RESEARCH PROTOCOL:

Defining subgroups of mitochondrial disease and dysfunction in autism spectrum disorder

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RESEARCH PLAN

*Background and Significance/Rationale

This research aims to better understand abnormalities in mitochondrial energy metabolism, and consequences of such abnormalities, in autism spectrum disorder (ASD). Mitochondrial disease (MD) is the most prevalent metabolic disorder affecting children with ASD (1). A recent meta-analysis by Rossignol and Frye found that 5% of children with ASD meet criteria for classic MD and that children diagnosed with ASD and MD (ASD/MD) have clinical characteristics distinct from the general ASD population, thereby supporting the existence of an ASD/MD subgroup (1). This meta-analysis also found that about 30% of children in the general ASD population exhibit biomarkers consistent with mitochondrial dysfunction, although it is not known how many of patients in these studies qualify for an MD diagnosis since additional clinical and laboratory findings supporting the diagnosis of MD were not reported. A recent study compared electron transport chain (ETC) function in lymphocytes between ASD and typically developing (TD) controls to determine if children with ASD manifested mitochondrial dysfunction (2). This study reported that 80% of the children with ASD demonstrated lower than normal ETC function, consistent with mitochondrial dysfunction. However, this lymphocyte study did not use standardized criteria to diagnose MD in the ASD children, leaving open the question of whether the children with ASD and mitochondrial dysfunction had classic MD or a level of mitochondrial dysfunction that was not severe enough to be considered MD.

This project will examine mitochondrial function in a wide range of ASD children with a minimally-invasive assay. The assay will use the Seahorse XF Extracellular Flux Analyzer 96 (Seahorse Bioscience, Inc, North Billerica, MA), a new state-of-the-art technology that simultaneously measures oxygen consumption (a measure of mitochondrial respiration) and glycolysis (a measure of anaerobic metabolism) in real-time in patient derived leukocytes. Our preliminary evidence below demonstrates that some children with ASD/MD demonstrate ETC complex over-activity, particularly complex IV over-activity, rather than ETC complex under-activity that is typical for classic MD. Additional preliminary evidence demonstrates that lymphoblastoid cell lines (LCLs) derived from ASD patients without known MD also demonstrate over-activity as manifested by high levels of oxygen consumption (measured by the Seahorse Analyzer) as compared to unaffected control LCLs. As complex IV activity is directly related to oxygen consumption these data suggest a unique profile of mitochondrial function in individuals with ASD regardless of whether or not they have MD. Whether or not this over-activity translates to greater ATP production is not known from this preliminary evidence but this question will be addressed in this project. Further preliminary evidence demonstrates that complex over-activity, particularly complex IV over-activity, in our ASD/MD cohort may be a compensatory process necessary for maintaining adequate redox metabolism. Parallel to this we demonstrate that LCLs have biomarkers consistent will higher levels of reactive oxygen species (ROS), suggesting that the over-activity in the LCLs may also be important for compensating for chronically increased ROS. We demonstrate that acutely challenging LCLs with increased ROS

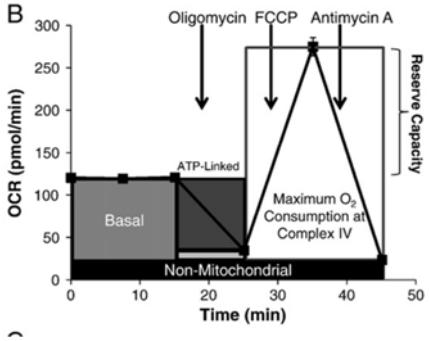
increases mitochondrial oxygen consumption but also produces a detrimental effect on mitochondrial function that is greater for ASD LCLs as compared to the control LCLs, suggesting that ASD LCLs are more metabolically fragile.

One of our goals is to develop a method using the Seahorse Analyzer to measure individual variations in mitochondrial function which can identify ASD children with MD and mitochondrial dysfunction without an invasive muscle biopsy. We believe that an assay that measures aerobic and anaerobic metabolism at baseline and during challenges of ROS will provide unprecedented new knowledge about mitochondrial function and dysfunction in ASD. We believe that this assay will allow us to answer important questions, such as whether mitochondrial dysfunction in the general ASD population is more prevalent and distinct from MD, and how mitochondrial dysfunction and secondary effects of mitochondrial dysfunction, specifically increased ROS, influence core ASD symptoms including language and social development. To this end we will develop profiles of mitochondrial function for individuals with ASD and known MD (ASD/MD) and individuals with ASD known not to have MD (ASD/NoMD), along with children that have a MD without ASD and developmentally delayed (DD) children or neurodevelopmentally atypical [NDA] (e.g. ADHD). These two groups (i.e., ASD/MD and ASD/NoMD) will serve as the two ends of the spectrum of mitochondrial function in children with ASD. We will then examine the profiles of mitochondrial function in a general population of individuals with ASD to determine how these individuals fall into this spectrum defined by the ASD/MD and ASD/NoMD groups, as well as how these subgroups differ from children that have a MD without ASD and children that are DD without either ASD or MD. Additionally, we will investigate the relationship between mitochondrial function, glutathione redox metabolism, and core ASD symptoms.

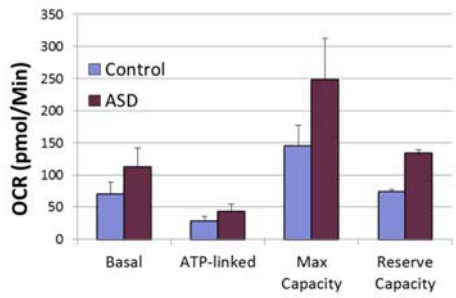
A Subset of Children with Autism Spectrum Disorder Demonstrate Electron Transport Chain Overactivity. Although the majority of studies that have estimated ASD/MD prevalence in the general ASD population have used the modified Walker criterion to define MD (3), there are some serious limitations to this criterion. This criterion relies on significant reduction in ETC complex function and known mitochondrial DNA mutations (3). However, only 23% of children with ASD/MD have a known mitochondrial DNA mutation (1) and reports have noted that some children with ASD/MD have ETC over-activity rather than deficiencies (4, 5). Thus, Frye and Rossignol (6) suggest that the Morava et al. criterion (7), a standard criterion that considers a wide variety of clinical, metabolic, imaging and morphological findings, many be more sensitive for diagnosing MD in children with ASD. ETC complex activity measurements were performed by the Baylor Medical Genetic Laboratory in Houston, TX (8) on frozen muscle from 20 individuals with ASD diagnosed with MD using the Morava et al. criterion (7) and normalized by citrate synthase activity. Examination of ETC function across these 20 individuals revealed significant variation in ETC function from low (i.e., <50% of normal) to very high (i.e., > 250% of normal). One subset of patients had low overall ETC activity, most consistent with a classic MD, while a second subset of patients had ETC complex over-activity (i.e., >= 150%) particularly in complex IV. Frye and Naviaux (4) recently published a case-series of ASD/MD children

with complex IV over-activity from this cohort. All cases had regressive autistic disorder with many having epilepsy, epileptiform EEGs, cerebral folate deficiency and potentially pathological mitochondrial DNA mutations. **Our preliminary data presented below links this over-activity with a more oxidized microenvironment. This many represent a compensatory process to counteract chronic increases in ROS or may reflect a greater level of ETC activity needed to maintain adequate ATP levels. Thus, ETC complex over-activity may not translate into increased energy (i.e., ATP) production (this notion will be considered within this project). This project will define mitochondrial metabolism profiles in leukocytes from children with ASD/MD using new state-of-the-art Seahorse Analyzer. This cohort will provide a positive control for which to compare leukocytes from a general population of children with ASD. This will allow us to determine whether there are specific subgroups of ASD children with MD and/or a milder form of 'mitochondrial dysfunction'.**

<u>Measuring Mitochondrial Energetics with the Seahorse Analyzer</u>



We used the Seahorse Analyzer to evaluate mitochondrial function in lymphoblastoid cell lines (LCLs) derived from individuals with ASD and unaffected TD controls. This new state-of-the-art instrument is capable of simultaneously measuring the rate of cellular oxygen consumption (OCR) and glycolysis (extracellular acidification rate) in real time in intact living cells. Several aspects of mitochondrial functional activity are defined in the assay by sequential addition of pharmacological inhibitors designed to probe individual components of mitochondrial function as diagrammed in the left Figure. Initially, Basal OCR is measured. Then, oligomycin, an ATP synthase inhibitor, is automatically injected to determine the proportion of the Basal OCR linked to ATP production. The Maximal Respiratory Capacity of the mitochondria is then determined by injecting the mitochondrial protonophore FCCP to collapse the inner membrane proton gradient, thus stimulating the ETC to work at maximum capacity to restore it. Reserve Capacity is then calculated by subtracting the Basal OCR from the Maximum Respiratory Capacity. Lastly, non-mitochondrial OCR is measured by automatic injection of antimycin A to inhibit electron flux through complex III.



