Investigation of CD4 and CD8 T-cells levels in vitiligo patients

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Abstract

There are several hypotheses explained the etiopathogenesis of vitiligo in patients. Various triggers increase the occurrence of vitiligo in people who are genetically predisposed to the disease. Autoimmunity is most plausible hypothesis. Numerous studies have revealed immune-mediated destruction of melanocytes in vitiligo. T-cells are essential in the progression of autoimmune diseases, including vitiligo, but really the initial steps that induce their activation are unknown. As a necessary consequence of proposed cellular immunity role in vitiligo, estimating CD3+CD4+ and CD3+CD8+ T-cells levels were conducted in a cohort of Iraqi vitiligo patients to support this hypothesis. Aim of the study Evaluation of peripheral blood CD3+CD4+, CD3+CD8+ T-lymphocytes levels and CD4/CD8 ratio in diagnosed vitiligo patients in comparison with matched healthy subjects using flowcytometry analysis. Methods Fresh peripheral blood samples from 45 vitiligo patients and 45 controls subjects were taken. Flow Cytometric analysis was used for assessment of CD3+CD4+ and CD3+CD8+ T-lymphocytes levels in all blood samples. Then CD4+/ CD8+ ratio was calculated, and comparison was done between vitiligo patients and controls subjects and among vitiligo patients' categories. Results The findings demonstrated that total peripheral blood lymphocytes count mean in vitiligo patients were significantly (p-value=0.01) higher than controls. Mean absolute count of CD3+CD4+ and CD3+CD8+ T- lymphocytes populations were close in value in patients and controls without significant differences even among patients' categories. CD4/CD8 ratio mean was elevated more than one and without significant differences (p-value=0.74) between patients and controls. Conclusion The significant increase in total lymphocyte count of patients compared with controls reinforces the findings of the other studies indicating the contribution of cellular immunity to disease development. It is suggested additional investigations regarding other peripheral lymphocyte subtypes such as B-lymphocytes and Natural killer cells, to give more appropriate correlative biomarkers for vitiligo progression assessment, and it is preferable to conduct researches on the respective T-cells in the skin as well as circulation, as this will be critical to better understanding the disease's pathogenesis.

Keywords: Vitiligo, Autoimmunity, Peripheral blood, T-lymphocytes, CD4/CD8 Ratio.

1. Introduction

Vitiligo is patchy skin depigmentation, can occur on any part of the body. It affects around 1% of the world's population, and there are no discernible differences in prevalence based on gender, race, or geographic region [1]. Vitiligo manifests as hypochromic or achromic macules and patches on the skin, which enlarge and increase over time. The lack of melanin is the clinical hallmark of vitiligo [2]. Numerous theories have been proposed to explain the pathogenesis of vitiligo. These theories considering the roles of innervation, microvascular abnormalities, oxidative stress-induced melanocyte degeneration, defects in melanocyte adhesion, autoimmunity, somatic mosaicism, and genetic influences [3]. The immune system's innate and adaptive responses appear to be implicated. None of these proposed ideas are sufficient to explain the various vitiligo phenotypes, and the overall contribution of each of these processes is still up for debate, despite the fact that the autoimmune nature of vitiligo is now widely accepted [4].

Adaptive immunity differs from innate immunity in that it produces antigen-specific responses and memorizing antigens. Adaptive immunity is responsible for the death of melanocytes in vitiligo [5]. T- lymphocytes, that are divided into CD4+ and CD8+ cells, are essential for cellular immunity. T helper cells are CD4+ T cells that can produce cytokines critical for humoral and cell mediated immune response. There are various subsets of CD4+ T cells including T helper cells (Th1, Th2, T-Reg and Th17) are available. CD8+ T cells, on the other hand, are characterized as cytotoxic T cells and are responsible for direct cellular destruction in the adaptive immune system [6]. T-lymphocytes were detected infiltrating close to melanocytes in patches, especially at the leading edge of vitiligo depigmentation. Isolated T-lymphocytes from biopsies showed autoreactivity to a number of melanocyte antigens, including melan A, gp100, and tyrosinase, as well as cytotoxicity to autologous melanocytes in vitro. CD4+T lymphocytes also participate to melanocyte loss by destroying MHC class II-expressing cells via the Fas–FasL interaction, because melanocytes express both MHC class II and Fas [5]. A critical role of CD8+ T cells

