Dermatology Practical & Conceptual

Serum Levels of IL-35, One of the Newest Members of Interleukin-12 Family of Cytokines, in Patients With Vitiligo

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ABSTRACT

Introduction: Vitiligo is a chronic skin disorder in which immune dysregulation has been reported as one of the major etiopathological factors. Interleukin-12 (IL-12), IL-23 and IL-27 of IL-12 cytokine family were identified as critical cytokines in the pathogenesis of many autoimmune and inflammatory skin diseases including vitiligo. IL-35 is one of the newest member of IL-12 cytokine family.

Objectives: The purpose of our study was to examine serum IL-35 levels in addition to serum IL-12, IL-23, IL-27 levels in the vitiligo patients and control group, and to investigate the relationship of these cytokines with the characteristics of vitiligo.

Methods: Serum IL-12, IL-23, IL-27 and IL-35 levels of 87 vitiligo patients and 70 healthy volunteers were analyzed using the enzyme-linked immunosorbent assay (ELISA). We compared the IL-12 cytokine family levels in the patient and control groups, and investigated the relationship of these levels with the characteristics of vitiligo.

Results: Patients had higher levels of IL-12 (31.2 versus 20.1, $P < 0.001$) and IL-35 (9.6 versus 8.1, $P = 0.031$). Patient and control groups had similar levels of IL-23 ($P = 0.78$) but were correlated with the Vitiligo Area Scoring Index (VASI) ($P = 0.022$, $r = 0.35$). Patients had lower levels of IL-27 (207.6 versus 258.7, $P < 0.001$). In addition, the levels of serum IL-27 were correlated negatively with the Vitiligo Disease Activity (VIDA), and positively with disease duration ($P = 0.007$, $r = 0.30$).

Conclusions: Differences of serum levels between Vitiligo patients and healthy controls, significant relationships with the characteristics of vitiligo suggest that the IL-12 cytokine family may play a role in the pathogenesis of vitiligo.

Introduction

Vitiligo is an autoimmune disorder characterized by depigmented patches on the skin, skin appendages and mucous membranes. Its frequency is approximately 1%. Although vitiligo most commonly develops before the second decade, it can occur in any age group [1]. Even if it is not mortal, it may cause many psychological problems including anxiety, depression and sleep disorders that seriously affects the quality of life [1,2].

The pathogenesis of vitiligo has not been fully defined but oxidative stress and immune dysregulation are thought to be the fundamental causes of vitiligo in genetically predisposed individuals [3]. Oxidative stress and similar other internal and external stimuli cause the release of inflammatory cytokines, which in turn stimulates the innate immune response, and a series of reactions that result in the activation of adaptive immune response and autoreactive CD8+ T cells [3]. The role of interleukins in the pathogenesis of vitiligo is also well known. Many interleukins including primarily IL-17, and IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-19, IL-23 and IL-33 have been examined in vitiligo patients. Significant relations have been shown between the dysregulation of interleukin levels and clinical factors such as the degree and extensiveness of the disease [4].

The IL-12 family consists of IL-12, IL-23, IL-27, and IL-35 [5,6]. These cytokines are heterodimeric and consist of an alpha subunit (p19, p35, or p28) and a beta subunit (p40 or Epstein-Barr virus induced gene 3, EBI3) [5]. Cytokines of the IL-12 family bind to heterodimeric receptors and act through the JAK-STAT signaling pathway [5]. Although, the members of this cytokine family have common subunits in its structure and receptors, they have different functions. IL-12, IL-23, IL-27 and IL-35 may have pro-inflammatory or anti-inflammatory effects by affecting different T lymphocyte subsets. IL-12 and IL-23 are mostly pro-inflammatory, IL-35 is mostly anti-inflammatory, while IL-27 shows pro-inflammatory and anti-inflammatory properties at different times [5,6].

Although the IL-12 cytokine family has been studied in many inflammatory and neoplastic diseases, few studies investigate its effects on vitiligo, and major differences were found between the results of those studies [7].

Objectives

The purpose of the present study was to investigate the levels of serum IL-12, IL-23, IL-27 and IL-35 in patients with vitiligo compared to healthy controls, and identify the relations between cytokine levels and clinical features.

