RESEARCH ARTICLE

Vitamin D Receptor Polymorphisms Associated with Autism Spectrum Disorder

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Vitamin D is endowed with a number of biological properties, including down-regulation of inflammation, and might contribute to the pathogenesis of autism spectrum disorders (ASD). Vitamin D binds to the vitamin D Receptor (VDR); the biological activity of the ensuing complex depends on VDR *FokI*, *BsmI*, *ApaI*, and *TaqI* gene polymorphisms. We evaluated such Single Nucletoide Polymorphismsm (SNPs) in a cohort of 100 Italian families with ASD children. *FokI* genotype distribution was skewed in ASD children compared with their healthy sibs (P_c = 0.03 2 *df*) and to a group of 170 Italian healthy women (HC) (P_c = 0.04 2 *df*). *FokI* genotype and allelic distribution skewing were also observed in mothers of ASD children compared to HC (P_c = 0.04 2 *df*). Both Transmission Disequilibrium Test for single loci and haplotype analysis distribution revealed a major *FokI (C)* allele-mediated protective effect, which was more frequently transmitted (73%) than not transmitted to healthy sibs (P = 0.02). A protective *FokI-*, *BsmI-*, *ApaI-*, and *TaqI* (*CCAG*) haplotype was more frequently carried by healthy sibs than by ASD children ($P = 1 \times 10^{-4}$; OR: 0.1, 95% CI: 0.03–0.4) too. Finally, a strong gene-dose association of *FokI (T)* allele with both higher Childhood Autism Rating Scale score (P_c = 0.01) and, particularly, with hyperactivity behavior (P_c = 0.006) emerged in ASD children. Because the protein produced by the *FokI (T)* allele is transcriptionally less active than that produced by the *FokI (C)* allele, the reduced biological activity of the vitamin D/VDR complex prevalent in ASD could favor ASD- and maternal immune activation- associated inflammation. Vitamin D supplementation might be useful in preventative and rehabilitation protocols for ASD. ***Autism Res*** *2020, 00: 1–11.* © 2020 International Society for Autism Research, Wiley Periodicals, Inc.

Lay Summary: Vitamin D deficiency and Vitamin D receptor (VDR) polymorphisms are associated with structural and functional brain abnormalities and behavioral disorders. We analyzed the association of VDR gene polymorphisms in a cohort of 100 Italian families with ASD children. A strong correlation between one of the VDR polymorphisms and hyperactivity behavior was evidenced in ASD children. In healthy mothers, the same VDR polymorphism was also correlated with an increased risk of giving birth to children with ASD.

Keywords: Autistic Spectrum Disorder; Vitamin D; VDR polymorphisms; Immune system, VDR FokI

Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by a deficit of social communication and distinctive behaviors [American Psychiatric Association, 2013]. Like all complex psychiatric conditions, ASD etiology involves both genetic and environmental factors, which interact to adversely affect brain development and functioning. A crucial predisposing role might be played by an altered embrio-fetal environment, as it occurs in the maternal immune activation (MIA). Indeed, polyabortivity [Altevogt et al., 2008; Atladóttir et al., 2009; Comi, Zimmerman, Frye, Law, & Peeden, 1999; Croen et al., 2005; Funderburk et al., 1983; Sweeten et al., 2003] and preterm births, both associated with MIA [Busse et al., 2019; Ito et al., 2010; Makhseed et al., 2003; Marzi et al., 1996; Piccinni et al., 1998; Saito et al., 2010], are significantly observed in mothers of children that later will be diagnosed with ASD [Guerini et al., 2014; Guerini et al., 2015; Guerini et al., 2018; Schendel and Bhasin, 2008; Zhang et al., 2018].

Several factors modulate the quality of the immune response during pregnancy, among which vitamin D plays a crucial role. Several studies indicate maternal

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vitamin D deficiency to increase the likelihood of recurrent miscarriage, preeclampsia, gestational diabetes, preterm birth, and low weight at birth [Harvey et al., 2014]. In ASD, the role played by vitamin D might get well beyond its mere immune-modulatory ability [Eyles et al., 2013], being also involved in neuronal differentiation, axonal connectivity, and dopamine ontogeny. In epidemiological terms, increased prenatal exposure to vitamin D levels correlates with improved cognition and reduced risk of both ASD and Attention Deficit Hyperactivity Disorder (ADHD) [Cannell, 2008, García-Serna and Morales 2019]; confirmingly, a recent systemic meta-analysis shows that decreased vitamin D levels during pregnancy might increase ASD risk [Wang et al., 2016]. Vitamin D biological activity is mediated by its binding to the vitamin D Receptor (VDR). The (1,25 $(OH)_2D$)-VDR complex translocates into the nucleus, generating heterodimers with the retinoid X receptor which, in turn, binds a specific sequence called vitamin D response element on DNA, thus activating a plethora of genes.