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has always been demonstrated in cell-specific autoimmune disorders development like multiple sclerosis, rheumatoid arthritis, and vitiligo [7]. In the pathophysiology of vitiligo, it is commonly believed that CD8+ T cell serves as the ultimate procedure in the process of extensive melanocyte destruction and confers the final and fatal assault on melanocytes. In fact, CD8+ T cells are described sometimes as the executioner in vitiligo. Vitiligo patients had a high frequency of melanocyte-specific CD8+ T cells in their lesional skin and blood [8,9]. Importantly, patients with vitiligo have activated CD8+ T cells that have been proven to be melanocyte-specific T cells both in vivo and in vitro [10]. In various approaches, CD8+ T cells mechanically trigger melanocyte death. The two most commonly mentioned ways include secreting perforin/ granzymes and using Fas–FasL mechanisms [11]. Furthermore, IFN-γ produced by CD8+ T cells promotes melanocyte apoptosis in a variety of ways. First, the IFN-CXCL9 /CXCL10 /CXCR3 axis attracts more CD8+ T cells and enhances their fatal function, resulting in a significant immunological response [12,13]. Based on the role of CD8 and CD4 in progression of vitiligo, the current study aims to estimate the peripheral blood CD3+CD4+ and CD3+CD8+ T-lymphocyte cells count and percentage of vitiligo patients using flow cytometric analysis in Basra, Iraq.

2. Materials and Methods

Patients group: The study included 45 patients with vitiligo, (27 female and 18 male), Age range was (7-65) years old, and 45 corresponding matched healthy individuals as control subjects (had no any evidence of vitiligo or any other disease whether autoimmune or not) conducted during period from February 2020 to July 2020 in Al-Bayan national medical laboratory /Basra province/Iraq. The patients were diagnosed clinically by dermatologist. There were non-segmental vitiligo patients at different stages and patients with segmental vitiligo. Some of patients were in active vitiligo status defined on the basis of progression or appearance of lesions in the last 3 months, and some were in stable disease (The course of vitiligo was defined as stable when new lesions had not appeared within 1 year). All patients that had received phototherapy or any specific topical and systemic steroids therapy were excluded, they were discontinued from treatment at least three months. A detailed history was taken from all patients, related to age, sex, age at onset disease, family history of vitiligo, disease duration and association of other diseases recorded. No one of them had presented any additional other autoimmune diseases. Informed consent of patients was obtained before being included in this study.

Method

Venous blood samples (5ml) of patients and controls were collected in vacuum tubes under sterile conditions from both patients and matched controls, taking 2ml of whole blood collected in a vial with ethylene-di-amine-tetra-acetic acid (EDTA) to enumerate absolute lymphocytes counts, using CBC, an automatic hematology analyzer (Sysmex Corp/ Japan). Evaluation the levels of total CD3+, CD4+, and CD8+ T-lymphocytes were performed by automated flow cytometry technique (BD BioScience FACSLyric TM/ USA), using the three-color antibody combinations. For immuno-phenotypical analysis the following fluorochromes conjugated monoclonal antibodies (anti-CD3-APC-H7-A, anti-CD4-PerCP-Cy5.5-A, and anti-CD8-FITC-A) to human lymphocytes cell-surface molecules of (CD3, CD4 and CD8) were used.

A minimum of 50.000 cells was measured in each analysis assessment on Flow Cytometer. Immediately after samples preparation data was collected using firstly dot plot diagram was plotted using Forward side scatter channel (FSC) on X axis, and Side Scatter channel (SSC) on Y axis on lymphocyte region. From this graph, lymphocyte population was gated and this population was plotted with CD3 on X axis and CD4+ on Y axis. Further gated and plotted on another graph with CD8+ on Y axis and CD3+ on X axis. CD4+ and CD8+ were counted and based on the CD4+/CD8+ ratio was estimated. Absolute CD3+ CD4+ and CD3+CD8+ lymphocyte counts were calculated by the absolute lymphocyte count.