The Figure to the left shows our preliminary data comparing Basal and ATP-linked OCR, Maximum Respiratory Capacity and Reserve Capacity in ASD and TD control LCLs (n=5/group). Each of these parameters was significantly elevated in the ASD cells, as compared to TD cells, under baseline conditions. Although the OCR is used to represent mitochondrial (aerobic) activity, only complex IV consumes oxygen, so OCR most closely represents complex IV activity. Thus, the LCLs from ASD

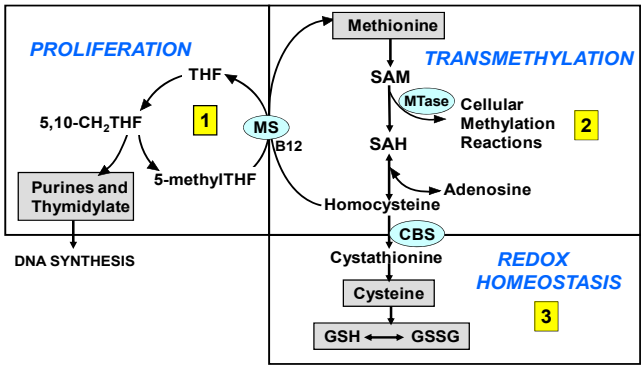
individuals parallel the complex IV over-activity identified in some ASD/MD children, suggesting that complex IV over-activity could be a unique characteristic of ASD mitochondria. Indeed, the Seahorse Analyzer may be an excellent instrument for measuring mitochondrial function in individual with ASD as over-activity may be particularly important. Since ASD individuals both with and without MD appear to have over-activity these two subgroups may or may not appear similar in Basal OCR. Thus, we will investigate a wide range of parameters of mitochondrial function derived from the Seahorse Analyzer, including parameters derived from a unique assay we will develop under this project which examines mitochondrial function under acute increases in ROS.

Oxidative Stress in Autism Spectrum Disorder

Glutathione is the major intracellular antioxidant responsible for maintaining redox homeostasis in the cytosol and mitochondria. Glutathione (GSH) is particular important in cells that have dysfunctional mitochondria, as mitochondrial dysfunction results in increased ROS. Three independent case-control studies conducted by Co-I James (9-11) documented that many ASD children have a GSH deficit in plasma and in the cytosol and isolated mitochondria from LCLs (Tables below). Further, the oxidized form of glutathione (GSSG) was significantly elevated in ASD children, resulting in a 2-fold reduction in the GSH/GSSG ratio (Table below).

Cytosolic and mitochondrial GSH/GSSG Redox Status in Control and ASD Lymphoblastoid cell lines (n=10/group)

	fGSH (nmol/mg protein)		GSSG (nmol/mg protein)		% oxidized GSH		fGSH/GSSG	
	Cytosol	**Mito**	**Cytosol**	**Mito**	**Cytosol**	**Mito**	**Cytosol**	**Mito**
Control	26.48±3.5	2.64±0.7	0.287±0.07	0.258±0.12	2.2±0.7	15.8±4.6	99.14±33.5	11.63±3.9
Autistic	21.72±4.3	1.75±0.3	0.356±0.06	0.366±0.11	3.2±0.6	29.1±4.7	61.81±10.6	5.06±1.3
p value	0.021	0.001	< 0.001	0.059	0.003	< 0.001	< 0.001	< 0.001

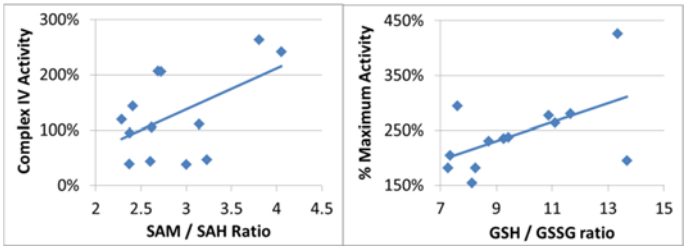


Several metabolic precursors for GSH synthesis were also lower suggesting that GSH synthesis may be insufficient to counteract oxidative stress. A deficit in GSH/GSSG antioxidant capacity is well known to promote oxidative stress and increase vulnerability to pro-oxidant environmental exposures and oxidative damage (12, 13). GSH synthesis is integrally related to folate-dependent one-carbon metabolism and methionine-dependent sulfur metabolism that provide the metabolic precursors for GSH synthesis. The three interconnected pathways leading to GSH synthesis are diagrammed in the Figure above. Pathway 1 is the folate cycle, Pathway 2 is the methionine trans-methylation cycle, and Pathway 3 is the

transsulfuration pathway leading to GSH synthesis. The vital importance of these interconnected pathways is underscored by their essentiality for error-free DNA synthesis (Pathway 1); for cellular methylation capacity (Pathway 2); and for the maintenance of cellular redox homeostasis (Pathway 3). The ratio of the methyl donor S-adenosylmethionine (SAM) to the product inhibitor S-adenosylhomocysteine (SAH) is a reflection of trans-methylation efficiency and cellular methylation potential. As diagrammed in the above Figure, methionine, SAM, SAH and cysteine are essential precursors for GSH synthesis and redox homeostasis. These data provide the basis to interpret the Seahorse Analyzer and mitochondrial complex over-activity data previously described. Based on our previous observations of elevated ROS and decreased mitochondrial GSH/GSSG in ASD LCLs relative to TD LCLs (14), we interpret our preliminary results to reflect an adaptive up-regulation of mitochondrial function in response to chronic elevations in ROS production in cells from ASD individuals.

Mitochondrial Electron Transport Chain Complex Overactivity Supports Redox Metabolism

The relationship between ETC function and biomarkers of redox metabolism was determined for a subset of the cohort of children with ASD/MD presented above. Measures of redox metabolism (GSH/GSSG and SAM/SAH ratio) as well as a measure of chronic oxidative stress (3-nitroTyrosine; 3NT) were investigated. The activity of each complex individually, the absolute maximum complex function, the relative maximum complex function (maximum activity / minimum activity) as well as the average complex function were investigated. Higher complex IV activity was associated with a higher SAM / SAH ratio (left panel in the figure below; r=0.53, p=0.05) and higher relative maximum complex activity was associated with a higher GSH/GSSG ratio (right panel in below figure, r=0.54, p<0.05). **Overall, these data point to ETC complex over-activity as a compensatory mechanism to support redox metabolism and are consistent with the preliminary ASD LCLs data described above. Indeed, both sets of data suggest that mitochondria from children with ASD, whether or not they have MD, demonstrate over-activity in attempt to maintain redox homeostasis in order to protect cells from oxidative damage. This project will examine these possibilities as understanding the exact relationships between cellular metabolic processes will improve treatment recommendations.**

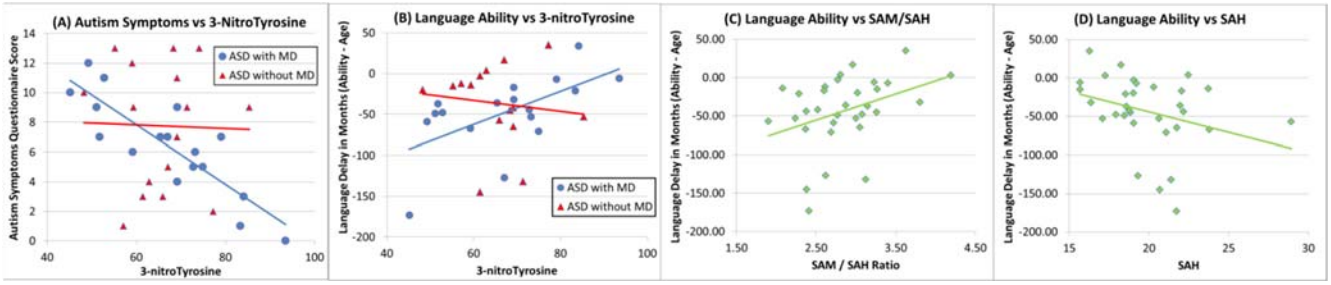


The Relationship Between Language Development and Autism Symptoms and Redox Metabolism is Moderated by Mitochondrial Dysfunction. The relationship between biomarkers of redox metabolism (GSH/GSSG and SAM/SAH ratios, 3NT) and ASD symptoms and language development was determined for a subset of the cohort of children with ASD/MD presented above and a comparison group of children with ASD without MD (ASD/NoMD) matched for gender and age. The difference between these relationships across groups was also considered. ASD symptoms were measured by the autism symptoms questionnaire, a checklist of DSM-IV symptoms developed by the

Center for Autism and Related Disorders, Inc (Tarzana, CA). To evaluate language we used the Preschool Language Scales-3 for children under 6 years of age and the Expressive One-Word Vocabulary Test for children older than 6 years. This combination of tests was used instead of a more extensive language assessment due to time constraints of the visit. To derive a measure of language delay that was comparable across the two language tests, the age equivalent score derived from either test was subtracted from the participant's age at testing. The studies proposed herein will use a comprehensive language assessment tool that spans a wide range of ages.

As seen in Panel A (figure below) the number of ASD symptoms was strongly related to 3-NT (protein oxidative damage marker) for the ASD/MD group [r=-0.85, r<0.01] but not the ASD/NoMD group [r=-0.03, p=ns; group x ASD symptoms interaction, F(1,28)=9.15, p<0.01]. Unexpectedly, higher 3NT levels were related to fewer ASD symptoms in the ASD/MD group. Similar findings were found for language ability (Panel B). Language ability was related to 3NT for the ASD/MD group (r=0.60,p<0.05), but not ASD/NoMD group (r=-0.12,p=ns; group x language interaction F(1,28)=4.43, p<0.05) such that better language development was associated with higher 3NT levels. These data demonstrate that the correlation between core ASD symptoms and redox metabolism differs between ASD children with and without MD.

In contrast, language ability was strongly associated with the SAM/SAH methylation ratio in both ASD/MD and ASD/NoMD groups (Panel C; SAM/SAH covariate F(1,28)=5.40,p<0.05) such that a higher SAM/SAH ratio was associated with better language ability in both groups. To understand whether this was due to SAM or SAH, we examined each independently. SAH, but not SAM, was found to be related to language ability such that lower levels of the methylation inhibitor SAH was associated



with better language ability [SAH covariate; F(1,28)=4.02, p=0.05]. Interestingly, none of the measures of ETC function in the ASD/MD group were significantly related to ASD symptoms or language ability.

