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Serum cytokine levels in children with spectrum autism disorder: Differences in pro- and anti-inflammatory balance

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ABSTRACT

Background: Autism Spectrum Disorders (ASDs) is a developmental and neurological disorder that affects all aspects of social communication, with limited and stereotypical interest, and atypical responses to sensory stimuli. Diagnosis of ASD is currently phenotype based with no reliable laboratory test available to assist clinicians. Researches have shown that individuals with autism often exhibit dysfunction of cytokines.

Methods: A total of 42 patients with ASD and 20 matched controls participants were recruited for the study. Diagnosis was conducted by medical specialists and based on the International Classification of Mental and Behavioral Disorders – ICD-10, DSM-5 and CARS sore. Whole blood samples were collected and serum IL's and chemokin levels were made using ELISA kits.

Results: Results demonstrated that in comparison to the controls, the individuals with autism showed significantly higher concentration of IL-1β, IL-4, IL-6 and IL-13. We also demonstrated significant correlations between the levels of cytokines which implies the presence of an interactive network between them. The results of ROC analysis indicated the 4-factors (IL-1β, IL-4, IL-6 and IL-13) could be potential biomarkers in diagnosis of ASD.

Conclusions: In this study, serum levels of cytokine differed among children with ASD. However, the findings of this support the possibility of using an appropriate selection of serum cytokine for the diagnosis ASD and emphasize the need to standardize quantitative methods for serum analysis.

1. Introduction

Autism Spectrum Disorders (ASDs) is a developmental and neurological disorder that affects all aspects of social communication and interactions, with limited and stereotypical interest, and atypical responses to sensory stimuli (Lochman et al., 2018). Although the exact underlying mechanism is not clear enough and remain controversial, evidences have supported a role for dysregulation of immune system (Masi et al., 2017). Numerous studies have demonstrated changes in the cytokine levels in the blood, brain, and cerebrospinal fluid (CSF) of ASD patients compared with healthy subjects (Xu et al., 2015). However, it is quite possible that ASDs are a manifestation of atypical development involving the nervous system, endocrine system, immune system (Ashwood et al., 2011; Goines & Ashwood, 2013). The findings of previously published studies have shown a role for the immune system in the pathogenesis of at least a subset of cases of ASD (ghjn Bjørklund et al., 2016; K Xu et al., 2015). Immunological factors contribute to ASD at multiple levels. In terms of genetic, immune molecules, including cytokines and genes within the major histocompatibility complex, have been associated with ASD susceptibility (Torres et al., 2016). Furthermore, many classic immune molecules have newly identified functions in neural development and plasticity (Estes and McAllister, 2015). However, regarding the possibility of using the measurement of serum or plasma cytokines for diagnosis and prognosis in patients with ASD, some of the findings of these studies have been contradictory (Ashwood et al., 2011).

Because the pathogenesis of ASD is a complex process that may begin in the prenatal period, this is a time that the immune system could play an important role. A study was designed with the framework of an ongoing project at the Regional Children's Hospital in Olsztyn and

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Table 1
Characteristics of serum cytokines evaluated in this study, Kordulewska et al., 2018a, 2018b.

Cytokine	Producing cells	Receptors and receptors-bearing cells	Function
IL-1β	Produced by variety of cell types but vast majority of studies have focussed on its production within cells of the innate immune system:monocytes and macrophages	IL-1RI; IL-1RII	IL-1 is primarily an inflammatory cytokine. It belongs to a group of cytokines with overlapping biologic properties (TNF-α and IL-6). IL-1 with TNF and IL-6 share the ability to stimulate T and B lymphocytes, increase cell proliferation, and initiate or suppress gene expression for proteins. IL-1 are ability to induce fever, sleep, anorexia and hypotension. What is more, stimulates the release of pituitary hormones, increases the synthesis of collagenases, resulting in the destruction of cartilage, and stimulates the production of prostaglandins, leading to decrease in the pain threshold. In addition IL-1 has some host-defense properties (Feghali and Wright, 1997, Dinarello, 2002).
IL-4	Produced by activated lymphocytes (Th2), mast cells, basophils and NK.	IL-2Rγ; IL-13Rα1	IL-4 has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into plasma cells. It is a key regulator in humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE, and up-regulates MHC class II production. IL-4 decreases the production of Th1 cells, macrophages, IFN-γ and dendritic cell IL-12 (Feghali and Wright, 1997; Braddock et al., 2018).
IL-6	IL-6 is secreted by T cells and macrophages to stimulate immune response.	IL-6Rα chain (CD126), and the signal-transducing component gp130 (called CD130).	IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier and initiating synthesis of PGE_2 in the hypothalamus, thereby changing the body's temperature set-point. In muscle and fatty tissue, IL-6 stimulates energy mobilization that leads to increase body temperature. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to a pathogen-associated molecular patterns (PAMPs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production. IL-6 is responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow (Feghali and Wright, 1997; Ghasemi, 2018).
IL-8	Is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells.	CXCRI; CXCRII	IL-8, also known as *neutrophil chemotactic factor*, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. IL-8 is also known to be a potent promoter of angiogenesis. In target cells, IL-8 induces a series of physiological responses required for migration and phagocytosis, such as increases in intracellular Ca^{2+}, exocytosis (e.g. histamine release). IL-8 is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory tract disease caused by viral infection (Feghali and Wright, 1997; Meniailo et al., 2018).
IL-10	Is primarily produced by monocytes and, to a lesser extent, lymphocytes Th2, mast cells, $CD4^+CD25^+Foxp3^+$ regulatory T cells, and in a certain subset of activated T and B cells	IL-10 signals through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins	IL-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway (Feghali and Wright, 1997; Moore et al., 2001)
IL-13	IL-13 is a cytokine secreted by Th2 cells, CD4 cells, Natural killer T cell, Mast cell, Basophil cells, Eosinophil cells and Nuocyte cells	IL-13Rα1	IL-13 is a central regulator in IgE synthesis, goblet cell hyperplasia, mucus hypersecretion, airway hyperresponsiveness, fibrosis and chitinase up-regulation. It is a mediator of allergic inflammation and different diseases including asthma (Feghali and Wright, 1997; Braddock et al., 2018).
TNF-α	It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4 + lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons	TNF can bind two receptors, TNFR1 (TNF receptor type 1; CD120a; p55/60) and TNFR2 (TNF receptor type 2; CD120b; p75/80).	The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via −1 and IL-6 producing cells. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease, cancer, major depression, psoriasis and inflammatory bowel disease (IBD).Though controversial, studies of depression and IBD are currently being linked to TNF levels (Feghali and Wright, 1997; Leung and Cahill, 2010).

