ORIGINAL ARTICLE

Diagnostic Potential of Serum TNFα and COX2 in ASD Disorder

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ABSTRACT

Background: *Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairment in social interactions, language delay and repetitive behavior of interest. Inflammation plays a key role in ASD, with pro-inflammatory cytokines such as interleukin TNFα implicated in disease pathogenesis. This cytokines are produced by activated microglia and contribute to neuroinflammation, amyloid-beta plaque formation, and tau hyperphosphorylation. The diagnostic potential of TNFα in ASD remains underexplored. A number of neurological and mental problems have been associated with elevated COX-2 expression, and the enzyme is well-known to play an important role in inflammatory processes.* ***Objectives:*** *This study aims to compare serum levels of TNFα and COX2 in ASD severity cases and healthy controls and investigates the diagnostic potential of these biomarkers using one way ANOVA.* ***Methodology:*** *The study enrolled 100 participants: 50 healthy controls and 50 ASD patients diagnosed according to CARS scal criteria. Serum TNFα and COX2 levels were measured using ELISA. Binary logistic regression, were employed to evaluate the diagnostic potential of these biomarkers.* ***Results:*** *ASD patients showed significantly (p-value <0.0001) elevated levels of TNFα, and COX2 compared to healthy controls. One way ANOVA analysis identified TNFα as a significant predictor of ASD with an TNFα levels in serum of the ASD group with its three stratified categories: mild, moderate, and severe. In mild cases the TNFα level 0.4740±0.072, the moderate cases 0.584±0.105 and for sever cases was 1.15186±0.498.* ***Conclusions:*** *The study concluded that elevated TNFα levels are strongly associated with ASD severity could serve as a promising diagnostic biomarker. Cox2, while elevated, did not show comparable diagnostic value.*

INTRODUCTION

In autism spectrum disorder (ASD), social interactions are impaired, language development is delayed, and repetitive behaviors are exhibited. ASD starts usually before the age of three years. [1]

The number of autistic subjects globally was estimated at 1-2 per one thousand people[2]. Indeed, in the developing country the picture of autism is almost the same. About 1.5% of children were estimated to have autism spectrum disorder in 2017[3] .Furthermore, Autism is believed to be a sex bias. ASD is also marked by many possible autistic characteristics which included, a lack of language and social skills, a narrow range of interests, bad eye contact, a repeated pattern of actions, sensory modulatory impairment, and differing degrees of cognitive and motor abnormalities [1]

Autism is considered as a syndrome rather than a disease with a multiple nongenetic and genetic causes though there is a convincing evidence to suggest that the interacting genetic factors is the sole causative determinant with estimated heritability of about 90%[4]. There are hundreds of genes though to be involved in the expression of this disorder and could be considered as a potential candidate gene.[5]

Immunological pathways play a critical role in ASD, particularly through the activation of microglia and the release of pro-inflammatory cytokines. Microglia, the central nervous system's immune cells, respond to amyloid-beta plaques by entering a chronic inflammatory state .[6]

ASD patients commonly exhibit altered socialization, information processing, and neural connectivity due to elevated TNFα levels, although commonly part of broader pro-inflammatory processes[7].

A pleiotropic cytokine, TNFα plays an important role in the development and function of the brain[8]. There is evidence that TNFα is related to ASD, major depressive disorder, bipolar disorder, schizophrenia pathobiology[9].

