

# Severity of ASD Symptoms and Their Correlation with the Presence of Copy Number Variations and Exposure to First Trimester Ultrasound

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Current research suggests that incidence and heterogeneity of autism spectrum disorder (ASD) symptoms may arise through a variety of exogenous and/or endogenous factors. While subject to routine clinical practice and generally considered safe, there exists speculation, though no human data, that diagnostic ultrasound may also contribute to ASD severity, supported by experimental evidence that exposure to ultrasound early in gestation could perturb brain development and alter behavior. Here we explored a modified triple hit hypothesis [Williams & Casanova, 2010] to assay for a possible relationship between the severity of ASD symptoms and (1) ultrasound exposure (2) during the first trimester of pregnancy in fetuses with a (3) genetic predisposition to ASD. We did so using retrospective analysis of data from the SSC (Simon's Simplex Collection) autism genetic repository funded by the Simons Foundation Autism Research Initiative. We found that male children with ASD, copy number variations (CNVs), and exposure to first trimester ultrasound had significantly decreased non-verbal IQ and increased repetitive behaviors relative to male children with ASD, with CNVs, and no ultrasound. These data suggest that heterogeneity in ASD symptoms may result, at least in part, from exposure to diagnostic ultrasound during early prenatal development of children with specific genetic vulnerabilities. These results also add weight to on-going concerns expressed by the FDA about non-medical use of diagnostic ultrasound during pregnancy. *Autism Res* 2016, 0: 000–000. © 2016 International Society for Autism Research, Wiley Periodicals, Inc.

**Keywords:** epidemiology; genetics; environmental influences; ASD severity; ultrasound

## Background

### *Heterogeneity of ASD*

Current research supports the view that incidence and the heterogeneity of symptoms of autism spectrum disorder (ASD) can arise through multiple factors.

In multifactorial disorders such as ASD, Casanova [Casanova, 2007] proposed a triple hit hypothesis where the convergence of three factors (1) the brain of a genetically at risk individual is (2) exposed during a time of critical neurodevelopment to (3) an exogenous stressor, may contribute to the incidence of ASD. First, the stressor occurs during a critical period of brain development. In ASD, it has been proposed that this insult occurs early in gestation (e.g. 1<sup>st</sup> or 2<sup>nd</sup> trimester) [Chess, 1977; Ivarsson et al., 1990; Rodier, 2002; Torrey et al., 1975] but not later [Yamashita et al., 2003]. Second, the individual has an underlying (genetic) vulnerability. In ASD, both specific genetic events [Bernier et al., 2014; O'Roak et al., 2012] and genetic risk factors [Klei et al., 2012; Werling & Geschwind, 2013] have

been proposed to play a role in the disorder. Third, there is an exogenous gestational stressor. Proposed gestational stressors are varied and have included (but are not limited to): anticonvulsants and other psychotropic medications [Christensen et al., 2013; Wood, 2014], maternal infection and immune activation [Malkova et al., 2012; Mazina et al., 2015; Patterson, 2011; Zerbo et al., 2013], and environmental toxins such as air pollutants [Ehrenstein et al., 2014], volatile organic compounds [McCanlies et al., 2012], pesticides [Roberts et al., 2007; Roberts & English, 2013], and plasticizers [Kalkbrenner et al., 2014; Stein et al., 2015; Testa et al., 2012].

Of interest to the present work is the possibility that diagnostic ultrasound could act as an exogenous stressor [Williams & Casanova 2010] and may be related to ASD severity.

### *Diagnostic Ultrasound Effects in Animal Models*

**Effects on brain structure, in vivo.** There exist a number of early studies that link ultrasound exposure

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Received September 01, 2015; accepted for publication July 21, 2016

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Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/aur.1690

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*in utero* in rodents to changes in neuro-anatomy, summarized in Stewart et al. [1985]. Ellisman, Palmer, & André [1987] observed that 30 minutes of diagnostic ultrasound applied to neonatal rat pups disrupted brain myelination compared to sham exposed animals. Ang et al. [2006] found disrupted cortical migratory patterns in mice exposed to a 30+ minutes of diagnostic ultrasound *in utero* relative to shams. Li et al. [2015] exposed rats to 20 minutes of diagnostic ultrasound *in utero* and observed changes in mRNA and protein expression levels of hippocampal N-methyl-D-aspartate (NMDA) receptor units, brain-derived neurotrophic factor (BDNF), and the presence of damaged hippocampal synapses.

