



Cytokines concentrations in serum samples from allergic children—Multiple analysis to define biomarkers for better diagnosis of allergic inflammatory process

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ABSTRACT

Background: Allergic diseases can expand at any age as a result of complicated interaction of environmental and genetic factors. Through the years, studies have found that allergic diseases are primarily described by elevated Th2 pathway activation, leading to increased serum IgE levels, allergen reactivity, blood eosinophil counts and secreted interleukins.

Methods: A total of 20 patients with allergy and 20 matched controls participants were recruited for the study. A study was designed with the framework of an ongoing project at the Regional Children's Hospital in Olsztyn on the analysis of the immune profile of children with allergy and asthma. Diagnosis was conducted by medical specialists. Whole blood samples were collected and serum IL's and chemokine levels were made using ELISA kits. **Results:** Results demonstrated that in comparison to the controls, the individuals with allergy showed significantly higher concentration of IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-13 and TNF- α . 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 3-factors (IL-1 β , IL-4, IL-8) could be additional, helpful biomarkers in better diagnosis of allergy.

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

1. Introduction

Allergic conditions perform a spectrum of disorders, including: eczema, certain forms of asthma, food allergies, eosinophil esophagitis, insect and drug allergies. The time between 20–30 years, the prevalence of allergic disease has raised exponentially throughout the world and now affects approximately 1 in 5 people (Pawankar et al., 2013). Allergic diseases can expand at any age as a result of complicated interaction of environmental and genetic factors. Usually allergic disease, are correlated with one another, further illustrating the overlapping underlying mechanisms of pathogenesis and disease. Through the years, studies have found that allergic diseases are primarily described by elevated Th2 pathway activation, leading to increased serum IgE levels, allergen reactivity, and blood eosinophil counts (Zheng et al., 2011). Allergen-induced IgE synthesis can trigger eosinophils, basophils and

mast cells to release cytokines for the differentiation of Th cells into Th2 cells to secrete interleukin-4 (IL-4), IL-5, IL-10 and IL-13 (Yssel and Groux, 2000). Numerous research in genetic contributed to the identification of large candidate genes in the pathogenesis of allergic inflammation and asthma. Many genes encode interleukins, which are produced by immune cells and mediating in the induction and the development of inflammatory responses.

Because the pathogenesis of allergic diseases, are 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 on the analysis of the immune profile of children with allergy and asthma. 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 allergic diseases. The

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Table 1
Characteristics of serum cytokines evaluated in this study.

Cytokine	Producing cells	Source	Receptors and receptors-bearing cells	Function
IL-1 β	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	Monocytes, macrophage, endothelium, natural killer	IL-1RI; IL-1RII	IL-1 is principally an inflammatory cytokine. It apply to a group of cytokines with overlapping biologic properties (TNF- α , IL-6, IL-8, GM-CSF, PGE2 by macrophage). IL-1 with TNF and IL-6 share the capability to increase cell proliferation, co-stimulate T activation by enhancing production of cytokines like IL-2, and it's receptor, enhanced also B proliferation and muration. In addition, suppress or initiate gene expression for proteins and enhanced Natural Killer (NK) cytotoxicity. IL-1 are ability to induce hypotension, sleep, anorexia, fever, bone resorption by osteoclasts and (acute phase proteins (APP). Pro-inflammatory activities by inducing chemokine and ICAM-1 and VCAM-1 on endothelium. Moreover, increases the synthesis of collagenases, resulting in the destruction of cartilage, stimulates the production of prostaglandins, leading to decrease in the pain threshold, encourage the release of pituitary hormones. In addition IL-1 has some host-defense properties (Feghali and Wright, 1997; Dinarello, 2002). IL-4 has act many biological functions, including the differentiation of B cells into plasma cells as well as stimulation of activated B-cell and T-cell and mast cell (MC) proliferation, and also is a key regulator in adaptive humoral immunity. Up-regulates MHC klas II on B and macrophages, and CD23 on B cells; down regulates IL-12 production and thereby inhibits Th1 differentiation. IL-4 induces switch to IgG1 and IgE. IL-4 reduces the production of IFN- γ , IL-12, macrophages, Th1 cells, and dendritic cell and increases macrophage phagocytosis (Feghali and Wright, 1997; Bradnock et al., 2018).
IL-4	Produced by activated lymphocytes (Th2), mast cells, basophils and NK.	Th2, Tc2, natural killer, mast cell	IL-2R γ ; IL-13R α 1	IL-6 is an critical mediator of fever and of the acute phase response. It is able of crossing the blood-brain barrier and beginning synthesis of Prostaglandin E2 (PGE2) in the hypothalamus, through developing the body's temperature set-point. In fatty tissue and also muscle, IL-6 prompt energy mobilization that leads to boost 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 activate intracellular signaling cascades that give rise to inflammatory cytokine production. IL-6 is responsible for differentiation of myeloid stem cells and of B into plasma cells, induces APP, enhances T proliferation (Feghali and Wright, 1997; Chasemi, 2018).
IL-6	IL-6 is secreted by T cells and macrophages to stimulate immune response.	Th2, monocyte, macrophage, bone marrow stroma	IL-6R α chain (CD126), and the signal-transducing component gp130 (called CD130).	IL-8, also known as <i>neutrophil chemotactic factor</i> , has two dominant functions. It cause 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. mediates chemotaxis and activation of neutrophils (Feghali and Wright, 1997; Meniallo et al., 2018).
IL-8	Is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells.	monocyte, macrophage, endothelium	CXCR1; CXCR2	

