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ORIGINAL ARTICLE

In the search for reliable biomarkers for the early diagnosis of autism spectrum disorder: the role of vitamin D

Afaf El-Ansary[1,2,3,4] · John J. Cannell[5] · Geir Bjørklund[6] · Ramesa Shafi Bhat[7] · Abeer M. Al Dbass[7] · Hanan A. Alfawaz[8] · Salvatore Chirumbolo[9] · Laila Al-Ayadhi[3,4,10]

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Abstract

Autism spectrum disorder (ASD) affects about 1% of the world's population. Vitamin D is thought to be essential for normal brain development and modulation of the immune system. Worldwide about 1 billion people are affected by vitamin D deficiency. High-sensitivity C-reactive protein (hs-CRP), cytochrome P450 2E1 (CYP2E1) and 8-hydroxy-2′-deoxyguanosine (8-OH-dG) are biomarkers related to inflammation and oxidative stress. In the present study, these biomarkers were together with serum 25-hydroxyvitamin D ($25(OH)D_3$) analyzed in 28 (mean age seven years) Saudi male patients with ASD. The study was conducted to determine if there is any relationship between vitamin D levels, the tested biomarkers and the presence and severity of ASD. The hope was to identify if these biomarkers may be useful for early ASD diagnosis. The Childhood Autism Rating Scale (CARS) and the Social Responsiveness Scale (SRS) were used to measure autism severity. The results of the ASD children were compared with 27 age and gender-matched neurotypical controls. The data indicated that Saudi patients with ASD have significantly lower plasma levels of $25(OH)D_3$ than neurotypical controls (38 ng/ml compared to 56 ng/ml, respectively; [$P = 0.001$]). Surprisingly, the levels of CYP2E1 were lower in the children with ASD than the neurotypical controls (0.48 ± 0.08 vs. 69 ± 0.07 ng/ml, respectively; $P = 0.001$). The ASD children also had significantly higher levels of hs-CRP (0.79 ± 0.09 vs. 0.59 ± 0.09 ng/ml, respectively; $P = 0.001$) and 8-OH-dG (8.17 ± 1.04 vs. 4.13 ± 1.01 ng/ml, respectively; $P = 0.001$, compared to neurotypical age and gender-matched controls. The values for hs-CRP and 8-OH-dG did not correlate [$P < 0.001$] with autism severity. There was found a relationship between autism severity on the CARS scale and the levels of $25(OH)D_3$ and CYP1B1. But this was not found for SRS. All four biomarkers seemed to have good sensitivity and specificity, but the sample size of the present study was too small to determine clinical usefulness. The findings also indicate that inadequate levels of vitamin D play a role in the etiology and severity of autism. Furthermore, the results of the present study suggest the possibility of using $25(OH)D_3$, CYP1B1, hs-CRP and 8-OH-dG, preferably in combination, as biomarkers for the early diagnosis of ASD. However, further research is needed to evaluate this hypothesis.

✉ Geir Bjørklund
bjorklund@conem.org

[1] Central Laboratory, Female Centre for Scientific and Medical Studies, King Saud University, Riyadh, Saudi Arabia

[2] Medicinal Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

[3] Autism Research and Treatment Center, Riyadh, Saudi Arabia

[4] Shaik AL-Amodi Autism Research Chair, King Saud University, Riyadh, Saudi Arabia

[5] Vitamin D Council, San Luis Obispo, CA, USA

[6] Council for Nutritional and Environmental Medicine, Toften 24, 8610 Mo i Rana, Norway

[7] Biochemistry Department, Science College, King Saud University, Riyadh, Saudi Arabia

[8] Department of Food Science and Human Nutrition, King Saud University, Riyadh, Saudi Arabia

[9] Department of Neurological and Movement Sciences, University of Verona, Verona, Italy

[10] Department of Physiology, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia

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Introduction

Autism spectrum disorder (ASD) is characterized by impaired social interaction, problems with verbal and nonverbal communication, and repetitive behaviors. Currently at least one of 68 children in the US has ASD (Zablotsky et al. 2015; Christensen et al. 2016). In the Gulf States, the prevalence of ASD ranges from 1.4 to 29 per 10,000 persons, therefore still representing a great health concern (Salhia et al. 2014). Diagnosis of ASD may appear problematic, especially early in the child's life. According to recent studies, the average age at diagnosis of ASD in the US is 53 months (ADDM Network 2014). Earlier diagnosis appears to improve the prognosis at least in higher functioning children (Anderson et al. 2014). In this perspective, clinically useful biomarkers are desperately needed, as they may improve ASD diagnosis at the earliest, although few molecules can be suitable for the purpose, such as the biomarkers of the glutamate excitotoxicity and oxidation found in ASD (El-Ansary 2016). Several authors have proposed various biomarkers of increased oxidative stress and impaired methylation capacity, such as methionine, homocysteine, cystathionine, cysteine, total glutathione, S-adenosyl-L-methionine, adenosine, and oxidized glutathione (James et al. 2004; James 2013; Hodgson et al. 2014). El-Ansary et al. (2016) used libraries of biomarkers to predict sensory abnormities in ASD accurately. In one study, all the 30 children with ASD undergoing the investigation, showed increased urinary kininogen-1 levels, when compared to neurotypical controls ($p < 0.001$). Interleukin-6 and serotonin have also been proposed as biomarkers with enough sensitivity and specificity to be useful, but sample size and failure of replication limited their clinical usefulness (Yang et al. 2015). Several other studies that have looked at biomarkers in ASD have also had too small sample sizes to determine specificity and sensitivity accurately (El-Ansary et al. 2011a; Ming et al. 2012; Emond et al. 2013; Kuwabara et al. 2013). Yusuf and Elsabbagh (2015) warned against the use of biomarkers to diagnose ASD due to the lack of adequate sensitivity and specificity in the current literature, so refreshing a concern still occurring in the research on autism pathology. ASD, which is regarded as a multifactorial disorder, is yet heterogeneous in its presentation. Therefore, it is unlikely that a single biomarker would be helpful to diagnose the disorder. A panel of biomarkers for ASD would have to be found that have enough specificity and sensitivity to be clinically useful (Pelphrey 2017).

Existing research indicates that vitamin D plays a crucial role in human health beyond the regulation of calcium homeostasis and bone mineralization (Holick 2007; Bjørklund 2016; Chirumbolo et al. 2017). Vitamin D is produced endogenously in the skin when ultraviolet light triggers its synthesis. For most people in the world, about 90% of the vitamin D is produced in this way, and about 10% is coming from food and dietary supplements (Holick et al. 2011; Mahmoud and Ali 2014). However, it is important for people living in high latitudes to get enough vitamin D from the diet. In Norway, the primary vitamin D sources in the diet are fatty fish, fortified butter and margarine (Løken-Amsrud et al. 2012), and supplementation with cod liver oil (Saltytė Benth et al. 2012). Vitamin D fortified milk and infant formula contain significant amounts of vitamin D for infants, but it is not clear that even 400 IU/day (10 μg) is considered as an adequate amount (Gross et al. 2013).

