UDC 616.5-003.829+612.017 doi: https://doi.org/10.15407/ubj96.03.066

NON-CODING RNA NEAT-1 AND INTERLEUKIN-6 AS DIAGNOSTIC INDICATORS FOR VITILIGO

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Received: 15 March 2024; **Revised:** 23 April 2024; **Accepted:** 31 May 2024

Vitiligo belongs to chronic autoimmune diseases and results in a loss of functioning melanocytes and skin depigmentation. Nuclear enriched abundant transcript 1 (NEAT-1) is a long non-coding RNA that has a vital role in the diagnostics and treatment of certain autoimmune and inflammatory diseases. It is suggested that NEAT-1 can increase the pro-inflammatory cytokine level via regulatory network. The aim of the work was to measure the serum level of NEAT-1 and IL-6 in vitiligo patients compared with healthy controls and to estimate its relation to disease activity. In the study, 60 individuals were enrolled subdivided into 40 vitiligo patients and 20 healthy controls of similar age and gender. NEAT-1 expression was detected by Quantitative real-time PCR, and IL-6 level was measured by ELISA. To assess the severity of the disease Vitiligo area scoring index (VASI) was calculated. Results showed that there was a significant increase in both NEAT-1 and IL-6 levels in vitiligo patients compared with the control group. A positive correlation between NEAT-1 and IL-6 levels and a negative correlation between NEAT-1 level and VASI score was revealed. The elevated serum levels of NEAT-1 and IL-6 suggest that these circulating biomarkers have promise as diagnostic indicators for vitiligo and possible targets for therapeutic interventions.

K e y w o r d s: vitiligo, non-coding RNA, NEAT-1, IL-6, serum.

Vitiligo is considered a chronic acquired pigmentation disorder that affects males and females equally. It is defined by white macular formation that results from the loss of epidermal melanocytes [1]. The etiological factors involved in the depigmentation process of vitiligo include immunological dysfunction, neurotoxic factor destruction, and genetic predisposition [2]. Various research indicates that the etiology and progression of vitiligo are affected by multiple polygenic factors. Furthermore, it is postulated that non-coding RNAs may significantly affect an individual's vulnerability to developing vitiligo [3] .

Numerous studies have indicated that the dysregulation of lncRNAs has a significant role in developing and progressing autoimmune and inflammatory diseases [4, 5].

Nuclear enriched abundant transcript 1 (NEAT-1) is a long non-coding RNA on chromosome 11q13.1. It is expressed consistently and extensively in various tissues and cell types [6]. NEAT-1 has a vital role in either the diagnosis or teatment of several autoimmune and inflammatory diseases such as rheumatoid arthritis, Systemic Lupus Erythematosus (SLE) and even other skin diseases [7, 8].

On the other hand, IL-6 has been found to elicit the activation of inflammatory and autoimmune mechanisms in various diseases, including multiple sclerosis, SLE [11] and vitiligo [12]. Increased IL-6 expression may cause IL-6R expression in target melanocytes to trigger downstream signaling, eventually altering melanocyte survival and homeostasis [12].

NEAT-1 was subsequently identified as an activator of the mitogen-activated protein kinase (MAPK) signaling pathway, resulting in the upregulation of IL-6. In general, overexpression of NEAT-1 can contribute to an increase in the production of cytokines and chemokines associated with the pathogenesis of SLE [8]. LncRNA NEAT1 can increase

the pro-inflammatory cytokine level (including IL6) by impeding the anti-inflammatory effect of miR-21. miR-21, as the targets of lncRNA NEAT1 via a competing endogenous RNA (ceRNA) regulatory network, are shown to participate in the pathological process and present the potential to be biomarkers of allergic inflammation diseases [9]. In RA, elevated lnc-NEAT1 but declined miR-21 was correlated with inflammation and disease activity [10].

Thus, in the present study, we aimed to measure and investigate their role in the early diagnosis of vitiligo and the determination of disease severity and its relation to other clinical data.

Materials and Methods

This case-control research included 60 people. Forty non-segmental vitiligo patients fulfiled the rules of the Declaration of Helsinki 1975. Written consent was obtained from each subject before the start of the study as well. Twenty healthy control studies were also included. Patients were recruited from the Dermatology outpatient clinic, Faculty of Medicine, Fayoum University Hospital. The study was approved by the ethical committee at the Faculty of Medicine, Fayoum University with ethical approval number M507 on 14/11/2020. A full laboratory test was performed for all patient groups.