3. Results

Table-1 Clinical characteristics of vitiligo patients.

Age of patients range	7-65 years
Mean of patients ages	23.7+15.6
Range of onset age	4-60 years
Mean of onset range	19.8+15.7
Men patients	18 (40%)
Men age range	8-52 years
Women patients	27 (60%)
Women age range	7-65 years
Active vitiligo	38 (84.4%)
Stable vitiligo	7 (15.5%)
Non-segmental vitiligo	34 (75.55%)
Segmental vitiligo	11 (24.44%)
Patients have vitiligo relatives	19 (42.2%)

This study was conducted on 45 patients with vitiligo. Table-1 illustrated the clinical characteristics and age distribution of the study included vitiligo patients; they were from Basra province/Iraq. Age was ranged from 7-65 years old, the mean of actual age (23.7±15.6 SD) years. Range of disease onset age was (4-60) years old, mean of onset range (19.8±15.7 SD). The largest proportion of patients were females 27(60%) while the males 18(40%), were less than females. The patients categorized into (non-segmental and segmental vitiligo), 34 (75.55%) were with non-segmental vitiligo, they represented the largest proportion of patients, while 11 patients were segmental vitiligo (24.44%). Disease was active in 38 (84.4%) of total patients, while only 7 (15.5%) were in stable status of disease.

19 patients at 42.2% percent had family history (1st and 2nd degree relative) affected with vitiligo.

Table-2 Results of the absolute counts and relative count (percent) of peripheral blood CD3, CD4, CD8 T lymphocytes and CD4/CD8 ratio in vitiligo patients compared with controls.

	Vitiligo patients (No=45)		Control group (No=45)		
T-cells	Range (cell*103/µl)	Mean ± SD	Range (cell*103/µl)	Mean ± SD	P-value
Lymphocyte count	(1.1-4.8)	2.60±0.89	(1.1-3.2)	2.14±0.60	0.01
Absolute CD3 count	(0.567-3.323)	1.24±0.52	(0.405-2.128)	1.21±0.47	0.79
Relative count CD3%		48.82±11.25		55.78±11.64	0.01
Absolute CD4 count	(0.211-1.756)	0.68±0.30	(0.213-1.549)	0.69±0.30	0.8
Relative count CD4%		26.71±8.03		31.74±7.99	0.003
Absolute CD8 count	(0.208-1.566)	0.56±0.27	(0.166-1.077)	0.52±0.23	0.49
Relative count CD8%		22.11±7.74		24.04±6.85	0.22
CD4/CD8	(0.53-3.88)	1.36±0.67	(0.75-3.66)	1.41±0.53	0.74

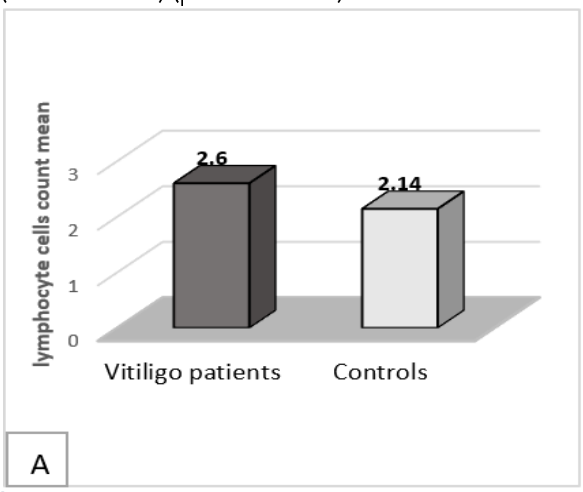
The data was discrete (categorical) in absolute and relative count, it was presented as Mean ± SD (standard deviation). T-test was used to compare study enrolled groups. A p-value < 0.05 with two tailed significance was considered statistically significant.

