioral and cognitive impairment in NF1 human studies and Nf1 animal models [7] [8]. Oligodendrocytes are the cells that make myelin in white matter tracts. Quantitative biomarkers of white matter structures may help measure and predict behavior in NF1 and facilitate interventions into clinical practice. Diffusion tensor imaging (DTI) shows global changes in NF1 participant brains which are consistent with white matter dysfunction [9]. White matter properties can also be evaluated using neurophysiological techniques, including Transcranial Magnetic Stimulation (TMS). TMS studies are generally brief, inexpensive, readily repeatable, and do not require sedation. TMS may be helpful for identifying clinically useful, specific brain biomarkers and assessments related to motor and learning difficulties in NF1 and, ultimately, other conditions involving RAS pathways (related RASopathies).

The biomarkers in Aim 2 have been developed and optimized in three NIH R01 grants. In collaboration with investigators at the Kennedy Krieger Institute in Baltimore, in studies that are ongoing, we demonstrated in a seminal publication that both the motor control assessment proposed for this study, the PANESS, and the primary physiological biomarker, TMS-evoked Short Interval Cortical Inhibition (SICI), correlate robustly with the presence and severity of ADHD [10]. We and others have also demonstrated that SICI is sensitive to medications, and, further that 4 weeks of treatment with a norepinephrine reuptake inhibitor can both improve ADHD symptoms and, in a statistically correlated way, alter TMS-evoked SICI [11]. In a preliminary study (unpublished data, see figure 2), we have evaluated these potential biomarkers in 11 children with NF1 and compared these to data in approximately 50 children with ADHD and 50 health controls. As shown in figure 2 (NF1 sample n=9 due to age matching), both motor scores with PANESS and TMS-evoked SICI appear to differ in NF1 children, with values as extreme or more extreme than those found in the ADHD cohort. Two other measures are also shown, whose differences were not significant in this study.

We have analyzed datasets from our untargeted metabolomics data for statistically significant metabolic pathways and identified abnormalities in the lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), Autotaxin axis. Autotaxin is a secreted phosphodiesterase that produces LPA from LPC. LPA is a lipokine that acts through specific guanine-nucleotide-binding protein (G protein)-coupled receptors to stimulate migration, proliferation, oxidative stress, survival and other functions in many cell types. Autotaxin has been shown to play a significant role in OLG development via 2 domains, the modulator of OLG remodeling and focal adhesion organization (MORFO) [12] and the catalytic enzyme domain that generates LPA [13]. Autotaxin is predominantly expressed and secreted in OLG cell lineage cells [14].

N-acetylcysteine (NAC) – properties, and rationale for use in NF1

We have treated 4 patients off-label with NAC at 900 mg twice daily with improved behaviors without side effects at our center and external collaborators at Children's National Medical Center. We will assess the safety and efficacy of N-acetylcysteine (NAC) in a phase II pharmacological intervention clinical trial in children with NF1. This drug choice is based on our findings from mouse models of the central nervous system manifestations of NF1, which reveal a role for myelin-forming oligodendrocytes in the control of nitric oxide synthases (NOS) and their product nitric oxide in maintenance of brain structure and function, including regulation of behavior and motor control (see below figure). Treating these mice with NAC corrected cellular and behavioral abnormalities [15] [16].

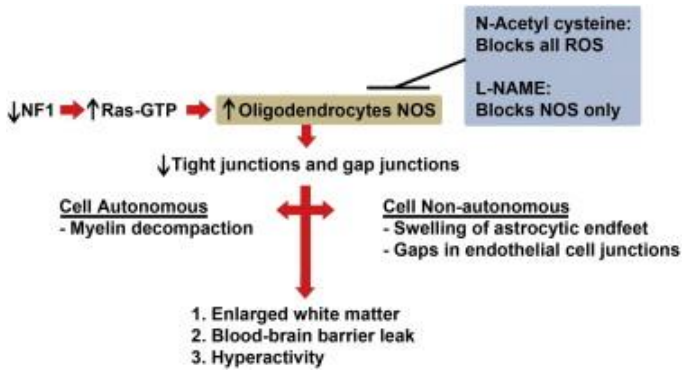

Figure 1. Nitric oxide (NO) can disrupt tight junctions and gap junctions altering the surrounding vasculature. NF1 loss and Ras hyperactivation can upregulate NO and NO synthases (NOS) in white matter. Treating mice with the antioxidant N-acetyl cysteine corrected cellular phenotypes. NAC is the N-acetyl derivative of l-cysteine. In the brain, NAC can influence oxidation. Once in the cell, the cystine is reduced to cysteine, the rate-limiting component of the endogenous antioxidant molecule glutathione.

NAC is a natural supplement that acts as an antioxidant and a glutamate modulating agent. It is widely available over the counter. Although no controlled safety studies have been published, NAC has been widely used in large doses for decades in the management of acetaminophen overdose. Its most common side effects are gastrointestinal, as noted in a recent study in children with Autism Spectrum Disorder. Relevant to this proposal, NAC was used to treat ADHD symptoms in systemic lupus erythematosus [17], Autism Spectrum Disorder (ASD), anxiety, and depression [18] [19] with promising responses [15] [16]. Dose of NAC will be used based on these prior published studies. As reported in these studies, there were no serious adverse events. Most side effects, such as nausea, were mild to moderate in severity and were transient and reversible.

Table 1: NAC Preliminary Data

Condition	Study design	Dose	Sample size	Result	Citation
SLE with ADHD	Randomized, Placebo controlled	2.4 or 4.8 gm/day	6 placebo, 9 each dose; adults, 3 months	Improved ADHD	Garcia 2013 [17]
Bipolar disorder	Open label	2 gm/day	149 adults, 2 months	Improved depression	Berk 2011 [20]
Autism Spectrum Disorder	12 week double blind randomized placebo controlled	900 mg TID	14 NAC, 15 placebo, children	Reduction in ABC irritability	Hardan 2012 [19]

TMS PRELIMINARY DATA: The goal of Aim 2 is to translate molecular, cellular, and animal model data to human NF1 clinical trials through evaluating human brain physiology. Our preliminary work comparing 9 NF1 children (mean IQ 94) with age-matched (all ages 8-12; mean for all groups 10.5 years) cohorts of 52 ADHD and 62 typically developing children (Figures 2a-d) has identified PANESS and TMS-evoked SICI as promising biomarkers.

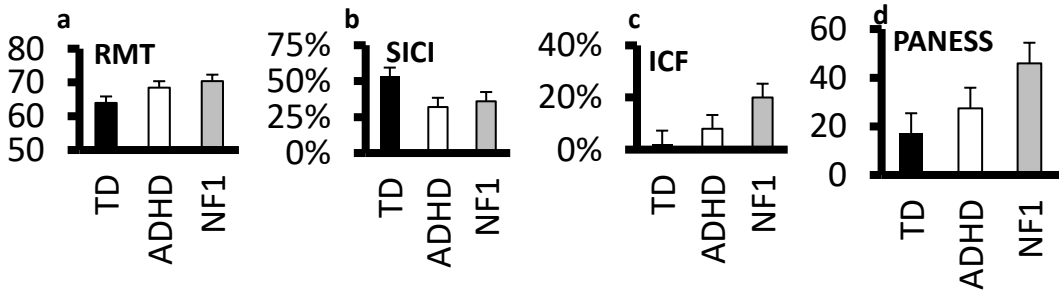

Figure 2. **a** TMS Resting Motor Threshold (RMT) ($F_{2,113} = 1.7$, $p = .2$); **b** Short Interval Cortical Inhibition (SICI) ($F_{2,94} = 10.3$, $p = .0001$); **c** Intracortical Facilitation (ICF) ($F_{2,97} = 1.6$, $p = .2$); **d** PANESS motor function battery ($F_{2,105} = 30.3$, $p < .0001$).