The VDR gene, located on chromosome 12q13, has several allelic variants (VDR gene polymorphisms) identified in different Restriction Fragments Length Polymorphism assays [Valdivielso and Fernandez, 2006]. Polymorphisms detected by restriction enzymes *ApaI* A/a: (Adenine -> Cytosine) (*rs7975232*) [Faraco, et al., 1989] and *BsmI* B/b (adenine -> guanine) (*rs1544410*) [Morrison, et al., 1992] are found in intron 8. *TaqI (rs731236*) polymorphism is placed in exon 9, T/t (cytosine -> thymine), as a silent mutation [Morrison et al., 1994]. Finally, missense *FokI (rs2228570)* polymorphism in exon 2 modifies the protein length, which results in a protein with either 427 (f -thymine- variant) or 424 amino acids (F -cytosine- variant), the latter being biologically more active [Gross et al., 1996; Saijo et al., 1991; Uitterlinden et al., 2004].

Some VDR polymorphisms are suggested to modulate susceptibility to a number of diseases [Abouzid et al., 2018]. In particular, *FokI (rs2228570)* associates with a higher risk of adverse pregnancy outcomes in a cohort of Italian women [Barchitta et al., 2018]. VDR SIngle Nucleotide Polymorphisms (SNPs) have also been suggested to associate with a variety of neuropsychiatric disorders [Lee et al., 2014; Jiang et al., 2015; Yan et al., 2005] including ASD. Different VDR SNPs were shown to be not univocally associated with ASD development and/or protection [Biswas et al., 2018; Cieślińska et al., 2017; Coşkun et al., 2016; Mobasheri *et al*, 2020; Zhang et al., 2018].

Reasonable grounds for such inconsistencies might be based on heterogeneous ethnicity and/or limited sample-size studies [Mobasheri *et al*, 2020; Zhang et al., 2018]. This led us to design a study in which the possible association between VDR SNPs and ASD could be verified in a larger and genetically well-defined population. We analyzed the distribution of *ApaI*, *TaqI*, *FokI*, and *BsmI VDR* polymorphisms on a cohort of Italian families with at least one child with ASD. The transmission rate from parents to both healthy and ASD children was analyzed to evaluate their risk- or protection-conferring potentials. Finally, we evaluated possible correlations between VDR polymorphisms and several areas of cognitive and behavioral impairments that characterize ASD.

Methods and Materials

Patients and Controls

A total of 100 Italian children (85 males; mean age 7.5 $\pm$ 4.1), with a ASD diagnosis according to DSM-5 criteria [American Psychiatric Association, 2013], born in peninsular Italy and of Italian descent, were enrolled at the IRCCS Don Gnocchi Foundation, the IRCCS Mondino Foundation National Neurological Institute, and the "Associazione Nazionale Famiglie di Persone con Disabilitá Intellettiva e/o Relazionale" (ANFFAS). Their 46 healthy sibs (15 males; mean age: 10 $\pm$ 5.7 years), 82 mothers, and 79 fathers were also recruited. All healthy siblings underwent clinical examination; none of them presented ASD symptoms and Childhood Autism Rating Scale (CARS) scores were all in the normal range (<30).

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Don Gnocchi foundation ethical committee. All clinical assessment was blinded to the genotypes of the subjects.

All ASD children underwent clinical, psychiatric, neurological, and neuropsychological evaluations, as well as mental status examinations (covering social interaction, imaginative play, and communication domains), as previously described [Guerini et al., 2015]; all evaluations were carried out by an experienced team Leiter-R [Roid et al., 2013]. Weschler Intelligence Scales [Wechsler, 2003] and Raven's Progressive Matrixes [Raven et al., 1984] were used to evaluate the global cognitive level. Inclusion criteria were the following (a) age between 3 and 12 years; (b) a primary diagnosis of ASD, and (c) the results of the Autism Diagnostic Observation Schedule 2 [ADOS-2; Lord et al., 2012] test that measures the clinical symptoms of autism. Other diagnostic tools employed in the evaluation to confirm the clinical diagnosis of ASD included the structured parent's reports Autism Diagnostic Interview-Revised [Lord et al., 1994], and the CARS [Schopler et al., 1980]. Learning, language, and attention-deficit/hyperactivity disorder were not considered as exclusion criteria.

Karyotype and DNA analysis for fragile-X and MECP2, screening for inborn errors of metabolism (phenylketonuria and amino and organic acidopathies), EEG, brainstem acoustic and visual evoked potentials as well as brain computerized tomography or magnetic resonance imaging were also performed in all included children.

EEG abnormalities were considered not as exclusion criteria unless a specific primary encephalopathy was diagnosed.

Exclusion criteria were the diagnosis of psychotic disorders and/or of intellectual disability without ASD according to DSM-5 [American Psychiatric Association, 2013]. Patients with a previously ascertained genetic syndrome were excluded from the study.

The following scales were used for the diagnostic evaluation of patients:

Cognitive assessment. Wechsler Intelligence Scale for Children [WISC-IV; Wechsler 2003]. WISC-IV administration provides four different indexes: Verbal Comprehension Index; Perceptual Reasoning Index; Working Memory Index; and Processing Speed Index.

Leiter-R [Roid et al., 2013]: This scale offers a nonverbal measure of intelligence and evaluates the ability to reason by matching and perceptual reasoning in general, irrespective of language and formal schooling.