(*continued on next page*)

Table 1 (*continued*)

Cytokine	Producing cells	Receptors and receptors-bearing cells	Function
IFN-γ	IFNγ is secreted by T helper cells (specifically, T_h1 cells), cytotoxic T cells (T_C cells), macrophages, mucosal epithelial cells and NK cells.	heterodimeric receptor consisting of Interferon gamma receptor 1 (IFNGR1) and Interferon gamma receptor 2 (IFNGR2).	IFN-γ has antiviral, immunoregulatory, and anti-tumor properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. Promotes NK cells activity, Increases antigen presentation and lysosome activity of macrophages, activates inducible nitric oxide synthase (iNOS), Induces the production of IgG2a and IgG3 from activated plasma B cells, promotes adhesion and binding required for leukocyte migration (Feghali and Wright, 1997; Harris, 2011).

IL – interleukin; IFN – interferon; TGF – transforming growth factor; TNF – tumor necrosis factor;

Center for Diagnosis, Treatment and Therapy of Autism at the Regional Children's Hospital in Olsztyn on the analysis of the immune profile of children with ASD. From these ongoing clinical studies and using data from the available published literature, a panel of cytokines were identified for serum measurements in children with ASD. The characteristics of the chosen cytokines are presented in Table 1, based on Kordulewska et al., 2018a, b.

The main aim of this study was to evaluate whether a cytokine and chemokine panel could be identified for the diagnosis and prognosis in children with diagnosed ASD, including typical autism spectrum disorder.

2. Material and methods

2.1. Ethic and consent

This study was approved by the Local Bioethics Committee (Approval No. 19/2016; 18/5/2016). For the study participants, parental informed consent was obtained. Written informed consent was obtained for the collection of serum samples and subsequent analyses from all participating families. A copy of the written consent is available for review.

2.2. Participants and autism diagnostic observation schedule

Initially, twenty control participants (mean ± SD age 7.35 ± 1.93; 7♀and 13 ♂) and forty – two children with ASD (mean ± SD age 6 ± 1.75; 7 ♀and 37 ♂) were recruited by the Center for Diagnosis, Treatment and Therapy of Autism at the Regional Children's Hospital in Olsztyn, Poland. Diagnosis was based on the International Classification of Mental and Behavioral Disorders – ICD-10 and DSM-5 and CARS sore (Table 2). F84 disease in children was identified on the basis of interdisciplinary differential diagnosis: psychiatric examination excluding mental illness; studies evaluating cognitive parameters in the respondents; neurological examination - EEG, evaluation of reflexes; speech therapy – evaluation study of the development of speech; passive and participatory observation lasting from 6 to 12 months; analysis of the documentation: names of parents, the opinions of educational institutions, video. The IQ level was evaluated by the test of cognitive development: the Leiter test and the Wechsler test (Leiter scale - standard IQ from 70 to 107; Wechsler – standard IQ from 90 to 104). This choice was dictated by the communication abilities of autistic children and their biological age. The Wechsler test was used in older children in full verbal contact with a recognized Asperger syndrome. The Leiter test was used for younger, not talking children with Autism. In ASD group were found most children with IQ's of 70–104 and 9 children with IQ's of < 70, which indicated mental retardation. The article was used to generalize the term ASD, as used in the DSM V nomenclature. In Poland, the diagnostic classification in medical centers is Class ICD-10. Typically developing children were screened for history of neurological, psychiatric and developmental disorders and all were un-medicated (including no psychotropic medication) and in good health at the time of participation. Each patient also has a basic neurological examination and an EEG. As a result of the diagnostic process, patients were afflicted with F84.0 code of ASD in the World Classification of Mental Disorders and Behavioral Disorders (ICD-10). Exclusion criteria were known neurological disorders including fragile-X syndrome and tuberous sclerosis, congenital metabolic disorders, chronic infectious diseases

Table 2
Characteristics of the study participants with autism spectrum disorder (ASD), and control group.

Children with diagnosed ASD					Control group		
ID sample	Age(yrs)	Sex	CARS	ASD	ID sample	Age (yrs)	Sex
ASD1	3	♂	–	F.84.0	C1	10	♀
ASD2	4	♂	–	F.84.0	C2	8	♂
ASD3	6	♂	–	F.84.5	C3	8	♂
ASD4	a	♂	–	F.84.0	C4	11	♂
ASD5	8	♂	–	F.84.0	C5	12	♂
ASD6	3	♂	–	F.84.0	C6	8	♂
ASD7	9	♂	–	F.84.0	C7	7	♂
ASD8	6	♂	–	F.84.5	C8	6	♂
ASD9	5	♂	–	F.84.0	C9	8	♀
ASD10	6	♂	–	F.84.0	C10	5	♂
ASD11	8	♂	–	F.84.5	C11	5	♂
ASD12	7	♂	–	F.84.5	C12	5	♀
ASD13	3	♀	–	F.84.0	C13	5	♂
ASD14	8	♂	–	F.84.0	C14	7	♂
ASD15	10	♀	–	F.84.0	C15	8	♀
ASD16	7	♀	–	F.84.0	C16	6	♂
ASD17	3	♂	–	F.84.0	C17	8	♀
ASD18	7	♂	–	F.84.0	C18	7	♂
ASD19	6	♀	–	F.84.0	C19	7	♀
ASD20	3	♀	–	F.84.0	C20	6	♀
ASD21	5	♂	45.5	F.84.1			
ASD22	4	♀	40.5	F.84.0			
ASD23	10	♂	30.5	F.84.5			
ASD24	10	♂	37.5	F.84.0			
ASD25	8	♂	31.5	F.84.0			
ASD26	5	♂	35.0	F.84.0			
ASD27	5	♂	44.5	F.84.0			
ASD28	6	♂	40.5	F.84.0			
ASD29	7	♀	40.5	F.84.0			
ASD30	3	♂	40.5	F.84.0			
ASD31	4	♂	31.5	F.84.0			
ASD32	6	♂	45.5	F.84.5			
ASD33	3	♂	31.5	F.84.0			
ASD34	8	♂	44.0	F.84.5			
ASD35	3	♂	31.5	F.84.0			
ASD36	9	♂	41.5	F.84.0			
ASD37	6	♂	42.5	F.84.5			
ASD38	3	♂	30.0	F.84.5			
ASD39	8	♂	30.0	F.84.0			
ASD40	3	♂	45.5	F.84.5			
ASD41	9	♂	30.5	F.84.1			
ASD42	6	♂	30.5	F.84.5			

