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Predictive Significance of Interleukins 17A and 33 in Risk of Relapsing–Remitting Multiple Sclerosis

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Abstract:

Cytokines are signaling molecules between inflammatory cells that play a significant role in the pathogenesis of a disease. Among these cytokines are interleukins (ILs) 17A and 33, and accordingly, the current case-control study sought to investigate the role of each of the two cytokines in the risk of developing multiple sclerosis (MS). Sixty-eight relapsing-remitting MS (RRMS) Iraqi patients and twenty healthy individuals (control group) were enrolled. Enzyme linked immunosorbent assay (ELISA) kits were used to determine serum levels of IL-17A and IL-33. Results revealed that IL-17A and IL-33 levels were significantly higher in MS patients than in controls (14.1 ± 4.5 *vs.* 7.5 ± 3.8 pg/mL; $p < 0.001$ and 65.3 ± 16.3 *vs.* 49.3 ± 20.0 pg/mL; $p < 0.001$, respectively). Receiver operating characteristic (ROC) curve analysis demonstrated that IL-17A was a very good predictor of MS (area under curve [AUC] = 0.869; 95% CI = 0.779 - 0.960; $p < 0.001$; cut-off value = 10.2 pg/mL; sensitivity = 80.8%; specificity = 75.0%). A similar prediction was presented by IL-33, but the AUC value was lower (AUC = 0.762; 95% CI = 0.63 - 0.89; $p < 0.001$; cut-off value = 56.4 pg/mL; sensitivity = 70.6%; specificity = 70.0%). Multinomial logistic regression analysis confirmed the significance of IL-17A and IL-33 in MS risk, and under three models of analysis, the estimated odds ratios for IL-17A (1.50, 1.49 and 1.50, respectively) and IL-33 (1.05, 1.05 and 1.06) were above 1.0. Patients stratified by gender (male and female), expanded disability status scale (EDSS: < 3 and ≥ 3) or medication (pre- and post-medication) showed no significant differences in serum levels of IL-17A and IL-33 for each stratum. However, with regard to response to medication, it was found that responding patients showed significantly higher levels of IL-33 than non-responders (70.9 ± 12.2 *vs.* 57.2 ± 18.2 pg/mL; $p = 0.018$). This difference was not observed when considering IL-17A. Pearson correlation analysis between IL-17A and IL-33 revealed that both cytokines were not significantly correlated. In conclusion, the study indicated that IL-17A and IL-33 were up-regulated in serum of MS patients, and this up-regulation was not influenced by age, gender, EDSS or medication status, but the elevated level of IL-33 was more pronounced in patients who responded to medication.

Keywords: Expanded disability status scale; IL-17A; IL-33; Logistic regression analysis; Multiple sclerosis; Receiver operating characteristic.

Introduction:

Multiple sclerosis (MS) is an autoimmune degenerative disorder featured by axon demyelination of neurons. The most prevalent clinical course of MS is relapsing-remitting multiple sclerosis (RRMS), which affects most patients[1]. Genetic and environmental factors are proposed to play a pivotal role in triggering the disease episodes, and their interaction may lead to immune dysregulation and development of disease.[2] Cytokines are among the immune components described to play a role in the pathogenesis of MS, and their significance in ameliorating or exacerbating episodes of the disease has been proposed.[3] IL-17A and IL-33 are two types of cytokines that have been the focus of recent research investigating the pathogenesis of MS.[4,5]

IL-17A is a pro-inflammatory cytokine produced by T helper (Th)-17 cell; human and

animal model studies proposed that this cytokine is a potential target for pharmaceutical interventions in several autoimmune diseases including MS.[6,7] Experimental observations demonstrated that IL-17A signaling is involved in recruiting inflammatory cells, neutrophils and macrophages, to the sites of inflammation.[8] In MS, increased mRNA expression of IL-17A in lymphocytes, oligodendrocytes and astrocytes has been described during the active phase of disease.[9] Besides, it has been indicated that the level of IL-17A in serum can be considered as one of the most distinct biomarkers among MS and other autoimmune diseases.[7] Furthermore, IL-17A has been associated with exaggerated inflammatory responses that mediate the pathogenesis of MS and other autoimmune diseases.[10]