METABOLOMICS DATA: untargeted global metabolomics profiling is a non-invasive technique that can help identify novel biomarkers that correlate with clinical disease and response in the setting of NF1. We have successfully performed untargeted metabolomics experiments using serum from healthy controls (n=12) and NF1 patients (n=18) collected from the NF1 center in our mass spectrometry laboratory. Patients with NF1 showed a distinct metabolomics signature compared to healthy age/sex match controls (Figure 3A). This preliminary data strongly supports our hypothesis that RAS-MAPK disorders can lead to detectable biochemical abnormalities. A clinical database with patient characteristics already exists at our center. We have input plexiform neurofibroma (PNF) growth to correlate with metabolomics data (Figure 3B). This technology will allow potential identification of candidate biomarkers that could correlate with different disease complications including behavioral abnormalities. We plan to collect samples from NAC treated individuals to identify compounds that mark response to therapy, and could serve as response biomarkers in a larger Phase II trial.

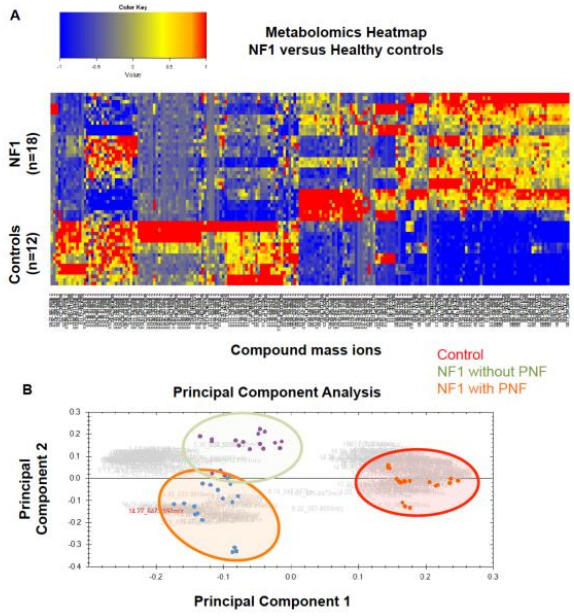

Figure 3. Neurofibromatosis type 1 metabolomics pattern is different from healthy controls.
A. Heatmap was based on 203 compounds out of 5,206 statistically significant compounds ($p<0.005$, fold change >2). Selected compounds includes diacylglycerol, lysophospholipids, ceramides species and other compounds. Blue indicates low abundance and Red increased abundance.

B. A principal component sub-analysis of these samples suggests a different biochemical profile and 10 candidate biomarkers in the NF1 patients who have progressive plexiform neurofibromas (PNFs) versus NF1 patients without PNFs.

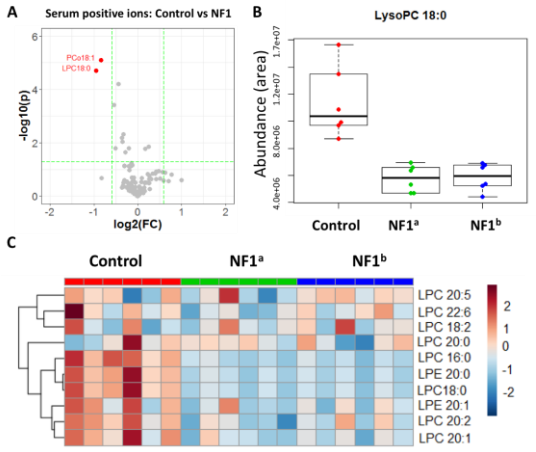

Figure 4. Lysophosphatidylcholine (LPC) species are depleted in serum from NF1 patients.
A. Volcano plot for biomarker discovery ($p<0.005$, fold change >2) shows low abundance of LPC18:0 and PCo18:1 in NF1 patients versus healthy controls.
B. Targeted mass spectrometry analysis of LPC species confirmed low LPC18:0 in NF1 patients ($NF1^a$) compared to controls ($p<0.005$). Replication cohort showed similar differences ($NF1^b$). **C.** Heatmap based on LPC species ($p<0.005$, fold change >2). All LPC species were low in the NF1 patient cohorts versus healthy. Blue indicates low abundance and Red increased abundance.

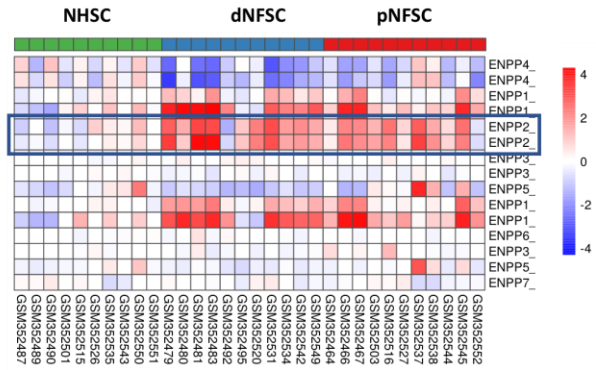

Figure 5. Log2 gene expression analysis of the ectophosphodiesterase/nucleotide phosphohydrolase (ENPP) nucleotidases family. These proteins catalyze the generation of bioactive lipids. Autotaxin (ENPP2) gene expression is elevated in dermal neurofibroma Schwann cells (dNFSC) and plexiform neurofibromas Schwann cells (pNFSC) versus control nerve human Schwann cells (NHSC).

STUDY DESIGN:

Overview of Design

The study design is essentially a cross-sectional survey and then longitudinal evaluation of cognition and behavior, motor function, cortical function, and metabolomics profiles in NF1 before and after 8 weeks of treatment with an FDA approved medication, N-acetylcysteine (NAC) or placebo. This is a cross-over double-blind placebo controlled study. As such, during the treatment observation period, subjects will be assigned to one of two treatment arms (NAC drug or placebo) in phase I. After 8 weeks of treatment and subsequent assessments described below, subjects will cross over to the alternate treatment arm for phase II. In this way, each participant will provide both control and experimental data. Aside from the treatment arm (NAC drug or placebo), parameters for phase I and phase II are identical.

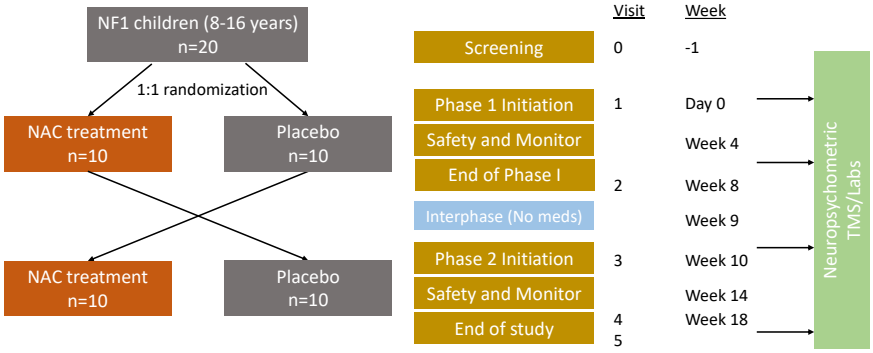

Figure 4: Study Design: a cross-over double-blind placebo controlled study

This is a single site, Co-PI mechanism collaboration. NF1 participants will be recruited from the NF1 and RASopathies Programs at Cincinnati Children's Hospital Medical Center as well as from local events (including philanthropic and education events). In brief, these include primarily web-based, email, and in-clinic advertisements. As before, NF1 and motor/learning behaviors abnormalities and severity assessment will involve validated, systematic, structured diagnostic interviews. Symptoms will be evaluated using standardized rating scales that are in wide clinical and research use. TMS and PANESS scale will be performed using established protocols at our institution.

Objective and Rationale for Aims 1-2. We propose a cross-sectional study where we will compare PANESS and SICI measures in 20 children with NF1 with data already acquired at CCHMC with those 2 measures. Next, these 20 children will be treated with NAC as described below. PANESS and TMS measures will be repeated after 8 weeks of treatment. Clinical symptom ratings will be performed independently. Motor and TMS ratings and clinical ratings will be blinded to one another until analyses are completed at the end of

the study. At present, there are no clinical characteristics, neuropsychologic variables, phenotypic variants or biomarkers which can accurately predict treatment response of abnormal motor/learning behaviors in NF1 to NAC therapy. We will assess ADHD attention and hyperactive/impulsive symptoms with the DuPaul DSM-5 based clinical rating scales and predict an improved rating after treatment with NAC.