ASD – autism spectrum disorder; ♂– male; ♀ – female; CARS – Childhood Autism Rating Scale; F84.0 – typical childhood ASD, according to the ICD-10; F84.1 – atypical ASD, according to ICD-10; F84.5 - Asperger syndrome (AS), according to ICD-10.

such as tuberculosis, acute infectious disease within the last 4 weeks, immunization within the last 8 weeks and immune-modulating medication in the previous four weeks.

All children who participated in our study underwent routine laboratory parameters, including serum biochemistry, and a complete blood count (CBC), including C-reactive protein (CRP). None of the children in our study were undergoing any treatment with medications for ASD children. Participants were excluded from the study if they had any other additional diagnoses of psychiatric or neurological disease. The characteristics of the chosen cytokines and growth factors, and their expected normal values are described in Table 1.

2.3. Serum collection and cytokine measurements

Whole blood samples were collected in Vacutainers (BD Bioscience, CA) with a clotting activator. Centrifuge at 2.000 *g* for 10 min at 17 °C, after that time we collected supernatants (serum) and aliquoted and stored at −80 °C, as describes before Kordulewska et al. (2015, 2016, 2017).

Serum IL's and chemokin levels were made using enzyme-linked immunosorbent assay (ELISA) kits (Diaclone, MabTech and BD) according to the manufacturer instructions. Triplicate samples were run and the results were equalised by comparison with standard curves expressed in pg/ml. Serum measurements in both tested group of study participants included: interleukin-1β (IL-1β); interlukin-4 (IL-4); interleukin-6 (IL-6); interlukin-8 (IL-8); interlukin-10 (IL-10); interlukin-13 (IL-13); tumor necrosis factor-?? (TNF-??); interferon-?? (IFN-??) (Table 1.)

2.4. Statistical analysis

Analysis and evaluation of measured data were performed using GraphPad Prism 7 (GraphPad Software, Inc., USA) and SPSS 17.0 statistical software (SPSS Inc., Chicago; IL); with results presented as mean ± SEM, and the mean values between control and ASD groups. A p value of < 0.01 was considered significant. We used Mann-Whitney nonparametric test for cytokin analyzed data. To test sensitivity and specifity of the biological markers of measured cytokines to detect ASD, receiver operating characteristic (ROC) analysis were perfomred. The correaltion between true positive rate (sensitivity) and false-positive rate (1-specificity) was represented as a curve. The cut-off point was choosen to minimize the sum of false-positive and false-negative test results. Efficient screening instruments are indicated by ROC curver with high area under the curve (AUC). Sperman rank order correlation analysis were used to dermine the relationship between analyzed parametres.

3. Results

3.1. Concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, TNF-α, IFN-γ

We observed significant 4-fold increase in IL-1β concentration in the ASD group compared to the control group ($p < .001$). In control group the mean level of IL-1β was 47 pg/ml while in ASD group 230 pg/ml (Fig. 1A).

Significant differences were noted in serum IL-4 between control and ASD groups ($P < .0001$) (Fig. 1B). The mean value in control group was 54 pg/ml while in ASD group 183 pg/ml.

We observed significant 2.2-fold increase in IL-6 concentration in the ASD group compared to the control group ($p < .001$). In control group the mean level of IL-6 was 210 pg/ml while in ASD group 479 pg/ml (Fig. 1C).

No significant difference was established in control and ASD group. While we noted highest level of IL-8 in ASD serum samples. Mean level of children with diagnosis ASD was 102 pg/mL, when in control participants 65 pg/ml (Fig. 1D).

No significant difference has been found in IL-10 concentration between control and ASD children. However, the highest level of IL-10 was observed in ASD children, where mean was 108 pg/ml, and in participants children was 61 pg/ml (Fig. 1E).

IL-13 concentration has been shown to be significantly 5.5-fold upregulated in children with diagnosed ASD ($p < .0001$) (Fig. 1F).

No significant differences had been found in TNF-α concentration in serum between children with ASD and control (Fig. 1G).

No significant difference was established in control and ASD group in concentration of IFN-γ (Fig. 1H).