Overall, these data demonstrate that chronically higher levels of ROS is related to better language development and fewer ASD symptoms in ASD/MD children but not in ASD/NoMD children. This may seem unexpected as ROS has been implicated in several neurodegenerative diseases, including Alzheimer, Parkinson and Huntington diseases as well as Friedrich's ataxia and amyotrophic lateral sclerosis (15) and has been implicated in synaptic loss in mitochondrial encephalopathies (16). However, it is important to note that elevations ROS also can have positive effects on development.

ROS is important for long term potentiation, a process that is essential for learning and memory, and can stimulate pathways responsible for increasing synaptic size and strength as well as synaptic vesicle recycling (16). In fact, ROS is necessary to stimulate cellular function, including mitochondrial function (17). These data suggest that an adaptive increase in ROS could facilitate development in children with ASD/MD, possibly by stimulating cellular pathways for growth, differentiation, synaptic development or by up-regulating protective mechanisms to counteract the destructive effects of ROS. It is also possible that this may simply reflect higher ROS levels as a by-product of abnormal ETC activity in ASD/MD children. Additionally, the relationship between language ability and the SAM/SAH ratio and SAH indicates that, in general, language development for children with ASD is related to adequate methylation capacity and/or the availability of glutathione precursors to provide adequate cellular redox buffering. **These data have important clinical implication as they point to the importance of adequate metabolic precursors for glutathione-mediated redox buffering but do not specifically support the reduction of ROS with extrinsic antioxidant treatment. In addition, these data indicate that mitochondrial dysfunction may have an indirect effect on language development and ASD symptom through alterations in redox metabolism. However, only a limited number of measures of mitochondrial function were investigated in our preliminary studies. This project will examine the relationship between a wide range of indexes of mitochondrial function and core ASD symptoms and redox metabolism to more definitively define individual mitochondrial dysfunction in relevant subsets of children with ASD.**

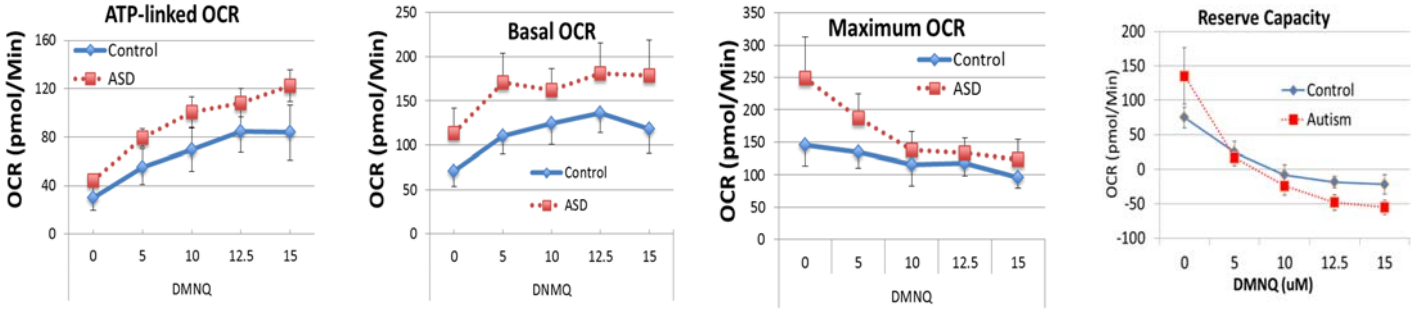
A Novel Assay Measures of Mitochondrial Response to Acute Increases in ROS

In order to increase the sensitivity of the Seahorse Analyzer assay for evaluating mitochondrial function in children with ASD and to confirm the hypothesis that mitochondria from children with ASD increase activity in response to ROS, we developed an assay that examines the effects of mitochondrial function in individuals with ASD to acute increases in ROS. ASD LCLs (and TD controls) were exposed to the redox cycling agent DNMQ for 1 hr before measurements. DNMQ enters cells and generates both superoxide and hydrogen peroxide similar to that generated by NADPH oxidase (18). In the Figures below we compared the impact of increasing DNMQ concentrations (5-15uM) on basal and ATP-linked OCR. In the ASD LCL cells, Basal and ATP-linked OCR remained significantly elevated at all DNMQ concentrations indicating that mitochondria from the ASD cells have a higher oxygen requirement for ATP synthesis at baseline and with increasing ROS. These data indicate that, as hypothesized, cells compensate for increased ROS by increasing activity and that mitochondrial energetics is indeed different in the ASD compared TD control cells, most likely due to increased ROS at baseline. These data provide strong support for our hypothesis.

Interestingly, baseline Maximum Respiratory Capacity (no DNMQ) was significantly higher in the ASD LCLs as compared to the TD LCLs (Figure above). One explanation is that the ASD LCLs have adapted to increased ATP synthesis in the context of a more oxidizing intracellular microenvironment. Another possibility is there is an adaptive increase in the number of mitochondria per cell (mitochondrial DNA copy number). In the experiments proposed within, we will examine both of these possibilities in leukocytes from children with ASD to better understand the basis for increased OCR and apparent mitochondrial over-activity.

The significance of increased mitochondrial activity in ASD LCLs is revealed by the fact that the apparent adaptive increase in Reserve Capacity is not maintained with increasing oxidative stress (Figure above). Indeed, the Reserve Capacity of the ASD LCLs, as compared to the TD LCLs, drops off more rapidly and to a greater extent as ROS is increased. This response indicates that ASD cells have an impaired ability to adapt to acute increases in ROS reflecting a more fragile redox homeostasis. We believe that cells from children with both ASD and MD will be more fragile since dysfunctional mitochondria results in a more oxidized microenvironment.

In the present study, we plan to adapt these measurements in LCLs to evaluate leukocytes in real time immediately after the blood draw. We do not anticipate difficulty in adapting these measurements to leukocytes from individually with ASD as the technique for measuring mitochondrial energetics using the Seahorse Analyzer in leukocytes has been developed (19). We will also measure intracellular GSH/GSSG, ATP production, mitochondrial DNA copy number, and the anaerobic metabolism under baseline conditions and after acute increases in ROS. Our studies will provide a better understanding of mitochondrial energetics and how cellular metabolism adapts to compensate for increased ROS in children with ASD. This project will establish the specific mitochondrial abnormalities and variation in mitochondrial function associated with ASD and lead to better care for the subgroup of children with mitochondrial dysfunction. This project will better define the proportion of children with ASD who have mitochondrial dysfunction which is currently estimated to between 5% and 80% of the general ASD population. This study will identify the impact of mitochondrial dysfunction on cognitive and behavioral symptoms in ASD and will shed light on potentially core pathophysiological processes that may contribute to ASD, thereby leading to more



targeted treatments for ASD. Most importantly, this technology can be easily adapted to screen infants to determine vulnerability to developing ASD and thus lead to preventive measures.

*Protocol Summary

Objective: The main objective of this proposal is to develop a minimally-invasive biological assay that can be widely used to identify variations in mitochondrial function and dysfunction in children with ASD. This assay will allow us to determine the true range of mitochondrial function and dysfunction across a population of children with ASD. The relationship between mitochondrial function and dysfunction in ASD, redox metabolism and core ASD symptomology will also be investigated.

Specific Aims: 1) To develop a minimally-invasive assay using immune cells and/or buccal cells that can accurately identify children with ASD/MD from the general ASD population. 2) To establish whether there is simply an ASD/MD subgroup or whether mitochondrial dysfunction occurs on a spectrum from mild to severe in ASD. 3) To determine the relationship between mitochondrial function and redox metabolism in children with ASD. 4) To evaluate the effect of mitochondrial dysfunction and glutathione redox status on language and social development and ASD symptoms. 5) To identify prenatal environmental factors associated with atypical mitochondrial activity in children with ASD using blood, buccal cell and tooth-based assays.
Study Design: Using the Seahorse Analyzer, we will develop profiles of mitochondrial function for individuals with ASD and known MD (ASD/MD) and individuals with ASD known not to have MD (ASD/NoMD). These two groups will serve as the two ends of the spectrum of mitochondrial function in children with ASD and will be compared to profiles from TD children. We will then examine the profiles of mitochondrial function in a general population of individuals with ASD to determine how these individuals fall into this spectrum defined by the ASD/MD and ASD/NoMD groups, as well as DD and no ASD/MD, and NDA and no ASD/MD. Glutathione redox metabolism will be measured in all participants. The relationship between mitochondrial function, redox metabolism and core ASD symptoms will be studied in the ASD groups with particular attention to whether ASD symptoms, language ability and adaptive behavior are directly related to mitochondrial function or indirectly related to mitochondrial function through its effect on redox metabolism.

Study Population: We will recruit several groups of children for this study: 50 children with ASD who have MD (ASD/MD); 50 children with ASD who do not have MD (ASD/NoMD); 50 no ASD/MD; 50 no ASD/no MD but DD or NDA; 100 TD controls; and a general population of 150 children with ASD.

Statistical Plan: Descriptive statistics, e.g., mean and standard deviation for continuous variables and number and percent for categorical variables will be obtained for all demographic variables, outcomes and predictors of interest. Data will be checked for errors, inconsistencies and missing values. Appropriate statistical methods will be used to test the specific hypotheses of interest.