Raven Progressive Matrices [Raven et al., 1984]: a nonverbal assessment of intelligence developed as a matrix-reasoning test with a piece missing in which the individual is asked to identify the correct response that completes the pattern choosing from six alternative possible response options.

Assessment of ASD symptoms. CARS [Schopler et al., 1980]: a semi-structured assessment tool, composed of 15 items lead by the clinician with the parents to measure interaction and communication social skill defects, repetitive behaviors and psychopathological comorbidities as well as hyperactivity traits.

ADOS-2 [Lord et al., 2012]: a semi-structured assessment tool lead by the clinician with the children, allowing systematic and standardized evaluation of the presence of ASD symptoms for collecting standardized and objective information about social communication skills, restricted interests, and repetitive behaviors, although insufficient on its own for a diagnosis.

The control group included a cohort of Italian women with no pregnancy-related disorder who gave birth to healthy and neurotypical offsprings [Barchitta et al., 2018]. Their VDR analysis was used as control value as their materno/fetal tolerogenicity had apparently been preserved. The sample of enrolled ASD children strictly reflected the number of patients followed by the pediatric clinical units taking part in the study.

VDR Genotyping

Genomic DNA was isolated from peripheral blood through the phenol/chloroform method or from saliva using the ORAgene-DNA (DNA Genotek, Ottawa, Canada).

The following *VDR* polymorphisms were analyzed: *rs731236 A/G(T/t) (aka TaqI)*, *rs10735810 C/T(F/f) (FokI)*, *rs1544410 C/T(B/b) (BsmI)*, and *rs7975232 A/C(A/a) (ApaI)*. They were selected based on their positions in the *VDR* gene or for their potential functional role. SNPs were evaluated by Allelic Discrimination Real-time PCR using pre-designed TaqMan probes (Applied Biosystems, Foster City, CA). PCR consisted of a hot start at 95°C for 10 min followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min. Fluorescence detection took place at 60°C. Assays were performed in 10 μL reactions, using TaqMan Genotyping Master Mix on 96-well plates using an ABI 7000 instrument (Applied Biosystems). Control samples, representing all possible genotypes, and a negative control were included in each reaction.

Statistical Analysis

Chi-square analysis was used to verify that populations were in Hardy–Weinberg Equilibrium (HWE) and to evaluate differences between groups. *VDR* allelic polymorphism distribution in children with ASD and their mothers, fathers and healthy siblings was analyzed by odds ratio (OR) and 95% confidence interval (95% CI). Analyses were carried out using SPSS 25.0 for Windows. *P*-value was considered significant when <0.05 after Bonferroni correction for two degrees of freedom (P_c) in 2×3 and 2×2 contingency tables. VDR SNPs haplotype comparisons between ASD children and their healthy sibs were performed using the SHEsis software [http://202.120.7.14/analysis/myAnalysis.php; Shi and He 2005].

Multinomial regression analysis was adopted to compare VDR polymorphisms distribution between ASD children and their sibs, calculating VDR genotypes as dependent variable and diagnosis and gender as covariates.

To assess allelic association, the Transmission Disequilibrium Test (TDT) [Spielman et al., 1993] was used. TDT simultaneously measures linkage and association by comparing the allele transmitted and the one that is not transmitted from a heterozygous parent to the child. Within each family, parental chromosomes transmitted to children were assumed to harbor a susceptibility allele, whereas non-transmitted chromosomes were assumed to harbor a normal allele. In the situation in which no linkage disequilibrium (LD) exists, the expected frequency of transmitted and non-transmitted marker alleles is 50% (1:1 ratio). The allele frequency differences between affected individuals and non-transmitted parental alleles at each locus were compared to the expected 1:1 ratio using the chi-square test with one degree of freedom (1 *df*) [He et al., 2002]. TDT was performed using the TDT-STDT Program 1.1 (http://spielman07.med.upenn.edu/TDT.htm). Parametric and nonparametric analyses were used to verify the presence of possible associations between neuropsychological and behavioral tests scores and *VDR* genotype distribution in ASD children, after Kolmogorov–Smirnov evaluation of Gaussian values distribution.

Results

Patients in this study were predominantly males (85:15) with a median age of 7.5 ± 4.1 years. The average CARS score was 38.09 ± 6.09. The average IQ was 64.8 ± 9.8. Major comorbidities were the following intellectual disability (32 patients), ADHD (11 patients), epilepsy (7), and anxiety traits (2).

VDR Genotype and Allelic Distribution

Genotype and allelic distribution of the *VDR ApaI*, *TaqI*, *FokI*, and *BsmI* polymorphisms were analyzed in all enrolled subjects and results compared to healthy controls (HC) represented by the cohort of healthy Italian women with no pregnancy disorders [Barchitta et al., 2018]. Genotype polymorphisms were equally distributed in male and female ASD children, as well as in their sibs, mothers, and fathers, and they were in HWE in all cases with the exception of the *BsmI* distribution in the control group (Table S1). For this reason, case controls comparison for *BsmI* distribution was not applied.