3.2. Correlation analysis between the levels of cytokines

As revealed by Spearman correlation analysis significant correlations have been found between cytokine levels. ASD patients for correlation analysis were placed in two groups: (Abdallah et al., 2013a) according to the CARS scale ($n = 22$) – Fig. 2A, (Abdallah et al., 2013b) based on ICD-10 classification ($n = 42$) – Fig. 2B and control participants ($n = 20$) – Fig. 3C. For autistic patients with define CARS scale we observed positive correaltion between level of IL-1β and IL-8 concentration ($R = 0.505$, $p < .05$) and IL-13 (0.657, $p < .01$), IL-6 and IL-13 (0.746, $p < .001$) (Fig. 2A). In ASD group according to ICD-10 classification we noted correlations in concentration of IL-1β and IL-6 ($R = 0.356$, $p < .05$), IL-8 ($R = 0.340$, $p < .05$), and IL-13 ($R = 0.511$, $p <$ 0,001). Negative correaltion was observed in concentration of IL-4 and Il-8 ($R = -0.334$, $p < .05$), and IL-6 concentration and IL-8 level ($R = -0.318$, $p < .05$). While positive correaltion between IL-6 and IL-13 ($R = 0.564$, $p < .001$). The same, positive correaltion was noted in IL-8 and IL-10 ($R = 0.454$, $p < .001$) and IFN-?? ($R = 0.610$, $p < .0001$), and IL-10 and IFN-?? ($R = 0.387$, $p < .05$) (Fig. 2B). We did not observe, any correlation in control group (Fig. 2C).

3.3. ROC curve analysis

To assess the usefulness of these factors as adjunct in the diagnosis of ASD, an ROC analysis was made. The optional cut-off for using IL-1β as a biomarker for ASD was 39.53 pg/mL. The cut-point was associated with a sensitivity of 81%and specificity 75% (AUC = 0.79; 95% Cl, 0.67–0.91, $p < .0003$) (Fig. 3A, Table 3). The optimal cut-off point for using IL-4 as a biomarker for ASD was 66.53 pg/ml. The cut-off point was associated with a sensitivity of 85%and specificity 90% (AUC = 0.86; 95% Cl, 0.75–0.95, $p < .001$) (Fig. 3B, Table 3). The optimal cut-off point for using IL-6 as a biomarker for ASD was 304.8 pg/ml. The cut-off point was associated with a sensitivity of 76% and specificity 85% (AUC = 0.79; 95% Cl, 0.67–0.90, $p < .001$) (Fig. 3C, Table 3). The optimal cut-off point for using IL-8 as a biomarker for ASD was 45.3 pg/ml. The cut-off point was associated with a sensitivity of 53% and specificity 75% (AUC = 0.54; 95% Cl, 0.40–0.68, $p = .632$) (Fig. 3D, Table 3). The optimal cut-off point for using IL-10 as a biomarker for ASD was 55.98 pg/ml. The cut-off point was associated with a sensitivity of 52%and specificity 55% (AUC = 0.53; 95% Cl, 0.39–0.67, $p = .735$) (Fig. 3E, Table 3). The optimal cut-off point for using IL-13 as a biomarker for ASD was 102 pg/ml. The cut-off point was associated with a sensitivity of 81% and specificity 80% (AUC = 0.90; 95% Cl, 0.81–0.98, $p < .001$) (Fig. 3F, Table 3). The optimal cut-off point for using TNF-?? as a biomarker for ASD was 91.07 pg/ml. The cut-off point was associated with a sensitivity of 63%and specificity 80% (AUC = 0.62; 95% Cl, 0.48–0.76, $p = .114$) (Fig. 3G, Table 3). The optimal cut-off point for using IFN-?? as a biomarker for ASD was 341 pg/ml. The cut-off point was associated with a sensitivity of 51% and specificity 65% (AUC = 0.54; 95% Cl, 0.40–0.68, $p = .580$) (Fig. 3H, Table 3).

In addition, the diagnostic ability was significantly improved by the combination of 4-factors were noted correlation between ASD patients and control participants and all 8-factors measured in plasma. The

