Disordered Cholesterol Metabolism in Autism Spectrum Disorders: Sterol and Genetic Analyses

An MPH thesis by

Joel Pearson, MD/MPH Candidate

Presented to the Department of Public Health and Preventive Medicine at Oregon Health & Science University in partial fulfillment of the requirements for the degree of Master of Public Health

May, 2010 (Defended April 23rd, 2010)

Thesis Committee:

Atif Zaman, MD, MPH, Thesis Chair
Robert Steiner, MD, Clinical Advisor
Thuan Nguyen, MD, PhD, Statistical Advisor

Department of Public Health and Preventive Medicine

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the master's thesis of

Joel Pearson

has been approved

_____
Thesis Chair- Atif Zaman, MD, MPH

_____
Thesis Advisor- Robert Steiner, MD

_____
Thesis Advisor- Thuan Nguyen, MD, PhD

Table of Contents

Abbreviations Used

7-DHC……..7-dehydrocholesterol
8-DHC……..8-dehydrocholesterol
24S…….24-hydroxycholesterol
ABAS……..Adaptive Behavior Assessment System
ADOS…….Autism Diagnostic Observation Schedule-generic
ASD…….....autism spectrum disorder
CBCL……..Child Behavioral Checklist
CDRC……...Child Development and Rehabilitation Center
DHCR7……7-dehydrocholesterol reductase gene
DMS-IV……..Diagnostic and Statistical Manual-IV
DNA……...deoxyribonucleic acid
GCRC……...General Clinical Research Center
HIPPA…….Health Insurance Portability and Accountability Act
IRB……institutional review board
LRCPS……..Lipid Research Clinics Prevalence Study
MSEL…….Mullen Scales of Early Learning
OCTRI……..…Oregon Clinical & Translational Research Institute
OHSU……..…Oregon Health & Science University
PCTRC…….Pediatric Clinical & Translational Research Center
SBV…….Stanford-Binet intellectual quotient- 5th edition
SLOS……..Smith-Lemli-Opitz syndrome
VABS……Vineland Adaptive Behavior Scales
WISC……Wechsler Intelligence Scale for Children

Acknowledgements

Many have helped me throughout the creation of this thesis. Special thanks to my thesis committee: Robert Steiner, Atif Zaman, and Thuan Nguyen, who showed much patience, helped me grow, and are truly wonderful mentors. Thanks as well to Jean-Baptiste Roullet, Louise Merkens, Clive Woffendin, Darryn Sikora, Erin Moran, Meaghan Peters, Andrea DeBarber, Anthony Gunsul, and Susan Dean, who helped with everything from laboratory data to restaurant recommendations. Thanks and infinite gratitude to John Stull for his boundless kindness and wisdom. Finally, thanks to Ed Keenan, a true gentleman and advocate for the MD/MPH program, to the stupendous MD/MPH Class of 2010, and to all the great folks in the Department of PHPM.

Abstract

Background The diagnosis of autism spectrum disorders (ASDs) has increased in prevalence 5 fold over the past two decades, leading to enormous medical costs and great public concern. These disorders may manifest in well described medical conditions, but their cause is usually unexplained. Recently, ASDs have been described in individuals with Smith-Lemli-Opitz syndrome (SLOS), which is caused by deficient cholesterol synthesis due to mutations of the 7-dehydrocholesterol reductase gene (*DHCR7*). This suggests a role for cholesterol metabolism in the pathophysiology of ASDs. Cholesterol is known to be necessary for brain development, including myelination, synaptogenesis, and neurosteroid signalling. This study will employ SLOS as a disease model by which to examine cholesterol's role in ASDs.

Objectives To analyze blood samples from children with and without an ASD for levels of cholesterol, a cholesterol precursor (7-dehydrocholesterol), a cholesterol metabolite indicating levels of cholesterol turnover in the brain (24S-hydroxycholestrol, "24S"), and mutations of *DHCR7* and to test the hypothesis that cholesterol metabolism is impaired in children with an ASD, specifically that plasma cholesterol and cholesterol metabolites are decreased, cholesterol precursors are increased, and mutations of *DHCR7* are more common.

Methods A case/control study was designed, with recruitment through the OHSU/CDRC Autism Clinic, consisting of children (≤18 years old) who were referred for a behavioral evaluation. Cases were defined as those who were diagnosed with an ASD and controls defined as those determined not to have an ASD. Levels of sterols and the frequency of nucleotide variants and pathologic mutations of *DHCR7* were compared across cases and controls and multiple regression analysis to compared sterol levels while controlling for the possible confounding variables of age, gender, race, and BMI.

Results Among 42 cases and 27 controls, baseline characteristics of age (78.5 vs. 93.4 months, p=0.15), gender (83% male vs. 81% male, p=1), race (85% white vs. 77% white, p=0.43), and BMI (17.3 vs. 19.0, p=0.11) did not differ significantly across case and control groups, respectively. In cases, blood levels of 24S (104.1 ng/mL vs. 78.6 ng/mL, p=0.002) and the 24S/Cholesterol ratio (71.6 vs. 57.4, p=0.015) were found to be significantly different, although cholesterol (146.6 mg/dL vs. 140.2 mg/dL, p=0.27) and 7-DHC (7.3 µg/dL vs. 13.8 µg/dL, p=0.22) levels were not. Cases were found to more frequently have nucleotide variants of the *DHCR7* gene (50% vs. 19%, p=0.01), specifically in the locations of Exon 4(189A>G) (43% vs. 15%, p=0.02) and Exon 9(1272 T>C) (43% vs. 19%, p=0.04), and although the proportion carrying a pathologic mutation did not differ statistically (cases: 7%, controls: 0%, p=0.28), it is notable that 3 cases carried pathologic mutations of *DHCR7* while no controls where carriers. This case carrier frequency of 1/14 is compared to a published carrier frequency of 1/30. Using multiple regression to control for the known confounder of age, 24S levels were significantly different between cases and controls (coefficient=17.01, p=0.01), although this difference became marginal when age was transformed to it natural logarithm (coefficient=13.10, p=0.051).

Conclusion Children with an ASD had more nucleotide variants and pathologic mutations of the *DHCR7* gene compared to those without an ASD (although the difference in pathologic mutations was statistically non-signficant). Also, 24S levels were found to be significantly greater in the ASD group when controlling for age. However, this differnce became marginal when age was transformed to better fit the relationship between age and 24S. No significant difference was observed in cholesterol or 7-DHC. No child met the diagnostic criteria for SLOS. These findings suggest that those with an ASD may have increased genetic variability in loci involved in cholesterol synthesis and that those with an ASD may experience greater cholesterol turnover or neuronal dengeneration in their brain.

Introduction

Background

Autism and ASDs Autism is a disorder of increasing concern, and it occurs with great costs to families and society. Autism spectrum disorders (ASDs) occur in an estimated 1/110 children in the US, and are present in all ethnic and socioeconomic groups.[1] The burden due to ASDs is immense, as caring for individuals with autism is estimated to cost $35 billion annually.[2]

Autism is a behaviorally-defined disorder featuring impairment of social and communication domains as well as rigid or repetitive behavior.[3] Autism, along with pervasive developmental disorder- not otherwise specified (PDD-NOS or "atypical autism") and Asperger's syndrome, comprise the autism spectrum disorders (ASDs), all of which feature autistic characteristics to varying degrees, but have diverse behavioral phenotypes. For example, an individual may show severe impairment of verbal communication but not meet full criteria for autism and receive the diagnosis of PDD-NOS. Asperger's syndrome features impairment of social interaction and restricted behaviors, but language and cognitive abilities are intact. All ASDs are classified within the larger diagnostic category of pervasive developmental disorders (PDD), which are defined by "severe and pervasive impairment in several areas of development".[3] Additional PDDs include Rett's disorder and childhood disintegrative disorder, which are not considered in this study.

Features of autism are often noted within the first year of life. Children with autism may have little interest in making friends and may bond poorly with family. Associated behaviors may include rigid preferences for food, hypersensitivity to photo or

touch stimuli, self-injurious behaviors, inappropriate affect, impulsivity, and aggressiveness. Mental retardation is commonly associated, ranging from mild to severe. However, intellectual prowess in computation, memory, and other realms has been noted in a small subset of patients.

Over the past two decades, the reported prevalence of ASDs has increased 5 fold.[4] The cause of the apparent increase in the diagnosis of autism is not clear, and although theories of toxicity due to thimerosal or measles-mumps-rubella vaccinations is widely disseminated in the media, the preponderance of evidence argues against them as a cause of autism.[5] Indeed, increased diagnosis of autism may be partially, if not largely, accounted for by greater provider awareness, improved referral services, and widened diagnostic criteria for the diagnosis.[6] A recent review of ASD prevalence surveys in the US and UK found wide ranging estimates, from 5 to 67 cases per 10,000 children, emphasizing the importance of the impact of testing methods and the composition of the sample population in the resulting estimate of prevalence.[7]

The cause of autism is unknown but appears multi-factorial, with a strong genetic component. Genetic studies find a 75% and 3% concordance rate for mono- and dizygotic twins, respectively.[1] Many genetic loci have been implicated in ASDs, and linkage studies have implicated nearly every chromosome, although few of these findings have been reproduced.[8] ASDs are more common in certain medical diseases, including congenital rubella syndrome and the genetic disorders of tuberous sclerosis, phenylketonuria, and Fragile X syndrome, although these likely account for 10% or fewer cases.[4] Inborn errors of metabolism which may feature autism spectrum behaviors, beyond phenylketonuria, include adenylosuccinatelyase deficiency, defects of creatine

synthesis, Smith-Lemli-Opitz syndrome (SLOS), and others.[9] Of these metabolic disorders, SLOS is comparatively common and well-described.

The association of Smith-Lemli-Opitz syndrome with ASDs Smith-Lemli-Opitz syndrome is a disorder of cholesterol metabolism in which the final step in cholesterol synthesis is disrupted due to inadequate activity of the necessary enzyme 7-dehydrocholesterol reductase (DHCR7). This is caused by mutations of the *DHCR7* gene.[10] The resulting biochemical profile features increased cholesterol precursors (7-DHC, 8-DHC) and decreased cholesterol.

Common features of SLOS include mental retardation, reduced growth, characteristic facies and syndactyly. The primary pathophysiologic cause of SLOS, decreased cholesterol or toxic excess of cholesterol precursors, or both, is unclear. The severity of SLOS can be graded by the variety of embryologic malformations present, and this correlates with both an increasing "DHC fraction" (7-DHC + 8-DHC expressed as a fraction of total sterols) and the type of *DHCR7* mutation (homozygous null/null being the most severe).[11]

Recent work has found overlap between SLOS and ASDs. Employing the instruments used to diagnose autism, as many as three quarters of children with SLOS meet criteria for an ASD.[12] Also, many children with SLOS demonstrate autism-associated behaviors such as self injury and sleep and temperament dysfunction.[13] In one reported case, further investigation of an established diagnosis of autism lead to the secondary identification of underlying SLOS.[14] Recently, sterols were measured in a

group of individuals with ASD, and although no sterol profiles were diagnostic of SLOS, unexpectedly low cholesterol levels were noted in some individuals.[15]

Links between SLOS and ASDs may not only include cholesterol metabolism but also abnormal serotonergic function. Waage-Baudet demonstrated a three to fifteen fold increase of serotonin immunoreactivity in the brains of a mouse model of SLOS.[16] This would imply that disordered cholesterol metabolism may lead to abnormal serotonergic function. Serotonin function abnormalities have been observed in autism. Specifically, autistic children have been noted to display altered serotonin synthesis capacity and express a serotonin transporter polymorphism.[17]

Cholesterol is essential for the nervous system As described above, it is not clear whether the nervous system pathology of SLOS results from inadequate cholesterol, toxicity of cholesterol precursors, both, or other factors, such as serotonergic derangement secondary to an impaired cholesterol metabolism. Importantly, cholesterol's roles for normal brain development, beyond descriptions of SLOS, are well-described. Cholesterol has been demonstrated to be a rate-limiting factor for myelination[18] as well as a limiting factor in synaptogenesis.[19] As it is a structural element in cell membranes, cholesterol affects membrane fluidity, morphology, and possibly signal transduction.[20] Also, cholesterol is a necessary precursor for neurosteriods, which modulate many, if not most, neurotransmitter receptors.[21] Cholesterol has a role in Alzheimer's disease, as the presence of APOE4 (a transporter of cholesterol) increases the risk of developing Alzheimer's, and individuals with Alzheimer's exhibit greater cholesterol flux from the brain as measured by the cholesterol metabolite, 24S-hydroxycholesterol.[22]

Roughly 25% of the body's cholesterol is found in the brain, and cholesterol does not freely pass the blood-brain-barrier, requiring endogenous synthesis of cholesterol within the brain and a means of cholesterol exit from the brain upon its breakdown, which is primarily in the form of 24S-hydroxycholesterol (24S).[23] Nearly all 24S in the blood originates from the brain, and blood 24S levels may reflect the number of active neurons in the brain as well as indicate neuronal damage.[23] As would be expected, infants with SLOS (and thus reduced cholesterol synthesis) have been observed to have lower plasma levels of 24S (by roughly 50%) compared to control infants.[24] Individuals with Alzheimer's and vascular dementia have higher levels of blood 24S,[25] which is thought to be due to increased breakdown of neurons. Finally, 24S has been demonstrated to be neurotoxic to *in vitro* cell lines,[26] although the *in vivo* meaningfulness of this finding is unknown.

