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# Study of *MTHFR* C677T Polymorphism and Associated Oxidative Stress Biomarkers among Autism Spectrum Disorder Patients in Jordan

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**Abstract**

Autism spectrum disorder (ASD) is a neurodevelopmental disorder caused by both genetic and environmental factors. The impact of the environmental triggers is associated with increases in oxidative stress. The aim of this study was to test the serum levels of oxidant/antioxidant status along with analysis of *MTHFR* C677T polymorphism (rs1801133) in autistic children. Twenty five patients (20 males and 5 females) diagnosed with ASD and twenty five healthy sex-matched and age-matched control participants were included in this study. *MTHFR* C677T polymorphism was analyzed by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The serum levels of reduced (GSH), total antioxidant capacity (TAC), cysteine, folate and vitamin $B_{12}$ contents in autistic patients were significantly lower than control group. In addition, high levels of serum oxidized glutathione (GSSG), malondialdehyde (MDA), homocysteine, total oxidant status (TOS) and carbonylate proteins were found in children with autism compared to control group. The *MTHFR* C677T frequency was significantly higher in autistic as compared to non-autistic children. The homozygous genotype CC of the *MTHFR* C677T was lower in patients with autism than the control group (28% vs 52%), while heterozygous CT genotype of the *MTHFR* C677T and the homozygous TT genotype were higher in patients with autism compared to control group (52%, 44%) and (20%, 4%), respectively. The *MTHFR* C677T polymorphism and reduction in cysteine, folate and vitamin B12 levels as well as increase in oxidative stress could contribute to ASD risk in Jordan. Further studies are needed on a larger scale to explore other genes polymorphisms and other risk factors that may be associated with ASD in Jordan.

**Keywords**: Autism, oxidative stress, glutathione, PCR-RFLP, MTHFR gene.

## 1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication, repetitive and restrictive behavior. Autism is most likely caused by multiple factors interacting in complex ways genetic, biological, environmental, and developmental. Therefore, ASD is not etiologically homogenous (Kroncke *et al*., 2016, Boucher, 2017). Autism is becoming a major public health issue in the world due to its yearly exponential increase in the numbers of individuals diagnosed with Autism (Boucher, 2017, Baio *et al*., 2018). The Center for Disease Control and Prevention (CDC) in the United States released a new report on the prevalence of ASD with an increase from 1 in 68 children from the previous report in 2016 to 1 in 59 children in the 2018 report (Baio *et al*., 2018). In the Arab world, there is a growing interest in autistic disorders due to global prevalence increases as reported by prevalence studies from different parts of the world over the past few years which come up with a more conservative median estimate of prevalence of 62 in 10,000 children (Elsabbagh *et al*., 2012). ASD is considered as a new field in the Arab world (Hussein and Taha 2013). The number of confirmed cases of ASD is unknown, and available reports suggest that the prevalence of ASD is 1.4, 29, and 59 per 10,000 children, respectively, in Oman (Al-Farsi *et al*., 2011), the United Arab Emirates (Eapen *et al*., 2007), and Saudi Arabia (Aljarallah *et al*., 2006). In a neighboring country to Jordan, a prevalence of 1 in 66 children was reported in Lebanon (Chaaya *et al*., 2016). In Jordan, a previous study was conducted between 2006 and 2007 reported that 5.2% of sample consist of 229 children with global developmental disorder (GDD) were diagnosed as autistic (Masri *et al*., 2011).

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* **Abbreviation list** : ASD: Autism Spectrum Disorder; MTHFR: Methylenetetrahydrofolate reductase; GSH: Reduced Glutathione; GSSG: Oxidized Glutathione; Cys: Cysteine; Hcy: Homocysteine; PCR-RFLP: Polymerase Chain Reaction– Restriction Fragment Length Polymorphism; MDA: Malondialdehyde; TOS: Total Oxidant status; TAC: Total Antioxidant Capacity; ELISA: Enzyme linked Immunosorbent Assay; SNPs: Nucleotide Polymorphisms

A recent study assessed the correlation between vitamin-D deficiency and autism spectrum disorder (ASD) in Jordan. Their results showed that Vitamin D levels in ASD patients were significantly lower, suggesting a possible role for vitamin D deficiency in the pathophysiology of ASD (Alzghoul *et al*., 2019a). Another related study aimed at identifying a possible association between levels of inflammatory markers among Jordanian children by comparing the plasma levels of selected cytokines in autistic children with those of their unaffected siblings and unrelated healthy controls. It was found that Interleukin-8 and TNF- α were exclusively elevated in autistic Jordanian children, while interlekin-6 was elevated in both autistic children and their siblings, potentially dismissing its significance. These results may be used for early testing and diagnosis of ASD as the researchers suggested (Alzghoul *et al*., 2019b).