A statistical different distribution of *FokI* genotype was observed between ASD and their healthy sibs (P_c = 0.03 2 *df*), as well as between ASD children and HC (P_c = 0.04 2 *df*) (Table 1). Namely, the *FokI* minor allele *(T)* was significantly more frequent in ASD children (37%) than in their healthy sibs (20.6%) (P = 0.008; OR: 2.2; 95% CI: 1.3–4.1). Importantly, the skewing of the *FokI* minor allele *(T)* frequency was statistically significant in comparison with HC as well (26.5%) (P = 0.01; OR: 1.63, 95% CI: 1.1–2.3). Since there was a significant discrepancy between ASD children and their sibs by gender stratification, a multinomial regression analysis to compare *VDR* polymorphism in ASD and their sibs was evaluated adjusting for gender as covariate variable. The association of *FokI (TT)* genotype was confirmed in ASD children (P = 0.03; OR: 6.3, 95% CI: 1.2–35.6). The possible correlation between *FokI* genotype and allelic distribution and ASD was further reinforced by results obtained in mothers of ASD children, thus *FokI* genotype distribution was statistically different in ASD mothers compared to HC (P_c = 0.04 2 *df*). Again the *FokI (T)* allele was significantly more frequently carried by ASD mothers (37.2%) compared to controls (P = 0.02; OR: 1.6, 95% CI: 1.1–2.5) (Table 1). Notably, the complementary *FokI (CC)* genotype was statistically less frequent in ASD children (41%) compared to their sibs (63%) (P_c = 0.04; OR: 0.4, 95% CI: 0.2–0.8) and in ASD mothers (41.8%) compared to HC (54.1%) (P_c = 0.04; OR: 0.5 95% CI: 0.3–0.9).

Finally, no statistical difference was observed between ASD fathers and controls regarding any of the *VDR* polymorphism distribution analyzed (Table 1 and Table S1).

VDR Fok1-BsmI-ApaI-TaqI Haplotype Analysis in ASD Children and their Sibs

A linkage haplotype study was conducted in all ASD children and their healthy sibs to evaluate both the linkage score between *VDR* variants and whether some associations between different haplotypes and ASD development

Table 1. *Fok-I* Genotype and Allele Distribution in 100 Italian ASD children, 46 Healthy Sibs, and Their Parents (82 mothers, 79 fathers)

	ASD children (100)[†]		ASD sibs (46)[‡]		ASD mothers (82)[§]		ASD fathers (79)[¶]		Controls (Barchitta et al., 2018) (170)[$]				
Fok-I	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	P_c value	OR	95% CI
CC	41	41.0	29	63.0	31	37.8	33	41.8	92	54.1	†‡**0.04**	0.4	0.2–0.8
											§**$0.04**	0.5	0.3–0.9
TC	44	44.0	15	32.6	41	50.0	40	50.6	66	38.8	ns		
TT	15	15.0	2	4.4	10	12.2	6	7.6	12	7.1	ns		
	HWE = 0.12		HWE = 0.97		HWE = 0.52		HWE = 0.19		HWE = 0.97		†**$0.04; †‡0.03; §$0.04; ¶$**ns		
C	126	63.0	73	79.4	103	62.8	106	67.1	250	73.5		†$0.6	0.2–0.8
												†‡0.4	0.4–0.9
												§$0.6	0.4–0.9
T	74	37.0	19	20.6	61	37.2	52	32.9	90	26.5		†$1.6	1.1–2.3
												†‡§2.2	1.3–4.1
												§$1.6	1.1–2.5
											†**$0.01; †‡0.008; §$0.02; ¶$**ns		

As a control group, the distribution data of Italian full-term women described by Barchitta et al. (2018) were reported. *N* = absolute number. % = percentage. HWE: Hardy–Weinberg equilibrium, *P* evaluation with 1° of freedom. If $P < 0.05$ the distribution is not consistent with HWE.

[†$]ASD children versus Controls.

[†‡]ASD children versus Sibs.

[§$]ASD mothers versus Controls.

[¶$]ASD fathers versus Controls.

Significant comparison was reported as *P*-value corrected for degree of freedom, Odds Ratio (OR), and 95% Confidence Interval (95% CI).

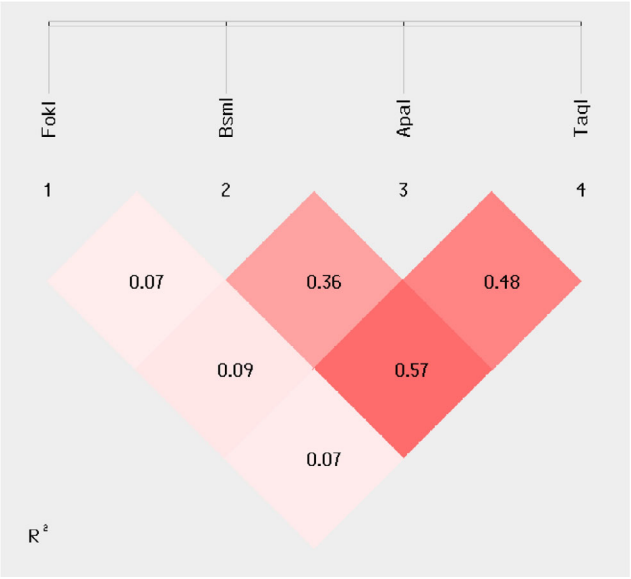

Figure 1. Linkage disequilibrium analysis (r^2) for the four analyzed SNPs. (1) *rs10735810* (FokI) chr12:46559162); (2) *rs1544410* (BsmI) *(chr12:46526102)*; (3) *rs7975232* (ApaI) (chr12:46525104); (4) *rs731236* (Taq1) (chr12:46525024).

