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Original article

Glucose transporter 1 deficiency, AMP-activated protein kinase activation and immune dysregulation in autism spectrum disorder: Novel biomarker sources for clinical diagnosis

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A R T I C L E I N F O

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A B S T R A C T

The neurophysiological basis of autism spectrum disorder (ASD) is still uncertain. Nevertheless, studies support the hypotheses that oxidative stress, neuroinflammation, immune dysregulation, and metabolic stress are contributors. In this study, the serum levels of 3-nitrotyrosine (3-NT), hypoxia-inducible factor 1 α (HIF-1 α), heat shock protein 70 (HSP-70), interleukin-17A (IL-17A), IL-35, vitamin D3 (VITD), glucose transporter-1 (GUT1), and AMP-activated protein kinase (AMPK) were estimated in Saudi ASD children versus age-matched neurotypical controls, aiming to investigate whether these parameters have potential roles in the pathophysiologic mechanisms of ASD and hoping to find a reliable marker for early ASD diagnosis. This study included 25 ASD children and 25 typically developing children (3–11 years old). The diagnosis of ASD cases was made based on the Autism Diagnostic Observation Schedule (ADOS) and the Statistical Manual of Mental Disorders (DSM-5). ASD subjects were commonly male and revealed an intelligence quotient (IQ) < 70.The results detected that ASD children have remarkable greater serum levels of nitrosative stress (3-NT), hypoxia (HIF-1 α), inflammatory (HSP-70, IL-17A, and AMPK) biomarkers and lower serum levels of anti-inflammatory (IL-35 and VITD) and metabolic stress (GUT-1) biomarkers versus age-matched controls ($P \leq 0.0001$). Pearson's correlation study revealed that 3-NT was positively associated with HIF-1 α and HSP-70. HIF-1 α was also positively correlated with HSP-70. AMPK was positively associated with GUT-1, however, IL-17A was negatively correlated with IL-35 and VITD.

Limitation:No specific therapeuticdrugs were administered in this study, and further studies are required to confirm the role of the selected biomarkers in ASD managements.

Conclusion: Changes in concentrations of different biomarkers indicate that they are involved in oxidative stress, metabolic stress, immune dysregulation and ASD pathogenesis. Hence, these parameters can prove to be promising biomarkers as well as therapeutic targets for the timely diagnosis and treatment of ASD patients.

1. Introduction

Around 1 % to 2 % of children all across the world are affected by the autism spectrum disorder (ASD) which has been established as a neurological and developmental disorder. It is characterized by a diverse set of behavioral conditions and clinical manifestations. The ratio of incidence between males and females is 4:1 and onset of the disorder is in early age (Brak et al., 2016). Though the exact mechanism behind ASD still needs to be deciphered, a huge body of evidence suggests that a blend of hereditary and environmental risk factors that affect the development of neural circuits makesan individual susceptible to developing ASD thereby resulting in defects relating to behavioral aspects like sociability, cognition, and communication (Kowalnik and Nowakowska, 2019). Researchers have reported a correlation between the etiopathogenesis of ASD and factors like metabolic disorders, nutritional deficiencies, immune disorders, neuronal inflammation, and oxidative stress (Yui et al., 2016, Arastoo et al., 2018, Rose and Ashwood, 2019).

Since the etiopathogenesis of ASD is not known, clinical markers have not been established for the diagnosis of this disorder. Accordingly, ASD is diagnosed based on clinical manifestations and definite history (Shen et al., 2020). For that reason, on-time diagnosis is usually not

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possible. Common symptoms of ASD are not treated because of the absence of established treatment plans (Farmer et al., 2013) and this is because ASD cannot be diagnosed until the age of 4.5 years (Shen et al., 2020). Yet, it has been reported that cases which were luckily diagnosed and treated before the age of three years demonstrated improved ASD outcomes and reduced disability (Landa, 2018). It can therefore be stated that the identification of reliable biomarkers allowing on-time diagnosis of this disorder can prove to be quite promising in clinical practice as these markers will allow to determine the chances of development of the same disorder in baby siblings of ASD affected children. Such markers can also facilitate the determination of underlying mechanisms involved in pathophysiology of the disorder and the development of different interventions for the timely treatment of the disorder (Ruggeri et al., 2014).

Researchers have identified certain risk elements which contribute tothe neurodevelopment of the disorder leading to ASD. These elements include cerebral injury (Yui et al., 2016, Manivasagam et al., 2020), neuroinflammation, oxidative modification of lipids and proteins and buildup of toxic ROS (Czaplinska and Pruska, 2016). Increased levels of several oxidative stress markers have been detected in ASD patients. An important marker in this regard is 3-nitrotyrosine (3-NT) whose association with the severity of ASD has been established. 3-NT is an indicator of protein oxidation (Ghezzo et al., 2013, Liu et al., 2022).

Literature contains some studies that report a connection between ASD and hypoxia (Şimşek et al., 2021, Da Silva et al., 2023). Hypoxic conditions are marked with an increment in a transcription factor namely Hypoxia-inducible factor-1 α (HIF-1 α). HIF-1 α works for homeostasis of cellular oxygen (Wenger, 2002). Accordingly, depending on the severity of hypoxia, HIF-1 α can serve to be the most important factor involved in harmful as well as protective effects occurring during the onset of several disorders associated with neurodegeneration (Zhang et al., 2006; Wu et al., 2020). Several reports indicating a correlation between HIF-1 α and disorders like bipolar disorder, major depressive disorder and mood disorders have been increasing (Shibata et al., 2013). To the best of the researcher's knowledge, literature is still deficient in reports investigating levels of HIF-1 α in ASD patients. In comparison to healthy controls, ASD patients demonstrated significantly low levels of HIF-1 α in serum during a study conducted by Şimşek et al. (2021).

Owing to its physiological characteristics, the heat shock protein 70 (HSP70, 70 kDa) has been established as an intracellular protein which demonstrates a neuroprotective effect on nervous system oxidative stress (Guo et al., 2007). On the other hand, extracellular HSP-70 which is discharged when there is cell injury is associated with harmful and inflammatory consequences for the nervous system (Turturici et al., 2011). A pro-inflammatory state is known to be derived by the HSP-70 as it activates the nuclear factor (NF)-κB leading to stimulation of the production of adhesion molecules over the surface of vascular cells. It also stimulates macrophages and monocytes to generate pro-inflammatory cytokines (IL-6, TNFα (Srivastava, 2002; Moore et al., 2012). Elevated serum levels of HSP70 in ASD patients have previously been reported (El-Ansary and Al-Ayadhi, 2012). Still, Tsukurova (2018) has found that decrement in the level of this protein.

ASD patients have been found to exhibit dysregulated levels of inflammatory and anti-inflammatory cytokines (Bjorklund et al., 2016). The neurodevelopmental disorder in patients affected by ASD is considerably affected by elevated levels of IL-6, IL-17, tumor necrosis factor (TNF) and other inflammatory proteins and also by reduced levels of IL-10, IL-35 and other anti-inflammatory proteins (Akintunde et al., 2015; Rose and Ashwood, 2019).

ASD has recently been found to be associated with a deficiency of vitamin D3 (1,25-dihydroxy vitamin D, VITD) (Cannell, 2017). It has been established that VITD is an important neuroactive steroid which is essentially required in optimum concentrations for the protection and development of the brain (Somma et al., 2017). The involvement of VITD in decreasing oxidative burden, immunomodulation, synaptic plasticity regulation, neuronal proliferation, differentiation, and apoptosis have been reported (Mazahery et al., 2016). Moreover, VITD canaugment the production of anti-inflammatory entities and suppressing the hazardous inflammatory entities thereby bringing about an anti-inflammatory impact on the brain (Mazahery et al., 2016; Cannell, 2017). Cannell (2017) has suggested that deficiency of vitamin D during pregnancy and early childhood can have a role in causing ASD. This is in agreement with the recent finding that reduced concentrations of VITD are demonstrated by ASD patients. D-hypovitaminosis can likely serve as a risk factor for ASD. It can therefore be asserted that VITD can serve as a potential biomarker allowing ASD diagnosis in early stages (Petruzzelli et al., 2020).