Preliminary studies of cholesterol in ASDs As detailed above, the understanding of cholesterol's role in autism has moved from the first description of autistic behaviors in SLOS,[13] to formal testing for autism in children with SLOS,[12] to measurement of sterols in children with autism.[15] Regarding the latter, Tierney observed that 19 of 100 patients with ASD had cholesterol levels below the 5th percentile, and although none met diagnostic criteria of SLOS, this does demonstrate a substantial difference between cholesterol levels for children with ASD and the published normal values.

The aim of this study is the comparison individuals with and without and ASD across behavioral domains and markers of cholesterol metabolism. Cholesterol metabolism will be assessed by both direct plasma measures and genetic testing, and

behavioral testing will use the "gold standards" for diagnosis of ASDs, making it the most comprehensive study of its kind to date and the first to compare these measures across children with ASDs and clinically-referred controls.

Study significance

Cholesterol plays multiple vital roles in brain development, and perturbation of its synthesis has far reaching effects, from the cellular and molecular level to gross physical malformations. Evidence is mounting for cholesterol's relevance to autism, and the SLOS model may provide insight into the etiology of autism and suggest therapeutic interventions. Dietary cholesterol supplementation in patients with SLOS improves blood cholesterol profiles,[27] and Elias noted improved growth and cognition and decreased irritability and self-injury for SLOS patients treated with cholesterol supplementation.[28] However, Sikora found that cholesterol supplementation did not improve developmental (motor and cognitive) outcomes of children with SLOS over 6 years of follow up.[29] Jira found that simvastatin therapy partially normalized sterol levels in SLOS patients, increasing cholesterol and decreasing cholesterol precursor levels in plasma.[30] If ASDs are found to feature disordered cholesterol metabolism, the aforementioned therapies used in SLOS may be useful in the treatment of the behavioral symptoms of ASDs.

Specific aims and hypothesis

1. Recruit 90 subjects evaluated at the OHSU/CDRC Autism Clinic, with 45 cases (diagnosed with an ASD) and 45 controls (determined not to have an ASD).

2. For all subjects, perform plasma analysis of cholesterol, a cholesterol precursor (7-DHC) and a cholesterol metabolite (24S), and sequencing of the *DHCR7* gene.
3. Test the hypothesis that cholesterol synthesis will be impaired in individuals with an ASD compared to those without an ASD, using SLOS as a disease model. This would be demonstrated by cases having:
    a. lower plasma cholesterol and cholesterol metabolites (24S)
    b. elevated plasma cholesterol precursors (7-DHC)
    c. more frequent mutations of the *DHCR7* gene

Methods

Brief review of study design

A case-control design was used to examine differences in cholesterol metabolism between children with an ASD and those without an ASD to test the hypothesis that cholesterol metabolism is impaired in children with an ASD relative to those without an ASD.

Timeline and approval

Subject recruitment began in the winter of 2007 and continued through the spring of 2009. Recruitment was halted due to time constraints upon personnel, and although not at goal, enrollment was deemed adequate (see power calculations in *Methods*). Laboratory analysis of samples was complete by winter 2009 and data analysis complete by spring 2010.

Our research protocol was approved by the Oregon Health & Science University's institutional review board on December 4th, 2006 (eIRB #1411).

Study subjects

Selection criteria All participants were children aged 1-18 years who had been seen at the OHSU/CDRC Autism Clinic. These children presented for diagnostic evaluation due to concern of parents, school counselors, etc, for autism or other related developmental disorders. The "case" group consists of children who received a diagnosis of an ASD, including autism, Asperger's disorder, and pervasive developmental disorder-NOS. Members of the "control" group may feature a behavioral disorder, but none received a diagnosis on the autism spectrum.

Exclusion criteria Individuals with non-ASD developmental disabilities are included *unless* the disability is due to an established genetic or metabolic disorder.

Sampling and recruitment When families present to the Autism Clinic for evaluation, they are queried as to their interest in participating in research studies. Those who are interested have their names and telephone numbers added to a "contact list" maintained by the clinic. Only those who indicated interest were asked to participate in this study, by direct telephone interaction or by investigators during clinic visits. Cases and controls were gathered from the contact list according to the diagnosis (ASD or non-ASD) received from their Autism Clinic evaluation. Attempts were made to match case and control groups for age, gender, and developmental level, but this goal could not be strictly met due to difficulty enrolling sufficient numbers of participants.

Human subjects protections

A. Risks Participation in this protocol involved a blood draw for each child and travel and time commitments (most visits last less than 1 hour) for parents and children. Blood draws may cause physical discomfort and temporary bruising may result. Infection or venous damage are possible but are extremely unlikely. Phlebotomy also frequently causes temporary emotional distress. It is possible that this exposure could lead to persistent fear of needles or medical procedures. Participants were able to withdraw from the study at any time if they so desired.

B. Benefits Subjects received no therapeutic intervention (procedure, medicine, etc.) in this protocol. However, some parents wish to maintain dialogues with our research staff after their child's initial clinical evaluation, and their informal questions are permitted and encouraged at visits for this study. Knowledge obtained by this informal consultation may enhance their interactions with their health care providers. Importantly, it was explained to each participant that individual study results cannot be released. However, participants can be informed of the collected results of the study and the investigators' conclusions. For many participants (most frequently parents), benefit is derived from the altruistic act of adding to the scientific body of knowledge of autism and developmental disorders. A small incentive was offered to participants in the form of a $20 gift card to local department stores ("Kroger family stores"). We felt this amount was adequate to partially defray the travel and time investments of participants but was not large enough to coerce individuals to partake in the study.

C. Special considerations This protocol's subjects are children, both with and without the diagnosis of a pervasive developmental disorder. Risks to subjects are minimal, and are not necessarily increased by a subject's ASD status, although some children with developmental disorders may respond with increased resistance to unfamiliar processes, such as a blood draw. All personnel, including phlebotomists, interacting with subjects have extensive experience with children, both those with and without disabilities. All investigators abide by OHSU IRB and HIPAA guidelines and will have undergone OHSU's Responsible Conduct in Research training. Throughout study visits, parental level of understanding of the protocol is assessed and their questions are encouraged. Parental consent is always attained and assent of the child is obtained whenever possible. The data of this study was not added to any patient's official medical record, but stored confidentially by the investigators (see *Data Management*, below).

Procedures

Autism evaluation All participants were evaluated by the Autism Clinic prior to enrollment in this study. Evaluation is done by an interdisciplinary team (psychologist, pediatrician, speech therapist, and others) using a battery of established metrics. The diagnosis of an ASD requires meeting both the ASD criteria of the Diagnostic and Statistical Manual-IV (DSM-IV)[3] and the Autism Diagnostic Observation Schedule-generic (ADOS),[31] as well as consensus among the evaluation team that the child's behaviors are consistent with ASD and not attributable to alternative diagnoses. Additional cognitive and behavioral data gathered include the Child Behavioral Checklist

(CBCL),[32] Stanford-Binet intellectual quotient- 5th edition (SBV),[33] Mullen Scales of Early Learning (MSEL),[34] Wechsler Intelligence Scale for Children (WISC),[35] Adaptive Behavior Assessment System (ABAS),[36] and the Vineland Adaptive Behavior Scales (VABS).[37] These metrics are discussed further in *Variables,* below.

Blood draw One non-fasting, whole blood draw was performed on each participant. The blood draw took place at OHSU's OCTRI/PCTRC outpatient facility. Blood draws were done by licensed phlebotomists with experience with special-needs children. Roughly one tablespoon was taken at each draw, and blood was handled as outlined in *Data management*, below.

Variables: predictor, outcome, and covariates

Predictor variables The primary predictor variable is the presence or absence of an ASD diagnosis. This is established using DSM-IV criteria, ADOS scales, and clinical judgment. Additional behavioral predictors are levels of development/intelligence (as measured by the MSEL, SBV, or WISC), adaptive behaviors (measured by the ABAS or VABS), and problem behaviors (measured by the CBCL). Metrics are described below.

A. The DSM-IV describes the autism spectrum disorders along 3 main axes: social interaction, communication, and restricted/stereotyped behavior.[3] A diagnosis of autism requires deficits in each of the 3 fields. The other ASD diagnoses include Asperger's disorder and pervasive developmental disorder-not otherwise specified. These are diagnosed using the same axes as autism, but inclusion criteria are less strict. Apserger's disorder involves social

disturbances and restricted behavior, but not language impairment. Pervasive developmental disorder-NOS is diagnosed when clinical impairments exist on the axes of autism but an insufficient number of criteria are met for the diagnosis of autism. Cases met criteria for an ASD, controls did not.

B. The ADOS is an interactive exam during which the clinician assigns scores to a child's behavior across multiple scales, including communication, social interaction, restricted behavior, and play/imagination.[31] Scores of the communication and social interaction scales are combined, and reaching a certain threshold score (>12) is considered diagnostic for ASD or autism. The threshold score for autism is greater than that for ASD, and increasing scores for autism are considered to indicate more severe impairment.

C. The CBCL is a survey administered to parents regarding their child's behavior. Behaviors of the child are scored, with increasing scores indicating increasingly problematic behavior.[32] These scores are compared to established normative data to describe the extent of problem behavior. Problems are primarily considered on 3 scales: internalizing problems (i.e., anxiety, depression), externalizing problems (i.e., acting out, violent behavior), and total problems.

D. The MSEL,[34] SBV,[33] and WISC[35] are interactive tests administered to children to determine their level of cognitive development or intelligence.

Each has been developed according to age-appropriateness, and children below their age-predicted level of development are administered the "development-appropriate" test (i.e., a child unable to complete the SBV will be administered the MSEL). Each test measures multiple axes of development (verbal comprehension, perceptual reasoning, etc.) and produces an overall score, and in this study we are interested in the latter "overall" score for development. This measure is normalized to a mean score of 100 for each test, with scores comparable across tests and across age. These tests are also diagnostic for mental retardation, which features a low intelligence score, and which commonly occurs in autism but more frequently exists independently.

E. The ABAS[36] and VABS[37] are standardized surveys given to parents/care providers to assess a child's "adaptive" behaviors (i.e., activities of daily living, social skills, etc.). Scores are compared to "normal values," with lower scores indicating fewer adaptive skills. The composite measures of each test are normalized to a mean score of 100, with scores comparable across tests and across age.

Outcome variables Plasma cholesterol, a cholesterol precursor (7-DHC), a cholesterol metabolite (24S), and the *DHCR7* gene will be evaluated as the indicators of deranged cholesterol metabolism.

A. Cholesterol is acquired both exogenously from food intake and synthesized endogenously in the body. Endogenously, it is the product of a chain of

enzymatic reactions. Normal values of cholesterol in the US were established by the Lipid Research Clinics Prevalence Study (LRCPS),[38] wherein 15,626 children aged 0-19 years underwent fasting blood draws. Cholesterol values were averaged over 4 bins of 5 years each (0-4, 5-9, 10-14, and 15-19 years), and generally found to increase slightly from the 0-4 year bin average to a small peak before 10 years of age, decline slightly prior to adolescence, and then climb to various adult levels (see Graph 3 in *Discussion*). Importantly, variation between bins over ages ≤19 years was subtle, less than 10 mg/dL. This is in contrast to cholesterol levels from birth to 1 year, which more than double[39] (note that recruitment for this study requires a minimum age of at least 1 year). The LRCPS found that for individuals 0-19 years of age, mean total cholesterol is roughly 160 mg/dL, with a 95th percentile of 200 mg/dL and a 5th percentile of roughly 120 mg/dL. In children with SLOS, Kelley[40] observed mean plasma cholesterol of 57 mg/dL, reflecting the much lower levels (often ½ or less) of total cholesterol in those with SLOS compared to those without SLOS. Finally, blood draws for this study are non-fasting, and our outcome of interest is total cholesterol, which is not affected by fed/fasting state (as opposed to cholesterol fractionated into LDL-C, HDL-C, etc.).[41] Thus, our cholesterol data should be comparable to national norms drawn during a fasting state.