Analyses will be adjusted for confounding variables such as age, gender, autism severity, etc. wherever necessary. Independence of predictor variables and homogeneity of variance assumptions will be checked prior to the analysis. P-values will be adjusted for multiplicity wherever appropriate. All tests will be two-sided and a P-value less than or equal to 0.05 will be considered to be significant. All analyses will be conducted using statistical software SAS v9.2 (SAS Inst, Cary, NC) or STATA 12 (STATA Corp, College Station, TX), or R (R Foundation for Statistical Computing, Vienna, Austria; (20)).

*Specific Aims/Objectives

Specific Aim 1: To develop a minimally-invasive assay using immune cells and/or buccal cells that can accurately identify children with ASD/MD from the general ASD population. The diagnosis of ASD/MD commonly requires an invasive muscle biopsy. This is particularly true in ASD as the majority of ASD/MD cases are not associated with mitochondrial DNA mutations (1) and many cases do not manifest lactate elevation (1, 2, 4, 20). Instead of using unreliable biomarkers and invasive muscle biopsies to diagnose MD in children with ASD, we will develop a simple method that allows the identification of ASD children with MD by examining mitochondrial function using the Goldenthal buccal cell assay and the new state-of-the-art Seahorse Analyzer in freshly obtained leukocytes. We will also examine ATP production, biomarkers of redox metabolism and/or mitochondria DNA copy number in these cells in case the energetic measurements cannot uniquely identify ASD/MD individuals. ***Hypothesis 1****: The buccal cells and leukocytes of children with ASD/MD, regardless of the specific mitochondrial defect, will demonstrate a unique profile of energy production defects, at baseline and/or with exposure to increased ROS, as compared to the buccal cells and leukocytes of ASD/NoMD individuals, that will be identifiable using the Goldenthal buccal cell assay and the Seahorse Analyzer.*

Specific Aim 2: To establish whether there is simply an ASD/MD subgroup or whether mitochondrial dysfunction occurs on a spectrum from mild to severe in ASD. We will approach this by comparing mitochondrial function in children with a wide range of children with ASD severities to children with ASD/MD and ASD/NoMD. ***Hypothesis 2****: Mitochondrial function in leukocytes and buccal cells from children recruited from the general ASD population will range from normal to severely dysfunctional.*

Specific Aim 3: To determine the relationship between mitochondrial function and redox metabolism in children with ASD. Both MD and ASD have been associated increased oxidative stress in in vivo and in in vitro models (14). Theoretically, glutathione should be depleted in children with ASD/MD or functionally significant mitochondrial dysfunction. Empirically demonstrating this in primary cells could lead to targeted treatments. ***Hypothesis 3****: In children with ASD/MD and the general ASD population, leukocyte GSH/GSSG redox status and glutathione precursor depletion will be related to mitochondrial function at baseline and after acute increases in oxidative stress as measured by the Seahorse Analyzer.*

Specific Aim 4: To evaluate the effect of mitochondrial dysfunction and glutathione metabolism status on language and social development and ASD symptoms. This will lead to a better understanding of how mitochondrial dysfunction is related to core symptoms of ASD. To facilitate this, we will recruit children with ASD with all degrees of severity. We approach this by determining if mitochondrial function and/or redox metabolism can predict cognitive development and ASD symptoms in ASD children. ***Hypothesis 4****: More severe mitochondrial dysfunction will be related to worse cognition and more severe ASD behavior both directly and indirectly through defects in redox metabolism.*

Specific Aim 5: To identify prenatal environmental factors associated with atypical mitochondrial activity in children with ASD using blood, buccal cell and tooth-based assays.
Environmental exposures will be compared between the two ASD mitochondrial groups and between ASD and controls. Exposures will be measured in two ways: 1) Gestational and early life exposures to heavy metals, pesticides, phthalates, acetaminophen, and inorganic and organic toxicants will be measured in baby teeth using mass-spectrometry based methods that have been well-validated; 2) The Environmental Exposure Questionnaire (EEQ) will obtain information on environmental, medication, infectious and inflammatory exposures prenatally and during infancy. ***Hypothesis 5:*** *exposure to environmental stressors result in changes in mitochondrial function that persist after the environmental stressor has dissipated.*

*Study Design and Procedures

Participants

Participants will consist of up to 250 children aged 0 years to 17 years 11 months. These 250 children will include up to 50 children with ASD diagnosed with a MD (ASD/MD), 50 children with ASD who do not have MD (ASD/NoMD), and a general population of 150 children with ASD and unknown MD status. Those children with ASD will have documentation of ASD (As defined by either a gold standard measure for ASD diagnosis: the Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview-Revised (ADI-R), and/or the minimum Arkansas state requirement for autism classification, as defined by a consensus diagnosis of ASD by a medical doctor, speech pathologist, and psychologist) or in an event where sufficient diagnostic information is lacking, and the PI believes that the clients meet all other inclusion criteria and a prospective diagnosis of an ASD is warranted, they may be considered potentially eligible. For those the PI believes a prospective diagnosis of ASD is warranted, a formal diagnosis will be scheduled to occur within a reasonable time frame from the date of study entry and prior to the conclusion of the trial. In addition this study will include 100 TD control children and 50 with MD but no ASD and 50 with no ASD or MD, but known DD or NDA.

Subject Recruitment

Children with ASD/MD, MD but no ASD, DD or NDA, general ASD, and ASD/NoMD will be recruited from children diagnosed at the Dennis Developmental Center (DDC) ATN Clinic whose families have

indicated in the ATN consent that they wish to be contacted about future research opportunities, from the AAA.org website for parents who have indicated that they are interested in research contact, from advertisement through local channels and BOOM text, by using social media through Arkansas Children's Hospital, flyers posted in the community (i.e. primary pediatric clinics), as well as referrals from pediatricians. Further description of these recruitment strategies can be found in the Recruitment Plan section. Potentially eligible children who wish to participate will be asked inclusion/exclusion questions. If the child qualifies, study staff will schedule the study visit and will attempt to mail or e-mail information regarding the scheduled visit to the parent(s). The consent form, parent questionnaires, and other helpful information (e.g. a map of the ACH and/or relevant published manuscripts to help better understand the trial) may be included with this information for review purposes. If the child has a diagnosis of ASD, DD, NDA, or MD, the parent will be asked to provide copies of diagnostic testing or to provide the names, address, and contact information of the place diagnostic testing was completed so that a release of information can be completed to allow study staff to obtain copies of diagnostic testing.

Recruitment may also occur for children (typically developing and non-typically developing) at conferences associated with neurodevelopmental disorders. Participant recruitment at these conferences will only be on the study populations listed in this protocol and no data collection will occur outside of what is approved and data collection will occur only after consent/assent is obtained.

Study Visits

After completing the screening interview where eligibility is determined, the study visit will be scheduled at which the parents will provide consent. The study visit does not require travel to the study site.

For study participants who do not visit the study site and who only complete questionnaires, we will obtain verbal consent from the parent using a phone script. The parent will be provided with an information sheet informing them about the study.

For study participants who complete more than just the questionnaires (e.g., provide baby teeth/buccal cells), the parents will sign the consent/HIPAA for the study after all of their questions have been addressed and return this signed form to research study staff. For study participants who are typically developing and 7 years of age or older (who complete more than just the questionnaires), the child will provide assent. The child will sign the assent after all of his/her questions have been addressed. If the child has a diagnosis of ASD, DD, NDA, or MD, the parent may be asked to provide diagnostic testing and/or to complete a release of information to allow study staff to obtain copies of diagnostic testing. For those who have been seen at ACH, diagnostic information may be obtained from medical records once the consent/HIPAA has been signed.

Following consent, each visit may include various general and/or cognitive-behavioral measures. The first time a measure is collected is to provide information for a cross-sectional relationship between autism symptoms and development to be correlated with mitochondrial function. The second time a measure is collected is to look at reliability. This will be enhanced with subsequent visits, up to 5, over the course of participation in this trial. Additional visits provide repeated measures to improve the fidelity of the analysis and look at reliability, but are not necessary for each participant. Due to the fact that many participants will be participating from out of state all visits and measures for each visits are optional. This is because we do not want to discourage potential enrollment due to the possibility of multiple visits or lengthy visits.

Subsequent visits (visits 2-5) will be scheduled when it is known if/when participants intend to return to ACH Campus and/or if the PI/parent requests an additional visit. If scheduled, visits 2-5 will occur within an approximate 1-72 months from visit 1. For visit 2-5, study staff may mail or e-mail information regarding the scheduled visit to the parent(s) prior to the scheduled date. If the consent form has been revised since the last informed consent discussion, the consent form and any new parent questionnaires may be included with this information for review purposes. In the event that the subsequent visit will only involve the completion of questionnaires, the parent will be provided an information sheet informing them about the study instead of the consent form. Any questionnaires which the parent already consented to may be included with instructions that the parent may complete these forms within 1 week of the scheduled re-consent discussion.

Measures which may be completed at each visit are listed below. All measures are optional and may be completed based on PI selection or the child/parent's willingness or ability to complete the measure. Data (questionnaires and/or samples) may be collected offsite at various conferences associated with neurodevelopmental disorders or completed at the parent's convenience and returned by mail/fax/e-mail.

<u>General Measures</u>

- Demographic Information including age, gender, ethnicity, and race,
- Information on Medication and Nutritional Supplements.
- Information on place of residence from prenatal to current

<u>Specimen Collections for Biochemical Measures</u>

- Baby teeth collection,
    - If the child has lost a tooth/teeth, the parent will be asked to provide baby tooth/teeth which may have been collected and stored after child lost by bringing them to the visit or mailing them to study staff.
    - If the child is still expected to lose a tooth/teeth, the parent will be asked to collect and store the tooth/teeth and provide them to the research site at the next visit or mail them to study staff.