Clinical assessment : to assess the severity of the disease Vitiligo area scoring index (VASI) [13] scores were calculated, and for the disease activity, vitiligo disease activity (VIDA) scores were calculated [14] as well as the extent of the disease was evaluated.

The inclusive criteria included adult subjects above 19 years old where all subjects were non-segmental vitiligo patients. Patients had not received any relevant systemic therapy for at least four weeks or suitable local treatment for at least two weeks before our study. Pregnant or lactating females; patients receiving immunosuppressive drugs and patients with any other inflammatory or autoimmune diseases or prominent skin diseases rather than vitiligo were excluded from the study. Patients who received PUVA therapy within the last 6 months and patients with leukoderma due to other causes were excluded.

Laboratory assessment and quantitation of NEAT-1 and IL-6 in serum were done in the Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Fayoum University.

For all patients, 10 ml of blood was obtained by venipuncture from the antecubital vein of every participant. They were divided into two vacutainer tubes; 2 ml was collected in a tube containing Ethylenediaminetetraacetic acid (EDTA) to determine the CBC, and 8 ml in plain tubes to separate the serum. They were then allowed to clot for 15 min at 37°C, centrifuged at 4000 rpm for 10 min, and the serum was separated. Immediately after collection, serum samples were kept at -80°C until they were utilized for laboratory investigations including alanine aminotransferase (ALT); aspartate aminotransferase (AST); creatinine; urea; cholesterol; triglycerides (TG) and high-density lipoprotein (HDL) as well as biomarkers interleukin-6 and NEAT-1.

Laboratory measurement:

1. For CBC: A cell counter detected the hemoglobin, leukocyte count (total/differential), and platelets count (Sysmex XN-1000 Automated Hematology Analyzer, Lincolnshire, IL, USA).

2. Parameters measured by colorimetric method using PHOTOMETER 5010 V5+:

• Determination of total cholesterol, triacylglycerols and HDL-cholesterol: Kits produced by (Human Gesellschaft fur Biochemica und Diagnostica mbh, Wiesbaden – Germany) were used.

• Determination of Serum Urea: Kits produced by (Linear Chemicals, Montgat – Barcelona, Spain) were used.

3. Parameters measured by kinetic methods using PHOTOMETER 5010 V5+:

• Determination of Serum Creatinine: Kits produced by (BioSystem S.A Costa Brava 30-Barcelona, Spain) were used.

• Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST): Kits produced by (Human Gesellschaft fur Biochemica und Diagnostica mbh, Wiesbaden – Germany) were used.

For the serum expression of NEAT-1, RNAs were extracted from the serum using the miRNeasy mini kit Cat. No.217004 (Qiagen, Valencia, CA, USA) following the manufacturer protocol. RNA samples were quantified and purified using the Nano Drop® [ND]IMPLE. GmbH, Munich, Germany Spectrophotometer. Followed by Reverse transcription on total RNA in a final volume of 20 µl RT reactions utilizing RT2 First Standard Kit (Cat. No.330404, Qiagen, Maryland, USA). The investigated lncRNA NEAT-1 expression levels were assessed using GAPDH as an internal control utilizing premade primers for NEAT-1 and GAPDH. NEAT-1's Catalog no. was 330701 LPH15809A, and its ref Seq Accession no. was NR 02272.1. According to the

manufacturer's procedure, GAPDH's catalog number was 330701 LPH31725A, its ref Seq Accession number was ENST00000496049.0, and RT2 SYBR Green ROX q PCR Master mix (Cat. No. 330520, Qiagen, Maryland, USA]. Real-time polymerase chain reaction (PCR) was performed on a 20 µl reaction mixture. The calculation of gene expression to an internal control was performed. The cycle threshold (Ct) refers to the number of amplification cycles needed for the fluorescence signal to cross a threshold during real-time PCR. The relative expression of NEAT-1 was quantified using the $2^{-\Delta\Delta Ct}$ method.