IL-33 is also a pro-inflammatory cytokine classified within the IL-1 family of cytokines. [11] It is released from stromal cells and innate immune cells, such as macrophages and dendritic cells, in response to inflammation.[12] IL-33 is considered as an "alarmin" that sends danger signals to the nearby tissues upon cell damage. [13] Further, IL-33 is recognized to have a dual function by acting as an extracellular cytokine and as a nuclear transcriptional factor.[14] Besides, the role of IL-33 in tissue repair and inflammation resolution has been described.[15] Correspondingly, dysregulated expression of IL-33 has been associated with the development of various inflammatory and autoimmune diseases including MS.[16]

In line with these findings, the predictive significance of circulatory IL-17A and IL-33 in risk of RRMS was investigated in a group of Iraqi patients. Equally important, effects of age, gender, clinical disability (defined by expanded disability status scale; EDSS), medication and response to medication, on the levels of these cytokines were also evaluated. To the best knowledge of the researchers, some of the latter evaluations have not well been elaborated on in the literature.

Materials and Methods:

Patients and controls

A retrospective case-control study was performed to determine the serum levels of IL-17A and IL-33 in 68 RRMS patients and 20 healthy subjects (control group). Patients were referred to the outpatient neurology unit (MS Clinic) at Bagdad Teaching Hospital during the period from December 2013 - March 2014. MS was diagnosed by consultant neurologist according to the revised McDonald criteria.[17] The classification of Lublin and Reingold was adopted to define the clinical form of RRMS. [18] Age, gender, clinical disability and medication were recorded using a standard questionnaire and medical records. The clinical disability of MS was evaluated by measuring the degree of ambulatory status, defined by the EDSS, at the time of patient enrollment. The EDSS ranges from 0 to10 i.e. from normal ambulatory status to complete disability and death. [19] For simplicity, the patients were distributed into two EDSS groups; < 3 (fully ambulant patients or have a minimum disability) and ≥ 3 (patients with moderate to high disability). Some patients were not on medication (pre-medicated group), while others were receiving the immunotherapy medication interferon beta, and these patients were considered post-medication group. Based on the clinical assessment of the neurologist consultant, the post-medicated patients were categorized as non-responders and responders. The control group included apparently healthy individuals, who had not received non-steroidal anti-inflammatory drugs, and they did not suffer from inflammatory, autoimmune or chronic diseases.

Measurement of serum IL-17A and IL-33 levels

Five milliliters of venous blood were drawn into a plain tube from each patient during the clinic visit. The blood was allowed to clot at room temperature (20-25 °C), and then centrifuged (3000 rpm for 15 minutes at 4 °C) to collect serum. The serum was kept frozen at -20 °C until assessment. Levels of IL-17A and IL-33 were measured in serum using enzyme linked immunosorbent assay (ELISA) kits. The procedure was carried out according to the manufacturer instructions (PeproTech Company, U.K.).

Statistical analysis

All data were analyzed using the statistical package IBM SPSS version 23.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 8.0.0 (San Diego, California USA). Categorical variables were given as number and percent, and significant differences were determined using Pearson Chi-square test. Test of normality was assessed for continuous variables using Kolmogorov-Smirnov test. The test revealed that all variables were normally distributed and therefore, they were expressed as mean ± standard deviation (SD). Differences between means were determined by the Student t-test. Receiver operating curve (ROC) analysis was applied to determine area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity and specificity. The cut-off value was optimized using Youden index. [20] Multinomial logistic regression analysis was performed to calculate odds ratio (OR) and 95% CI. The analysis

was either unadjusted (Model I), adjusted for age (Model II), or adjusted for age and gender (Model III). Pearson correlation analysis was performed to assess the correlation between IL-17A and IL-33. A probability (p) value ≤ 0.05 was considered significant.

Results:

Baseline characteristics

MS Patients and controls were characterized in terms of age and gender. The mean age was approximated in both group and no significant difference was observed (34.8 ± 9.7 *vs*. 35.0 ± 8.9 years; $p = 0.923$). In the case of gender, MS patients were presented more frequently by females than males (66.2 *vs*. 33.8%), but the difference was not significant compared to controls (75.0% females and 25% males; $p = 0.456$). The patients were further defined in terms of EDSS, medication and response to medication. About two-thirds of patients had an EDSS of < 3 (62.5%), while 37.5% were classified under EDSS of ≥ 3. For medications, 44.1% of patients were classified in the pre-medication group, while 55.9% were examined after receiving their medication (post-medication group). It was possible to follow up 38 patients in terms of their response to medication, and it was found that 39.5% of the patients were non-responders, while 60.5% were responders. (Table 1).

