Zagazig University Medical Journal
www.zumj.journals.ekb.eg

Volume 29, Issue 2, March 2023, page (242-247) Supplement Issue

Manuscript ID ZUMJ-2011-2026 (R3)
DOI **10.21608/ZUMJ.2021.51028.2026**

ORIGINAL ARTICLE

Serum Interleukin 23 and Nitric Oxide in Vitiligo: New insights into the emerging association with VASI Score

Bessar H[1*], AbdelMoez B[1], Sharaf SM[2] MD, H. Ghanem A[1]

1 Dermatology , Venereology and Andrology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

2 Clinical Pathology Department , Faculty of Medicine, Zagazig University, Zagazig, Egypt

***Corresponding author:**
Hagar Bessar
Dermatology, Venereology and Andrology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Email:
dr_hagarawadbessar@yahoo.com

Submit Date 2020-12-07
Revise Date 2021-01-18
Accept Date 2021-02-10

ABSTRACT

Background: Vitiligo is a depigmentation disease of the skin caused by selective melanocyte degradation. In recent years, vitiligo has become known as an autoimmune disorder. This study aimed to determine serum levels of interleukin 23 (IL-23) and nitric oxide (NO) in vitiligo patients and their possible role in the pathogenesis of vitiligo, in an attempt to identify new treatment strategies.

Methods: The autoimmune and autocytotoxic mechanisms of vitiligo development were investigated in a case-control study comparing 30 vitiligo patients to 30 healthy controls. Serum levels of IL-23 and NO were measured in vitiligo patients and healthy controls. The interactions between IL-23 and NO with different parameters in the vitiligo group were also examined.

Results: Serum IL-23 and NO levels were higher in the vitiligo group compared with healthy controls ($P < 0.001$ and $P = 0.02$, respectively). In the vitiligo group, there was a significant positive correlation between serum IL-23 and NO levels and vitiligo area scoring index (VASI).

Conclusions: Our findings revealed a correlation between elevated serum IL-23 and NO in the vitiligo group that was positively linked to VASI scores. This suggests that these two factors are involved in the disease pathogenesis and inhibition of these pathways might be a novel strategy in vitiligo therapy.

keywords: Vitiligo, IL-23, Nitric oxide.

INTRODUCTION

Vitiligo is a skin depigmentation condition caused by selective melanocyte degradation [1]. Increasing evidence confirms the theory of autoimmune etiology. Vitiligo development includes various factors, including infections, stress, neurological defects, alterations in melatonin receptors, and genetically susceptible populations. Vitiligo is clinically characterized by the persistent and gradual depletion of functional melanocytes accompanied by the appearance of white macules or patches in areas of the skin, hair, and mucous membranes [2]. To investigate the autoimmune and auto-cytotoxic hypotheses in patients with vitiligo, we tested serum interleukin (IL)-23 and nitric oxide (NO) levels. Limited studies investigated the role of NO in vitiligo pathogenesis or studied the role of interleukins in vitiligo pathogenesis. In this ongoing study, we sought to determine serum levels of IL-23 and NO in vitiligo patients and their possible combined role in the pathogenesis of vitiligo in an attempt to identify new treatment strategies.

METHODS

This study was fulfilled in the out-patient clinic of the Dermatology, Venereology, and Andrology Department plus Clinical Pathology Laboratory at Zagazig University Hospitals from September 2019 to March 2020. The study consisted of 30 patients who had vitiligo and 30 healthy age- and sex-matched controls. Informed consent was given from every patient and control before the start of the study. All the patient's data were approved for use by the Institutional Review Board of Faculty of Medicine, Zagazig University Hospital. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. The selection requirements for patients included: clinical diagnosis of vitiligo confirmed by woods light examination, aged above 12 years, all systemic therapies stopped 1 year before the beginning of the study, and all topical therapies stopped three months before the beginning of the study. We excluded patients complaining of active medical conditions, including malignancies, HIV,

or hepatitis B or C positive, or otherwise immunocompromised. In addition, we excluded pregnant or lactating women.