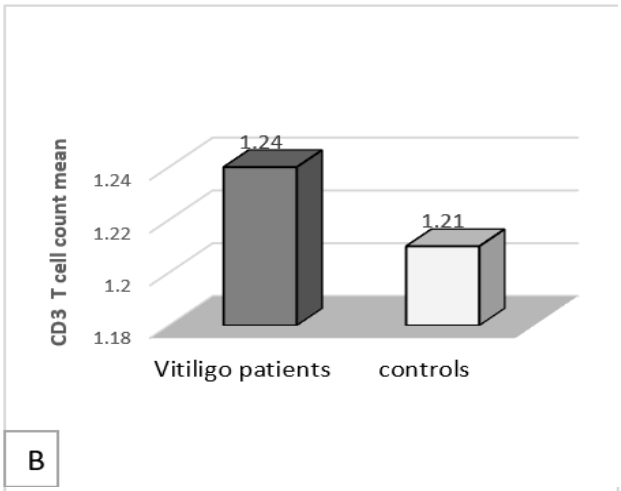
Between patients and controls groups, there was statistically significant difference in the total lymphocytes' cells count of vitiligo patients compared with control group (p-value=0.01), as the mean of total lymphocytes count in the patients was (2.60±0.89) and in controls (2.14±0.60).

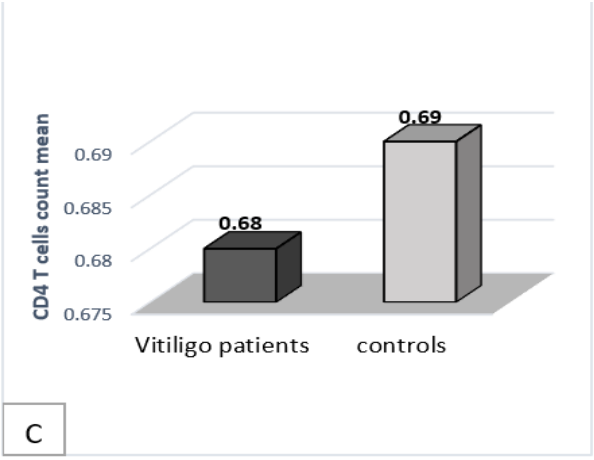
Mean value of absolute CD3 T-cells count were observed to be not statistically different in patients (1.24±0.52) in comparison to control group (1.21±0.47) (p-value=0.79), but the mean of total relative count CD3 value within total lymphocytes count is observed statistically significant lower in vitiligo patients (48.82±11.25) than control group (55.78±11.64) (p-value=0.01).

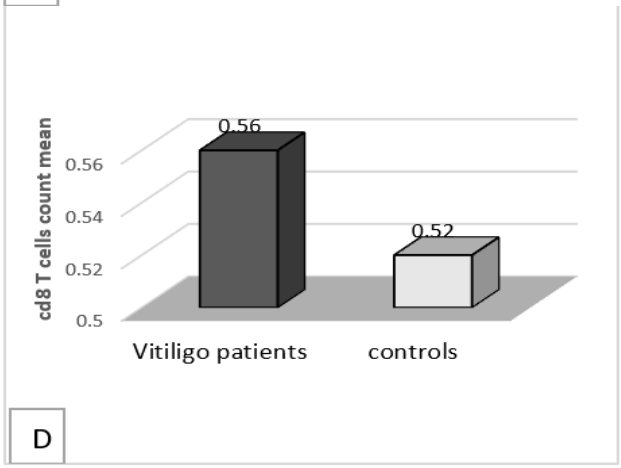
There was no statistically significant variation in the mean of total absolute count of CD4+ T-cells population comparatively in vitiligo patients (0.68±0.30) to control group (0.69±0.30) (p-value=0.8), but it was found a statistically significant decrease in the mean of total relative count CD4+T lymphocytes in vitiligo patients (26.71±8.03) than control group (31.74±7.99) (p-value=0.003).