Table 1. Baseline characteristics of MS patients and controls.

Characteristic	MS patients (N = 68)	Controls (N = 20)	*p*-value
Mean age ± SD (years)	34.8 ± 9.7	35.0 ± 8.9	0.923
Gender; N (%)			
Male	23 (33.8)	5 (25.0)	0.456
Female	45 (66.2)	15 (75.0)	
EDSS group; N (%)			
< 3	40 (62.5)	NA	NA
≥ 3	24 (37.5)	NA	NA
Medication; N (%)			
Pre-medication	30 (44.1)	NA	NA
Post-medication	38 (55.9)	NA	NA
Response to medication; N (%)			
Non-responder	15 (39.5)	NA	NA
Responder	23 (60.5)	NA	NA

MS: Multiple sclerosis; SD: Standard deviation; EDSS: Expanded disability status scale; *p*: Probability of student t-test or Pearson Chi-square test; NA: Not applicable.

Serum levels of IL-17A and IL-33

Serum levels of IL-17A and IL-33 were tested for normality, and were found to be normally distributed in MS patients and controls. Therefore, their levels were given as mean ± SD, and significant differences were evaluated by Student's t-test. Levels of IL-17A and IL-33 were significantly higher in MS patients than in controls (14.1 ± 4.5 *vs*. 7.5 ± 3.8 pg/mL; $p < 0.001$ and 65.3 ± 16.3 *vs*. 49.3 ± 20.0 pg/mL; $p < 0.001$, respectively) (Fig. 1). ROC curve analysis revealed that IL-17A was a very good predictor in discriminating between MS patients and controls (AUC = 0.869; 95% CI = 0.779 - 0.960; $p < 0.001$; cut-off value = 10.2 pg/mL; sensitivity = 80.8%; specificity = 75.0%). A similar prediction was presented by IL-33, but the AUC value was lower (AUC = 0.762; 95% CI = 0.63 - 0.89; $p < 0.001$; cut-off value = 56.4 pg/mL; sensitivity = 70.6%; specificity = 70.0%) (Fig. 2). Multinomial logistic regression analysis confirmed the significance of IL-17A and IL-33 in the risk of MS. Under the three models of analysis, the estimated ORs for IL-17A (1.50, 1.49 and 1.50, respectively) and IL-33 (1.05, 1.05 and 1.06) were above 1.0, and this may suggest that both cytokines were risk factors of MS, independent of age and gender (Table 2).

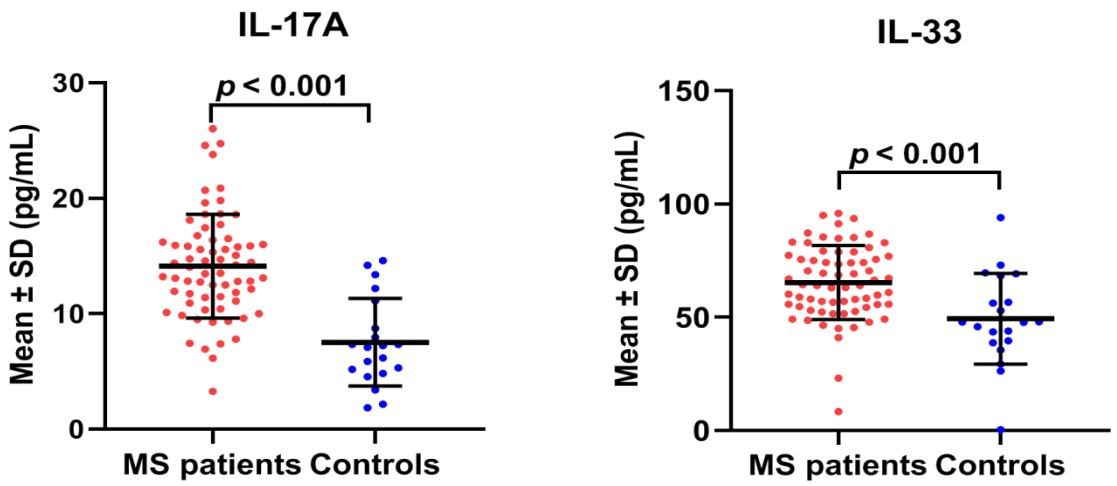

Figure 1. Scatter dot plots of IL-17A and IL-33 serum levels in MS patients and controls.