Whatever the source, vitamin D is biologically inert in the human body and needs two hydroxylations to be activated. The first of these is a liver hydroxylation that converts 25-hydroxyvitamin D ($25(OH)D_3$, calcidiol). This is the primary circulating form of vitamin D. Next, the metabolite $25(OH)D_3$ is hydroxylated in the kidneys and by some other body cells to 1,25-dihydroxy vitamin D ($1,25(OH)_2D_3$, calcitriol), which is a secosteroid hormone. A CYP enzyme (CYP27B1) catalyzes the hydroxylation of $25(OH)D_3$ to its active hormonal form (calcitriol), and another two CYP enzymes (CYP2R1 and CYP24A1) metabolize vitamin D_3 to $25(OH)D_3$ (Schuster 2011). The half-life of $25(OH)D_3$ varies, but the half-life is relatively long. Its half-life has been found to be 2–3 weeks when in both a high ultraviolet B atmosphere (Gambia) and a low one (the UK) (Jones et al. 2015; Holick 2006).

Plasma vitamin D metabolites are mostly transported by vitamin D-binding protein (DBP), also known as group-specific component (GC). Albumin is the secondary vitamin D binding protein, especially in patients with a low DBP concentration (Bikle et al. 1985; Currenti 2010). In addition to vitamin D transport, DBP is an essential glycoprotein macrophage-activating factor (GcMAF). It also scavenges actin and has fatty acid binding properties (Carpenter et al. 2013). Genotypes of DBP have been found to correlate with differences in plasma DBP and $25(OH)D_3$ levels (Chun et al. 2016). An interaction between vitamin D status and some maternal variants of DBP (GC) as well as birth weight (Delanghe et al. 2015) has been reported. A high metabolic activity of GcMAF has been demonstrated in the blood of patients with ASD (Siniscalco et al. 2014).

Research indicates that vitamin D protects cells by up-regulating DNA-repair genes that stabilize the genome and helps in the protection against oxidative stress and toxic effects (Gonzalez-Suarez et al. 2011; Graziano et al. 2016). The most common genetic finding reported in ASD is a wide-

spreading of de novo mutations, the location of which varies from child to child and does not allow a full elucidation of autism genomics. Evidence showed that vitamin D prevents and repairs these small mutations (Kinney et al. 2010). Interestingly, vitamin D is also involved in the glutathione cycle through its upregulation of γ-glutamyl transpeptidase, which is the rate-limiting enzyme in the glutathione metabolic pathway (Garcion et al. 1996, 2002). Many children with ASD have low glutathione levels (Ghanizadeh et al. 2012). The nuclear activity of vitamin D depends on the interaction with vitamin D receptor (VDR).

There are vitamin D receptors in most areas of the brain. Vitamin D appears to protect neurons and promote neural growth (Ali et al. 2016). Vitamin D deficiency has been associated with cognitive pathology, including ASD (Cherniack and Troen 2011; Mazahery et al. 2016). It has been hypothesized that gestational and early childhood vitamin D deficiency might be a cause of ASD (Cannell 2008). Further, it has been suggested that high-dose vitamin D treatment has beneficial effects in ASD subjects (Cannell 2013; Saad et al. 2016a).

Vitamin D deficiency during pregnancy is widespread and results in infants who have 33–50% less vitamin D than their mothers (Wegienka et al. 2016). In a mostly white cohort in Iowa, 70% of 4-month-old breastfed infants had mean $25(OH)D_3 < 12$ ng/ml (ideal range 40–60 ng/ml) (Dawodu and Wagner 2012). The prevalence of levels <15 ng/ml in the infants was 50% in the summer and 79% in the winter periods of the year. Fifty-seven percent of the infants who were followed for six months still had vitamin D deficiency. In a study from Cincinnati, 18% of exclusively breastfed infants aged 1 month had vitamin D levels <10 ng/ml (in the rachitic range); 76% of the infants and 17% of their mothers had serum $25(OH)D_3 < 20$ ng/ml (Dawodu et al. 2014). The recommended range of vitamin D by the Endocrine Society is 40–60 ng/ml (Holick et al. 2011). In Boston, 58.0% of newborns and 35.8% of the mothers had $25(OH)D_3 < 20$ ng/mL and 38.0% of the infants and 23.1% of the mothers had $25(OH)D_3 < 15$ ng/mL.

If vitamin D deficiency were one of the causes of ASD, then ASD would be common in children with rickets. This has first relatively recently been studied. Zaky et al. (2015) reported that 25% of children with rickets also have ASD. A recent meta-analysis of 11 studies found significantly lower $25(OH)D_3$ levels measured in ASD children compared to neurotypical controls (Wang et al. 2016). Several studies have reported an inverse correlation between the levels of vitamin D and autism severity measured by standard rating scales (Mostafa and Al-Ayadhi 2012; Gong et al. 2014; Du et al. 2015). Vitamin D deficiency may in some children with ASD contribute to the production of serum anti-MAG autoantibodies (Mostafa and Al-Ayadhi 2012).

Furthermore, one study showed that siblings with ASD had lower $25(OH)D_3$ levels at birth (Fernell et al. 2015). A Swedish population study ($N = 509{,}639$) found an association between diagnosed lifetime maternal vitamin D deficiency (< 10 ng/ml) and risk of ASD, especially of ASD with intellectual disability (Magnusson et al. 2016). In another population study ($n = 4229$), Australian women with low vitamin D levels (< 10 ng/ml) in mid-gestation had at birth twice as much risk to bear a child with autistic traits compared to women with vitamin D levels >20 ng/ml (Vinkhuyzen et al. 2016). Chinese researchers investigated 68 children diagnosed with ASD, and 68 sex and age-matched neurotypical children (Chen et al. 2016). They found that women with the lowest quartile of vitamin D levels in their first trimester were four times more likely to give birth to a child with ASD. In the same study, first-trimester vitamin D levels were associated with autism severity ($R = -0.302$; $P = 0.001$) (Chen et al. 2016).

One of the most frequent findings in ASD is a widespread de-novo genetic damage (Kinney et al. 2010). DNA damage is a critical cause of cellular dysfunction and cell death. For example, 8-hydroxydeoxyguanosine (8-OH-dG) is the product of free radical attack to DNA-bound guanosine. It is the most abundant product of cellular DNA oxidation (Helbock et al. 1999) and is a potent mutagen (Calderón-Garcidueñas et al. 1999) and hence a marker for oxidative damage from DNA. As indicators that ASD might be an inflammatory disorder, reduced glutathione, increased malondialdehyde (a biomarker for oxidative stress), and higher 8OHdG may be found in ASD children compared with neurotypical controls (Sajdel-Sulkowska et al. 2008; Sajdel-Sulkowska et al. 2009).