Genetic bases have been shown to play a strong role in the development of ASD; 65-90% of twin studies can be explained by genetic factors and most of the genetic data for ASDs depends on protein products (Bralten *et al*., 2018). Some evidence for a genetic risk comes from having specific Single Nucleotide Polymorphisms (SNPs) associated with ASD (Wu *et al.*, 2017, Mohmuda *et al*., 2016). Many studies found that autism is associated with C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene, which regulates folate/homocystine pathway (Boris *et al*., 2004, Guo *et al*., 2012, Divyakolu *et al*., 2013, Shawky *et al*., 2014, Sener *et al*., 2014, and Rai, 2016). The location of *MTHFR* gene is on chromosome 1, and the C667T polymorphism is located at exon 4 which causes the conversion of alanine to valine at codon 222 (Liew and Gupta, 2015). Several investigators reported that individuals with ASDs have a deficiency in serum level of $B_{12}$ and folate (Kałużna-Czaplińska, 2013, Zou *et al.*, 2019).

On the other hand, individuals with ASD have been found to have lower GSH/GSSG ratio compared to normal individuals as well as significantly lower levels of serum cysteine, the limiting substrate in GSH synthesis (Main *et al*., 2012, Han *et al*., 2015). There is overwhelming evidence that the dysregulation of GSH homeostasis is implicated in the etiology and progression of several diseases such as neurodegenerative diseases (Johnson *et al.*, 2012). Moreover, a decrease in the glutathione redox ratio has been reported in individuals with autistic disorder and a possible role of oxidative stress and mitochondrial dysfunction in the pathophysiology of this disease (Main *et al*., 2012, González-Fraguela, 2013, Ozturk *et al*., 2016, and Ugur *et al*., 2018). It was suggested that abnormalities in glutathione metabolism and imbalance in GSH concentration may lead to neuro-developmental problems in Autism (Rossignol and Frye, 2014, Kern *et al*., 2011, Main *et al*., 2012, and Hodgson *et al*., 2014). In Jordan, few studies have been reported to identify some biomarkers which can be measured to predict early diagnosis and possible causes of autism in children. The present study aims to investigate *MTHFR* C677T polymorphism and the possible association between oxidative stress biomarkers among Autism spectrum disorder Jordanians by comparing the plasma levels of selected biomarkers in autistic patients with healthy sex-matched and age-matched controls.

## 2. Materials and Methods

### *2.1. Participants*

A total of 25 individuals (with age range from 7 to 18 years) from both sexes with autistic spectrum disorder (ASD) were recruited from the Autism Academy of Jordan, Amman, Jordan for this case-control study, and 25 age and sex matched normal children from Med labs medical laboratory, Amman, Jordan participated as controls. For ASD group, exclusion criteria were the presence of neurological diseases (such as cerebral palsy, bipolar disorder and epilepsy) or metabolic disorders (e.g. phenylketonuria). Exclusion criteria for control group were language disability, autoimmune disorders or genetic disorder. At the time of blood draw, children in both groups were in good health and not taking any vitamin supplementation.

The autistic individuals were diagnosed previously by other psychiatrist, psychologist, and developmental pediatrician. A written consent was provided by parents of the children for the participation of their respective children in this study. The study was conducted in accordance with Declaration of Helsinki, and the protocol was approved by the Ethics committee of Graduate Studies School in The University of Jordan, Amman, Jordan **(**Project code: 4/ 2018/2017/3/137).

### *2.2. Blood Sample collection*

About five milliliters of blood were withdrawn from each subject using plane tubes. Serum was extracted and stored at -20 ºC for a maximum of a week or stored at -70 ºC for longer period unless the test required freshly prepared samples. For DNA extraction, 2 ml blood was collected using heparin tubes.

### *2.3. Study measurements*

#### *2.3.1. Genotyping*

Genomic DNA was extracted from blood sample using the Promega Wizard™ DNA Purification System (Promega). The quality of DNA was verified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its concentrations was determined at 260/280 nm (should be around 1.8) and then it was examined using a 2% agarose gel. C677T (rs1801133) polymorphism of *MTHFR* gene was studied in both autistic and normal age matched control individuals using polymerase chain reaction– Restriction Fragment Length Polymorphism (PCR-RFLP) assay with the following primers to amplify a 497-bp region from genomic DNA:

5'-TGGGGTCAGAAGCATATCAGTCA-3' (forward)
5'-CTGGGAAGAACTCAGCGAAC-3' (reverse).