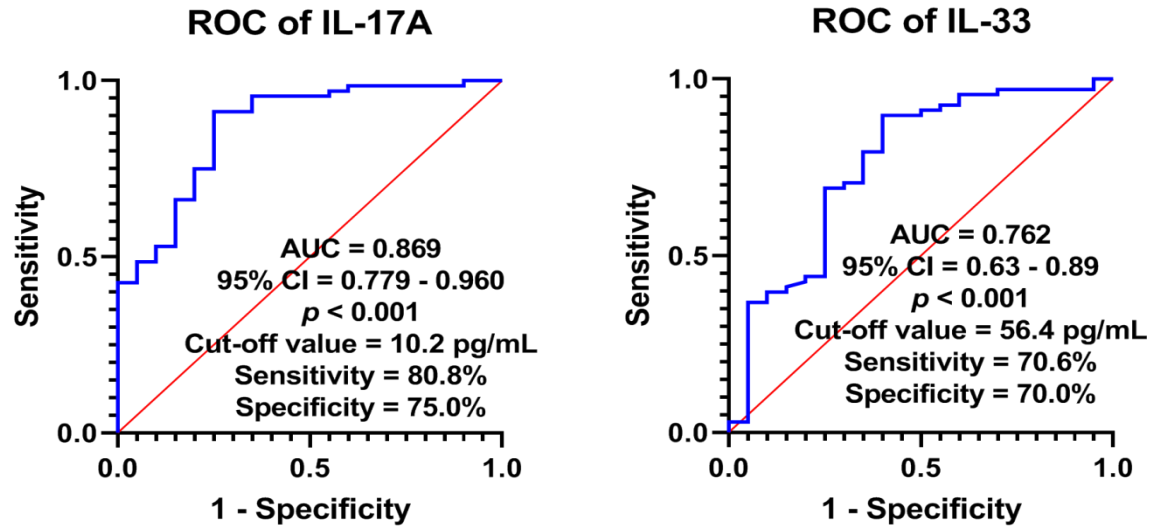

Figure 2. ROC curve analysis IL-17A and IL-33 in serum of MS patients *versus* controls showing area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity and specificity.

Table 2. Logistic regression analysis of IL-17A and IL-33 in MS patients *versus* controls.

Analysis Model†	IL-17A			IL-33		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Model I	1.50	1.24 - 1.80	< **0.001**	1.05	1.02 - 1.09	**0.002**
Model II	1.49	1.24 - 1.80	< **0.001**	1.05	1.02 - 1.09	**0.002**
Model III	1.50	1.24 - 1.80	< **0.001**	1.06	1.02 - 1.10	**0.002**

†Controls were the reference category; Model I: Unadjusted; Model II: Adjusted for age; Model III: Adjusted for age and gender; OR: Odds ratio; CI: Confidence interval; p: Probability.

Serum levels of IL-17A and IL-33 stratified by characteristics of patients

Serum levels of IL-17A and IL-33 were stratified by gender, EDSS, medication and response to medication in MS patients. Patients stratified by gender (male and female), EDSS (< 3 and ≥ 3) or medication (pre- and post-medication) showed no significant differences in serum levels of IL-17A and IL-33 for each stratum (Figs. 3, 4 and 5 respectively). However, with regard to response to medication, it was found that responding patients showed significantly higher levels of IL-33 than non-responders (70.9 ± 12.2 *vs*. 57.2 ± 18.2 pg/mL; $p = 0.018$). This difference was not observed when considering IL-17A (Fig. 6).