**Effects on behavior, in vivo.** Ultrasound exposure to rodents *in utero* can also alter their subsequent behavior. For example, decreased locomotor and exploratory activities, as well as decreased learning ability was found in mice exposed to prenatal ultrasound [Devi et al., 1995; Suresh et al., 2002, 2008]. Similarly in macaques exposed to prenatal ultrasound, the authors reported reduced levels of physical activity as compared with control monkeys, with all effects normalizing by age 5–6 months [Tarantal & Hendrickx, 1989]. McClintic et al. [2013] found that that 30 minutes of prenatal ultrasound resulted in juvenile mice that were less social and more hyperactive in social situations than sham exposed mice. Finally, Li et al. [2015] observed a reduction in spatial learning and memory abilities of rats after their exposure to prenatal diagnostic ultrasound. It is important to mention that there exist studies showing negative results after prenatal ultrasound exposure. As an example, Jensch et al. [1995] found that rats exposed to 35 minutes of ultrasound *in utero* did not exhibit any changes in memory and anxiety tests.

#### *Diagnostic Ultrasound in Prenatal Clinical Practice*

Each decade since the introduction of ultrasound to obstetric medicine, its popularity and use has escalated. In modern obstetrics, it is standard clinical practice to utilize ultrasound to diagnose, date, and monitor the growth of the fetus. From an obstetric perspective, first trimester ultrasound leads to improved pregnancy dating, reducing antenatal testing and labor inductions [Caughey, Nicholson, & Washington, 2008; Abramowicz, 2013]. Socio-emotional concerns may also guide ultrasound requests – to provide reassurance of fetal health, to increase paternal and familial involvement, and to provide pictorial momentos [Gudex, Nielsen, & Madsen, 2006]. While rates of use vary based on insurance type and provider [O’Keeffe & Abuhamad, 2013], there has been a significant increase in the utilization of obstetric ultrasound in the last 5 years, including increases in

multiple general ultrasounds in low-risk pregnancies and targeted ultrasound examinations intended for higher risk concerns [You et al., 2010].

While generally considered safe, recent FDA statements regarding the use of prenatal diagnostic ultrasound as well as on-going attention in the scientific literature [Abramowicz, 2013] continue to stress its conservative use for medical purposes. Ultrasound consists of high frequency sound, which when absorbed may lead to thermal (hyperthermia) and non-thermal (cavitation, radiation pressure) effects within tissue [Mourad, 2012]. Energy absorption depends on tissue type, exposure, and ultrasound mode, such as for imaging or blood-flow monitoring. The early FDA restriction on intensity alone was replaced during the 1990s with a nearly eight-fold increase in intensity along with imposition of maximum values of a thermal index (TI) to measure the tendency of ultrasound to warm tissue and a mechanical index (MI) related to the likelihood of ultrasound to produce cavitation. This is particularly concerning as ultrasound technicians and maternal fetal medicine fellows demonstrated poor knowledge regarding safety levels [Meizner 2012; Houston, Allsworth, & Macones, 2011; Sheiner, Shoham-Vardi, & Abramowicz, 2007]. For example, although less established in humans than in animal models [reviewed in Edwards, 2006], the developing central nervous system, particularly during the 1st trimester may be particularly susceptible to hyperthermic injury [Edwards, 2006], with maternal hyperthermia associated with increased neural tube defects [Milunsky et al., 1992; Moretti et al., 2005].

**Effects on general child outcomes.** In humans, a number of non-randomized and randomized studies have investigated ultrasound exposure and found no significant (or consistent) relation to congenital anomalies, birth size, cancer/tumors, heart disease, general neonatal and child outcomes, and specific psychopathology such as schizophrenia and psychosis [reviewed in Houston et al., 2009]. A few notable differences were found in case-control designs suggesting increased presence of speech delay [Campbell, Elford, & Brant, 1993] but not in randomized trials [Kieler et al., 1998a,b; Newnham et al., 2004; Salvesen et al., 1994]. There have been several reports of increased left handedness in males in relation to fetal ultrasound exposure [Kieler et al., 1998a,b, 2002; Salvesen et al., 1993].