The regulation of the uptake of glucose through the blood–brain barrier (BBB) essentially requires GLUT1 which serves as the main regulator (Chen et al., 2015). Zeller et al., (1997) have reported an association between GLUT1 levels in cerebral micro-vessels and brain glucose uptake. Similarly, Ulne et al., (2009) have reported that GLUT1 deficiency results in reduced levels of glucose in cerebrospinal fluid. According to Winkler et al., (2015), such reduced glucose levels may lead to decreased blood flow, cerebrovascular disintegration, BBB breakdown, progressive neuronal loss, behavioral modification, and damaged neuronal activity eventually resulting in neurodegeneration. Researchers have detected reduced levels of GLUT1 in individuals affected by cognitive deficits, movement problems, epilepsy and Alzheimer's disease (Pearson et al., 2013; Winkler et al., 2015).

AMP-activated protein kinase (AMPK) has turned into the chief player in sustaining a suitable energy level in the brain. It is a metabolic serine/threonine protein kinase (Chen et al. 2021). It has proven to be an intracellular energy monitor whose activation has been reported in conditions of cellular ATP depletion during various stressful scenarios like ischemia, hypoxia and glucose deficiency (Shackelford and Shaw, 2009). The energy balance is sustained by AMPK as it triggers ATP-generating catabolic mechanisms like glucose uptake and fatty acid oxidation and inhibits ATP-consuming anabolic pathways (Hardie et al., 2016). Attenuation of neuroapoptosis induced by deficiency of glucose has been found to occur after activation of AMPK during an in vitro study (Culmsee et al., 2001). Besides this, Park et al., (2018) have reported an essential role played by AMPK in brain neuro-inflammatory disease. AMPK causes microglia to turn into an anti-inflammatory phenotype from an inflammatory phenotype. Yaku et al., (2013) have found the upregulation of numerous antioxidant genes by AMPK through stimulation of Nrf2 which regulates the production of antioxidant proteins involved in defense against oxidative damage brought about by inflammation and tissue damage. Although AMPK activation has neuroprotective effects, Ullah et al., (2014) have detected an association between prolonged activation of AMPK and neuronal cell death.

In the light of facts stated above it can be asserted that quantification of certain entities relating to hypometabolism, immune dysregulation, neuroinflammation and oxidative stress in individuals affected by ASD can facilitate comprehension of different pathophysiological mechanisms involved in ASD as well as identification of potential markers allowing early diagnosis of this disorder. Literature is still deficient in reports investigating concentrations of IL-17A, IL35, HIF-1α and 3NT and their association with the etiopathogenecity of ASD. Though the connection between ASD and VITD levels has been researched in the past, its link with IL-17A and other auto-inflammatory cytokines in ASD patients still needs to be deciphered. Understanding this link can facilitate the elucidation of etiopathological mechanisms underlying an inflammatory autoimmune brain lesion in individuals affected by ASD. Regrettably, the literature contains no information relating to AMPK and GUT1 levels and their link with ASD.

This research therefore aims to determine levels of IL-17A, IL-35, AMPK, GUT1, VITD, HSP-70, HIF-1α and 3-NT in Saudi kids affected by ASD in comparison with gender and age-matched neurotypical controls. This will facilitate i) understanding the correlation of these entities with the pathogenicity of ASD; ii) identification of potential ASD biomarkers allowing early diagnosis thereby achieving improved outcomes for ASD

affected children and iii) development of improved early treatment interventions.

2. Patients and methods

This research has been conducted in compliance with the ethical guidelines provided in the Declaration of Helsinki (Edinburgh, 2000). 25 ASD children and 25 age- and gender-matched healthy subjects were participated in this study. The ages of both ASD children and healthy subjects were between three and eleven years.The majority of subjects were male. All subjects were selected through the King Abdulaziz University Hospital. Diagnosis for ASD was made based onthe Statistical Manual of Mental Disorders (DSM-5) and the Autism Diagnostic Observation Schedule (ADOS). ASD subjects demonstrated an intelligence quotient (IQ) less than 70. Registration for the healthy controls was made at the well-baby center, the King Abdulaziz University Hospital. Controls were subjected to a physical and mental examination so that any disease conditions could be ruled out. Informed consent was obtained from parents or legal guardians of all the participants before they could participate in this research.

2.1. Exclusion criteria

Subjects affected by any neurodevelopmental issues (seizures, bipolar disorder, fragile X syndrome), autoimmune disorders (multiple sclerosis, asthma etc.) or metabolic disorders (phenylketonuria) were excluded from the study. The same is the case with children having pulmonary, cardiovascular, kidney, liver, endocrine or any other medical condition.

2.2. EthicalApproval

Approval was taken from the Ethics Committee of the King Abdulaziz University Hospital for conducting this research (approval number 68–22). As per the guidelines of this committee, written consent was taken from the parents/guardians of all subjects included in this research.

2.3. Samples collection

5 ml of blood were drawn fromeach subject, including tests and controls following overnight fasting.Before the collection of blood samples, each child was treated with an anesthetic spray to relieve pain. These samples were taken in sterile test tubes which were then kept at room temperature for two hours to allow clotting of blood. This was followed by centrifugation of samples at 3000 rpm for 20 min. In this way, serum was separated and kept at −80C until further use for biochemical tests (El-Ansaryand Ayadhi, 2012).

2.4. Biochemical serum assays

2.4.1. Enzyme-linked immunosorbent assay (ELISA)

Quantitative analysis has been carried out using the Human Sandwich ELISA kitsaccording to the instructions of manufacture.AMPK (MyBioSource Inc., San Diego, USA), GUT1 (MyBioSource Inc., San Diego, USA), VITD (MyBioSource Inc., San Diego, USA), IL-35 (Antibodies.com, Cambridge, UK), IL-17A (Abcam, ab119535, Shanghai, China), HSP-70 (Kamiya Biomedical Company, Washington, USA), HIF-1α (MyBioSource Inc., San Diego, USA) and 3-NT (MyBioSource Inc., San Diego, USA) were used for quantification. Wells of a microtiter plate which were coated with human-specific antibodies were added with test/standard specimens and biotin-linked antibodies having specificity for the target protein. The plate was incubated for two hours at 37C. Wells were then washed to allow the removal of unbound entities. Each well was then added with streptavidin-horse radish peroxidase (HRP) conjugate followed by incubation for 20–25 min at 37C. Wells were again washed after incubation and 3,3′, 5,5′ tetramethylbenzidine (TMB) substrate was introduced. A blue-colored product was formed because of the reaction between substrate and enzyme. When sulfuric acid was added to cease the enzyme reaction, this product turned yellow. Color intensity was measured using a microplate reader at 450 nm. Color intensity was proportional to the quantity of protein present in the plate. Sensitivities of the assay were 0.039 ng/ml, 0.5 pg/ml, 0.21 pg/ml, 1.27 ng/ml, 31.25 pg/ml, 0.19 ng/ml and < 0.123 ng/ml for 3NT, IL-17A, IL-35, HSP-70, HIF-1α, AMPK and GUT1 respectively.

2.5. Statistical analysis

Data analysis was carried out using SPSS version 22. Mean ± standard error (SE) was computed for different entities. Means were compared using an independent *t*-test. The degree of correlation between two variables was computed through Pearson's correlation 'r' test. The level of significance used in this study was $P \leq 0.05$.

3. Results

3.1. Serum level of nitrosative stress marker

3.1.1. 3-Nitrotyrosine

A significant anincrease in serum 3-nitrotyrosine (3-NT) of ASD children (14.00 ± 0.47 nmol/ml) versus age and gender-matched control individuals (6.76 ± 0.38 nmol/ml, $P \leq 0.0001$) (Fig. 1A), with a percentage change of 107.1 % (Fig. 1B).

3.2. Serum level of hypoxia marker

3.2.1. Hypoxia Inducible Factor-1α

As shown in Fig. 2A, the serum concentration of Hypoxia Inducible Factor-1α (HIF-1α) was significantly greater (168.20 ± 2.70 pg/ml) in ASD subjects in comparison to relevant controls (109.88 ± 1.14 pg/ml, $P \leq 0.0001$). The percentage change was found to be + 53.1 % as shown in Fig. 2B.