Sajdel-Sulkowska et al. (2009) reported an increase in 8-OH-dG in cerebella of ASD individuals, a finding that later was confirmed by Rose et al. (2012). However, two more recent studies failed to find elevated 8-OH-dG in individuals with ASD (Ming et al. 2005; Yui et al. 2016). Chauhan et al. (2004) found remarkably increased urinary levels of 8-OH-dG in ASD patients. Also, children with brain damage have been found to have increased urinary 8-OH-dG levels (Fukuda et al. 2008). In a randomized controlled clinical trial, even the small dose of 800 IU/day of vitamin D was enough to reduce 8-OH-dG by 25% (Fedirko et al. 2010).

The cytochrome CYP-450 (CYP) family of enzymes is responsible for anabolism and catabolism of both exogenous and endogenous materials (Nebert and Russell 2002). Studies have shown that increased CYP1B1 plays a significant role in oxidative stress (Dey 2013). Increased CYP1B1 is associated with increased oxidative stress, mainly in alcoholic liver disease. People who are co-exposed to toluene and tobacco smoke are due to a possible CYP1B1 repression at higher risk of oxidative stress (Jiménez-Garza et al. 2015). Also, CYP1B1 gene mutation is associated with the abnormal social-emotional behavior as autistic phenotype (Chakrabarti

et al. 2009). Moreover, abnormal CYP1B1 is correlated with altered lipid metabolism repeatedly reported in ASD patients (Lin et al. 2015).

High-sensitivity C-reactive protein (hs-CRP) is a highly conserved plasma protein that participates in the systemic response to some forms of inflammation. Some, but not all, studies have found elevated hs-CRP in children with ASD as a marker of inflammation, which is thought to be a mechanism involved in the neuropathology of ASD (Singh 2005). Mellenthin et al. (2014) reported that different inflammatory biomarkers are differently associated with 25(OH)D_3. They also found an interesting U-shaped association between 25(OH)D_3 and hs-CRP (Mellenthin et al. 2014).

It was hypothesized that serum 25(OH)D_3 might be related to cytochrome P450 1B1 (CYP1B1), high-sensitivity C-reactive protein (hs-CRP), and 8-OH-dG in Saudi patients with ASD compared to neurotypical controls. If a relationship were found, this could help to better understand the etiological mechanism and the possible role of vitamin D in the control of oxidative stress and inflammation in ASD. In the present study, it was expected to find significantly elevated levels of hs-CRP, CYP1B1, and 8-OH-dG, as well as a decreased level of 25(OH)D3 in individuals with ASD, compared to neurotypical controls.

Materials and methods

Subjects

The present cross-sectional study was performed on 28 male children with ASD. The patients were recruited from the Autism Research and Treatment Center at the Faculty of Medicine, King Saud University in Riyadh, Saudi Arabia. Of these individuals, 18 were verbal, and 10 were nonverbal. Their ages ranged between 3 and 12 years (mean SD = 7.0 ± 2.34 years). The patients were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR) criteria (APA 2000). The control group comprised 27 age- and sex-matched neurotypical children with a mean age of 7.2 ± 2.14 years. The controls were normally developing, neurotypical children, unrelated to the ASD subjects. The control children were neurotypical older siblings of healthy infants who were attending the Well Baby Clinic at King Khalid University Hospital for a routine check-up of their growth parameters. They had no clinical indications of infectious disease or neuropsychiatric disorders. Participants were excluded from the study if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. All the participants had normal results for urine analysis and sedimentation rate. Written consent was obtained from the parents of each subject, and the Ethics Committee at King Khalid Hospital, King Saud University approved the study.

Evaluation of autism severity

In the present study, the Childhood Autism Rating Scale (CARS) and the Social Responsiveness Scale (SRS) were used to measure autism severity. CARS rates the child on a scale from one to four in each of 15 areas relating to people (emotional response; imitation; body use; object use; listening response; fear or nervousness; verbal communication; non-verbal communication; activity level; level and reliability of intellectual response; adaptation to change; visual response; taste, smell and touch response; and general impressions). Children who score 30–36 on the CARS scale have mild to moderate autism, while those with scores ranging between 37 and 60 points have severe autism (Mick 2005). On the SRS, a total score of 76 or higher is considered very severe autism and strongly associated with a clinical diagnosis of autistic disorder. A score of 60–75 is interpreted as mild to moderate range of social impairment (Constantino et al. 2003).

Blood sampling and processing

After an overnight fast, blood samples were collected in the morning from both groups. The samples were taken from the antecubital vein in 10 mL tubes containing sodium heparin. The tubes were at room temperature and centrifuged at 3500 rpm for 15 min within the first 4 h following the blood withdrawal. Plasma and red blood cells were obtained and eventually stored at −80 °C until analysis.

Biochemical analysis

The plasma concentration of vitamin D_3, 25(OH)D_3, was measured by a commercially available HPLC kit (Eagle Biosciences, Nashua, NH, USA) according to the manufacturer's instructions. 400 μL sample, calibration or control, was mixed with another 400-μL ice-cold internal standard, and briefly mixed. The sample was then mixed with 500 μL precipitation reagent (PREC) and centrifuged at 10.000 rpm for 5 min. For HPLC injection 50 μl of the upper liquid layer was used. The HPLC separation uses "reversed phase" (IC3401rp) column at 30 °C. Chromatograms are produced by UV-detector. The separation took 15 min. A plasma calibrator (Eagle Biosciences, Nashua, NH, USA) was used to quantify the results. The internal standard method was used to calculate the concentration of 25(OH)D_3 via integration of peak and height areas, respectively. The results were expressed as ng/mL. Vitamin D deficiency was defined as serum 25(OH)D_3 levels <20 ng/mL and insufficiency at serum levels <30 ng/mL; knowing that the Endocrine Society recommends levels between 40 and 60 ng/ml (Holick et al. 2011).

Assay of cytochrome P450 1B1

Quantitative detection of cytochrome P450 1B1 (CYP1B1) in plasma was done with an ELISA kit (Biomatik Corporation, Cambridge, Ontario, Canada). The microtiter plate provided in this kit is pre-coated with an antibody specific to CYP1B1. Standards or samples are added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for CYP1B1. Next, avidin-conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. After 3,3′,5,5′ tetramethylbenzidine (TMB) substrate solution is added, only those wells that contained CYP1B1, biotin-conjugated antibody, and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution, and the color change is measured spectrophotometrically via wavelength of 450 nm ± 10 nm. The concentration of CYP1B1 in the samples is then determined by comparing the optical density of the samples to the standard curve. Detection range is 0.156–10 ng/mL.

Assay of high-sensitivity C-reactive protein

High-sensitivity C-reactive protein (hs-CRP) was measured using a solid phase direct sandwich diagnostic kit (GenWay Biotech, San Diego, CA), according to the manufacturer's instruction. The samples and anti-CRP-HRP conjugate were added to the wells coated with monoclonal antibody (mAb) to CRP. Hs-CRP in the patient's plasma sample binds to anti-CRP mAb on the well and the anti-hs-CRP second antibody then binds to hs-CRP. Unbound protein and HRP conjugate are washed off with wash buffer. Upon the addition of the substrate, the intensity of the color is proportional to the concentration of hs-CRP in the sample. A standard curve is prepared relating color intensity to the level of the hs-CRP with hs-CRP undetectable under 0.3 mg/L.