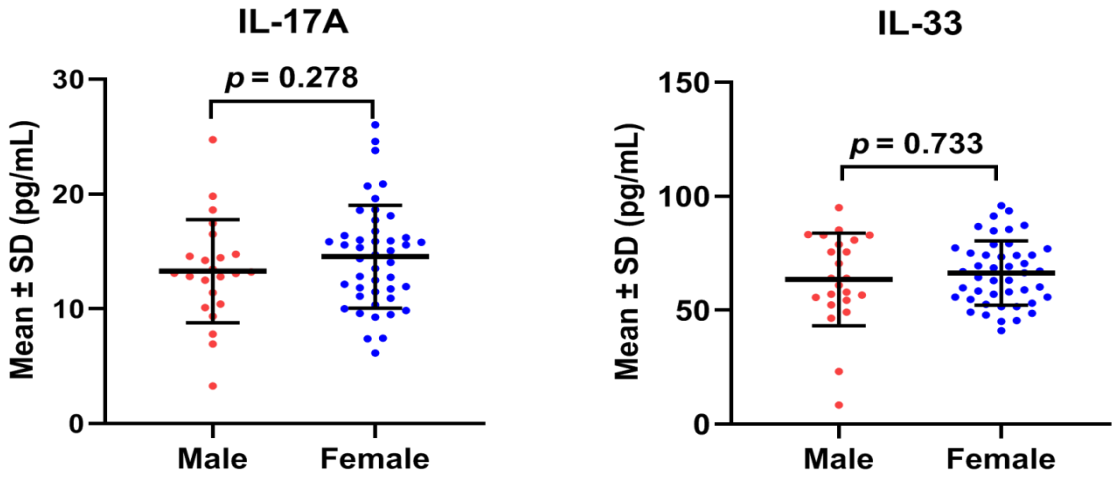

Figure 3. Scatter dot plots of IL-17A and IL-33 serum levels in MS patients stratified by gender.

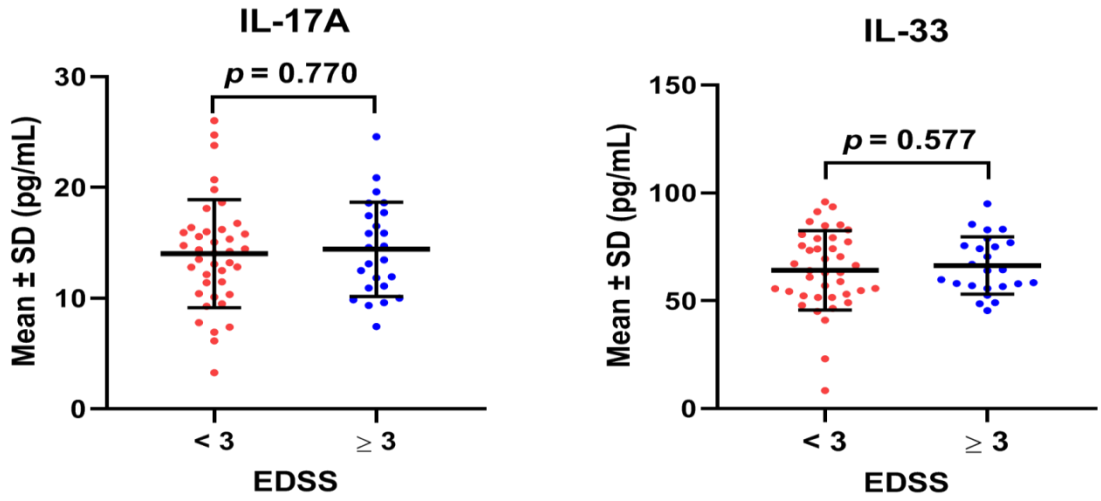

Figure 4. Scatter dot plots of IL-17A and IL-33 serum levels in MS patients stratified by expanded disability status scale (EDSS).

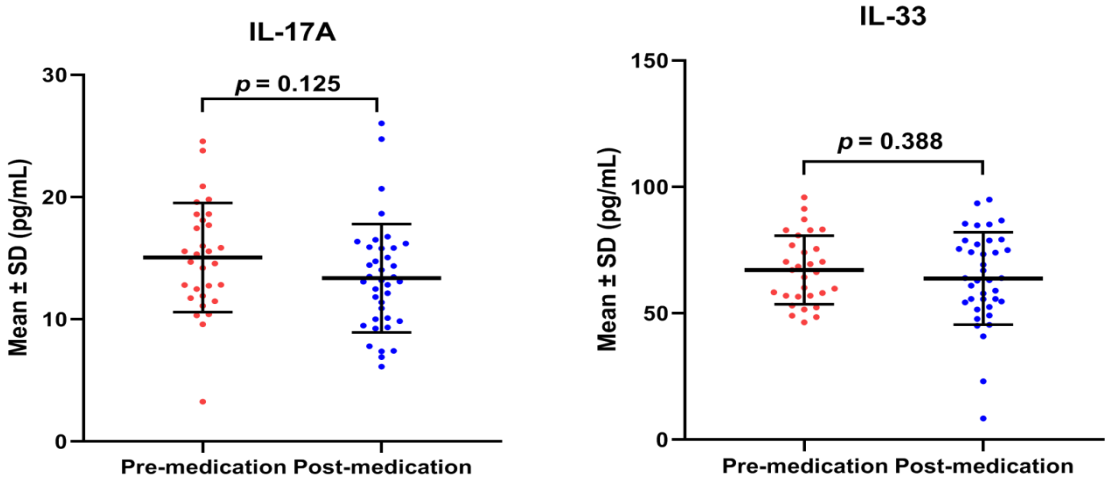

Figure 5. Scatter dot plots of IL-17A and IL-33 serum levels in MS patients stratified by medication.