Methods

Eighty-seven (52 females, 35 males) vitiligo patients and 70 healthy controls were included in the study.Patient and control groups were similar in terms of age and sex (P = 0.51 and 0.39, respectively).

The patients were clinically diagnosed with vitiligo. Patients who came to the dermatology outpatient clinic with complaints of skin discoloration were evaluated. Patients with hypopigmented/depigmented macules and patches were examined under WOOD light, and patients with depigmented patches matching the definition of vitiligo were included in the study. Patients with lesions spreading segmentally were excluded from the study even if they were diagnosed with vitiligo. Vitiligo Activity and Severity Index (VASI) was used to determine the extent of vitiligo, and vitiligo disease activity score (VIDA) was used to evaluate the disease activity of vitiligo. The Vitiligo Disease Activity Score (VIDA) is a six-point scale that is used to evaluate the vitiligo disease activity. The VIDA score is evaluated as "+4 = active for the last 6 weeks, +3 = active for the last 3 months, +2 = active for the last 6 months, +1 = active for the last 1 year, 0 = stable over the last year, -1 = stable with spontaneous re-pigmentation over the last year" [8]. The age of onset, duration of the disease, presence of additional autoimmune disease, presence of vitiligo in the family, and presence of additional autoimmune disease in the family were recorded based on a detailed history taken from the patients. All participants gave written informed consent prior to the study.

Examination of IL-12 Cytokine Family Level

Venous blood samples were collected from all participants after 12 hours of fasting. Blood samples collected for analysis were centrifuged at 4000 rpm for 10 minutes. Separated sera were aliquoted into Eppendorf tubes and stored at -80 °C until the time of analysis. Serum IL-12, IL-23, IL-27 and IL-35 levels were detected with human ELISA (double antibody sandwich ELISA method) kits according to manufacturer instructions (manufacturer: USCN Life Sciences). All values are expressed as pg/mL. Intra-Assay CV values were less than 10% and inter-assay CV values were less than 12% for all parameters.

Statistical Methods

The data were analyzed using SPSS/IBM for windows 21.0. Descriptive statistics such as percentage, mean, median, standard deviation and interquartile range (IQR) were used to define the sample. The assumption of conformity to the normal distribution was examined using the Shapiro Wilk test. The difference between the means of two independent groups was analyzed by the Student t test in cases where parametric test assumptions were met, and the difference

between the medians of two independent groups was analyzed by Mann-Whitney U test in cases where parametric test assumptions were not met. Spearman or Pearson tests were used to evaluate the correlation between two numerical values. Categorical data were analyzed with the Chi-square significance test or Fisher Exact test. A 95% level of significance (error margin: $\alpha = 0.05$) was used to determine the statistically significant differences in the analyses.

Results

Demographic and Clinical Features of Study Population

Fifty-nine point eight percent of the vitiligo patients were female and 40.2% were male. The mean age of the patients was 39.8 years (SD=13.2 years). 55.2% (N = 48) of the patients had generalized vitiligo and 34.5% (N = 30) had localized vitiligo. The median VASI was 4 (IQR = 1.18-10.3). The age of onset was 26 (IQR = 13-40) and the duration of the disease was 8 months (IQR = 2-21 months). The VIDA score was "-1" for 23%, "0" for 12.6%, "1" for 10.3%, "2" for 19.5%, "3" for 8.4%, and "4" for 3.4% of the patients. While 29.9% of the patients had a history of non-vitiligo autoimmune disease, 21.8% had a family history of vitiligo, and 21.8% had a family history of non-vitiligo autoimmune disease.