B. 7-DHC is the precursor directly preceding cholesterol in the synthetic chain, and accumulates in individuals with SLOS due to mutation of *DHCR7*, which would normally convert 7-DHC to cholesterol. Levels of 7-DHC are much

greater than normal in individuals with SLOS, from 10 to over a thousand fold. Kelley[40] found mean plasma 7-DHC in patients with SLOS to be 148 μg/mL (sd= 119 μg/mL), compared to control children of 0-12 years with 0.10 μg/mL (sd= 0.05 μg/mL) and controls aged 12 years-adult with 0.13 μg/mL (sd= 0.04 μg/mL). Please note that the current study will report 7-DHC as μg/dL, with the above values equal to 14,800 μg/dL and 10 μg/dL for SLOS patients and control children, respectively. The severity of metabolic cholesterol derangement in SLOS is often presented as the "DHC fraction" which = 7-DHC + 8-DHC (8-DHC is an isomer of 7-DHC) expressed as a fraction of total sterols. This measure has been found to correlate with both increasing severity of clinically graded embryologic malformations and genetic mutations in SLOS (with the most severe genetic cases being homozygous null/null for *DHCR7*).[11] We are not able to report a DHC fraction in this study as we did not quantitate 8-DHC.

C. 24S is a metabolite of cholesterol via the enzyme 24S-hydroxylase, which is expressed primary in the brain, in the adrenal glands to a lesser extent, and scantily in other organs.[42] As cholesterol does not readily cross the blood brain barrier, 24S serves as a means of excretion of brain cholesterol into the circulation. Levels of 24S in the circulation are found to decrease exponentially with age, from a peak in infancy to a plateau past 20 years of age (see *Appendix A*), with 24S/cholesterol ratios of 157 ng/mg for children and 35 ng/mg in adults.[42]

D. The *DHCR7* gene encodes the enzyme that is necessary for the final enzymatic step of cholesterol synthesis. Mutations of exons 4-9 of the *DHCR7* gene result in a variety of alterations throughout the DHCR7 enzyme, but which often occur in 3 regions: the transmembrane domain, the fourth cytoplasmatic loop, and the C-terminus.[43] Of the over 100 mutations known, most mutations are missense mutations, although deletions, insertions, nonsense, and splice site mutations are described as well.[44]

In this study, deviation from established *DHCR7* nucleotide sequence will be defined as 1) a "pathologic mutation" of the type known to cause SLOS (causal when paired with another pathologic mutation), or 2) a "nucleotide variant" which is a non-protein altering nucleotide change. Various combinations of pathologic mutations (when present in homozygous or compound heterozygous states) have been correlated with both the severity of malformations and cholesterol derangement in SLOS.[11] Although we hypothesize an increased frequency of *DHCR7* mutations and nucleotide variants among the ASD group, we do not speculate as to which type of variants/mutations will be most common. The carrier frequency of SLOS mutations is estimated to be 1/30 in Oregonians,[45] with estimates in some European populations even higher.[46]

Covariates This study's cases and controls are relatively closely related groups of subjects, as all displayed behavioral dysfunction, prompting them to be seen at the Autism Clinic and evaluated. That is, control subjects are "clinically-referred," and may

be affected by disorders (for example, global developmental delay) which may co-occur in ASD cases. This sample design will help minimize confounding that may occur by the presence of neurologic dysfunction beyond our current knowledge. Individuals with established genetic or metabolic disorders will be excluded from recruitment.

As BMI may affect blood cholesterol levels,[47] it will be modeled for in analysis if necessary. Age, as described above, strongly influences 24S levels, and will be modeled for accordingly in regression analysis. Gender and race, although not known to strongly influence our outcomes, will be assessed for confounding as well.

Table A: Brief description of predictor and outcome variables

Variable	Characterization
Predictors	
DSM-IV	Clinical diagnosis: non-ASD, autism spectrum, autism
ADOS	Test to diagnose ASD using communication & social scales
CBCL	Survey of problems, higher score= more problems
MSEL/SBV/WISC	Tests of development and intelligence quotient (IQ)
ABAS/VABS	Surveys of adaptive behaviors, lower score= less adaptive
Outcomes	
Cholesterol	Range: normal mean= 160mg/dl, SLOS mean= 70mg/dl
7-DHC	Elevated in SLOS, level increases with severity of SLOS
24S	Metabolite of brain cholesterol
DHCR7	SLOS features homozygous or compound heterozygous mutations

Analyses

Laboratory analysis Specimens consist of a single non-fasting, whole blood draw of approximately 15ml from each subject. Cholesterol and 7-DHC from participant plasma will be measured in the Steiner laboratory by gas chromatography/mass spectrometry analysis,[40] using 5α-cholestane as an internal standard.[48] Calibration will use an "authentic" cholesterol standard.[49] 24S will be measured by liquid

chromatography-tandem mass spectrometry,[50] using a racemic 24-hydroxycholesterol internal standard. Genetic analysis will take place at the OHSU/OCTRI Genetics Core laboratory. Genetic material will be extracted from peripheral lymphocytes (Gentra PureGene Cell Kit) and amplified by polymerase chain using ABI BigDye v3.1 terminator sequencing mix (Applied Biosystems, ABI) on an ABI 9800 Thermal Cycler. The purified sequencing product will be run on an ABI 3130XL Genetic Analyzer and analyzed using Lasergene sequencing software (DNASTAR Inc.).

Statistical analysis

A. Baseline variables of age, gender, race, BMI, and behavioral testing will be compared between cases and controls using 2-sample *t*-tests, χ^2, or Fisher's exact tests, where appropriate, with customary significance levels of p=0.05.

B. Mean sterol levels will be compared between the case and control groups using 2-sample *t*-tests, and then modeled using linear regression to control for potentially confounding variables, such as age, gender, race and BMI. Any variables found to be significant in simple linear regression will be including in model building exercises to determine the most predictive yet parsimonious multiple linear regression models for blood cholesterol and 24S levels (modeling is not planned for 7-DHC). Significance will be defined as p-values equal to or less than 0.05. Sterol comparisons will be used for determination of power and sample size (see below).

C. Analysis of *DHCR7* sequencing will compare proportions of cases and controls by 1) presence of nucleotide variants 2) presence of pathologic mutations 3) presence of any nucleotide variant or pathologic mutation 4) mutations/variants at specific loci or 5)

having a specific unique combination of mutations/variants, by Fisher's exact, given that expected frequencies of mutations are low. This analysis is considered exploratory and will not be subject to linear regression and will not influence power/sample size calculations.

D. Sample size and power will be determined using SLOS as a model of deranged cholesterol metabolism. Studies of SLOS affected children have reported average blood cholesterol of 57 mg/dl with a standard deviation of 53 mg/dl, compared to an average of 155 mg/dl with a standard deviation of 56 mg/dl for unaffected children.[40] Such a large difference, approximately 100 mg/dl, is not expected between our cases and controls, however, and analysis will be designed to detect a difference of 45 mg/dl. Using a difference of 45 mg/dl, the expected standard deviations described, and a power of 80%, 12 subjects in each group would be needed to detect a significance level of $\alpha=0.05$ using 2-sample *t*-tests (Stata 10.1). However, given the exploratory nature of this study, and given the wide spectrum of severity in ASDs, additional data would prove helpful, and we set a goal of 45 of both cases and controls for recruitment.

Data management

Recruitment is executed via lists of subjects who indicated interest in participating in studies at the Autism Program. These lists are maintained by the Autism Clinic and are accessible only to personnel directly involved in either patient care or Program research.

Blood/plasma samples will be stored in coolers and "de-identified," with only assigned subject numbers as identification. Samples will be banked for future studies with the agreement of subjects.

All laboratory data will be gathered by the appropriate investigator and transferred to a common database maintained in de-identified form by this protocol's Principal Investigator at the CDRC. Only investigators of this protocol will have access to the data. All behavioral data is maintained in a de-identified database by the OHSU/CDRC Autism Clinic.

Results

Baseline behavioral and demographic data

Satisfactory data was gathered in a total of 69 participants, 42 cases and 27 controls (see *Inclusion/exclusion of participants,* below, for further discussion). Demographic data is summarized in Table 1. Age was recorded twice: at initial Autism Clinic evaluation and at the time of blood draw. The average time elapsed between these events was 9 months (not different between groups, with cases mean difference = 9.6 months and controls mean difference = 8.5 months, p=0.06). Given that sterol levels are known to vary with age, the age at blood draw is of greater interest than age at evaluation. At time of blood draw, cases were found to be younger (mean= 78.5 months) than controls (mean= 93.4 months), but this difference was not significant (p= 0.16).

Body-mass index levels did not significantly differ between cases and controls (mean= 17.3 and19.0, respectively, p=0.11). Trends of BMI over age were noted to generally follow the percentiles reported by the CDC: decreasing from birth to a nadir lasting approximately from 48 to 84 months, and then rising toward adult levels[61] (see *Appendix B* for graphical summary).

Sex was found to be predominately male and did not differ significantly between groups, with cases 83.3% male and controls 81.5% male (p=1.0).

Race was found to be predominately white and did not differ significantly between groups, with cases 85.4% white and controls 76.9% white (p=0.43). Additional races included African American (N=1, 1.5% of total), Hispanic (N=4, 6.0% of total), Asian/Pacific Islander (N=2, 3.0% of total), and "other" (N=5, 7.5% of total).

Table 1: Participant characteristics

	Total (N=69)	+ASD (N=42)	-ASD (N=27)	P-value
Age at blood draw in months (+/-SD)	84.4 (44.5)	78.5 (47.9)	93.4 (37.8)	0.16
Age at clinic evaluation in months (+/- SD)	75.2 (44.5)	68.9 (47.7)	84.9 (37.8)	0.13
Body-mass index (+/- SD)	17.9 (3.6)	17.3 (2.7)	19 (4.7)	0.11
Male sex N (%)	57 (82.6)	35 (83.3)	22 (81.5)	1
White race N (%)	55 (82.1)	35 (85.4)	20 (76.9)	0.43

Autism Clinic behavioral testing focused on four primary measures: ADOS classification, developmental levels, adaptive behaviors, and behavioral problems (summarized in Table 2). As ASD classification is part of this study's case definition, ADOS scores accordingly were greater in cases (total score= 16.15) than controls (total score= 4.2) to a significant degree ($p < 0.01$). The communication and social measures were also each significantly greater in cases than controls ($p < 0.01$).

Developmental scores were found to be significantly lower in cases than in controls (mean= 64.9 and 90.8, respectively, $p < 0.01$). However, total behavioral problems were found to be significantly greater for controls than cases (mean=71.9 and 64.8, respectively, $p < 0.01$), with internalizing problems also greater in controls compared to cases (mean=70.0 and 62.3, respectively, $p < 0.01$), and externalizing

problems marginally greater in controls than cases (mean=68.0 and 62.5, respectively, p= 0.06). Adaptive behaviors were not significantly different between groups (cases mean=68.1 and control mean=72.7, p=0.10).

Table 2: Behavioral testing

	Total (N=69)	+ASD (N=42)	-ASD (N=27)	P-value	
Developmental (+/- SD)	75.5 (24.0)	64.89 (21.5)	90.8 (19.0)	<0.01	*
Adaptive (+/-SD)	69.9 (12.3)	68.14 (13.7)	72.7 (9.5)	0.10	
Problems-Internal (+/-SD)	65.5 (8.5)	62.33 (7.8)	70.0 (7.4)	<0.01	*
Problems-External (+/-SD)	64.7 (11.6)	62.53 (11.0)	68.0 (11.8)	0.06	
Problems-Total (+/-SD)	67.7 (8.9)	64.78 (8.6)	71.9 (7.7)	<0.01	*
ADOS-Communication (+/-SD)	3.9 (2.8)	5.71 (2.0)	1.2 (1.2)	<0.01	*
ADOS-Social (+/-SD)	7.5 (4.5)	10.44 (2.6)	2.9 (2.4)	<0.01	*
ADOS-Total (+/-SD)	11.5 (6.7)	16.15 (4.1)	4.2 (3.1)	<0.01	*

Inclusion/exclusion of participants

All subjects included were aged 1-18 years and received sufficient behavioral testing to determine the presence or absence of an ASD and had a blood draw sufficient for cholesterol measurement and sequencing of *DHCR7*. Exclusion criteria of known metabolic or genetic defects were not observed for any subjects. Recruitment was more successful for cases than controls, and the goal of a 1:1 case and control ratio was not met due to inability to enroll sufficient controls. Enrollment ceased due to time constraints.

