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Hyperhomocysteinemia among Omani autistic children: a case-control study

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High serum homocysteine (Hcy) level is regarded as an indicator for impairment of folate-dependent methionine cycle and is associated with oxidative stress. In a case control study, we evaluated eighty 3–5 years old Omani children (40 diagnosed with Autism Spectrum Disorder and 40 their age and gender matched controls) for their fasting serum homocysteine levels as a biomarker of Autism Spectrum Disorder (ASD). Serum folate and vitamin B_{12} status were also evaluated. The serum homocysteine was measured using an enzyme immunoassay (EIA) technique whereas folate and vitamin B_{12} were measured using an automated random access immune-assay system. The results indicated that mean serum Hcy levels were significantly ($P<0.05$) higher in autistic children (20.1 ± 3.3 μmol/L) as compared to controls (9.64 ± 2.1 μmol/L). Significantly ($P<0.05$) lower serum folate (1.8 ± 0.4 μg/L) and vitamin B_{12} (191.1 ± 0.9 pg/mL) levels were observed in autistic children as compared to controls (6.1 ± 0.6 μg/L and 288.9 ± 1.3 pg/mL, respectively). The levels of homocysteine in autistic children were also much higher as compared to normal reference values (5–15 μmol/L). The results suggest that high fasting serum homocysteine and low folate and vitamin B_{12} levels could be used as clinical biomarkers for an early diagnosis and management of ASD.

Keywords: serum homocysteine, folate, vitamin B_{12}, autistic children, Oman

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INTRODUCTION

Autistic spectrum disorder (ASD) is a complex and mysterious neurodevelopmental disorder that appears in the early years of life (Santangelo & Tsatsanis, 2005; Keller & Persico, 2007; Blaylock, 2008). Autistic children are characterized by impaired social interaction and communication and fail to respond to certain stimuli exhibiting some restricted and repetitive behavior or actions (Reading, 2008; Buehler, 2011). The incidence of autism has increased rapidly in recent decades and the pattern of increase is not influenced by maternal age, race/ethnicity or child gender (Weiser *et al*., 2008; Towle *et al*., 2009). The data from the USA and other Western countries indicates the prevalence of autism spectrum disorder of 1 in 110 children (Towle *et al*., 2009; Daniels *et al*., 2011). Very limited published data is available about the prevalence of autism spectrum disorder in Omani children. A latest study has reported a total of 113 cases of ASD in Oman indicating an overall prevalence of only 1.4 cases per 10000 children aged 0–14 years (Al-Farsi *et al*., 2011). The low prevalence has been attributed to under reporting or lack of diagnosis. The parent's report of a child's professional ASD diagnosis has been considered as the most vital piece of information (Daniel *et al*., 2011).

A number of factors such as genetic, environmental, autoimmune function, redox potential, oxidative stress, and inflammatory biomarkers have been implicated in the etiology of ASD (Reading, 2008; Weiser *et al*., 2008) that is, however, still poorly understood. The recent rise in autism cases cannot only be explained from genetic causes and therefore certain environmental factors may likely be associated with ASD (Bernard *et al*., 2002; Adams *et al*., 2009; Blaylock, 2008; 2009a, 2009b; Kern *et al*., 2010). Although metabolic abnormalities have been implicated in the pathogenesis of many neurological disorders, the metabolic pathology of autism has been less explored as compared to broad-scale genomic approaches (Miller, 2003; Muntjjewerff *et al*., 2003). Abnormalities involving the folate-dependent homocysteine methylation reactions, oxidative stress, and genetic predisposition have been implicated as potential causes. It has also been proposed that pro-inflammatory cytokines arising from maternal inflammation, infection and possibly from autoimmunity after passing through the placental and blood-brain barriers may cause aberrant neuronal growth and plasticity within the fetal brain via a "cytokine-storm" (Buehler, 2011). However, the underlying mechanism or a specific metabolic target relevant to ASD has not yet been identified. Clinical biomarkers are of great significance in establishing the diagnosis of many diseases. Identification of the metabolic profile of ASD may therefore represent an effective tool for its early diagnosis and treatment.