PCR conditions were done as in (Delshadpour *et al*., 2017). PCR reactions were prepared in 25 µl reaction volume containing: 1 µl from each primer (100 pmol/µl), 12.5 µl master mix (New England $BioLabs_{Inc}$), and 4 µl genomic DNA (80 ng/µl) and 6.5 µl deionized water. PCR cycle condition was as follows: initial denaturation step of 94 °C for 5 min, and 35 cycles at 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 45 s followed by final extension step at 72 °C for 5 min. Then, 5 µl of the PCR product was run on 2% Agarose gel and 1% TBE (Tris-Borate-EDTA) buffer. For genotyping, restriction enzyme digestion was carried

out following the PCR using Taq *I* restriction enzyme (20,000 units/ml, New England BioLabs$_{Inc}$). For 2 h at 65 °C, the digestion enzyme mixture (50 µl volume) was: 1µl restriction enzyme, 5 µl NEBuffer, 10 µl DNA PCR product (about 1 µg) and 34 µl free nuclease water. Then 10 µl of the mixture was checked with 2% agarose gel electrophoresis (at 100 volt for 2 hr).

*2.3.2. Biochemical assays*

*2.3.2.1. Determination of Folate, B12, Cysteine and Homocysteine.*

In our study, the serum levels of folate, $B_{12,}$ Cysteine and homocysteine were measured by a quantitative sandwich enzyme linked immunoassay technique (ELISA) using commercial kits (Mybiosource, USA) and the ELISA reader (AccuReader version1.10).

*2.3.2.2. Determination of Oxidative stress indicators.*

Reduced glutathione (GSH), Oxidized Glutathione (GSSG), Glutathione redox (GSH/GSSG), Malondialdehyde level (MDA), Total Oxidant Status (TOS), Total Anti-oxidant Capacity (TAC) and Carbonylated protein concentration were measured using a commercially available assay kit for each test and the ELISA reader (AccuReader version1.10).

*2.3.2.2.1. Reduced glutathione (GSH)*

This Quantitative Sandwich ELISA kit is to be used for determination the level of GSH in fresh undiluted human serum (Mybiosource, USA).

*2.3.2.2.2. Oxidized Glutathione (GSSG)*

This ELISA kit applies the competitive enzyme immunoassay technique to determine GSSG in fresh human serum (samples were diluted with sample diluents as recommended by the Kit) (Mybiosource, USA).

*2.3.2.2.3. Glutathione redox (GSH/GSSG)*

In this part, the ratio between GSH and GSSG for each sample was measured.

*2.3.2.2.4. Malondialdehyde level (MDA)*

This experiment used double-sandwich ELISA technique; the pre-coated antibody is human MDA monoclonal antibody. If samples are not timely detected, they should be divided according to single usage amount and frozen reserved at -20 ºC (Mybiosource, USA).

*2.3.2.2.5. Total Oxidant Status (TOS)*

In this kit, the TOS was measured in fresh prepared undiluted human serum sample. The absorbance was read in three interval times (after 30 sec, after 5 min and after 10 min.) then delta absorbance (Δ Abs) was measured (Mybiosource, USA).

*2.3.2.2.6. Total Antioxidant Capacity (TAC)*

This assay measures total antioxidant capacity in fresh prepared human serum (detect directly). The sample was diluted with the sample diluents immediately before the test as recommended in the kit (Mybiosource, USA).

*2.3.2.2.7. Carbonylated protein concentration*

It is colorimetric assay that utilizes the DNPH reaction to measure the protein carbonyl content in the serum. If the sample could not test at the same day, it could be stable for 1 month at -70 ºC.

*2.4. Statistical analysis*

For the biomedical results, unpaired Student's t-test was used to compare between two groups and the level of statistical significance was set at $P < 0.05$. To compare the differences in allele and genotype frequencies between cases and controls Chi-square ($\chi^2$) test were applied. The strength of association was assessed by calculating odd ratio (OR) and 95% confidence interval. A $p$ value < 0.05 was considered statistically significant. Hardy-Weinberg Equilibrium was assessed for genotype and allele type. All the statistical analyses were performed using GraphPad Prism (7) statistical package.