Assay of 8-hydroxy-2′-deoxyguanosine

8-Hydroxy-2′-deoxyguanosine (8-OH-dG) was measured in blood plasma from the patients with ASD and the neurotypical controls using the OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA, USA). The unknown 8-OH-dG samples or 8-OH-dG standards were first added to an 8-OH-dG/bovine serum albumin conjugate preabsorbed microplate. After a brief incubation, an anti-8-OH-dG mAb was added, followed by an HRP conjugated secondary antibody. The 8-OH-dG content in unknown samples was determined by comparison with the predetermined 8-OH-dG standard curve. Normal 8-OH-dG levels are 4.13 ± 1.01 ng/ml.

Statistical analysis

Data were analyzed using IBM SPSS 16 statistical software. The results were expressed as mean ± SD, and all statistical comparisons were made using an independent *t*-test with $P \leq 0.05$ considered as significant, through a Wilcoxon-Mann Whitney test when data were not normally distributed (Shapiro-Wilk's test negative). For normal distribution data were elaborated with an ANOVA (Tukey's post hoc test). The utility diagrams of the measured parameters were drawn with the X-axis representing percentile rank of the biomarker, the Y-axis representing the probability of identifying the disease, and the horizontal line the prevalence of the disease. Multiple regression analysis was also performed using vitamin D as a dependent variable and CRP, CYP1B1 and 8-OH-dG as independent variables. Qualitative variables were elaborated using covariance analysis (Marusteri and Bacarea 2010). Predictiveness curves for the risk models were calculated on IBM SPSS 16 and based on existing literature (Pepe et al. 2008; Huang et al. 2007; Vickers and Elkin 2006).

Results

Table 1 shows that when autism was described as mild or severe in CARS but not in SRS, the suggested markers, i.e., vitamin 25(OH)D_3, hs-CRP, CYP1B1 and 8-OH-dG indicated a significant marked difference with respect to neurotypical children (control) (Table 1). In severe autistic subjects, as emerging from CARS, 25(OH)D_3 plasma levels were 57.45% lower than controls, hs-CRP higher of about 34%, CYP1B1 decreased as expected (about 70%) and 8-OH-dG increased by about 2-fold (Table 1). In SRS autistic individuals such differences were not significant. As emerging from results shown in Table 1, the marker 8-OH-dG resulted as the more stable in effect either in mild, moderate or in severe ASD, both for CARS and SRS. In order to select the predictive variable in the series of parameters considered in Table 1, we fitted different regression models by using an SPSS software using a stepwise regression procedure, in the form of sequences of F test. Table 2 shows that 8-OH-dG resulted in the best independent marker of ASD resulting from the stepwise regression analysis on vitamin D deficiency as the dependent variable. The frequency distribution of 25(OH)D_3, in the 28 ASD pediatric subjects matched to the 27 neurotypical children is reported in Fig. 1.

It can be easily observed that all control subjects demonstrate vitamin D levels higher than the known average normal level [56 ng/ml] (Holick et al. 2011), while, in ASD patients, 10/28 demonstrate an insufficient level while 18/28 demonstrate significantly lower levels than those the normal values [56 ng/ml].

Table 1 Mean and standard deviation of plasma levels of 25-(OH)D_3, hs-CRP, CYP1B1and 8-OH-dG in blood samples drawn from 28 boys with ASD compared to 27 age and sex matched neurotypical children. Data were elaborated following an ANOVA test (Tukey's post hoc test)

Parameters	Group	N	Mean ± S.D.	Percent Change	Two-tailed *P*-value t-test
25(OH)D_3 (ng/ml)	Control	27	140.43 ± 17.68	100.00	0.001
	Autistic patients	28	95.63 ± 26.63	68.10	3.3901
	Autism (mild to moderate in CARS)	7	100.61 ± 24.05	71.64	0.046
	Autism (severe in CARS)	21	80.68 ± 22.27	57.45	2.0402
	Autism (mild to moderate in SRS)	12	95.32 ± 30.79	67.88	0.777
	Autism (severe in SRS)	11	98.68 ± 24.52	70.27	0.28400
hs-CRP (mg/l)	Control	27	0.59 ± 0.09	100.00	0.001
	Autistic patients	28	0.79 ± 0.09	133.90	3.4103
	Autism (mild to moderate in CARS)	7	0.78 ± 0.12	132.20	0.745
	Autism (severe in CARS)	21	0.79 ± 0.08	133.90	0.3267
	Autism (mild to moderate in SRS)	12	0.81 ± 0.10	137.29	0.323
	Autism (severe in SRS)	11	0.78 ± 0.08	132.20	0.9967
CYP1B1 (ng/ml)	Control	27	0.69 ± 0.07	100.00	0.001
	Autistic patients	28	0.48 ± 0.08	69.57	3.4530
	Autism (mild to moderate in CARS)	7	0.56 ± 0.09	81.16	0.003
	Autism (severe in CARS)	21	0.46 ± 0.07	66.67	2.2302
	Autism (mild to moderate in SRS)	12	0.47 ± 0.06	68.12	0.980
	Autism (severe in SRS)	11	0.47 ± 0.08	68.12	0.0201
8-OH-dG (ng/ml)	Control	27	4.13 ± 1.01	100.00	0.001
	Autistic patients	28	8.17 ± 1.04	197.82	3.3921
	Autism (mild to moderate in CARS)	7	8.75 ± 0.84	211.86	0.090
	Autism (severe in CARS)	21	7.98 ± 1.05	193.22	1.7343
	Autism (mild to moderate in SRS)	12	8.00 ± 1.04	193.70	0.393
	Autism (severe in SRS)	11	8.37 ± 0.97	202.66	0.9931

Figure 2 shows the frequency distribution of the markers analyzed in the study. Regarding mild-moderate and severe sub-grouping, it can be observed that overlapping was absent in the case of vitamin D and CYP1B1 but was observed in case of hs-CRP and 8-OH-dG (Fig. 2). If we consider a defined marker for the predictiveness of a binary outcome, such as, e.g., serum concentration of 25(OH)D_3, in order to be adapted to calculate the risk of finding ASD in investigated children in a diagnostic approach, we must calculate a predictiveness curve of the risk model. When we calculated the predictiveness curves of each marker, any marker appeared to show the ability to predict risk in a quite comparable way (Fig. 3). The correlation plots also showed that hs-CRP was positively correlated with 8-OH-dG and negatively correlated with 25(OH)D_3 and CYP1B1, while, on the other hand, 25(OH)D_3 was positively correlated with CYP1B1 and negatively correlated with hs-CRP and 8-OH-dG as markers of neuroinflammation and oxidative stress respectively (Fig. 4).