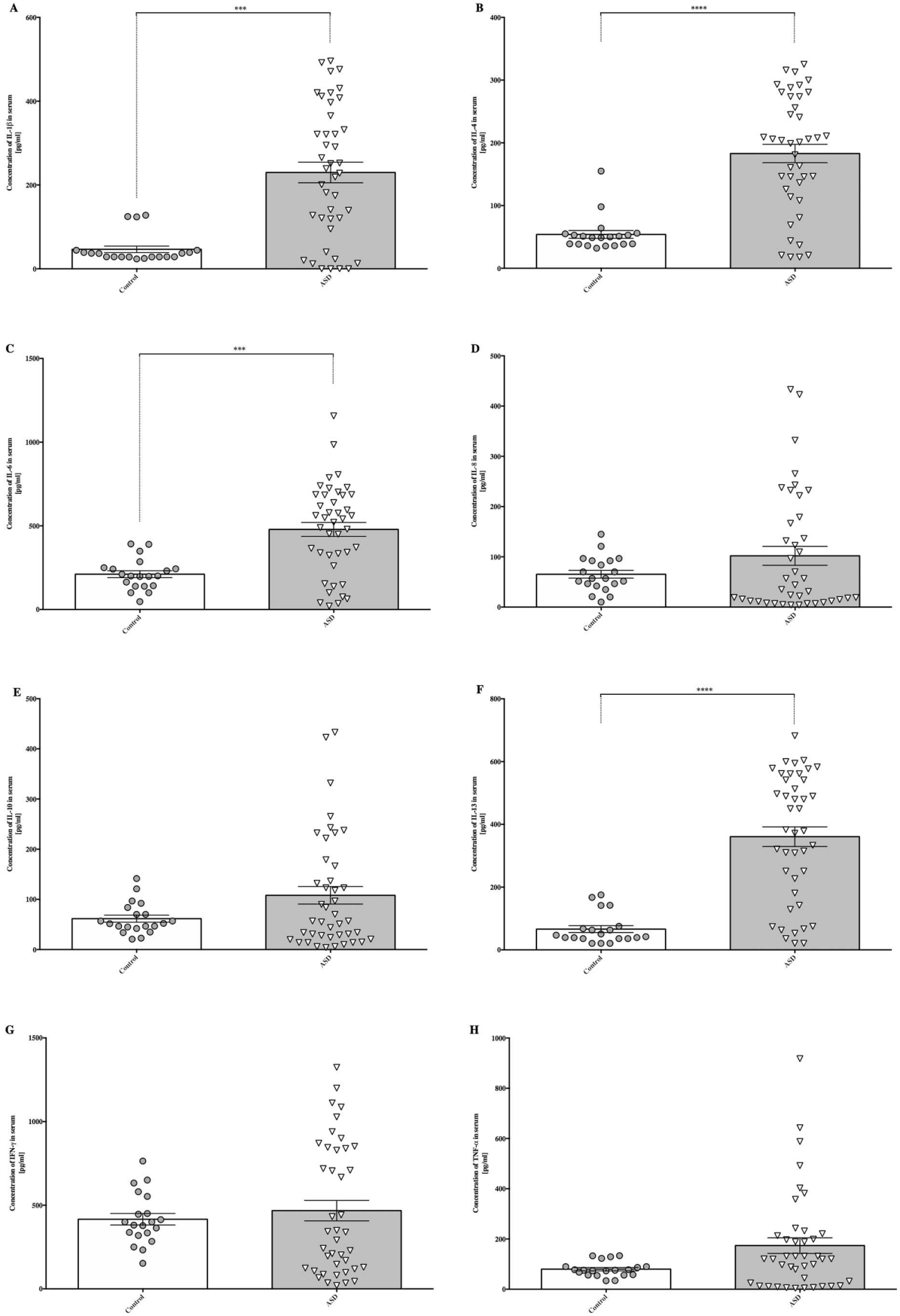

Fig. 1. Mean serum and individual results of the subject concentrations of interleukin: (A) IL-1β, (B) IL-4, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) TNF-??, (H) IFN-??. Data are presented as the mean ± S.E.M. *** $p < .001$, **** $p < .0001$ vs. control.

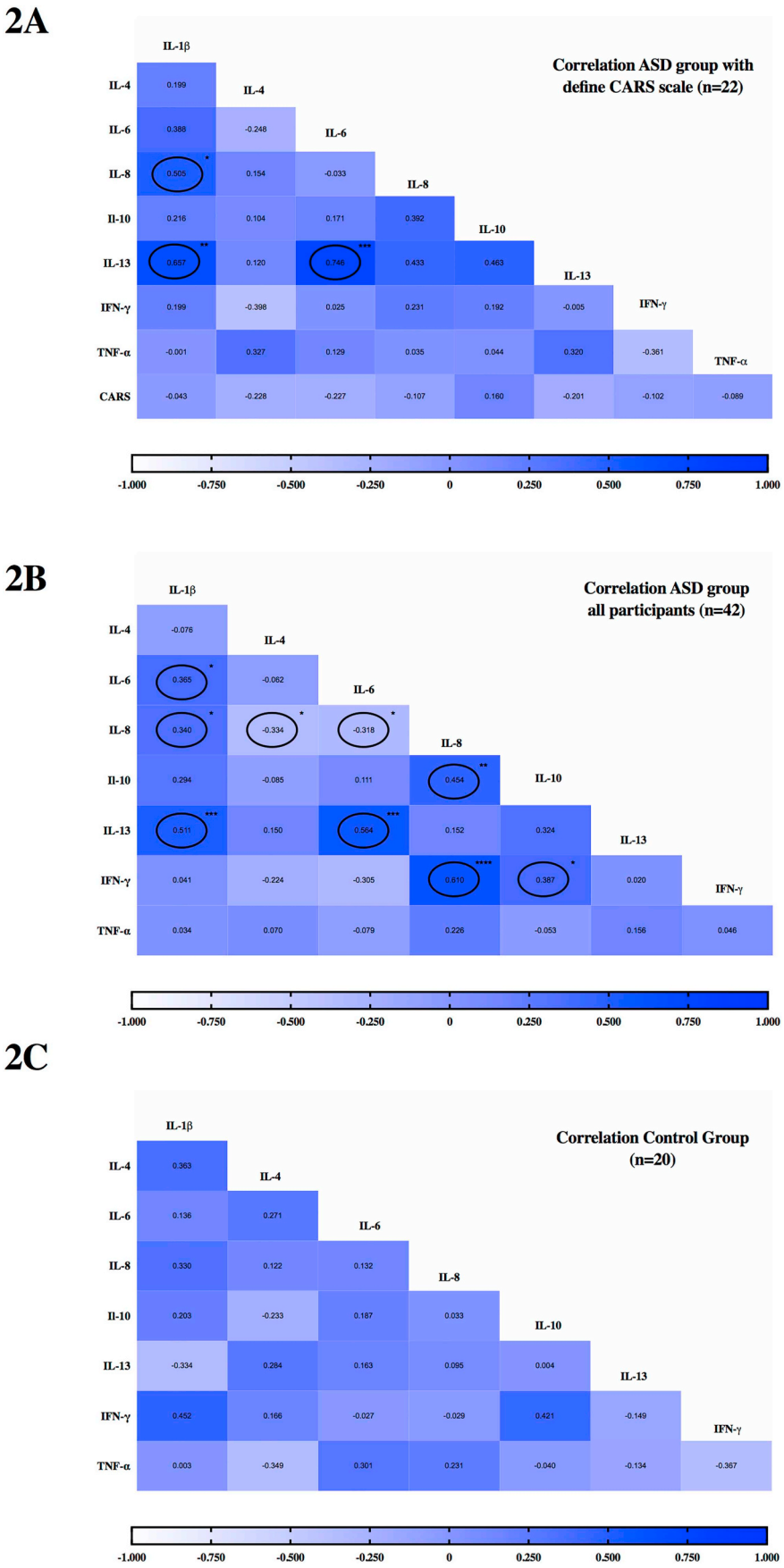

Fig. 2. Correlation of cytokine levels using the Childhood Autism Rating Scale (CARS) in children with autism spectrum disorder (ASD), and healthy, control participants. Positive correlations are shown in blue and negative in white. The intensity of the color increases with the significance of correlations. Correlation coefficients are given inside the squares. Statistically significant correlations are highlighted by white circles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