(continued on next page)

Table 1 (continued)

Cytokine	Producing cells	Source	Receptors and receptors-bearing cells	Function
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	Th (Th2 in mouse), T cell, B cell, monocyte, macrophage	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 down-regulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. Inhibits IFN- γ secretion by mouse, and IL-2 by human. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF- κ B activity, and is involved in the regulation of the JAK-STAT signaling pathway (Feghali and Wright, 1997; D Moore et al., 2010)
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	Th2, mast cell	IL-13R α 1	IL-13 is a central regulator in IgE synthesis, goblet cell hyperplasia, mucus hyper-secretion, airway hyper-responsiveness, fibrosis and chitinase up-regulation. It is a mediator of allergic inflammation and different diseases including asthma. Inhibits activation and cytokine secretion by macrophage. Coactivates B proliferation, up-regulates MHC klas II and CD23 on B and monocyte, induced switch to IgG1 and IgE, induces VCAM-1 on endothelium (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	T cell, monocyte, macrophage, mast cell, natural killer, B cell	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 (cytotoxicity), cachexia (weight loss), inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via -1 and IL-6 producing cells. Induces cytokine secretion and E-selection on endothelium. Activates macrophage, anti-viral. 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).
IFN- γ	IFN- γ is secreted by T helper cells (specifically, Th1 cells), cytotoxic T cells (T _C cells), macrophages, mucosal epithelial cells and NK cells.	Th1, natural killer	heterodimeric receptor consisting of Interferon gamma receptor 1 (IFNGR1) and Interferon gamma receptor 2 (IFNGR2).	IFN- γ has anti-viral (inhibits viral replication), immunoregulatory, and anti-tumor properties. Enhances MHC klas I and II, activates macrophage, antagonizes several IL-4 actions, inhibits proliferation of Th2. 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.

characteristics of the chosen cytokines are presented in Table 1.

The main aim of the study was to evaluate the serum cytokines concentrations in children with diagnosed allergy and asthma and evaluated whether this result could be identified for additional diagnosis in children with allergy.

2. Material and methods

2.1. Participants

The patients were recruited by specialists in the Regional Specialized Children's Hospital in Olsztyn, Poland, informed consent was obtained from all children's parents and the study was approved by the Local Bioethics Committee (No. 19/2016; 18/5/2016).

Our control group comprised 20 healthy children with no history of behavioral disorders and the same amount of tested group 20 children with diagnosed allergies (20 children with food allergies with: 1) co-existing asthma $n = 5$; 2) atopic dermatitis $n = 5$). Children with fever, infections and skin problems and those taking steroids or antibiotics were excluded from the study. The patients were allowed β_2 antagonists when necessary, but no medications were taken 72 h prior to blood collection and all other medications, including anti-histamines, were excluded during the study. Patients were selected based on established criteria: diagnosed asthma, skin prick test, means of cIgE and a-sIgE in the blood as well as blood eosinophilia. Demographic and clinical characteristic of the study population are shown in Table 2.

2.2. Serum collection and cytokine measurements

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

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

Table 2

Characteristics of the study participants with diagnosed allergy and control group.