Tests of hypothesis

Comparison of means and frequencies Blood cholesterol levels were found to not differ significantly between cases (mean= 146.6 mg/dL) and controls (mean=140.2

mg/dL, p=0.27), nor were levels of 7-DHC (cases mean= 7.3 μg/dL, control mean= 13.8 μg/dL, p=0.22). However, levels of 24S were found to be significantly greater in cases (mean= 104.1 ng/mL) than controls (mean=78.6 ng/mL, p <0.01), as was the 24S/cholesterol ratio (case mean= 71.6 ng/mg, control mean= 57.4 ng/mg, p < 0.02). Sterols between groups are summarized in Table 3.

Table 3: Sterols by group

	Total (N=69)	+ASD (N=42)	-ASD (N=27)	P-value	
Cholesterol (mg/dL)(+/-SD)	144.1 (22.0)	146.6 (19.0)	140.2 (26.0)	0.27	
7-DHC (μg/dL) (+/-SD)	9.8 (16.8)	7.3 (2.1)	13.8 (26.5)	0.22	
24S (ng/mL)(+/-SD)	94.6 (35.8)	104.1 (37.6)	78.6 (26.1)	<0.01	*
24S/Cholesterol ratio (ng/mg)(+/-SD)	66.3 (24.9)	71.6 (26.3)	57.4 (19.9)	0.02	*

Sequencing of the *DHCR7* gene was first described by pathogenicity and location of variants or mutations (summarized in Table 4). Twenty-six total subjects were found to have a nucleotide variant or mutation, with this proportion significantly greater in cases (21 subjects, 50%) than controls (5 subjects, 19%, p= 0.01). These proportions are the same as those having a nucleotide variant, while fewer had a pathologic mutation. Only children with an ASD were found to have pathologic mutations of *DHCR7*, although the proportion of subjects having a pathologic mutation did not differ significantly between cases (7.1%) and controls (0%, p=0.28). Of the observed pathologic mutations, one is known to lead to a frameshift {Exon 9 (IVS8-1 G>C)},[51] one results in a stop codon {Exon 6 (452 G>A)},[11] and one is a missense mutation {Exon 7 (670 G>A)},[52] each altering the amino acid sequence and final protein. All subjects with pathologic mutations were SLOS carriers (simple heterozygotes); no subjects were

diagnosed with SLOS (i.e., none exhibited two mutations in a homozygous or compound heterozygous pattern).

Table 4: Nucleotide variants and mutations, by exon

	Total (N=69)	+ASD (N=42)	-ASD (N=27)	P-value	
Subjects with any variant or mutation N(%)	26 (37.7)	21 (50)	5 (19)	0.01	*
Subjects with a pathologic mutation N(%)	3 (4.4)	3 (7.1)	0 (0)	0.28	
Subjects with a nucleotide variant N(%)	26(37.7)	21 (50)	5 (19)	0.01	*
Subjects with variants and/or mutations, by exon					
Exon 3 N(%)	0 (0)	0 (0)	0 (0)	NA	
Exon 4 (189 A>G) N(%)	22 (31.9)	18 (42.9)	4 (14.8)	0.02	*
Exon 4 (231 C>T) N(%)	3 (4.4)	3 (7.1)	0 (0)	0.28	
Exon 5 (336 G>A) N(%)	2 (2.9)	2 (4.8)	0 (0)	0.52	
Exon 6 (438 C>T) N(%)	8 (11.6)	5 (11.9)	3 (11.1)	1	
Exon 6 (452 G>A) N(%)	1 (1.5)	1 (2.4)	0 (0)	1	
Exon 7 (670 G>A) N(%)	1 (1.5)	1 (2.4)	0 (0)	1	
Exon 9 (IVS8-1 G>C) N(%)	1 (1.5)	1 (2.4)	0 (0)	1	
Exon 9 (1008 C>T) N(%)	1 (1.5)	1 (2.4)	0 (0)	1	
Exon 9 (1158 C>T) N(%)	10 (14.5)	7 (16.7)	3 (11.1)	0.73	
Exon 9 (1272 T>C) N(%)	23 (33.3)	18 (42.9)	5 (18.5)	0.04	*

Considered by exon location, a significantly larger proportion of cases had nucleotide variants at Exon 4(189 A>G) (cases= 42.9%, controls= 14.8%, p=0.02) and Exon 9(1272 T>C) (cases=42.9%, controls=18.5%, p=0.04). The proportion having variants or mutations at other locations did not significantly differ (Table 4). Variants/mutations occurred in 11 unique combinations, and the proportion in which these combinations occurred in cases and controls was not significantly different ($p \geq 0.30$ for each comparison).

Finally, carrier frequencies of pathologic *DHCR7* mutations ("SLOS mutations") have been published, with a carrier frequency of IVS8-1 mutations observed to be 1% and the carrier frequency of all SLOS mutations estimated to be 3.4%.[39] The current study's case carrier frequencies were 2.4% and 7.1% while control carrier frequencies were 0% and 0% (IVS8-1 and all SLOS mutations, respectively), although none of the

differences between case/control and expected carrier frequencies were statistically significant (see Table 5 below).

Table 5: Carrier frequencies for SLOS pathologic mutations in Oregon children

Battaile et al.		Current study					
	Carrier (%)	+ASD	Carrier (%)	P-value	-ASD	Carrier (%)	P-value
IVS8-1	1.06	IVS8-1	2.38	0.36	IVS8-1	0.0	1.0
All mutations†	3.37	All mutations‡	7.14	0.17	All mutations‡	0.0	1.0

† "Total" carrier frequency extrapolated from observed frequency of IVS8-1 mutation

‡ All pathologic mutations observed in the current study

Regression analysis Outcome variables of interest for regression analysis include cholesterol and 24S. Relevant predictors include the presence/absence of an ASD (this study's variable of primary interest), age (which is known to influence cholesterol and 24S levels), gender, race, and BMI (which is known to influence cholesterol levels).[47]

A. Cholesterol

1. Simple linear regression

Cholesterol was found be distributed close enough to normal so as to be suitable for regression (p=0.054, Shapiro-Wilk test). Regressing cholesterol on all predictors individually, no variables were found to be predictive of cholesterol levels (see Table 6).

Table 6: Simple linear regression of cholesterol on single predictors

		95% CI				
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared
ASD	6.41	-4.39	17.21	1.40	0.24	0.02
Age	-0.05	-0.17	0.07	0.57	0.45	0.01
BMI	-0.60	-2.14	0.94	0.61	0.44	0.01
Gender	-9.18	-23.05	4.69	1.74	0.19	0.03
Race_2*	-9.13	-54.55	36.29	0.53	0.72	0.03
Race_3*	-1.38	-24.69	21.93	0.53	0.72	0.03
Race_4*	17.87	-14.53	50.28	0.53	0.72	0.03
Race_5*	-8.33	-29.35	12.69	0.53	0.72	0.03

*Note that race regressed while comparing to "white" and controlling for all other races.
Race 2= Black, 3= Hispanic, 4=Asian/Pacific Islander, 5=Other

2. Model building and diagnostics

The lack of significance of individual predictors is not due to failing to satisfy the assumption of linearity, nor are there any apparent outliers, as illustrated in the scatter plots of cholesterol by each variable (see *Appendix C*). When including all variables in forward selection and backward elimination procedures, no variables were found to significantly predict cholesterol at p=0.05 (see *Appendix D*).

3. Final model

When cholesterol was regressed on all variables in one model, no variables were found to be significantly predictive of cholesterol (see Table 7) at the level of p=0.05, and the in the full model F(8,53)=0.98, p=0.46.

Table 7: Multiple linear regression of cholesterol on all predictors

		95% CI			
Predictor	β coef.	Lower	Upper	t statistic	P-value
ASD	5.98	-6.49	18.46	0.96	0.34
Age	-0.01	-0.19	0.17	-0.11	0.91
BMI	-0.21	-2.33	1.92	-0.20	0.85
Gender	-11.52	-26.40	3.36	-1.55	0.13
Race_2*	-1.67	-48.27	44.92	-0.07	0.94
Race_3*	1.40	-22.17	24.96	0.12	0.91
Race_4*	16.75	-16.38	49.88	1.01	0.32
Race_5*	-23.99	-51.02	3.04	-1.78	0.08

*Note that race regressed while comparing to "white."
Race 2= Black, 3= Hispanic, 4=Asian/Pacific Islander, 5=Other

B. 24S

1. Simple linear regression

24S was found be distributed close enough to normal so as to be suitable for regression (p=0.15, Shapiro-Wilk test). Regressing 24S on all predictors individually, ASD status, age, natural log transformation of age, and BMI were

found to be predictive of 24S levels (see Table 8). Transformation of age was

Table 8: Simple linear regression of 24S on single predictors

		95% CI				
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared
ASD	25.53	8.48	42.57	8.95	<0.01*	0.12
Age	-0.53	-0.68	-0.38	52.29	<0.01*	0.45
Ln(Age)	-48.72	-61.01	-36.44	62.73	<0.01*	0.49
BMI	-4.41	-6.66	-2.17	15.43	<0.01*	0.20
Gender	-2.08	-25.00	20.84	0.03	0.86	0.00
Race_2*	-37.80	-110.96	35.36	0.74	0.57	0.05
Race_3*	-1.65	-39.23	35.94	0.74	0.57	0.05
Race_4*	32.98	-19.23	85.19	0.74	0.57	0.05
Race_5*	8.38	-25.53	42.29	0.74	0.57	0.05

*Note that race regressed while comparing to "white" and controlling for all other races.
Race 2= Black, 3= Hispanic, 4=Asian/Pacific Islander, 5=Other

done given the known logarithmic decrease in 24S levels with increasing age from infancy to adulthood (see *Appendix A*).

2. Model building and diagnostics

Individual predictors were found to satisfy the assumption of linearity and lack outliers, as illustrated in the scatter plots of 24S on each variable (see *Appendix E*). In particular, the transformation of age is noted to be slightly more linear than non-transformed age. When including all variables (including either age or transformed age, but not both simultaneously) in forward selection and backward elimination procedures, age, natural log transformed age, and ASD status were found to significantly predict 24S at p=0.05 (see *Appendix F*), although, as will be illustrated below, the significance of ASD status depends on whether age or transformed age is used. Thus, two models were considered for model diagnostics: 1) 24S regressed on age and ASD status and 2) 24S regressed on natural log transformed age and ASD status. Viewing the jackknife residuals of the models plotted against the two models' fitted values suggests they fulfill the linear regression assumptions of linearity and homoscedasticity (see *Appendix*

G). Given that both of the above two models are sound and are predictive of 24S, each is considered in discussion below.

3. Final models

The general forms of the two models found to be predictive of 24S are given as "expected 24S" or "E(24S)":

1) $E(24S)=\beta_0+ \beta_1*ASD+ \beta_2*Age+ E$

2) $E(24S)=\beta_0+ \beta_1*ASD+ \beta_2*$Natural log transformed age$+ E$

Performing linear regression of the above equations finds the output as given in Table 9, with the final models:

1) $E(24S)=109.02+ 17.01*ASD - 0.50*Age$

2) $E(24S)=269.48+ 13.10*ASD -$ 45.51*Natural log transformed age

Table 9: Multiple linear regression of 24S on two predictors, in two different models

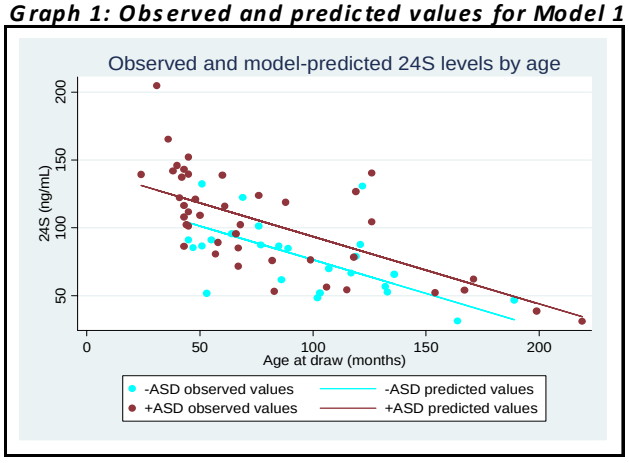
Model 1		95% CI						
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared	t statistic	Prob >|t|
ASD	17.01	3.79	30.23	31.70	<0.01	0.50	2.57	0.01*
Age	-0.50	-0.64	-0.35	31.70	<0.01	0.50	-6.93	<0.01*
Model 2		95% CI						
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared	t statistic	Prob >|t|
ASD	13.10	-0.03	26.23	34.78	<0.01	0.52	1.99	0.051
Ln(Age)	-45.51	-57.95	-33.07	34.78	<0.01	0.52	-7.31	<0.01*

In summary, both models are predictive of 24S levels, and transformed age is slightly more predictive of 24S than non-transformed age. ASD status is significantly predictive of 24S levels when modeled with age, but is only marginally predictive of 24S when modeled with transformed age. Or, said another way, when controlling for age, 24S levels are significantly greater in subjects with an ASD, however, when controlling for natural log transformed age, 24S levels are marginally greater in subjects with an ASD. The difference in 24S

by age between ASD and non-ASD subjects is illustrated in Graphs 1 and 2, below.