Data management: We obtained demographic history, full dermatologic history (including onset, course, and duration of vitiligo, site, type, extension, and therapies), history of any other skin conditions (such as psoriasis, dermatitis, multiple atypical moles, sun sensitivity, sunburn, or rash within a couple of months of de-pigmentation, or premature hair graying), history of autoimmune disease, medical problems, physical illness or stress, and family history. Dermatological examination data were recorded, including the number of vitiligo lesions, anatomical distribution of the vitiligo, and type of vitiligo. Laboratory results were collected. Serum IL-23 and NO levels were obtained. We compared the data between the vitiligo and control groups.

Sample collection and storage: Five milliliters of venous blood were collected in complete aseptic conditions from the antecubital vein. Samples of serum were collected in a special tube for separation. Samples were allowed to clot for 30 minutes before centrifugation (10 minutes at 3000 rpm); then serum aliquots were stored at −20 °C.

Determination of serum IL-23 level: We conducted a case-control association analysis using an Enzyme-linked Immunosorbent Assay (ELISA) to investigate whether IL 23 imbalance plays a role in pathogenesis of Vitiligo. Plasma IL-23 level was measured using an ELISA kit (R&D System Europe, Abingdon, UK) based on the manufacturer's instructions (Invitrogen-Life Technologies). The absorbance was read on an ELISA reader at 450nm (Labsystems).

Determination of serum NO Serum NO levels in blood samples were evaluated using sandwich ELISA detection. NO production was assessed by the determination of the products of NO oxidation. After the reduction of nitrate (NO_3-) to nitrite (NO_2-), the total nitrite was quantified by spectrophotometry (at 540 nm) in samples after reaction with Griess reagent, as described by Touil-Boukoffa (1998) [3].

STATISTICAL ANALYSIS

Continuous variables were expressed as mean ± standard deviation (SD) and compared using t tests or expressed as median (range) and compared using Mann–Whitney U tests depending on whether they were normally distributed or not. Categorical variables were expressed as percentages then compared using Chi-Square tests. Then using The Pearson correlation coefficient was to calculate the correlation between quantitative variables. Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of different parameters with maximum sensitivity and specificity for prediction of the outcome. Accuracy was measured by area under the ROC curve. A p-value ≤ 0.05 was considered significant. Statistical calculations were done using SPSS (Statistical Package for the Social Science) version 20 for Microsoft windows.

RESULTS

In this study, the mean age of our patients was 27.67 ± 14.2 years and 73.3% were female. The median age of the healthy age- and sex-matched control group was 29.33 ± 10.37 years and 53.3% were female. There were no significant differences in demographic data (age, gender, race), family history, past medical, or surgical history between the two groups.

Clinical findings in the vitiligo group the median duration of the disease was 2.5 years (range: 0.16–23). The median vitiligo area scoring index (VASI) was 2.75 (range: 0.75–40.5). Seventeen (56.7%) cases were generalized nonsegmental vitiligo. Eighteen (60%) cases had previous treatment (7 cases: 0.03% topical tacrolimus and corticosteroid; 3 cases: topical salicylic acid; 5 cases: systemic phototherapy, pimecrolimus, and corticosteroid; 3 cases: systemic phototherapy, pulse therapy, and corticosteroid).

IL-23 and NO levels :Serum IL-23 levels were higher in the vitiligo group (median: 259.18 ng/L; range: 101.82 – 1506.96) compared with the control group (154.27 ng/L; 107.4 – 879.1) ($P < 0.001$) (Figure 1(a)). Serum NO levels were higher in the vitiligo group (12.4 Mmol/L; 3.08 – 66.7) compared with the control group (8.8 Mmol/L; 2.1 – 38) ($P = 0.02$) (Figure 1(b)). There were no statistical significance levels of serum IL-23, or NO levels based on gender, family history, or previous treatment.Correlation between serum biomarkers and age, disease duration, and VASI in vitiligo patients Neither IL-23 nor NO significantly correlated with patient age or disease duration (Table 2). We noticed a significant positive correlation between serum IL-23 levels and VASI in the vitiligo group ($P = 0.03$) (Figure 2 (a)). There was a highly significant positive correlation between serum NO levels and VASI scores in the vitiligo group ($P = 0.005$) (Figure 2 (b)).