Regarding the count of CD8+ T-cells, also there was no variation in the level of mean CD8+ T lymphocytes absolute count in vitiligo patients (0.56±0.27) compared to controls (0.52±0.23) (p-value=0.49) and its mean of total relative count (p-value=0.22) between vitiligo patients (22.11±7.74) and controls group (24.04±6.85), so based on these results there was no statistically significant difference in the mean of CD4+/CD8+ ratio (p-value=0.74) between the two groups.









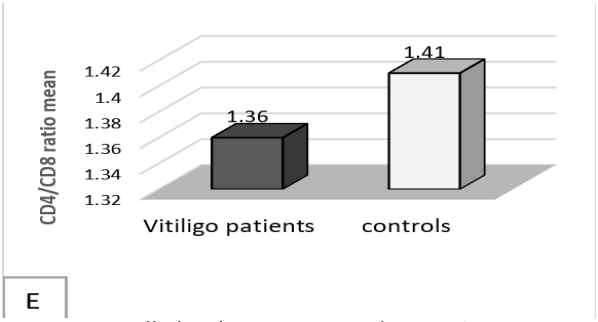


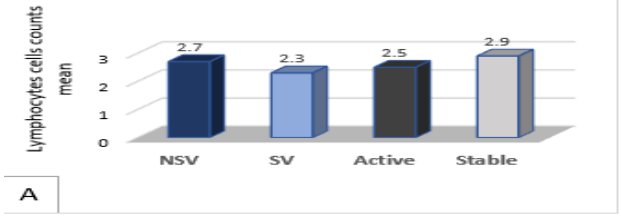
Figure-1/ Mean of CD3+, CD4+, CD8+ T-cell absolute count, and CD4+/CD8+ ratio in vitiligo patients and controls. A- Total lymphocytes cells count mean in vitiligo patients (no=45) and controls (no=45), values are given as mean ± SD; patients versus controls (2.6±0.89 versus 2.14±0.60). B- Mean of total absolute CD3+ T cells count in patients versus controls (1.24±0.52) versus (1.21±0.47).

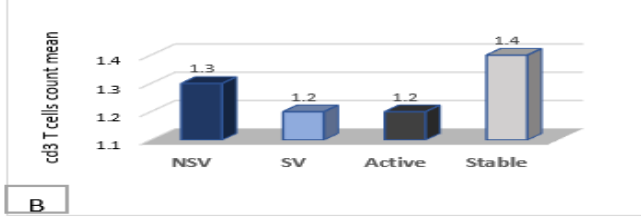
C-Mean of total absolute CD4+ T cells count in vitiligo patients versus controls (0.68±0.30 versus 0.69±0.30). D- Mean of total absolute CD8+ T cells count in patients versus controls (0.56±0.27 versus 0.52±0.23). E- Mean of CD4+/CD8+ ratio count in patients versus controls (1.36±0.67 versus 1.41±0.53).

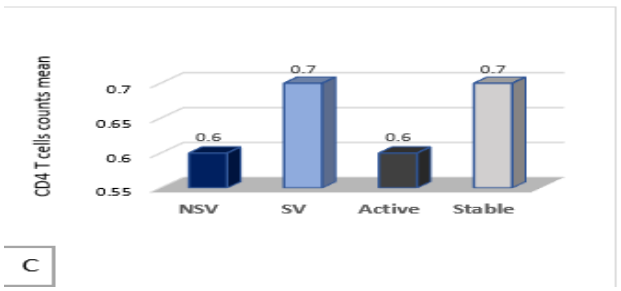
Table-3 Comparison the absolute and relative count of CD3, CD4, CD8 T-lymphocytes and CD4/CD8 ratio regarded vitiligo patients' categories.