Homocysteine (Hcy) is a sulfur-containing amino acid that is known to be implicated in the pathogenesis of many clinical conditions (Newton *et al*., 2010). Homocysteine in the body is derived from demethylation of

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Abbreviations: AD, Alzheimer disease; APA, American Psychiatric Association; ASD, autism spectrum disorder; CARS, childhood autism rating scale; CVD, cardiovascular diseases; DS-IV-TR, Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision; EIA, enzyme immunoassay; Hcy, homocysteine; GSH, glutathione; MTHFR, methylenetetrahydrofolate reductase; MTHFR C677T, methylenetetrahydrofolate reductase allele variant; SOD, superoxide dismutase; SQUH, Sultan Qaboos University Hospital; tHcy, plasma total homocysteine.

exogenous methionine and is metabolized along two pathways, remethylation to methionine or transsulfuration to cysteine (Miller & Kelly, 1996; Miller, 2003; James *et al*., 2004). Folate and vitamins B_6 and B_{12} also play an important role in homocysteine metabolism and any defect in these pathways can lead to accumulation of homocysteine in the body (Herrmann *et al*., 2007). Since one source of Hcy is S-adenosylmethionine, the methyl group donor for DNA-methylation, the use of DNA-methylation based biomarkers may therefore be possible for detection, diagnosis, prediction of response to therapy and prognosis of outcomes for various diseases, including neurodegenerative and psychiatric disorders (Levenson, 2010). The role of homocysteine as a potential and predictive biomarker in age-related neurodegenerative diseases has been well recognized. Increased concentration of total plasma homocysteine is now considered a candidate risk factor for dementia that can predict Alzheimer's disease (AD) or dementia several years before their manifestation (Hermann and Obeid, 2011). Kaluzna-Czaplinska *et al*. (2011a) reported higher levels of homocysteine (2.36 ± 1.24 mmol/mole creatinine) in the urine of autistic children as compared to their age matched healthy children (0.76 ± 0.31 mmol/mole creatinine). Higher levels of homocysteine have also been reported in the serum and plasma of autistic children (James *et al*., 2004; Pasca *et al*., 2006). No such biochemical data is available for normal and autistic children in the Sultanate of Oman. The present study was therefore conducted to compare the serum homocysteine, folate and vitamin B_{12} levels in normal and autistic Omani children.

MATERIALS AND METHODS

Study design. This is a case-control study designed to determine the serum Hcy, folate and vitamin B_{12} levels in normal and autistic Omani children. The study was conducted over the period from December 2009 to August 2010. A total of eighty children (40 confirmed autism spectrum disorder (ASD) cases and 40 their age and gender matched control subjects) participated in this study. Ascertainment of ASD diagnosis was made according to the criteria of the American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) (APA, 2000). All subjects diagnosed with ASD exhibited symptoms within the typical triad of autistic traits: communication impairment, social deficits, and ritualistic interests. Clinical DSM-IV-TR consensus diagnosis was corroborated using the Childhood Autism Rating Scale (CARS). All enrolled cases in this study were newly diagnosed with ASD. This was done to avoid any casual-relationship between the effect of medications on the measured biochemical parameters (Hcy, B_{12} and folate). All ASD cases were examined by a family physician and a child psychiatrist, who were well trained and experienced in clinical management of ASD. The control subjects were randomly selected from the eligible outpatients at the Department of Child Health at Sultan Qaboos University Hospital (SQUH). All the eligible control subjects were defined as children 3–5 years old who were not known to be autistic or to have any other neurodevelopmental or behavioral disturbances that could be related to or confused with ASD. In order to exclude the possibility that the controls could have any sub-clinical autistic features, each control subject was also clinically examined by the family physician and pediatrician. The study was approved by the Medical Research Ethics Committee of Sultan Qaboos University.

Serum homocysteine (Hcy), folate and vitamin B_{12} measurements. Fasting blood samples were collected from both the cases and controls at 8:00 am during their visit to the hospital in the outpatient department. Ten ml of venous blood was collected from the median cubital vein by venipuncture into a plain tube. Following centrifugation, the serum was transferred to an Eppendorf tube and stored at –80 °C prior to Hcy measurements. The serum homocysteine was measured using an enzyme immunoassay (EIA) technique using an Immulite 2000 homocysteine analyzer (Quillard *et al*., 2003). The principle of the procedure is based on competitive immunoassay. Normal fasting serum Hcy reference values reported in the literature are 5–15 μmol/L (Frantzen *et al*., 1998). Serum folate and vitamin B_{12} were measured using an automated random-access immunoassay system (Siemens Medical Solution Diagnostics, ADVIA Centaur Chemistry Analyzer, Bohemia, NY 11716, USA).

Statistical analysis. The data was analyzed statistically using GraphPad Prism statistical software package for personal computers version 5. Results are presented as means ± standard deviation (S.D.). Student's unpaired t-test was used to compare the values in normal and autistic Omani children. The level of statistical significance was set at $P < 0.05$ (Snedecor & Cochran, 1989).