Characteristics	Control	ASD with co-existing allergies
Patient's samples, no.	20	20
Age (y), mean $\pm$ SD	7.60 $\pm$ 1.93	5.4 $\pm$ 1.95
Female sex, no. (%)	10 (35,7%)	8 (27,6%)
Diagnosed Allergy, no. (%)	0 (0%)	29 (100%)
Moderate/severe asthma, no. (%)	0 (0%)	12 (41%)
Skin prick test positivity, no. (%)	0 (0%)	29 (100%)
cIgE [IU/mL] mean $\pm$ SD	90.13 $\pm$ 43.93****	338.9 $\pm$ 177.2
a-sIgE [IU/mL] class	Negative predictive value, class 0	Positive predictive value, class 4 - 6
Blood eosinophilia (mean % of eosinophils in the blood smear) mean $\pm$ SD	3.668 $\pm$ 1.104****	25.83 $\pm$ 5.027

**** $p < 0.0001$ control group vs. allergy group.

2.3. 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 $\pm$ SEM, and the mean values between control and allergy groups. A p value of < 0.01 was considered significant. We used Mann-Whitney nonparametric test for cytokine analysed data. To test sensitivity and specificity of the biological markers of measured cytokines to detect allergy, receiver operating characteristic (ROC) analysis were performed. The correlation between true positive rate (sensitivity) and false-positive rate (1-specificity) was represented as a curve. The cut-off point was chosen 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 analysed parametres.

3. Results

3.1. Production of cytokines

3.1.1. IL-1 β

We noted significantly 5.5-fold increased in IL-1 β concentration in the allergy group compared to the control group ($p < 0.0001$). In control group the mean level of IL-1 β was 62 pg/ml while in allergy group 337 pg/ml (Fig. 1A).

3.1.2. IL-4

Significant differences were noted in serum IL-4 between control and allergy groups ($P < 0.0001$) (Fig. 1B). The mean value in control group was 55 pg/ml while in allergy group 278 pg/ml.

3.1.3. IL-6

We observed significant 1.8-fold increase in IL-6 concentration in the allergy group compared to the control group ($p < 0.01$). In control group the mean level of IL-6 was 210 pg/ml while in allergy group 374 pg/ml (Fig. 1C).

3.1.4. IL-8

We established 3.6-fold development in allergy group compared to control ($p < 0.0001$) (Fig. 1D). Mean level of children with diagnosis allergies was 68 pg/mL, when in control participants 248 pg/ml (Fig. 1D).

3.1.5. IL-10

We detected 2.4-fold significantly increased of concentration of IL-10 in allergy group compared to control ($p < 0.0001$) (Fig. 1E). Mean level of children with diagnosis allergies was 146 pg/mL, when in control participants 62 pg/ml.

3.1.6. IL-13

IL-13 concentration has been shown to be significantly 3-fold up-regulated in children with diagnosed allergy ($p < 0.0001$) (Fig. 1F). Mean level of children with diagnosis allergies was 198 pg/mL, when in control participants 66 pg/ml (Fig. 1F).

3.1.7. TNF- α

We noted 2-fold increased serum levels of TNF- α (Fig. 1G) in children with diagnosed allergies compared to control participants ($p < 0.0001$). Mean level of children with diagnosis allergies was 168 pg/mL, when in control participants 82 pg/ml.

3.1.8. IFN- γ

We established 3-fold decreased IFN- γ in allergies group compared to control group ($p < 0.0001$) (Fig. 1H). Mean level of children with