Serum IL-12, IL-23, IL-27, and IL-35 Concentrations in Vitiligo Patients and Control Group

Serum IL-12, IL-23, IL-27, and IL-35 concentrations in vitiligo patients and control group are summarized in Table 1. Serum IL-12 and IL-35 levels were higher in vitiligo patients compared to healthy controls (31.2 versus 20.1, P < 0.001 and 9.6 versus 8.1, P = 0.031, respectively) (Figure 1). Serum IL-27 levels were 207.6 (SD=71.3) in vitiligo patients

Table 1. Serum IL-12, IL-23, IL-27, and IL-35 concentrations in vitiligo patients and control group

	Vitiligo	Control	P value
IL-12 (pg/mL), median (IQR)	31.2 (25.4-42.1)	20.1 (13.3-32.8)	<0.001
IL-23 (pg/mL), median (IQR)	25.1 (19.9-31.8)	21.8 (18.9-72.2)	0.78
IL-27 (pg/mL), mean (SD)	207.6 (71.3)	258.7 (85.5)	<0.001
IL-35 (pg/mL), median (IQR)	9.6 (5.8-20.8)	8.1 (5.1-11.3)	0.031

IQR = interquartile range; SD = standard deviation.

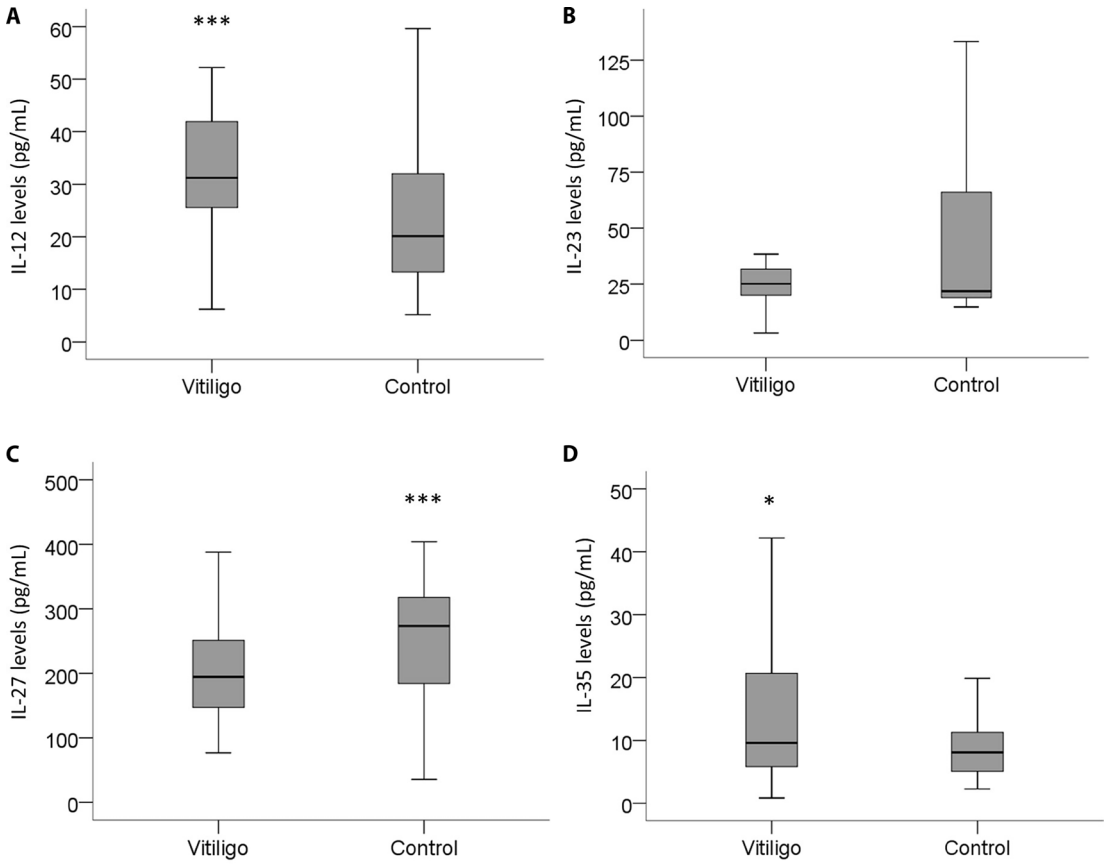

Figure 1. Comparison of serum IL-12, IL-23, IL-27, and IL-35 concentrations in vitiligo and control groups (***P < 0.001, *P < 0.05).

and 258.7 (SD=85.5) in controls. Serum IL-27 levels were statistically lower in patient group compared to healthy controls (P < 0.001) (Figure 1). Serum IL-23 levels were similar among patient and control groups (P = 0.78) (Figure 1).