For determination of the concentration level of IL-6, an ELISA kit(BT LAB Human Interleukin 6 ELISA Equipment Cat. No. E0090Hu) was provided.

Data and statistical analysis. Data were collected and coded into Microsoft Excel sheets, data analysis was performed using the Statistical Package of Social Science (SPSS) software version 22.0 on Windows 8.1 (SPSS Inc., Chicago, IL, USA). Data were subjected to the Kolmogorov-Smirnov test to determine the normality distribution and method of analysis. Skewed data were expressed as median (range) where the statistical significance was tested using the Mann-Whitney U test at *P*-value < 0.05.

For normal quantitative parametric data Student *t*-test was used to compare measures of 2-independent groups. One-way ANOVA test was used for comparing more than 2-independent groups.

While for non-parametric data, the Kolmogorov-Smirnov test was used. The Mann-Whitney U test was used to compare outcomes between two independent groups. For comparing more than 3 groups, the Kruskall-Wallis test was used. For measuring the correlation between qualitative data, the Bivariate Pearson correlation test to find out the association between different groups with a two-tailed to test the significance. Sensitivity and specificity tests were generated for testing a new test with ROC

T a b l e. Laboratory and clinical investigations for the studied groups

Variable	Vitiligo, *n* = 40	Control, *n* = 20	*P*-value
Laboratory investigations, (mean ± SD)			
ALT	17.5 ± 6.16	13.70 ± 6.52	0.03*
AST	19.93 ± 5.85	19.20 ± 6.04	0.6
Urea	25.9 ± 7.7	23.68 ± 6.59	0.2
Creatinine	0.89 ± 0.13	0.79 ± 0.21	0.04*
TG	115.15 ± 61.56	104.60 ± 43.02	0.49
Cholesterol	192.80 ± 32.53	186.80 ± 26.95	0.48
HDL	41.70 ± 5.83	43.40 ± 8.81	0.37
Hb	12.70 ± 1.59	13.19 ± 1.65	0.2
Platelets	272.33 ± 68.31	283.9 ± 31.7	0.4
WBCs	6.29 ± 2.13	6.51 ± 1.43	0.6
Clinical investigations (Median (IQR))			
Age of onset (years) Median (IQR)	28.5 [16.50-43.75]	–	–
Disease duration (years) Median (IQR)	9.50 [4.0-15.0]	–	–
VASI Median (IQR)	3.85 [1.57-8.43]	–	–
VIDA Median (IQR)	4 [1.50-4]	–	–

Note. Laboratory investigation data is shown as mean ± SD and clinical investigation data is shown as Median (IQR). Independent Student *t*-test was used. *Significant at $P \leq 0.05$. ALT – alanine aminotransferase; AST – aspartate aminotransferase; TG – triglycerides; HDL – high-density lipoprotein; Hb – hemoglobin; WBCs – white blood cells; VASI – vitiligo area scoring index; VIDA – vitiligo disease activity

curve (receiver operating character). *P*-value < 0.05 was considered as a cut-off value for significance.

Result

In this case-control study, forty non-segmental vitiligo and twenty healthy controls with a mean age of 41.4 ± 8.7 and 38.6 ± 8.6 respectively. For the vitiligo group, 72.5% of the patients were females and 27.5% were male, while for the healthy group, the percentage of females and males was equally 50 to 50%. There were no significant differences between both groups as regards to age and gender. For all individuals, laboratory and clinical investigations were performed. No significant difference between vitiligo patients and controls regarding Hb, platelets, and WBCs count (*P*-value > 0.05) (Table).

The expression level of NEAT-1 and the concentration level of IL-6 were measured and compared accordingly to both groups. Results revealed that there is a statistically significant difference between both groups as regards to NEAT-1 with mean and standard deviation of 41.058 ± 34.030 and 0.996 ± 0.040 for vitiligo patients and healthy controls respectively (*P* = 0.0001). There is also a significant difference as regards to IL-6 for both groups (*P* = 0.002), where the concentration level of IL-6 tends to increase in vitiligo patients at 58.40 ± 31.33 than controls 35.07 ± 3.31 (Fig. 1).