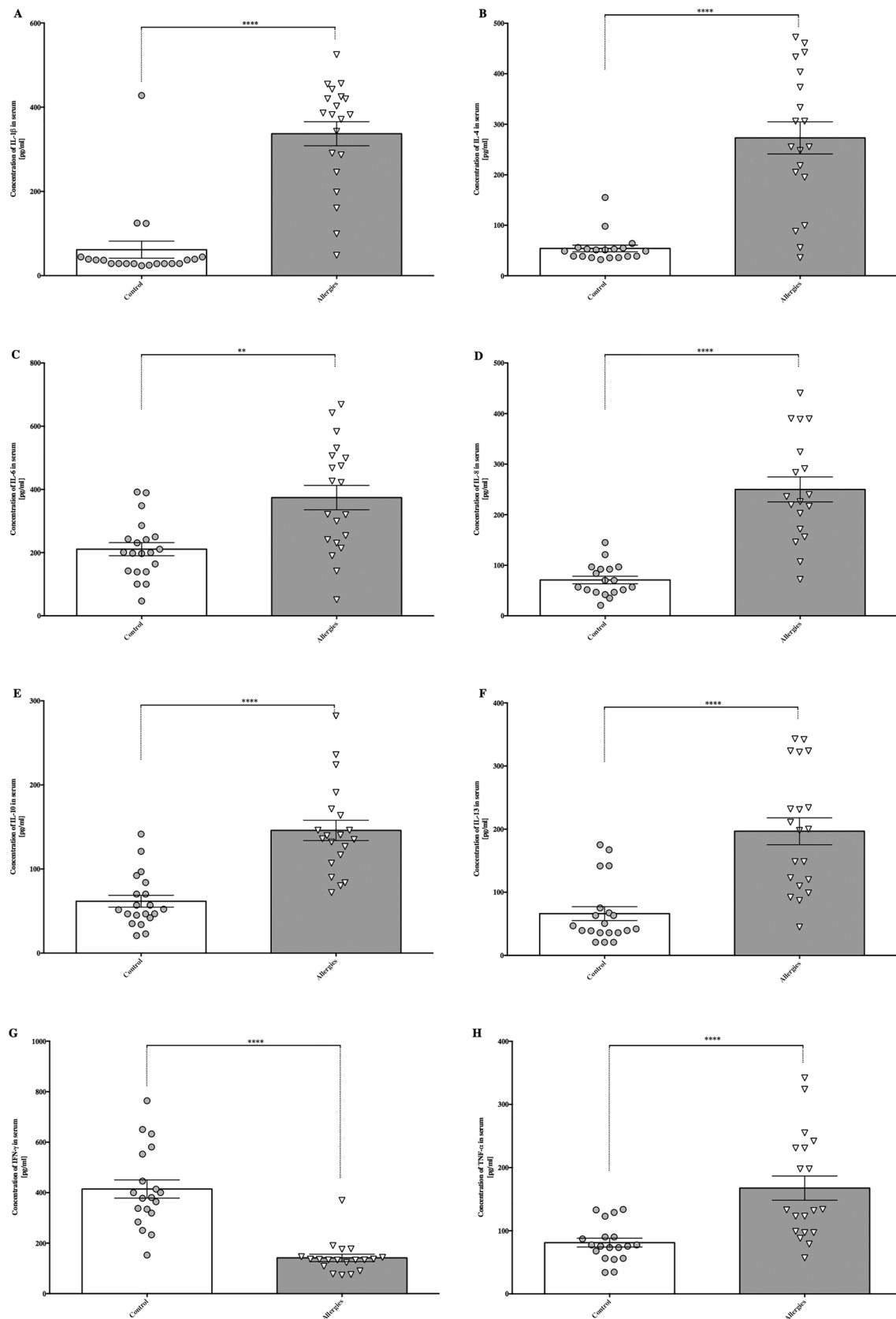


Fig. 1. Mean serum and individual results of the subject concentrations of interleukin: (A) IL-1 β , (B) IL-4, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) TNF- α , (H) IFN- γ . Data are presented as the mean $\pm$ S.E.M. *** $p < 0.001$, **** $p < 0.0001$ vs. control.

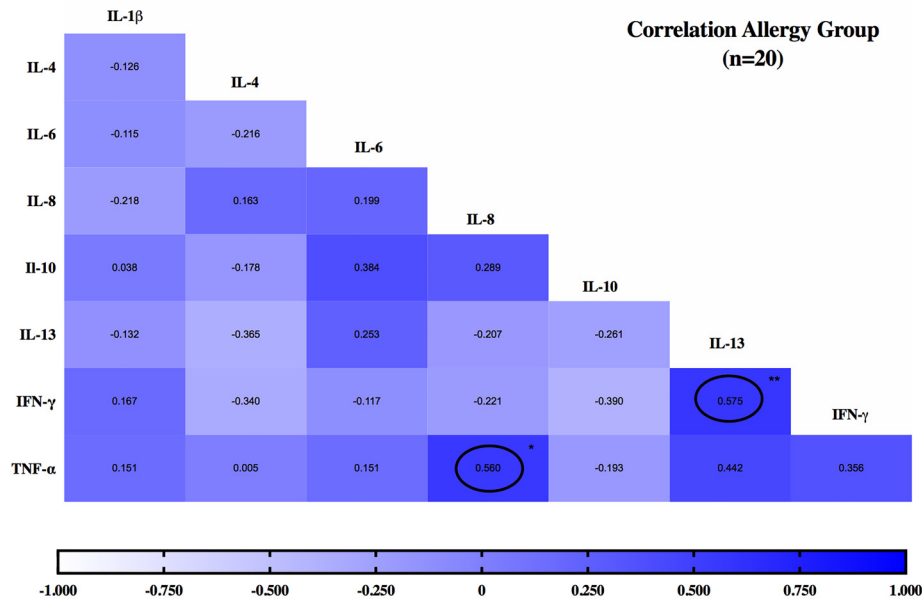
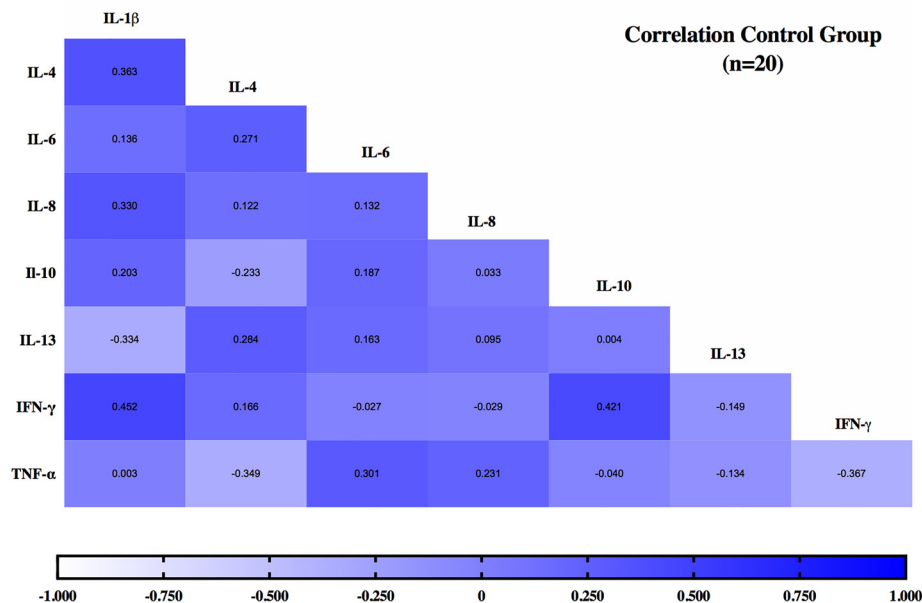
A**B**