**ASD incidence and ultrasound exposure.** The relation between ultrasound as a primary etiological factor and ASD diagnostic outcome has not been supported [Grether et al., 2010; Stoch et al., 2012]. Stoch et al. [2012] did not find a relation between child ASD

**Table 1. Proband Age, Mean Standard Test Scores (SD), and Regression Results for All Children with ASD with Known Absence or Presence of Identified CNV, and Absence ('No U1') or Presence ('U1') of First Trimester Ultrasound**

Total (N = 1749)	ASD		Beta	95%CI	P-value
	No U1	U1			
N	668	1081			
Age (mos)	117.7 (44.2)	101.6 (40.3)			
Adaptive and cognitive					
VABS-II	72.6 (11.5)	73.6 (12.1)	-0.73	-1.87 to 0.43	0.22
Verbal IQ	77.5 (31.4)	78.5 (30.4)	0.13	-3.02 to 3.28	0.94
Nonverbal IQ	85.3 (26.3)	84.9 (26.1)	-1.28	-3.94 to 1.38	0.35
Social affective behaviors					
<b>ADOS SA</b>	<b>11.3 (4.0)</b>	<b>11.0 (4.0)</b>	<b>-0.45</b>	<b>-0.86 to -0.04</b>	<b>0.03</b>
ADI-R SD	20.4 (5.6)	20.1 (5.8)	0.28	-0.29 to 0.85	0.33
SRS	79.9 (9.8)	79.5 (10.5)	0.53	-0.50 to 1.56	0.31
Repetitive behaviors					
ADOS REP	3.9 (2.1)	4.0 (2.0)	-0.02	-0.23 to 0.18	0.84
<b>ADI-R RRB</b>	<b>6.4 (2.5)</b>	<b>6.6 (2.5)</b>	<b>0.34</b>	<b>0.09 to 0.60</b>	<b>0.008</b>
RBS-R	26.7 (17.0)	27.7 (17.0)	1.36	-0.38 to 3.10	0.13

diagnosis and randomized prenatal exposure to a single second trimester ultrasound versus multiple second and third trimester scans (at 18, 24, 34, 38 weeks); nor to levels of adult autism traits in a primarily “neurotypical” population. Grether et al. [2010] analyzed antenatal ultrasound exposure (primarily 2<sup>nd</sup> trimester exposure) as a risk factor for ASD using medical data from Kaiser Permanente of Northern California Health Care System; there was no difference in number of exposures during the entire gestation or by trimester in 393 controls and 362 autism cases. Critical to the triple hit hypothesis and not addressed by previous reports, ultrasound as an exogenous stressor would have the most significant impact during the 1<sup>st</sup> trimester and only in those with specific genetic risk factors.

#### Current Project

Given the increase in ultrasound intensity and use, extant animal studies, and lack of human data addressing ASD characteristics and ultrasound exposure during the 1<sup>st</sup> trimester of pregnancy, we sought here to explore a variant of Casanova’s triple hit hypothesis, assessing factors that may influence the severity of ASD symptoms rather than ASD incidence. Specifically, we sought to study how (1) the presence of ultrasound (2) within a period of high neural vulnerability (1<sup>st</sup> trimester) in a group (3) with underlying vulnerabilities (males versus females; presence or absence of CNVs) may relate to the severity of ASD symptoms. We used linear regression models with autism severity outcomes (including verbal and non-verbal IQ, adaptive skills, social ability, and repetitive behaviors) to test if the presence of an ultrasound, maleness, and CNVs are significant risk factors. In this analysis, we calculated propensity scores to assess the predicted probability of a first trimester ultrasound in order to adjust for

differences in baseline factors that influence prenatal care and subsequent ultrasound exposure. We predicted that first trimester ultrasound would be a statistically significant risk factor for increased autism severity in children made genetically vulnerable by CNVs, even after adjusting for confounding factors because of differences in likelihood of ultrasound.

## Methods

### Participants

This study uses data from the SSC (Simon’s Simplex Collection) funded by the Simons Foundation Autism Research Initiative [Fischbach & Lord, 2010]. Briefly, 2,644 families were successfully recruited from 12 sites across the USA and included families with one child with ASD aged 4 to 18 years (a “proband”), one unaffected full sibling (>80% of cases), and the biological mother and father. Inclusion/exclusion criteria can be found at <http://sfari.org>. Approval was obtained at each local human subject’s institutional review board and parents and participants completed informed consent.

All probands were assessed on the Autism Diagnostic Interview-Revised (ADI-R) [Lord, Rutter, & Le Couteur, 1994], the Autism Diagnostic Observation Schedule (ADOS) [Lord et al., 2000], and DSM-IV [American Psychiatric Association, 2000] as administered by experienced clinicians. A description of the instruments can be found at <https://sfari.org/resources/simons-simplex-collection/ssc-instruments>.