Association of the IL-12 Cytokine Family With Patient Demographics and Vitiligo Disease Characteristics

Serum IL-12, IL-23, IL-27, and IL-35 levels were similar among male and female patients (P = 0.93, 0.25, 0.79, and 0.96, respectively). Serum IL-23 levels positively correlated with age (P = 0.025, r = 0.34) but no statistically significant correlation was observed between age and serum IL-12 (P = 0.19, r= -0.11), IL-27 (P = 0.29, r = 0.12), and IL-35 (P = 0.91, r = -0.01) levels.

Serum IL-12, IL-23, IL-27, and IL-35 levels were similar among vitiligo patients with localized and generalized vitiligo (P = 0.44, 0.16, 0.49, 0.32, respectively). While there was a significant correlation between the VASI value and IL-23 (P = 0.022, r= 0.35), VASI and Serum IL-12 (P = 0.74, r=-0.04), IL-27 (P=0.11, r=0.18), and IL-35 (P=0.49, r=-0.08) levels were not correlated (Figure 2). VIDA scores and IL-27 levels were negatively correlated. Patients with high VIDA scores and active disease had significantly lower levels of IL-27 (P = 0.024, r = -0.26). No correlation was observed between VIDA and serum IL-12 (P = 0.79, r = -0.03), IL-23 (P = 0.58, r = -0.08) or IL-35 (P = 0.91, r = -0.01) levels.

Vitiligo age of onset and the levels of IL-12, IL-23, IL-27, or IL-35 were not found to be correlated (all P values > 0.05), but IL-27 levels and disease duration were positively correlated (P = 0.007, r = 0.30). The correlation between the disease duration and IL-23, IL-27 or IL-35 level is shown in the Figure 3.

Patients with and without a non-vitiligo autoimmune disease, patients with and without a family history of vitiligo, and patients with and without a family history of non-vitiligo autoimmune disease had similar levels of serum IL-12, IL-23, IL-27, and IL-35 (all P values > 0.05).