## 3. Results

*3.1. MTHFR genotyping*

Genotyping of *MTHFR* C677T was done by PCR-RFLP method (Figs. 1 and 2). The *MTHFR* C677T genotypes frequencies in the ASD children are shown in Table 1. The homozygous TT genotype was present in (20%) of the ASD children and in (4%) of the controls (<0.05). The heterozygous CT genotype occurred in 52% of children in the ASD group and in 44% the control group (0.285). The T allele frequency in the ASD children was 46% compared to an allele frequency of 26% among the controls (<0.05). These results indicate that *MTHFR* C677T polymorphism (rs1801133) was associated with autism in this population sample from Jordan.

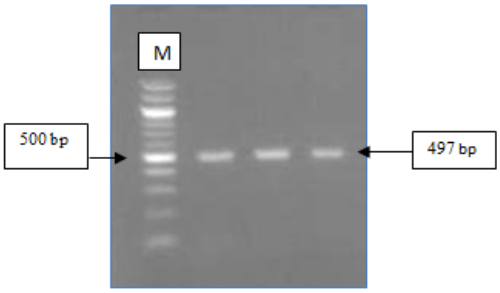


**Figure 1.** The products of MTHFR gene PCR amplification, an agarose gel electrophoresis.
Fragments of 497 bp are for the MTHFR gene.
M- marker 100 bp DNA ladder. 2% Agarose gel

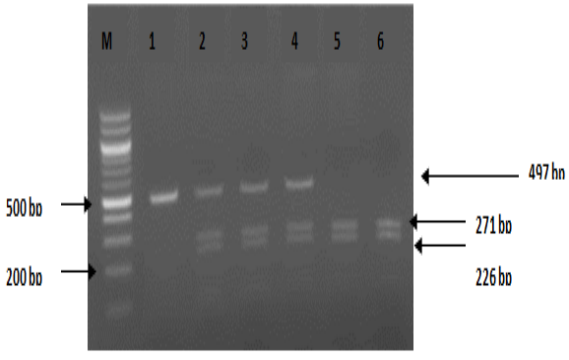


**Figure 2**. The products of MTHFR gene PCR-RFLP amplification on 2% Agarose gel electrophoresis.
CC homozygote had a single band (1) of 497 bp. TC heterozygote had three bands of 497, 271 and 226 bp (2,3,4) and TT homozygote had a two fragment of 271 and 226 bp (5,6). M-marker 100 bp DNA ladder.

**Table 1**: Genotype and the distribution of alleles of MTHFR C677T SNP in people with and without autism

| Genotype | Autism n (%) | Control n (%) | *P* value | OR (95% CI) |
|---|---|---|---|---|
| CC | 7 (28%) | 13 (52%) | 0.0416 * | 0.359 (0.11-1.179) |
| CT | 13 (52%) | 11 (44%) | 0.286 | 1.379 (0.4489-4.449) |
| TT | 5 (20%) | 1 (4%) | 0.0409* | 6 (0.6683-73.17) |
| At least one C model | 20 (80%) | 24 (96%) | 0.0409 | 0.167 (0.0137-1.49) |
| CC and CT | | | | |
| TT | 5 (20%) | 1 (4%) | | |
| At least one T model | 18 (72%) | 12 (48%) | 0.0416* | 2.786 (0.848-9.091) |
| TT and CT | | | | |
| CC | 7 (28%) | 13 (52%) | | |
| Allele C | 27 (54%) | 37 (74%) | 0.0186* | 0.413 (0.1855-0.9369) |
| Allele T | 23 (46%) | 13 (26%) | | |
| Expected genotypes (H.W. Freq.) | Autism n (%) | Control n (%) | *P* value for Autism | *P* value for control |
| CC | 7.29 (29.16%) | 13.69 (54.76%) | 0.055 | 0.515 |
| CT | 12.42 (49.68%) | 9.62 (38.48%) | | |
| TT | 5.29 (21.16%) | 1.69 (6.76%) | | |

*Significant level $p<0.05$, *p* value calculated by Chi-square. CC: homozygous wild type, CT: heterozygous, TT: homozygous mutant. OR: odd ratio; CI 95% confidence interval; H.W freq: Hardy-Weinberg frequencies.

### *3.2. Serum levels of vitamin B12, cysteine, Homocysteine and folate*

The mean serum levels of $B_{12}$, Cystiene, homocysteine and folate were evaluated for both autistic and control subjects in this study. The results were summerized as mean±SD in table 2.