Discussion

To achieve better outcomes for children with ASD, it is of critical importance to diagnose the disorder as early as possible. In the first year of life, many infants show both biological and behavioral signs that something is abnormal. For other children with ASD, the autistic symptoms seem to be

Table 2 Stepwise regression analysis in which vitamin D was used as the dependent variable against the three other measured parameters as independent markers, demonstrating that 8-OH-dG was the most contributed marker to 25(OH)D_3 deficiency

Predictor variable	Beta	*P* value	Adjusted R square	Modelt	
				F value	P value
8-OH-dG	−0.543	0.001	0.288	43.473	0.001

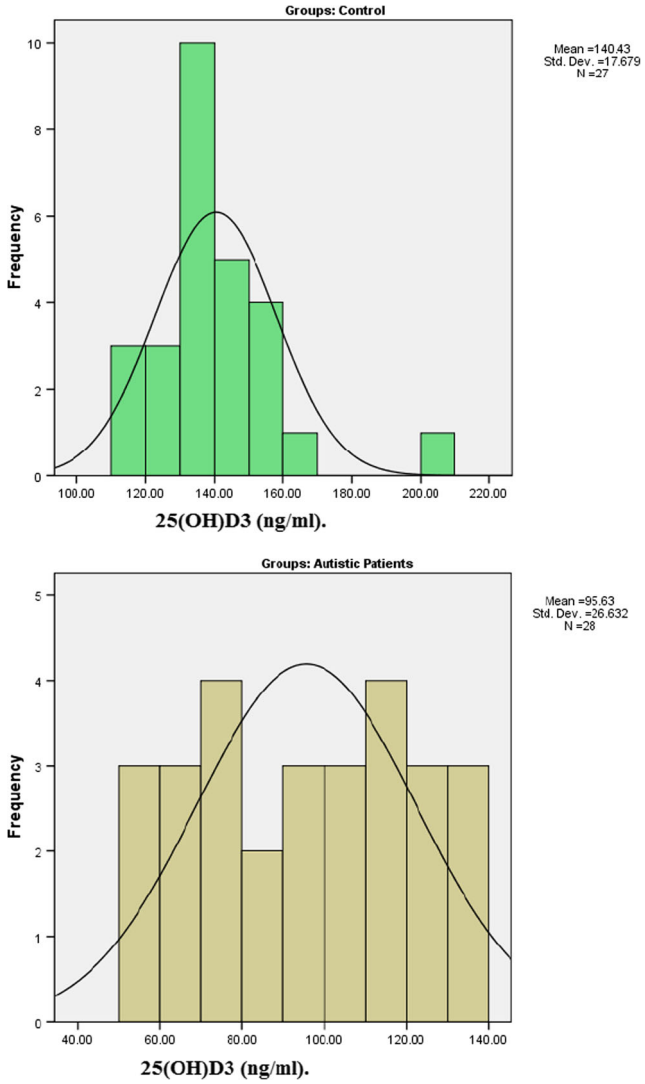

Fig. 1 Frequency histograms of the plasma calcidiol (25(OH)D_3) in neurotypical controls (panel **a**) and children with ASD (panel **b**). Distributions were plotted according to the standard level of reference. Lognormality curves were fitted on the histograms (black curve) and used to calculate data in a goodness of fitness way (Anderson test and Shapiro-Wilk test positive, $p < 0.01$). Data from 28 boys with ASD and 27 age and sex matched neurotypical children. A line designates the mean value for each group. It shows the normal distribution of 25(OH)D_3 in control and ASD patients. It can be easily observed that 21/27 of control subjects demonstrate vitamin D levels higher than the average mean (56 ng/ml), while, in ASD patients, 10/28 demonstrate an insufficient level while 18/28 demonstrate significantly lower levels than those of control subjects (i.e., less than 56 ng/ml)

clinically absent during infancy, but they regress by age two **(**Werner and Dawson 2005**)**. Psychological assessments designed to detect behavioral signs of children as young as 12 months have shown promise. Examples of this are the interactive measure Autism Observation Scale for Infants (Stone et al. 2004; Bryson et al. 2008), and the First Year Inventory, which is a questionnaire for a parent (Baranek et al. 2003; Watson et al. 2007; Turner-Brown et al. 2013). However, additional research is needed to assess the clinical utility of these diagnostic instruments.

As already mentioned, 25(OH)D_3 levels between 40 and 60 ng/mL is recommended by the Endocrine Society (Holick et al. 2011). In the present study, some of the study subjects, even some of the children with ASD, showed concentrations as high as 40 ng/ml. However, the normal ranges of vitamin D vary substantially between different laboratories (Lai et al. 2012). Therefore, it is best to think of them relatively, not categorically. Nevertheless, the patients with ASD in the present study had much lower 25(OH)D_3 levels compared to age and gender-matched neurotypical controls. This finding is in agreement with previous studies (Mostafa and Al-Ayadhi 2012; Wang et al. 2016).

The present study found a barely significant association between vitamin D levels and autism severity measured by CARS ($P = 0.046$). The observation that the levels of