As a precursor to prostaglandins and thromboxanes, cyclooxygenase (COX) converts arachidonic acid into prostaglandin H2. COX exists in two major isoforms: COX-1 is consistently termed and participates in

prostaglandin synthesis for the functions of cellular housekeeping, whereas COX-2 is an inducible form. COX-2 contributes to carcinogenesis and inflammation by catalyzing the formation of prostaglandins E2, which promote cell proliferation, inhibit apoptosis, and encourage angiogenesis [10].Growing factors, cytokines, and proinflammatory agents induce COX-2 during inflammation, and COX-2 is involved in epilepsy, seizures, normal neuronal function, neurotoxicity, synaptic plasticity, and prostanoid production in neurodegenerative processes[11]. There is constitutive expression of COX-2 in the neuronal tissues in areas that have been significantly affected by psychiatric disorders, such as the amygdala, hypothalamus, hippocampus, and forebrain.[12]

Immune and Endothelial cells in the cerebral vasculature release cytokines that negatively diffuse or straightly interact with the receptors of BBB triggered via central noradrenergic receptors, triggering cyclooxygenase2 (Cox-2) inflammatory signaling in the tissues of the brain.[13]

METHODOLOGY

In the current study, 100 participants were enrolled, comprising 50 healthy controls (HC) and 50 ASD patients .Specimens has been collected from the psychiatric Department at Al-Hakim Hospital in AL-Najaf Governorate; Hamaeem Alsalam center for autistic and slow-speaking children ; specialist Sura Rashid Haroun center for the care of people with special needs in Babel Governorate, Dr. Mustafa Al-Yasiri institute for speech and communication in Diwaniyah Governorate. According to the Diagnostic criteria of the autism CARS scale, all subjects considered typical for ASD were conscripted. The diagnosis was carried out by a consultant psychiatrist in association with the researcher in person. All the patients were sub-grouped again according to the severity of the symptoms into the following sub-groups: 1- Mild ASD (n=39) 2- Moderate ASD (n=13) 3- Severe autistic patients (n=8). The CARS scaling tool was adopted in the current study. It is a diagnostic technique that categorizes people into levels of normal and severe levels of autism. controls were children who were normal and mentally healthy, not related to the autistics. The control group included hospital staff and acquaintances of the authors. Participants were excluded if they had chronic inflammatory diseases such as rheumatoid arthritis, as well as thyroid disorders. Additionally, all individuals were free from any Axis I or II psychiatric disorders. C-reactive protein levels in all participants were below 6 mg/L, indicating no evidence of inflammation.

Clinical measurement

5 ml of venous blood using a plastic syringe with a disposable needle. The blood was stored in a serum gel tube and left at room temperature for 15 minutes before being centrifuged at 3500 rpm for 10 minutes. The resulting serum was aliquoted into small Eppendorf tubes and stored at -80°C until ELISA analysis.

ELISA kits were obtained from the manufacturing company (Sun long, China). The plate has been pre-coated with human TNFα and COX2 antibodies. To affix to antibodies coated on the wells, TNFα and COX2 are inserted into the sample. Further binding to these proteins is made possible with the addition of biotinylated human TNFα and COX2 antibodies in the sample. Finally, with a view to binding the Biotinylated TNFα and COX2 antibodies, Streptavidin-HRP is added. A wash is performed beyond incubation to remove unbound Streptavidin-HRP, a substrate solution is added, and the color is developed according to human TNFα and COX2 levels. Several minutes later, an absorbance measurement at 450 nm is performed in a Microplate Reader after the addition of the acidic stop solution to terminate the reaction.

Statistical analysis

We performed statistical analysis for this study using SPSS, version 30. Data normality was assessed with the Kolmogorov–Smirnov test, indicating that cytokine levels did not follow a normal distribution. The one-way ANOVA for continuous variables was utilized to compare them between groups. In the whole cohort (n=100), this study applied chi-square ($\chi 2$) testing.

RESULTS

A total of 50 ASD patients who were clinically diagnosed by the psychiatrist's consultant in association with the researcher in person were recruited to the present investigation. In addition, another 50 children clinically assessed as healthy were included as matched controls in both the age and gender aspects. Both samples were run in parallel to avoid any unexpected experimental errors. The ages of the ASD patients and the normal healthy control were in the range of (3-13years). The parents who agreed to allowed their children to participate in the study were asked to fill pre-written questioners form as stated in the materials and methods section. The details of the questioners and the parent's answers are grouped in Appendix 2.