3.3. Serum level of inflammatory markers

3.3.1. Heat shock protein- 70

As shown in Fig. 3A, serum concentrations of heatshock protein-70 (HSP-70) were considerably higher (6.60 ± 0.26 ng/ml) in ASD subjects in comparison to the relevant controls (1.85 ± 0.095 ng/ml, $P \leq 0.0001$). The percentage change was found to be + 256.75 % as shown in Fig. 3B.

3.3.2. Serum level of interleukin −17A (IL-17A)

Fig. 4A shows a remarkable increase in the serum level of IL-17Ain ASD children (32.04 ± 1.3 pg/ml) with respect to age and gender-matched control subjects (14.37 ± 0.44 pg/ml, $P \leq 0.0001$), with a percentage change of + 122.96 % (Fig. 4B).

3.3.3. Serum level of AMP-Activated protein kinase

As shown in Fig. 5A, serum concentrations of AMP-activated protein kinase (AMPK) were considerably higher (123.32 ± 3.74 ng/ml) in ASD subjects in comparison to the relevant controls (57.6 ± 2.94 ng/ml). The percentage change was found to be + 114.1 % as shown in Fig. 5B.

3.4. Serum level of anti-Inflammatory markers

3.4.1. Serum level of Interleukin-35 (IL-35)

Fig. 6A illustrates a remarkable decrease in the serum level of

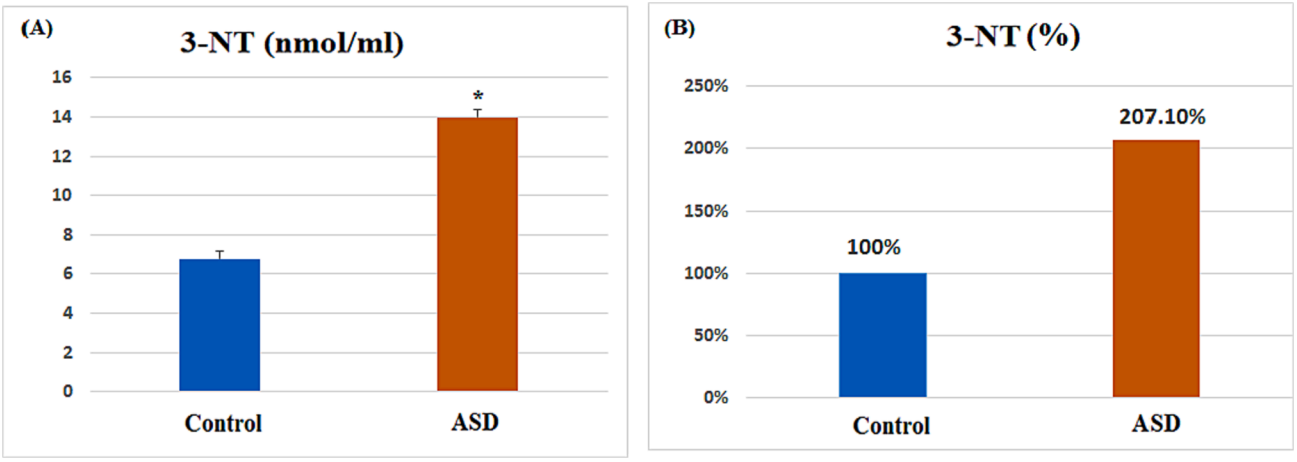

Fig. 1. Serum level of 3-NT in children with ASD (n = 25) and control individuals (n = 25). (A): Values were graphed as histograms with a mean ± SE.*$P \leq 0.0001$ compared with the control. (B): Percentage change of serum 3-NT in ASD children with respect to controls.

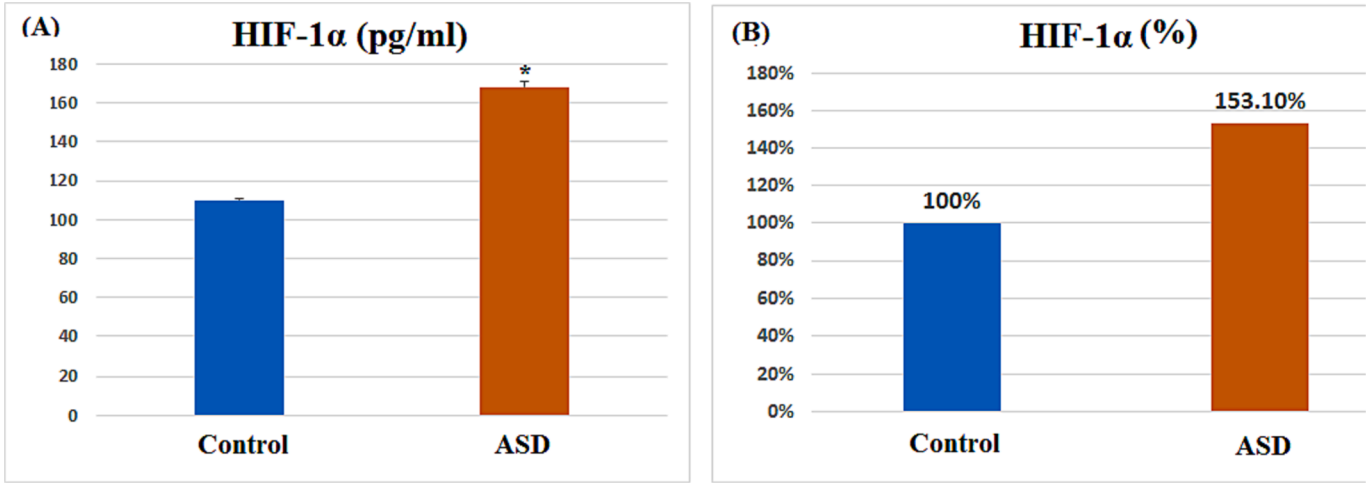

Fig. 2. Serum level of HIF-1α in ASD children (n = 25) and control subjects (n = 25). (A): Values were graphed as histograms with a mean ± SE.*$P \leq 0.0001$ compared with the control. (B): percentage change of serum HIF-1α in ASD children with respect to control.

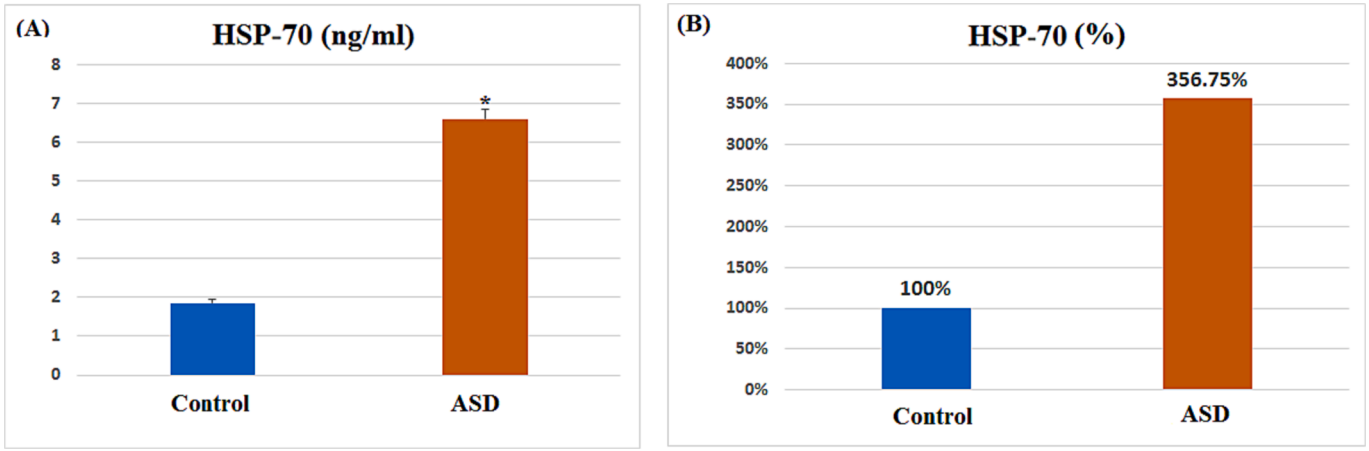

Fig. 3. Serum level of HSP-70 in ASD children (n = 25) and control individuals(n = 25).(A): Values were graphed as histograms with a mean ± SE.*$P \leq 0.0001$ compared with control. (B): Percentage change of serum HSP-70 in children with ASD with respect to control healthy individuals.

interleukin-35(IL-35) of children with ASD (2.64 ± 0.16 ng/ml) with respect to age and gender-matched control subjects (7.2 ± 0.22 ng/ml, $P \leq 0.0001$), with a percentage change of − 70.27 % (Fig. 6B).