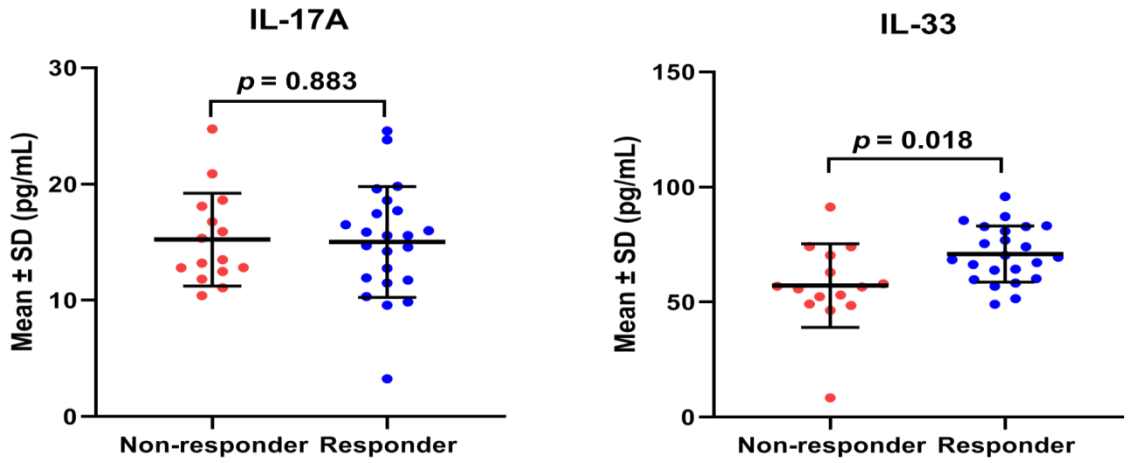

Figure 6. Scatter dot plots of IL-17A and IL-33 serum levels in MS patients stratified by response to medication.

Pearson correlation

Pearson correlation analysis between IL-17A and IL-33 serum levels revealed that both cytokines were not significantly correlated in MS patients or controls. However, it was observed that the correlation coefficient was higher in controls than in patients (0.330 *vs*. 0.042) (Fig. 7).

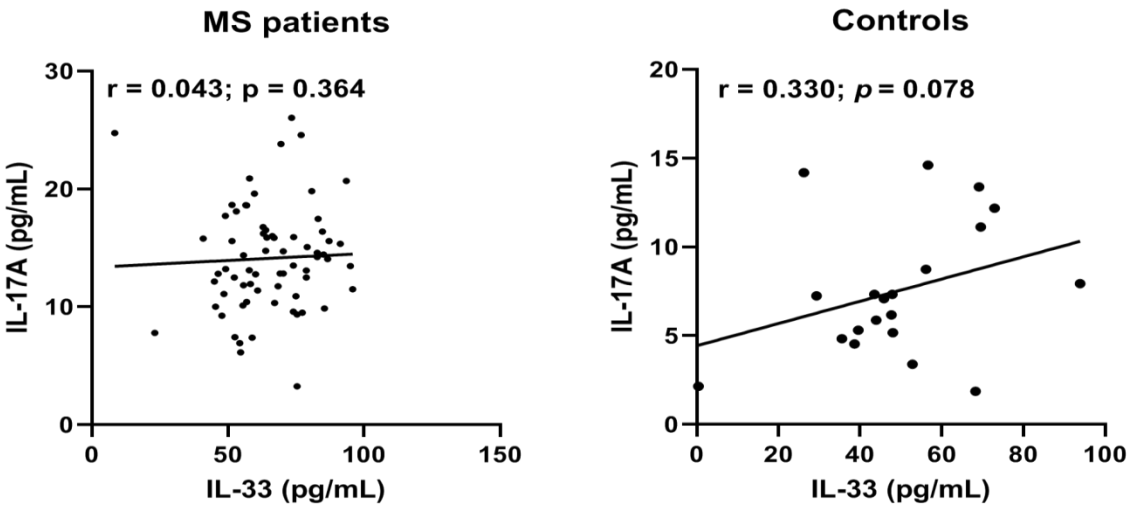

Figure 7. Scatter plot and Pearson correlation coefficient (r) for analysis between IL-17A and IL-33 serum levels in MS patients and controls.