Correlation between serum IL-23 and NO in vitiligo patients IL-23 and serum NO significantly and positively correlate in the vitiligo group ($r = 0.34$, $P = 0.04$) (Table 3 & Figure 3).

Validity of IL-23 and NO in the diagnosis of vitiligo As shown in Table 4, the sensitivity of IL-23 in diagnosing vitiligo at a cut-off of ≥184.47 ng/L was 80%, the specificity was 86.7%, and the accuracy was 83.3%. The sensitivity of NO in diagnosing vitiligo at a cut-off of ≥11.07 Mmol/L was 60%, the specificity was 63.3%, and the accuracy was 61.7% (Table4) (Figure 4).

Table 1: Comparison of IL-23 and NO in the two studied groups.

Variable	Cases (n=30)	Control (n=30)	MW	P
IL-23 (ng/L): *Mean ± SD* *Median* *Range*	 429.20 ± 360.47 259.18 101.82 – 1506.96	 179.33 ± 135.65 154.27 107.4 – 879.1	4.41	<0.001**
Nitric oxide (Mmol/L): *Mean ± SD* *Median* *Range*	 25.20 ± 21.81 12.4 3.08 – 66.7	 11.64 ± 8.39 8.8 2.1 – 38	2.28	0.02*

SD: Stander deviation.
MW: Mann Whitney test.
*: Significant (P<0.05).
**: Highly significant (P<0.01).

Table 2: Correlation between serum biomarkers (IL-23 and Nitric Oxide) levels and age, duration and VASI score of the vitiligo group.

	Age (years)		**Duration (years)**		**VASI score**	
	R	P	r	P	r	P
IL-23	0.28	0.14	0.03	0.87	0.36	0.03*
Nitric Oxide	0.02	0.93	0.05	0.78	0.52	0.005*

r: Pearson's and Spearman's correlation.
*: Significant (P<0.05).

Table 3: Correlation between IL-23 and nitric oxide serum levels of the vitiligo group

Variable	IL-23 (n=30)	
	R	P
Nitric oxide (Mmol/L)	0.34	0.04*

r: Pearson's and Spearman's correlation.
*: Significant (P<0.05).

Table 4: Validity of IL-23 and nitric oxide in diagnosis of Vitiligo:

Marker	Cutoff	AUC	CI	Sens.	Spec.	+PV	-PV	Accuracy	p-value
IL-23	≥184.47	0.83	0.72 – 0.94	80	86.7	85.7	81.3	83.3	<0.001**
Nitric oxide	≥11.07	0.67	0.53 – 0.81	60	63.3	62.1	61.3	61.7	0.02*

*: Significant (P<0.05).
**: Highly significant (P<0.01).

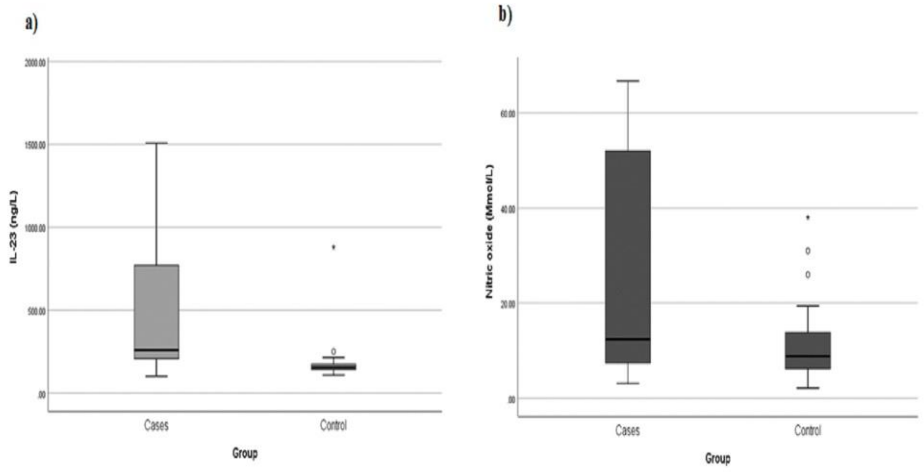

Figure 1: a) IL-23 in the two studied groups, b) Nitric oxide in the two studied groups