3.4.2. *Serum level of vitamin D3*

Fig. 7A demonstrates serum vitaminD3(VITD) level was lower in ASDchildren (7.71 ± 0.40 ng/ml)than age and gender-matched control subjects (19.26 ± 0.70 ng/ml, $P \leq 0.0001$), with a percentage change of

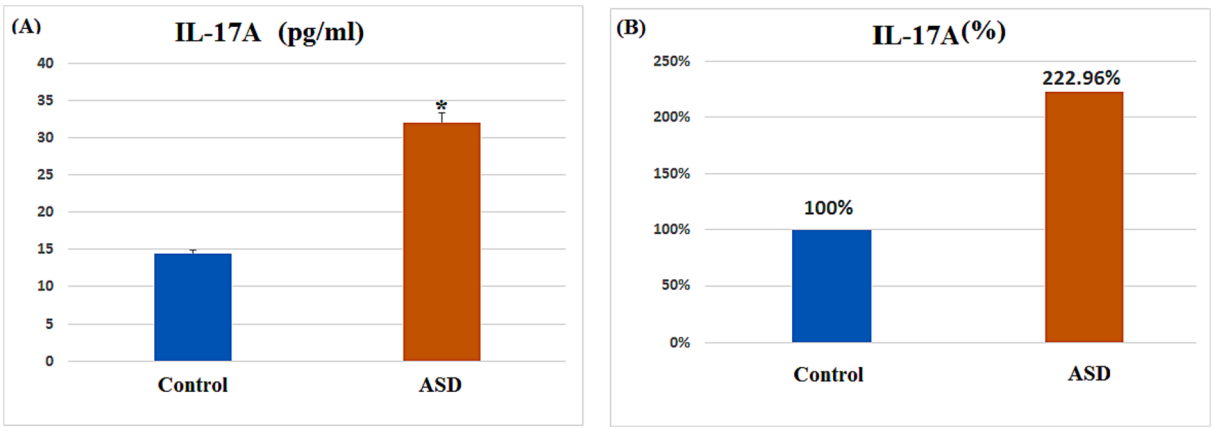

Fig. 4. Serum level of IL-17A in children with ASD (n = 25) and control individuals(n = 25).(A): Values were graphed as histograms with a mean ± SE. *$P \leq 0.0001$ compared with control. (B): Percentage change of serum IL-17A in ASD children with respect to control healthy individuals.

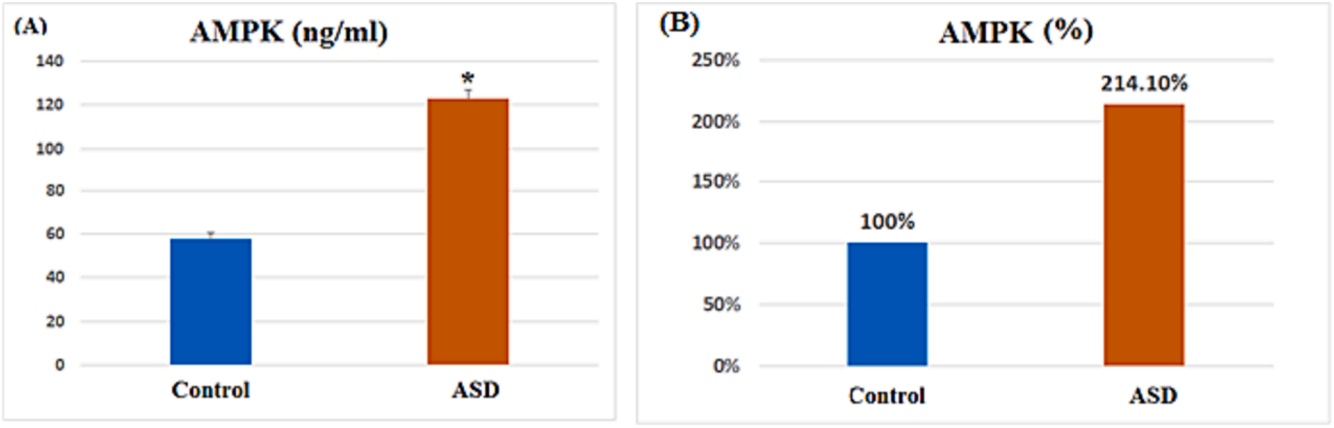

Fig. 5. Serum level of AMPK activity in ASD children (n = 25) and control individuals (n = 25). (A): Values were graphed as histograms with mean ± SE.*$P \leq 0.0001$ compared with control. (B): Percentage change of serum AMPK in ASD children versus control healthy individuals.

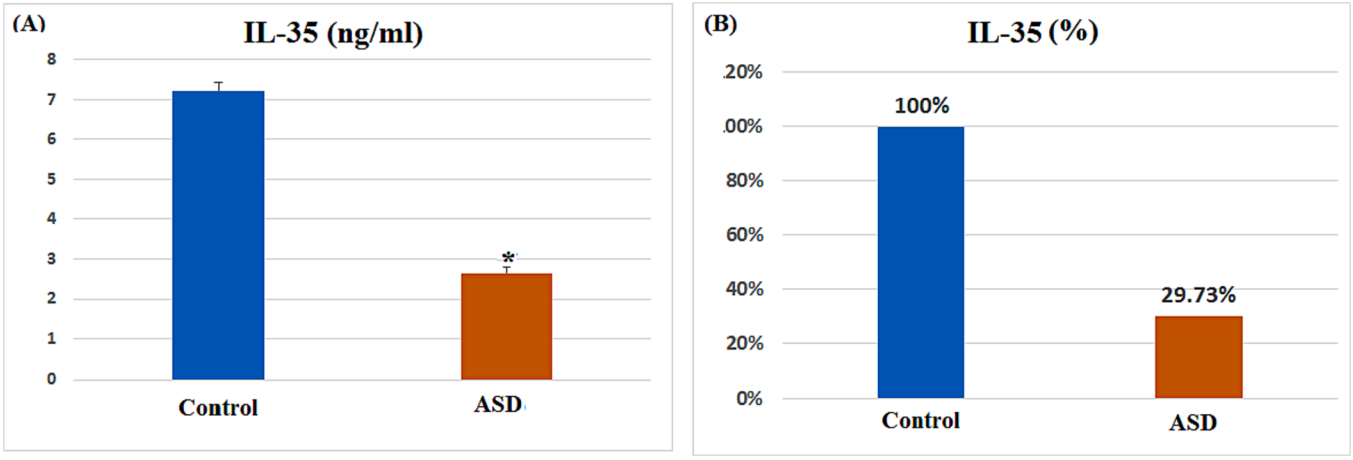

Fig. 6. Serum level of IL-35 in children with ASD (n = 25) and control individuals(n = 25).(A): Values were graphed as histograms with mean ± SE. *$P \leq 0.0001$ compared with control. (B): Percentage change of serum IL-35 in ASD children versus control healthy individuals.

− 59.97 % (Fig. 7B).

3.5. Serum level of metabolic stress marker

3.5.1. Glucose transporter −1

As shown in Fig. 8A, serum concentrations of glucose transporter −1 (GUT1) were considerably lower (0.28 ± 0.017 ng/ml) in ASD subjects in comparison to the relevant controls (0.68 ± 0.022 ng/ml, $P \leq 0.0001$). The percentage change was found to be −58.82 % as shown in Fig. 8B.