- Buccal cell collection,
    - Buccal cell collection may occur during the visit at ACH or ACHRI in an enclosed room, in order to maintain confidentiality during collection.
    - Study staff will use a soft swab to gently swipe the inside of the child's cheek bilaterally for one minute each, twice per cheek. Upon collection, the swabs will be placed into a small plastic centrifuge tube labeled with the study ID number of the participant and collection date. These tubes will then be placed on ice (or ice bath) until it can be delivered to the research site laboratory.
    - Blood, urine, stool, GI biopsies, and muscle biopsies were previously collected as part of this study; however, they will no longer be collected from current participants.

<u>Cognitive-Behavioral Measures</u>

- Vineland Adaptive Behavior Scale (VABS)
    - May be completed as interview or questionnaire based on PI discretion, study staff availability, or parent willingness/ability to complete.
- Parent Questionnaires
    - Each questionnaire is optional, based on PI selection and parent willingness/ability to complete
    - If a questionnaire was completed for another purpose (e.g. clinical reasons or a different research study), study staff will verify that it was completed within the valid time frame for the questionnaire per industry standards. Time frames are indicated in the brief description of each questionnaire below. If valid, the original questionnaire or a photocopy will be obtained for research purposes and the parent will not be asked to repeat the questionnaire.
    - If applicable, study staff will provide the selected questionnaires along with a postage-paid, addressed envelope to the parent. The parent will be asked to complete the questionnaires and return them in the provided envelope.
    - Possible questionnaires to be completed include:
        - Aberrant Behavior Checklist (ABC)
        - Autism Impact Measure (AIM)
        - Child Behavior Checklist (CBCL)
        - Diet Diary
        - Environmental Exposure Questionnaire (EEQ)
            - only completed 1 time, not completed at subsequent visits if previously completed
        - Gastrointestinal Severity Index (GSI)
        - Health History and Mitochondrial Symptoms Questionnaire
        - Repetitive Behavior Scale – Revised (RBS-R)
        - Sensory Questionnaire
        - Social Communication Questionnaire (SCQ)
            - not completed by those with a confirmed ASD diagnosis

        - only completed 1 time, not completed at subsequent visits if previously completed
    - Social Responsiveness Scale (SRS)
- Teacher/Provider Questionnaires
    - Each questionnaire is optional, based on PI selection, availability of teacher or provider, and parent willingness
    - If applicable, study staff will provide the selected questionnaires along with a postage-paid, addressed envelope to the parent. The parent will be asked to give these items to the teacher/provider and request they complete the questionnaires and return them in the provided envelope.
    - Possible questionnaires to be completed include:
        - Aberrant Behavior Checklist (ABC)
        - Child Behavior Checklist (CBCL)
        - Repetitive Behavior Scale – Revised (RBS-R)
        - Social Responsiveness Scale (SRS)

Biochemical Measures

The following measures may be conducted on obtained specimen(s). Previously obtained specimens include: blood, urine, stool, GI biopsies, and muscle; however, these will no longer be collected from current participants. Specimens which may be obtained from current participants or have been previously obtained include: baby teeth and buccal cells. Measures will be selected based on PI decision as well as amount collected.

1. Mitochondrial Energetics*:* When possible, leukocytes and platelets will be isolated from a blood sample. One of several seahorse assays of mitochondrial respiration will be conducted these blood cells using the Seahorse Analyzer (Seahorse Bioscience, Inc, North Billerica, MA). When possible we will conduct the cell mitochondrial stress test. By using standardized compounds to modulate mitochondrial function (as described in the introduction), Basal mitochondrial respiration, ATP-linked mitochondrial respiration, Maximal Respiration Capacity and non-mitochondrial respiration will be measured. Reserve Capacity will be calculated from these measures. We will obtain these measures at baseline and during a ROS challenge using DNMQ at several concentrations (5 uM, 10 uM, 15 uM, 20uM, 25uM, and/or 30uM). We have already optimized parameters of this assay using LCLs derived from ASD and control subjects (see Background). In some cases, additional Seahorse assays may be performed on the isolated leukocytes and platelets, including electron transport chain (ETC) function in permeabilized cells, the fuel flex test and/or the glycolytic stress test. The ETC function assay is conducted by first permeabilizing the cells using a novel permeabilization reagent available from Seahorse Bioscience (PMP). Then the cells are fed ADP and substrates for ETC complexes (complexes I, II, III/IV, and IV). Using the same pharmacological inhibitors as the cell mito stress test, a coupling assay is performed to derive State 3, State 4, State 3 uncoupled and the respiratory control ratio (RCR) for each of the complexes. The fuel flex test measures the

dependency, capacity, and flexibility of cells to oxidize any one of three critical mitochondrial fuels (glucose, glutamine, or fatty acids) by measuring mitochondrial oxygen consumption of cells in the presence or absence of fuel pathway inhibitors, in live cells, in real time. The glycolysis stress test measures glycolysis in live cells in real time while inducing acute metabolic stress and allows for the measure of basal glycolysis, glycolytic capacity and glycolytic reserve.

2. Redox Metabolism*:* Active reduced glutathione (GSH); oxidized disulfide inactive form of glutathione (GSSG); cysteine (rate-limiting amino acid for glutathione synthesis); 3-nitroTyrosine and 3-chloroTyrosine (biomarkers of protein damage due to oxidative stress); 8-oxo-deoxyguanine (biomarker of DNA damage due to oxidative stress); methylation metabolites and precursors for glutathione synthesis (methionine, SAM, SAH, adenosine); These tests are routinely done in plasma and leukocytes in the James Laboratory (9, 10, 14).
3. Blocking Folate Receptor α Autoantibody: The autoantibody titers are measured by the laboratory of Dr. Edward Quadros, Ph.D. at the State University of New York, Downstate (Brooklyn, NY). Samples that are sent will be coded.
4. ATP Measurement*:* Quantitative determination of mitochondrial ATP (pmole/10^6 cells) is determined using a bioluminescence assay with a LMAX II luminometer (Molecular Devices) using the ATP Bioluminescence Assay kit CLS II (Roche Diagnostics). This assay is based on luciferase requirement for ATP in producing light emission and is used in the James Laboratory (21).
5. Mitochondrial Copy Number*:* The Novoquant Human Mitochondrial to Nuclear DNA Ratio Kit (Novogen) will be utilized to determine whether there are differences in mitochondrial numbers between the ASD groups. Real-time PCR with primers specific for mitochondrial DNA and nuclear DNA will be used as described by the manufacturer to calculate mitochondrial/nuclear copy number ratio for each sample.
6. Clinical Laboratory Testing: In order to verify that certain blood based immunological factors are not skewing the interpretation of the results, we will conduct random clinical laboratory testing on a select subset of samples, in order to address this potential confounding variable. The laboratory testing may consist of free cortisol, ACTH, Corticotropin Releasing Hormone (CRF), CBC w/ differential, CD3 T cell, CD19 B Cell, CD4 T Helper, CD8 T suppressor, IgG, and CD56 NK cell testing.
7. Urine Testing: Testing for the presence of metabolites associated with oxidative stress in plasma requires very careful, quick, and precise attention to the blood sample that is collected. In order to ensure accuracy and validity associated with these metabolites, very close attention to the details associated with collection, preprocessing, handling, storage, and processing must be implemented. Due to the very nature of the complexities and potential complications associated with the many steps associated with ensuring accuracy of the test results, other testing mechanisms are sorely needed. For collection procedures, see above. Up to approximately 5mL may be collected. The reason for urine testing is simple. Urinary metabolites are much more stable than plasma; however this technique has not been

validated to date. Urine testing will allow an attempt to validate this potential mechanism for testing oxidative stress in urine. This methodology could lead to a great technological advance in the field of oxidative stress and it would be the first time that urine samples have validated the stability of metabolites associated with oxidative stress in the study population.

8. Stool Testing: Upon receipt to the research site laboratory, the stool will be stored in -70 ◦C in order to flash freeze the sample. All data sent to other facilities will either be de-identified or coded.
9. Teeth Collection: Provided teeth will be sent to the Icahn School of Medicine in Mount Sinai, New York along with copies of the EEQ. This group is developing and evaluating procedures to analyze the presence of environmental exposures in teeth samples and how these potential exposures may contribute to neurodevelopmental outcomes. Copies of the completed EEQs and teeth will be stored at ACHRI in dedicated lab space until ready to ship to the Icahn School of Medicine for further analysis. Teeth will be individually wrapped and placed in bags and coded with patient ID, date of collection, and DOB and stored at room temperature. All data sent to other facilities will either be de-identified or coded.
10. Buccal Cell Collection: Electron transport chain (ETC) function will be measured in buccal cells collected. The de-identified or coded samples will be sent to Dr. Michael Goldenthal at St. Christopher's Hospital for Children in Philadelphia, PA to assess ETC function. The samples will be shipped in closed tubes on frozen cooling packs (such as Nordic Ice, Freez-Paks, etc.) in a sealed Styrofoam container or a closed insulated cooler (such as Arctic Zone) by overnight delivery. Samples will be mailed to:

    Dr. Michael Goldenthal
    St. Christopher's Hospital for Children
    Room 318
    3601 A Street
    Philadelphia, PA 19134

    If possible, samples will be collected on M-W to give sufficient time for shipment to be received and processed. Samples will not be collected on or the day before holidays.
11. GI Biopsy: GI symptoms are very common in children with autism and GI disorders have been documented in many studies. In order to better understand the bioenergetics, redox, and metabolic characteristics of GI tissue, GI tissue will be assessed using the same markers that are being measured in blood and urine. The purpose of this is to determine if there is tissue specific physiological abnormalities or if the abnormalities seen in blood and urine are generalizable to other tissue types as well. In Pediatric GI, it is standard practice to take endoscopic pinch biopsies in any child who undergoes upper and/or lower endoscopy. Routinely, in the upper GI tract, up to 3 extra duodenal and up to 2 additional gastric pinch biopsies may be taken. In the lower GI tract (terminal ileum and colon) up to 6 additional pinch biopsies may be taken. Furthermore, a rectal sample may be obtained, if the physician performing the biopsy agrees for clinical and/or research purposes. If the sample is to be obtained for research purposes only, the family can opt in or out to having this occur.