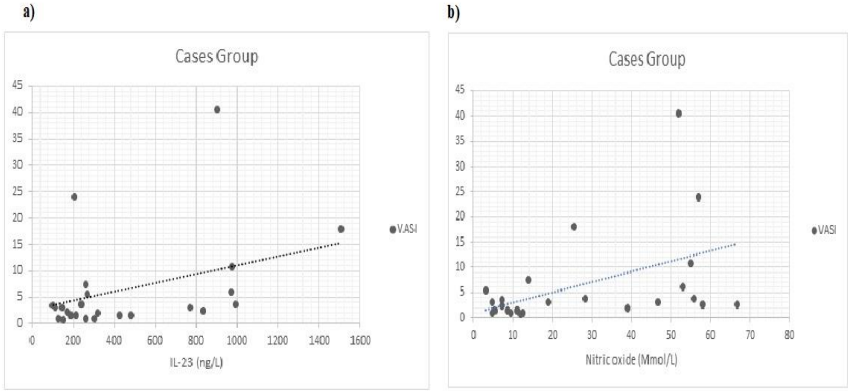

Figure 2: Correlation between a) IL-23, b) Nitric oxide and VASI score among the cases group.

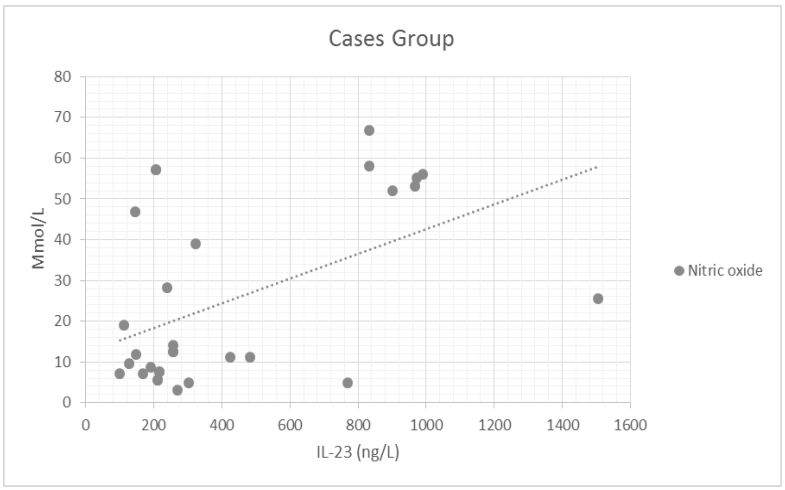

Figure 3: Correlation between IL-23 and Nitric oxide among vitiligo group cases.

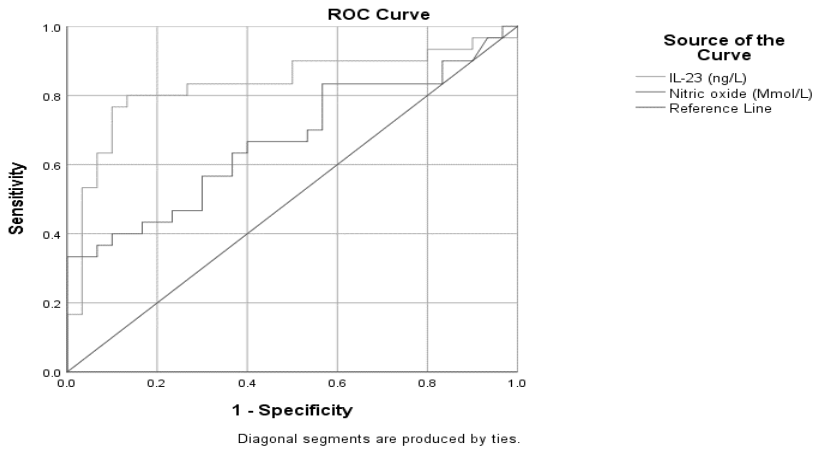

Figure 4: Roc curve of IL-23 and nitric oxide validity in diagnosis of Vitiligo.