The levels of cysteine, folate and vitamin $B_{12}$ were significantly lower in the ASD group compared to the control group, while significantly higher level of homocysteine was found in the ASD patients compared to the control group (Table 2).

**Table 2:** The level of vitamin $B_{12}$, cysteine, Hcy and folate in serum between autistic and control, Amman, Jordan, results are mean±SD (n=25).

| **Metabolite** | **Autism** | **Control** | ***P*-value** |
|---|---|---|---|
| Vitamin $B_{12}$ (ng/ml) | 335.64 ± 86.63 | 461.16 ± 8.25 | <0.05 |
| Cysteine (Cys) µmol/l | 192.35 ± 22.15 | 247.112 ± 44.012 | <0.05 |
| Homocysteine (Hcy) µmol/l | 15.27 ± 3.517 | 8.984 ± 2.04 | <0.05 |
| Folate ng/ml | 4.464 ± 1.30 | 8.604 ± 1.25 | <0.05 |

### *3.3. Serum oxidative stress biomarkers in autistic and control groups*

The levels of GSH, TAC and Glutathione were statistically significant lower in the ASD group than in the control group, while the levels of GSSG, TOS and carbonylated protein values were statistically significant higher in the ASD group than in the control group ($p<0.05$). The results are given as mean ± SD for each measured parameters as shown in table 3.

**Table 3:** Oxidative stress biomarkers in serum of autistic and control children, Results are expressed as mean ±SD.

| | Autistic | Normal | *P* value |
|---|---|---|---|
| **MDA (µmol/l)** | 2.094 ± 0.44 | 0.82 ± 0.20 | <0.05 |
| **GSH (µmol/l)** | 2.84 ± 0.86 | 8.69 ± 1.48 | <0.05 |
| **GSSG (µmol/l)** | 0.56 ± 0.08 | 0.25 ±0.058 | <0.05 |
| **TAC (mmol/l)** | 0.49 ± 0.09 | 1.36 ± 0.25 | <0.05 |
| **TOS (µmol H2O2 Equiv./L)** | 47.48 ± 1.46 | 42.80± 3.14 | <0.05 |
| **Glutathione redox (GSH/GSSG) (µmol/l)** | 5.23 ± 1.77 | 29.51 ± 3.98 | <0.05 |
| **Carbonylated protein concentration (µmol/l)** | 1.25 ± 0.17 | 0.83± 0.097 | <0.05 |

MDA-Malondialdehyde, GSH-Reduced glutathione, GSSG-Oxidized glutathione, TAC-Total antioxidant capacity, TOS-Total oxidant status.

## 4. Discussion

This is the first study to examine several parameters that were found to be associated with autism in Jordan. Several investigators studied oxidative stress in autism by measuring products of lipid peroxidation and antioxidants such as glutathione (Main *et al*., 2012, Han *et al*., 2015, Johnson *et al*., 2012, González-Fraguela *et al*., 2013, Hodgson *et al*., 2014). Lipid peroxidation biomarkers such as MDA are usually increased in autism along with altered glutathione levels as well as the homocysteine/methionine metabolism, which indicates that oxidative stress in this disease is elevated. The results indicated that the ASD group had significantly higher levels of GSSG, TOS, Hcy, MDA and carbonylated protein concentrations as well as low levels of Cys, GSH, GSH/GSSG, TAC, folate and $B_{12,}$ which suggested that antioxidant capacity and redox homeostasis were significantly decreased in children with ASD. The present findings are in agreement with a previous study that reported that children with ASD have weakness in glutathione (GSH) redox metabolism and chronic oxidative stress (Rose *et al.*, 2012). Several studies showed that decreased (GSH) and increased (GSSG) in plasma of children with ASD indicate that oxidative stress may play a central role in the pathogenesis of ASD due to accumulation of toxic materials, which can promote neuronal damage in genetically predisposed individuals (González-Fraguela *et al*., 2013, Rose *et al*., 2012). Moreover, Al-Gadani *et al*., 2009 reported significantly lower GSH in plasma of Saudi autistic children as compared to age matching controls. Weak antioxidant response and increase in total oxidants as measured by the blood level of TOS can cause neuronal cell damage (Ozturk *et al.*, 2016, Ugur *et al*., 2018). In agreement with this notion, the present study reported a significant elevation in TOS level in children with autism. Previously reported data are in line with our results that suggested significantly higher levels of serum Hcy in autistic children, compared to non-autistic children (Zou *et al*., 2019, Ali *et al*., 2011). Methionine synthase enzyme remethylates Hcy to methionione by using 5-methyltetrahydrofolate as a methyl donor and vitamin $B_{12}$ as cofactor. So, deficiency in vitamin $B_{12}$ leads to metabolic disturbances of Hcy, and an elevation of Hcy levels in serum. In addition, the enzyme methylenetetrahydrofolate reductase (MTHFR) regulates folate availability and acts at the cross road between

methyl group transfer and biosynthesis of nucleotides (Boris *et al*., 2004, Divyakolu *et al*., 2013).