Fig. 2. Correlation of cytokine levels in children with diagnosed allergy 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 colour in this figure legend, the reader is referred to the web version of this article).

diagnosis allergies was 142 pg/mL, when in control participants 414 pg/mL.

3.2. Correlation analysis between the levels of cytokines

As revealed by Spearman correlation analysis significant correlations have been noted between cytokine levels. In children with diagnosed allergies we found positive correlation in concentration of IL-8 and TNF- α ($R = 0.560$, $p < 0.05$) and IL-13 and IFN- γ ($R = 0.575$, $p < 0.001$) (Fig. 2A). On the other hand, we did not observe, any correlation in control group (Fig. 2B).

3.3. ROC curve analysis

To assess the practicality of these factors as adjunct of additional biomarkers for allergies, ROC analysis was prepared. The optional cut-off for using IL-1 β as a biomarker for allergies was 124.4 pg/mL. The cut-point was associated with a sensitivity of 90% and specificity 90% ($AUC = 0.95$; 95% CI, 0.87–1.0, $p < 0.0001$) (Fig. 3A and Table 3). The optimal cut-off point for using IL-4 as a biomarker for allergies was 87.04 pg/mL. The cut-off point was associated with a sensitivity of 90% and specificity 90% ($AUC = 0.93$; 95% CI, 0.85–1.1, $p < 0.001$) (Fig. 3B and Table 3). The optimal cut-off point for using IL-6 as a biomarker for allergies was 252 pg/mL. The cut-off point was associated with a sensitivity of 70% and specificity 80% ($AUC = 0.79$; 95% CI,