For the purpose of the present study, five additional pinch biopsies during the upper and six during the lower endoscopy will be obtained for a total of up to 11 additional pinch biopsy specimens. Depending on the pathological findings, several additional upper and lower GI biopsies could be obtained. Each pinch biopsy is about 6mg in weight. Therefore, a maximum of up to 66 mg of GI tissue solely for research purpose could be taken. There is minimal additional inherent risk to obtaining these biopsies and the amounts taken will not be more than during standard procedures. Once collected, the samples will be placed into a small plastic centrifuge tube labeled with the study ID number of the patient and collection date. GI biopsies will be either flash frozen upon collection or put into a preservation tube for fresh tissue analysis and will be de-identified or coded.

12. Muscle Biopsy: This tissue will be used to complete ETC measurements through collaboration with the ACNC. The tissue will be submerged in an ice-cold preservation buffer (BIOPS) containing 10mM CaK_2-EGTA; 7.23mM K2-EGTA; 20mM imidazole; 20mM taurine 50mM K-MES; 0.5mM dithithreitol; 6.56 MgCl2; 5.77mM ATP and 15mM creatine phosphate (pH of 7.1). Samples will be kept in ice-cold BIOPS buffer and transferred to the laboratory high-resolution respirometry (HRR) at the ACNC. Once collected, the samples will be placed into tubes labeled with the study ID number of the patient and collection date.

Cognitive-Behavioral Measures

Psychological assessment

Adaptive Behavior: The Vineland Adaptive Behavior Scale (VABS) will measure functional abilities with several domains including communication, daily living skills, socialization, and motor skills. The VABS has been used widely in ASD assessments and provides a measure of core symptoms and adaptive function across a wide age range. All sections of the VABS up through physical activity "using small muscles" may be completed. Subsequent sections consisting of problem behaviors may not be completed, as these are not needed in order to obtain an adaptive behavior composite score. The VABS can be administered as a survey interview or as a questionnaire per the Vineland II Manual.

Medical Questionnaires

1. Health History and Mitochondrial Symptoms Questionnaire: We have developed a questionnaire that reviews the signs, symptoms, laboratory abnormalities and comorbid medical conditions that define mitochondrial disease. These questions will be derived from the major criteria used to define mitochondria disease including the Modified Walker (3) and Morava (6, 7) criteria. We will provide a score based on the number of indicators endorsed.

Parent-Teacher Questionnaires

1. Aberrant Behavior Checklist (ABC): The ABC is a behavioral questionnaire developed to rate symptoms of hyperactivity, irritability, lethargy, and stereotypic behavior in individuals with developmental disabilities. The ABC may be completed by the parent and/or teacher/provider. If completed by the consenting parent within 2 weeks of the visit, this form may be considered valid

and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.

2. Social Responsiveness Scale (SRS): The SRS is a quantitative scale that measures the severity and type of social impairments that are characteristic of ASD. The SRS may be completed by the parent and/or teacher/provider. If completed by the consenting parent within 6 months of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
3. Autism Impact Measure (AIM): The AIM has been recently developed across 8 ATN sites. The AIM assesses both frequency and impact of current core ASD symptoms during the past 2-weeks. Initial studies have demonstrated excellent psychometric properties and construct validity. The SRS may be completed by the parent. If completed by the consenting parent within 2 weeks of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
4. Child Behavior Checklist (CBCL): The CBCL is an easy to complete standardized questionnaire that can assess a wide range of behaviors commonly associated with ASD symptoms, including anxiety, depression, withdrawal, sleep problems, somatic problems, and aggressive and destructive behavior. The CBCL may be completed by the parent and/or teacher/provider. If completed by the consenting parent within 2 months of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
5. Social Communication Questionnaire (SCQ): The SCQ is an instrument used to routinely screen for Autism Spectrum Disorders. For children without a confirmed ASD diagnosis this questionnaire will be used to rule out ASD by confirming an SCQ score of <12. The Current for will be used for children ≤ 5 years of age and the Lifetime form will be used for children > than 5 years of age. The SCQ may be completed by the parent. If completed by the consenting parent within 3 months of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
6. Environmental Exposure Questionnaire (EEQ): The EEQ is a comprehensive form designed to evaluate potential environmental exposures and how they may contribute to neurodevelopmental outcome. This data will be attempted to be correlated with teeth that are collected on children that are participants in this study. The EEQ may be completed by the parent.
7. Repetitive Behavior Scale – Revised (RBS-R): In order to evaluate repetitive behaviors, we will use the RBS-R in order to account for this autism symptoms. The RBS-R measures a breadth of repetitive behaviors in children, adolescents, and adults with Autism Spectrum disorders. The RBS-R provides a quantitative, continuous measure of the full spectrum of repetitive behaviors. The RBS-R consists of six subscales including: Stereotyped Behavior, Self-injurious Behavior, Compulsive Behavior, Routine Behavior, Sameness Behavior, and Restricted Behavior, which have no overlap of item content. The RBS-R is a parent or provider report with a standardized Likert scale for a variety of stereotyped and repetitive behaviors. To be completed by parents and providers. The RBS-R may be completed by the parent and/or teacher/provider. If completed by

the consenting parent within 1 month of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.

8. Gastrointestinal Severity Index (GSI) Questionnaire: Researchers at Arizona State University have developed an index to assess gastrointestinal disorders. The GSI will be used in order to collect supplemental information to assess gastrointestinal disorders on select children. The GSI may be completed by the parent. If completed by the consenting parent within 1 week of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
9. Diet Diary: To track dietary habits of select children, we will use the Block diet questionnaire developed by the NutritionQuest Group. The Diet Diary may be completed by the parent. If completed by the consenting parent within 1 week of the visit, this form may be considered valid and the original or a photocopy obtained in place of asking the parent to repeat the questionnaire.
10. Sensory Questionnaire: the sensory questionnaire was developed by Schoen and Miller (2008). This questionnaire is a parent-report measure to assess sensory processing. If completed by the consenting parent within 6 months of the visit, this form may be considered valid and the original or a photocopy may be obtained in place of asking the parent to repeat the questionnaire.

Future Studies: If the family decides to opt in to allowing their samples to be stored for future studies, the following will be done. Samples will be stored at Arkansas Children's Hospital Research institute for future autism research projects looking at biomarkers derived from the blood. Samples will be given a unique identifier and stored at –80°C in the Arkansas Children's Hospital Research Institute. Samples will be stored until they are used up or destroyed. The Principal Investigator, Dr. Shannon Rose, will retain control over the collected samples unless it is requested that the sample be destroyed and then it will not be used for any future studies. There is a possibility that the leftover de-identified or coded blood, stool, and/or urine samples may be sent to Dr. Edward Quadros, Ph.D. at SUNY-Downstate in NYC, Dr. Robert Naviaux at UCSD, or to Dr. Daewook Kang at Arizona State University for further analysis. If the participant wishes to have the sample removed from storage and destroyed at any time, they may contact a study staff at (501) 364-4519 or 1-800-283-7428 Monday through Friday 8:00 a.m. to 4:30 p.m. IRB permission will be obtained prior to sharing samples for future studies. These samples and possibly clinical research data will be de-identified and no PHI will be shared with collaborators. If samples or clinical research data are shared, a log of those who withdraw samples will be documented thoroughly.

Collaborators and future studies of de-identified leftover samples and clinical research data are listed below. As additional collaborators/studies are identified they will be added to this list.

1. Dr. Michele Jacob, who will measure changes, related to neurodevelopmental outcomes, in levels of cellular proteins and messenger RNA related to the beta-catenin and WNT cellular regulation pathways in her laboratory in immune cells.

Michele Jacob
Dept. of Neuroscience
Tufts University, Sch. of Med.
136 Harrison Ave
Boston, MA 02111
Phone: 617-636-2429

2. Dr. Harland Winter, who will examine serum samples using advanced proteomics techniques to identify proteins unique to individuals with autism.

   Harland Winter
   Digestive Function Lab
   Massachusetts General Hospital
   50 Blossom Street
   GRJ 1324
   Boston, MA 02114

3. Dr. Juergen Hahn, who will analyze de-identified clinical research data to understand the effect of comorbidities on biochemical and cognitive-behavioral measures and understand when biochemical measures can be used to distinguish individuals with autism from those with typical development and those with developmental delays without autism.

   Juergen Hahn
   Center for Biotechnology and Interdisciplinary Studies, Room 4213
   110 8th Street
   Troy, NY 12180

4. Dr. Stephen Edelson, who will analyze de-identified clinical research data to see if certain underlying perturbations in mitochondrial function correlate with sensory integration problems in children with ASD. Furthermore, he will also assess hypersensitivities to chemical exposures and chemical intolerances of mothers to see if this is a possible contributing factor as well.