Viewing the plotted values in Graphs 1 and 2 one can see the clustering of subjects with an ASD in the younger age range. Restricting models to include only subjects of the same age range {and Ln(age) range} finds that 24S remains significantly different between ASD status groups when regressed with age, and remains marginally significant when regressed with natural log transformed age (see Table 10).

Graph 1: Observed and predicted values for Model 1



Graph 2: Observed and predicted values for Model 2

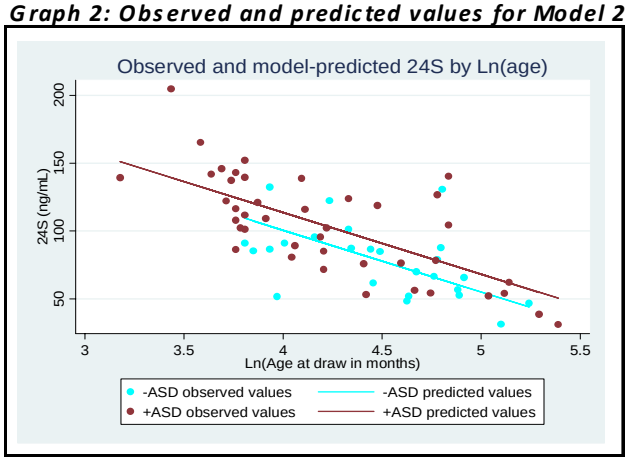


Table 10: Multiple linear regression of 24S on two predictors, in two different models, with ages restricted to the same range

Model 1		95% CI						
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared	t statistic	Prob > |t|
ASD	13.83	0.17	27.48	12.60	<0.01	0.34	2.03	0.05*
Age	-0.39	-0.57	-0.21	12.60	<0.01	0.34	-4.30	<0.01*
Model 2		95% CI						
Predictor	β coef.	Lower	Upper	F statistic	Prob >F	R-squared	t statistic	Prob > |t|
ASD	13.33	-0.60	27.27	10.84	<0.01	0.31	1.92	0.060
Ln(Age)	-34.56	-51.84	-17.29	10.84	<0.01	0.31	-4.02	<0.01*

Discussion

Baseline comparability of cases and controls

Baseline data of gender, race, age, and BMI were not found to differ significantly between groups, and pertinent issues regarding these variables (i.e., controlling for them as covariates, issues of generalizability, etc.) are discussed below.

As discussed in *Methods,* the cases and controls were all individuals referred to the Autism Clinic for evaluation, which suggests at least somewhat similar behavioral characteristics of cases and controls. Differences observed between cases and controls include lower developmental scores in cases and more problematic behavior in controls. Lower developmental scores in subjects with an ASD is not surprising, given that developmental delay often occurs with ASDs.[3] More problematic behavior in non-ASD subjects may suggest that the severely problematic behavior was itself sufficient to prompt caregivers to seek an evaluation for autism, and that caregivers may not know or may misunderstand the defining features of autism. As discussed further below, our use of a clinically-referred control group provides a robust comparison and is a strength of the current study.

Discussion of sterol findings

Cholesterol and 7-DHC As noted in *Background*, there has been increasing evidence of disordered cholesterol metabolism in ASDs, particularly that cholesterol levels are lower in ASDs than expected.[15] Using SLOS as a model of inhibited cholesterol synthesis, we hypothesized that cholesterol would be lower and 7-DHC (a cholesterol precursor) would be higher in subjects with ASDs. However, our data found

no significant difference in cholesterol or 7-DHC between the groups. This is not due to lack of study power when given that SLOS was our disease model. However, it is true that if very subtle differences in levels did exist, this study may have lacked sufficient enrollment to detect them.

This study's average observed 7-DHC levels were 7.3 μg/dL for cases and 13.8 μg/dL for controls. Although the finding that 7-DHC levels in controls were twice that of cases is initially striking, both groups are far below the levels expected for individuals with SLOS. Comparing our data to the child controls in Kelley[40] of 10 μg/dL, we see good agreement, and stark contrast to the levels in Kelley's SLOS patients, which averaged 14,800 μg/dL. As well, the higher level of our controls was largely the result of two subjects with 7-DHC levels of 52 and 140 μg/dL. Importantly, these levels are still well below what would be expected for cases of SLOS and are likely attributable to individual or laboratory variation. Neither subject had any nucleotide variants or pathologic mutations. That no subjects of the current study had 7-DHC levels suggestive of SLOS is to be expected, as no subjects had two pathologic SLOS mutations, so all would be expected to have sufficient *DHCR7* activity to convert 7-DHC to cholesterol.

Cholesterol was not significantly predicted by BMI or any other of our baseline variables. There is not extensive data correlating BMI and cholesterol in children, but a recent study observed only a weak correlation (Spearman rho =0.13) between BMI and non-HDL cholesterol.[53] It may be that our study had insufficient power to detect such a small relationship.

We found no difference in cholesterol and 7-DHC levels between cases and controls, and although this does not rule out disordered cholesterol metabolism in ASDs,

it does suggest that using SLOS as a disease model may be incorrect. It is possible, however, that a confounder may be influencing our data, such as exposure to the common anticonvulsant drug valproate, which has been observed to inhibit cholesterol synthesis in rat brains.[54] Exposure to valproate was not recorded in the present study, but could theoretically represent a positive or negative confounder of our findings if its experimental effects were disproportionately present in cases or controls, respectively. Finally, even though we found cholesterol and its immediate precursor 7-DHC in normal quantities in blood, it is possible that cholesterol utilization, destruction, or turnover is affected by case status—processes which may not perturb total cholesterol and 7-DHC but which may help explain the difference in 24S levels observed in this study.

24S

A. Findings and modeling If SLOS was a fitting disease model for cholesterol metabolism in ASDs, we would expect lower 24S in cases than controls, which was not observed. Given the younger age of cases, we would expect greater 24S levels in them, which was observed. We found that even when controlling for age, 24S levels were different between cases and controls. However, when age was controlled for and transformed by its natural logarithm to better account for the exponential change of 24S over age, 24S became marginally different between cases and controls. When further controlling for age by restricting analysis to subjects of the same age range, similar results were observed.

Considering which of the above models is most predictive, we see that the greatest amount of variance of 24S, 52% (r-squared=0.52), is explained by Model 2,

which uses the natural log-transformed age over full age ranges (see Table 9). The model which explains the least amount of variance, 31% (r-squared=0.31), is Model 2 when using restricted age ranges. When ASD status is removed from Models 1 and 2 (not restricted by age), the variability of 24S explained by the models decreases from 50% and 52%, respectively, to 45% and 50%, illustrating that ASD status accounts for 5% and 2% of the variance of 24S, depending on the model. We can conclude that although age is most important in predicting 24S levels, the presence of an ASD meaningfully influenced 24S levels as well. Although statistical significance is alternatively present and borderline for ASD status, potential clinical significance is present throughout.

In terms of the magnitude of 24S change, case status increases 24S by 17.01 ng/mL in Model 1 and by 13.10 ng/mL in Model 2. Regarding age, Model 1 finds for each month increase in age, 24S will fall 0.5 ng/mL. In contrast, the magnitude of 24S change over age predicted by Model 2 is not constant, but rather varies with age. This is illustrated by finding the velocity of 24S change over age, that is, its derivative. In Model 1, derivation of the term 0.50*Age equals 0.5, meaning that the change in 24S due to age is constant, at 0.5 ng/mL per month. In Model 2, derivation of the term 45.51*Ln(age) equals 45.51/age, meaning that the change in 24S varies with age, and is inversely proportional to age. For example, Model 2 predicts that when increasing age from 5 to 6 months, 24S= -45.51*Ln(6/5)= -8.3 ng/mL, while when increasing age from 99 to 100 months, 24S= -45.51*Ln(100/99)= -0.46 ng/mL. Note the substantial difference in the change of 24S, even though each change in age was = 1 month. Thus it is seen that Model 2 accounts for the logarithmic nature of 24S over age while Model 1 assumes a linear relationship.

B. Possible physiologic and pathophysiologic pathways Our finding of greater 24S levels in children with an ASD is novel, and suggests a greater flux of 24S from the brains of children with an ASD, perhaps by increased cholesterol turnover, increased brain cholesterol due to increased brain volume, or increased neuronal destruction.

Although no known anatomic or physiologic change in the brain defines ASDs, research has suggested a variety of differences between autistic and non-autistic brain form and function. Most relevant to our study are differences observed in brain size. In children with an ASD, head circumference, an indicator of brain size, was observed to be larger, with increased rates of macrocephaly, compared to controls.[55] Similarly, Courchesne found cerebral and cerebellar grey and white matter volumes to be greater in children with ASDs than controls between ages 2-4 years, but by 16 years of age, volumes were similar (cerebellar white matter) or greater (remaining 3 areas) in controls for each measure.[56] In the same study, Courchesne also observed an increased prevalence of macrocephaly (37%) in children with autism at ages of 2-4 years. Head circumference and brain volumes were not measured in our study, but it is conceivable that greater brain volumes, with a greater absolute level of cholesterol turnover than smaller brains, could account for increased levels of 24S, especially considering the high cholesterol content of myelin.[18]

As discussed in *Background,* 24S is low in children with SLOS (presumably from low brain cholesterol turnover) and increased in individuals with dementia (possibly from increased neuronal destruction). An additional pathway impacting 24S levels is that via histone deacetylase inhibitors, which in rat brains have been shown to increase the

expression of 24-hydroxylase, the enzyme which produces 24S.[57] Interestingly, valproate (a commonly used anticonvulsant) is a histone deacetylase inhibitor, and fetal intrauterine exposure to it has been used in creating rat models of autism,[58] has been shown to decrease IQ in human children,[59] and has been reported to increase the risk of autism in children.[60] Taken together, such literature suggests a potential role for valproate in causing autism and increasing levels of 24S, and thus valproate exposure represents a possible, albeit extremely unlikely, positive confounder in the present study. Data on the maternal or subject use of valproate was not gathered in our study.

Discussion of genetics findings

No cases of SLOS were identified. However, single pathologic mutations were observed in three children with an ASD (and none were observed in controls): Exon 6 (452G>A), stop codon; Exon 7 (670 G>A), missense; Exon 9 (IVS8-1 G>C), splice-site frameshift. The mutations at Exon 6 (452G>A) and Exon 9 (IVS8-1 G>C) are reported to be present in 6.4% and 31.5%, respectively, of published cases of SLOS,[43] while Exon 7 (670 G>A) was described more recently[52] and its prevalence is not established.

Each subject with a pathologic mutation in our study is a carrier of SLOS. Carrier frequencies of SLOS mutations have been established in the literature, with the carrier rate of any SLOS mutation estimated at 3.4% in Oregon.[45] Interestingly, we observed pathologic mutations in 7% of cases (double the expected frequency) and no controls. While the carrier frequencies in our cases and controls did not statistically significantly differ compared to the expected prevalence in Oregon (see Table 5), it is striking that we observed twice the expected carrier rate for SLOS in our ASD group and no carriers in

our controls. Our small sample size limits the potential of this finding to approach statistical significance.

The proportion of subjects with nucleotide variants was significantly different, at 50% of cases and 19% of controls. Analysis of such sites of increased variation may prove useful in future genetic studies as established single nucleotide polymorphism maps grow and increase in power.

The implications of these genetics findings are unclear. No genotypes were consistent with SLOS, however, SLOS mutations were observed more frequently in the ASD group than would be expected by published estimates (although "non-significantly"). As well, more variability, in the form of nucleotide variants, was observed in cases. Taken together, these data may suggest increased genetic instability in children with ASDs, particularly in genes involved in cholesterol metabolism.