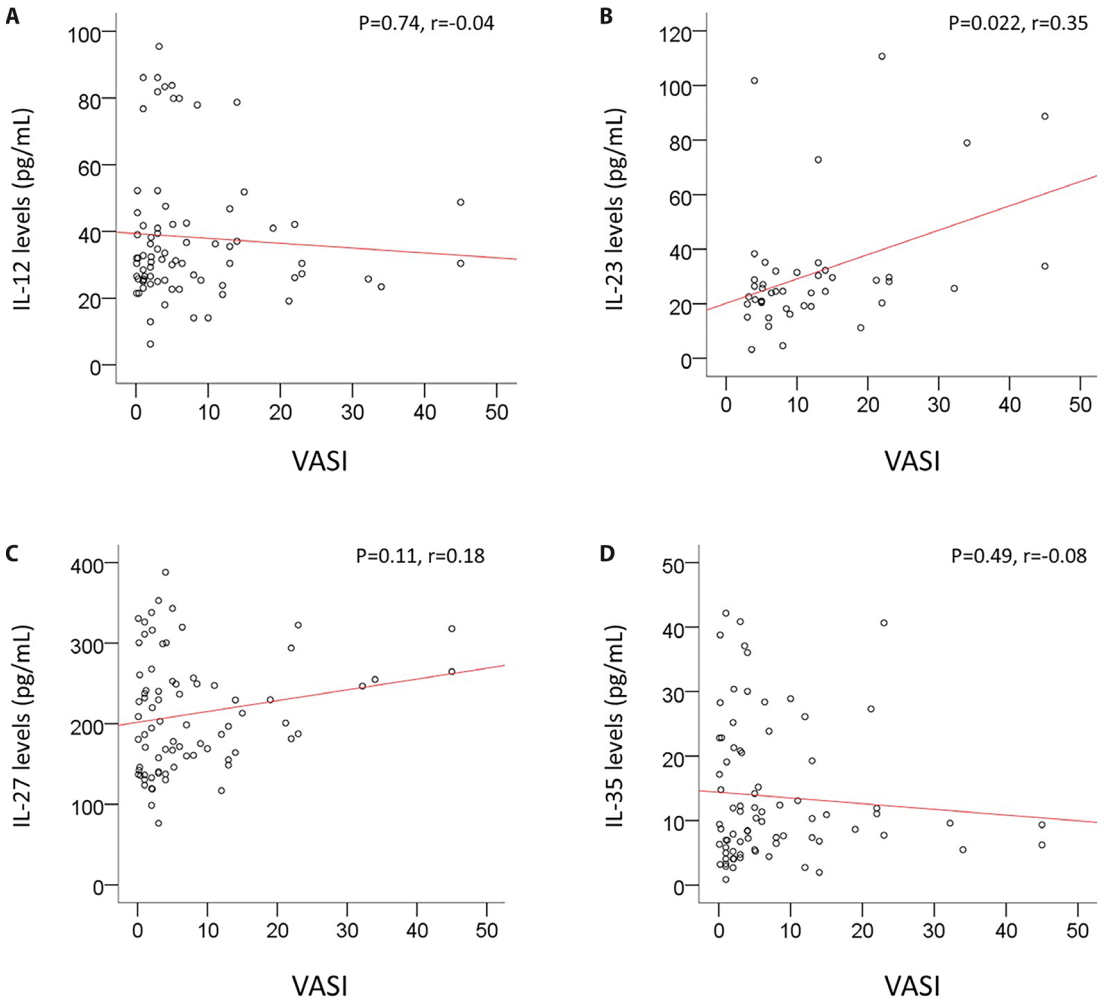

Figure 2. The correlation between Vitiligo Area Scoring Index (VASI) and the interleukin-12 family of cytokines.