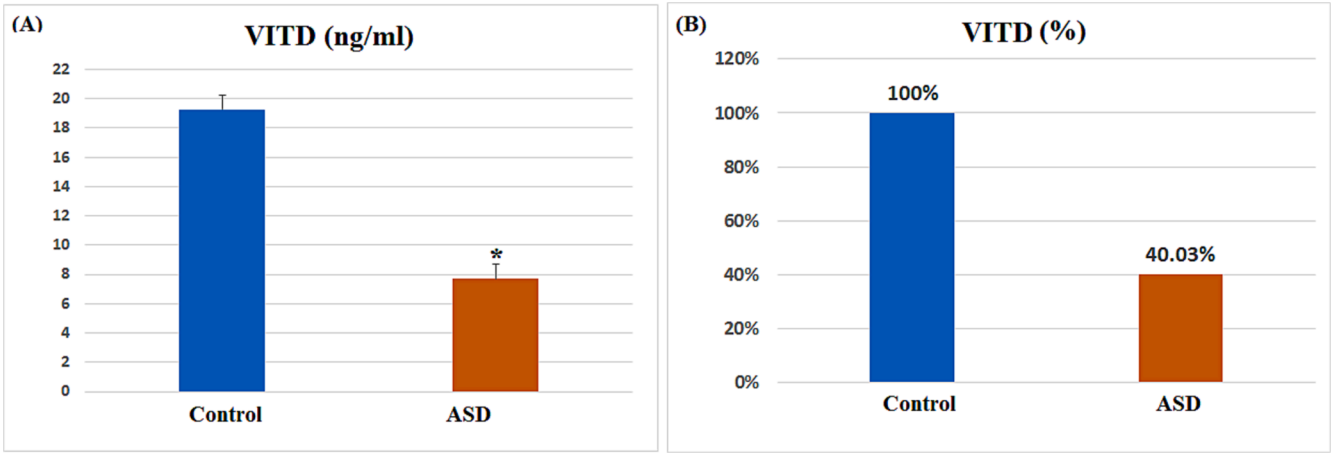

Fig. 7. Level of VITD in serum of ASD children (n = 25) and control subjects(n = 25). (A): Values were graphed as histograms with mean ± SE. *$P \leq 0.0001$ compared with control. (B): Percentage change of serum VITD in ASD children versus control healthy individuals.

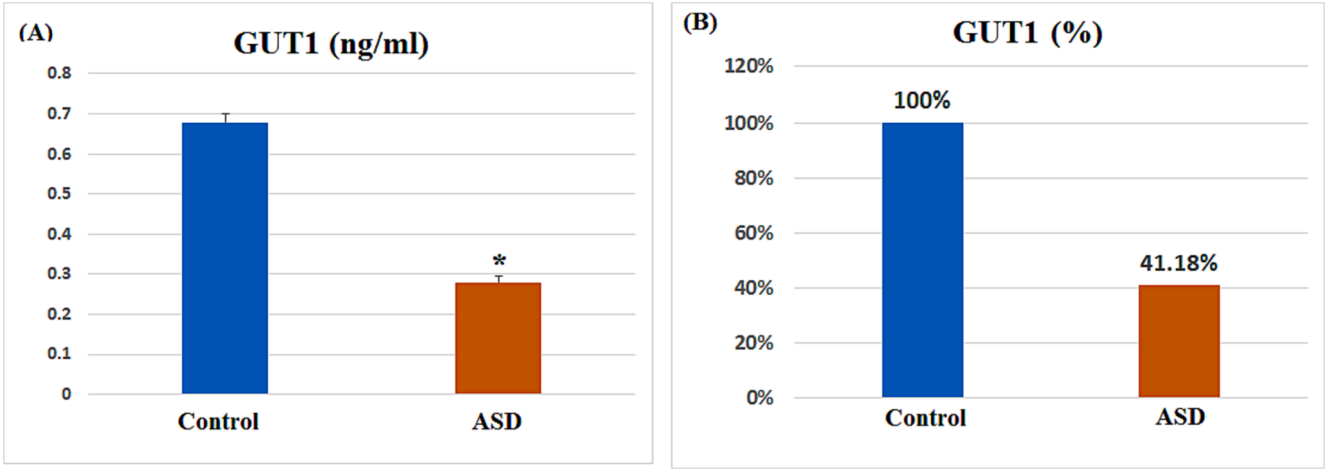

Fig. 8. Serum level of GUT1 in ASD children (n = 25) and control individuals(n = 25). (A): Values were graphed as histograms with mean ± SE. *$P \leq 0.0001$ compared with control. (B): Percentage change of serum GUT1 in ASD children versus control healthy individuals.

3.6. Pearson's correlation between different biomarkers

The correlation analysis showed a positive correlation between serum levels of 3-NT and serum levels of HIF-1α as shown in Fig. 9B (r = 0.785, P = 0.0001). Serum levels of 3-NT were also found to be positively correlated with HSP-70 levels as shown in Fig. 10B (r = 0.489; P = 0.013). Similarly, a significantly positive correlation has been detected between serum levels of HSP-70 and those of HIF-1α as shown in Fig. 11B (r = 0.52, P = 0.004). In contrast to this, a negative correlation was found between serum concentrations of IL-17A and IL-35 as shown in Fig. 12B (r = -0.878, P = 0.001). Similarly, serum concentrations of IL-17A demonstrated a negative correlation with serum concentrations of VITD as shown in Fig. 13B (r = -0.732, P = 0.001). Moreover, a positive correlation also existed between serum concentrations of GUT-1

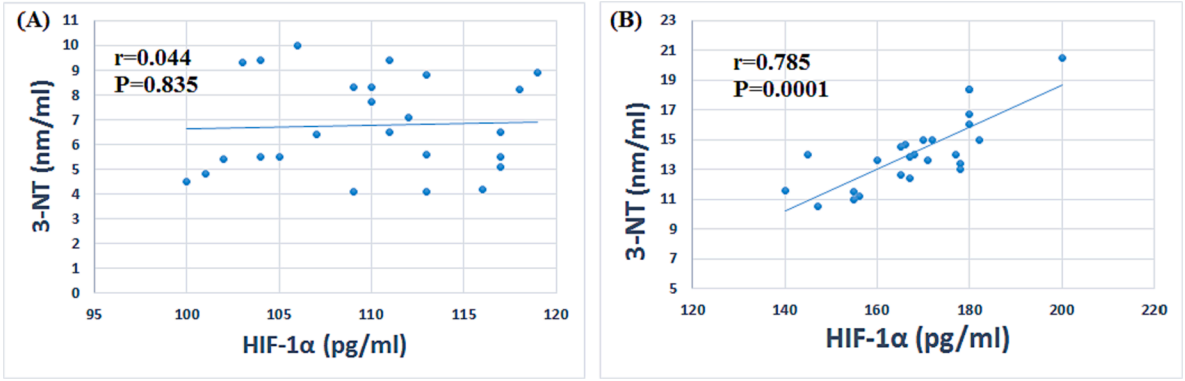

Fig. 9. Correlation between serum levels of 3-NT and HIF-1α. (A): Control children (n = 25). (B): ASD children (n = 25).