Demographic parameters

Gender

Gender has a strong significant association with ASD. The number of autistic male patients (41) was significantly higher than that of autistic female patients (9). Figure 1 demonstrated the distribution of autistic patients in relation to the gender among the ASD group. Remarkably, the percent of male autistic patients and female autistic patients 82%, 18% respectively among the total autistic 50 patients. Male patients which represent about four time higher than that of the female's patients. The male to female ratio is 5:1.

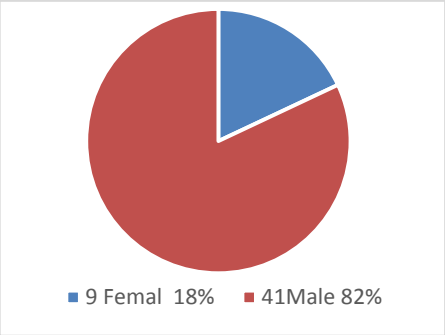

Fig. 1: Demonstrated the distribution of autistic patients in relation to the gender among the ASD group

1. TNFα

The level of TNFα, were compared among cases with different severity of autism. (mild, moderate and severe). TNFα levels among the ASD group was estimated to investigate high correlation with the degree of severity of the symptoms. Table (1) showed Data are presented as a mean ± SD of 50 autistic cases with autism divided into three subgroups the TNFα levels in serum of the ASD group with its three stratified categories: mild, moderate, and severe was reported . In mild cases the TNFα level was 0.4740±0.072, the moderate cases. 0.584±0.105 and for sever cases 1.15186±0.498. Remarkably, significant difference in the levels TNFα was estimated .The p-value for the comparison between the case and control groups is 0.0001, this indicates that the difference in TNFα levels between the case and control groups is statistically high significant.

Table 1: Serum TNFα level in patients according to the severity of autism

	Mean ± SD				P-value
	Case			Control	
TNFα	Mild ASD	Moderate ASD	Sever ASD	0.310±0.040	0.0001
	0.4740±0.072	0.584±0.105®	1.15186±0.498®		
	±	±	±	±	

2. COX2

Levels of COX2, was compared among cases with different severity of autism. (mild, moderate and severe). **COX2** levels among the ASD group was estimated to investigate high correlation with the degree of severity of the symptoms. Table (2) showed Data are presented as a mean ± SD of 50 autistic cases with autism divided into three subgroups the **COX2** levels in serum of the ASD group with its three stratified categories: mild, moderate, and severe . The p-value for the comparison between the case and control groups is 0.0001, this indicates that the difference in **COX2** levels between the case and control groups is statistically high significant.

Table 2: Serum COX-2 level in patients according to the severity of autism

	Mean ± SD				P-value
	Case			Control	
COX2	Mild	Moderate	Severe	0.392±0.135	0.0001
	0.517±0.295	0.585±0.334	1.188±0.630		
	±	±	±	±	

DISCUSSION

TNFα is one of the most studied cytokines, which has several crucial physiological and pathophysiological functions, such as in cell differentiation, neurogenesis, cell survival/apoptosis, and brain development[14]. Accordingly, TNFα can affect major mechanisms of ASD pathophysiology including cognitive performances[15]. High TNFα levels have been detected in the gastrointestinal tract, brain, and plasma in subjects with ASD[16]. Heightened TNFα levels contribute to altered socialization, information processing, and neural connectivity despite being commonly seen as a part of wider pro-inflammatory processes.[17]

TNFα as a pleiotropic cytokine plays an important role in controlling the development and function of the brain[8]. It had been revealed that ASD, major depression, bipolar disorder, and the pathobiology of schizophrenia have all been linked to TNFα[18].

Moreover, high expression of TNFα in serum has already been detected in ASD patients. Researchers found elevated levels of IL-6, TNFα, and interleukin-1-beta (IL-1-β) in the brain and cerebrospinal fluid of postmortem individuals with autism spectrum disorders[19] .