The low TAC and high Hcy levels shown in our study in ASD children indicate that Hcy can cause oxidative stress by affecting the redox signaling pathways of neuronal cells causing imbalance in the antioxidant/oxidant system. Our findings are in agreement with those reported by Zou *et al*., 2019.

Another significant outcome of the present investigation is the association between the polymorphism of Methylenetetrahydrofolate reductase (*MTHFR*) gene and autism. 5,10-Methylenetetrahydrofolate reductase - a key enzyme in methionine-homocysteine metabolism - maintains the folate pool between the methylation pathways and DNA synthesis. To our knowledge, there is no previous study in our population that investigated the polymorphism of the *MTHFR* gene and autism. There is statistically significant correlation between autism and the C677T polymorphic genotypes of *MTHFR* gene among autistic individuals in several studies (Boris *et al*., 2004, Rai, 2016, Liu *et al*, 2011). *MTHFR* C677T (rs1801133) is a risk factor for ASD especially in the homozygous state. Many studies have reported that autism could be associated with metabolic abnormalities in the folate/homocysteine pathway, which is contributed in DNA methylation, thus altering gene expression (Pasca *et al*., 2009, Divyakolu *et al*., 2013, Pu *et al*., 2013). These polymorphism at position C677T of *MTHFR* gene and the disturbances of the folate metabolic pathway in autism have been reported in many populations such as Egypt (Meguid *et al*., 2015, Shawky *et al*., 2014), Saudia Arabia (Elhawary et al., 2016) , Turkey (Sener *et al*., 2014), Iran (Delshadpour *et al*., 2017) and China (Guo *et al*., 2012). *MTHFR* gene C677T polymorphism could predict higher levels of Hcy concentrations in populations with mild folate deficiency (Han *et al*., 2015).

Our results are in agreement with the result reported by Boris *et al*., 2004 and Shawky *et al*., 2014. There is more prevalence of the homozygous 677TT allele of the MTHFR gene in the typical autism group (Boris *et al*., 2004, Guo *et al*., 2012, Shawky *et al*., 2014). It was found that the individuals with homozygous (TT) have an approximately 50% decrease in the activity of MTHFR enzyme, and a 30% decrease in enzyme activity of individuals with the heterozygous (CT) (Guo *et al*., 2012). However, Delshadpour *et al*., 2017 reported that there was no significant correlation between *MTHFR* 677T gene polymorphism and autism and that MTHFR role in folate metabolism may participate in epigenetic mechanisms that modify complex gene expression which can cause autism. The mechanisms of *MTHFR* C677T polymorphisms as a risk factor of autism are still unclear and need more investigation.

## 5. Conclusion

In conclusion, oxidative stress in combination with the C677T polymorphism of *MTHFR* gene might play a crucial role in the etiology and wide spread of ASD in Jordan. However, we cannot exclude other risk factors; and more research is still needed with larger sample size to fully characterize the potential causes of ASD in Jordan.

**Declarations of interest:**

none

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## References

Al-Farsi YM, Al-Sharbati MM, Al-Farsi OA, Al-Shafaee MS, Brooks DR and Waly MI. 2011. Brief report: Prevalence of autistic spectrum disorders in the Sultanate of Oman. *J Autism develop dis.* **1 (6)**:821-825.

Al-Gadani Y, El-Ansary A, Attas O and Al-Ayadhi L. 2009. Metabolic biomarkers related to oxidative stress and antioxidant status in Saudi autistic children. *Clin Biochem*, **42**:10-11, 1032-1040.

Ali A, Waly M I, Al-Farsi Y M, Essa M M, Al-Sharbati M M and Deth, R C. 2011. Hyperhomocysteinemia among Omani autistic children: a case-control study. *Acta Biochimica Polonica*, **58**(4): 547-551.

Aljarallah A, Alwaznah T, Alnasari S, and Alhazmi M. 2006. A study of autism and developmental disorders in Saudi children. Riyadh: Report, King Abdulaziz City for Science and Technology http:// www.kacst.edu.sa. Accessed 13 June 2014.