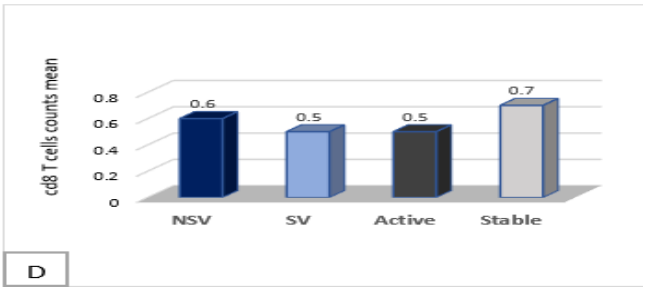
	Non-Segmental vitiligo No=34	Segmental vitiligo No=11		Active vitiligo No=38	Stable vitiligo No=7	
T-cells	Mean ± SD	Mean ± SD	P-value	Mean ± SD	Mean ± SD	P-value
Lymphocyte count	2.7±0.9	2.3±0.8	0.216	2.5±0.9	2.9±0.8	0.348
Absolute CD3 count	1.3±0.5	1.2±0.5	0.792	1.2±0.5	1.4±0.6	0.432
Relative count CD3%	47.5±11.6	52.9±9.6	0.170	48.9±10.9	48.3±13.9	0.904
Absolute CD4 count	0.6±0.2	0.7±0.3	0.597	0.6±0.3	0.7±0.3	0.422
Relative count CD4%	25.1±7.3	31.8±8.4	0.015	27.1±8.2	24.6±7.2	0.460
Absolute CD8 count	0.6±0.3	0.5±0.2	0.277	0.5±0.3	0.7±0.3	0.217
Relative count CD8%	22.4±8.1	21.1±6.7	0.637	21.8±7.4	23.7±10.1	0.556
CD4/CD8	1.3±0.7	1.6±0.7	0.263	1.3±0.6	1.0±0.6	0.223

Vitiligo patients categorized according to the distribution of depigmented macules into non-segmental vitiligo that characterized with symmetrical distribution, and segmental vitiligo, an uncommon form of localized vitiligo, characterized by unilateral distribution of depigmented macules. According to the basis of progression or appearance of lesions in the last 3 months, they categorized into active vitiligo, while the stable vitiligo defined when new lesions had not appeared within 1 year. The same parameters (total lymphocytes, CD3, CD4, CD8 and CD4/CD8 ratio) that were discrete into count and percentage, presented as Mean ± SD (standard deviation) were compared in this study among vitiligo patients' groups. It was observed no statistically significant results regarded all data (p-value >0.05) as illustrated in Table-3, except the mean of total CD4 lymphocytes relative count was statistically significant higher in segmental vitiligo patients (31.8±8.4) than non-segmental vitiligo patients (25.1±7.3), with p-value=0.015.









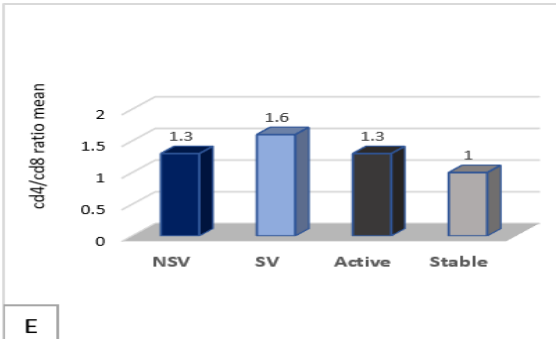


Figure-2/ CD3+, CD4+, CD8+ T-cells absolute count mean, and CD4+/CD8+ ratio mean in vitiligo patients' categories (NSV= non-segmental vitiligo (no=34); SV= segmental vitiligo (no=11); Active vitiligo (no=38); Stable vitiligo (no=7). Values are given as mean ± SD.

A- Total lymphocytes cells count mean in NSV patients versus SV patients (2.7±0.9 versus 2.3±0.8), and in active vitiligo patients versus stable vitiligo patients (2.5±0.9 versus 2.9±0.8). B- Mean of total absolute CD3+ T cells count in NSV versus SV (1.3±0.5 versus 1.2±0.5); active versus stable (1.2±0.5 versus1.4±0.6).