Brain tissue, among other tissues, is heavily dependent on COX-2 for inflammatory reactions[19]. Pro-inflammatory molecules induce COX-2 expression, which produces prostanoids in acute and chronic inflammation [20]

The catalytic activity of COX-2 may be involved the pathological processes of carcinogenesis and inflammation by synthesizing prostaglandin E2, which stimulates cell proliferation, inhibits apoptosis and encourages angiogenesis [21]

Within inflammation, growth factors, cytokines, and proinflammatory agents induce COX-2, which has been implicated in the neurodegenerative formation of prostanoids in processes, synaptic plasticity, neurotoxicity, normal neuronal functioning, and seizures [22]

The neuronal tissues of COX-2 constitutive expression are regions that have critical involvement in psychiatric disorders, including the hypothalamus, hippocampus, and forebrain [13].

Circulating cytokines liberated by the vessel endothelium and circulating immune cells can freely diffuse or directly engage BBB receptors that are activated by the central noradrenergic system to induce cyclooxygenase2 (Cox-2) inflammatory signaling within the brain tissues [13]. Furthermore, peripheral cytokines can likewise affix to receptors on the nodose ganglion, liver, or spleen to transmit cytokine signals to the brain through afferent fibers of the vagus nerve, which will trigger neural activity or stimulate microglia to produce IL-6.[23]

The bidirectional correspondence between the brain and the immune system has been taken as the underlying rational concept wherein inflammation, when dysregulated in nature, is responsible for psychiatric and neurological disorders. The nonsteroidal anti-inflammatory drugs (NSAIDs), and in particular celecoxib, a selective Cox-2 inhibitor, have been suggested to improve the efficacy of conventional psychiatric medications since they inhibit cyclooxygenase-2[24 25].

CONCLUSIONS

The highest distribution of ASD were fuighen in males than females, the male to female ratio is 5:1 .Family history is still an effective independent predictor of ASD onset. These discoveries can aid in ASD risk assessment, ASD prediction tools improvement, and ASD pathogenesis studies. The interleukins TNFα, COX2 were found in considerably greater concentrations among participants than healthy controls, proposing these tragedies a key heroine progression through ASD.

Ethical Approval Declaration

The procedures followed in this study were in accordance with the regulations of the relevant clinical research ethics committee In addition, each participant provided written consent following a concise overview of the project.

Declarations:

Consent for publication: Not applicable Availability of data and material: Data are available upon request. Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article. This manuscript has not been previously published and is not under consideration in another journal.

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REFERENCES

1. Hirota T, King BH. Autism spectrum disorder: a review. 2023; 32(9) :157–168 .
2. Zhong C, Rando J, Patti MA, Braun JM, Chen A, Xu Y, Lyall K. Gestational thyroid hormones and autism-related traits in the EARLI and HOME studies. Autism Research. 2024;(4):716-727.
3. Shaw KA. Early identification of autism spectrum disorder among children aged 4 years—Early Autism and Developmental Disabilities Monitoring Network, six sites, United States, 2016. MMWR. Surveill. Summ. 2020; 69:434-438.
4. Saxena A, Chahrour M. Autism spectrum disorder. in Genomic and Precision Medicine Elsevier.2017; 8 :301–316.
5. Zhu M, Zhao S. Candidate gene identification approach: progress and challenges. Int. J. Biol. Sci .2007;3:420-422.
6. Gupta N. et al. Recent progress in therapeutic strategies for microglia-mediated neuroinflammation in neuropathologies. Expert Opin. Ther. Targets.2018;2(2) :765–781.
7. Holston H, Karvat A, Lallian S, Wu P. Anti-Inflammatory Interventions for Autism Spectrum Disorder. Berkeley Pharma Tech J. Med.. 2024;4: 59–82 .
8. Gonzalez Caldito, N. Role of tumor necrosis factor-alpha in the central nervous system: A focus on autoimmune disorders. Front. Immunol. 2023;14:1213-448.
9. Sethi R, Gómez-Coronado N, Walker AJ, Robertson ODA, Agustini B, Berk M, Dodd S. Neurobiology and therapeutic potential of cyclooxygenase-2 (COX-2) inhibitors for inflammation in neuropsychiatric disorders. Front.

psychiatry.2019; 10:605-607.