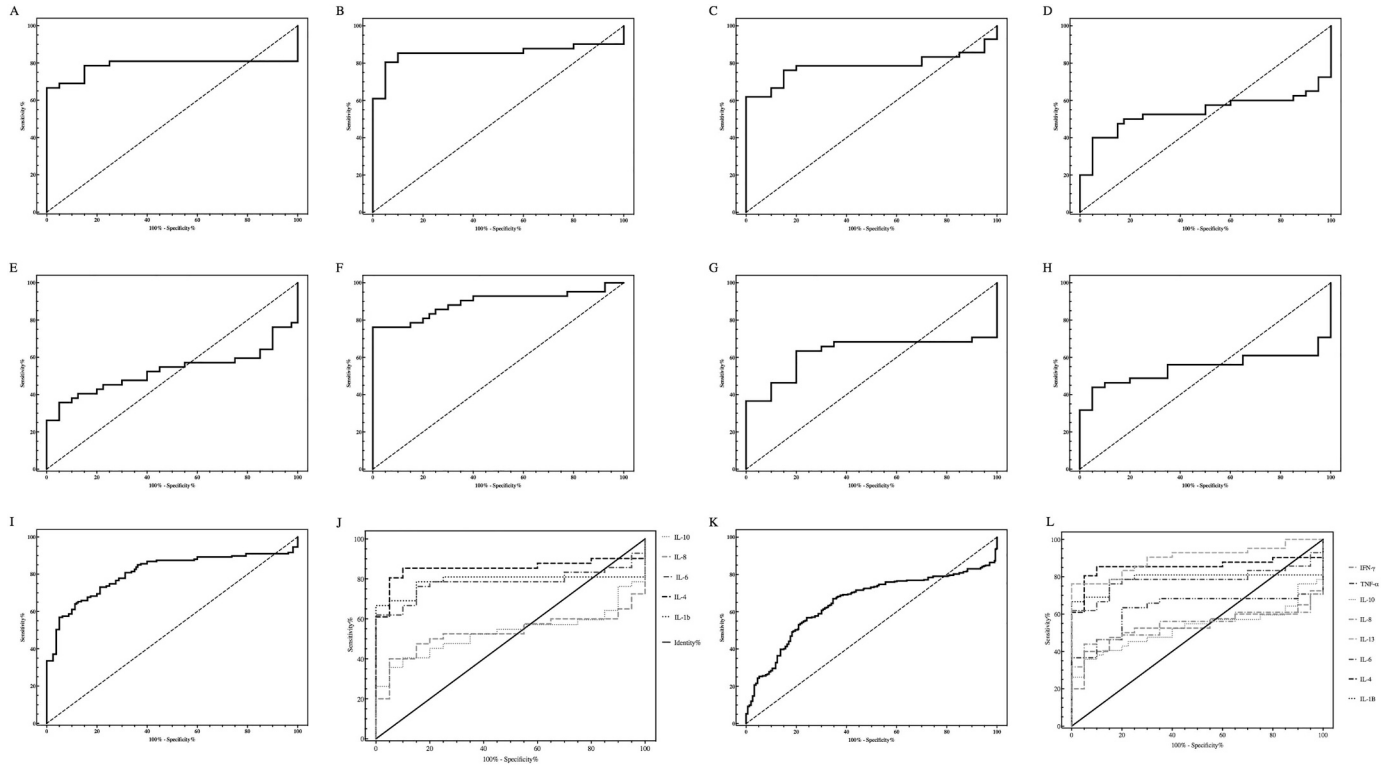

Fig. 3. Receiver operating characteristic (ROC) curves for: (A) IL-1β, (B) IL-4, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) TNF-??, (H) IFN-??, (I) combined of 4-factors (IL-1β, IL-4, IL-6, IL-13) as a biomarkers for ASD – together, (J) separate every analysis and (K) combined of all 8-factors (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, TNF-??, IFN-??) as a biomarkers for ASD – together and (K) separate every analysis and (L).

combination of 4-factors had a sensitivity of 88% and specificity 81% (AUC = 0.85; 9 5% Cl, 0.75–0.90, p < .001(Fig. 3I, J, Table 3).). While, the combination of 8-factors had a sensitivity of 67% and specificity 66% (AUC = 0.68; 95% Cl, 0.60–0.70, p < .001) (Fig. 3K, L, Table 3).

4. Discussion

The purpose of this study was to explore the plausible use of biomarkers in assisting the diagnosis of ASD and define levels of cytokine concentration in serum from ASD patients and control participants. The results of this study showed that serum levels of ILs are different in healthy controls and children with diagnosed ASD. We demonstrated a trend toward higher concentration of all tested ILs, while significant differences in IL-1β, IL-4, IL-6, IL-13. We also observed correlations between the levels of cytokines which implies the presence of an interactive network between them. The ROC analysis indicated the IL-1β, IL-4, IL-6, IL-13 were the potential biomarkers in diagnosis ASD.

Elevations of these cytokines suggest that the children with ASD have a heightened immune response. With the significant elevations observed in IL-4, IL-13 and IL-1β and IL-6, this response appears to be predominantly, but not exclusively, Th2 in origin.

Such changes in IL values can contribute in systemic inflammation observed in ASD children. Previously, animal studies have revealed a role for severe inflammatory pain and sustained inflammatory responses in development delays and psychiatric disorders such as ASD (Lee et al., 2016). In recent days, findings show evidence of inflammation in brain tissue in ASD, are evidenced by biomarkers of inflammation in the Cerebrospinal fluid (CSF) and blood of individuals diagnosed with an ASD (Chez et al., 2007). Neuroinflammation, could be characterized by increased expression and/or release of cytokines and chemokines (Monnet-Tschudi et al., 2011). Furthermore, several evidences pointed to the fact that peripheral cytokines can come into the brain (Kern et al., 2016).

In our study we demonstrated that level of IL-1β is significantly higher in ASD children than in control participants. The same results

Table 3
Values of area under the ROC curve (AUC), sensitivity and specify for the optional cut-off point.