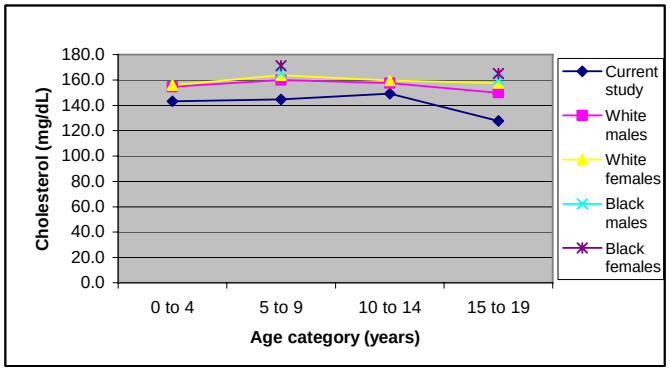
Generalizability of the findings

Our subjects were mostly boys, which is consistent with a greater prevalence of ASDs in boys than girls,[1] and mostly white, which is consistent with the racial make up of this study's setting in Portland, Oregon. We acknowledge that this study's small number of non-white participants limited our ability to perform sub-analyses by race, and thus that we may not be able to identify different outcomes by race, if they were to exist.

Of interest, cholesterol norms established using nation-wide data[38] at ages 0-4, 5-9, 10-14, and 15-19 years were found to be similar to averages in this study (see Graph 3). As well, when the BMI levels and trend with age found in this study are compared to

nationally-established BMI by age percentiles,[61] there is good agreement (see *Appendix B*). This is reassuring for the generalizability of this study.

Graph 3: Cholesterol levels from current study and national sample



Limitations

This is the most comprehensive investigation of the role of cholesterol metabolism in autism to date. The complexity of the variables involved, however, create many challenges.

First, when considering an "autism spectrum disorder," the inherent variability of the phenotype therein makes classification challenging. To make our results as valid and generalizable as possible, the "gold standards" of diagnosing autism, the DSM-IV and ADOS, were used. Bias was avoided by the interdisciplinary (pediatricians, clinical psychologists, geneticists, etc., must be in agreement on the diagnosis) nature of evaluations at the Autism Clinic.

Second, our control group consisted of children without an ASD or known genetic or metabolic disorders who were referred for an evaluation for autism. This "clinically-referred" control is desirable in that it is a rigorous comparison between those with, and those suspected of having, an ASD. This allows us to compare groups across strict criteria for ASDs, and also allows behavioral comorbidities to be present in each group,

thus loosely "matching" for potential confounders (known and unknown) therein. This is in contrast to comparing ASD cases to "normal" controls with no behavioral comorbidities. Such a comparison lacks overlap of comorbidities and would be subject to more confounding unless the comorbidities are strictly controlled for. Our similar comparison groups may also both harbor pathology of cholesterol metabolism, and our comparison should find only that difference in cholesterol metabolism attributable to ASD status, although it may also make such a difference more difficult to detect than comparing ASD cases to "normal" controls, as a greater difference in outcome variables would perhaps be observed comparing ASD cases and "normal" controls. Accordingly, comparison between our cases and controls can be considered conservative and potentially statistically shifted toward the null hypothesis. Certainly, small sample size may limit this study's ability to find subtle differences in our outcome variables.

Third, even though strict controlling for age was attempted in efforts to appropriately compare levels of 24S, our enrollment was small and we were unable to match ages between cases and controls, and the control group was older. One to one age matching between groups would significantly decrease the concern for age's effect on 24S levels and greatly assist in the comparison of 24S levels between groups.

Finally, the role of genetics in ASDs is broad, beyond *DHCR7* mutations alone. Furthermore, although our study is the first to describe *DHCR7* mutations across the ASD spectrum, we examined the sequence of only a single gene, coding for only one enzyme, and additional genes involved in cholesterol metabolism and beyond must be considered. Also, our study was powered to find moderate to mild changes in plasma cholesterol, and

may not have had adequate power to detect differences between the frequency of *DHCR7* mutations among our cases, controls, and published population data.

Future research

There is a growing body of knowledge describing disordered cholesterol metabolism in ASDs, although at present a consensus of pathology does not exist. Our data suggest more genetic variability in a gene coding for an enzyme vital for cholesterol synthesis and suggest greater flux of cholesterol (via 24S) from the brains of children with an ASD compared to controls. Our data neither firmly supports nor refutes previous findings, but rather expand the general theory that cholesterol metabolism is perturbed in autism.

The much anticipated DSM-V is scheduled for release in 2013, and proposed changes to DSM-IV definitions include subsuming the diagnoses of autism, Asperger's disorder, childhood disintegrative disorder, and PDD-NOS under the single diagnosis of "autism spectrum disorders."[62, 63] The rationale for this change includes findings that while the aforementioned conditions are well-distinguished from other developmental disorders, they are not well-differentiated from each other, and would be best classified under one diagnosis understood to entail a spectrum of pathology, with established modifiers (i.e. for severity, specific deficits, etc.) to describe heterogeneity within the diagnosis.[62] Such a re-classification would not greatly impact the implications of the current study, as our case definition corresponds exactly to that of the newly proposed diagnosis of "autism spectrum disorders," except for childhood disintegrative disorder, which is not included in the current study and is very rare (2/100,000).[63] This agreement of the current study's focus with the possible re-defining of autism spectrum disorders in

the DSM-V suggests satisfactory applicability of our data to future neuropsychiatric research.

Future directions suggested by our findings include both expanding upon our methods and looking to new horizons. Increased sample size may be necessary to find subtle perturbations of cholesterol metabolism in ASDs, and comparing across cases, clinically-referred controls, and "normal" controls may potentially illustrate gradations of such pathology. Accounting for age, head circumference/brain volume, and exposures (such as valproate) will be essential.

The interplay of sterols and histone deacetylase inhibitors (i.e., valproate), which inhibit cholesterol synthesis[54] and induce 24S production,[57] warrants both further animal model and human study, and suggests possible interesting avenues in fetal programming due to intrauterine exposure. Analysis of genes involved in cholesterol metabolism should continue, but expand to encompass a more complete picture of its synthesis, use, and breakdown, and how these may vary with age. Corbett found decreased levels of apolipoprotein (B-100 and A-IV) in the blood of autistic versus non-autistic children.[64] Apolipoproteins are necessary for cholesterol transport and homeostasis and their decreased levels could be either causal or in response to alterations of cholesterol metabolism. This finding illustrates that we must look beyond cholesterol itself to consider the full scope of sterol, lipid, and isoprenoid metabolism.

Hopefully, with further effort, effective forms of medical management will be developed for ASDs, and it is feasible such treatment may grow from understanding gained of cholesterol's role in autism.

References

1) Centers for Disease Control and Prevention. Autism Spectrum Disorders Homepage. Facts About ASDs [Online]. (March 2010). Available: http://www.cdc.gov/ncbddd/autism/facts.html

2) Harvard School of Public Health. Press Releases. Autism Has High Cost to U.S. Society [Online]. (April 2006). Available: http://www.hsph.harvard.edu/news/press-releases/2006-releases/press04252006.html

3) Pervasive developmental disorders. In Diagnostic and statistical manual of mental disorders. 4th ed (DSM-IV-TR). 2000; Washington, DC: American Psychiatric Association.

4) Muhle R, Trentacoste SV, Rapin I. Genetics in Autism. Pediatrics. 2004;113:472-486.

5) Centers for Disease Control and Prevention. CDC Studies on Vaccines and Autism Spectrum Disorders [Online]. (September 2008). Available: http://www.cdc.gov/ncbddd/autism/documents/vaccine_studies.pdf

6) Fombonne E. Is There an Epidemic of Autism? Pediatrics. 107 (2), 2001:411-412.

7) Fombonne E. Epidemiology of Autistic Disorder and Other Pervasive Developmental Disorders. Journal of Clinical Psychiatry. 2005; 66 [suppl 10]: 3-8.

8) Kumar RA, Christian SL. Genetics of Autism Spectrum Disorders. Current Neurology and Neuroscience Reports. 2009;9: 188-197.

9) Manzi B, Loizzo AL, Giana G, Curatolo P. Autism and Metabolic Disease. Journal of Child Neurology. 2008; 23(3):307-314.

10) Wassif CA, Maslen C, Kachilele-Linjewile S, Lin D, Linck LM, Connor WE et al. Mutations in the Human Sterol 7-Reductase Gene at 11q12-13 Cause Smith-Lemli-Opitz Syndrome. American Journal of Human Genetics. 1998; 63:55-62.

11) Witsch-Baumgertner M, Fitzky BU, Ogorelkova M, Kraft HG, Moebius FF, Glossmann H et al. Mutational Spectrum in the 7DHC Reductase Gene and Genotype-Phenotype Correlations in 84 Patients with Smith-Lemli-Opitz Syndrome. American Journal of Human Genetics. 2000; 66: 402-412.

12) Sikora DM, Pettit-Kekel K, Penfield J, Merkens LS, Steiner RD. The Near-Universal Presence of Autism Spectrum Disorders in Children With Smith-Lemli-Opitz Syndrome. American Journal of Medical Genetics Part A. 2006; 140: 1511-1518.

13) Tierney E, Nwokoro NA, Porter FD, Freund LS, Ghuman JK, Kelley RI. Behavior Phenotype in the RSH/Smith-Lemli-Opitz Syndrome. American Journal of Medical Genetics. 2001; 98: 191-200.

14) Bukelis I, Porter FD, Zimmerman AW, Tierney E. Smith-Lemli-Opitz Syndrome and Autism Spectrum Disorder. American Journal of Psychiatry. 2007; (164)11:1655-1661.

15) Tierney E, Bukelis I, Thompson RE, Ahmed K, Aneja A, Kratz L et al. Abnormalities of Cholesterol Metabolism in Autism Spectrum Disorders. Americal Journal of Medical Genetics Part B. 2006; 141B: 666-668.

16) Waage-Baudet H, Lauder JM, Dehart DB, Kluckman K, Hiller S, Tint GS et al. Abnormal Serotonergic Development in a Mouse Model for the Smith-Lemli-Opitz Syndrome: Implications for Autism. International Journal of Developmental Neuroscience. 2003;21: 451-459.

17) Chugani DC. Role of Altered Brain Serotonin Mechanisms in Autism. Molecular Psychiatry. 2002; 7:S16-S17.

18) Saher G, Brugger B, Lappe-Siefke C, Mobius W, Tozawa R, Wehr MC et al. High Cholesterol Level is Essential for Myelin Membrane Growth. Nature Neuroscience. 2005 ; 8 :468-475.

19) Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A et al. CNS Synaptogenesis Promoted by Glia-Derived Cholesterol. Science. 2001 ; 294 : 1354-1357.

20) Porter FD. Malformation Syndromes due to Inborn Errors of Cholesterol Synthesis. Journal of Clinical Investigations. 2002; 110:715-724.

21) Gibbs TT, Farb DH. Dueling Enigmas: Neurosteroids and Sigma Receptors in the Limelight. Science Signaling. 2000; 60:pe1.

22) Papassotiropoulos A, Lutjohann D, Bagli M, Locatelli S, Jessen F, Buschfort R et al. 24S-hydroxycholesterol in Cerebrospinal Fluid is Elevated in Early Stages of Dementia. Journal of Psychiatric Research. 2002; 36:27-32.

23) Leoni V. Oxysterols as Markers of Neurological Disease- a Review. The Scandinavian Journal of Clinical & Laboratory Investigation. 2009;69(1): 22-25.

24) Bjorkhem I, Starck L, Andersson U, Lutjohann D, von Bahr S, Pikuleva I et al. Oxysterols in the Circulation of Patients with the Smith-Lemli-Optiz syndrome: Abnormal Levels of 24S- and 27-hydroxycholesterol. Journal of Lipid Research. 2001; 42:366-371.

25) Lutjohann D, Papassotiropoulos A, Bjorkhem I, Locatelli S, Bagli M, Oehring RD et al. Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. Journal of Lipid Research. 2000; 41: 195-198.

26) Kolsch H, Ludwig M, Lutjohann D, Rao ML. Neurotoxicity of 24-hydroxycholesterol, an important cholesterol elimination product of the brain, may be prevented by vitamin E and estradiol-17B. Journal of Neural Transmission. 2001; 108 (4):475-488.

27) Linck LM, Lin DS, Flavell D, Connor WE, Steiner RD. Cholesterol Supplementation With Egg Yolk Increases Plasma Cholesterol and Decreases Plasma 7-Dehydrocholesterol in Smith-Lemli-Opitz Syndrome. American Journal of Medical Genetics. 2000 ; 93 :360-365.

28) Elias ER, Irons MB, Hurley AD, Tint GS, Salen G. Clinical Effects of Choesterol Supplementation in Six Patietns With the Smith-Lemli-Opitz Syndrome (SLSO). American Journal of Medical Genetics. 1997 ; 68 :305-310.

29) Sikora DM, Ruggiero M, Petit-Kekel K, Merkens LS, Connor WE, Steiner RD. Cholesterol Supplementation Does Not Improve Developmental Progress In Smith-Lemli-Opitz Syndrome. Journal of Pediatrics. 2004; 144: 783-91.

