

Exposure to Varicella Zoster Virus Is Higher in Children with Autism Spectrum Disorder than in Healthy Controls. Results from a Case-control Study

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Abstract. *Background/Aim: Autism spectrum disorder (ASD) is a group of central nervous system disorders lacking a definite etiology. The aim of the present study was to compare the exposure rate and titer of antibodies to Varicella Zoster Virus (VZV) in children with ASD and in healthy controls. Patients and Methods: We enrolled 54 children with ASD and 46 control individuals. Results: The exposure rate and titer of anti-VZV antibodies were significantly higher in children with ASD compared to controls (59% vs. 39% and 694 mIU/ml vs. 94 mIU/ml, respectively). Conclusion: In the present case-control study, exposure to VZV was found to be independently associated with ASD.*

Autism Spectrum Disorder (ASD) is a cognitive and developmental disorder characterized by abnormal or deficient social interaction, impaired communication and language abilities and a narrow pattern of interests and activities (1). ASD includes Autistic Disorder (AD), Rett Syndrome, Asperger Syndrome, PDD-NOS (Pervasive Developmental Disorder, Not Otherwise Specified), and Childhood Disintegrative Disorder. However, the recently available fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) by the American Psychiatric Association (2) modified the diagnostic criteria for autism spectrum disorder, with the consequence that all the sub-diagnoses have been nullified and included in the

definition of ASD. Two data regarding ASD are worrisome: i) its increasing prevalence, which was estimated to be 1:2,000 children before the 80s (3) and is now 1:88 newborns in USA (4), even if lower figures (annual incidence of about 1.2/1,000 boys and 0.2/1,000 girls) have been shown in an UK study (5); ii) the absence of a definite etiological factor in the majority of cases (4, 6). What is currently known is that the aetiology of ASD derives from an interaction between genetic and environmental factors (7). This generic sentence reveals our ignorance regarding the specific malfunction. In absence of a definite aetiology, no preventive measure can be employed to revert the current increasing trend of prevalence.

Several different aetiologies have been hypothesized to contribute to ASD onset. Hypotheses include infections (8-13), vaccine exposure (14, 15), vitamin D deficiency (16-20), autoimmune diseases (21, 22). Our group has recently proposed a unifying hypothesis for the etiopathogenesis of ASD (23). We suggested that ASD is a disorder of the immune system due to multiple causes: in individuals with a background of genetic predisposition and environmental susceptibility (likely associated with vitamin D deficiency), an infection (perhaps a viral infection) triggers an immune-mediated damage to specific areas of central nervous system that results in symptoms of ASD (23).

Several studies have associated viral infections to ASD (15, 24-29). Many of them are case reports that deal with herpes virus infections: Herpes Simplex Virus (30-33), Epstein Barr virus, Cytomegalovirus (34-42), Human Herpes Virus 6 (15, 43) or Human Herpes Virus 8 (43). However, no study has assessed the prevalence of anti Varicella-Zoster Virus (VZV) antibodies in children with ASD. The aim of the present study was to compare the prevalence and the titer of anti-VZV antibodies in patients with ASD and in healthy controls.

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Key Words: Autism spectrum disorder, etiopathogenesis, prevalence, titre, VZV.

Patients and Methods

Patients. We recruited patient cases among those admitted as to the Child and Adolescent Neuropsychiatry Unit at the Second University of Naples and to the Department of Pediatrics of the University of Naples “Federico II”, Italy between January 2010 and January 2013. The parents/legal guardians supplied informed consent and identification information was removed from each sample. The Ethics Committee of the University of Naples “Federico II” approved the study (protocol number: 85/09). Inclusion criteria for cases were diagnosis of ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition, Text Revision (DSM-IV-TR) (1) (as the V edition was released only after the conclusion of enrollment) and informed consent signed by parents/guardians; the only exclusion criterion was the inability to sign an informed consent form.

Controls were enrolled at the Division of Pediatric Surgery of the University of Naples “Federico II” where they were admitted for minor surgical treatments (*e.g.* phimosis, hernias, cryptorchidism, vesicoureteral reflux, hydrocele testis, *etc.*). They underwent an interview to rule-out possible ASD and, if such a disorder was identified, the affected children were excluded from the study.

To authenticate the diagnosis of autism, cases were administered the Autism Diagnostic Interview, Revised version (44), the Childhood Autism Rating Scales (CARS) (45) and the Autism Diagnostic Observation Schedule (ADOS)-Generic (46). In order to assess adaptive functioning, Vineland Adaptive Behavior Scales (47) were utilized whereas developmental quotient was estimated by the means of the Griffiths Mental Developmental Scales (48).