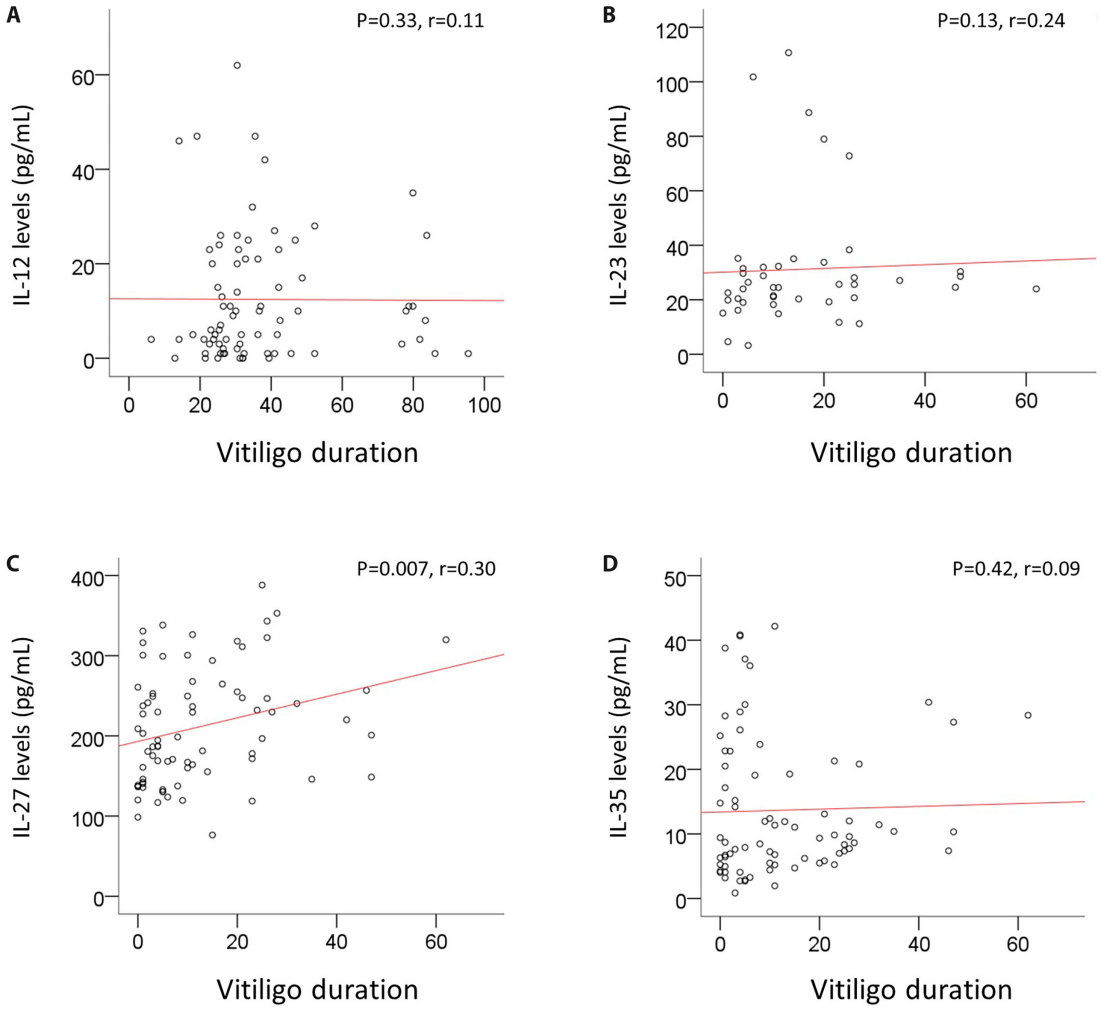

Figure 3. The correlation between vitiligo duration and the interleukin-12 family of cytokines.

Conclusions

We compared the levels of IL-12, IL-23, IL-27 and IL-35 in vitiligo patients and healthy volunteers. Patients had higher levels of IL-12 and IL-35, and lower levels of IL-27. While patient and control groups had similar IL-23 levels, serum IL-23 levels were correlated with the extensiveness of the disease. We observed a negative correlation between serum IL-27 levels and VIDA scores, and a positive correlation between disease duration and serum IL-27 levels.

IL-12 is released from dendritic cells, macrophages and B cells in response to internal and external stimuli. Comprised of p40 and p35 subunits, IL-12 exerts its effect by binding to the heterodimeric receptor that is made up of the IL-12Rβ1 and IL-12Rβ2 subunits. Binding of IL-12 to the receptor activates Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2), phosphorylating STAT4 for biological effects [9]. IL-12 is a proinflammatory cytokine and it performs these functions mainly through its effects on T cell differentiation and functions. Recognition of pathogens by an antigen-presenting cell stimulates the production of IL-12, which in turn stimulates the release IFN-γ and the development of T helper type 1 (Th1) immune response, eliminating intracellular bacteria and viruses and stimulating inflammation [9,10].

In our study, we found significantly higher levels of serum IL-12 in Vitiligo patients compared to healthy volunteers. In a study on innate pro-inflammatory cytokines in patients with vitiligo, Gholijani et al found high levels of serum IL-12, which was consistent with our findings [11].

IL-12 may play a role in the pathogenesis of vitiligo by affecting many pathways. IL-12 is known to activate type 1 cytotoxic CD8 T cells to direct the granzyme and perforin-mediated cytotoxic activity [10]. IL-12 may also contribute to the formation of vitiligo by affecting the presentation of autoantigens. In a study on generalized vitiligo patients, Singh et al investigated the relationship between IL-12 levels and dendritic cell counts of the vitiligo and control groups. They found an increase in dendritic cells in addition to an increase in serum IL-12 levels in the vitiligo group. The authors stated that increased dendritic cell frequencies and proinflammatory cytokines including IL-12 may be associated with defective antigen presentation [7].

Additionally, IL-12 helps the differentiation of naive CD4+ T cells into interferon (IFN)-γ producing Type 1 T helper (Th1) cells and stimulates IFN-γ production from natural killer (NK) cells [5]. The role of IFN-γ in the pathogenesis of vitiligo is known well. IFN-γ increase the expression CXCL9, CXCL10 and their receptor CXCR3 which results in recruitment and localization of melanocyte-specific autoreactive T cells within epidermis as well as stimulation of melanocyte apoptosis [3].