RESULTS AND DISCUSSION

The results on the serum homocysteine levels in both autistic Omani children and their age-matched healthy controls are presented in Fig. 1. The results indicated that the mean serum Hcy levels were significantly ($P < 0.01$) higher in autistic children (20.1 ± 3.3 μmol/L) as compared to their age and gender matched healthy controls (9.64 ± 2.1 μmol/L). The level of serum homocysteine in Omani autistic children observed in this study was also much higher than the reported normal reference values (5–15 μmol/L) (Frantzen *et al*., 1998). The results of the present study are in line with the previously reported data of Pasca *et al*. (2006) who observed significantly higher levels of plasma homocysteine in autistic children (9.83 ± 2.75 μmol/L) as compared to normal healthy children (7.51 ± 0.93 μmol/L). Some other studies have also reported higher levels of homocysteine in the serum and plasma of autistic children (Boris *et al*.,

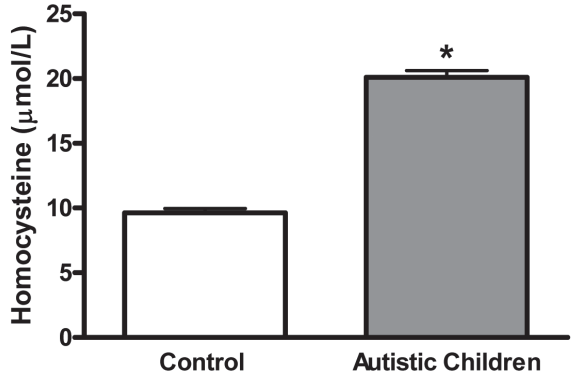

Figure 1. Serum homocysteine levels in control and autistic children.
*Significantly higher in autistic children as compared to controls ($P < 0.05$).

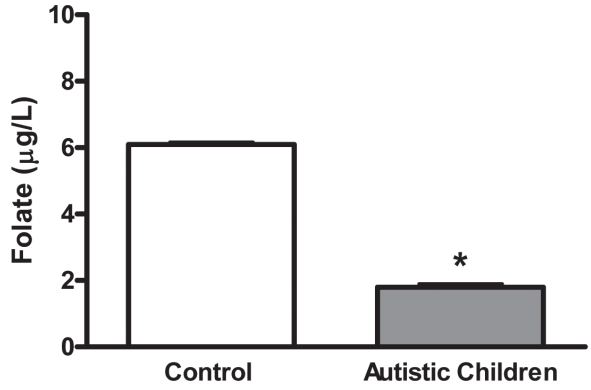

Figure 2. Serum folate levels in autistic children as compared to controls.
*Significantly lower in autistic children as compared to controls ($P < 0.05$).

2004; James *et al*., 2004; Waly *et al*., 2004; Moretti *et al*., 2005), which suggests that hyperhomocysteinemia could be present in children with ASD. Higher levels of homocysteine have also been reported in the urine of autistic children as compared to their age and gender matched healthy controls (Kaluzna-Czaplinska *et al*., 2011a).

It has been suggested that the elevated serum levels of Hcy can be both due to genetic as well as nutritional factors (Dhonukshe-Rutten *et al*., 2009; Wijsman *et al*., 2011). In the body, homocysteine (Hcy) is derived from demethylation of exogenous methionine (Miller & Kelly, 1996; Miller, 2003). Methionine synthase is responsible for recycling the toxic levels of homocysteine into methionine. Methylenetetrahydrofolate reductase (MTHFR) is the central enzyme in folate metabolism and acts at the crossroads between methyl group transfer and biosynthesis of nucleotides. Any reduction in its concentration may affect DNA synthesis (Ryan & Weir, 2001; James *et al*., 2008). Decreased methionine synthase activity and increased frequency of methylenetetrahydrofolate reductase allele variant (MTHFR C677T) can favour increased levels of plasma homocysteine (James *et al*., 2008; Pasca *et al*., 2008). Studies have, however, shown that MTHFR C677T alone is not a risk factor for ASD (Santos *et al*., 2010). High levels of plasma homocysteine and increased oxidative stress have generally been associated in the pathophysiology of many neuropsychiatric disorders

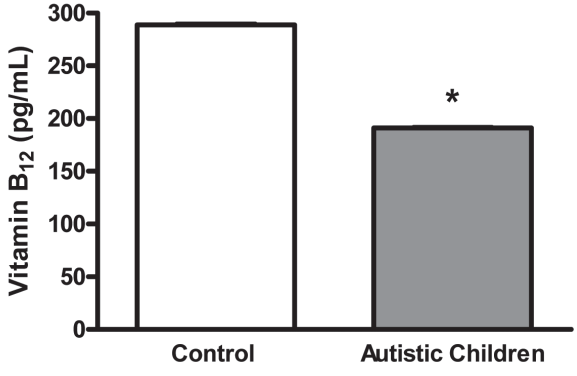

Figure 3. Serum vitamin B12 levels in autistic children as compared to controls.
*Significantly lower in autistic children as compared to controls ($P < 0.05$).

including ASD (Chauhan & Chauhan 2006; Suh *et al*., 2008; Ientile *et al*., 2010; Tu *et al*., 2010). Homocysteine and especially its metabolic products are powerful excito-toxins, which can reduce energy production by mitochondria and may greatly enhance the excito-toxicity of many environmental neurotoxins such as mercury, lead, cadmium, aluminum, fluoride, etc. (Blaylock, 2009a).