Alzghoul L, Abdelhamid S S, Yanis A H, Qwaider Y Z, Aldahabi M, and Albdour S A. 2019. The association between levels of inflammatory markers in autistic children compared to their unaffected siblings and unrelated healthy controls. *Turkish J Medical Scie*. **49**(4):1047-1053

Alzghoul L, AL-Eitan LN, Aladawi M, Odeh M, and Hantash OA. 2019. The Association Between Serum Vitamin D3 Levels and Autism Among Jordanian Boys. *J Autism develop dis.* **16**:1-6.

Baio J, Wiggins L, Christensen D L, Maenner M J, Daniels J, Warren Z and Durkin,M S. 2018. Prevalence of autism spectrum disorder among children aged 8 years—autism and developmental disabilities monitoring network, 11 sites, United States, 2014. *MMWR Surveillance Summaries*, **67**:1-23

Boris M, Goldblatt A, Galanko J and James S J. 2004. Association of MTHFR gene variants with autism. *J Ame Phy Sur*, **9**: 106-8.

Boucher J M .2017. Autism spectrum disorder: Characteristics, causes and practical issues. *SAGE*.

Bralten J, Van Hulzen K J, Martens M B Galesloot T E, Vasquez A A, Kiemeney L A & Poelmans G. 2018. Autism spectrum disorders and autistic traits share genetics and biology. *Mol psych*, **23**: 1205-1212.

Chaaya M, Saab D, Maalouf F T and Boustany R M. 2016. Prevalence of autism spectrum disorder in nurseries in Lebanon: a cross sectional study. *J Autism Dev dis*, **46**: 514-522.

Delshadpour M, Mashayekhi F, Bidabadi E, Shahangian S & Salehi Z. 2017. MTHFR rs1801133 Gene polymorphism and autism susceptibility. *Cas J Neu Sci*, **3**: 39-45.

Divyakolu S, Tejaswini Y, Thomas W, Thumoju S, Sreekanth V R, Vasavi M and Ahuja Y. R. 2013. Evaluation of C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene in various neurological disorders. *J Neu Dis.* http://dx.doi.org/10.4172/2329-6895.1000142

Eapen V, Mabrouk AA, Zoubeidi T, and Yunis F. 2007. Prevalence of pervasive developmental disorders in preschool children in the UAE. *J Tropical Pediatrics*. **53(3)**:202-205.

Elhawary N, Tayeb M, Bogari N and Qotb N. 2016. Vulnerability of genetic variants to the risk of autism among Saudi children. *Hum Geno*, **10**: 236.

Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcín C, Montiel-Nava C, Patel V, Paula CS, Wang C and Yasamy MT. 2012. Global prevalence of autism and other pervasive developmental disorders. *Autism Res*. **5(3)**:160-79.

González-Fraguela M E, Hung M L D, Vera H, Maragoto C, Noris E, Blanco L and Robinson M. 2013. Oxidative stress markers in children with autism spectrum disorders. *J Adv Med Medi Res*, 307-317.

Guo T, Chen H, Liu B, Ji W and Yang C. 2012. Methylenetetrahydrofolate reductase polymorphisms C677T and risk of autism in the Chinese Han population. *Gen Tes Mol Biom*, **16**: 968-973.

Han Y, Xi Q Q, Dai W, Yang S H, Gao L, Su Y Y and Zhang X. 2015. Abnormal transsulfuration metabolism and reduced antioxidant capacity in Chinese children with autism spectrum disorders. *Inter J Develop Neuro*, **46**: 27-32.

Hodgson N W, Waly M I, Al-Farsi Y M, Al-Sharbati M M, Al-Farsi O, Ali A and Deth R C. 2014. Decreased glutathione and elevated hair mercury levels are associated with nutritional deficiency-based autism in Oman. *Exper Bio Med*, **239**: 697-706.

Hussein H and Taha G R. 2013. Autism spectrum disorders: A review of the literature from Arab countries. *Midd East Cur Psychi*, **20**: 106-116.

Johnson W M, Wilson-Delfosse A L and Mieyal J. 2012. Dysregulation of glutathione homeostasis in neurodegenerative diseases. *Nutri*, **4**: 1399-1440.

Kałużna-Czaplińska J, Żurawicz E, Michalska M and Rynkowski J. 2013. A focus on homocysteine in autism. *Acta Biochimica Polonica*, **60 (2):**137-142.