Vitiligo-like depigmented patches in mice in studies investigating the effects of IL-12 on melanoma cells also suggested that IL-12 may play a role in the pathogenesis of vitiligo. In a study conducted by Nagai et al., mice were inoculated with locally IL-12-producing transfected melanoma cells, vitiligo was observed in half of the mice whose CD4+ T cells depleted [12]. In further analyses where the cause of vitiligo was investigated, it was reported that the Th1/Th2 cytokine profile shifted in the Th1 direction and that CD8+ T lymphocyte accumulations were observed around the hair follicles in lesions with vitiligo. Even though IL-12 is known to trigger a Th1 response, observation of vitiligo in mice with depleted CD4+ T cells raised the question of whether IL-12 alone is enough for the development of vitiligo and whether additional pathways are needed for the development of vitiligo [12].

In our study, patient and control groups had similar serum IL-23 levels but there was a significant correlation between IL-23 levels and disease severity. Only a limited number of studies examine serum IL-23 levels and their relations with disease characteristics. In two different studies conducted by Vaccaro et al. and Nieradko-Iwanicka et al, vitiligo patients had higher serum IL-23 levels than the control group [13, 14]. On the other hand, Osman et al. found in a study comparing 42 adult vitiligo patients and 43 healthy controls that the patient and control groups did not have any significant difference in terms of IL-23 levels, which was consistent with our study [15]. Studies also differ greatly in terms of the relationship between serum IL-23 levels and disease characteristics. While Vaccaro et al. reported a positive correlation between serum IL-23 levels and disease duration, extent of vitiligo and severity of the disease, Osman et al. did not find a correlation between IL-23 and disease duration [13,15]. Nieradko-Iwanicka et al. investigated the association between IL-23 and diseases severity and reported that patients with an involved body surface area <10% and >10% were similar in terms of serum IL-23 levels [14].

IL-23 (comprised of p40 and p19 subunits) exerts its effect by binding to the heterodimeric receptors that are made up of the IL-12Rβ1 and IL-23R subunits. Once IL-23 binds to the receptor, JAK2 and TYK2 are activated. Activated JAK2 and TYK2 than phosphorylate STAT3 and STAT4 accomplishing its biological effects [9]. The biological activity of IL-23 is generally proinflammatory. IL-23 stimulates IL-17 and/or IL-22 production from Th17 cells, group 3 innate lymphoid cells (ILC3), γδ T cells and IL-17-producing CD8+ T (Tc17) cells, and plays a role in stabilization of Th17 cells [9, 10]. Although the main role of IL-23 in the pathogenesis of vitiligo has not been fully understood, there are abundant data supporting the effect of IL-17 in the development of vitiligo [16,17]. Vitiligo patients have higher levels of IL-17 and more Th17 cells in peripheral blood than controls. Vitiligo tissues have Th17 cell infiltration and increased IL-17 mRNA expression. In addition, in-vitro studies showing that IL-17 reduces melanin production and causes shrinking in melanocytes supports the possible role of IL-17 in the pathogenesis of vitiligo [16,17]. Stimulating Th17 cell differentiation, hence production of IL-17 cytokines, IL-23 may contribute to the pathogenesis of vitiligo directly or through Th17/IL-17 pathway.

Although IL-12 and IL-23 are thought to play a role in the pathogenesis of vitiligo, whether IL-12/IL-23 blockers can be used in the treatment of vitiligo is not clear. IL-12/23 inhibitors have very different effects on vitiligo lesions. Patients administered IL-12/23 inhibitors have reported formation of de novo vitiligo and deterioration of vitiligo as well as improvement in vitiligo and repigmentation of depigmented patches [18,19].

Serum IL-27 levels were lower in vitiligo patients compared to controls. IL-27 levels of vitiligo patients had been analyzed in a previous study. Hosseini et al. compared IL-27 levels of 79 vitiligo patients and 45 healthy controls, and reported lower IL-27 levels in vitiligo patients in parallel with our results. The study did not report any correlation between IL-27 levels and gender, extensiveness of the disease in terms of body surface area, type of vitiligo, and treatment responses [20]. We could not find any correlation between IL-27 levels and extensiveness of the disease either, but IL-27 levels were negatively correlated with the disease activity. Patients with active vitiligo had lower levels of IL-27, patients with a stable course of disease had higher IL-27 levels. IL-27 levels were also correlated with disease duration. The fact that IL-27 was lower in patients with a shorter disease duration and a higher activity score suggests that IL-27 may play a role in formation of vitiligo patches at early stages of the pathogenesis of vitiligo.