### Variables

Proband descriptive characteristics include measures of cognitive ability (non-verbal and verbal subdomains of the Differential Ability Scale [DAS], Elliott, 2007), social ability (ADI-R Social Domain score [ADI-R SD], Lord

**Table II. Proband Age, Mean Standard Test Scores (SD), and Regression Results for All Children with ASD, with an Identified CNV, and Absence ('No U1') or Presence ('U1') of First Trimester Ultrasound**

ASD with CNV ( <i>N</i> = 133)	ASD + CNV		Beta	95%CI	<i>P</i> -value
	No U1	U1			
<i>N</i>	49	84			
Age (mos)	118.5 (42.5)	111.4 (41.4)			
Adaptive and cognitive					
VABS-II	73.5 (10.1)	71.0 (13.4)	-4.11	-8.56 to 0.35	0.07
Verbal IQ	81.8 (32.6)	73.6 (30.5)	-9.39	-21.37 to 2.59	0.12
<b>Nonverbal IQ</b>	<b>87.7 (27.6)</b>	<b>76.8 (25.8)</b>	<b>-11.16</b>	<b>-21.08 to -1.24</b>	<b>0.03</b>
Social affective behaviors					
ADOS SA	10.7 (3.8)	11.4 (3.9)	0.90	-0.59 to 2.38	0.23
ADI-R SD	20.9 (5.3)	20.9 (5.9)	0.42	-1.73 to 2.56	0.70
SRS	77.9 (12.3)	78.6 (11.0)	2.24	-2.13 to 6.62	0.31
Repetitive behaviors					
ADOS REP	3.5 (2.1)	4.2 (1.9)	0.69	-0.04 to 1.43	0.06
ADI-R RRB	6.5 (2.6)	7.0 (2.8)	0.71	-0.36 to 1.77	0.19
RBS-R	23.2 (14.1)	26.6 (15.2)	4.01	-1.72 to 9.74	0.17

et al. 1994; ADOS Social Affective Domain score [ADOS SA], Gotham, Risi, Pickles, & Lord, 2007; Social Responsiveness Scale [SRS], Constantino & Gruber, 2003], and repetitive behaviors (ADI-R Restrictive and Repetitive Behaviors score [ADI RRB], Lord et al., 1994; ADOS Repetitive Domain score [ADOS REP], Gotham et al. 2007; Repetitive Behavior Scale-Revised [RBS-R], Bodfish, Symons, & Lewis, 1999; Vineland Adaptive Behavior Scale, Second Edition [VABS-II], Sparrow, 2011]. Observed child behaviors are derived from the DAS and the ADOS. Parent report of behaviors is derived from the ADI-R, VABS-II, SRS, and RBS. These tests were not applied to the family members of probands.

Information regarding pregnancy and general medical history for all probands was collected by a clinician in a semi-structured interview. Specific to this study, we assessed ultrasound exposure as whether or not the biological mother received an ultrasound during a time of critical neurodevelopment of the fetus: the first trimester.

Genetic data included analysis of the presence or absence of ASD-associated CNVs of a subset (1749) of the SCC children, made available via Girirajan et al. [2013]. In order to set the context for the analysis of that subpopulation that is at the heart of this paper, we analyzed (SPSS Statistical Software, IBM Corporation, Armonk, NY) our data for phenotype differences related to the known presence ( $n = 133$ ) or absence ( $n = 1616$ ) of a CNV [Mazina et al., 2015] and for differences related to duplications ( $n = 56$ ) or deletions ( $n = 77$ ) [Girirajan et al., 2013]. Children with ASD and an identified CNV compared with those without a CNV had mothers and fathers of similar age ( $F(1,147) < 0.56, P > 0.46$ ), no differences in maternal and paternal education ( $\chi^2(3) < 4.2, P > .33$ ) or family income ( $\chi^2(3) = 0.82, P = 0.66$ ), no differences in Verbal IQ ( $F(1,1747) = 1.55, P = 0.21$ ) or

Vineland Adaptive Behavior Composite ( $F(1,1747) = 1.78, P = 0.18$ ), nor did they differ on any of the other autism symptom variables ( $F_s < 2.36, P > 0.13$ ). Relative to ASD children without identified CNVs, ASD children with identified CNVs did trend towards a lower Nonverbal IQ ( $F(1,1747) = 3.73, p = .054$ ). Also, with regard to children with a duplication compared with a deletion, they did not differ in any of the phenotypic variables reported above ( $F_s(1,131) < 1.34, P > 0.25$ ). This last result differs from those of Girirajan et al who found more impairment in the restricted and repetitive behavior domain in children with duplications compared to deletions.