DISCUSSION

Vitiligo, a widespread depigmenting skin disease, has an estimated global prevalence of 0.5 –2.0%. Selective depletion of melanocytes contributes to the non-scaly, milky white macules that characterize the condition. An important progress has been made in understanding the pathogenesis of vitiligo, now known as an autoimmune disorder [2].Awareness of the important role of T lymphocyte helper 17 (Th17) cells in the pathogenesis of multiple autoimmune diseases is increasing. IL-17A and IL-17F production from T-lymphocytes is regulated by IL-23 which in its turn developed from dendritic-cell, independent of cell-

to-cell connections, or conventional TCR activation. IL-23 stimulates the development of Th17 cells, so IL-23 is thought to mediate autoimmunity through induction of IL-17 secretion. In vitiligo patients, elevated serum IL-17 levels have been remarked, indicating that IL-17 participates in the immune response to the early-onset of the disease [4]. Nitric oxide is an important mediator of various physiological processes, as melanogenesis, keratinocytes response to UV, and cell proliferation and differentiation. Evidence suggests that NO plays a critical role in many inflammatory, hyperproliferative, and autoimmune diseases. Several cytokines (IL-1, IL-6, IL-23, IL-17, IL-33, IFN-g, TNF-a) stimulate inducible nitric oxide synthase (iNOS) expression and play an important role in establishing depigmentation [5]. Increased serum concentrations of IL-23 and IL-33 are responsible for intensifying the condition in vitiligo patients. Both NO and IL-23 may trigger an autocrine loop within the innate immune system, supporting the emerging related theory between vitiligo as an autoimmune disorder, and the T helper-17 axis [6]. The current study analyzed the serum levels of IL-23 and NO in vitiligo patients and compared the results with a group of healthy donors. The present work was performed on 30 patients with vitiligo and 30 healthy age- and sex- compared controls. In the present study, all systemic therapies had been stopped for 1 year and all topical therapies had been stopped for 3 months prior to the beginning of the study. This was a longer period than the study by Koca and his colleagues, in which patients stopped systemic and topical treatments 2 months prior to the study. In our study, no statistically significant differences in serum IL-23 and NO levels were detected in treated patients versus patients who were not previously treated. These results indicate that serum IL-23 and NO levels were not affected by vitiligo treatments, other than biological therapy, even after a short time after ending treatment. However, this might change if tested after biological treatment [7]. The significantly higher serum IL-23 levels ($P < 0.001$) in vitiligo patients (mean ± SD: 429.20 ± 360.47 ng/l, median: 259.18 ng/l) in comparison to controls (mean ± SD: 179.33 ± 135.65 ng/l, median: 154.27 ng/l) in our study was also demonstrated by other studies. Vaccaro et al, reported that IL-23 serum levels were considerably higher in vitiligo patients (median: 5.25 pg/ml) compared with controls (median: 2.44 pg/ml). Wang et al, also reported significantly higher IL-23 serum levels in vitiligo patients than in the healthy controls. In contrast, Osman et al., found lower, but not statistically significant, IL-23 plasma levels in vitiligo patients (median: 0.044pgm/ml) than in controls (median: 0.045pgm/ml) ($P = 0.19$). The difference between our study and Vaccaro et al., compared with the results of the Osman et al., study can be attributed to the different numbers of patients and controls enrolled in each study. The geographic and cultural diversity may also play a role in the discrepancy. Nevertheless, the latter study did mention that they excluded patients with autoimmune diseases, while Vaccaro et al. included patients with autoimmune diseases. Such controversy about IL-23 levels warrants further study in a larger number of vitiligo patients [8-10]. A significant positive correlated IL-23 serum levels with VASI score of the vitiligo group ($P = 0.003$) was shown in our study. These findings agreed with the study conducted by Vaccaro et al., who noticed a significant positive correlation between IL-23 serum levels and the extent of vitiligo ($P < 0.05$), although his results were based on BSA scoring. In other words, a higher disease extent might account for higher IL-23, which is a proinflammatory cytokine that might take a significant role the complex pathogenesis of vitiligo [8]. A relationship between IL-23 levels and the disease duration has been suggested by Vaccaro et al., who found a significant positive correlation between IL-23 serum levels and duration of the disease as ($P < 0.05$). However, we found no statistically significant correlation between IL-23 levels and disease duration in our patients ($P = 0.87$). The lack of such a correlation does not exclude its possible existence in the multi-factorial pathogenic mechanisms in vitiligo. The relatively limited number of recruited patients and controls in our study may account for our inability to demonstrate such association [8]. We observed a statistically significant increase in serum NO levels ($P = 0.02$) in vitiligo patients (mean: 25.20 ± 21.81 Mmol/L; Median: 12.4Mmol/L) compared to controls (mean: 11.64 ± 8.39 Mmol/L; Median: 8.8Mmol/L). This observation agrees with multiple studies. Koca et al., reported higher levels of serum NO in vitiligo patients (mean: 12.06 ± 4.78 nmol/L) compared with controls (mean: 10.90 ± 1.79 nmol/L) but the differences were not significant ($p > 0.05$). Similarly, Vaccaro and his group reported that tissue iNOS expression and NO_2/NO_3 levels have been increased in vitiligo lesions in comparison with healthy controls. Increased iNOS activity in all layers of the epidermis of vitiligo patients can result in increased production of NO, responsible for the reduction/death of melanocytes leading to loss of pigmentation [7,11]. In contrast to our results, Yildirim et al., found no significant difference in NO levels ($P > 0.05$) in the tissue of vitiligo patients compared with controls, although they observed a disturbance in the oxidant–antioxidant