Virological tests. The LIAISON® VZV IgG assay uses chemiluminescence immunoassay (CLIA) technology for the quantitative determination of specific IgG antibodies to VZV in human serum or plasma samples. VZV antigen was used for coating magnetic particles (solid phase) and a mouse monoclonal antibody to human IgG was linked to an isoluminol derivative (isoluminol-antibody conjugate). During the first incubation, VZV antibodies present in calibrators, samples or controls bound to the solid phase. During the second incubation, the antibody conjugate reacts with VZV IgG already bound to the solid phase. After each incubation, the unbound material was removed with a wash cycle. Subsequently, the starter reagents were added and a flash chemiluminescence reaction was thus induced. The light signal, and hence the amount of isoluminol-antibody conjugate, was measured by a photomultiplier as relative light units (RLU) and was indicative of VZV IgG concentration present in calibrators, samples or controls.

Test of assay-specific calibrators allowed the detected RLU values to adjust the assigned master curve. An analyser automatically calculated VZV IgG antibody concentrations expressed as mIU/ml and graded the results. The assay range corresponded to 10-4,000 mIU/ml VZV IgG. The cut-off value chosen to achieve the highest diagnostic specificity and sensitivity in the tested population was 150 mIU/ml VZV IgG. Sample results were interpreted as follows: samples with VZV IgG concentrations below 150 mIU/mL were graded negative; samples with varicella-zoster virus IgG concentrations equal to or above 150 mIU/mL were graded positive.

Statistical analysis. Kolmogorov-Smirnov test was used to check for Gaussian distribution of quantitative variables. In case of Gaussian distribution, data are reported as mean±standard deviation (SD) and

Table I. *Demographic and clinical features of children with Autism Spectrum Disorders.*

Classification	Median (interquartile range)
VABS (Adaptive quotient)	50 (38-58)
CARS (Total score)	36 (33-40)
GMDS (Developmental quotient)	50 (43-59)
ADOS (Total score)	16 (13-18)
ADOS (Language)	5 (4-6)
ADOS (Interaction)	10 (8-12)

GMDS: Griffith mental developmental scales; VABS: Vineland adaptive behavior scales; ADOS: Autism diagnostic observation schedule; CARS: Childhood autism rating scales.

tests for comparisons were the Student's *t*-test for unpaired variables in case of two groups and ANOVA test in case of 3 or more groups. In cases of non-Gaussian distribution, data are given as median and interquartile range (IQR), tests for comparisons were the Mann-Whitney *U*-test in case of 2 groups and Kruskal-Wallis test for 3 or more groups. The χ^2 test (or Fisher's exact test) was used for qualitative or categorical variables. Spearman's rho test was used for correlations between rates of scales and antibody titers.

Age and gender were included in a logistic regression analysis model together with the antibody presence for the prediction of disease status. A *p*-value of 5% or less in the two-sided test was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, ILL, USA).

Results

In the present study we enrolled 100 children, 54 with ASD (19 with Autistic disorder (AD) and 35 with non-AD ASD) and 46 healthy controls. Mean age was 6.1 (SD=2.5) years for cases and 5.9 (SD=2.8) years for controls (*p*=0.775). Males out-numbered females, equally among cases (41/54, 75.9%) and controls (39/46, 84.8%, *p*=0.270). The neuropsychiatric and clinical features of the children with ASD are shown in Table I.

We assessed the rate of seropositivity for VZV in cases and controls. As shown in Table II, the rate of seropositivity was significantly higher in cases than in controls. Moreover patients with AD tended to have a higher prevalence of anti-VZV antibodies than those with milder forms. We measured and compared antibody titres for VZV in cases and controls and, as shown in Table III, they were significantly higher in cases than in controls and tended to be higher in children with the severe forms than those with milder forms.

As age may impact on antibody prevalence and can even be a confounding factor for grading the severity of the disease, we performed a logistic regression analysis, which included age, gender and seropositivity for VZV as independent variables and health condition (ASD *vs.*

Table II. Rate of seropositivity to VZV-IgG in cases and controls and in patients with autistic disorder or non-autistic disorder autism spectrum disorders.