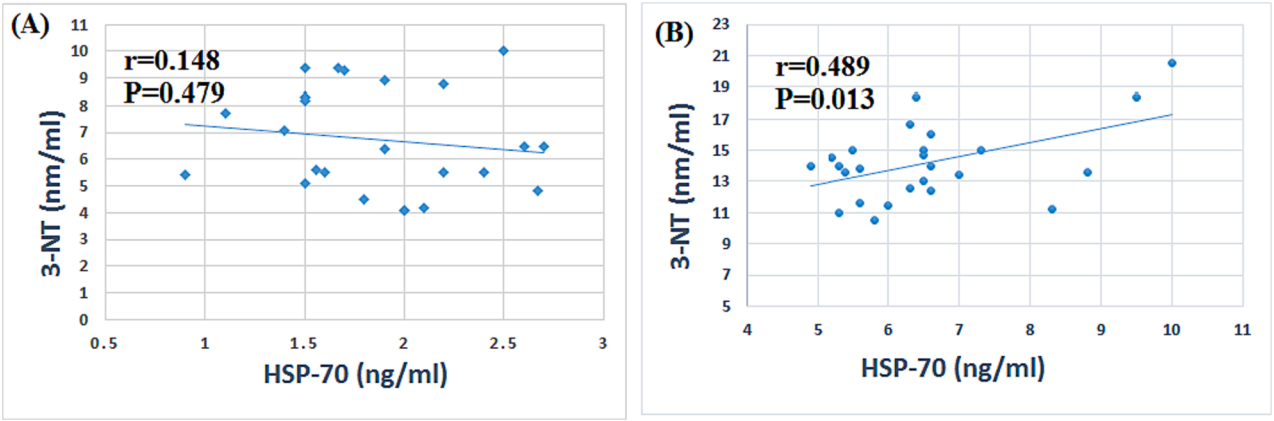

Fig. 10. Correlation between serum levels of 3-NT and HSP-70. (A):Control children (n = 25).(B): ASDchildren (n = 25).

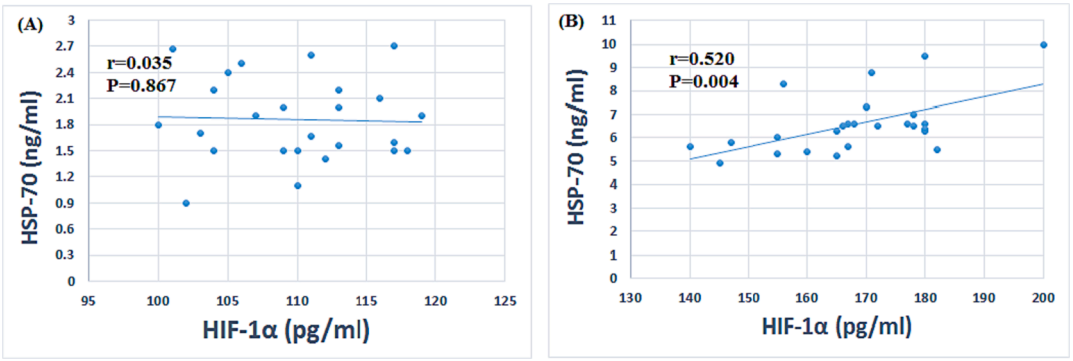

Fig. 11. Correlation between serum HSP-70 level and HIF-1α. (A): Control children (n = 25). (B): ASD children (n = 25).

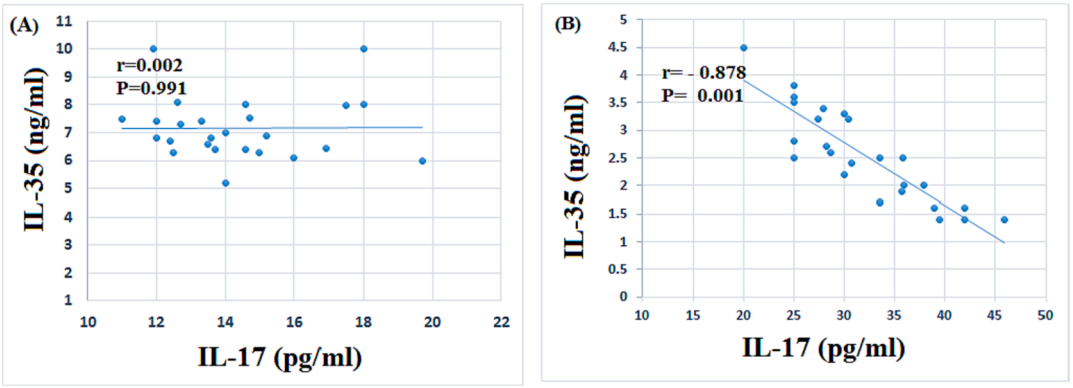

Fig. 12. Correlation between serum IL-35 level and IL-17.(A): Control children (n = 25). (B): ASD children (n = 25).

and AMPK as shown in Fig. 14B (r = 0.456, P = 0.022). In the case of healthy controls, IL-17A was found to be negatively correlated with VITD (r = -0.8, P = 0.001).

4. Discussion

It has been established that oxidative stress is associated with the etiology of ASD (Manivasagam et al., 2020). Researchers detected elevated levels of oxidative stress markers in ASD patient's peripheral body fluids and these levels have been associated with the severity of the disease (Ghezzo et al., 2013). During this research, ASD subjects had demonstrated increased levels of oxidized protein tyrosine derivative i.e. 3-NT (tyrosine nitration marker) in comparison to healthy controls thereby pointing towards the fact that vulnerability of these subjects to oxidative stress can be associated with their contact with different environmental factors. This is in concordance with findings of Melnyk et al., (2012). The researchers reported increased levels of 3-NT plasma in subjects affected by autism thereby pointing towards an association between etiology of ASD and oxidative protein damage. 3-NT has been established as the main product generated by the nitration of tyrosine through peroxynitrite, a potent agent of oxidization (Ischiropoulos et al., 1992).

In a scenario of oxidative stress, the reaction between superoxide anion and nitric oxide (NO) results in the formation of peroxynitrite. 3-NT generation can bring about conformational changes in proteins leading to permanent structural and functional damages which end up with damage to cells (Souza et al., 2008). It has now been established that 3-NT is a reliable marker indicating chronic immune system activation as well as neuronal death brought about by oxidative protein

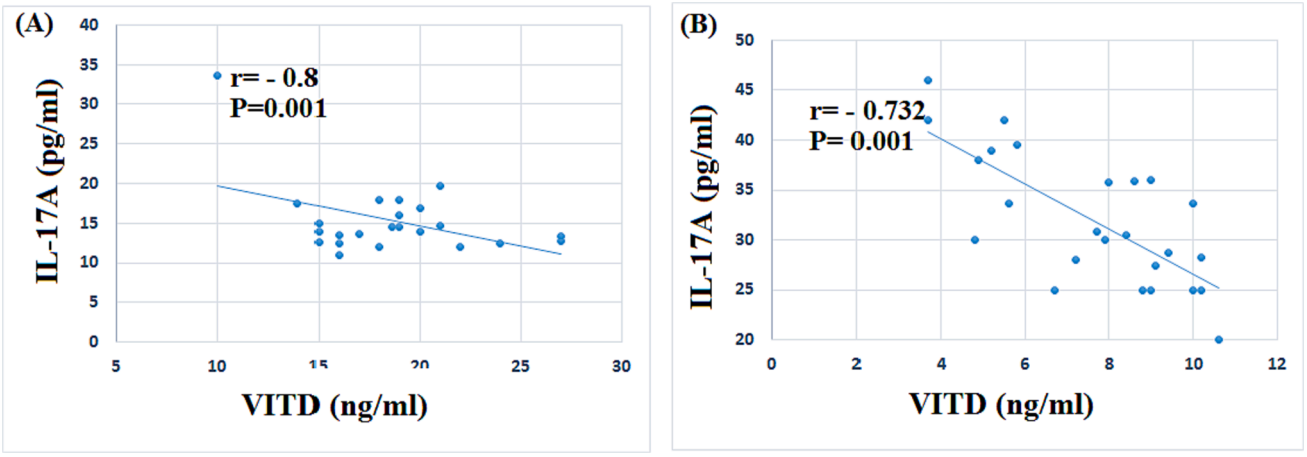

Fig. 13. Correlation between serum levels of IL-17A and VITD. (A): Control children (n = 25). (B): ASD children (n = 25).