could be observed. Haplotype analysis evidenced, as expected, the presence of a LD between *ApaI*, *BsmI*, and *TaqI* ($r^2 > 0.3$); in contrast, no LD could be detected between *FokI* and any of the other *VDR* polymorphisms ($r^2 < 0.10$), therefore, *FokI* can be considered to be an independent marker within the *VDR* gene (Fig. 1).

Haplotype analyses evidenced an increased risk to ASD development spanning from the shorter *FokI-BsmI (TT)* (OR: 2.14, 95% CI: 1.1–4.4) to the extended *FokI-BsmI-ApaI-TaqI (TTAG)*(OR: 3.05; 95% CI: 1.4–6.8) haplotype. Thus, the extended *FokI-BsmI-ApaI-TaqI (TTAG)* haplotype was significantly more frequently carried by ASD children (22.5%) compared to their healthy sibs (8.1%) ($P = 4 \times 10^{-3}$). This result was further confirmed by the observation that the complementary *FokI-BsmI (CC)* haplotype was protective against ASD development in healthy sibs (OR: 0.59, 95% CI: 0.4–0.9) and, once again, the complementary extended *FokI-BsmI-ApaI-TaqI (CCAG)* haplotype showed an additive protective effect (OR: 0.1, 95% CI: 0.03–0.4) ($P = 1 \times 10^{-4}$) (Table 2).

TDT of VDR Alleles to ASD Children and their Non-ASD Siblings

A TDT analysis was performed next by comparing the number of transmitted and non-transmitted *FokI* minor allele *(T)* (corresponding to the transmission of the *FokI* major allele *(C)*). A total of 45 mothers and 46 fathers were heterozygous for the *FokI* genotypes and were used for TDT analysis in 79 ASD children (62 males) and 16 sibs (eight males). Results indicated that both *FokI* alleles were

Table 2. *VDR* Haplotype Association Risk to ASD Development in 100 AD Children and 46 Healthy Sibs

		At-risk haplotype							Protective haplotype						
		ASD		Sibs					ASD		Sibs				
VDR SNPs		*N*	%	*N*	%	P_c value	OR	95% CI		*N*	%	*N*	%	P_c value	OR	95% CI
FokI-BsmI	TT	45	22.5	11	11.9	**3×10^{-2}**	2.14	1.1–4.4	CC	85	42.5	51	55.4	**4×10^{-2}**	0.59	0.4–0.9
FokI-BsmI-ApaI	TTA	45	22.5	11	11.9	**3×10^{-2}**	2.14	1.1–4.4	CCA	27	13.5	22	23.9	**3×10^{-2}**	0.49	0.3–0.9
FokI-BsmI-TaqI	TTG	45	22.5	8	8.1	**4×10^{-3}**	3.05	1.4–6.8	CCG	3	1.5	12	13.0	**1×10^{-4}**	0.1	0.03–0.4
FokI-BsmI-ApaI-TaqI	TTAG	45	22.5	8	8.1	**4×10^{-3}**	3.05	1.4–6.8	CCAG	3	1.5	12	13.0	**1×10^{-4}**	0.1	0.03–0.4

N = absolute number. % = percentage. Only significant comparison was reported as *P*-value corrected for degree of freedom, Odds Ratio (OR), and 95% Confidence Interval (95% CI).

Table 3. *Fok-I* Transmission Disequilibrium Test from Heterozygous Parents to ASD Children and Their Healthy Sibs

	ASD children (from all informative families) $N = 79$					ASD children (from complete informative families) $N = 16$					Healthy Sibs $N = 16$				
	T		C			T		C			T		C		
Alleles	*N*	(%)	*N*	(%)	*P*-value	*N*	(%)	*N*	(%)	*P*-value	*N*	(%)	*N*	(%)	*P*-value
Total	45	49.5	46	50.5	0.92	7	35.0	13	65.0	0.18	**6**	**27.3**	**16**	**72.7**	**0.03**
From father	25	54.6	21	45.7	0.56	3	27.3	8	72.7	0.13	**3**	18.2	9	81.8	0.08
From mother	20	44.4	25	55.6	0.46	4	44.4	5	55.6	0.74	3	30.0	7	70.0	0.21
Male	37	50.0	37	50.0	1	6	35.3	11	64.7	0.22	3	21.4	11	78.6	**0.03**
Female	8	47.0	9	52.9	0.8	1	33.3	2	66.7	0.56	3	37.5	5	62.5	0.6

Only transmission of the T and C alleles is reported. *N* = absolute number of transmitted alleles. % = percentage of transmitted alleles. Total ASD children from trios families with heterozygous parents both with and without healthy sibs ($N = 79$); ASD children from complete informative families ($N = 16$): composed of heterozygous parents and both one ASD and one healthy sib ($N = 16$). Significant *P* values are in bold.

equally transmitted to ASD children from both parents and that the *FokI* minor allele *(T)* was significantly less frequently transmitted to ASD siblings (27.3%) compared to the major allele *(C)* (72.7%) ($P = 0.03$) (Table 3).

