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Abstract. Background/Aim: Autism spectrum disorders (ASD) are neurodevelopmental disorders without a definitive etiology in most cases. Environmental factors, such as viral infections, have been linked with anomalies in brain growth, neuronal development, and functional connectivity. Congenital cytomegalovirus (CMV) infection has been associated with the onset of ASD in several case reports. The aim of this study was to evaluate the prevalence of congenital CMV infection in children with ASD and in healthy controls. Patients and Methods: The CMV genome was tested by polymerase chain reaction (PCR) on dried blood spots collected at birth from 82 children (38 with ASD and 44 controls). Results: The prevalence of congenital CMV infection was 5.3% (2/38) in cases and 0% (0/44) in controls (p=0.212). Conclusion: The infection rate was about 10-fold higher in patients with ASD than in the general Italian population at birth. For this reason, detection of CMV-DNA on dried blood spots could be considered in the work-up that is usually performed at ASD diagnosis to rule-out a secondary form. Given the potential prevention and treatment of CMV infection, this study could have intriguing consequences, at least for a group of patients with ASD.

According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by impairments in two core domains: social-communication and restricted and repetitive patterns of behavior, interest or activities (1). ASD presentation is very heterogeneous depending on the severity of impairment in the two core domains. Furthermore, clinical features vary depending on the presence/absence of intellectual disability and verbal skills.

The prevalence of ASD has considerably increased in recent decades. In fact, at the beginning of the century the prevalence of ASD was estimated at around 60-70/10,000 (2), with a male to female ratio of 4:1 (3), whereas studies conducted in the past two decades revealed an increase of 100-250/10,000 (4-6) and a decrease in male predominance (ratio 2.0-2.6:1) (7, 8). Only in about 20% of cases is a definite etiology recognized. These cases are called “secondary ASD” (9). No definitive cause is found in the remaining 80% of cases, and these are designated “primary ASD” (10-12). Primary ASD is believed to result from the interaction of environmental factors, which probably occur during intrauterine life, with the genetic profile. This interaction leads to aberrant changes in brain growth, neuronal development, and functional connectivity that finally give rise to the disorder (11, 13-15).

There are no instrumental diagnostic tests for ASD. Thus, its diagnosis is based on clinical findings. However, a series of
tests are routinely performed to rule-out secondary ASD. They include genetic tests (recommended in their case of familiality for genetic diseases, intellectual disability, and malformations in various organs), metabolic tests (in case of familiality for metabolic disorders, cyclical vomit, precocious epilepsy, mental retardation, clinical evidence or suspicion of metabolic disorders) (16). Several treatments have been proposed, both pharmacological and non-pharmacological, but their efficacy is currently disappointing, because of the lack of knowledge of the etiopathogenetic mechanisms of ASD (17-20).

In some cases, ASD has been linked to infection (21-39). A viral infection may per se damage the central nervous system or it might trigger an autoimmune reaction against some cerebral regions (11). Several case reports have associated cytomegalovirus (CMV) infection with ASD (40-48). We previously demonstrated that exposure to CMV was similar in a cohort of children with ASD and in same-aged healthy controls (49). However, this type of study does not allow for a precise timing of viral exposure (11). To overcome this drawback, in the present study, as samples we used dried blood spots (DBS) collected at birth from children with ASD and healthy controls. The use of DBS for studies on ASD provides a unique picture of the clinical situation at birth, which is considered a key moment in the development of ASD. CMV-DNA detection on DBS has been widely used in the retrospective diagnosis of congenital infection when no neonatal sample was available (50, 51). This test has also been used to evaluate the prevalence of congenital CMV infection in children with cortical development disorders (such as pachigiria and leucodystrophy of unknown origin) (52). The same test revealed that 20-30% of all cases of infantile deafness were due to congenital CMV infection (53, 54).

The aim of this study was to evaluate the rate of congenital CMV infection assessed via detection of viral genome in DBS by polymerase chain reaction in children with ASD and in healthy controls.

Materials and Methods

Participants. Children with ASD were enrolled at the Child and Adolescent Neuropsychiatry Unit at the Second University of Naples, Italy, between January 2010 and January 2013. Inclusion criteria for cases were: diagnosis of primary ASD [at the time performed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition, Text Revision (DSM-IV-TR)] (55)]. Diagnosis was confirmed based on the criteria of the new DSM-5 classification (1). The only exclusion criterion was the inability of parents or legal guardians to sign an informed consent form.