Variables	AUC	95% Cl	*p*-Value	Sensitivity (%)	Specificity (%)	Cut-off point
IL-1β (pg/mL)	0.79	0.67–0.91	< 0.0003	81	75	> 39.53
IL-4 (pg/mL)	0.86	0.75–0.95	< 0.001	85	90	> 66.53
IL-6 (pg/mL)	0.79	0.67–0.90	< 0.001	76	85	> 304.8
IL-8 (pg/mL)	0.54	0.40–0.68	0.632	53	75	< 45.3
IL-10 (pg/mL)	0.53	0.39–0.67	0.735	52	55	< 55.98
IL-13 (pg/mL)	0.90	0.81–0.98	< 0.001	81	80	> 102
TNF-α (pg/mL)	0.62	0.48–0.76	0.114	63	80	> 91.07
IFN-γ (pg/mL)	0.54	0.40–0.68	0.580	51	65	< 341
Combination of 4-factors where we noted correlation (IL-1β; IL-4; IL-6; IL-13 pg/mL)	0.85	0.75–0.90	< 0.001	88	81	< 100
Combination of all factors where we noted correlation (IL-1β; IL-4; IL-6; IL-8; IL-13; IL-10; TNF-α; IFN-γ pg/mL)	0.68	0.60–0.70	< 0.001	67	66	< 104

obtained and Jyonouchi et al. (2001) and his group in peripheral blood mononuclear cells (PBMCs) from children with ASD. Incubated cells secreted significantly higher amounts of soluble IL-1 β compared with controls. IL-1 β has also been shown to play a key role in mediating severe placental damage and neurodevelopmental anomalies. IL-1 β showed the highest concentration levels in fetal brains and was the only cytokine that was significantly upregulated 24 h after maternal poly (I:C) injection, suggesting that IL-1 β may have a deleterious impact on central nervous system development (Xu et al., 2015). The same result noted Suzuki et al. (2011) who had reported considerable elevated IL-1 β plasma levels in patients with ASD compared with healthy participants, what we also confirmed. On the other hand, another studies demonstrated no differences in the serum levels of IL-1β, soluble IL-1 and IL-1 receptor between children with ASD and the control subjects (Basheer et al., 2018; Croonenberghs et al., 2002; Singh, 1996).

In our study we noted higher concentration of IL-4 in ASD patients than controls, which is in contrast with results obtained by Basheer et al., 2018. However, Goines et al. (2011) made study levels of cytokines and chemokines in archived maternal serum collected during pregnancy and analyzed whether these levels were related to ASD in the child. They have provided evidence for increased IL-4 in mothers bearing a child with ASD. This funding was also confirmed by Abdallah et al. (2013a, 2013b) where they detected increase level of IL-4 in CSF children with ASD.

The observed elevation of IL-6 in serum samples of ASD children in our experiment is in line with Li et al. (2009); Ashwood et al. (2011); Ricci et al. (2013) and Theoharides et al. (2016) study which shows higher levels of this cytokine in the serum of ASD patients. While, Singh (1996) first examined the plasma levels of IL-6 and found no significant differences between subjects with ASD and controls. However, Vargas' group demonstrated that IL-6 was increased in autistic brains. Studies made by Xu et al. (2015) found that IL-6 was significantly increased in the cerebella of autistic subjects. The cerebellum was suggested as a main focus of neuroinflammation in autism, and the selective vulnerability of the Purkinje cells may play a role in the etiopathogenesis of ASD. In further studies, they investigated how IL-6 affects neural cell development and function by transfecting cultured mouse cerebellar granule cells with an IL-6 viral expression vector. They demonstrated that IL-6 overexpression in granule cells caused impairments in granule cell adhesion and migration but had little effect on the formation of dendritic spines or granule cell apoptosis (Xu et al., 2015). These findings suggest that IL-6 not only plays an important role in the etiology of ASD but may provide a potential biological marker that enables the early diagnosis of the disorder and earlier therapeutic intervention, what we also indicated.

Abnormal immune responses, including self-reactive antibodies, have been reported in individuals affected with ASD and found either increases plasma concentrations of IL-8 in patients compared to the matched normal controls have recently been detected (Ashwood et al., 2011) or no change (Schwarz et al., Nelson et al., 2006) in peripheral IL-8 levels. In our research we also did not noted significantly higher concentration of IL-8 in ASD patients compared to controls participant, but we observed tendency to grow up IL-8 level in ASD group. This finding corresponds to similar increases of IL-8 seen in the brain and CSF of individuals with ASD (Vargas et al., 2005; Li et al., 2009). The reason why these chemokines are increased in subjects with ASD is currently unknown. However, IL-17 is known to be a potent mediator of production of IL-8 from epithelial cells (Eyerich et al., 2010). Since IL-8 function as chemotaxin of these chemokines, its elevation in the peripheral circulation suggests an activation of innate immunity.

In our research we observed no significant differences in IL-10 levels this results, are in line with study Molloy et al. (2006), where the levels of IL-10 produced by PBMC from ASD children were similar to the levels of IL-10 in the controls. In contrast, Abdallah et al. (2012, 2013a) reported decreased levels of many cytokines (i.e., IFN-γ, IL-2, IL-4, IL-6, IL-10) in neonatal samples from children with ASD and no differences in chemokine levels. Similarly, we supposed that the increase in proinflammatory cytokines such as IL-1β, IL-4, IL-6 and the same level like in control anti-inflammatory cytokines such as IL-10 demonstrates a possible hyperimmune state in ASD (Gesundheit et al., 2013).

In this case-control study we found that serum samples from children with diagnosed ASD had significantly higher level of the Th2 cytokines IL-4 and IL-13 than in control participants. Elevations of these cytokines at baseline suggest that the children with ASD have a heightened immune response or allergies problems which was published by Kordulewska et al. (2019). Our findings indicate an activated adaptive response at baseline in children with ASD that is predominantly Th2, as evidenced by the significantly higher levels of IL-4 and IL-13. However, we also observed an absence of an IL-10 regulatory response. This supports the concept of a dysregulation of the adaptive immune responses in ASD children. These results are consistent with those reported by Jyonouchi et al. (2002) of a heightened and dysregulated innate immune response in children with ASD as evidenced by elevated proinflammatory cytokine levels in serum.