Screening Period: Screen candidates will be solicited from clinic referrals and from advertisements. If, after the screening visit, the candidate is determined eligible to participate, the candidate will then be entered into the baseline testing portion of the study.

Baseline Testing Period (Visit 0): Baseline testing may occur remotely, may coincide with the first study visit, or split among both options and will consist of clinical assessments and ADHD severity testing with evaluation by an independent rater blinded to study procedures as well as parent ratings of ADHD, assessment of anxiety, and depressive symptoms. Some participants may undergo a short, optional pre-treatment, unsedated research MRI for structural and DTI. All participants will receive a full psychometric and neurological examination and be screened with a comprehensive assessment battery designed to guarantee that they meet inclusion/exclusion criteria for the study, that they are candidates to receive NAC being offered in the trial, and that a robust database is available regarding NF1 motor/learning behavior symptomatology, comorbid symptomatology, broader psychosocial function, neuropsychological function, academic performance, severity and impairment, and medical status. Per FDA Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials, an assessment for suicide (Columbia Suicide Severity Rating Scale, C-SSRS) will be performed at every visit and monitoring visits or phone calls.

Treatment Observation Period:

Phase I:
Study initiation will consist of motor PANESS and TMS measures (AIMS 1 and 2) and blood will be drawn for metabolomics profiles (AIM 3). Subjects will then return to the outpatient RASopathies clinic where they will be assigned to one of two treatment arms (NAC drug or placebo) of this study. Each subject will receive drug or placebo (visits 1 and 3) without feedback regarding treatment arm assignment to Dr. Gilbert until the end of the study. At each of these outpatient visits (visits 1 and 3) subjects will be assessed for vital signs, adverse events, pill counts and concomitant meds, and for motor/learning behaviors severity and improvement. At the final phase I and final study visits (visits 2 and 4, respectively), treatment outcomes will be rated by an independent rater as well as by the study physician (Dr. Prada, or qualified designee) for motor/behavior improvement. A repeat longitudinal TMS study will occur at weeks 8 and 18 (at the end of phase I and phase II, respectively) while on a stable treatment dose of NAC or placebo (visits 2 and 4) by Dr. Gilbert within 8-10 hours following AM dosing. At end of the final phase I study visit (visit 2), subjects will undergo an 'interphase' for 1 week during which they will abstain from medication (drug or placebo). After interphase, subjects will enter phase two of this cross-over double-blind placebo controlled study and commence the alternative treatment arm.

Phase II:
In phase two, subjects will cross over to the opposite treatment arm (NAC drug or placebo) than they were assigned during phase one. All other details outlined in phase I will remain unchanged.

After both phases of the treatment observation period are complete, there will be a debriefing interview, in which children and their families will review both their performance during the trial and tolerability data (visit 5). A plan for follow-up care will be created by Dr. Prada, or qualified designee, with referral for follow-up treatment. If needed, subjects will be given transition medication (atomoxetine or otherwise depending on effectiveness of study drug) until subject can establish appropriate care with a clinician.

Emergency Study Extension

In light of the recent coronavirus pandemic, CCHMC has implemented significant restrictions on in-clinic study visits and other non-essential activities that prohibit normal operations. Below is a two-option contingency plan to accommodate currently enrolled and active (on drug/placebo) participants (all are currently in phase II of the study, between week 14 and 18).

Contingency Option 1:

Study participants whom are currently enrolled and on study drug (or placebo) are to maintain a stable dose (no change in dose). At week 18, when an in-person study visit would typically occur, a virtual visit will take place. This week 18 virtual visit can occur over the telephone, via email, or videoconference. Virtual assessments will include surveys and PANESS (performed via videoconference when possible).

After the in-clinic study visit restrictions have been lifted all study visit assessments will be performed as outlined in this protocol. If restrictions to hospital access are still in place at week 26 (8 weeks after the originally-planned visit 4), a virtual visit will take place and the TMS assessment will be omitted. During this virtual study visit, participants will complete surveys and PANESS remotely as described above and undergo a research physical exam/consultation with the study physician via telemedicine. At that point, the participant will discontinue study drug/placebo and officially conclude their participation in this study.

Contingency Option 2:

Study participants whom are currently enrolled and on study drug (or placebo) are to maintain a stable dose (no change in dose). If a participant has an essential clinic visit during or after week 18 of this study (the point of a typical visit 4), the family and research team may consider conducting the week 18 research visit with significant contact restrictions. An essential clinic visit will be defined as a clinic visit that has not been rescheduled as part of the current hospital restrictions. While on campus for an essential clinic visit, participants will receive visit 4 safety labs/EKG and TMS. All assessments that can be done remotely (surveys, PANESS, telemedicine physical exam) will be done remotely as described under contingency 1.

Precautions will be taken to minimize potential exposure. The study coordinator will review the current pre-visit mandates outlined on Centerlink with the participant/family. Communication via email or phone will be made the day prior to the study visit to confirm that the participant and parent are healthy (symptom free). If the participant or any member of their household has a fever, cough or colds, body aches, or stomach virus symptoms, the study visit will be cancelled and contingency option 1 will be implemented. Only one family member (a parent/guardian and no siblings) will be permitted on campus with the study participant. Additional restrictions will be implemented as appropriate per hospital and government policy/recommendations.

CLINICAL SUICIDE IDEATION PLAN:

Rationale:

Per FDA *Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials*, an assessment for suicide will be performed at each visit.

Suicide assessment:

Columbia Suicide Severity Rating Scale, C-SSRS

Review of C-SSRS Responses:

Study doctor (or designee) will verbally administer and then review each suicide assessment. The 'Columbia-Suicide Severity Rating Scale Screen with Triage Points' document outlines the planned course of action based on the participant's response. In particular, if a participant discloses any suicide

ideation, the study staff will contact the participant's mental health provider or PCP and/or *Psychiatric Intake Response Center (PIRC)* at Cincinnati Children's Hospital for further assessment and guidance.

> *The Pediatric Intake Response Center (PIRC) manages psychiatric patient consultation and intake for Cincinnati Children's Hospital Medical Center. Staff members are available 24 hours a day, seven days a week at 513-636-4124 or psychiatryresponse@cchmc.org .*

DURATION:

The current plan is for the study to last for 3 years. Data analysis and completion of study reports/papers will occur in the last 6 months of the study (see timeline below).

Timetable	
Sept 2018	IRB submission
Oct 2018 – Jan 2018	Operations Staff Preparation/ Prepare materials for Orientation and Training Meeting; CRF Dataform Generation; Prepare Study Medication Packets and Materials for Study Initiation
Feb 2018 - Jun, 2019	First Subjects recruited
June 2019 - Aug, 2020	Subjects recruited, complete the study
Sept 2020 - Dec 2020	Data closeout and analysis, submit manuscript

Investigator Experience

Dr. Gilbert has been principal investigator or site investigator on a number of open-label and placebo-controlled clinical trials and has conducted all the TMS studies described in this protocol. Dr. Prada has been principal investigator in studies for neurofibromatosis, RASopathies and metabolomics.