   Stephen Edelson, MD
   Director - Autism Research Institute
   Autism Research Institute
   4182 Adams Ave.
   San Diego, CA 92116

5. Dr. Janet Cakir, who will analyze de-identified clinical research data to test for correlations between various environmental exposures and the development of autism/occurrence of regression. Dr. Cakir is a geographer with vast experience working with environmental databases and analyzing environmental data. Dr. Cakir has children enrolled in the study. To manage this potential conflict of interest I will have an independent reviewer and myself carefully monitor her data analysis.

   Janet Cakir, PhD
   NPS SER Climate Change, Socioeconomics, and Adaptation Coordinator

NCSU Applied Ecology
South Atlantic Landscape Conservation Cooperative
1751 Varsity Dr.
Raleigh, NC 27606

6. Dr. Manish Arora, who will analyze baby teeth using the technology developed at his laboratory. He will be able to provide exposure information on a wide range of chemicals, including metals and organic toxicants, over the prenatal and early childhood periods. The tooth-matrix biomarkers can be used to reconstruct past exposure history during key developmental periods including prenatal life. The association of metals in teeth and mitochondrial function may provide novel insight into the molecular mechanisms linking environmental metal exposures and autism severity.

   Manish Arora BDS, MPH, PhD
   Associate Professor
   Division Chief of Environmental Health
   Director of Exposure Biology
   Department of Preventive Medicine
   One Gustave L. Levy Place
   Box 1057
   New York, NY 10029-6574

7. Dr. Haiwei Gu, who conducts research in metabolomics in metabolic diseases. He will analyze Urine / Plasma pairs from the mitochondrial cohort in order to better characterize metabolic aspects of our autism cohort and examine the correlation between urine and plasma metabolites.

   Dr. Haiwei Gu
   Center for Metabolic and Vascular Biology
   College of Health Solutions
   Arizona State University-Mayo Clinic Campus
   13208 E Shea Blvd, CRB 2-228
   Scottsdale, AZ 85259

*Study Population/Data Source

Inclusion Criteria for ASD children

1. Autism Spectrum Disorder (As defined by a gold standard measure for ASD diagnosis: the Autism Diagnostic Observation Schedule, Autism Diagnostic Interview, and/or the minimum Arkansas state requirement for autism classification, as defined by a consensus diagnosis of ASD by a medical doctor, speech pathologist, and psychologist.).
2. 0 years through 17 years 11 months of age

Inclusion Criteria for TD, DD, MD, NDA children

1. 0 years to 17 years 11 months of age

Exclusion Criteria (all children)

1. Any historical event/information that may, in the opinion of the PI, be a reason to exclude the child from participation.

*Ethical Considerations

Subject Screening:

Children with ASD may be recruited from our Autism Treatment Network (ATN) site whose families have signed a HIPAA release to be contacted about research. The UAMS ATN site is the state hub for the evaluation of ASD in children. Our ATN site is part of the national ATN organization which is sponsored by Autism Speaks. Our site has been approved for funding for the next 3 years before a renewal application is necessary. The clinic evaluates 4 new patients per week and follows children diagnosed with ASD on a yearly basis. We also have more than 270 participants in the ATN registry who have signed consent to be contacted for research studies such as the proposed study. Children may also be recruited from the AAA.org website for parents who have indicated that they are interested in research contact, from advertisement through local channels and BOOM text, by using social media through Arkansas Children's Hospital, flyers posted in the community (i.e. primary pediatric clinics), as well as referrals from pediatricians.

When a parent inquires about the study or a potentially eligible child is identified, study staff will contact the parent(s) and describe the study. Study staff will screen by asking inclusion/exclusion criteria questions. If the child qualifies from the screening interview, study staff will schedule the study visit.

Consents:

For those whose participation will only include completion of questionnaires, a phone script will be used to obtain verbal consent. For those who previously participated in this study, and their subsequent visit will only involve complete of questionnaires, a phone script will be used to obtain verbal re-consent. For these participants, an information sheet informing them about the study will be mailed, e-mailed, or faxed to the parent/LAR(s) at the time of determining eligibility so that they can reference it prior to deciding to participate. The conversation and parent/LAR(s) verbal consent will be documented.

For those whose participation will include more than just the completion of the questionnaires (e.g., provide baby teeth/buccal cells), the consent form will be mailed, e-mailed, or faxed to the parent/LAR(s) at the time of determining eligibility so that any questions can be addressed before the visit. If the parent/LAR is coming to the study site to complete study tasks, the signing of the IRB-approved consent will be done in person at the study visit. If the parent/LAR will not travel to the study site (e.g. parent/LAR is sending in questionnaires/specimens and/or completing interview by

phone) the consent discussion will occur by phone once the parent has received the consent/HIPAA form. For study participants who are typically developing and 7 years of age or older (who complete more than just the questionnaires), an assent discussion will also occur by phone. If consent/assent (if applicable) is obtained, the parent/LAR will be asked to mail, scan and e-mail, or fax the signed consent/assent/HIPAA form to study staff with the questionnaires. If the parent chooses to mail the consent form they can do so in the postage-paid, addressed envelope provided for the questionnaires. In the event questionnaires are returned without a consent form or the consent form is not fully completed, study staff will attempt to follow-up with parent up to 1 year following the date of the consent discussion. After 1 year, if a signed consent form is not obtained, the received questionnaire data will not be entered and used in data analysis.

Seventeen year old subjects with ASD, DD, or NDA may be recruited into this study. We neither feel it is appropriate nor justified to have them re-consent when they turn 18 years of age, due to the fact that we are dealing with a cognitively impaired population and it would be unknown if they truly gave an informed consent or not. Thus, we believe that the parent/LAR signature at the time of study entry it is the most appropriate. For typically developing controls, assent will be obtained, if 7-17 years of age, inclusive. We anticipate that the study visit can be completed in less than 2 hours. If typically developing controls turn 18 while enrolled in the study, they will be asked to consent themselves at the time of their next study visit, if they are interested in doing so.

*Risks and Benefits

The known risks of this study are related to confidentiality research information.

Loss of confidentiality is a slight risk. This risk is minimized by assigning a unique study number to identify the subject instead of using the subject's name on study documents. The assigned study number will be used on the study information collected and when analyzing the data. Information collected about the subject (demographics, study assessments, lab results) will not be kept in health records or shared with physicians, employers, and insurers. All subjects' personal information will be kept secure in a locked environment, and/or a computer file that is protected with limited password-access for only authorized researchers directly involved in this study. All research records will be kept confidential to the extent provided by law.

There are no direct medical benefits to participation. The new knowledge gained from this study may help in the development of a minimally-invasive biological assay that can be used to identify variations in mitochondrial function and dysfunction in children with ASD.

*Statistical Plan

Statistical Analysis

Descriptive statistics, e.g., mean and standard deviation for continuous variables and number and percent for categorical variables will be obtained for all demographic variables, outcomes and predictors of interest. Data will be checked for errors, inconsistencies and missing values. Appropriate statistical methods will be used to test the specific hypotheses of interest. Analyses will be adjusted for confounding variables such as age, gender, autism severity, etc. wherever necessary. Independence of predictor variables and homogeneity of variance assumptions will be checked prior to the analysis. P-values will be adjusted for multiplicity wherever appropriate. All tests will be two-sided and a P-value less than or equal to 0.05 will be considered to be significant. All analyses will be conducted using statistical software SAS v9.2 (SAS Inst, Cary, NC) or STATA 12 (STATA Corp, College Station, TX), or R (R Foundation for Statistical Computing, Vienna, Austria; (22)).

Specific Aim 1: To determine if children with ASD/MD can be differentiated from children with ASD/NoMD by their mitochondrial energy profile defined by the Goldenthal buccal cell array and/or the Seahorse XF Analyzer. A multivariable logistic regression model will be used to model the probability of a child with ASD falling into ASD/MD group. Four oxygen consumption variables, four acidification variables, ATP production and DNA copy number among others will be included as candidate predictor variables in the model in addition to the covariates age and gender. Additional variables characterizing the change in the oxygen consumption and acidification variables (such as the slope) with increasing DNMQ dose will also be investigated. We will use data reduction methods that guard against overfitting before model development to make it more likely that the statistical model will validate on independent data. These methods include principal components analysis, variable clustering, and battery reduction (22) and penalized regression (23). In addition to the steps above, the modeling strategies of Harrell (22) and Steyerberg (24) will be followed, including but not limited to imputing missing values using the *aregImpute* function in the RMS library of the R package, checking for overly influential observations, checking for linearity assumptions, and checking additivity assumptions by testing prespecified interaction terms. Finally, we will develop a reduced model (a parsimonious model) that will be manageable for the modeling and the cluster analysis in Aim 2. Model discrimination will be assessed using the C-statistic and by plotting the area under the receiver operating characteristic (ROC) curve. The ROC curve will be further examined to determine the cutoff value with the highest sensitivity and lowest specificity.