We then classified patients as regards to disease severity based on VASI score into two subgroups:

- Mild: 32(80%)
- Moderate: 8(20%)

Results revealed that the expression level of NEAT-1 tends to increase in the mild patients group compared to the moderate with significant *P*-value of 0.026. The concentration level of IL-6 tends to increase in moderate patients compared to mild patients, but there is no significant difference between mild and moderate groups (Fig. 2).

After using the Pearson correlation test, results revealed that there are positive correlations between VASI and duration (r = 0.321, P = 0.044); NEAT-1 and WBC (r = 0.330, P = 0.038) and also between NEAT-1 and IL-6 (r = 0.50, P = 0.017). There is a negative correlation between the expression level of NEAT-1 in serum and VASI score (r = -0.358, P = 0.013) (Fig. 3).

Diagnostic performance of NEAT-1 and IL-6 as markers of vitiligo were calculated where for NEAT-1, the calculated sensitivity and specificity to discriminate vitiligo patients from healthy control were 87.5 and 98.1%, respectively, and accuracy of 92.80% (*P* < 0.0001). Also, for IL-6, the sensitivity and specificity were 85.0 and 99.8% with an accuracy percentage of 92.4% (*P* < 0.0001). In the present study, the cut-off values for NEAT-1 and IL-6 seem

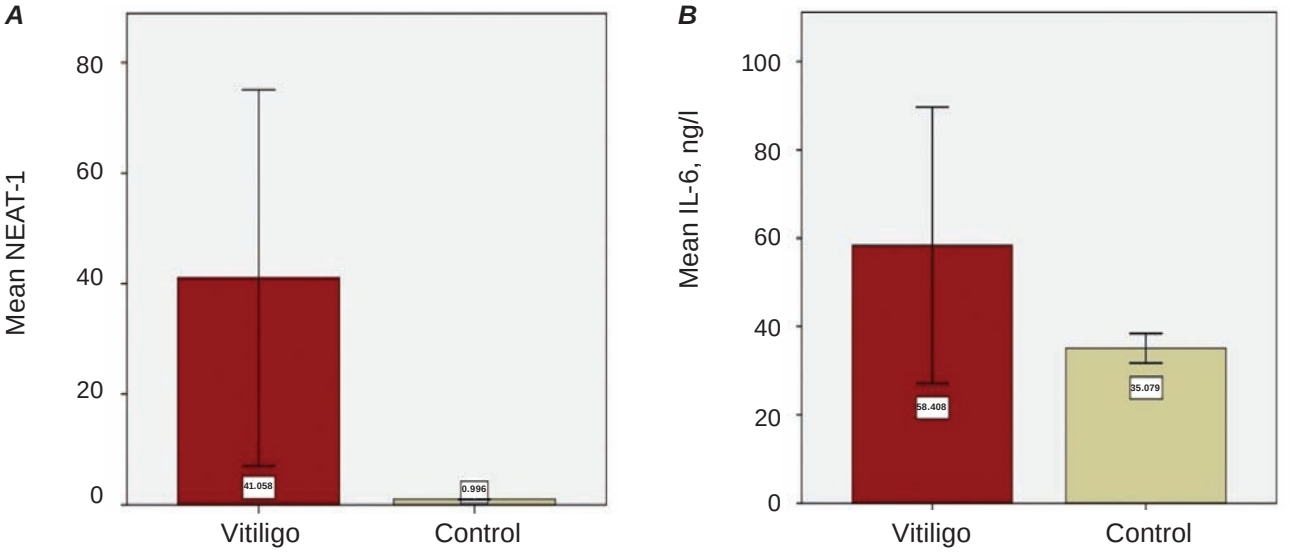

Fig. 1. Comparison between serum biomarkers among vitiligo patients and healthy controls. ***A*** *– Expression level of NEAT-1 there is a statistically significant difference between both groups with mean ± SD 41.058 ± 34.03 and 0.996 ± 0.04 for vitiligo patients and healthy controls respectively (P = 0.0001).* ***B*** *– Concentration level of IL-6 there is also a significant difference for both groups with mean ± SD 58.40 ± 31.33 and 35.07 ± 3.31 for vitiligo patients and healthy controls respectively (P = 0.002)*