30) Jira PE, Wevers RA, de Jong J, Rubio-Gozalbo E, Janssen-Zijlstra FS, van Heyst AF et al. Simvastatin: a New Therapeutic Approach for Smith-Lemli-Opitz Syndrome. Journal of Lipid Research. 2000; 41:1339-1346.

31) Lord C, Rutter M, DiLavore P, Risi S. Autism Diagnostic Observation Schedule-Generic. 1999. Western Psychological Services.

32) Achenbach TM. Child Behavioral Checklist. 2000. Achenbach System of Empirically Based Assessment.

33) Roid GH. Stanford-Binet Intelligence Scales, Fifth Edition (SB:V). 2003. Itasca, IL: Riverside Publishing.

34) Mullen EM. Mullen Scales of Early Learning, AGS Edition. 1995. Bloomington, MN: Pearson Assessments.

35) Wechsler D. Wechsler Intelligence Scale for Children, 4th edition. 2003. Bloomington. MN: Pearson Assessments.

36) Harrison P and Oakland T. Adaptive Behavior Assesesment System, 2nd Edition. 2003. Bloomington. MN: Pearson Assessments.

37) Sparrow SS. Vineland Adaptive Behavior Scales, Interview Edition. 1984. Bloomington. MN: Pearson Assessments.

38) Lipid Research Clinics Program. The Lipid Research Clinics Population Studies Data Book, Vol. 1. Bethesda, MD: National Institutes of Health, Lipid Metabolism Branch; 1980.

39) Darmady JM, Fosbrooke AS, Lloyd JK. Prospective Study of Serum Cholesterol Levels during First Year of Life. British Medical Journal. 1972; 2: 685-688.

40) Kelley RI. Diagnosis of Smith-Lemli-Opitz syndrome by gas chromatography/mass spectrometry of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. Clinica Chimica Acta. 1995;236:45-58.

41) Cohn JS, McNamara JR, Schafer EJ. Lipoprotein Cholesterol Concentrations in the Plasma of Human Subjects as Measured in the Fed and Fasted States. Clinical Chemistry. 1988; 34(12): 2456-2459.

42) Lutjohann D, Breuer O, Ahlborg G, Nennesmo I, Siden A, Diczfalusy U et al. Cholesterol homeostasis in human brain: Evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proceedings of the National Academy of Sciences. 1996; 93: 9799-9804.

43) Jira PE, Waterham HR, Wanders RJ, Smeitink JA, Sengers RC, Wevers RA. Smith-Lemli-Opitz Syndrome and the *DHCR7* Gene. Annals of Human Genetics. 2003; 67: 269-280.

44) The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff. Search gene symbol: DHCR7. [Online]. (April 2010). Available: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=DHCR7

45) Battaile KP, Battaile BC, Merkens LS, Maslen CL, Steiner RD. Carrier Frequency of the Common Mutation IVS8-1G>C in *DHCR7* and Estimate of the Expected Incidence of Smith-Lemli-Opitz Syndrome. Molecular Genetics and Metabolism. 2001; 72: 67-71.

46) Nowaczyk MJ, Waye JS, Douketis JD. *DHCR7* Mutation Carrier Rates and Prevalence of the RSH/Smith-Lemli-Optiz Syndrome: Where Are the Patients? American Journal of Medical Genetics Part A. 2006; 140A: 2057-2062.

47) Williams PT, Hoffman K, La I. Weight-Related Increases in Hypertension, Hypercholesterolemia, and Diabetes Risk in Normal Weight Male and Female Runners. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27:1811-9.

48) Corso G, Rossi M, De BD, Rossi I, Parenti G, Dello RA. Effects of storage on 7- and 8-dehydrocholesterol levels analysed on whole blood spots by gas chromatography-mass spectrometry-selected ion monitoring. Journal of Chromatography B. 2002;766:365-370.

49) Lipid Research Clinics Program: Manual of Laboratory Operations, Lipid and Lipoprotein Analysis. 1982, 2nd ed: Dept of Health and Human Services (NIH).

50) DeBarber AE, Lutjohann D, Merkens L, Steiner RD. Liquid chromatography-tandem mass spectrometry determination of plasma 24S-hydroxycholesterol with chromatographic separation of 25-hydroxycholesterol. Analytical Biochemistry. 2008; 381: 151-153.

51) Fitzky BU, Witsch-Baumgartner M, Erdel M, Lee JN, Paik YK, Glossman H et al. Mutations in the Δ7-sterol reductase gene in patients with the Smith-Lemli-Opitz syndrome. Proceedings of the National Academy of Sciences. 1998; 95: 8181-8186.

52) Witsch-Baumgartner M, Clayton P, Clusellas N, Haas D, Kelley RI, Krajewska-Walasek M et al. Identification of 14 Novel Mutations in *DHCR7* Causing the Smith-Lemli-Opitz Syndrome and Delineation of the *DHCR7* Mutational Spectra in Spain and Italy. Human Mutation. 2005; 799:1-8.

53) Srinivasan SR, Myers L, Berenson GS. Distribution and Correlates of Non-High-Density Lipoprotein Cholesterol in Children: The Bogalusa Heart Study. Pediatrics. 2002;110; e29.

54) Bolanos JP, Medina JM, Williamson DH. Inhibition of sterol but not fatty acid synthesis by valproate in developing rat brain *in vivo*. Biochemical Journal. 1990; 272: 251-253.

55) Lainhart JE, Bigler ED, Bocian M, Coon H, Dinh E, Dawson G et al. Head circumference and height in autism: a study by the Collaborative Program of Excellence in Autism. American Journal of Medical Genetics Part A. 2006; 140(21):2257-74.

56) Courchesne E, Karns CM, Davis HR, Ziccardi R, Carper RA, Tigue ZD et al. Unusual brain growth patterns in early life in patients with autistic disorder: An MRI study. Neurology. 2001; 57: 245-254.

57) Shafaati M, O'Driscoll R, Bjorkhem I, Meaney S. Transcriptional regulation of cholesterol 24-hydroxylase by histone deacetylase inhibitors. Biochemical and Biophysical Research Communications. 2009; 378(4):689-94.

58) Schneider T, Przewlocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology. 2005;30(1):80-9.

59) Meador KJ, Baker GA, Browning N, Clayton-Smith J, Combs-Cantrell DT, Cohen M et al. Cognitive Function at 3 Years of Age after Fetal Exposure to Antiepileptic Drugs. New England Journal of Medicine. 2009;360(16):1597-1605.

60) Landrigan PJ. What causes autism? Exploring the environmental contribution. Current Opinions in Pediatrics. 2010;22:219-225.

61) Centers for Disease Control and Prevention. Clinical Growth Charts, Children 2 to 20 years [Online]. (August, 2009). Available: http://www.cdc.gov/growthcharts/clinical_charts.htm

62) American Psychiatric Association. DSM-V Work Group Reports. Report of the DSM-V Neurodevelopmental Disorders Work Group. [Online]. (April 2010). Available: http://www.psych.org/MainMenu/Research/DSMIV/DSMV/DSMRevisionActivities/DSM-V-Work-Group-Reports/Neurodevelopmental-Disorders-Work-Group-Report.aspx

63) American Psychiatric Association. DSM-5 Development. Proposed Revisions. 299.10 Childhood Disintegrative Disorder. [Online]. (April 2010). Available: http://www.dsm5.org/ProposedRevisions/Pages/proposedrevision.aspx?rid=96#

64) Corbett BA, Kantor AB, Schulman H, Walker WL, Lit L, Ashwood P et al. A Proteomic Study of Serum from Children with Autism Showing Differential expression of Apolipoproteins and Complement Proteins. Molecular Psychiatry. 2007; 12: 292-306.

Appendices

Appendix A: 24S levels by age*

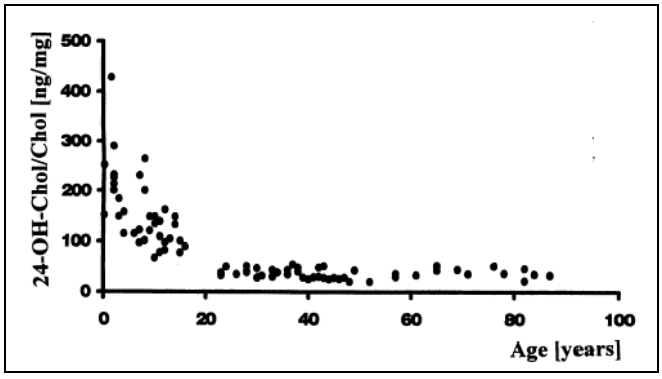
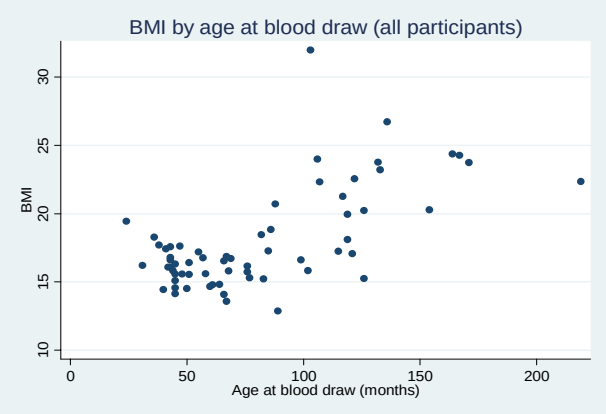


*Image reproduced with permission from the authors: from Lutjohann et al.[42]

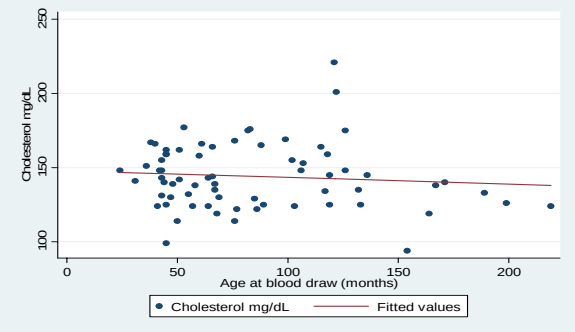
Appendix B: BMI by age



Scatter plot of BMI by age in current study.

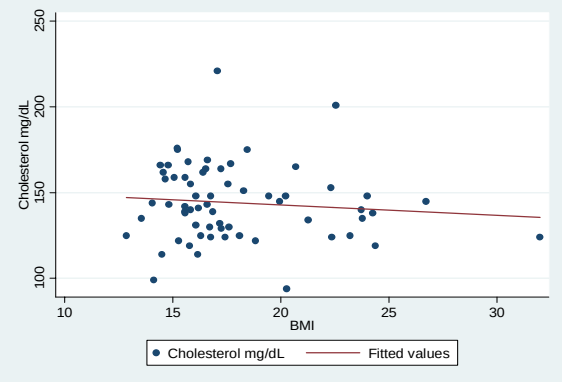
CDC BMI percentiles by age (2-20 years) in boys (left) and girls (right).[44]

Appendix C: Plots of cholesterol by single predictors

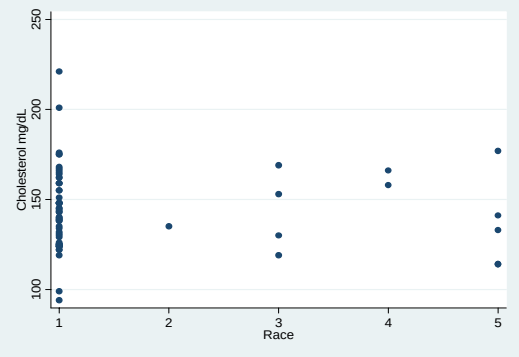


Cholesterol plotted by age, with fitted line.

Cholesterol plotted by gender (1=girls, 2=boys).



Cholesterol plotted by BMI, with fitted line.



Cholesterol plotted by race. (1=White, 2= Black 3=Hispanic, 4=Asian/Pacific Islander, 5=Other)

Cholesterol plotted by ASD status (1= -ASD, 2=+ASD).