C- Mean of total absolute CD4+ T cells count in NSV versus SV (0.6±0.2 versus 0.7±0.3); active versus stable (0.6±0.3 versus 0.7±0.3). D- Mean of total absolute CD8+ T cells count in NSV versus SV (0.6±0.3 versus 0.5±0.2); active versus stable (0.5±0.3 versus 0.7±0.3). E- Mean of CD4+/CD8+ ratio count in NSV versus SV (1.3±0.7 versus 1.6±0.7); active versus stable (1.3±0.6 versus 1.0±0.6).

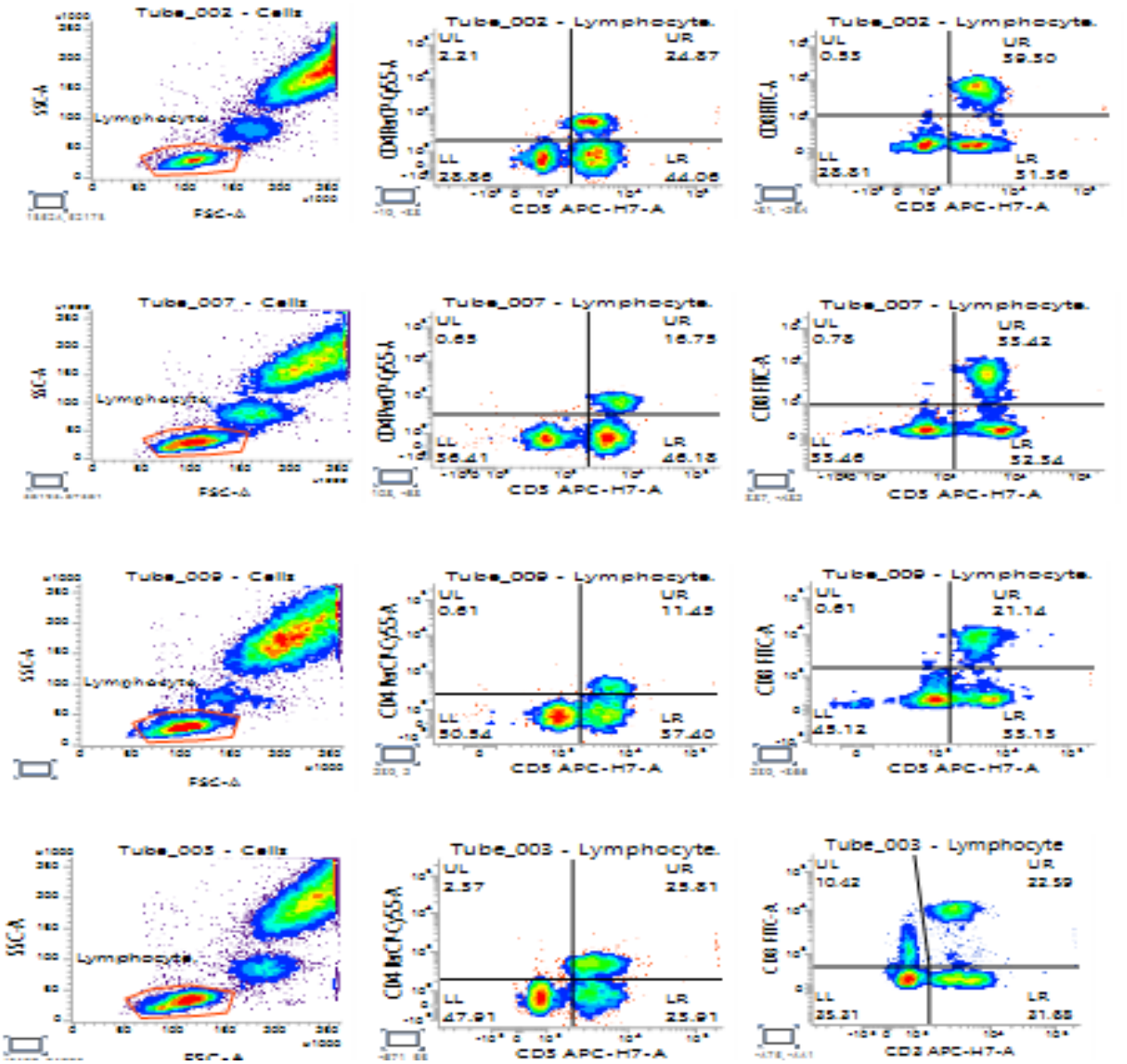


Figure-3/ Flow cytometry analysis indicates peripheral blood CD3+CD4+, CD3+CD8+ T lymphocytes subset levels from a representative vitiligo cases and healthy subject. The plots show the gating strategy in A-healthy subject (Tube 2) B- vitiligo patient (Tube 3,7,9).

4. Discussion

Although the precise pathophysiology of vitiligo is unknown, there are a number of theories explain it, but the autoimmune hypothesis has been raised in recent years, according to a multitude of experimental and clinical studies [14,15]. The evidences have raised the possibility that vitiligo is a T-cell mediated autoimmune disease. Investigations of peripheral blood T-cells lend considerable backup to this hypothesis [16]. On the basis of many studies supported T-cells involvement in the pathogenesis of vitiligo, so in this study the peripheral blood circulating T- lymphocytes (CD4 and CD8) absolute count and relative count of patients with matched controls were analyzed by flow cytometry to search for any significant correlation for a peripheral blood T-cells distribution to vitiligo etiology.

According to this study investigation, it was in accordance with studies that confirmed the significance of cellular immunity role in pathogenesis of vitiligo, as observed in the present study findings, that revealed there were statistically significant increase in total lymphocyte count mean of the study enrolled vitiligo patients' group (2.60±0.89) in comparison with matched healthy individuals' group (2.14±0.60), (p-value =0.01), Table-2.