This analysis was confirmed selecting 16 complete families with one ASD child (13 male, three female) and one healthy sib (eight male, 8 female): nine mothers and 11 fathers had the criteria of heterozygosity necessaries to perform TDT analysis (Table 3). TDT analysis evaluated in this subgroup, clustered by gender, evidenced a significantly reduced transmission of the *FokI* minor allele *(T)* to male sibs (21.4%) than in male ASD (35.3%). No significant discrepancy was observed in females. No other association for any of the other *VDR* SNPs was observed by TDT analysis.

VDR Genotype Correlation with Neuropsychological Score

Scores obtained in neuropsychological and behavioral evaluation scales, as well as EEG results, were correlated with the *VDR* SNPs. A significant association of CARS total score with the *VDR FokI* SNPs ($P_c = 0.01$) was evidenced by the ANOVA test. A multinomial regression analysis was performed next, considering *VDR FokI* genotype distribution as the dependent variable and age and gender as covariates. The mean CARS total scores distribution and significant difference between ASD children carrying either the *FokI (TT)*($N = 15$) (mean: 42.3; *SD*: 7.15) or the *(CC)* genotypes ($N = 41$) (mean: 36.9; *SD*: 5.5) ($P = 0.004$; OR: 1.19; 95% CI: 1.1–13) after adjusting for gender and age is reported in Figure 2, Panel A. CARS total scores were skewed in ASD children carrying the *(TT)* or, though to a lower level, the *(TC)* genotypes ($N = 41$) (mean: 37.8; *SD*: 5.7; $P_c = 0.02$; OR: 1.1, 95% CI: 1.0–1.3), suggesting the presence of a gene-dose effect. No differences in CARS scores were observed between *(CC)* and *(CT)* genotypes.

An in-depth analysis of possible associations between *FokI* genotypes and specific CARS items was finally performed. Multinomial regression analysis, as shown above, adjusted for age and gender provided a significant association of VDR *FokI* genotypes with CARS item 13, that is, hyperactivity ($P_c = 0.006$). Thus, a significantly higher hyperactivity score was observed in ASD children carrying *VDR FokI (TT)* genotype (mean: 2.96; *SD*: 0.62) compared to those carrying either the *FokI(TC)* (mean: 2.37; *SD*: 0.7) ($P_c = 0.01$; OR: 3.7, 95% CI: 1.3–10.5) or the *FokI(CC)* genotypes (mean: 2.2; *SD*: 0.78) ($P = 0.003$; OR: 5.14, 95% CI: 1.7–15.1), strongly suggesting a gene-dose effect of the *(T)* allele on hyperactivity score in ASD children (Fig. 2, Panel B).

Discussion

Vitamin D, by binding its specific receptor, mediates a plethora of biological effects, including immune modulation. The activity of the vitamin D/VDR complex is