The results on the serum folate and vitamin B_{12} levels in Omani autistic and normal children are shown in Figs. 2 and 3. Significantly ($P < 0.05$) lower serum folate (1.8 ± 0.4 μg/L) and vitamin B_{12} (191.1 ± 0.9 pg/mL) levels were observed in autistic children as compared to controls (6.1 ± 0.6 μg/L and 288.9 ± 1.3 pg/mL respectively). The serum concentrations of folate and Vitamin B_{12} in autistic children were much below the values defined as deficient levels (3.0 μg/L and <250 pg/ml respectively) for these nutrients (Shils *et al*., 2006). Our results, however, are not in agreement with the previously reported data by Pasca *et al*. (2008) who did not show any difference in serum folate levels between ASD patients and their control samples. Folate and vitamins B_6 and B_{12} play an important role in the metabolism of homocysteine and any defect in Hcy turnover due to either dietary deficiencies or mal-absorption or inappropriate metabolic utilization of these nutrients can lead to accumulation of homocysteine in the body (James *et al*., 2008; Herrmann *et al*., 2007). It has therefore been hypothesized that higher homocysteine level should not only be considered as a mere marker of vitamin deficiency but also as a risk factor or an indicator of disease (Ientile *et al*., 2010). The use of DNA-methylation-based biomarkers has also been proposed for detection, diagnosis, prediction of response to therapy and prognosis of outcomes for various diseases, including neurodegenerative and psychiatric disorders (Levenson, 2010).

Many children with ASD show selective food choices (Ahearn *et al*., 2001; Paul *et al*., 2007) that could cause vitamin deficiencies because of improper diet (Kaluzna-Czaplinska *et al*., 2009) resulting in aggravation of some autistic symptoms. Saudi autistic children showed impaired energy metabolism that was correlated with oxidative stress, which was reported to occur due to glutathione (GSH) depletion and over-expression of superoxide dismutase (SOD) activity in these children (Al-Mosalem *et al*., 2009; Al-Gadani *et al*., 2009). Waskiewicz *et al*. (2010) observed an inverse association between the consumption of vitamins B_6, B_{12} and folate and Hcy concentration and prevalence of hyperhomocysteinemia in Polish population. Supplementary intake of vitamins B_6, B_{12} and folate were effective in lowering the urinary homocysteine levels (Kaluzna-Czaplinska *et al*., 2011b). Xia (2011) reported that a 9-year-old boy with autism responded positively to nutritional supplements. A role and efficacy of nutritional supplements in autism-spectrum disorders has been suggested (James *et al*., 2009; Xia, 2011). In autistic children a specific diet is therefore considered of great significance.

Data from various studies suggests a relationship between high levels of Hcy and an increased risk of CVD, thrombosis, stroke and neurodegenerative diseases (Hoţoleanu *et al*., 2007; Humphrey *et al*., 2008; Lubinska *et al*., 2006; Tu *et al*., 2010; Hermann & Obeid, 2011; Remacha *et al*., 2011). Plasma total homocysteine (tHcy) levels could also be associated with rapid cognitive decline and higher behavioral disturbances and depression in Alzheimer disease (Seshadri *et al*., 2002; Chen *et al*., 2010; Tu *et al*., 2010). Plasma Hcy level has already been recognized as a potential and predictive biomarker

in age-related neurodegenerative diseases (Hermann & Obeid, 2011). Altered plasma fatty acid pattern in Saudi autistic patients has also been proposed as a diagnostic biomarker for autism (El-Ansary *et al*., 2011). The results of our study are the first of its kind reported from Oman and confirm the hypothesis that increased serum levels of homocysteine could be implicated in the pathophysiology of ASD in Omani children. The lower serum folate and vitamin B_{12} levels observed in Omani autistic children also need further attention to explain their role in the complex etiology of ASD. The serum homocysteine, folate and vitamin B_{12} levels can therefore be used as clinical biomarkers not only for diagnosing the possible nutritional deficiencies but also to identify alterations in metabolic pathways. The outcome of this study suggests role of homocysteine as a potential biomarker in the progression and early detection of autism in the Sultanate of Oman. Further studies with larger cohort are required to validate these preliminary results.

CONCLUSIONS

The results of our study confirm the hypothesis that increased levels of fasting serum homocysteine, and lower levels of folate and vitamin B_{12} could be implicated in the pathophysiology of ASD and may be used as clinical biomarkers for an early diagnosis and management of Omani autistic children.

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