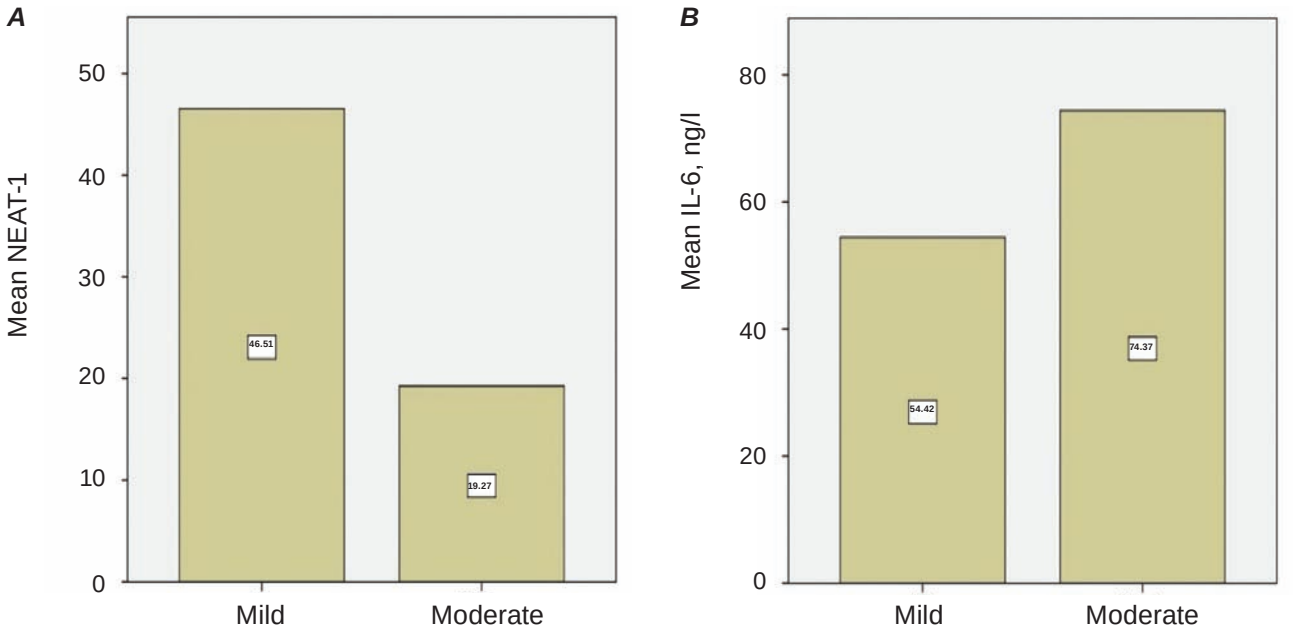

Fig. 2. Expression level of serum biomarkers among patients' groups as regards to disease severity. ***A*** *– The expression level of NEAT-1 tends to increase in mild patients group compared to moderate with a significant P-value of 0.026.* ***B*** *– The concentration level of IL-6 tends to increase in moderate patients compared to mild patients but there is no significant difference between mild and moderate patients*

to be 21.17 and 41.89 respectively. In order to find the true cut-off values that would make both biomarkers potential assessment tools with prospect for a therapeutic target, more extensive trials are warranted. The ROC curves analysis of serum levels of NEAT-1 and IL-6 for vitiligo patients are shown in Fig. 4.

Discussion

Vitiligo is a depigmentation disease of unclear etiology that is multifactorial. It is defined by the development of strongly demarcated white maculae on the skin, which occurs due to melanocyte loss [15].

Previous studies have established a correlation between NEAT-1 and the inflammatory mechanism by which the overexpression of NEAT-1 stimulates the release of several inflammasomes and pro-inflammatory cytokines [CXCL10, IL-6, and IL-1] that triggers an immunological response in inflammatory and immune diseases [16, 17]. It has been proposed that NEAT-1 might be suggested as a therapeutic target for inflammatory and autoimmune diseases because of its potential role(s) in immune response [17].

On the other hand, IL-6 is a cytokine with pro-inflammatory properties that has a potential role in developing and progressing autoimmune disorders [18].

In the present study, we detected that the expression levels of NEAT-1 and IL-6 were higher in vitiligo patients compared to controls.