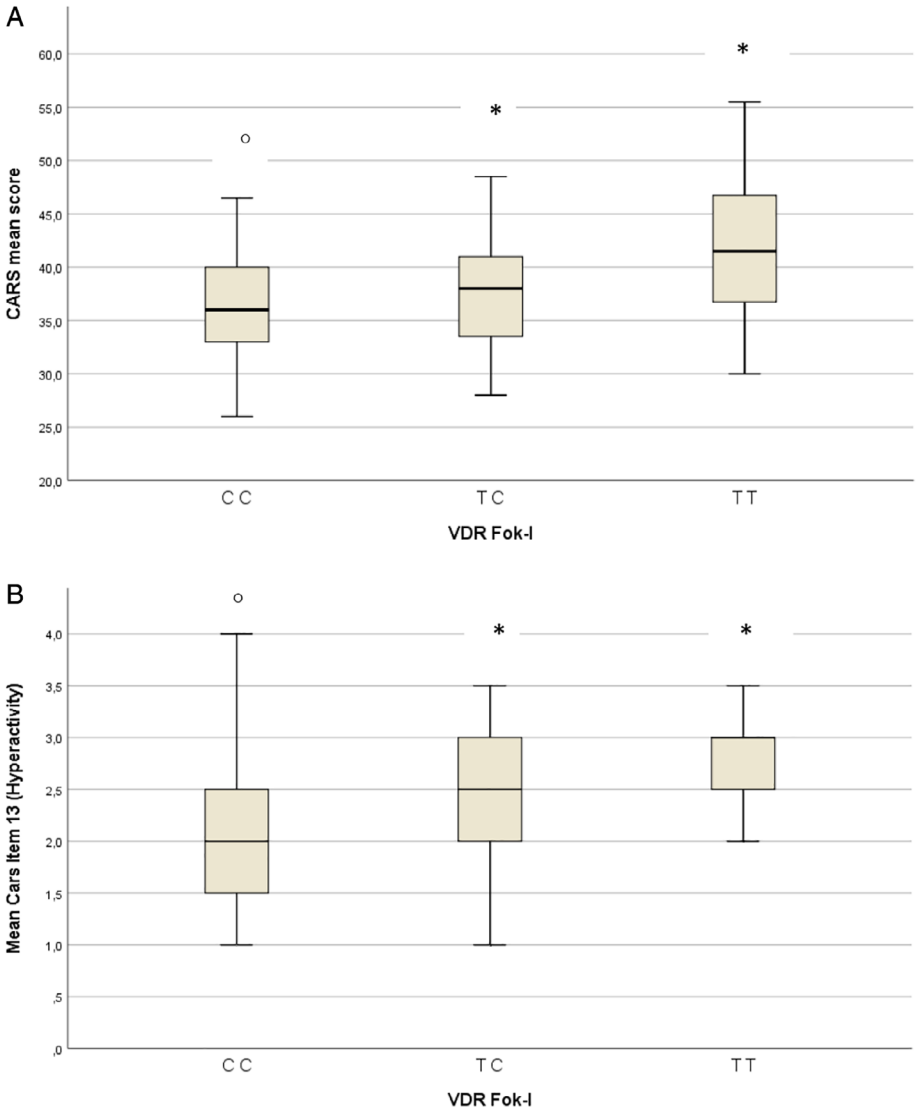

Figure 2. Panel **A**: Mean CARS scores distribution in ASD children clustered for *FokI* genotype. *TT* (*N* = 15; mean: 42.3; *SD*: 7.15), *TC* (*N* = 42; mean: 37.8; *SD*: 5.7), *CC* (*N* = 41; mean: 36.9; *SD*: 5.5). Multinomial logistic regression analysis adjusted for age and gender: ° *TT* vs. *CC* (P_c = 0.004; OR: 1.2, 95% CI: 1.1–1.3); * *TT* vs. *TC* (P_c = 0.02; OR: 1.1, 95% CI: 1.0–1.3).Panel **B**: Mean CARS item 13 (hyperactivity) scores distribution in ASD children clustered for *FokI* genotype. *TT* (*N* = 15; mean: 2.96; *SD*: 0.62), *TC* (*N* = 42; mean: 2.37; *SD*: 0.7), CC (*N* = 41; mean: 2.2; *SD*: 0.78). Multinomial logistic regression analysis adjusted for age and gender: ° *TT* vs. *CC* (P_c = 0.003; OR: 5.1, 95% CI: 1.8–15.0); **TT* vs. *TC* (P_c = 0.01; OR: 3.7, 95% CI: 1.3–10.5).

modulated by several single nucleotide polymorphisms within the *VDR* gene, the best characterized of which are *FokI*, *BsmI*, *ApaI*, and *TaqI*. We analyzed such SNPs in the context of ASD, a neurodevelopmental disorder in which vitamin D deficiency may play a pathogenic role [Biswas et al., 2018]. We found a strong correlation of *VDR FokI* polymorphism with ASD, and in particular, with hyperactivity behavior. Notably, results obtained in mothers of ASD children showed that *VDR FokI* polymorphism is also correlated with an increased risk of giving birth to children who will develop ASD. The observed association involves the *FokI* minor allele *(T)*, which was more frequently carried by both ASD children and their mothers. This association was confirmed when *VDR* SNPs distribution was analyzed in ASD children and their healthy siblings; thus, a higher frequency of the major *FokI* allele *(C)* was seen in healthy children compared to their ASD sibs. An association study of *VDR* polymorphisms with clinical and neuropsychological measures of cognitive and behavioral impairments evidenced that the *FokI (T)* allele impacts specifically on hyperactivity with a gene-dose effect. Finally, family TDT analysis confirmed a preferential transmission of the major *FokI (C)* allele to healthy sibs, whereas no difference in allelic transmission was evidenced in ASD, suggesting a protective role of *FokI (C)* allele in ASD development. Interestingly, this protective

effect appears to be stronger in healthy brothers than in sisters. Despite limited by the small number of sibs, this observation suggests that in sibs sharing the same environmental condition and gender risk, *VDR* polymorphisms play a role in conferring either susceptibility or protection toward ASD development.

Notably, whereas no differences could be detected for the individual *BsmI*, *ApaI*, and *TaqI VDR* SNPs, *FokI-BsmI-ApaI-TaqI (TTAG)* haplotype was significantly associated with ASD risk while its complementary *(CCAG)* haplotype conferred protection to healthy sibs.