Therefore, from the total of 2,644 SSC probands with ASD, the final sample for our analysis consisted of 1749 children (66.1% of the total data set) with ASD for whom we had information on the presence or absence of CNVs and the presence or absence of a first trimester ultrasound. Proband characteristics are in Supporting Information Table 1; a descriptive list of CNVs by sex and ultrasound exposure is presented in Supporting Information Table 2. Rates of ultrasound use in the first trimester did not differ between individuals with specific CNV types (duplications vs deletions or inherited maternal/paternal or de novo mutations) as presented in Supporting Information Table 3.

#### Statistical Analysis

We applied a multiple linear regression model (Stata SE, version 12) for analysis of autism severity with first trimester ultrasound as a dichotomous primary predictor, and the propensity score as an additional covariate, along with child sex and age in months at assessment. Given that we hypothesize that child sex and CNV status may be effective modifiers in the relationship between first trimester ultrasound and ASD severity, we

**Table III. Proband Age, Mean Standard Test Scores (SD), and Regression Results for Male Children with ASD, with an Identified CNV, and Absence ('No U1') or Presence ('U1') of First Trimester Ultrasound**

Males with CNV ( <i>N</i> = 111)	Male + CNV		Beta	95%CI	<i>P</i> -value
	No U1	U1			
<i>N</i>	38	73			
Age (mos)	115.8 (39.6)	113.7 (42.2)			
Adaptive and cognitive					
VABS-II	74.7 (10.3)	71.5 (13.0)	-4.15	-9.04 to 0.75	0.10
Verbal IQ	86.5 (32.5)	75.1 (29.7)	-11.29	-24.32 to 1.73	0.09
<b>Nonverbal IQ</b>	<b>92.6 (26.8)</b>	<b>78.6 (24.4)</b>	<b>-12.43</b>	<b>-23.11 to -1.76</b>	<b>0.02</b>
Social affective behaviors					
ADOS SA	10.6 (3.6)	11.5 (3.6)	1.14	-0.39 to 2.67	0.14
ADI-R SD	20.7 (5.0)	20.7 (6.0)	-0.052	-2.39 to 2.89	0.97
SRS	77.1 (11.8)	78.0 (11.2)	1.87	-2.94 to 6.68	0.44
Repetitive behaviors					
<b>ADOS REP</b>	<b>3.4 (2.2)</b>	<b>4.2 (1.8)</b>	<b>0.90</b>	<b>0.12 to 1.69</b>	<b>0.02</b>
ADI-R RRB	6.3 (2.6)	7.1 (2.8)	0.92	-0.25 to 2.09	0.12
RBS-R	22.0 (12.9)	26.8 (15.4)	5.47	-0.73 to 11.67	0.08

included sub-analyses limited to: (1) all ASD children with identified CNVs and (3) ASD males with identified CNVs, and we formally tested for effect modification using interaction terms and the Wald statistic.

We performed covariate adjustment using propensity scores, as described by Austin [2011a,b] and as employed by other researchers [Arabi et al., 2013; Goldin, Sawin, Garrison & Christakis, 2007; Gagliardi, Bellu, Zanini, Dammann, 2009; Lund et al., 2013 but see Brooks & Ohsfeldt, 2013; Weitzen et al., 2004]. Propensity scores were developed to adjust all regression analysis for potential confounding because of the likelihood of a mother receiving a first trimester ultrasound. Specifically, maternal and pregnancy characteristics may influence the likelihood of receiving a first trimester ultrasound [Caughey et al., 2008] and may therefore influence child outcomes. Variables included in the development of the propensity scores were limited to those that would have been known during the first trimester such as demographic characteristics (e.g., race, income), maternal characteristics (e.g., age, education), prior pregnancy history (e.g., use of fertility treatments, pregnancy loss, preterm birth), and current pregnancy information (e.g., weight, weight gain, timing of prenatal care initiation, and complications or procedures occurring during the first trimester) [Baxter et al. 2007; Ben Itzhak, Lahat, & Zachor, 2011; Zachor & Ben Itzhak, 2011].

## Results

For all 1749 SSC children with ASD (Table II), exposure to 1<sup>st</sup> trimester ultrasound compared with those with no exposure was related to lower observed social affective symptoms (ADOS SA), but greater parent reported restrictive and repetitive behaviors (ADI-R RRB).