Appendix D: Forward selection and backward elimination of variables of cholesterol

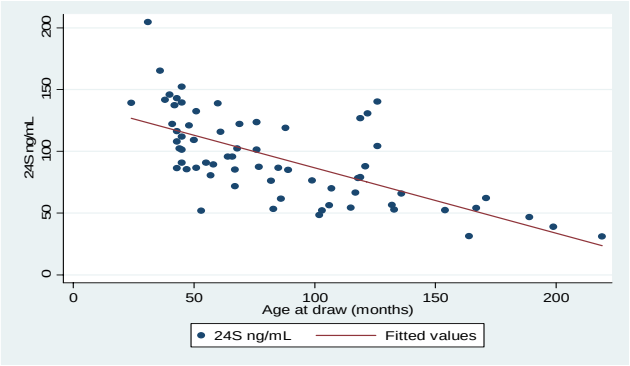
```
. xi: stepwise, pe(.2): regress Chol_mg_dL asd  age_at_draw BMI gender i.race
i.race            _Irace_1-5         (naturally coded; _Irace_1 omitted)
                      begin with empty model
p = 0.0978 <  0.2000  adding  _Irace_5
p = 0.1307 <  0.2000  adding  gender
p = 0.1956 <  0.2000  adding  asd

      Source |       SS       df       MS              Number of obs =      62
-------------+------------------------------           F(  3,    58) =    2.34
       Model |  3276.95099     3  1092.317              Prob > F      =  0.0827
    Residual |  27076.0974    58  466.829265           R-squared     =  0.1080
-------------+------------------------------           Adj R-squared =  0.0618
       Total |  30353.0484    61  497.590957           Root MSE      =  21.606

------------------------------------------------------------------------------
  Chol_mg_dL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Irace_5 |  -23.81824   12.8314    -1.86   0.069    -49.50307    1.866591
      gender |  -11.18078   6.975653    -1.60   0.114    -25.14407    2.782504
         asd |   7.446906   5.687804     1.31   0.196    -3.938471    18.83228
       _cons |   153.0414   15.56876     9.83   0.000     121.8771    184.2056
------------------------------------------------------------------------------
```

Forward selection of variables for modeling cholesterol (Stata 10.1)

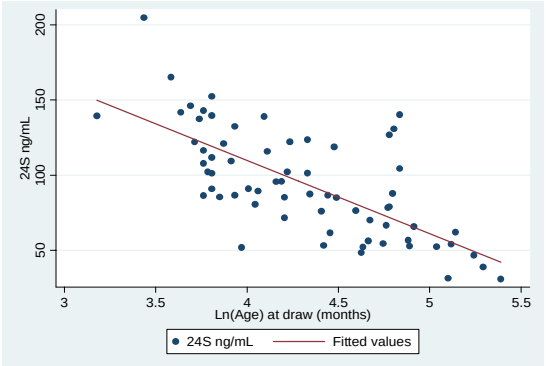
```
. xi: stepwise, pr(.2): regress Chol_mg_dL asd  age_at_draw BMI gender i.race
i.race            _Irace_1-5         (naturally coded; _Irace_1 omitted)
                      begin with full model
p = 0.9428 >= 0.2000  removing _Irace_2
p = 0.9098 >= 0.2000  removing age_at_draw
p = 0.9046 >= 0.2000  removing _Irace_3
p = 0.7181 >= 0.2000  removing BMI
p = 0.2713 >= 0.2000  removing _Irace_4

      Source |       SS       df       MS              Number of obs =      62
-------------+------------------------------           F(  3,    58) =    2.34
       Model |  3276.95099     3  1092.317              Prob > F      =  0.0827
    Residual |  27076.0974    58  466.829265           R-squared     =  0.1080
-------------+------------------------------           Adj R-squared =  0.0618
       Total |  30353.0484    61  497.590957           Root MSE      =  21.606

------------------------------------------------------------------------------
  Chol_mg_dL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         asd |   7.446906   5.687804     1.31   0.196    -3.938471    18.83228
      gender |  -11.18078   6.975653    -1.60   0.114    -25.14407    2.782504
    _Irace_5 |  -23.81824   12.8314    -1.86   0.069    -49.50307    1.866591
       _cons |   153.0414   15.56876     9.83   0.000     121.8771    184.2056
------------------------------------------------------------------------------
```

Backward elimination of variables for modeling cholesterol (Stata 10.1)

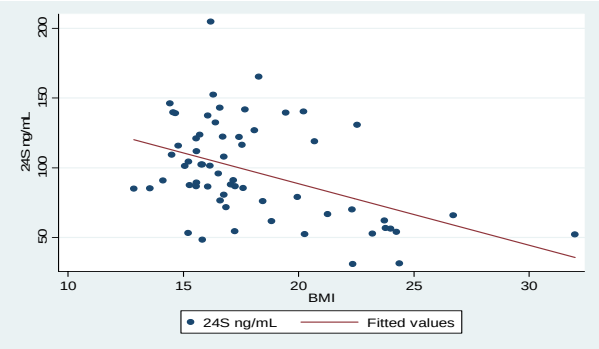
Appendix E: Plots of 24S by single predictors



24S plotted by age, with fitted line.



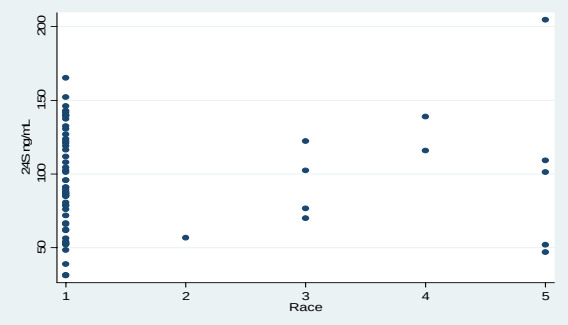
24S plotted by Ln(age), with fitted line.



24S plotted by BMI, with fitted line.

24S plotted by gender (1=girls, 2=boys).

24S plotted by ASD status (1= -ASD, 2=+ASD).



24S plotted by race. (1=White, 2= Black 3=Hispanic, 4=Asian/Pacific Islander, 5=Other)

Appendix F: Forward selection and backward elimination of variables on 24S

```
. xi: stepwise, pe(.2): regress  _24S_ng_mL asd  age_at_draw BMI gender i.race
i.race            _Irace_1-5         (naturally coded; _Irace_1 omitted)
                  begin with empty model
p = 0.0000 <  0.2000  adding  age_at_draw
p = 0.0379 <  0.2000  adding  asd
p = 0.0834 <  0.2000  adding  _Irace_5

      Source |       SS       df       MS              Number of obs =      60
-------------+------------------------------           F(  3,    56) =   19.52
       Model |  38069.1781     3   12689.726           Prob > F      =  0.0000
    Residual |  36409.3137    56  650.166316           R-squared     =  0.5111
-------------+------------------------------           Adj R-squared =  0.4850
       Total |  74478.4917    59  1262.34732           Root MSE      =  25.498

------------------------------------------------------------------------------
  _24S_ng_mL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 age_at_draw |   -.489186   .0827759    -5.91   0.000    -.655006    -.323366
         asd |   15.96425   7.167369     2.23   0.030     1.606281    30.32221
    _Irace_5 |   26.99693   15.31399     1.76   0.083    -3.680671    57.67453
       _cons |   110.3788   15.57345     7.09   0.000     79.18146    141.5762
```

Forward selection of variables for modeling 24S (Stata 10.1).

```
. xi: stepwise, pr(.2): regress  _24S_ng_mL asd  age_at_draw BMI gender i.race
i.race            _Irace_1-5         (naturally coded; _Irace_1 omitted)
                      begin with full model
p = 0.9910 >= 0.2000  removing BMI
p = 0.9687 >= 0.2000  removing _Irace_3
p = 0.8003 >= 0.2000  removing _Irace_2
p = 0.5423 >= 0.2000  removing gender
p = 0.4079 >= 0.2000  removing _Irace_4

      Source |       SS       df       MS              Number of obs =      60
-------------+------------------------------           F(  3,    56) =   19.52
       Model |  38069.1781     3   12689.726           Prob > F      =  0.0000
    Residual |  36409.3137    56  650.166316           R-squared     =  0.5111
-------------+------------------------------           Adj R-squared =  0.4850
       Total |  74478.4917    59  1262.34732           Root MSE      =  25.498

------------------------------------------------------------------------------
   _24S_ng_mL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
          asd |    15.96425   7.167369     2.23   0.030     1.606281    30.32221
 age_at_draw |   -.489186   .0827759    -5.91   0.000     -.655006    -.323366
     _Irace_5 |    26.99693   15.31399     1.76   0.083    -3.680671    57.67453
        _cons |    110.3788   15.57345     7.09   0.000     79.18146    141.5762
```

Backward elimination of variables for modeling 24S (Stata 10.1).

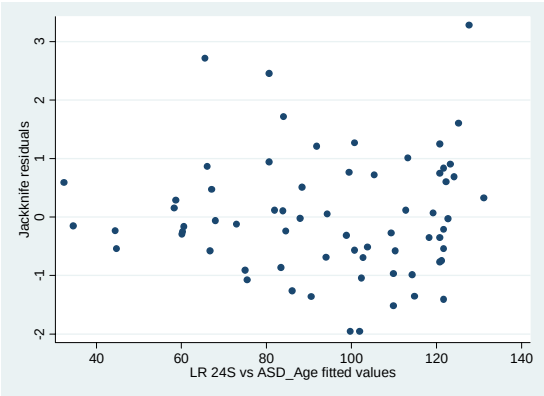
```
. xi: stepwise, pe(.2): regress _24S_ng_mL asd log_agedraw BMI gender i.race
i.race            _Irace_1-5          (naturally coded; _Irace_1 omitted)
                      begin with empty model
p = 0.0000 <  0.2000  adding  log_agedraw
p = 0.1342 <  0.2000  adding  asd
p = 0.1105 <  0.2000  adding  _Irace_5

      Source |       SS       df       MS              Number of obs =      60
-------------+------------------------------           F(  3,    56) =   21.21
       Model |  39617.6654     3  13205.8885           Prob > F      =  0.0000
    Residual |  34860.8264    56  622.514756           R-squared     =  0.5319
-------------+------------------------------           Adj R-squared =  0.5069
       Total |  74478.4917    59  1262.34732           Root MSE      =  24.95

------------------------------------------------------------------------------
   _24S_ng_mL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
 log_agedraw |   -43.75175   7.009109    -6.24   0.000    -57.79268   -29.71082
          asd |    11.75301   7.202954     1.63   0.108    -2.676241    26.18226
     _Irace_5 |     24.3975    15.0439     1.62   0.110    -5.73905    54.53404
        _cons |    264.6889   36.17866     7.32   0.000     192.2143    337.1635
```

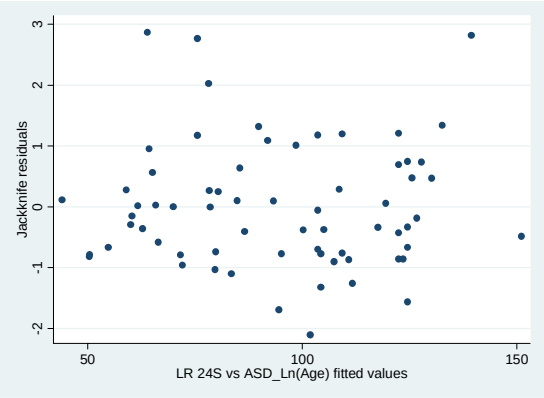
Forward selection of variables for modeling 24S, using Ln(age) (Stata 10.1).

```
. xi: stepwise, pr(.2): regress _24S_ng_mL asd log_agedraw BMI gender i.race
i.race            _Irace_1-5          (naturally coded; _Irace_1 omitted)
                      begin with full model
p = 0.8149 >= 0.2000  removing _Irace_2
p = 0.7813 >= 0.2000  removing BMI
p = 0.7407 >= 0.2000  removing _Irace_3
p = 0.6447 >= 0.2000  removing gender
p = 0.2811 >= 0.2000  removing _Irace_4

      Source |       SS       df       MS              Number of obs =      60
-------------+------------------------------           F(  3,    56) =   21.21
       Model |  39617.6654     3  13205.8885           Prob > F      =  0.0000
    Residual |  34860.8264    56  622.514756           R-squared     =  0.5319
-------------+------------------------------           Adj R-squared =  0.5069
       Total |  74478.4917    59  1262.34732           Root MSE      =  24.95

------------------------------------------------------------------------------
   _24S_ng_mL |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
          asd |    11.75301   7.202954     1.63   0.108    -2.676241    26.18226
 log_agedraw |   -43.75175   7.009109    -6.24   0.000    -57.79268   -29.71082
     _Irace_5 |     24.3975    15.0439     1.62   0.110    -5.73905    54.53404
        _cons |    264.6889   36.17866     7.32   0.000     192.2143    337.1635
```

Backward elimination of variables for modeling 24S, using Ln(age) (Stata 10.1).

Appendix G: Jackknife residuals of multiple linear regression of 24S





Jackknife residuals for full models plotted against the models' fitted values:

Model 1 (left): 24S on ASD status and age

Model 2 (right): 24S on ASD status and Ln(age)