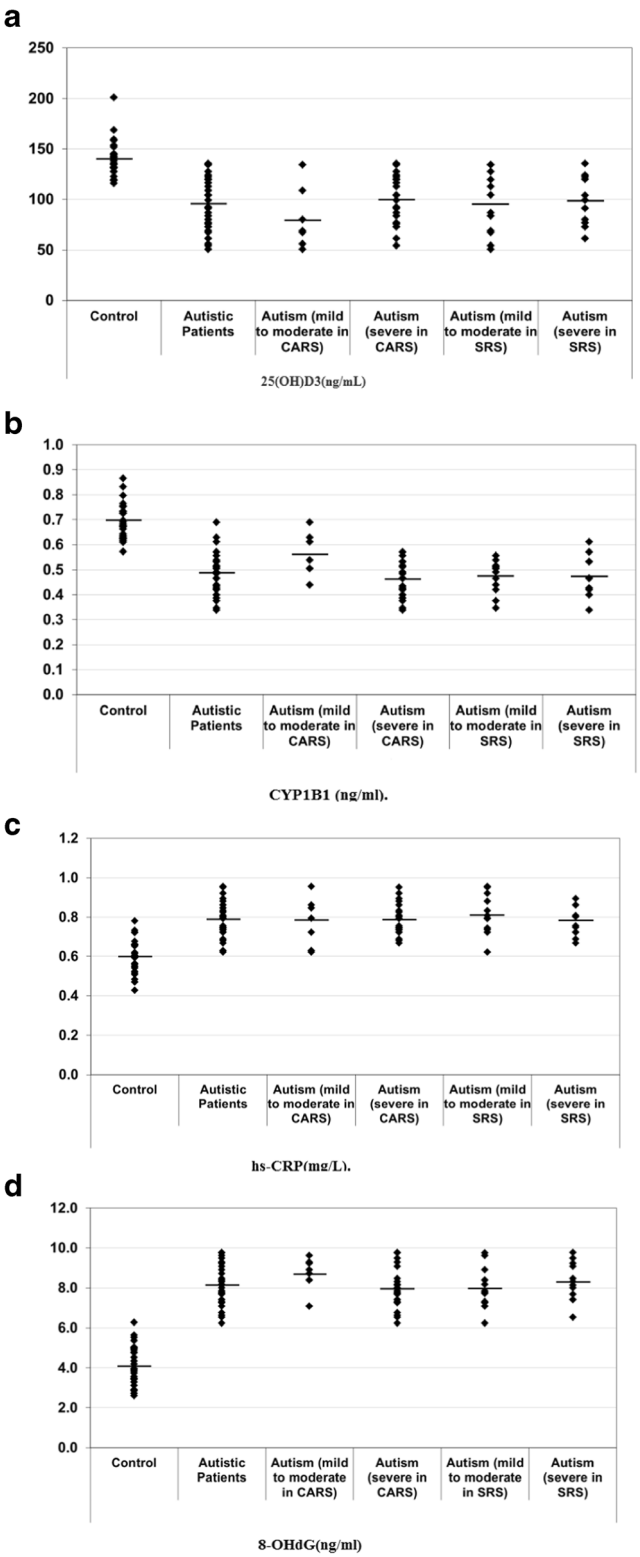

Fig. 2 **a-d** Scatter plot points graphs of 25(OH)D_3, CYP1B1, hs-CRP, and 8-OH-dG. It demonstrates individual data distribution around the mean value represented by a straight line for the four parameters. The absence of overlapping between 28 boys with ASD and 27 age- and sex-matched neurotypical children in the four measured parameters is noted. Regarding mild-moderate and severe sub-grouping, it can be observed that overlapping was absent in the case of vitamin D and CYP1B1 but was observed in case of hs-CRP and 8-OH-dG

25(OH)D_3 are inversely related to autism severity is supported by several studies (Mostafa and Al-Ayadhi 2012; Gong et al. 2014; Chen et al. 2016; Saad et al. 2016b). Vanlint and Nugent (2006) reported a significant relationship between vitamin D

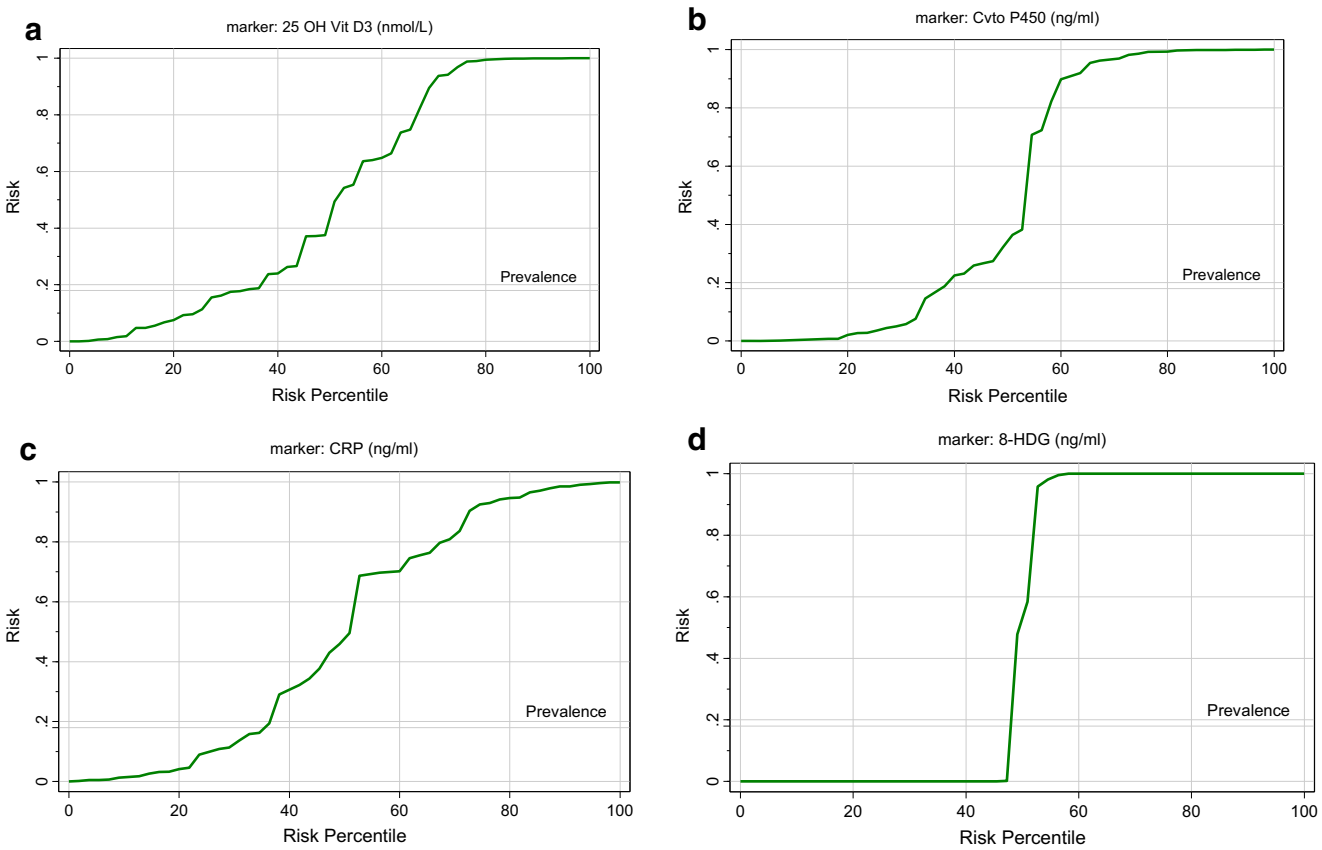

Fig. 3 Predictiveness curves of the four measured parameters as an assessment of the performance of 25(OH)D_3 CYP1B1, CRP and 8-OH-dG in regards to ASD prevalence in a Saudi population of autistic males

deficiency and intellectual disabilities in 337 individuals with cognitive impairments, even after control of the sun exposure.

Researchers in Sweden have shown that estrogen makes female brains more responsive to neurohormonal growth-stimulating effects of calcitriol. This suggests that estrogen enhances the beneficial effects of vitamin D on the brain (Fan et al. 2006). At the same time, testosterone significantly inhibits CYP27B1 (the gene that metabolizes 25(OH)D_3 into a steroid hormone) while stimulating CYP24A1 (the gene responsible for catabolizing vitamin D to be excreted in the bile)(Olmos-Ortiz et al. 2016).

As estrogen potentiates the effect of vitamin D, and testosterone retards it, the differences in the levels of sex steroids during early brain development may show protective effects of estrogen on developing female brains due to vitamin D deficiency, while testosterone cannot protect ASD males as Cannell (2008) first hypothesized.