For all 133 SSC children with ASD and identified CNVs (Table II), exposure to 1<sup>st</sup> trimester ultrasound compared to those with no exposure was related to statistically significantly lower Nonverbal IQ. In addition, there were trends towards more impaired adaptive behaviors (VABS-II) via parent report, and trends toward increased observed repetitive behaviors (ADOS REP).

For all 111 SSC male children with ASD and identified CNVs (Table III), exposure to 1<sup>st</sup> trimester ultrasound compared to those with no exposure was related to statistically significantly lower nonverbal IQ and significantly increased observed repetitive behaviors (ADOS REP). In addition, there were trends in reduced verbal IQ and in more parent reported repetitive symptoms (RBS-R).

Wald tests for interaction between ultrasound and gender were statistically significant for parent report of adaptive behaviors (VAB-II: beta -4.26, 95% CI -6.71 to -1.80, *P* = .001), social ability (SRS: beta 5.3, 95% CI 3.11 to 7.50, *P* < .001). Wald tests for the interaction between ultrasound and CNVs within males were statistically significant for Verbal IQ (beta -13.3 95% CI -25.8 to -0.78, *P* = 0.04) and Nonverbal IQ (beta -12.0, 95% CI -21.5 to -2.5, *P* = 0.01).

The small sample size of females with identified CNVs precluded us from applying our model to this cohort. Information on measurement means and standard deviations for males or females, with and without identified CNVs, and with and without 1<sup>st</sup> trimester ultrasound is provided in Supporting Information Table 4.

## Discussion

Supporting the hypothesis that ultrasound may act as an exogenous stressor related to ASD severity, for male children with ASD and an identified CNV, exposure to

1<sup>st</sup> trimester ultrasound was associated with poorer Nonverbal IQ and increased repetitive behaviors. In addition, we found that ultrasound may also contribute to variability in outcome in children with ASD in a double hit model. Specifically, exposure to 1<sup>st</sup> trimester ultrasound in children with ASD, regardless of the known presence or absence of an identified CNV, was associated with less observed social affective severity, but increased parental reports of repetitive behaviors.

### *Triple Hit Hypothesis*

We could not test the original triple hit hypothesis of Casanova, which speaks to the incidence of ASD, because we did not have the requisite medical information for the siblings of our probands.

In support of our modified triple hit hypothesis, however, males with ASD and an identified CNV with first trimester ultrasound exposure had lower Nonverbal IQ and increased repetitive behavior, with trends toward lower Verbal IQ, all relative to males with an identified CNV but not exposed to first trimester ultrasound. Given that lowered IQ and greater repetitive behaviors are found in a number of other neurodevelopmental disorders [Lewis & Kim, 2009] and in children with genetic vulnerabilities [Girirajan et al., 2011, 2012; Pinto et al., 2014], the specificity to ASD is unclear. Expanding consideration of our modified triple hit hypothesis to other neurodevelopmental disorders is therefore warranted.

As females with ASD with available data for this analyses made up only 12.6% of the sample, and with females with an identified CNV representing only 1.2% of cases, addressing the impact of first trimester ultrasound on girls was not possible. Relative to the entire sample, girls in the SSC sample were found to have higher levels of social and communication symptoms as well as lower verbal and non-verbal IQ [Frazier et al., 2014], and more likely to have CNVs in deleterious regions related to neurodevelopmental disorders [Jacquemont et al., 2014; Robinson et al., 2013; Werling & Geschwind, 2013]. The extent to which female sex interacts with environmental hits remains to be tested.

### *All Children with ASD, Identified Absence/Presence of CNVs, and 1<sup>st</sup> Trimester Ultrasound*

All children with ASD known absence or presence of CNVs and exposure to 1<sup>st</sup> trimester ultrasound had increased repetitive behaviors, suggesting that an exogenous stressor (here, diagnostic ultrasound), delivered during a time of critical neurodevelopment can increase the severity of at least one class of ASD symptom, consistent with a 'double hit' hypothesis. This result holds true when considering gender and the presence of CNVs, discussed below.