1. **Gilbert DL**, Murphy T, Jankovic J, Budman CL, Black KJ, Kurlan R, Coffman KA, McCracken J, Juncos J, Grant J, and Chipkin RE. A randomized, double-blind, placebo-controlled study of the D1 receptor antagonist ecopipam for children and adolescents with Tourette syndrome. *Presented at the 21st International Congress of Parkinson's Disease and Movement Disorders, June 8, 2017*.
2. **Gilbert DL**, Budman CL, Singer HS, Kurlan R, Chipkin RE. A D1 Receptor Antagonist, Ecopipam, for treatment of tics in Tourette Syndrome. Clinical Neuropharmacology, 2014. 37:26-30. PMID: 24434529.
3. Chen TH, Wu SW, Dixon S, Shahana N, Huddleston DA, Sarvis AR, Sallee FR, **Gilbert DL**. Reduced short interval cortical inhibition correlates with atomoxetine response in children with attention-deficit hyperactivity disorder (ADHD). Journal of Child Neurology, 2014. 29:1672-9. PMID: 24413361.
4. **Gilbert DL**, Isaacs KM, Augusta M, MacNeil LK, Mostofsky SH. Motor Cortex Inhibition: a marker of ADHD Behavior and Motor Development in Children. Neurology 2011; 76:615-621. PMID: 21321335.
5. **Gilbert DL**, Zhang J, Lipps TD, Natarajan N, Brandyberry J, Wang Z, Sallee FR, Wassermann EM. Atomoxetine Treatment of ADHD in Tourette Syndrome: Reduction in Motor Cortex Inhibition Correlates with Clinical Improvement. Clinical Neurophysiology 2007; 118:1835-1841. PMID: 17588810.
6. 4. **Prada CE**, Rangwala F, Lovell A, Schorry EK, Saal HM, Hopkin RJ. (2012). Pediatric plexiform neurofibromas: management and clinical outcomes. The Journal of pediatrics. 2012; 160(3):461-7. PubMed [journal] PMID: 21996156
7. **Prada CE**, Jousma E, Rizvi TA, Wu J, Dunn RS, Mayes DA, Cancelas JA, Dombi E, Kim MO, West BL, Bollag G, Ratner N. (2013). Neurofibroma-associated macrophages play roles in tumor growth and response to pharmacological inhibition. Acta neuropathologica. 2013; 125(1):159-68. NIHMSID: NIHMS417791. PMID: 23099891, PMCID: PMC3547628

8. **Prada CE**, Hufnagel R, Trent H, Lovell A, Hopkin RJ, Saal HM, Schorry EK. (2015). The use of magnetic resonance imaging screening for optic pathway gliomas in neurofibromatosis type 1. The Journal of pediatrics. 2015; 167(4):851-856.e1. PMID: 26233602

SELECTION AND RECRUITMENT OF PARTICIPANTS:

Recruitment will be primarily from the NF1 and RASopathies Clinics using advertising materials we will submit for approval through the CCHMC IRB. These include emails and small flyers which are posted at CCHMC locations. We have controlled data from age matched children ages 8-16 years. We will use this data for comparison.

- 20 children ages 8-16 years with NF1

Inclusion criteria:

1. Males and females aged 8 - 16 years at time of enrollment whom meet NIH diagnostic criteria for NF1.
2. Participants must have a full-scale IQ of 70 or above, as determined by neurocognitive testing within the last 3 years or during the enrollment process.
3. Participants on stimulant or any other psychotropic medication should stay on a stable dose for at least 30 days before entering the study.

Exclusion criteria:

1. Participants should not be receiving chemotherapy currently, or have received chemotherapy in the 6 months prior to entering the study.
2. No active intracranial lesions (stable low grade glioma are acceptable) or epilepsy diagnosis.
3. Major Depression, Bipolar Disorder, Conduct Disorder, Adjustment Disorder, other major Anxiety Disorders, or other developmental psychiatric diagnoses, based on the child's history or on parent and child responses from the KSADS. Note that while this is an exclusion for participation in the study if there is a prior evaluation available, this becomes a criterion, *after inclusion,* for the investigator to withdraw the child from the study prior to completion if we identify this on the first study day.
4. For females, pregnancy.
5. Current use of antidepressants, non-stimulant ADHD medications, dopamine blocking agents, mood stabilizers.
6. Implanted brain stimulator, vagal nerve stimulator, VP shunt, cardiac pacemaker, or implanted medication port.
7. Asthma (bronchospasm has been reported as occurring infrequently and unpredictable when acetylcysteine is used as a mucolytica agent).
8. High risk of upper gastrointestinal (GI) hemorrhage. Examples: presence of esophageal varices or peptic ulcers

Inclusion of women and minorities

We will include males and females in numbers consistent with the distribution expected in the clinic populations. We will not exclude any participant based on race or ethnicity.

PROCESS OF OBTAINING INFORMED CONSENT:

The informed consent process will involve a member of the study team and the parent and child. If the individual is recruited over the telephone or by email (e.g., responder to advertisement) he or she will be mailed a consent form and a booklet of questionnaires, and scheduled to attend an evaluation. At the evaluation, the research staff will again explain the study goals and procedures and obtain written consent.

Participation in this study will not change current medical treatment. Permission of the primary care/attending physician will not be sought.

Prior to study entry, all children recruited will undergo an initial screening lasting up to 15 to 20 minutes with a parent to determine their general suitability for inclusion or exclusion. The primary diagnostic screen will be the KSADS-PL. This screening may be done in person or on the phone. If determined to be eligible during the initial screen, consent will occur in person. Following the consent process, a set of behavioral and neuropsychometric assessments will be administered and families will be informed if they are eligible for the next phase of the study. The questionnaires include the DuPaul ADHD rating scale, PARS (Pediatric Anxiety Rating Scale), CDRS (Children's Depression Rating Scale), C—SSRS (Columbia Suicide Severity Rating Scale), and KBIT-2 (Kaufman Breif Intelligence Test, Second Edition).

Parents will deliver an enclosed teacher rating scale packet, with questionnaires/rating scales targeted to the classroom environment, to their child's teacher. Teachers will return completed rating scales to the lab via US mail using an addressed/stamped envelope provided by the lab. A letter will be included within the teacher packet indicating that, by completing the questionnaires and mailing them in, they provide implicit consent.

After initial screening and baseline (completed only on initial entry to the study) there will be approximately two on-site study visits completed per phase arm (NAC drug or placebo arm) (Table 2), involving approximately 120 minutes of diagnostic, motor, and neuropsychological assessment, a 90 minute TMS session, and 60 minutes of actual scan time (total = 5-6 hours). The administration of all interviews and cognitive testing will also be standardized with respect to personnel. A third day of testing may be added to accommodate the subjects' schedules and all research evaluations.

Short Form Consenting Process:
For participants who do not speak English, we will utilize the short form consent process. A translator or qualified bilingual staff member will be present for the consent process. Dr. Prada may conduct the consent process with Spanish-speaking participants. The participant will sign both a short form and the full consent document.

A separate consent form will be signed by each participant. Subjects 11 years and older will provide documented assent via a signature on the consent form.

<u>Documentation of informed consent when using a short form consenting process:</u>

1. The participant/LAR signs and dates the short form on the appropriate line.
2. The person authorized to obtain the consent signs and dates the summary document on the appropriate line.
3. The 'witness' (as defined above) signs and dates both the short form and the summary document on the appropriate line.

Recruitment	Screening: Visit 0	Determine Eligibility	Study Visits 1 and 3 (Phase Initiation)	Study Visits 2 and 4 (Phase End)	Study Visit 5 (Debriefing Interview)
	- Consent/Assent - Telephone screen - MRI (optional)		- Physical Exam - Vitals - Urine pregnancy test	- Physical Exam - Vitals	- Review performance and tolerability data

	- Behavioral and Neuropsychometric Assessments: • KSADS (w/parent) • PARS • CDRS DuPaul ADHDPARSCDRS • KBIT-2 • PANESS • C-SSRS		- Blood draw for metabolomics and safety labs - AE Assessment - Med Log Review - Behavioral and Neuropsychometric Assessments: • ADHD-RS • PARS • CDRS DuPaul ADHDPARSCDRS • PANESS • C-SSRS • BRIEF (optional) - TMS rSICI/fSICI	- Blood draw for metabolomics and safety labs - AE Assessment - Med Log Review - Behavioral and Neuropsychometric Assessments: • ADHD-RS • PARS • CDRS DuPaul ADHDPARSCDRS • PANESS • C-SSRS • BRIEF (optional) - TMS rSICI/fSICI	- Follow-up care plan with referral for follow-up treatment - Transition medication (if needed)

STUDY PROCEDURES:

Screening and Baseline Assessment (Visit 0):

All participants will receive a full psychometric and neurological examination at baseline, and be screened with a comprehensive assessment battery designed to guarantee that they meet inclusion/exclusion criteria for the study, that they are candidates to receive NAC being offered in the trial, and that a robust database is available regarding NF1 motor/learning behavior symptomatology, comorbid symptomatology, broader psychosocial function, neuropsychological function, academic performance, severity and impairment, and medical status. Some participants may undergo a short, optional pre-treatment, unsedated research MRI for structural and DTI. Baseline assessments may be collected at screening visit 1. Per FDA *Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials*, an assessment for suicide (Columbia Suicide Severity Rating Scale, C-SSRS) will be performed at this visit.