The present study indicates that vitamin D may play a role in ASD. About 50% children with ASD have elevated levels of serum peripheral serotonin but low central serotonin (Coutinho et al. 2004; Patrick and Ames 2014). Vitamin D in the brain has been shown to downregulate the transcription of the serotonin-synthesizing gene tryptophan hydroxylase (TPH) 2 (Patrick and Ames 2014). However, vitamin D upregulates TPH1 in tissues outside the blood-brain barrier. According to this mechanism, the lower level of vitamin D reported in the present study may be a cause of the significant increase of serotonin seen in the plasma of patients with ASD (El-Ansary et al. 2011b). Vitamin D may also be related to the remarkable alteration of oxytocin and vasopressin that has been reported in patients with ASD (Xu et al. 2013). Genes that encode the oxytocin-neurophysin 1 preproprotein are upregulated by vitamin D. Both oxytocin and vasopressin receptor genes are to some extent regulated by vitamin D (Olmos-Ortiz et al. 2016).

In the present study, elevated levels of hs-CRP in patients with ASD were found compared to neurotypical controls (see also Table 1). This is consistent with at least one previous study, which demonstrated that hs-CRP might be a biomarker for ASD (Khakzad et al. 2012).

The reported insufficiency of vitamin D in ASD could also be related to the significant elevations of alpha-N-acetylgalactosaminidase (Nagalase) as a marker of secondary immune dysregulation in ASD patients compared to controls (Griffin et al. 2003). Much higher Nagalase activity inhibits, through a deglycosylation reaction, the macrophage activation of vitamin D binding protein (DBP), and suppresses the immune response. The impact of Nagalase on VDBP

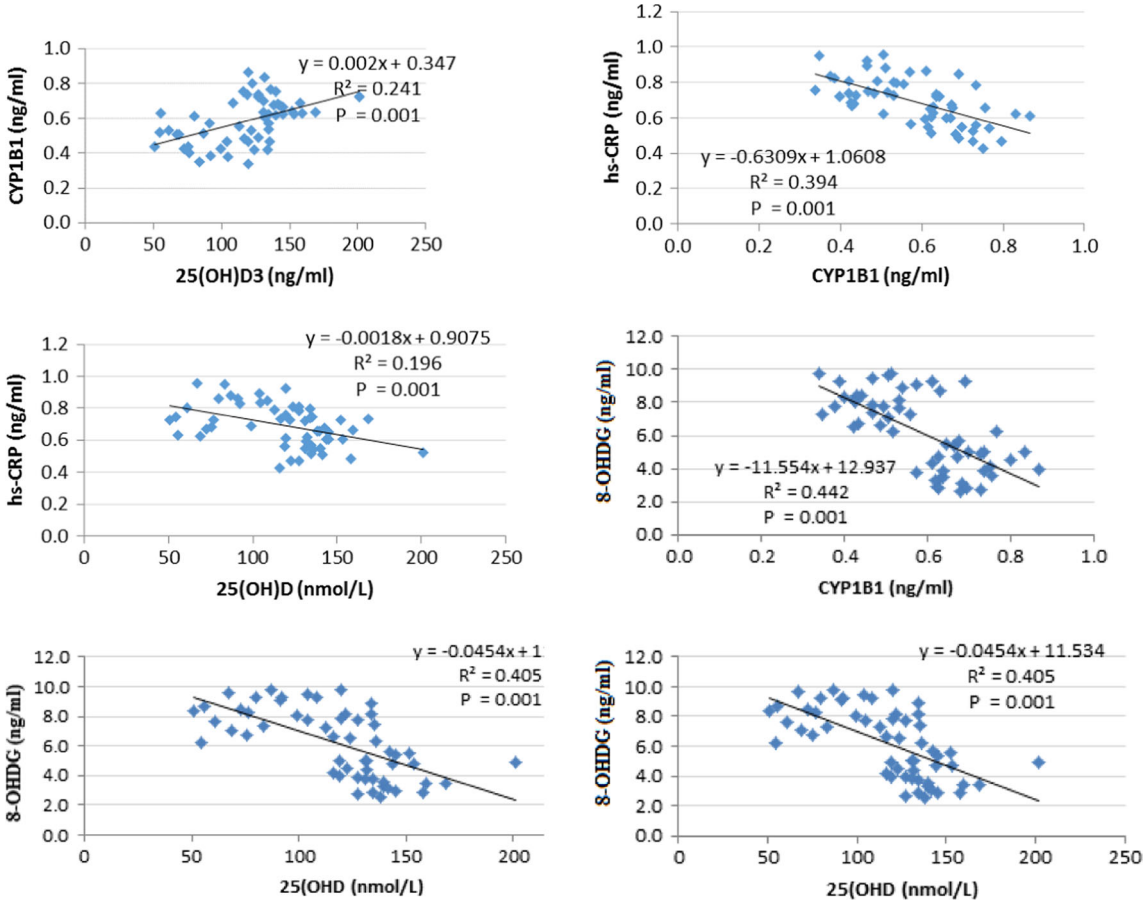

Fig. 4 These plots demonstrate the correlation between four measured parameters in 28 boys with ASD and 27 age and sex matched neurotypical children. Hs-CRP was positively correlated with 8-OH-dG and negatively correlated with 25(OH)D_3 and CYP1B1. On the other hand, 25(OH)D_3 was positively correlated with CYP1B1 and negatively correlated with CRP and 8-OH-dG as markers of neuroinflamation and oxidative stress respectively. Statistics obtained from rank groups with a Wilcoxon-Mann Whitney test. (Kolmogorov-Smirnov indicated that data were nonparametric)

transportation of vitamin D is not understood. However, the contribution of vitamin D insufficiency to autoimmunity suggests a relationship between elevated Nagalase, GC-MAF dysfunction, and vitamin D insufficiency in ASD individuals. Therefore, Nagalase should be given more attention as a possible diagnostic agent.

It is well known that cytochrome P450 (CYP) enzymes are crucial for the bioactivation of vitamin D in the formation of the secosteroid calcitriol, which requires a 25-hydroxylation followed by a 1α-hydroxylation catalyzed by the liver and kidney cytochrome P450 enzymes (Wikvall 2001). The association between the levels of CYP1B1 and 25(OH)D_3 and autism severity measured by CARS ($P = 0.046$ and 0.003 respectively) may show that vitamin D insufficiency and lower CYP1B1 activity are both predisposing developmental factors. To the best of our knowledge, the present study is the first study that reports a significantly lower level of CYP1B1 in patients with ASD (Table 1). However, the significance of this finding is unclear. CYP1B1 may be epigenetically silenced in ASD. Kouzmenko et al. (2010) hypothesized that epigenetic silencing of CYP27B1, the gene that encodes for calcitriol, may explain one of the mechanisms in which vitamin D influences ASD.