In contrast to our hypothesis, however, children with ASD, known absence or presence of identified CNVs and exposure to 1<sup>st</sup> trimester ultrasound had a small but statistically significant reduction in social affective symptoms. The presence of a 1<sup>st</sup> trimester ultrasound may also be related to numerous maternal variables that influence child outcomes. Specifically, mothers in our sample who received 1<sup>st</sup> trimester ultrasounds were more likely to be older at time of conception, which has been shown to increase risk for ASD [Sandin et al., 2013], and more likely to have a higher household income, found to be related to increased diagnostic rates but also access to resources [Croen, Grether, & Selvin, 2002; Johnson & Myers, 2007; Thomas et al., 2012a,b]. In non-clinical samples, older maternal age, higher parental education, and higher income are strongly associated with better academic achievement, cognitive ability, and social competence [Edwards & Roff, 2010; Pati et al., 2011]. Similarly, utilization of prenatal care is more likely in mothers with higher income, more educated, or live in urban settings [Braveman et al., 2003; Chiavarini, Lanari, Minelli, & Salmasi, 2014; Stativa et al., 2014]. Pre-conception and pregnancy health is related to better child outcomes [Liu et al., 2015], with some disagreement [Noonan et al., 2012; for review – Alexander, 2001]. Thus, it is possible that despite our use of a propensity score to correct for likelihood of 1<sup>st</sup> trimester ultrasound, there remain residual confounding factors. Disentangling the effect of ultrasound *per se* versus ultrasound as a proxy for other variables related to outcomes will be critical in future studies.

### *Neural Vulnerability and External Hits*

In this report, we focused on the 1<sup>st</sup> trimester as a period of neural vulnerability as supported by other studies of environmental factors and their relevance to autism [Dufour-Rainfray et al., 2011]. For example, the chemicals valproate [Arndt, Stodgell, & Rodier, 2005], thalidomide [Miller et al., 2005], and misoprostol [Bandim et al., 2003] cause up or down regulation of certain genes involved in neural proliferation and migration [Dufour-Rainfray et al., 2011]. Maternal infection and fever generally [Atladdottir et al., 2010; Lee et al., 2014; Zerbo et al., 2013] and specifically during the first trimester [Atladdottir et al., 2010] have also been associated with autism. Supporting a gene by environment interaction, Schwartzer et al. [2013] found maternal immune activation was strain dependent in mice, and male mice born to maternal immune activated mothers had more disrupted social behaviors [Malkova et al., 2012]. Similarly, Mazina et al. [2015] found that maternal infection during pregnancy in children with CNVs resulted in greater severity of autism symptoms.

## Mechanism

The rodent studies of Li et al. [2015] suggest one potential mechanism by which ultrasound may act. They observed reduced expression of mRNA and proteins associated with the NMDA receptor pathway and BDNF in the hippocampus of rats exposed to three 20-minute applications of ultrasound. Interestingly, a lower dose exposure (4 minutes) resulted in increased expression in NMDA (GluNR1 and GluNR2B subunits) and BDNF. This interaction with dose and expression suggests an overall sensitivity of these systems to ultrasound. The NMDA system has a broad impact on variability in social and cognitive functioning [Burnashev & Szepietowski, 2015; Lee, Choi, & Kim, 2015]. Alterations of NMDA receptors disrupt response inhibition and social interaction in mice [Chung et al., 2015; Finlay et al., 2015]. *GRIN1* encodes for the NMDA GluN1 subunit and is related to decreased social interaction in knockout mice [Gandal, Anderson, & Billingslea, 2012; Saunders et al., 2013]; *GRIN2B* encodes for the GluN2B subunit, which has been found in rare mutations in autism [O’Roak et al., 2011; Myers et al., 2011; Talkowski et al., 2012; Yoo et al., 2012]. This complex pathway is susceptible to disruption via both increases and decreases in protein expression [Zito et al., 2009]. Additional animal studies are needed to elucidate the vulnerability of the NMDA system to genetic and environmental disruption and their role in social and non-social learning and memory.

BDNF is a neurotrophic factor related to neuronal maturation and synaptic synthesis. BDNF is elevated in children with ASD [Bryn et al., 2015; Kasarpalkar et al., 2014; Zhang et al., 2014] and genes associated with neurodevelopmental disorders impact BDNF expression [Briz et al., 2013; Koh, Lim, Byun, & Yoo, 2014; Louhivuori et al., 2011; Ouyang et al., 2013]. Prenatal exposure to other environmental toxins impact BDNF: Bisphenol A altered DNA methylation in mice [Kundakovic et al., 2014] and valproate increased BDNF mRNA and protein levels in mice and rats [Almeida, Roby, & Krueger, 2014]. A relation between BDNF levels (both hyper and hypo) and neural connectivity in ASD has been proposed [Koh et al., 2014].