Phase Initiation (Visits 1 and 3):

The second visit will be for baseline testing in a med-free state. All participants will receive a full physical and TMS testing (visits 1 and 3) as well as psychometric and neurological examination (at visit 3 only if baseline measurements were obtained at visit 1). At the end of visit 1 and 3, participants will be placed on 70 mg/kg/dose (max dose 900 mg) three times per day of NAC or placebo. Per FDA *Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials*, an assessment for suicide (Columbia Suicide Severity Rating Scale, C-SSRS) will be performed at this visit.

Monthly Medication Monitoring and Safety Assessment (Phone calls,e-mails, and/or visits).

The study physician (Dr. Prada, or qualified designee) is responsible for evaluating the participant's clinical state each month, including both the progress of treatment and the occurrence of adverse events/tolerability problems. The study physician will interview child and family regarding level of clinical improvement and tolerability. They may obtain the participant's weight, pulse and blood pressure. Concomitant medications and adverse effects (e.g. SERS) will be reviewed (in person or via phone or email). Dr. Prada or designee will also question the subjects as to compliance with study medication. If needed Dr. Prada or designee may ask for the participant to bring in medication bottles to check pill count and can also check to see if the prescription has been filled/refilled at the reported times. At a monthly medication visit, the study physician or designee will: 1) do vitals; 2) evaluate adverse events both spontaneous and elicited, 3) and fill out medication log/assess compliance. Per FDA *Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials*, an assessment for suicide (Columbia Suicide Severity Rating Scale, C-SSRS) will be performed.

End of Phase I and End of Study visits (Visit 2, 4).
Participant will undergo a TMS evaluation at completion of 8 weeks of NAC or placebo. An independent rater will assess clinical outcome with the primary outcome measure (PANESS scale), the same rater that performed the baseline neuropsychometrics assessment (whenever possible). Participants will also meet with Dr. Prada and Dr Gilbert to conduct the study closeout visit which consists of 1) vitals; 2) evaluate adverse events both spontaneous and elicited, 3) fill out medication log/assess compliance, 4) assess collateral symptoms of depression/anxiety which may have developed during the trial, 5) evaluate parent ratings, 6) write a progress note documenting the visit, 7) assign motor/learning behavior improvement, 8) meet with participant and family to review the child's performance during the trial to present a set of clinical recommendations for future care (not restricted to medication, and not for a specific medication). Per FDA *Guidance for Industry: Suicidal Ideation and Behavior: Prospective Assessment of Occurrence in Clinical Trials*, an assessment for suicide (Columbia Suicide Severity Rating Scale, C-SSRS) will be performed.

Table 3: SCHEDULE OF STUDY VISITS									
	Visit 0 Screening	**Visit 1 Initiation Phase I**	**Remote monitor and safety**	**Visit 2 End Phase I**	**Interphase (No meds)**	**Visit 3 Initiation Phase II**	**Remote monitor and safety**	**Visit 4 End Phase II**	**Visit 5 Debriefing Interview**
Task	**Week -1**	**Week 0**	**Week 4**	**Week 8**	**Week 9-10**	**Week 10**	**Week 14**	**Week 18**	
Review Eligibility Criteria	X								

Informed Consent	X								
KSADS PL Interview	X								
KBIT-2 (Kaufman Breif Intelligence Test, Second Edition).	X								
MRI (optional)**	X								
PARS (Pediatric anxiety rating scale)	X		X	X		X	X	X	
CDRS (Children's depression rating scale)	X		X	X		X	X	X	
Physical Exam*	X	X		X		X		X	
Vitals*	X	X		X		X		X	
Urine Pregnancy (Females only)	X	X				X			
C-SSRS (Columbia Suicide Severity Rating Scale)	X	X	X	X		X	X	X	
ADHD-RS (DuPaul) - Parent		X	X	X		X	X	X	
Safety labs*		X		X		X		X	
TMS assessment		X		X		X		X	
Med Log		X	X	X		X	X	X	
NAC/Placebo Prescription		X				X			
PANESS scale		X		X		X		X	
Metabolomics		X		X		X		X	
Behavioral Rating Inventory of Executive Function (BRIEF) - Parent (optional)		X		X		X		X	
AE assessment (MedDRA)		X	X	X		X	X	X	X
Follow-up plan				X					X

* Indicates clinical procedures performed as standard of care

**Optional procedures: Study doctor to determine which participants are offered these procedures

Treatment with NAC: For this double-blind placebo controlled study without dose modification of NAC, each subject will be dosed with 70 mg/kg/dose (max dose 900 mg) three times per day of NAC for 8 weeks. The maximum daily dose will be 2700 mg (900 mg per dose, three times per day). We have treated 5 patients off-label with NAC at 900 mg twice daily with improved behaviors without side effects. NAC is a natural supplement that acts as an antioxidant and a glutamate modulating agent. It is widely available over the counter.

Monitoring and Managing Adherence to Treatment:
Pill counts and/or review of the medication log will be used to estimate compliance, along with queries about missed doses. We will require a standard of four days out of seven on treatment to obtain a rating, but will counsel the participant and family about the importance of adherence in such a situation. We will require a minimum of 5 days per week on treatment in each of the final two weeks of each treatment block, so that the end-of-treatment ratings are an accurate reflection of treatment. For the final study visit and pTMS assessment, subjects must have 100% compliance for the two days prior to testing or testing will be rescheduled within a reasonable 3-4 day window so that compliance can be assured.

Medical/safety assessments:
During screening, children will receive a review of systems and physical examination, laboratory measures (CBC, basic chemistries with liver transaminase, thyroid function test, urinalysis), and pregnancy test (if

relevant). Pregnancy tests will be administered when deemed appropriate by Dr. Prada or designee. Results of the pregnancy test will be provided to the parent/guardian of the participant. If an uninterpretable urine pregnancy test is obtained or if the participant cannot/refuses to provide a urine sample, then a serum pregnancy test will be performed. All values outside the reference range will be assessed by the investigator, Dr. Prada, for clinical relevance.

Measurement and Management of Adverse Effects:
Participants and parents/guardians will be given the opportunity to report AEs to the investigator spontaneously in response to a general prompt (e.g. "Any changes in your health since the last visit?"). Terminology for adverse events (AEs) will follow the Medical Dictionary for Regulatory Activities (MedDRA) verson 20.1. (https://www.meddra.org). If AEs occur, there investigator will rate the seriousness of the event, whether the event was anticipated, and the likelihood that it is related to NAC. Serious events will be reported to the IRB and the study monitor within 24 hours of awareness. All other adverse events will be reviewed quarterly by the safety monitor.

Table 4: Medical Dictionary for Regulatory Activities (MedDRA)

MedRA System organ class	**MedDRA preferred term**
Nervous system disorders	Headaches, Dyskinesias, Disturbances in initiating and maintain sleep,
Psychiatric Disorders	Anxiety symptoms, decreased or increased physical activity levels, depressive symptom
Gastrointestinal Disorders	Nausea, vomiting, constipation

MRI (optional):
The MRI protocol will be comprised of imaging sequences providing high-resolution detail of brain tissue structure. A 3D T1-weighted volume will be acquired with ≤ 1 mm isotropic resolution and strong contrast between gray matter and white matter tissue. This image volume will be used for anatomic reference and may also serve to provide voxel-level volumetric assessments. A diffusion-tensor imaging (DTI) sequence (≤ 2 mm isotropic resolution) will follow, which is directionally sensitive to the rate of water diffusion in brain tissue. This sequence will measure diffusion rates in 32 or more directions to allow voxel-wise determination of diffusion anisotropy. This is especially useful in white matter, where restricted diffusion of axonal structures leads to a high degree of anisotropy that reflects tissue integrity.