To the best of our knowledge, no previous studies illustrated the role of NEAT-1 in vitiligo patients. However, studies agreed with our results that the expression of NEAT-1 tends to increase in autoimmune diseases such as RA, SLE, multiple sclerosis and Behçet's disease which are autoimmune diseases like vitiligo [7, 8, 19, 20].

In contrast with our results, Mohammed et al., 2022 found that NEAT-1 levels were significantly down-regulated in Behçet's disease patients compared to controls [21].

Results also illustrated that NEAT-1 negatively correlated with illness severity score VASI, suggesting that increased inflammation could reduce NEAT-1 expression. There are also positive correlations between VASI and duration; NEAT-1 and WBC.

Similarly agreeing with our results, studies found that the down-regulation of NEAT-1 was associated with severe Behcet Disease [21]. Also, there was a correlation between NEAT-1 and RA disease activity [22]. A study on SLE patients detected that there was a positive association between NEAT-1 expression and disease activity in SLE [8].

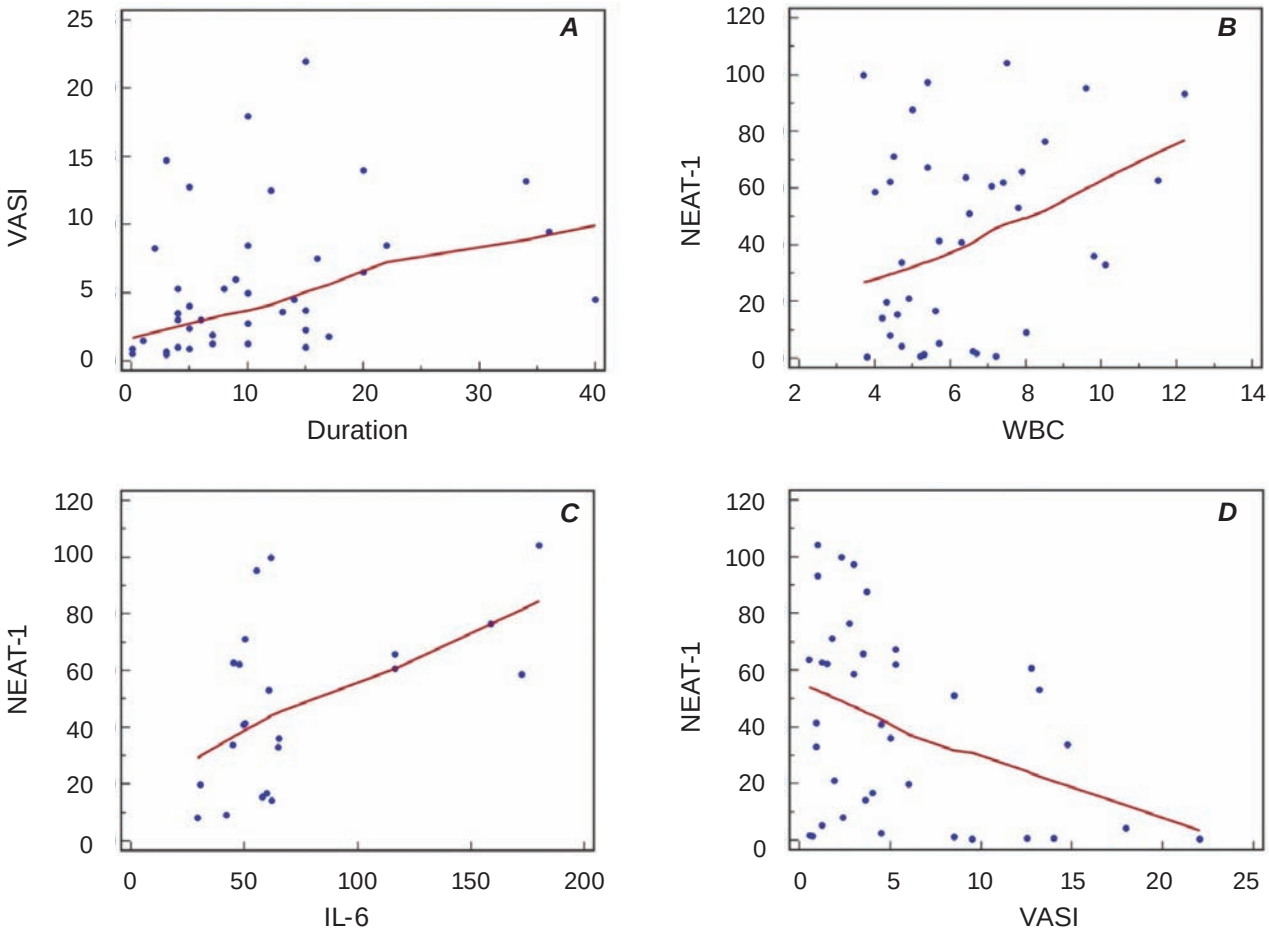

Fig. 3. Correlations between parameters and biomarkers among vitiligo patients. ***A*** *– There are positive correlations between VASI score and disease duration.* ***B*** *– There are positive correlations between NEAT-1 and WBC.* ***C*** *– There are positive correlations between NEAT-1 and IL-6 in serum.* ***D*** *– The correlation between NEAT-1 and VASI score is a negative correlation*