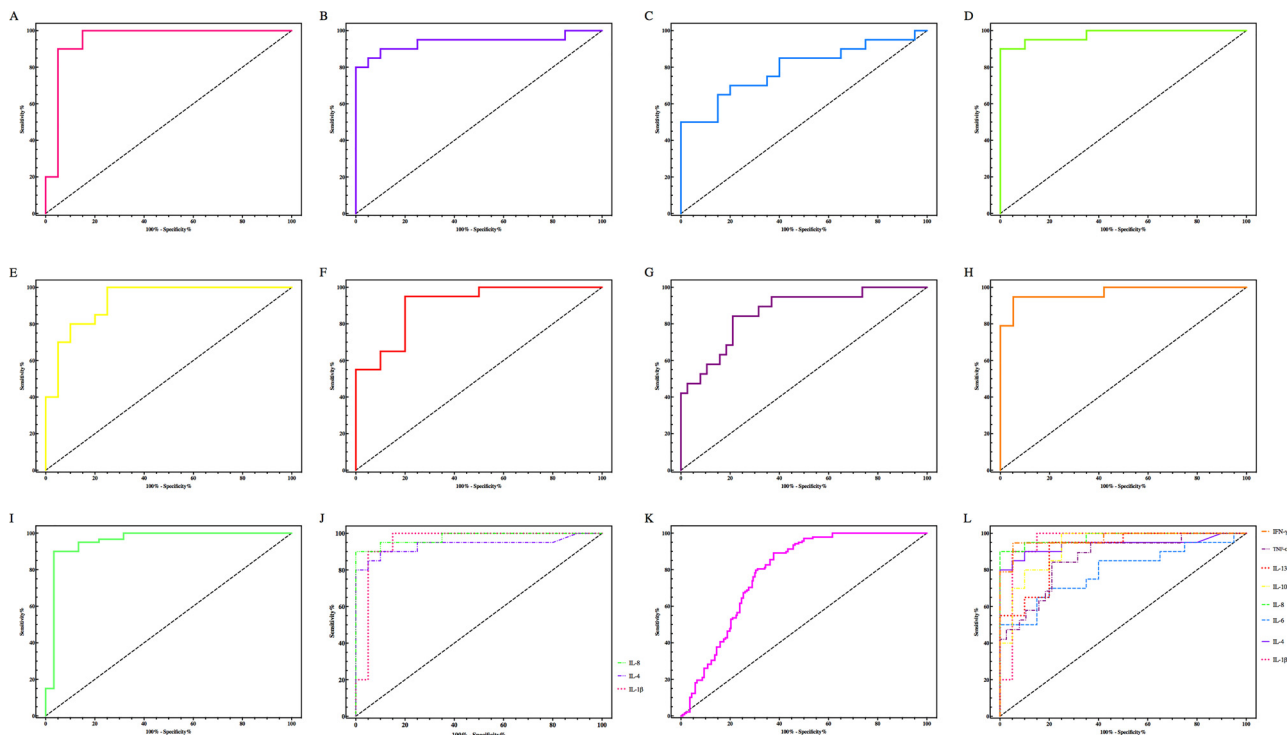


Fig. 3. Receiver operating characteristic (ROC) curves for: (A) IL-1 β , (B) IL-4, (C) IL-6, (D) IL-8, (E) IL-10, (F) IL-13, (G) TNF- α , (H) IFN- γ , (I) combined of 4-factors (IL-1 β , IL-4, IL-6, IL-13) as a biomarkers for allergy – together, (J) separate every analysis and (K) combined of all 8-factors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

0.65 – 0.94, $p = 0.002$) (Fig. 3C and Table 3). The optimal cut-off point for using IL-8 as a biomarker was 133 pg/ml. The cut-off point was associated with a sensitivity of 90% and specificity 95% (AUC = 0.98; 95% CI, 0.94 – 0.68, $p = 1.1$) (Fig. 3D and Table 3). The optimal cut-off point for using IL-10 as a biomarker was 87 pg/ml. The cut-off point was associated with a sensitivity of 85% and specificity 80% (AUC = 0.93; 95% CI, 0.85–1.0, $p < 0.001$) (Fig. 3E and Table 3). The optimal cut-off point for using IL-13 as a biomarker was 81.06 pg/ml. The cut-off point was associated with a sensitivity of 95% and specificity 80% (AUC = 0.90; 95% CI, 0.81–1.0, $p < 0.001$) (Fig. 3F and Table 3). The optimal cut-off point for using TNF- α as a biomarker was 93.57 pg/ml. The cut-off point was associated with a sensitivity of 85% and specificity 79% (AUC = 0.86; 95% CI, 0.75 – 0.98, $p < 0.001$) (Fig. 3G and Table 3). The optimal cut-off point for using IFN- γ as a biomarker was 211.5 pg/ml. The cut-off point was associated with a sensitivity of 95% and specificity 95% (AUC = 0.97; 95% CI, 0.92–1.0, $p < 0.001$) (Fig. 3H and Table 3).

In addition, the diagnostic capability was significantly enhanced by the combination of 3-factors were noted correlation between allergies patients and control participants and all 8-factors measured in plasma.

The combination of 3-factors had a sensitivity of 95% and specificity 87% (AUC = 0.97; 95% CI, 0.92–1.0, $p < 0.0001$) (Fig. 3I, J and Table 3). While, the combination of 8-factors had a sensitivity of 76% and specificity 70% (AUC = 0.78; 95% CI, 0.72 – 0.84, $p < 0.001$) (Fig. 3K, L and Table 3).

4. Discussion

The purpose of this study was to analyze the plausible use of peripheral biomarkers in assisting the diagnosis of allergies and define levels of cytokine concentration in serum from children with diagnosed allergies and control participants. The results of this case showed that serum levels of IL's are different in healthy controls and children with allergies. We demonstrated a trend toward significantly higher concentration of tested IL's in allergies sample, only IFN- γ concentration was lower in those group than control. 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-1 β , IL-4, IL-8 were the potential helpful biomarkers in diagnosis allergies.