The T1-weighted image will be processed using one of the commonly available software packages for MRI, including FSL (http://www.fmrib.ox.ac.uk/fsl/) or SPM (Wellcome Trust Centre for Neuroimaging). Processing will include segmentation into gray matter, white matter, and cerebrospinal fluid tissue types and normalized by transformation into a common spatial template (MNI). DTI volumes will have been aligned to each other and with the T1 anatomic image and normalized to the same template space before processing in FSL to extract mean diffusivity (MD) and fractional anisotropy (FA) on a voxel-wise basis. In healthy white matter, diffusion will be greatest along axonal fibers and FA describes the degree to which axial diffusion exceeds transverse diffusion. Images may be processed in other ways to extract additional structural information. The T1 segmentations may be analyzed as tissue probability maps for voxel-based morphometry. The DTI data may allow axonal fiber tractography to ascertain structural connectivity between brain regions.

Since all images will have been normalized to the same space, voxel-wise comparisons across subjects will be possible. Our primary imaging outcomes in white matter will be voxel-wise comparison of FA

between NAC and Placebo groups and regional correlation between FA and neuropsychometric outcomes. Other imaging measures, some outlined above, may also undergo these comparisons.

Clinical Scales:

ADHD – Dupaul ADHD-RS, parent form.

This 18 question scale is based on the DSM-5 and is widely used in clinical trials and routine care.

Behavioral Rating Inventory of Executive Function (BRIEF)

The Behavioral Rating Inventory of Executive Function (BRIEF) is a parent-reported symptom rating form that is used regularly in NF1 research. This assessment measures executive function behaviors at home and at school for children and adolescents (age 5–18). This scale would give a sense of if any changes are occurring in real-world executive function.

Pediatric Anxiety Rating Scale (PARS) [21]

The PARS is a clinician rated scale that is completed following interview with the parent and child. The PARS has two sections, a symptom checklist and severity items, each of which is linked to the past week. We chose PARS over several possible self-report scales because young children may not be able to complete a self-report scale.

Children's Depression Rating Scale (CDRS) [22]

The CDRS is a clinician-completed scale that rates presence and severity of depressive symptoms, based on interviews with the parent and child separately. There are 17 behavior items of affective state, rated dimensionally normed for children 6-12 years.

Cognitive Function.

Each child will be administered a psychological screening battery by trained research assistants under the supervision of a licensed psychologist, to assist in evaluation of inclusion and exclusion criteria, and to identify co-morbid disorders (e.g. learning disorder). Assessment of cognitive function will be used to assess suitability for the study and to describe the sample. Within the constraints of limited time and resources, we have selected the following tests to evaluate cognitive functioning and learning problems. ***KBIT-2 (Kaufman Breif Intelligence Test, Second Edition)*** [23] validated for use in 6-12 year olds, consisting of 2 subtests (Vocabulary, Similarities) assessing verbal IQ and 2 subtests (Block Design, Matrix Reasoning) assessing performance IQ will be used with an administration time of 30 minutes which will provide an estimate for performance IQ, verbal IQ and full scale IQ.

PANESS scale:

The primary battery of interest for this study is the PANESS, as described elsewhere. This test measures timed movements, lateral preference, motor overflow, dysrhythmia, coordination, gait, balance, and motor persistence. The PANESS will be administered by a research assistant in Dr. Gilbert's lab. Dr. Gilbert's staff was trained in PANESS administration by the team at the Kennedy Krieger/Johns Hopkins, where the PANESS was originally developed, and has performed this examination as part of multiple NIH funded R01 studies. This will be performed independently of and blinded to clinical ratings.

Transcranial Magnetic Stimulation-paired pulse paradigm (pTMS):

After application of rigorous exclusion and safety criteria, as employed in prior studies, informed consent will be obtained for the procedure, as is standard of care when the procedure is performed. This includes

discussion with children and families of the possibility of transient side effects such as headache. pTMS will be performed using a Magstim 200® stimulator (Magstim Co., New York, NY, USA) connected through a Bistim® module to a 90 mm circular coil. The coil is placed with its center near the vertex in the optimal position and orientation for producing a motor evoked potential (MEP) in the right first dorsal interosseous (FDI) muscle. The EMG is recorded from the right (or dominant) FDI with surface electrodes, amplified, and filtered (100/1000 Hz) (Coulbourn Instruments, Allentown, PA) before being digitized at 2 kHz and stored for analysis using Signal® software and a Micro1401 interface (Cambridge Electronic Design, Cambridge, UK).

The resting MEP threshold (RMT) is measured using a method similar to that described elsewhere [25] by setting the stimulator to 20 % of maximum output and increasing by 10 % increments until a MEP is obtained. The intensity is then decreased in increments of 1 % of maximum stimulator output until an intensity is reached where 10 averaged stimuli failed to produce a MEP. Active MEP threshold (AMT) is determined by having the participant moderately contract the APB with auditory feedback to maintain a constant level of EMG. The stimulator output is decreased in increments of 1 % of maximum output from the RMT until 10 averaged stimuli fail to show a MEP above background.

SICI inhibition is measured in resting muscle with a paired-pulse paradigm using paired vs. single pulse conditions [26]. The first condition is a single test pulse, delivered at an intensity that consistently produces a 500-1500 mV MEP. In the second condition, a subthreshold stimulus (60% of RMT) is followed after by a test pulse. Twenty trials are performed for the single-pulse condition, and 20 each for the other interstimulus intervals 3 ms, 10 ms. Relative to the single pulse response, the 3 ms interval is inhibitory (SICI) and the 10 ms interval is excitatory (intracortical facilitation). The order of the intervals is varied randomly, and the interval between trials varies randomly by <20% around a mean of 6 seconds. SICI is expressed as a ratio of the mean MEP amplitude after the conditioned pulses divided by the mean MEP amplitude after the single pulses. A detailed review of systems will be obtained before and after TMS, and participants will be provided the number of the investigator and study coordinator to call if there are concerns about possible side effects after the laboratory visit.

A complementary functional neuroimaging technique is Magnetoencephalography (MEG). MEG tracks neuronal activity in the brain with respect to time. Clinical and research applications of MEG include the identification of regions affected by pathology as well as analysis of motor, cognitive, and behavioral brain processes. To further elucidate the relationship between neuronal activity and specific mental functions, MEG data will be collected in addition to the TMS data. This additional subset of data will be useful if TMS data collection does not provide statistically significant results.

Metabolomic Analyses:
To account for bioanalytical complexity and variance from experimental and instrumental noise, we will use randomized sample repeating design and data preprocessing procedures to validate ion features and signal intensity. A computational pipeline built in-house will be used to prioritize ion features for identification of biomarkers of the NF1 [27]. At first, univariate data analysis was applied in parallel on all features using both parametric and nonparametric statistical methods to discover those features significantly altered between different phenotypes. Bonferroni and false discovery rate (FDR) correction are used to control family wise error in multiple hypothesis testing. Multivariate data analysis including random forest and partial least square discriminant analysis (PLS-DA) are applied to select informative biomarkers using covariance and correlation characteristics of features. To model and describe the constraint stoichiometry, network analyses are conducted to extract meaningful associations. A ratio correlation network and a weighted correlation network analysis (WGCNA) are used by measuring topological characteristics for biomarker candidate selection and identification. After identification of these putative signature metabolites, a targeted approach will be developed to acquire accurate levels of these markers. With the

comparative intensity/concentration results of identified metabolites, a subsequent metabolic network reconstruction will be carried out to reveal the underlying biochemical pathways perturbed in NF1 and motor and learning behavior.