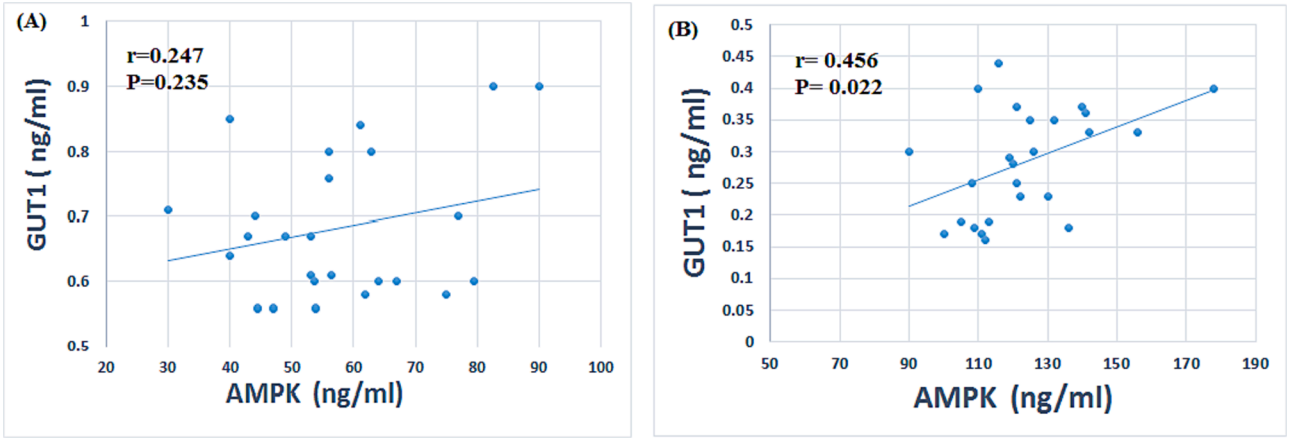

Fig. 14. Correlation between serum levels of GUT1 and AMPK activity.(A): Control children (n = 25). (B): ASD children (n = 25).

damage in living organisms with inflammatory disorders (Meredith et al., 2014). It has therefore been proposed to be an initial marker pointing towards neurodegenerative disorders (Yeo et al., 2015). It can therefore be asserted that increased serum levels of 3-NT in ASD subjects recorded in this research might have contributedtoneuronal damage in the ASD subjects.

Based on epidemiological data, it can be stated that fetal or neonatal hypoxia is involved in ASD etiology (Kolevzon et al., 2007). Increased serum concentrations of HIF-1α in ASD subjects recorded during this research indicate that this change can participate in ASD etiology. While on the other hand, Simşek et al., (2021) detected reduced serum concentrations of HIF-1α in ASD subjects. HIF-1α has therefore been suggested to have twofold activity as a dangerous or protective transcription factor involved in the causation of several diseases associated with neurodegeneration. However, this activity is dependent on the extent of hypoxia (Piret et al., 2002). HIF-1α can protect against neuronal cell death induced in response to glucose deficiency through augmenting expression of GLUT1 and GLUT3 thereby allowing enhanced uptake of glucose by neurons subjected to hypoxia (Wu et al., 2020). Moreover, HIF-1α has proven to be a crucial regulator for hippocampal neurogenesis involved in cognitive activities (Braun and Jessberger, 2014). Nevertheless, increased expression of HIF-1α induced by extreme hypoxic conditions may cause activation of p53 thereby inducing cellular apoptosis since p53 is a tumor suppressor gene (Piret et al., 2002). Promotion of neuroinflammation by HIF-1α through the production of inflammatory cytokines like IL-1β, IL-6, IL-8, and TNF-α has also been reported. It also does so through the production of NFκB which can trigger the transcription of genes relating to inflammation (Zhang et al.,2006, Eltzschig and Carmeliet, 2011). When IL-1β is upregulated, it can result in hyperexcitability as well as neuronal injury through augmented Ca2 + flow (Viviani et al., 2003).

According to Pearson's correlation mentioned in this study, the serum concentration of HIF-1α was found to be positively correlated with that of 3-NT in ASD subjects. This association suggests that augmented production of HIF-1α in ASD subjects can be linked with excessive production of 3-NT. It has recently been reported that activation of HIF-1α is capable of instigating excessive generation of ROS which is essentially required by generation of 3-NT and oxidation of proteins (Tang et al., 2023). Based on these findings, it can be suggested that increased serum concentration of HIF-1αcan serve as an indicator pointing towards nitrosative stress, neuroinflammation and neuronal cell death in individuals affected by ASD.

As far as HSP70 is concerned, its concentration in serum was found to be greater in the case of ASD subjects as compared to relevant controls during this research. This is in concordance with a previous study which reported an increased HSP 70 concentrations in autistic children (El-Ansary and Al-Ayadhi, 2012). However, another study reported reduced serum concentration of HSP-70 in ASD subjects as compared to controls (Tsukurova, 2018). Augmented concentrations of HSP-70 recorded in this research can be caused by its excessive generation and/or discharge outside the tissue as a consequence of tissue injury. Researchers have already demonstrated increased expression of the HSP gene after several injuries like trauma, epilepsy, neurodegenerative disorder, and stroke

(Turturici et al., 2011).

According to Matzinger (1994), extracellular HSP70 serves as a signal indicating danger for the innate immune system. Srivastava (2002) has reported the interaction of HSP70 with antigen-presenting cells (APC) thereby stimulating these cells to generate proinflammatory cytokines as well as activating NF-κB leading to the instigation of the adaptive immune response. Also, antigens are presented to Tc cells.

According to Zhang et al., (2009), binding between HSP70 and Toll-like receptors (TLR4) results in the generation of proinflammatorycytokines..Ran et al., (2004) have demonstrated that TNF-mediated apoptosis can be instigated by increased levels of HSP70 through binding of IκB kinase (IKK) in response to certain stimuli. Moreover, the induction of some genes by HSP70 has also been reported. These genes contribute to psychotic disorders and depression (Silverman and Sternberg, 2012; Beck et al., 2013). Since serum concentrations of HSP70 are strongly correlated with serum concentrations of 3NT and HIF-1α in ASD subjects, it can be stated that increased production of HSP70is linked with generation of 3NT as well as HIF1α and this can lead to augmented oxidative stress together with neuroinflammation in ASD affected individuals. Results obtained during this study indicate that HSP70 makes a considerable contribution to ASD etiopathogenecity. For that reason, it can serve as a potential predictor for neuroinflammation development associated with ASD.

Inflammatory as well as anti-inflammatory cytokines are unbalanced in ASD patients (Bjorklund et al., 2016). In concordance with past research studies, this research has found significantly higher serum concentrations of inflammatory cytokine IL-17A together with reduced serum concentration of anti-inflammatory cytokine IL-35 in ASD subjects in comparison to relevant controls (Akintunde et al., 2015; Rose and Ashwood, 2019). Patients with autoinflammatory conditions like multiple sclerosis had demonstrated elevated concentrations of IL-17 and reduced concentrations of IL-35 in previous studies (Teymouri et al., 2018; Akiyama and Sakuraba, 2021). The disturbance in the balance of inflammatory and anti-inflammatory cytokines observed during this research can validate the autoimmune reactions and neuroinflammation which had been reported in the past in ASD-affected individuals (Hughes et al., 2018; Nadeem et al., 2022). Antagonistic outcomes of IL-17 and IL-35 have been established (Keijsers et al., 2014; Teymouri et al., 2018). T-helper 17 (Th17) cells have been reported as chief cells secreting IL-17 (Moynes et al., 2014). Conversely, IL-35 is largely generated by Treg cells (regulatory T lymphocytes).

The disturbed balance between IL17 and IL35 can be associated with a disturbance in the balance of Th17 and Treg cells which has been detected in ASD subjects in the past (Rose et al., 2018). It has been asserted that a reduction in the population of Treg cells or their activity in ASD-affected individuals can cause reduced levels of IL-35 in them (Rose and Ashwood, 2019). The disturbance in the immune system brought about by IL-35 may lead to an imbalance involving augmented immune activation and neurodevelopment can be affected by this imbalance (Rose and Ashwood, 2019). Since serum levels of IL17A are negatively correlated with serum levels of IL35 in ASD patients during this research, it can be stated that there exists an association between IL35 downregulation and IL17A overexpression.