Discussion:

Multiple sclerosis (MS) is a neurodegenerative disorder that has been extensively studied in terms of cytokine effect on disease progression or amelioration. Our current findings referred to a significant elevation in IL-17A serum level in RRMS patients compared to controls and that was consistent with previous studies.[21, 22, 23]. Moreover, our study did not find any correlation between serum IL-17A level and clinical features of MS, including EDSS, medication and gender; and this was consistent with previous studies.[21,24]. It was stated that serum level of IL-17A was significantly higher in non-responders to IFN-β therapy than responders,[24] while our results showed no correlation between IL-17A level and response to therapy. It was assumed that IL-17A is a signature cytokine of activated Th-17 cells; these cells are differentiated from naïve CD+4 T-cells and contribute to tissue inflammation and host defense against infections and autoimmune diseases, such as MS and rheumatoid arthritis by inducing the expression of pro-inflammatory cytokines and chemokines.[9] Moreover, CNS resident cells, such as microglia and astrocytes also produce IL-17A, therefore, inhibiting IL-17A production can result in the suppression of tissue inflammation and

enhancing repair mechanism.[5] Increased gene expression of IL-17A in CNS plaques collected from autopsy of MS patients was reported.[7] The current results also revealed that there was no significant difference in serum IL-17A level between patient subgroups based on EDSS and this was in agreement with a former study performed by Matusevicius et al, 1999[25], who referred to an increased production of IL-17 in mononuclear cells of CSF compared to blood and this expression was at the highest level in blood upon disease exacerbation. Furthermore, a body of evidence pointed to the importance of IL-17A in experimental autoimmune encephalitis (EAE) and RRMS development and the increased level in CSF was associated with increased permeability of blood-brain barrier.[26] Our results also demonstrated that there was a slight decreased serum level of IL-17A in treated patients comparing to untreated patients; but this did not reach to a significant level which indicated that the immunotherapy might have a slight impact on this biomarker or it can be owing to remission phase of the patients. Contrarily, it was referred to the role of IFN-β therapy in reducing T-lymphocyte cell migration to CNS and induction of Th-17 cell apoptosis that encounter disease severity.[27] The current findings referred to the importance of IL-17A as a very good predictor for distinguishing MS affected individuals from those unaffected as revealed by ROC curve analysis.

The current study also showed that there was a significant increased IL-33 level in MS patients compared to controls, and ROC curve analysis confirmed the predictive significance of IL-33 in MS. Studies of other world groups of MS have revealed consistent findings.[28, 29, 30] Additionally, the difference in IL-33 serum level between pre- and post-medicated patients was not significant and that was consistent with other observations.[28] Nevertheless, the level of IL-33 in responders to IFN-β therapy was significantly higher than those non-responders; this may be owing to the larger sample size in treated patients than those untreated. It was stated that higher plasma level of IL-33 in RRMS patients with mild severity may contribute in remyelination and suppression of inflammation.[31] The present findings also showed that there was no correlation between IL-33 serum levels and EDSS and this was in agreement with Mado et al, 2021. [31]

It was documented that CNS is the main site for IL-33 mRNA expression and IL-33 protein is elevated in active areas of demyelinated white matter plaques.[32] Furthermore, the transcription factor NF-*k*B that promotes the expression of IL-33 showed an increased expression in the leukocytes and astrocytes of the CNS in MS patients.[32] Despite the non-significant difference, serum IL-33 level was slightly lower than in treated patients as compared to untreated MS group, and this may indicate that the drug did not have a remarkable effect on the level of this biomarker. A previous study also shared these findings.[33]

Conclusion:

The study indicates that IL-17A and IL-33 are up-regulated in serum of MS patients, and this up-regulation is not influenced by age, gender, EDSS or medication status, but the elevated level of IL-33 is more pronounced in patients who responded to medication. However, it should be noted that the study is limited by the low sample size of the controls.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours.
- Authors sign on ethical consideration's approval
- Ethical Clearance: The study was approved by Iraqi Ministry of Health. Informed consent was obtained from participants.
- Funding: This research is self-funded and received no grant from any funding agency in the public, commercial, or not-for-profit sectors