Recent studies have reported an association of produced excessive proinflammatory cytokine like IL-6 and TNF-α from PBMC and lymphoblasts from individuals with ASD both basally (N Malik et al., 2011) and after lipopolysaccharide (LPS) stimulation (Jyonouchi et al., 2001) as compared with controls. The alternation of TNF-α were reported in most studies about ASD but only in the PBMCs, CSF and brain not plasma (Xu et al., 2015). Jyonouchi et al. (2001) tested 71 autistic children aged 2–14 years and compared them with healthy siblings and other controls. The authors found that TNF-α was elevated in the autistic subjects. Their study showed that PBMCs activated by LPS produced higher levels of TNF-α, IL-1α, and/or IL-6 in most autistic children (83.1%) compared with the control group. The investigators concluded that a majority of the autistic children in the group, especially those with increased TNF-α, exhibited excessive or poorly regulated innate immune responses. In addition, Chez et al. (2007) detected elevated TNF-α in the cerebrospinal fluid of autistic children. Additionally, Li et al. (2009) found that TNF-α was significantly increased in the brains of autistic subjects. Further Ashwood et al. (2011) used polyhydroxyalkanoates (PHA) and tetanus to stimulate PBMCs from autistic subjects and controls to compare group- associated cellular responses. They found that TNF-α production was significantly increased in the autistic subjects. In contrast, Singh (1996) demonstrated that plasma TNF-α did not significantly differ between autistic subjects and normal controls. The results of this study were consistent with the finding above.

Singh (1996) found significantly elevated IFN-α levels in the plasma of 20 autistic children compared with 20 healthy controls. Subsequently, Croonenberghs et al. (2002) confirmed the results. Further, Li et al. (2009) also demonstrated that the concentration of IFN-γ was significantly increased in the brains of ASD patients compared with control participants. Another study detected elevated serum IFN-γ in women who had given birth to a child who was later diagnosed with autism (Goines et al., 2011). It is known that under normal circumstances, pregnancy shifts the maternal immune system toward a more tolerant stage, causing an overall decrease in proinflammatory cytokine trajectories in the innate and adaptive arms of the immune system and an increase in counter regulatory cytokines. Mothers of children with autism demonstrated increased levels of the inflammatory cytokine IFN-γ, which may indicate an atypical immune state during gestation (Xu et al., 2015). However, Sweeten et al. (2004) demonstrated that the plasma levels of IFN-γ did not differ between the autistic group and control group. Results of our study were consistent with the finding above – we did not observe significant differences between ASD patients and control participants.

4.1. Correlation analysis between the levels of cytokines

We also demonstrated significant correlations between the levels of

cytokines which implies the presence of an interactive network between them. In addition, our study is one of the most comprehensive evaluations of cytokine levels in ASD patients in the terms of the number of assessed cytokines as well as correlation analysis between their levels and ROC analysis.

There were no statistically significant correlations between the plasma levels of analytes and clinical variables, including age, weight, height, BMI, IQ (full, verbal and performance), but some correaltion had been noted between concentration cytokines in serum ASD patients (Fig. 2A, B and C). However, we did not find significant correlations with the CARS scale. This could be because most of the children recruited were at the moderate to severe end of the scale and we have only 22 children define by CARS scale, because in Poland we do not diagnosis ASD by this method. In future, it would be important to recruit more children with mild symptoms, to fully cover the severity scale.

4.2. ROC curve analysis

A biomarker can be defined as a biological variable associated with the disease of interest across and within individuals, measurable directly in a given patient or in his/her biomaterials using sensitive and reliable quantitative procedures (Gabriele et al., 2014). Although some researches regarded cytokines (Abdallah et al., 2013a, 2013b) as the potential biomarkers for ASD patients, only association analysis was used in most of studies. However, it is not sufficient to define the biomarker only by correlation analysis. In the present study ROC analysis was performed to assess the usefulness of these biomarkers. We found that the combination of 4-factors (IL-1β, IL-4, IL-6 and IL-13) performed the good sensitivity and specificity in diagnosis of ASD, and the AUC is 0.85. The AUC provides a useful metric to compare different biomarkers. Whereas an AUC value close to 1.00 indicates an excellent diagonal and predictive marker. AUC close to 1.00 is always accompanied by satisfactory values of specificity and sensitivity of the biomarker. The high sensitivity means that in most cases the ASD will be identified, while the high specificity means that few individuals whose positive test results on the ASD prediction were false. This shows their usefulness as predictive biomarkers. This could be supported by the high sensitivity and specificity recorded through ROC analysis. To our knowledge, this is the first study to use the combined ROC curve to analysis the potential biomarkers for assisting the diagnosis of ASD.

Of course, we know that the present study has some limitations. Firstly, diagnostic procedures applied in Poland are using scale according ICD-10, not CARS scale, that is why we have only 22 patients diagnosed in that way. Secondly, this study used a small sample ($n = 42$), because we choose to restrict participation to a small, well-characterized sample of ASD. Findings require replication in a larger group of individuals with autism. It is very possible that a closer analysis of the deep relations of those analyzed 4-factors will offer insights for novel pharmacologic treatments in ASD.

5. Conclusions

In this study, serum levels of cytokine differed among children with ASD. However, the findings of this support the possibility of using an appropriate selection of serum cytokine for the diagnosis ASD and emphasize the need to standardize quantitative methods for serum analysis. In brief, in the present study we demonstrated dysregulation of cytokine levels in serum samples of ASD patients compared with healthy, control participants which is in line with previous reports which highlighted the importance of immune responses in the development of ASD.

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Ethical approval

All procedures performed in our studies with human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments and comparable ethical standards.

Informed consent

Informed consent was obtained for all individual study participants.

Declaration of Competing Interest

None of the authors reported financial interest or potential conflict of interest, nor have financial relationship with commercial entities who have interest in the subject matter of this manuscript.

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