IL-27 was thought to be a pro-inflammatory cytokine since it stimulates the production of interferon-γ (IFN-γ) from T cells and natural killer cells (NK), increases adhesion molecules on the T cell surface, and improves the function of CD8+ T cells [21]. However, a growing number of studies have supported the anti-inflammatory properties of IL-27 in recent years. IL-27 shows a suppressive effect by stimulating the production of IL-10, supporting the growth and survival of Treg cells, stimulating the expression of inhibitory

receptors on the T cell surface, and suppressing dendritic cells [21]. Vitiligo patients having lower levels of IL-27 than controls suggests that IL-27 may play an immunosuppressive role in vitiligo.

Although serum levels of IL-12, IL-23 and IL-27 have been studied in vitiligo, the effect of IL-35 in vitiligo is yet to be identified. Serum IL-35 levels were also higher in vitiligo patients compared to controls in our study. IL-35 is mainly produced by regulatory T cells (T_{reg}), active macrophages and B cells. Comprised of EBI3 and p35 subunits, IL-35 operates by binding to its heterodimeric receptor (GP130–GP130, IL-12Rb2–IL-12Rb2, IL-12Rb2–GP130, and GP130-IL-12Rb1). IL-35 binding to the receptor activates JAK1 and JAK2. STAT1, STAT3, and STAT4 are than phosphorylated for biological effect [22]. Different receptors on the surfaces of cells may have different biological effects by activating different JAK/STAT pathways. IL-35 receptors on T_{reg} cells and B_{reg} cells enable IL-35 to stimulate expansion in T_{reg} and B_{reg} cells, creating immunosuppressive effects [23].

IL-35 serum levels have been previously studied in many autoimmune and inflammatory diseases and different outcomes reported. Compared to healthy controls lower serum IL-35 levels were reported in patients with systemic lupus erythematosus and rheumatoid arthritis, whereas higher levels of IL-35 were seen in patients with systemic sclerosis [23,24].

Even though the reason for the increased levels of IL-35, which has an anti-inflammatory and immunosuppressive cytokine, in autoimmune/inflammatory diseases is not fully known, several studies have attempted to explain this contradiction. In a study investigating whether IL-35 has different properties from IL-10, transforming growth factor (TGF)-β or similar other anti-inflammatory cytokines, Li et al. showed that IL-35 was a stimulable anti-inflammatory cytokine unlike other anti-inflammatory cytokines [25]. IL-35 prevents inflammation from reaching its maximum impact rather than preventing inflammation from forming by being expressed in response to inflammatory stimuli [25]. Qiu et al. found higher levels of serum IL-35 in patients with active Lupus compared to controls, and observed a reduction in IL-35 levels after treatment. The authors argued that IL-35 was an anti-inflammatory factor that increased to antagonize the negative impact of the pathological inflammation to a certain extent [26]. Correlation of IL-35 with sedimentation levels in patients with primary Sjögren syndrome backs the arguments that IL-35 is an anti-inflammatory cytokine that is secreted secondarily to inflammatory stimuli to antagonize the effects of maximal inflammation [24].

Although vitiligo patients had higher levels of IL-35 than healthy controls, there was not any significant correlation between serum IL-35 levels and the extensiveness or activity of the disease. In vitiligo IL-35 may be expressed to antagonize the maximal effects of pro-inflammatory cytokines as is the case with lupus and primary Sjögren disease.

Levels of IL-12, IL-27 and IL-35 from the IL-12 cytokine family were not similar for vitiligo patients and healthy volunteers. Vitiligo patients had higher levels of IL-12 and IL-35, and lower levels of IL-27. Also, lower levels of IL-27 were clearer in patients with a higher disease activity and a shorter duration of vitiligo. IL-23, which had similar serum levels in the patient and control groups, was correlated with VASI. This study showed that in addition to previously studied IL-12 cytokines (IL-12, IL-23, IL-27), IL-35, one of the newest members of IL-12 family, may also play a role in the pathogenesis of vitiligo.

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