Controls were recruited by a general pediatrician during routine examination at their medical office in Torre del Greco, Naples, Italy or at the Division of Pediatric Surgery of the Federico II University of Naples, where they were admitted for minor surgical procedures (phimosis, hernia, cryptorchidism, vesicoureteral reflux, hydrocele tests, etc.). All controls were aged between 0-8 years. They were enrolled between January 2010 and January 2013 and underwent an interview to rule out the presence of neuropsychiatric disorders. To verify the diagnosis of ASD, cases were assessed with the Autism Diagnostic Observation Schedule-Generic (ADOS-G) (56), performed by a licenced clinician. Griffiths Mental Development Scales (GMDS-ER) (57) were administered to all cases to determine the level of development. The Autism Diagnostic Interview, Revised version (ADI-R) (58), the Childhood Autism Rating Scales (CARS) (59), and the Vineland Adaptive Behavior Scales (VABS) (60) were administered to the parents of all patients. A revised algorithm of ADOS was adopted to evaluate ASD severity (61-63). Based on a standardized clinical evaluation (obtained using ADOS, GMDS and VABS), three levels of ASD severity were identified as specified by DSM-5 criteria. Level 1 refers to clinical conditions in which the patient needs support for adequate adaptation; level 2 refers to clinical conditions in which they need significant support for adequate adaptation; and level 3 refers to clinical conditions in which they need very significant support for adequate adaptation.

Samples. All newborns in Italy are screened for some congenital disorders at 2-5 days of age. The DBS obtained during this procedure are stored at room temperature and are not exposed to light.

All procedures were performed in compliance with the ethical standards of relevant laws and institutional guidelines and in accordance with the with the 1964 Helsinki declaration and its later amendments. The research was prospectively reviewed and approved by the Ethics Committee of the Federico II University of Naples and the Ethics Committee of ASL NA3 SUD approved the study (85/09). Informed consent was obtained from the children’s parents or legally authorized representatives.

Procedure. A 3-mm diameter circle punched out of the DBS was used for CMV-DNA extraction, and nucleic acid was amplified as previously described (64) by an operator blinded to the characteristics of the cases. Stringent control measures were applied to prevent both carryover and contamination (65). In brief, DNA was extracted from the circle of DBS by adding 35 μl of cell culture medium (minimum essential medium) followed by thermal shock (55˚C for 60 min and 100˚C for 7 min, centrifugation at 11.200 rcf, and the supernatant was frozen at −80˚C overnight) and amplified using a nested polymerase chain reaction (PCR) designed to amplify one region in the GP58 gene (66). The first round of CMV-DNA amplification was carried out starting with 5 μl extract in 45 μl PCR mixture (10 mM Tris – HCl, 1.5 mM MgCl2, 50 mM KCl, 0.1% triton X-100, 200 mM dNTP) containing 1U Taq polymerase (DyNAzyme™ II DNA Polymerase; Finnzymes, Thermo Fisher Scientific Inc. Rataastie, Vantaa, Finland) and the primers (1 mM) necessary for amplification. The second round of CMV-DNA amplifications consisted of 2 μl amplicons in 48 μl PCR mixtures. The amplification conditions for the first round were as follows: denaturation at 94˚C for 2 min followed by 35 cycles at 94˚C for 30 s, 55˚C for 30 seconds and 72˚C for 30 s with a 5 min final extension at 72˚C. The second amplification differed from the first amplification in that 30 cycles were performed and the annealing temperature was 53˚C.

To verify extraction from DBS, a house-keeping gene was amplified with a PCR designed to amplify human β-globin with primers GH20 and PC04 (67). The final PCR mixture in a 50-μl reaction included 3 μl of extracted template and the PCR conditions were as follows: denaturation at 94˚C for 2 min followed by 40 cycles at 94˚C for 30 s, 55˚C for 30 s, 72˚C for 1 min and a 5 min final extension at 72˚C. The PCR products were separated by
electrophoresis on a 2% agarose gel and visualized by SYBR® Safe DNA Gel Stain (Invitrogen™ by Life Technology, Carlsbad, CA, USA) with transillumination.