Authors' contributions statement:

M. A. S. A. N.: acquisition of data analysis, interpretation, drafting the MS, A. H. A.: design, conception, data analysis, revision and proofreading, E. D. S.: data analysis, revision and proofreading

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الاهمية التنبؤية للانترلوكينات 17أ و 33 في خطورة مرض التصلب العصبي من النوع الانتكاسي-المتحسن

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الخلاصة:

تعتبر الحركيات الخلوية جزيئات مرسلة للإشارات بين الخلايا المناعية وتلعب دوراً اساسياً في الامراضية ومنها الانترلوكينات 17A و 33 . لقد هدفت دراسة المرضى-السيطرة الحالية التحري عن دور هذين الحركيين الخلويين في خطورة تطور مرض التصلب العصبي. لقد تمت مشاركة ثمان وستون مريضاً مصاباً بالتصلب العصبي المتعدد من نوع الانتكاسي-المتحسن وعشرون شخصا من الاصحاء (مجموعة سيطرة) واستخدمت طريقة الادمصاص المناعي المرتبط بالأنزيم (الاليزا) لقياس المستوى المصلي. لقد اظهرت النتائج بوجود زيادة معنوية في المستويات المصلية لكل من IL-17 A و IL-33 مقارنة مع مجموعة السيطرة (14.1 ± 4.5 مقابل 7.5 ±3.8 ، $P <$ 0.001 و65.3 ± 16.3 مقابل 49.3 ± 20.0 ، $P <$ 0,001) بيكوغرام/ مل على التوالي. لقد اظهر تحليل منحنى خصائص تشغيل المستقبِل ROC بان الحركي الخلوي IL17A يمثل عامل تنبؤ جيد لمرض التصلب العصبي (المنطقة اسفل المنحنى AUC =0,869، 95% CI =0.779- 0.965، $p <$ 0,001 ، القيمة النهائية = 10.2 بيكوغرام/ مل ، الحساسية= 80.8%، النوعية= 75%. كما وان هناك تنبؤاً مشابهاً للانترلوكين 33 ولكن هناك انخفاضاً في قيمة المنطقة أسفل المنحنى (AUC =0,762 , 95% CI =0,63 - 0,89 ، $P <$ 0,001 ، القيمة النهائية = 56,4 بيكوغرام/ مل ، الحساسية= 70.6%، النوعية= 70%. لقد تم اثبات اهمية الانترلوكينات 17 و33 في خطورة مرض التصلب العصبي من خلال تحليل الانحدار المنطقي المتعدد، وبوجود ثلاث نماذج تحليلية، لقد قُدرت قيمة الارجحية اعلى من 1 لكل من الانترلوكين 17 (1.5، 1.49 ،1.5) والانترلوكين 33 (1.05، 1.05، 1.06). لم تظهر فروقاً معنوية للمرضى المصنفين حسب الجنس (الذكر والانثى)، مقياس حالة العجز الحركي الموسّعة (EDSS > 3 و EDSS ≤ 3) أو العلاج (ما قبل العلاج وما بعده) وفقاً للمستوى المصلي لكلا الانترلوكينين. ووفقاً للاستجابة للعلاج، فقد تبين بأن المستوى المصلي للانترلوكين 33 كان اعلى للمرضى المستجيبين للعلاج من هؤلاء الذين لم يستجيبوا له (70,9 ± 12,2 مقابل 57,2 ± 18.2 بيكوغرام/ مل، $p =$ 0.018 ، وهذا الفرق لم نلاحظه للانترلوكين 17 A. وقد اظهر تحليل بيرسون للعلاقة بان كلا الانترلوكينين غير مرتبطين معنوياً مع بعضهما. وكاستنتاج نهائي، لقد بينت الدراسة بان الانترلوكينين 17A و33 يزداد انتاجهما في مرضى التصلب العصبي غير ان هذا الانتاج غير متأثر بالعمر والجنس وقيمة EDSS او العلاج ولكن ازدياد المستوى المصلي للانترلوكين 33 كان واضحاً بالنسبة للمرضى المستجيبين للعلاج.

الكلمات المفتاحية: معيار العجز الحركي الموسَّع، انترلوكين-17أ، انترلوكين-33، تحليل الانحدار المنطقي، التصلب العصبي المتعدد، منحنى خصائص تشغيل المستقبِل