Our results are, at least in part, in agreement with previously reported association of the *VDR Fokl (T)* minor allele with ASD and suggest its impact on vitamin D metabolism in ASD children [Coşkun et al., 2016]. Moreover, data herein might explain the controversial correlations between *VDR* SNPs and ASD [Biswas et al. 2018]. Possibly due to its role in functional VDR protein delivery, *FokI* could be the SNP more directly associated with ASD, though a supplementary effect of other surrounding *VDR* polymorphisms linked to other risk loci cannot be excluded in principle [Coşkun et al., 2016]. We found no association of *ApaI* or *TaqI* polymorphisms with ASD, as also previously reported [Cieślińska et al., 2017; Zhang et al., 2018]. However, we observed *FokI-BsmI-ApaI*, *FokI-BsmI-TaqI*, and *FokI-BsmI-ApaI-TAqI* haplotypes to be associated with ASD when co-segregation was evaluated in ASD children and their sibs. The role of these SNPs in linkage disequilibrium with other SNPs in conferring susceptibility to ASD development cannot thus be excluded. On one hand, our results are consistent with recent meta-analytical data suggesting cognitive development and ASD disorders to directly relate to vitamin D prenatal exposure [García-Serna and Morales 2019]; on the other hand, they suggest that normal amounts of vitamin D can lead to different pregnancy outcomes as a consequence of the *VDR* gene polymorphisms.

FokI (T) allele, the most frequently seen in ASD, is responsible for the transcription of a VDR protein containing 427 aa, which is less active than the 424 aa protein coded by *FokI (C)* [Gross et al., 1996; Saijo et al., 1991; Uitterlinden et al., 2004[. In principle, a dysfunctional VDR protein would impair the neurobiological effects of vitamin D during pregnancy. Recent results on BALB/c mice showed that maternal vitamin D deficiency induces neuroanatomical abnormalities in fetuses (on lateral ventricles and ASD-related neuronal pathways) and affects neurotrophin gene expression [Hawes et al., 2015]. Thus, *FokI (T)* allele, which might relate to a reduced vitamin D activity, can result in adverse neurodevelopment and immune-mediated events in pregnant mothers and *FokI (T)*-positive children.

Besides its direct effects on fetal brain development, a reduced vitamin D activity may induce detrimental immunostimulatory effects, which, in pregnancy, would contribute to MIA, a known risk factor for ASD. Mothers of ASD children bear higher risk of repeated miscarriages [Altevogt et al., 2008; Atladóttir et al., 2009; Comi et al., 1999; Croen et al., 2005; Funderburk et al., 1983; Sweeten et al., 2003] and preterm birth, conditions known to be associated with immune activation [Busse et al., 2019; Ito et al., 2010; Makhseed et al., 2003; Marzi et al., 1996; Piccinni et al., 1998; Saito et al., 2010]. In addition, these mothers have a higher prevalence of autoimmune conditions [Onore et al., 2012] and ASD children have increased concentration of pro-inflammatory cytokines along with inflammasome activation [Alabdali et al., 2014; Saresella et al., 2009; Saresella et al., 2016; Suzuki et al., 2011]. Finally, significant expression of class II-MHC from activated astrocytes and microglia are found in ASD brains [Vargas et al., 2005]. The immunological scenario seen in MIA and ASD is characterized by an increased functionality of TH1 and TH17 T-lymphocytes and a reduced activity of TH2 and Treg cells. As vitamin D stimulates TH2 and Tregs lymphocytes differentiation and reduces that of TH1 and TH17 [Ji et al. 2019; Sharif et al., 2018], the overall immunological effects of a peculiar VDR genetic configuration that reduces vitamin D biological activity would favor the immune activation as seen in ASD mothers and their children.

We acknowledge that our study does have some methodological limitations. One such concerns the enrollment criteria. The control group was selected on the basis of the available results obtained in healthy Italian women with full-term pregnancy who gave birth to healthy newborns whereas the ASD group was based on ASD children afferent to the pediatric and neuropsychiatric clinical units involved in the recruitment. Also, the number of healthy sibs was lower than that of their ASD counterpart because, in many cases, they were only children and for some of them (eight out of 54 siblings) parents denied their consent to participate to the study based on the will to prevent healthy sibs and themselves from unnecessary medical procedure, or even without providing specific motivations. This lead to a discrepancy between the number of ASD children and that of their parents and sibs; we performed TDT familial analysis only in complete trios families and, again, only in those families with both one ASD child and at least one healthy sib. Another possible biasing factor is that ASD and healthy sibs were not gender matched. However, being ASD predominantly affecting males [Chiappedi et al., 2010] it is not unlikely that female healthy sibs are more numerous than males. Nonetheless, the regression multinomial analysis confirmed the genotype *FokI* association with ASD after gender correction.

Taken together, data herein reinforce the potential that *VDR* polymorphisms play a role in the pathogenesis of ASD. If this is the case, since genetic polymorphisms cannot be easily modified, vitamin D supplementation might be one easy possible strategy to compensate for a

functional loss of VDR. More specifically, given the very low risk of side effects, it would be interesting to verify whether ASD symptoms, hyperactivity, in particular, would be favorably modulated by vitamin D administration and whether this could play its part in ASD-tailored rehabilitation protocols.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Table S1 TaqI, BsmI, ApaI genotype and allele distribution in 100 Italian ASD children 46 out of their healthy sibs and their parents (82 mothers, 79 fathers). As control group distribution data of Italian full term women described by Barchitta et al., was reported. N = absolute number, % percentage. HWE: Hardy Weinberg equilibrium, p evaluation with 1° of freedom. If $p < 0.05$ the distribution is not consistent with HWE.