Each sample was tested on three series of three DBS. All cases that tested positively in at least two out of the three tests were considered positive. New series of punches were tested from the cards of cases that were positive in only one of the triplicate amplifications (50). Negative and positive controls for CMV were included in all PCR runs and consisted of blank cards with minimum essential medium and mock DBS, respectively.

**Statistical analysis.** The Kolmogorov–Smirnov test was applied to determine whether distribution was Gaussian or non-Gaussian. We report the data as mean±standard deviation (SD) or as median and interquartile range (IQR), respectively. For comparisons between quantitative data, Student’s *t*-test for unpaired variables or the Mann–Whitney *U*-test was applied in case of Gaussian or non-Gaussian distribution, respectively. For categorical variables, the chi-squared test with Yates correction (Fisher’s exact test where appropriate) was applied. A *p*-value of 5% at two-sided test was considered statistically significant. Statistical analysis was carried out with the Statistical Package for the Social Sciences version 18.0 (SPSS Inc. Chicago, IL, USA).

**Results**

A total of 82 children were enrolled in this study, 38 with ASD and 44 controls. None of the controls was affected by a neuropsychiatric disorder. The mean age was 5 years for cases (SD=1.3 years) and 4 for controls (SD=1.7 years) (*p*=0.007). Boys outnumbered girls, both among cases (29/38, 76.3%) and controls (30/44, 68.2%, *p*=0.414). The main clinical scores of patients with ASD are reported in Table I. Sixteen out of the 38 patients were diagnosed with ‘ASD with associated intellectual disability’. The rate of CMV positivity as assessed by viral genome detection on DBS was 5.3% (2/38) in cases and 0% (0/44) in controls (*p*=0.212).

The two patients (patient 1 and patient 2) who tested positively for CMV-DNA had a mild intellectual disability. Compared with the 38 patients without CMV-DNA, the two patients with CMV-DNA had significantly lower CARS scores (median of 30 vs. 38, *p*=0.034) and showed a trend towards having lower total ADOS scores (median of 12.5 versus 17, *p*=0.069). Patient 1 was a boy born by vaginal delivery at 38 weeks of gestation (birth weight: 3,000 g). His psychomotor development was regular, except for language and sphincter control (not yet acquired at age 3 years). At 24 months of age, because of language delay, parents requested a medical consultation. A child neuropsychiatrist diagnosed relational and communication problems. The boy underwent a hearing evaluation through auditory brainstem response audiometry, which yielded normal results. He had no history of convulsion or any other neurological problems. His electroencephalogram was normal. During clinical evaluations, the patient showed partially preserved communication skill and willingness to interact with his parents, but the quality of this relationship was very poor. He presented a high level of hyperactivity and anxiety at separation from his parents and had self-injurious and restricted and repetitive behaviors. His development quotient evaluated by GMDS-ER, was 54; the adaptive quotient, evaluated by VABS, was 72; his ADOS scores were indicative of autistic features, both in communication and in social interaction areas. His diagnosis, at that time made according to DSM-IV criteria, was “Pervasive Developmental Disorder not otherwise specified”. Patient 2 was also a boy. He was born by Cesarean section at 38 weeks of gestation (body weight: 3,150 g). At birth, he did not cry immediately, but other perinatal events were regular. He underwent a transfontanellar ultrasound at birth which did not show any pathological finding. He presented a mild delay in psychomotor development at about 24 months of life, and a regression of language skills. In fact, he experienced a loss of the use of language for communication. At this age, he began to show a behavior characterized by a tendency for isolation and for difficulty in interacting with peers. Consequently, the child’s parents requested a medical consultation with a child neuropsychiatrist who diagnosed relational and communication problems. The boy underwent hearing evaluation through auditory brainstem response audiometry, which yielded normal results. He had no history of convulsion or any other neurological problems. His electroencephalogram was normal. During clinical evaluations, the patient showed some communication skills, presented a functional use of objects and was able to play appropriately with various objects, but the quality of communication with others was impaired and variable. His development quotient, evaluated by GMDS-ER, was 54; adaptive quotient, evaluated by VABS, was 56; the ADOS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>GMDS (Developmental quotient)</td>
<td>54 (35-62)</td>
</tr>
<tr>
<td>VABS (Adaptive quotient)</td>
<td>51 (40-63)</td>
</tr>
<tr>
<td>ADOS (Language)</td>
<td>6 (4-7)</td>
</tr>
<tr>
<td>ADOS (Interaction)</td>
<td>12 (10-13)</td>
</tr>
<tr>
<td>ADOS (Total score)</td>
<td>17 (15-20)</td>
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<tr>
<td>CARS (Total score)</td>
<td>37 (33-40)</td>
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<tr>
<td>Gravity score</td>
<td></td>
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<tr>
<td>1: 10%</td>
<td></td>
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<tr>
<td>2: 35%</td>
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<td>3: 55%</td>
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scores were indicative of autistic features, both in communication and in social interaction areas. His diagnosis, at that time made according to DSM-IV criteria, was “Pervasive Developmental Disorder not otherwise specified”.