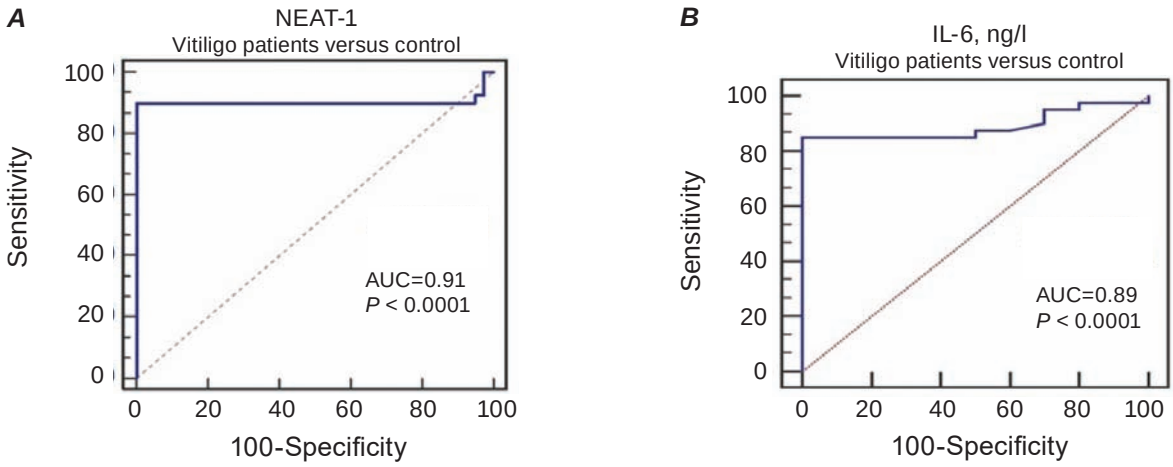

Fig. 4. ROC curve analysis. ***A*** *– Diagnostic performance of NEAT-1 in serum of vitiligo patients, the calculated sensitivity and specificity were 87.5 and 98.1%, respectively, and accuracy of 92.80% (P < 0.0001) with a cut-off value of 21.17.* ***B*** *– Diagnostic performance of IL-6 in serum of vitiligo patients, the calculated sensitivity and specificity were 85.0 and 99.8% with an accuracy percentage of 92.4% (P < 0.0001) with cut-off value 41.89*

Regarding the positive correlations between NEAT-1 and WBC, NEAT-1, a constitutively and widely expressed lncRNA involved in innate immunity and highly expressed in peripheral blood mononuclear cells (PBMCs) and monocytes in SLE patients. It was identified as activating the (mitogen-activated protein kinase) MAPK signalling pathway with upregulation of IL-6 expression. In addition, the skin is a large and important peripheral lymphoid organ in which many immune cells migrate from the blood circulation. In patients with vitiligo, PBMCs can secrete proinflammatory cytokines such as IL-1b, IL-6, IL-8, and tumor necrosis factor (TNF-α) [23] and infiltrate around the lesions of vitiligo [24] suggesting that PBMCs play important roles in the pathogenesis of vitiligo.