Many toxins have been associated with ASD (Lanphear 2015). Toxins are one class of xenobiotics. Other examples of xenobiotics include drugs, pesticides, cosmetics, flavorings, fragrances, food additives, industrial chemicals, and environmental pollutants. Humans are exposed to thousands of xenobiotics in their lifetimes but seemingly catabolize them. Many researchers are on a quest to find xenobiotics that cause ASD, without apparently considering the health of the body's systems that excrete xenobiotics.

Air pollution dramatically reduces vitamin D produced from the UVB in sunlight (Agarwal et al. 2002; Windham et al. 2006; Vieira 2015) and has been suggested as a cause of ASD (Hosseinpanah et al. 2010; Baïz et al. 2012; Kelishadi et al. 2014). If air pollution caused ASD, would not the autism epidemic in the USA have occurred during the 1950s and 60s when the air pollution was much worse? Perhaps the difference is that "in the day" we were weaned on vitamin D

enriched cow's milk and played in the sun. Toddlers today are protected from sunlight (Gies et al. 2013) and are often weaned on unfortified fruit juice (Walker et al. 2006).

The human body gets rid of xenobiotics, including toxins, via the CYP3A4 system. CYP3A4 is the most active gene in the cytochrome P450 system that codes for detoxifying of xenobiotics. CYP3A4 is very active in the brain (Ferguson and Tyndale 2011) and codes for proteins mainly involved in cellular detoxification. CYP3A4 is directly genetically upregulated by vitamin D (Thompson et al. 2002; Wang et al. 2013). However, ASD children with vitamin D deficiency may not be able to upregulate CYP3A4 entirely, and thus their brains are at risk from toxins. Likewise, the body's master antioxidant, glutathione is also directly upregulated by vitamin D (Rose et al. 2012; Jain and Micinski 2013). Glutathione has been shown to be low in autistic cerebellums (Jain and Micinski 2013). The administration of vitamin D significantly increases serum glutathione levels (Zhang et al. 2016). Therefore, impaired defense may contribute to ASD, and not just the toxins.

The neuroinflammation in ASD (El-Ansary and Al-Ayadhi 2012; Bjørklund et al. 2016) may be caused by chronic overproduction of pro-inflammatory cytokines and oxidative stress occurring in genetically susceptible vitamin D deficient children (Mazahery et al. 2016). Very severe inflammation, such as sepsis, significantly depresses the activity of several enzymes including the cytochrome CYP450 family responsible for the catabolism of xenobiotics (Jacob et al. 2009). Also, at least one other study found hs-CRP is elevated in ASD and may be used by clinicians to help diagnose ASD (El-Ansary et al. 2011b). The reported decrease in detoxification capacity in Saudi ASD patients reported by Al-Yafee et al. (2011) may contribute to the etiology of ASD and is consistent with the finding of elevated inflammatory markers in the present study and the severity of autism as measured by the CARS and SRS.

DNA damage is an underlying cause of much cellular dysfunction. If the DNA goes unrepaired, it can drive mutagenesis that disrupts normal gene expression and produces proteins with abnormal functions. 8-OH-dG is the most abundant product of cellular DNA oxidation (Prabhulkar and Li 2010). Table 1 demonstrates the significant increase of 8-OH-dG in ASD patients compared to neurotypical control subjects. Also, multiple regression analysis using $25(OH)D_3$ as the dependent variable (Table 2) demonstrates a correlation between vitamin D deficiency and the significant increase of 8-OH-dG. This may support the recent work of Gordon-Thomson et al. (2012) in which they reported that DNA strand breaks in UV-irradiated human keratinocytes where 8-oxoguanine DNA glycosylase digests increased more than 2-fold but was decreased by calcitriol treatment. An analog of calcitriol significantly reduced 8-OH-dG levels in rats who had a cyclosporine-induced renal injury (Piao et al. 2012).

Based on the relationship between vitamin D deficiency and the increase of DNA damage clinically as evidenced by a significant increase of 8-OH-dG, the remarkable increase in autism prevalence could be related to the prevalence of vitamin D deficiency, which has grown considerably during recent years, perhaps due to sun avoidance, sunscreen use and an indoor lifestyle (Cannell 2017). The use of sunscreen is relatively common in pregnant women, to whom strict avoidance of sun is often recommended for prevention of chloasma (Lakhdar et al. 2007).

The suggested relationship between vitamin D deficiency and elevated 8-OH-dG might also be useful as biomarkers for ASD. Peripheral hyperserotonemia in ASD supports the recent work of Alfawaz et al. (2014) who demonstrated significant protective and restorative effects of vitamin D on low serotonin, interferon-gamma (IFγ) and glutathione-s-transferase (GST) induced by the administration of propionic (PPA) acid to produce a rodent model of autism.

While the early ASD diagnosis is important for the institution of behavioral therapy, a recent randomized controlled trial (RCT), published in *the Journal of Child Psychiatry and Psychology*, found high dose vitamin D (300 IU/KG/day) had a significant treatment effect on the core symptoms of ASD (Saad et al. 2016b). The authors also reported that younger children and those children whose final $25(OH)D_3$ exceeded >40 ng/ml responded best. The finding of this RCT is supported by positive open-label trials (Cannell 2017). If replicated, it would make the early diagnosis of ASD critical, because effective treatment with vitamin D may be possible.

Limitations

The findings of the present study are hindered by a relatively small sample size, and will, therefore, need to be replicated by other groups before clinical utilization. Also, all the subjects in this study were males, so the findings cannot be assumed to be present in females with ASD. The $25(OH)D_3$ levels of both the subjects and controls in the present study were much higher than in other studies of children with ASD. Saudi Arabia is relatively close to the equator and has a sunny climate, which may explain some of the findings of the present study. Also, operator error is a possibility. In addition, as cited above, $25(OH)D_3$ levels vary from lab to lab and should be thought of relatively rather than absolutely.

Conclusions

The current problem with biomarkers is that numerous small studies have found multiple, but different, biomarkers for ASD. Researchers studying biomarkers for ASD need to meet

and collaborate more as it appears that a meta-analysis cannot be done now because such a wide variety of biomarkers have been studied, and so a useful meta-analysis cannot be done.

The present study demonstrates lower vitamin D levels in Saudi children with ASD, which agrees with a recent meta-analysis as mentioned above. We found a significant inverse association between hypovitaminosis D and impaired cognitive development as measured on the CARS. This is compatible with the hypothesis that vitamin D plays a role in the etiology of ASD. We replicated the reports of others in finding that $25(OH)D_3$ and hs-CRP are abnormal in ASD. Two previous studies (see above) found that 8-OH-dG was not elevated in ASD. However, it was elevated in the sample of the present study. As far as we know, we are the first to report that CYP1B1 is reduced in ASD.

Further studies should be conducted with a larger number of participants to confirm these preliminary findings and to advance towards the goal of a set of biomarkers that will lead to earlier ASD diagnosis and intervention.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest with respect to the authorship, and/or publication of this article.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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