At follow-up examinations, both children presented similar development trajectories: both language development and cognitive abilities improved, whereas behavioral features remained poor and indicative of an autistic disorder. A diagnosis of level 2 ASD according to the DSM-5 severity index was made in both cases.

Discussion

Our study shows that the rate of CMV congenital infection assessed by viral genome detection on DBS was 5.3% (2/38) in children with ASD, whereas no control child was affected. The two children who tested positively for CMV-DNA, showed no signs of congenital CMV infection at birth and a milder form of ASD than the CMV-negative children with ASD. The prevalence of congenital CMV infection found in our study is 30-fold higher than that of the general Italian population [which is estimated to be 0.18 % (68)] and is similar to those of the few studies conducted so far in children affected by ASD. In detail, in a Japanese study, CMV-DNA on DBS or preserved umbilical cord blood samples was detected in two of 27 (7.4%) patients with ASD (without other neurological manifestations) compared with 0.31% in the control group (p=0.004) (69). In a study conducted in Sweden, 1/115 (0.9%) children with ASD tested positively for CMV-DNA on DBS compared to 0.2% in the general population (p=0.206). This rate increased to 3% when only children with ASD and intellectual disability were considered (1/33, 3%, p=0.064 compared to the general population) (70). However, given the small sample size of all the studies performed so far, caution should be exercised in interpreting these results.

To determine if there is an association between ASD and congenital CMV infection, one should first consider the biological plausibility of this association. CMV is a teratogenic virus that can directly damage key structures in the developing brain when contracted during pregnancy (71). Several in vitro studies show that CMV infection can cause chromosomal damage (72), modulate the expression of developmental genes, inhibit neuronal differentiation and induce apoptosis in neural precursor cells (71, 73, 74). In addition, CMV infection can contribute to central nervous system disorders via viral-related inflammation which, in turn, can directly injure the brain, enhance brain vulnerability (75) and act as a trigger of autoimmune disorders (76, 77).

In humans, both symptomatic and asymptomatic congenital CMV infection has been repeatedly linked to ASD (26, 40, 41, 45-48). However, in most cases, children had other neurological manifestations such as epilepsy, palsy, and deafness (41, 45, 47). Sakamoto et al. reported ASD as the only late manifestation of an asymptomatic congenital CMV infection (69). Our two patients are similar to those reported by Sakamoto et al., namely, late ASD onset as an isolated disorder. Notably, auditory brainstem response audiometry, which is an objective measure of auditory function, did not reveal hearing loss in our two patients. Interestingly, both our patients were diagnosed with Pervasive Developmental Disorder not otherwise specified, because although their clinical features at onset were suggestive of ASD, they had slightly preserved communicative and social tasks which differed from classic autism.

The rate of prevalence of congenital CMV infection in children with ASD (about 5%) is comparable to the prevalence rate of fragile X syndrome and tuberous sclerosis, which are the disorders that are currently investigated in these individuals to rule out a secondary form of ASD. Therefore, we concur with Engman et al. (70) and Eriksson et al. (78) that clinicians could include the detection of CMV-DNA via DBS in the work-up that is usually performed in cases of ASD diagnosis (70, 78).

The link between CMV infection and ASD could have intriguing consequences because this infection is potentially preventable in pregnant women and because of the existence of specific antiviral treatment against CMV. In conclusion, the prevalence of congenital CMV infection in our cohort of children with ASD was much higher than that in the general population (5% versus 0.6%), which supports an etiological association between ASD and CMV infection. Consequently, detection of CMV-DNA via DBS can be considered in the work-up of children with ASD. Given the potential prevention and treatment of CMV infection, this study could have intriguing consequences, at least for a group of patients with ASD.

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Conflicts of Interest

All the Authors report no financial or other conflict of interest relevant to the subject of this article.

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