Kern J K, Geier D A, Adams J B, Garver C R, Audhya T and Geier M R. 2011. A clinical trial of glutathione supplementation in autism spectrum disorders. *Medical science monitor: International Med J Experi Clini Res*, **17**: CR677.

Kroncke A P, Willard M and Huckabee H. 2016. The Causes of Autism. In *Assess Autism Spec Dis*,11-21. Springer, Cham.

Liew SC and Gupta ED. 2015. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: epidemiology, metabolism and the associated diseases. *Eur J Med Gen*. **58(1)**:1-0.

Liu X, Solehdin F, Cohen I L, Gonzalez M G, Jenkins E C, Lewis M S and Holden J J. 2011. Population-and family-based studies associate the MTHFR gene with idiopathic autism in simplex families. *J Autism Develop Dis*, **41**: 938-944.

Mahmuda N, Yokoyama S, Huang J J, Liu L, Munesue T, Nakatani H and Higashida H. 2016. A study of single nucleotide polymorphisms of the SLC19A1/RFC1 gene in subjects with autism spectrum disorder. *Inter J Mole Scie*, **17**: 772.

Main P A, Angley M T, O'Doherty C E, Thomas P and Fenech M. 2012. The potential role of the antioxidant and detoxification properties of glutathione in autism spectrum disorders: a systematic review and meta-analysis. *Nutr Meta*, **9**: 35-37.

Masri A, Hamamy H, and Khreisat A. 2011. Profile of developmental delay in children under five years of age in a highly consanguineous community: a hospital-based study–Jordan. *Brain and Develop*. **1(10):**810-5.

Meguid N, Khalil R, Gebril O and El-Fishawy P. 2015. Evaluation of MTHFR Genetic Polymorphism as a Risk Factor in Egyptian Autistic Children and Mothers. *J Psych*, **18**: 1-4.

Ozturk O, Basay O, Basay B K, Alacam H, Buber A, Kaptanoglu B and Kardesler A C. 2016. Oxidative imbalance in children and adolescents with autism spectrum disorder. *Klinik Psikofarmakoloji Bülteni-Bulletin Clini Psychopharma*, **26**: 257-264.

Paşca S P, Dronca E, Kaucsár T, Crăciun E C, Endreffy E, Ferencz B K and Dronca M. 2009. One carbon metabolism disturbances and the C677T MTHFR gene polymorphism in children with autism spectrum disorders. *J Cel Mol Med*, **13**: 4229-4238.

Pu D, Shen Y and Wu J. 2013. Association between MTHFR Gene Polymorphisms and the Risk of Autism Spectrum Disorders: AM eta-Analysis. *Autism Res*, **6**: 384-392.

Rai V. 2016. Association of methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism with autism: evidence of genetic susceptibility. *Meta Brain Dis.*, **31**: 727-735.

Rose S, Melnyk S, Pavliv O, Bai S, Nick T G, Frye R E and James S J. 2012. Evidence of oxidative damage and inflammation associated with low glutathione redox status in the autism brain. *Trans Psych*, **2**: e134.

Rossignol, D A and Frye R E. 2014. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. *Front Physi*, **5**: 150.

Sener E F, Oztop D B and Ozkul Y. 2014. MTHFR gene C677T polymorphism in autism spectrum disorders. *Gene Res Inter*. http://dx.doi.org/10.1155/2014/698574

Shawky R M, El-baz F, Kamal T M, Elhossiny R M, Ahmed M A and El Nady G H. 2014. Study of genotype–phenotype correlation of methylene tetrahydrofolate reductase (MTHFR) gene polymorphisms in a sample of Egyptian autistic children. *Egyp J Med Hum Gene*, **15**: 335-341.

Uğur Ç, Tunca H, Sekmen E, Üneri ÖŞ, Alişik M, Erel Ö and Solmaz E. 2018. A comparative study of the oxidative stress indices of children with autism and healthy children. *Anatolian J Psych*, **19**:314-22.

Wu Z, Qin J, You Y, Ma Y, Jia M, Wang L and Li J. 2017. Genetic variants in the transcription regulatory region of MEGF10 are associated with autism in Chinese Han population. *Scie Rep*, **7**: 2292.

Zou M, Sun C, Liang S, Sun Y, Li D, Li L and Xia W. 2019. Fisher discriminant analysis for classification of autism spectrum disorders based on folate-related metabolism markers. *J Nutr Bioch*, **64**:25-31.