	VZV-IgG		p-Value
Cases	32/54 (59.3%)	AD 13/19 (68.4%) Non-AD ASD 19/35 (54.3%)	0.045 (χ^2) [†] 0.082 (χ^2) [‡]
Controls	18/52 (39.1%)		

χ^2 : χ^2 test; [†]Cases vs. controls; [‡]AD vs. non-AD ASD vs. controls.

Table III. VZV antibody titers in cases and controls and in patients with autistic disorder or non-autistic disorder autism spectrum disorders.

	VZV-IgG (mIU/ml)		p-Value
Cases	694.25 (10.83-1802.75)	AD 712.3 (22.09-1742) Non-AD ASD 610.8 (10-1823)	0.046 (U) [†] 0.089 (KW) [‡]
Controls	27.03 (10-951.95)		

Data are median (interquartile range). U: Mann-Whitney U-test; KW: Kruskal-Wallis test. [†]Cases vs. controls; [‡]AD vs. non-AD ASD vs. controls.

Table IV. Logistic regression model to identify independent predictors for presence of the disorder.

	B	SE	OR	95% CI	p-Value
Gender	0.592	0.561	1.807	0.602-5.429	0.291
Age	0.064	0.093	1.067	0.890-1.279	0.486
Presence of VZV antibodies	0.975	0.492	2.651	1.010-6.959	0.048

SE: Standard error. OR: Odds ratio. CI: Confidence interval.

controls) as the dependent variable. Results are summarized in Table IV. Based on this analysis, the presence of VZV antibodies was an independent predictive factor for the presence of the disorder.

We compared GMDS, VABS, ADOS and CARS scores with VZV antibody levels using Spearman's rho test. *p*-Values were 0.013 (*p*=0.927) for GMDS, 0.162 (*p*=0.248) for VABS, 0.276 (*p*=0.045) for CARS, and -0.098 (*p*=0.487) for ADOS.

Discussion

With the exception of few cases reported in which a viral infection can be the sole cause of autism (32, 38, 40, 49), ASD can be considered a complex immune disorder (21, 23, 50-52). Viral infections are one of the possible triggers of this deranged immune response (9, 23, 25, 29, 33, 53-55). In this "hit-and-go" hypotheses, viral infection triggers the autoimmune disorder that is then able to self-maintain it even without the persistence of viral infection (23). This implies

that it is hard to find the infection in patients with ASD as it could last for a little time. However it is possible to provide evidence of a viral exposure by the means of specific antibody detection. For this reason several researchers have investigated exposure markers to different viruses (HSV, HHV6, CMV, Rubella virus, EBV) in subjects with ASD (15, 30, 33, 43, 56, 57).

To the best of our knowledge, ours is the first study to systematically evaluate the exposition to VZV in children with ASD and compared it to that of healthy controls. VZV is a herpesviridae family member which causes chickenpox at its first infection and Zoster at its reactivations. It is noteworthy that VZV has been implicated in multiple sclerosis, which is considered an immune-mediated disease of the central nervous system, and that can share some pathologic features with ASD (58).

The results of our study clearly show a higher rate of exposure to VZV in ASD than in controls. Moreover the titre of anti-VZV was significantly higher in ASD children than

in controls. Finally, a trend toward a higher exposure rate and higher titres in patients with the more severe form (AD) than in those with the mild form (Non-AD ASD) and a positive significant correlation between a gravity score (CARS) and the titre of the VZV antibodies were also found. At multivariate analysis, anti-VZV antibody presence was found to be an independent risk factor for ASD (OR: 2.651, 95% CI: 1.010-6.959) while age and gender were not.

It is noteworthy that, as expected, approximately 40% of healthy children had exposure markers to VZV. This datum further confirms that infection itself is not sufficient to arouse the disease but a constellation of genetic and environmental factors play a predisposing role in ASD onset (23). The findings of the present study can have dramatic consequences in the discovery of the etiopathogenesis of ASD. In fact, without a clear picture on the causes and pathogenesis of ASD, all preventive and even biologically curative strategies are condemned to fail. The type of study and methods used do not allow us to determine when this higher exposure to VZV occurred in children with ASD. Only large studies with molecular biology techniques applied on specific substrates (*e.g.* amniotic fluid) can give this relevant answer.

In conclusion, in a case control study, exposure to VZV and high titres of specific anti-VZV antibodies were significantly associated with ASD. Future studies are urgently warranted to confirm these results and provide a deeper analysis on this association.

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Received January 7, 2014

Revised March 10, 2014

Accepted March 11, 2014