Measurement of LPC-Autotaxin-LPA axis:
We will perform serum targeted analysis for LPC, autotaxin activity and LPA measurements pre and post-treatment. We will perform a highly specific mass spectrometry target analysis of LPC species from serum for clinical correlation. We will use authentic standards to accurately measure low and high levels of these biomarkers. This technique will be more reliable and reproducible for clinical applications. We will quantify autotaxin enzyme activity using a fluorometric assay to correlate with LPC measurements (Echelon Biosciences, K-4100). Samples will be collected in EDTA free tubes to avoid interference with analysis per manufacturer recommendations. We will quantify LPA using serum ELISA using the validated kit from Biosource (Reference MBS2024826). In the event that a significant change in autotaxin levels is not observed, we will evaluate untargeted global metabolomics profiling to identify possible disease biomarkers that are affected by NF1 and by treatment with NAC.

9. DATA ANALYSIS/METHODS:

Data Analysis and Data Monitoring-
Data will be maintained on the central servers of Neurology and Human Genetics at Cincinnati Children's Medical Center.

Statistical Analysis:
The purpose of this study is to generate preliminary estimates of effects NAC in NF1 on both behavior and potential biomarkers. Therefore we are not proposing a sample size calculation.

In general, our statistical analysis plan is based upon generalized linear models, particularly linear and logistic regression. The primary dependent variables for Aims 1 and 2 are continuous. For Aim 1, the primary behavioral outcome, which is also the primary outcome for the study, is the Dupaul ADHD rating scale score which rates ADHD behaviors, per the DSM5, in 18 domains, from 0 to 3, for a maximum of 54. Pre and post treatment ADHD will be evaluated with paired t test, with regression analysis to explore additional variables as per below. For Aim 2, PANESS scores and motor evoked potential amplitudes are the primary biomarker measures in the motor system. Analyses of these variables will also be performed pre- and post- treatment with paired t test. These variables will also be correlated, using Pearson or Spearman as appropriate, with the behavioral changes from Aim 1.

For analyses of change over time, mixed models which include subject-specific random effects will be used to account for the correlation of multiple measurements on each subject and capture between-subject variation in rates of change. Unless otherwise noted, the Type I error rate for hypothesis tests and confidence intervals will be controlled at α=0.05. To assist interpretation, the size of effects of interest in each model will be represented by R^2 (proportion of outcome variation explained by predictors) and/or Cohen's *d* (difference in group means, in standard deviation units). These effect measures are mathematically related, $d=2r / \sqrt{(1-r^2)}$. In the case of logistic regression, effect sizes will be given in terms of odds ratios, while predictive accuracy will be assessed using cross-validation procedures.

Exploratory/descriptive data analysis will precede all inferential analyses to ensure that distributional assumptions are satisfied. In addition to computation of basic descriptive sample statistics (measures of central tendency, variability, and bivariate association), graphical tools such as histograms and scatterplots will be used to assess, e.g., approximate normality, linearity, and presence of influential outlying observations. If substantial violations of parametric assumptions are detected, we will implement non-parametric alternatives, including classical rank-based procedures as well as computationally-intensive sample re-use procedures such as the non-parametric bootstrap. Non-parametric procedures generally have

less power than parametric procedures when the assumptions of the latter are met, but often have markedly better properties when the assumptions fail.

In the event that a participant is lost to follow-up, an incomplete series of observations can still be analyzed using the mixed-model methodology, provided that the dropout can be assumed to be missing at random in the sense that the probability that data are missing depends only on observed covariates (e.g., prior values of the outcomes, treatment assignment), rather than the unobserved values themselves. This assumption cannot be directly verified from the data. When it holds, however, mixed-model procedures yield the most efficient unbiased estimates, and implicitly impute the missing data using a subject-specific linear trend estimated from the non-missing observations. This effectively assumes that observed trends would have continued, in contrast to the "last observation carried forward" assumption that future observation would remain at a constant level equal to the last value. Neither assumption can be empirically proven correct. As a sensitivity analysis, therefore, the last available observation will be substituted for the protocol-defined last observation, provided that the participant had received at least one dose of study medication prior to the last observation. It is possible to determine whether participants who drop out are systematically different from those who do not with respect to observed data (e.g., baseline values, treatment assignment, demographic and clinical covariates). Logistic regression analyses will be used to explore whether such variables are predictive of dropout.

10. FACILITIES and PERFORMANCE SITES:
Division of Human Genetics
Cincinnati Children's Hospital Medical Center

Division of Neurology; TMS laboratory
Cincinnati Childrens Hospital Medical Center

11. POTENTIAL BENEFITS:
There is a potential for direct benefit by participating in this study if the treatment improves the symptoms of motor behaviors and learning associated with NF1. However, there is a possibility of no direct benefit to the participant.

12. POTENTIAL RISKS, DISCOMFORTS, INCONVENIENCES AND PRECAUTIONS:
a. Known and potential discomforts or hazards of single and paired pulse TMS:
Single and paired pulse TMS has been used at CCHMC under Dr. Gilbert's direction since 2001 for research only. Potential discomforts are mild and transient. In a prior study of 40 healthy and ADHD children, Garvey et al asked children to rank TMS compared to other childhood activities. TMS was ranked preferable to 1) a "shot"; 2) going to the dentist; and 3) a long car ride. The following mild, transient effects were reported in our prior study of 35 children and adults: scalp discomfort (12%), hand weakness (9%), headache, neck pain, arm pain, and arm tingling (6%), hand pain, decreased hand dexterity, hearing changes, and tiredness (3%). All of these had resolved by the following day. There were no physical findings after TMS supporting the subjective descriptions of loss of strength or dexterity. A prior common concern about use of TMS was the risk of seizures [28]. We follow recommended guidelines [28] and have seen no seizures in children or adults studied at our center. In addition, more recent studies even in children with epilepsy suggest that the risk of TMS inducing seizures is extremely low.

b. Known and potential discomforts or hazards of NAC
N-acetylcysteine (NAC) is FDA approved for treatment of acetaminophen overdose, respiratory disease due to mucous obstruction in acute and chronic settings in children and adolescent. Common side effects includes pruritus, rash, urticaria, diarrhea, nausea, and vomiting. NAC may not be effective for every participant, therefore participants may experience a continuance or worsening of their condition, including school performance or social interaction, while in the study.

c. Precautions, risk minimization:
The protocols include a number of precautions to minimize risk. The TMS laboratory was established in 2001 in consultation with Dr. Eric Wassermann, an internationally known researcher in transcranial magnetic stimulation. Dr. Gilbert or Dr. Wu will be present during TMS. All subjects will wear 34 NRR earplugs or headphones during TMS if the head is stimulated at an intensity of over 90%. Standard exclusion criteria are applied for participation in this study. Detailed questioning for any adverse events will occur after the experiment and the next day after the study. Access to the Magstim Stimulator is Limited – these TMS devices are kept in room 4 of the EEG lab on the seventh floor of the A tower. The laboratory is continually monitored by CCHMC during business hours and is locked at all other times.

d. *The method of monitoring study conduct.*
Adverse events will be reviewed using direct questions with a detailed review of systems on the day of the study. The principal investigator or designee is responsible for reporting adverse events to Cincinnati Children's Hospital Medical Center Institutional Review Board. Unanticipated events will be reported to both IRBs, according to the individual IRBs' requirements.

e. Methods for maintaining data quality and confidentiality:
Data are maintained in case report folders identified only by an anonymous subject ID. They will be kept in a secured area of Cincinnati Children's Hospital. All data kept in computerized files are in computers or a server with restricted, password-protected access. Only the primary investigator and designated study staff within his laboratory have access to case report folders and computer passwords (restricted by access level). A participant will be identified throughout the central database by his or her unique subject identification number (SID). Information which could identify a subject, such as name, address or social security number, will either not be stored electronically. A database audit trail of all data element changes over the life of the study will be maintained.

Metabolomics data will be kept in a separate data base without demographic and clinical information, and will be merged during the analytic stage of the study. Data entry, verification and validation will be carried out using our proprietary clinical data management system. We have a fire-wall-protected and local-network-based database management system for paper-based data collection, reports processing and information handling.