## Limitations

Ultrasound exposure was assessed via parent report with a 4 to 18 year old sample. Thus, recall error may have influenced our results in regard to reporting on the presence, absence, and the timing of ultrasounds, although Olson et al. [1997] suggest that memory for pregnancy related medical procedures is relatively high. While presence/absence of an ultrasound is more likely to be reported correctly, reporting an “early” ultrasound as first versus second trimester may have induced

random error. Specifically, the first trimester spans weeks 1 through 12 of pregnancy, with first trimester ultrasound recommended between “10 to 13” weeks [Salomon et al., 2012]. However, if a systematic recall bias existed, it is unlikely this would differentially impact reporting by child genotype, as genotype was not known at time of ultrasound or at assessment. Future research based on medical records that provide exact dates and nature of ultrasound exams is necessary to determine the influence of ultrasound timing and therefore identify a specific window of vulnerability.

Lastly, there is some controversy regarding the use of propensity scores to adjust for confounding in observational studies [Brooks & Ohsfeldt, 2013; Weitzen et al., 2004]. Although it would have been theoretically possible to include all of the covariates used in creation of the propensity score as individual covariates in the final regression model, this would have resulted in even smaller cell sizes throughout the regression matrices for our models, especially given the relative rarity of CNVs in the sample. The small cell size problem is a well-documented rationale for using propensity scores to address confounding in observational studies [Braitman & Rosenbaum, 2002; Cepeda, Boston, Farrar, & Strom, 2003; Ali, Groenwold, & Lungel, 2014; Glynn, Schneeweiss & Sutmer, 2006] as use of propensity scores adds only a single dimension to the regression model. Of note, at least one study found that outliers among the propensity score distribution may considerably skew effect estimates [Kurth et al., 2006]. However, post hoc testing found that this was not the case in our analyses. Specifically, sensitivity analyses excluding all subjects whose predicted probability of first trimester ultrasound was less than 20% resulted in no notable differences in regression results. Moreover, the effect size associated with exposure to first trimester ultrasound changed by less than 10% in all cases such that no model predictions shifted between statistically significant and non-significant. We also note that propensity score adjustment does not help reduce bias due to unmeasured confounders [Brooks & Ohsfeldt, 2013; Weitzen et al. 2004]. This, of course, is an inherent limitation of observational studies, one that would remain true if we had chosen regression models that only adjusted directly for measured covariates without the use of propensity scores.

## Conclusion

In a national sample of children with ASD that included detailed, quantifiable post-natal childhood outcomes and genetic information, we found that the combination of first trimester ultrasound and presence of CNVs in male children with ASD correlated with poorer

cognitive outcomes and increased repetitive behavior. Moreover, across gender and independent of the presence of identified CNVs, first trimester ultrasound correlated with increased repetitive behaviors. Better understanding of the range and types of environmental stressors that may interact with genetic vulnerabilities is needed in order to fully understand contributions to heterogeneity in ASD functioning. If we can better identify the neural development periods most vulnerable to environmental insults, particularly in regard to exposures that can be limited, modified, or regulated by changing practice such as non-medical use of diagnostic ultrasound imaging, it may be possible to quickly reduce the severity of ASD in future children. Further replication both in larger epidemiological populations and in animal models that can address mechanisms are needed.

### Competing Interests

The authors declare that they have no competing interests.

### Acknowledgments

This project was supported by the National Institutes of Health (R01HD064820 & P50HD055782 Webb) and the Simons Simplex Collection (SFARI #89368 Bernier). The authors are grateful to all of the families at the participating Simons Simplex Collection (SSC) sites, as well as the principal investigators (A. Beaudet, R. Bernier, J. Constantino, E. Cook, E. Fombonne, D. Geschwind, R. Goin-Kochel, E. Hanson, D. Grice, A. Klin, D. Ledbetter, C. Lord, C. Martin, D. Martin, R. Maxim, J. Miles, O. Ousley, K. Pelphrey, B. Peterson, J. Piggot, C. Saulnier, M. State, W. Stone, J. Sutcliffe, C. Walsh, Z. Warren, E. Wijsman). They appreciate obtaining access to phenotypic data on SFARI Base. Approved researchers can obtain the SSC population dataset described in this study by applying at <https://base.sfari.org>. They also thank Dr. Eric Handler, Public Health Officer of Orange County, California, for valuable discussions. Correspondence: Pierre D. Mourad, Department of Neurological Surgery; Box 356470 University of Washington; Seattle WA 98195; [doumitt@uw.edu](mailto:doumitt@uw.edu).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article.