10. Faki Y, Er A. Different chemical structures and physiological/pathological roles of cyclooxygenases. Rambam Maimonides Med. J.2021; 12:34-37.
11. Vishwakarma S, Singh S, Singh TG. Pharmacological modulation of cytokines correlating neuroinflammatory cascades in epileptogenesis. Mol. Biol. Rep.2022; 49:1437–1452.
12. Stachowicz K. Deciphering the mechanisms of regulation of an excitatory synapse via cyclooxygenase-2. A review. Biochem. Pharmacol.2021; 192:714-729.
13. Ballaz S, Bourin M. Anti-inflammatory therapy as a promising target in neuropsychiatric disorders. Neuroinflammation, Gut-Brain Axis Immun. Neuropsychiatr. Disord. 2023;45: 459–486.
14. Parkitny L, Maletic-Savatic M. Glial PAMPering and DAMPening of Adult Hippocampal Neurogenesis. Brain Sci. 2021 11:1299 -2002.
15. Singh D, Vignat J, Lorenzoni V, Eslahi M, Ginsburg O, Lauby-Secretan B, Vaccarella S. Global estimates of incidence and mortality of cervical cancer in 2020: a baseline analysis of the WHO Global Cervical Cancer Elimination Initiative. lancet Glob. Heal.2023 1 : e197–e206 .
16. Ashwood P. Preliminary findings of elevated inflammatory plasma cytokines in children with autism who have co-morbid gastrointestinal symptoms. Biomedicines.2023; 11:436-440.
17. Suprunowicz M, Tomaszek N, Urbaniak A, Zackiewicz K, Modzelewski S, Waszkiewicz N. Between Dysbiosis, Maternal Immune Activation and Autism: Is There a Common Pathway? Nutrients. 2024;16: 549-552.
18. Saleki K, Alijanizadeh P, Javanmehr N, Rezaei N. The role of Toll-like receptors in neuropsychiatric disorders: Immunopathology, treatment, and management. Med. Res. Rev.2024;44 :1267–1325 .
19. Ghaffari M, Ganji M, Nikkhah Ghamsari N. A reflection in sociology in Iran; systematic review of the studies. Q. J Res Plan High. Educ.2023; 27 : 173–205.
20. Vishwakarma RK, Negi DS. The development of COX-1 and COX-2 inhibitors: a review. Int J Pharm Sci Res .2020;11: 3544-3549.
21. Mahboubi-Rabbani M, Abdolghaffari AH, Ghesmati M, Amini A, Zarghi A. Selective COX-2 inhibitors as anticancer agents: a patent review (2018-2023). Expert Opin. Ther. Pat.2024; 34 : 733–757.
22. Hiskens MI, Schneiders AG, Fenning AS. Selective COX-2 Inhibitors as Neuroprotective Agents in Traumatic Brain Injury. Biomedicines. 2024;12 :1930-1934.
23. Garrett AD. A Toolbox for Observing and Modulating the Gut-Brain Axis. at (2022).p/67.
24. Bottaccioli AG, Bologna M, Bottaccioli F. Psychic life-biological molecule bidirectional relationship: pathways, mechanisms, and consequences for medical and psychological sciences—a narrative review. Int J Mol Sci.2022; 23 : 3932-3936.
25. Perrone MG, Centonze A, Miciaccia M, Ferorelli S, Scilimati A. Cyclooxygenase inhibition safety and efficacy in inflammation-based psychiatric disorders. Molecules.2020; 25 : 5388-5391.