system in vitiligo and suggested further studies [12]. Our result revealed that there is a highly significant positive correlation between VASI scores and NO serum levels in vitiligo patients (P = 0.005). However, Zayed et al., reported no statistical differences between patients with vitiligo harboring different genotypes with regards to disease extent. We observed higher serum IL-23 and NO levels that might be affected by the greater disease extent correlating with more cytokines [13]. In the current study, no statistically significant differences were found between age, sex, disease duration, and family history and NO serum levels in vitiligo patients. This finding agrees with Zayed et al., who reported that there were no statistically significant differences found between patients with vitiligo harboring the wildtype or the genotypes of iNOS_954 G/C single nucleotide polymorphisms, with regards to age, sex, disease duration, and family history [13]. Both NO and IL-23 can cause an autocrine loop within the innate immune system, confirming the current concepts between vitiligo as autoimmune disorders and T-17 axis. In addition, IL-23/IL-17A axis plays a crucial role in IBD pathogenesis through the NO pathway [14]. In our study, we observed with interest the positive correlation between IL-23 and NO in the serum of patients with vitiligo (P = 0.04). To our knowledge, this is the first study to show a positive correlation between IL-23 and NO in vitiligo patients that was positively related to VASI scoring. This implies that both factors are involved in the disease pathogenesis and targeting them may be a new approach in vitiligo therapy.

Conflict of interest None

Financial disclosures None

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To Cite:

Bessar, H, AbdelMoez, B., Sharaf,SM, Ghanem,A. Serum Interleukin 23 and Nitric Oxide in Vitiligo: New insights into the emerging association with VASI Score. Zagazig University Medical Journal, 2023; (242-247): -.doi: 10.21608/ZUMJ.2021.51028.2026.