Table 3

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

Variables	AUC	95% CI	p-Value	Sensitivity (%)	Specificity (%)	Cut-off point
IL-1 β (pg/mL)	0.95	0.87–1.0	< 0.0001	90	90	> 124.4
IL-4 (pg/mL)	0.93	0.85–1.1	< 0.001	90	90	> 87.04
IL-6 (pg/mL)	0.79	0.65–0.94	0.002	70	80	> 252
IL-8 (pg/mL)	0.98	0.94–1.1	< 0.001	90	95	< 133
IL-10 (pg/mL)	0.93	0.85–1.00	< 0.001	85	80	< 87
IL-13 (pg/mL)	0.90	0.81–1	< 0.001	95	80	> 81.06
TNF- α (pg/mL)	0.86	0.75–0.98	< 0.001	85	79	> 93.57
IFN- γ (pg/mL)	0.97	0.92–1.0	< 0.001	95	95	< 211.5
Combination of 3-factors(IL-1 β ; IL-4; IL-8 pg/mL)	0.97	0.92– 1.0	< 0.0001	95	87	< 98
Combination of all factors where we noted correlation (IL-1 β ; IL-4; IL-6; IL-8; IL-13; IL-10; TNF- α ; IFN- γ pg/mL)	0.78	0.72–0.84	< 0.001	76	70	< 133.4

4.1. Immune system

Recent findings indicate that interleukins can influence biological responses through the increased/decreased production of them and suggest that many allergic reactions and other biological processes may be regulated by a "cytokine cascade" (Galli et al., 1994). Allergen-induced IgE synthesis triggers eosinophils, basophils and mast cells to release cytokines for differentiation of Th cells into Th2 cells to secrete IL-4, IL-5, IL-10 and IL-13. Moreover, basophils, mast cells and eosinophils act as effectors of allergic inflammation through the release of pro-inflammatory, vasoactive and fibrogenic factors including histamine, peptide leukotrienes, platelet activating factor tryptase and chymase. These are responsible for allergy and inflammatory response symptoms (Kordulewska et al., 2016).

4.2. IL-1 β activity

Our results obtained from this study was in line with Kordulewska et al., (2016) where they observed increased secretion of IL-1 β in 30 people with diagnosed allergy in serum and peripheral blood mononuclear cells (PBMC's) sample. In addition, Shoham et al. (2003) indicated that monocytes from patients with auto-inflammatory diseases produce increased IL-1 β levels than control monocytes (Shoham et al., 2003). In addition, researchers report that IL-1 β serum levels can be elevated in inflammatory and non-inflammatory conditions as different as sepsis, chronic rheumatoid disease, allergic inflammation and non-inflammatory tissue injury (Ulrich et al., 2001).

4.3. IL-4 activity

Research made by Matsumoto et al., (1991) measured concentration of IL-4 in children serum samples with diagnostic allergies and confirmed significantly increased levels. In addition, with all clinical manifestations, including food anaphylaxis, bronchial asthma and atopic eczema. Our results were similar, with a significantly higher IL-4 level in allergic children than in non-allergic participants. Additional support is provided by: (1) Pawankar et al. (1997) described that nasal mast cells (NMC) from patients with perennial allergic rhinitis (PAR) expressed significantly higher IL-4 levels than cells from patients with chronic infective rhinitis (CIR); (2) Hashimoto et al. (1993) who observed increased serum IL-4 in patients with allergic asthma in comparison to normal control subjects and (3) Fujishima et al. (1995) found that although their control group had small mean IL-4 concentrations in their tears, patients with diagnosed seasonal allergic conjunctivitis and vernal keratoconjunctivitis had significantly higher IL-4.

4.4. IL-6 activity

In our research study we noted significantly higher concentration of IL-6 in allergy group in comparison to control. Given the multifunction nature of IL-6 and its involvement in many category of inflammation, including allergy inflammation, a possible involvement in asthma and allergies has been suggested, what we confirmed in our study. This also has been supported by elevated IL-6 concentrations in the sputum of patients with diagnosed asthma. In addition, was noted higher IL-6 and sIL-6 in serum at the same time when asthma attacks were confirmed (Morjaria et al., 2010). What is more, murine models of asthma have pointed out a beneficial reaction of inhibition of IL-6R, which was shown to change the balance between Th2 cell feedback with increased number of Treg cells formed that had immunosuppressive activity (Rath et al., 2015). Similar results obtained Salem et al., (2018), where IL-6 was highly expressed by fibroblasts from severe asthmatics compared to both cells from the mild asthmatics and healthy controls.