Specific Aim 2: A cluster analysis will be used to determine whether there are several groups of ASD patients or whether there are only two groups, one with MD and one with no MD. The cluster analysis will be based on the recent work of Hastie et al (25) which has been successful in finding valid clusters (subtypes) in breast cancer patients in DNA microarray studies. As recommended by these authors, we will use K-means clustering to find the smallest within-group sum of squares over the variables for k groups and determine the optimal number of groups by using the validation methods discussed below. The cluster analysis will be validated according the procedure described in Kapp and Tibshirani (26). The R package *clusterRepro* will be used to implement this procedure. The prediction equation from the logistic regression model obtained in Aim 1 will then be used to estimate the

probability of having mitochondrial dysfunction for each patient in the general ASD population. The cutoff value determined in Aim 1 will be used to classify each patient as ASD/MD and ASD/NoMD and the proportion of patients classified as ASD/MD will be compared across different clusters to finally establish the number of levels of MD severity within the general ASD population. If the cluster analysis combined with the logistic model does not perform well to establish levels of MD severity, linear discriminant analysis will also be explored to distinguish between several groups.

Specific Aim 3: To determine if mitochondrial dysfunction is associated with redox metabolism, a linear regression model will be performed using mitochondrial dysfunction as the dependent variable and redox metabolism as the primary independent variable along with ASD group classification and their interaction. Tests for the interaction effect and contrasts will determine if the association between mitochondrial dysfunction and redox metabolism depends on the ASD group classification. We will examine these relationships for the various biomarkers of mitochondrial function and redox metabolism.

Specific Aim 4: Using ANCOVA, we will evaluate whether mitochondrial function and/or redox metabolism predicts cognitive development and/or ASD symptoms, whether the influence of mitochondrial function on cognitive development and/or ASD symptoms is indirect through redox metabolism and whether these relationships are different for individuals with ASD/NoMD and ASD/MD. We will conduct both moderation and mediation analysis. Indirect effects will be assessed through mediation analyses which will use the procedures of Baron and Kenny (27) with the exception that bootstrapping procedures (28) will be used to test for statistical significance of the indirect effects.

Specific Aim 5: Box and Q-Q plots will be used for data visualization of chemical analytes with and without log transformations and used as appropriate. Our experience with the distribution of chemicals in teeth is that it is approximately log-normal. While we do not anticipate problems resulting from missing data, multiple imputation will be used when the assumption of missing-at-random can be justified should these procedures be needed. Comparisons of chemical concentration between the participant groups (ASD/NoMD, ASD/MD, typically developing) will be made using a Kruskal-Wallis test. The Dunnet test will be used to adjust for multiple comparisons between the groups across chemicals with $p < 0.05$ as the critical cutoff for significance. All analysis will be performed with SAS software. Relevant design variables will be included as covariates if justified. Lastly, as a secondary analysis, we will determine whether ASD severity (in terms of adaptive behavior and ASD symptoms) depends on exposures using a linear regression model including aforementioned covariates. Restricted cubic spline may be used if independent variables demonstrate non-linear relationships.

Power Analysis

We intend to sample 50 children in the ASD/MD group, 50 children in the ASD/NoMD group. These sample sizes are dictated by the availability of ASD patients with MD. Also 150 children in the general ASD group, 50 noASD/noMD (i.e. DD) and 100 for the TD group will be sampled. It is imperative for us to establish here that this sample size will produce sound inferences. For Aim 1, an overall sample of size 100 assuming equal number of cases and controls will have 83.7% power to detect a change in probability from 0.50 to 0.67 (an odds ratio of 2) when the independent variable is increased from its mean to one standard deviation above the mean. For the cluster analysis in Aim 2, the number of groups will be limited to three, given the sample size of 150. For Aims 3-5, an ANCOVA model with sample sizes 50, 50 and 150 for the three groups has 84% power to detect a significant difference in group means assuming a standard deviation of 1 for the group means and a standard deviation of 5 for observations within each group. All sample size calculations were done using PASS 11 (29) assuming a two-sided significance level of 5%

*Data Handling and Recordkeeping

Methods Used for Data Collection
All subjects will be given a unique identifier. All data (cognitive, behavioral, and biochemical) will be generated with this number. All biomaterials will be stored with this unique identifier.

Data generated from sample analysis consists of:
- HPLC and Mass Spectrometer results and source data including source chromatograms
- Excel file recording of plasma and leukocyte metabolite concentrations for intracellular redox status using oxidized and reduced glutathione and results of nutritional biomarkers.
- Excel file recording of leukocyte mitochondrial respiration, ATP content and mitochondrial copy number.

Data Quality Assurance
Steps to ensure the accuracy and reliability of data include selection of qualified research team members, review of protocol procedures with the study team, and periodic monitoring of the data for accuracy. Data will be entered into a study specific database and verified for accuracy.

Volunteer Identification
Unique identifiers for this study will be assigned to each subject enrolled.

Confidentiality / Disposition of Data
All data additionally coded as described above (volunteer identification). Access to this link, study specimens, and research generated from this study will be password controlled and the identity of the samples will be records will be restricted to the research team and institutional (e.g. IRB, Research Compliance) and regulatory oversight personnel.

Data will be stored in hard copy and/or in password protected database(s). Research records are maintained in a locked and secure environment with restricted access.

Study records will be maintained for the period of time the investigator file is required to be retained by local institutional policy or regulatory requirements, whichever is greater. Biomaterials will be stored with the unique identifier until the end of the study and kept for future studies (if consent for this is provided) until they are either used or destroyed.

Sharing Research Results

Any findings from these studies that are unanticipated will result in Dr. Kahler referring the participant to the appropriate provider for evaluation. Research results from oxidative stress, mitochondrial function, the language assessment, and Vineland will be provided to the families in the form of a research results letter. The letter will refer families to their child's regular doctor if they are concerned and that we will assist them in finding a doctor if they do not have one. The results are indicated to be from a research study and have not been validated in the clinical population. They are based on limited and unpublished data. This information is intended for the sole purpose of providing the recipient the information from an on-going confidential research study. This information is confidential in nature and due to the proprietary nature of study it should not be disclosed to persons outside of the scope of the research study.

*Study Registration and Publication

The completed study will be published in peer-reviewed journals and presented at scientific conferences.

Additional Safeguards to Protect Vulnerable Populations

IRB approved flyers and advertisements may be utilized for participant recruitment. ACH, UAMS, ACNC, and community physicians will be encouraged to refer potential participants and all may be utilized for possible participant recruitment and referrals. Previous autism study participants, whose parent(s) have given written permission with a HIPAA release to be contacted about future research opportunities, will be contacted for interest in participation.

Written or verbal (whichever is appropriate) informed parental/LAR consent will be obtained by appropriately trained study personnel. Parent(s)/LAR(s) will be provided a copy of the informed consent or study information sheet (whichever is appropriate) to review prior to participation. A signed copy of the consent will be provided to parent(s)/LAR(s), as applicable. Data will be entered into the database directly from source documents and/or study specific case report forms. For typically developing controls, assent will be obtained, if applicable. Participants will be given as much time as needed, in order to determine if they want to participate in the study or not.

Recruitment Plan

Children with idiopathic ASD will be recruited from our Autism Treatment Network (ATN) site and the registry we have developed over the past 4 years. The University of Arkansas for Medical Sciences (UAMS) ATN site is the state hub for the evaluation of ASD in children. Our ATN site is part of the national ATN organization which is sponsored by Autism Speaks. Our site has been approved for funding for the next 3 years before a renewal application is necessary. The clinic evaluates 4 new patients per week and follows children diagnosed with ASD on a yearly basis. We also have more than 270 participants in the ATN registry who have signed consent to be contacted for research studies such as the proposed study. The ATN sites routinely perform the cognitive and behavioral measures proposed in this study.

Children with ASD/MD, DD, NDA, noASD/MD and ASD/NoMD may be recruited from outpatient clinics at ACH such as the pediatric GI clinic. Groups will try to be balanced across study groups on important medical factors, such as seizures, gastrointestinal disorders, and epilepsy, in order to provide a robust cohort.

Children (controls and non-controls) may be recruited by using social media through Arkansas Children's Hospital, advertisement through local channels and BOOM text, word of mouth, ACNC, flyers posted in the community (i.e. primary pediatric clinics), as well as referrals from pediatricians. Children may also be identified through parent and patient centered online resources, such as post0218pediatrictherapies.com, Facebook, http://babyfoodsteps.wordpress.com, Arkansas Autism Alliance website, etc. IRB approved flyers and postings will be posted on these outlets for interested families.

When a parent inquires about the study or a potentially eligible child is identified, study staff will describe the study to the parent to determine interest. If interested, study staff will screen by asking inclusion/exclusion criteria questions. If the child qualifies from the telephone interview, study staff will schedule the consent discussion phone call and provide the parent with the consent form or study information sheet depending on what tasks will be completed.

No compensation will be offered for completing study tasks.

Monitoring Plan

Adherence to the monitoring plan will be documented. The research study staff will be responsible for monitoring the following items in the study every 3 months. Results and deviations will be reported to the PI. All results will be kept as a hard copy and kept secured in a file folder or binder in a secure, locked space of the research site.

Subjects File Documents to be reviewed (located in a locked space of the research site):

- Initial visit data inquiry
- Statement of informed consent using the correct, IRB approved version of signed Consent Form with all necessary signatures and/or documentation of verbal consent.
- Exclusion /Inclusion documentation
    - Age
    - If diagnosis of ASD, documentation obtained
- Cognitive and Behavioral Questionnaires (all present in locked files)

2. The following are password controlled/or identity is coded:

- Enrollment Log
- HPLC results and source data including source chromatograms
- Excel file recording of plasma and leukocyte metabolite concentrations for methionine, S-adenosylmethionine, S-adenosylhomocysteine, adenosine, homocysteine, cystathionine, cysteinylglycine, oxidized and reduced glutathione.
- Excel file recording of leukocyte mitochondrial respiration, ATP content and mitochondrial copy number.

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