13. RISK/BENEFIT ANALYSIS
In the opinion of the investigative team, this study, based on our experience with NAC and with administering TMS as well as the published literature on NAC and TMS, involves minimal risk, with potential for direct benefit.

14. DATA SAFETY AND MONITORING
Data Safety Monitoring Plan:
14.1 Steps to be taken to assure the accuracy of the study data: The study team will review case report forms, source data, and spreadsheet entries once annually to ensure accuracy of data entry.
14.2 Adverse events and unanticipated events will be classified based on severity and attributed to individual components of study participation. For example, if a subject reports nausea at the end of the TMS baseline visit, this will be classified based on severity rating from the subject (mild, moderate, severe in the case report forms) and relationship to study tasks will be ascertained as well as possible (related to TMS, related to study medication, related to both, related to neither, unsure) on case report form.
14.3 A safety monitoring review will occur quarterly. The study team will submit a list of enrolled participants and any reported adverse events reported in the prior quarter. The study monitor will review these and produce, sign, and date a report summarizing safety and adverse effects and provide a conclusion

supporting one of 3 options: 1) continue the study unchanged; 2) modify the protocol/informed consent or other operational procedures; 3) stop the study. Either of the last two options will result in a report to the IRB and modifications as necessary. Jeff Tenney MD PhD has agreed to serve as the study monitor.

15. PRIVACY AND CONFIDENTIALITY

Privacy of the individuals participating in this proposed study will be maintained through non-identifying subject ID codes (SID), locked storage and password protected files. The study log will be maintained in a password protected folder on the desktop computer of the principal investigator. Case reports and spreadsheets will refer to subjects by number only. Subjects will be informed that, if necessary, the IRB or FDA may review the data.

16. COST OF PARTICIPATION

There will be no charges to participants or to third party payers related to study visits or procedures in this study. NAC is an FDA approved medication. NAC will be provided for the 8 week duration of the study to all participants.

17. PAYMENT FOR PARTICIPATION

Families will be reimbursed for transportation costs at $15 per visit completed during this study for a total of $75 per subject.

References

[1] G. G. A. S. M.T. Acosta, "Neurofibromatosis type 1: new insights into neurocognitive issues," *Curr Neurol Neurosci Rep,* pp. 136-143, 2006.

[2] M. D. B. M. e. a. Ballester R, "The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins.," *Cell. ,* pp. 851-859, 1990.

[3] P. R. Rosser TL, "Neurocognitive dysfunction in children with neurofibromatosis type 1.," *Curr Neurol Neurosci Rep. ,* pp. 129-136., 2003.

[4] J. K. v. d. F. F. B. N. K.-T. B. Casnar CL, "Relations between fine motor skill and parental report of attention in young children with neurofibromatosis type 1.," *J Clin Exp Neuropsychol. ,* pp. 930-943, 2014.

[5] J. J. W. P. e. a. Walsh KS, "Neurocognitive outcomes in neurofibromatosis clinical trials: Recommendations for the domain of attention.," *Neurology. ,* pp. S21-30, 2016.

[6] B. C. C. F. e. a. Acosta MT, " The Learning Disabilities Network (LeaDNet): using neurofibromatosis type 1 (NF1) as a paradigm for translational research.," *Am J Med Genet A,* pp. 2225-2232, 2012.

[7] P. J. W. R. S. E. R. C. N. K. Pride N, "Corpus callosum morphology and its relationship to cognitive function in neurofibromatosis type 1," *J Child Neurol,* pp. 834-841, 2010.

[8] K. E. W. X. e. a. Wang Y, "ERK inhibition rescues defects in fate specification of Nf1-deficient neural progenitors and brain abnormalities.," *Cell,* pp. 816-830, 2012.

[9] R. T. L. E. C. T. S. A. B. C. Karlsgodt KH, "Alterations in white matter microstructure in neurofibromatosis-1.," *PLoS One,* p. e47854, 2012.

[10] I. K. A. M. M. L. M. S. Gilbert DL, "Motor cortex inhibition: a marker of ADHD behavior and motor development in children.," *Neurology.,* pp. 615-621, 2011.

[11] W. S. W. J. e. a. Chen TH, " Reduced Short Interval Cortical Inhibition Correlates With Atomoxetine Response in Children With Attention-Deficit Hyperactivity Disorder (ADHD).," *Journal of child neurology.,* 2014.

[12] M. W. A. F. L. Y. L. N. F. A. M. F. B. F. J. Dennis, "Phosphodiesterase-Ialpha/autotaxin's MORFO domain regulates oligodendroglial process network formation and focal adhesion organization," *Mol Cell Neurosci,* pp. 412-424, 2008.

[13] E. M. Y. K. K. T. K. K. K. Y. K. F. A. Tokumura, "Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase," *J Biol Chem,* pp. 39436-39442, 2002.

[14] R. C. W. M. B. F. M.A. Fox, "Phosphodiesterase-Ialpha/autotaxin: a counteradhesive protein expressed by oligodendrocytes during onset of myelination," *Mol Cell Neurosci,* pp. 507-519, 2003.

[15] L. H. N. M. e. a. Chen YW, "Activation of mGluR2/3 underlies the effects of N-acetylcystein on amygdala-associated autism-like phenotypes in a valproate-induced rat model of autism.," *Front Behav Neurosci,* p. 8:219., 2014.

[16] R. T. T.-M. H. e. a. Mayes DA, "Nf1 loss and Ras hyperactivation in oligodendrocytes induce NOS-driven defects in myelin and vasculature.," *Cell Rep.,* pp. 1197-1212., 2013.

[17] F. L. D. M. L. Z. F. S. P. Garcia RJ, "A. Brief report: attention deficit and hyperactivity disorder scores are elevated and respond to n-acetylcysteine treatment in patients with systemic lupus erythematosus.," *Arthritis & Rheumatology,* pp. 1313-1318, 2013.

[18] D. O. D. S. M. G. B. M. Fernandes BS, "N-Acetylcysteine in depressive symptoms and functionality: a systematic review and meta-analysis.," *J Clin Psychiatry.,* pp. e457-466, 2016.

[19] F. L. L. R. e. a. Hardan AY, "A randomized controlled pilot trial of oral N-acetylcysteine in children with autism.," *Biol Psychiatry.,* pp. 956-961., 2012.

[20] D. O. C. S. e. a. Berk M, "The efficacy of N-acetylcysteine as an adjunctive treatment in bipolar depression: an open label trial.," *Journal of affective disorders.,* pp. 389-394., 2011.

[21] G. RUiPPAS., "Fluoxetine for the treatment of Anxiety Disorders in Children and Adolescents.," *NEJM.,* pp. 1279-1285., 2001.

[22] M. H. Poznanski E, "Children's Depression Rating Scale-Revised.," *Los Angeles: Western Psychological Association,* 1999.

[23] A. BN., "Validity of the Wechsler abbreviated scale of intelligence and other very short forms of estimating intellectual functioning.," *Assessment.,* pp. 17-23., 2002.

[24] W. D. W. I. A. T.--.-S. Edition., *San Antonio, Texas: Psychological Corporation,* 2001.

[25] N. K. Mills KR, "Corticomotor threshold to magnetic stimulation: normal values and repeatability.," *Muscle and Nerve.,* pp. 570-576., 1997.

[26] Z. U., "Intracortical inhibition and facilitation in the conventional paired TMS paradigm.," *Electroencephalogr Clin Neurophysiol Suppl.,* pp. 51:127-136., 1999.

[27] Z. X. S. K. D. P. Erkan E, "Distinct urinary lipid profile in children with focal segmental glomerulosclerosis.," *Pediatr Nephrol.,* pp. 581-588., 2016.

[28] W. EM., "Risk and safety of repetitive transcranial magnetic stimulation: report and suggested guidelines from the International Workshop on the Safety of Repetitive Transcranial Magnetic Stimulation.," *Electroencephalogr Clin Neurophy,* pp. 1-16, 1996.