We hypothesized that NEAT-1 might be implicated in vitiligo pathogenesis as an autoimmune disease through its effect on IL-6. Various studies on vitiligo patients reported that there was a statistically significant difference between the serum levels of IL-6 among vitiligo patients and controls where IL-6 tended to increase in patients [22-28].

The present study demonstrated a positive correlation between NEAT-1 and IL-6. Previous study on RA patients showed that there were positive correlations between the two biomarkers in patients' tissue [29]. Xu et al., 2019 found that NEAT-1 could regulate LPS inflammation genes and NEAT-1 positively correlated with IL-6 and it was found that LPS could upregulate lncRNA NEAT-1 expression [30]. LPS exposure also increased releases of proinflammatory cytokines IL-6 [8]. LPS can inhibit melanogenesis and enhance autophagy in vitiligo melanocytes [31]. In addition, NEAT-1 also regulates the expression of a group of chemokines and cytokines, including IL-6 and CXCL10, through the MAPK pathway [16]. Thus, it is obvious that LPS increases both NEAT-1 and IL-6 and decreases melanin synthesis in addition NEAT-1 increases the level of IL-6. The latter plays a role in the development of vitiligo.

Our results showed a significant increase only in serum ALT and creatinine in vitiligo patients compared to controls (*P*-value = 0.03, 0.045, respectively); no significant difference regarding AST, urea, TG, cholesterol, HDL, Hb, platelets, and WBCs count (*P* value > 0.05). This result agreed partly with Al Houssien A.O. et al., who found that vitiligo patients had an increased risk of developing renal diseases compared with the control group and disagreed with his result regarding dyslipidemia. He found it was significantly associated with vitiligo compared to the control group, and the LDL/HDL ratio was significantly higher among the patients' group than the control group [32].

Thus, NEAT-1 can upregulate key players (CXCL10, IL-6, and IL-1β) in vitiligo development. Cytokines are essential for the onset and progression of the disease. Not only will our findings help to clarify the pathophysiology of vitiligo, but they will also provide further opportunities for developing a targeted immunological intervention to treat this challenging disease.

Conclusions. The findings in this study provide novel insights into the potential role of lncRNA NEAT-1 and IL-6 in predicting vitiligo. The elevated serum levels of NEAT-1 and IL-6 suggest that these circulating biomarkers are promising as diagnostic indicators for vitiligo and possible targets for therapeutic interventions.

Limitations. The use a group of patients with long medical story of the disease, not people with freshly developed disorder

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Funding. No financial support was provided for this study.

НЕКОДУЮЧА РНК NEAT-1 ТА ІНТЕРЛЕЙКІН-6 ЯК ДІАГНОСТИЧНІ ІНДИКАТОРИ ВІТИЛІГО

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Вітиліго – хронічне аутоімунне захворювання, яке призводить до втрати функціонуючих меланоцитів та депігментації шкіри. NEAT-1, довга некодуюча РНК, відіграє важливу роль у діагностиці та лікуванні деяких аутоімунних та запальних захворювань. При-

пускають, що NEAT-1 може підвищувати рівень прозапальних цитокінів через регуляторну систему. Метою роботи було визначення рівня NEAT-1 та IL-6 у сироватці крові пацієнтів із вітиліго порівняно зі здоровими особами та оцінка його зв'язку з перебігом захворювання. У дослідженні взяли участь 60 осіб, яких було розподілено на групи, а саме 40 пацієнтів із вітиліго та 20 здорових осіб аналогічного віку та статі. Експресію NEAT-1 визначали методом ПЛР у реальному часі, рівень IL-6 визначали за допомогою ELISA. Оцінювання тяжкості захворювання здійснювали розраховуючи індекс площі вітиліго (VASI). Показано, що пацієнти із вітиліго мали значне підвищення рівнів NEAT-1 і IL-6 порівняно з контрольною групою. Виявлено позитивний кореляційний зв'язок між рівнями NEAT-1 та IL-6 і негативний кореляційний зв'язок між рівнем NEAT-1 та індексом VASI. Підвищені рівні NEAT-1 та IL-6 у сироватці крові свідчать про те, що ці біомаркери є перспективними діагностичними критеріями вітиліго та можливими мішенями для терапії.

Ключові слова: вітиліго, некодуюча РНК, NEAT-1, IL-6, сироватка крові.

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