4.5. IL-8 activity

We showed that children with diagnosed allergies had higher concentrations of IL-8 in serum than healthy controls. This finding confirms, at least in part, previous observations that in patients with allergic asthma IL-8 levels were increased both in sputum (Jatakanon et al., 1999) and in peripheral blood (Shute et al., 1997). This results of increased concentrations of IL-8 are in agreement with the study of Marini et al., (1992), who noted elevated levels of IL-8 and IL-8 mRNA in freshly isolated bronchial epithelial cells of patients with asthma. Furthermore, they also noted that the epithelial cells of people with diagnosed asthma spontaneously produced more amount of IL-8 than the epithelial cells obtained from healthy participants.

4.6. IL-10 activity

Our findings indicated a tendency towards increased IL-10 concentration in children with diagnosed allergy, what is in line with previously study made by Kordulewska et al., (2016) in asthmatic patients. Further, our increased concentration in serum of IL-10 in children with allergy concurs with Robinson et al.'s. (1996) observation that IL-10 mRNA expression is increased in allergy and atopic asthma and in plasma from asthmatic patient (Wong et al., 2001). Furthermore, some authors demonstrated that bronchoalveolar lavage fluid from asthmatic patients contains an increased level of IL-10 compared to control (Robinson et al., 1996). However, reduced IL-10 expression in peripheral mononuclear cells (PBMC) of asthmatic patients was also demonstrated by Borish et al., 1996 and Wong et al., (2001). It seems that IL-10 possesses pleiotropic effects in addition to induce allergic reaction. However, our research results of increased plasma cytokine level confirm that Th2 cytokine IL-10 plays a central role in allergic diseases.

4.7. IL-13 activity

Animal models and many clinical trials have indicated an important role of concentration of IL-13 in the pathogenesis of allergic and asthma (Yssel et Groux, 2000). In our study we observed higher levels of IL-13 in allergic children in comparisons to healthy participants, this is in line with results made before by Kordulewska et al., (2016) in asthmatic patients. These data, and the observations that IL-13 expression is up-regulated in atopic skin, in the lungs of patients with asthma, in patients with chronic sinusitis. Is also downregulated in response to corticosteroid therapy, underscore the unique and important role of IL-13 in the regulation of atopic and allergic responses (de Vries, 1998).

4.8. IFN- γ activity

We observed significantly decreased levels of IFN- γ in children with allergies in comparison to healthy participants. Similarly, the same results obtained Wong et al., (2001); Litonjua et al., (2003) and Liu et al., (2016) in patients with diagnosed asthma.

4.9. TNF- α activity

Our findings reported a significant increase of TNF- α in serum of allergic patients compared to control, the same results were described by Kordulewska et al., (2016). What is more, similar results obtained Zhou and Liu (2005) in inflammatory response in asthmatic airways by increased TNF- α mRNA and supported by higher level of protein (Miller et al., 1998) in asthma patients' airways, and also by Ying et al.'s. (1991) research which reported increased TNF- α in asthmatic and allergic airways (Cazzola and Polosa, 2006). In addition, Zipperlen et al. (2005) indicated over-expression of TNF- α in both normal and inflamed intestinal mucosa in patients with inflammatory bowel disease.

5. 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 allergy 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 symptoms or: age, weight, height, BMI but some correlation had been noted between concentration cytokines in serum allergy patients (Fig. 2A).

5.1. 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 (Duramad et al., 2007) as the potential biomarkers for allergy 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 3-factors (IL-1 β , IL-4, IL-8) performed the very good sensitivity and specificity in diagnosis of allergy, and the AUC is 0.97. 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 allergy will be identified, while the high specificity means that few individuals whose positive test results on the allergy 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 allergy.

Of course, we know that the present study has some limitations. Firstly, this study used a small sample ($n = 20$), because we choose to restrict participation to a small, well-characterized sample of allergy. Findings require replication in a larger group of individuals with diagnosed allergy. It is very possible that a closer analysis of the deep relations of those analyzed 3-factors will offer insights for novel pharmacologic treatments in diagnostic allergy when there are any uncertainties in the diagnosis.

6. Conclusions

In this study, serum levels of cytokine differed among children with allergies. However, the findings of this support the possibility of using an appropriate selection of serum cytokine for the better diagnosis of allergies 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 allergy patients compared with healthy, control participants which is in line with previous reports which highlighted the importance of immune responses in the development and treatment of allergy.

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Conflict of 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.

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.

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