Generation of IL17A has been reported to be suppressed by IL35 (Yokota et al., 2015). The increment in serum concentrations of IL17A recorded in ASD subjects during this study can be associated with the buildup of amyloid β peptides (Aβ) in the tissues of the brains of these subjects. This has been reported in previous studies involving ASD individuals as well (Bailey et al., 2008). It has been reported in the past that accumulation of β peptides (Aβ) in brain tissue can trigger Th17 cells thereby causing the generation of IL-17 (Sun et al., 2019). Through activating microglia and NFκB, neuroinflammation can be induced in ASD patients by 1L17 generation (Sun et al., 2015; Nadeem et al., 2018). Besides this, when IL17A binds to specific receptors present on endothelial cells of BBB results in enhanced permeability of these cells. This causes immune cells to infiltrate and this infiltration has proven to be an important factor in the causation of autoimmune inflammatory damage to the brain (Kermode et al., 1990). Hence, the disturbance in the balance of IL17A and IL35 can be viewed as an indicator pointing towards autoimmune neurodegeneration in ASD subjects.

The number of reports suggesting the role played by deficiency of vitamin D in ASD etiology is increasing with time (Cannell, 2017). Significantly reduced levels of VITD demonstrated by ASD subjects can be due to insufficient exposure to sunlight, inadequate dietary intake of VITD or its reduced absorption as reported in previous studies (Wang et al., 2020).

It can therefore be asserted that D-hypovitaminosis is involved in etiopathogenesis of ASD. The impact of VITD on the development and function of the brain has already been established (Karras et al., 2018). Moreover, VITD can demonstrateanti-inflammatory and antioxidant characteristics. It can also serve as a neuroprotective entity by inhibiting nitric oxide synthase, generating inflammatory cytokines, suppressing activation of glial cells and upregulating antioxidants (DeLuca, 2016; Cannell, 2017).A negative correlation between serum concentrations of VITD and serum concentrations of IL17A has been detected through the statistical analysis of the obtained data. This shows that reduced levels of VITD in ASD subjects account for excessive generation of IL17. As per the researcher's best knowledge, this is novel research demonstrating an association between VITD and IL17A levels in ASD-affected individuals. Zhghaier et al., (2020) have also proposed that administration of VITD is capable of suppressing IL17 and other Th17-related cytokines in experimental cases of autoimmune encephalomyelitis.

According to Mak (2018), VITD canpromote theproliferation of Treg cells and diminishing autoimmune reactions as well as excessive immune responses. Hence, it can be stated that the imbalance between IL17 and IL35 recorded during this research could be due to D-hypovitaminosis in subjects with ASD. It is noteworthy that this is the first study during which serum concentrations of VITD, IL17, IL35 and 3NT have been studied at the same time in ASD subjects thereby allowing researchers to assert that D-hypovitaminosiscan contributetoinflammatory autoimmune neurodegeneration and oxidative stress associated with ASD.

Hypo-metabolism has turned into a considerable risk factor which can make an individual susceptible to neurodegenerative diseases. Characteristic features of hypo-metabolism include disturbed uptake and consumption of glucose. Reduced serum concentrations of GUT1 recorded in ASD subjects during this study indicate a deficiency of this glucose transporter in the brains of subjects. Only a small number of researchers have investigated concentrations of GUT1 in individuals affected by ASD. This research has determined serum concentrations of GUT1 in ASD children for the first time. Previously, Harik (1992) has reported reduced levels of GUT1 in the brains of Alzheimer's disease patients. The reduction in serum concentrations of GUT1 in ASD patients recorded in this research could be due to the buildup of Aβ in brain tissue as asserted by Bailey et al., (2008). Likewise, Kouznetsova et al., (2006) reported low levels of GUT1 in brain cortical regions demonstrating increased amyloid concentrations in mice with AD.

According to Kawai et al. (1990) that decreased expression of GUT1 could be a consequence of degeneration of capillaries because of Aβ plaques in individuals having AD. When low levels of glucose are delivered to the brain (neuroglycopenia) because of insufficient glucose transporters could lead to conditions like metabolic stress, progressive neuronal loss, neurodegeneration, hypometabolismandBBB breakdown (Winkler et al., 2015; Zilberter&Zilberter, 2017). Reduced uptake of glucose by the brain and reduced glucose utilization have been reported as an initial indicator of neurodegeneration (Butterfield and Halliwell, 2019). The disturbed activity of cerebral GLUT1 has been found to cause epilepsy, developmental delay, movement disorder, intellectual disability and cognitive impairment (De Vivo et al., 1991).

AMPK has proven to be a metabolic sensor which participates in the

regulation of cellular energy metabolism as well as chronic inflammatory disorders (Wang et al., 2018; Chen et al., 2021). Increased AMPK activity was recorded in the case of ASD patients during this research. The AMPK activity was found to be positively correlated with serum levels of GUT1 in ASD individuals. This is the first research which has investigated AMPK activity in ASD subjects. Increased AMPK activity recorded in this research could be due to insufficient brain glucose, inflammation, hypoxia, and metabolic stress.

Earlier research studies affirm these hypotheses (Concannon et al., 2010; Wang et al., 2018). Since serum AMPK concentrations were found to be positively correlated with serum concentrations of GUT1 among ASD subjects, it can be asserted that increased levels of AMPK in ASD subjects have caused increased expression of GUT1 although low levels of this transporter had been recorded in sera of ASD subjects as compared to healthy controls. Earlier, it had been proposed that activation of AMPK is capable of promoting GLUT1 expression thereby augmenting the rate of uptake and consumption of glucose which leads to attenuation of neuronal death (Culmsee et al., 2001; Chen et al., 2021). Neuron energy homeostasis can be restored by AMPK activity through switching to reduced consumption of ATP and elevated generation of ATP (Hardie et al., 2012).

Besides this, activation of AMPK has been reported to cause attenuation of neuroinflammation through suppression of inflammatory mediator generation (Wang et al., 2018) and augmentation of AMPK/Nrf2 signaling which contributes to anti-inflammatory activities (Park et al., 2018). Conversely, prolonged or excessive activation of AMPK can bring about neuronal apoptotic cell death through Bim (Bcl-2 homology domain 2 (BH3)-only protein) upregulation and caspase-3 activation (Ullah et al., 2014; Eom et al., 2016). Besides these, increased activation of AMPK in a scenario of energy insufficiency augments astrocytic glycolysis and ketosis to recompense neuronal energy insufficiency. Still, prolonged glycolysis in astrocytes results in progressive acidosis and inhibits neurons from utilizing lactate for energy thereby ending up with neuronal death (Li and McCullough, 2010). Lastly, the extent of oxidative stress can be increased by AMPK activation (Ju et al., 2014) thereby causing neurodegeneration.

4.1. Limitations

No particular therapeutic interventions were applied in this study, limiting the evidence about how the selected biomarkers track or predict outcomes of treatment.

5. Conclusion

ASD has been proven to be a lifelong neurodevelopmental disorder. Prolonged and excessive production of 3NT, HIF1α, HSP-70, IL17A and AMPK and reduced production of VITD, IL35 (anti-inflammatory indices) and GUT1 (an indicator of metabolicstress and hypometabolism) can play a role in causing neuronal cell death leading to neurodegeneration in ASD children. Disturbed levels of IL17A and IL35 together with D-hypovitaminosis can serve as promising indicators of inflammatory autoimmune neurodegeneration. Considering the results obtained during this research, it can be stated that these parameters which demonstrated changes in ASD subjects are essentially involved in the etiopathogenicity of ASD. Hence, these parameters can be used as potential markers and therapeutic targets allowing early diagnosis as well as treatment of ASD affected individuals. This study did not apply any specific therapy procedures, which may limit the evidence regarding how the chosen biomarkers track or predict treatment effects. Further work is needed to certify the role of these biomarkers in the management of ASD.

5.1. Recommendations for future research

The current biomarkers are being developed as diagnostic tools. Further studies are needed to explore the mechanisms underlying the observed biomarker changes. Also, further longitudinal studies are required to validate the role of these biomarkers in the early detection of ASD, its severity, and prognosis, as well as aid in the selection of effective treatments or monitoring the response to therapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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