However, in terms of the T-cells levels (CD3), there was no statistical difference (p-value=0.79) in the mean of absolute CD3+ count values between the two groups. even in T-cell subtypes CD4, and CD8 levels findings, and consequently there was no statistically significant differences (p-value=0.74) in the mean of CD4/CD8 ratio of patients in comparison with controls. Patients with vitiligo have been reported to have decreased levels of CD4+ to CD8+ lymphocytes in their blood and perilesional skin when compared to healthy skin [17-21], furthermore, as disease progresses, the CD4+/CD8+ratio decreases [22], but in the present study, it was found an elevated CD4/CD8 ratio more than one, to be a marker of an imbalanced lymphocyte immune response. It is also observed in several studies that the absolute and relative lymphocyte subtypes count in the blood of vitiligo cases were normal, so consequently the ratio of CD4+/CD8+ T-cells showed an elevated median value [23,6].

Regarding the data on peripheral blood CD8+ T-cells levels in the present study, it was not in side with most studies that reported higher blood levels of cytotoxic CD8+ T lymphocytes in vitiligo patients when compared to healthy individuals, and the activity of the disease is correlated with these levels [22,24-26]. As illustrated in Table-2, it was found that the mean of absolute CD8+T cells count was slightly higher in vitiligo patients than healthy controls group without statistical significance (p-value=0.49). A decrease in peripheral blood CD8+ lymphocytes could be related to increased homing of these cells to vitiligo-affected skin sites; once in the active disease, lymphocytes migrate to the skin due to the expression of the cutaneous lymphocyte-associated antigen (CLA) molecule [18]. Many studies indicated to the CD8 T-cells dominance in most vitiligo patients' intra- and perilesional skin, as in the skin of active vitiligo lesions, the presence of more CD8+ T lymphocytes confirmed by flow cytometry analysis [27]. The activated cytotoxic CD8+ T cells cause melanocyte malfunction, depigmentation, and apoptosis [8,22,28].

Moreover, no statistically significant variation (p-value=0.8) was found in CD4 T cells levels in vitiligo patients with their corresponding controls comparatively, Table-2, but there was significantly elevated CD4 relative count related segmental vitiligo patients compared with non-segmental vitiligo patients. Although no statistical differences were found even among vitiligo patients' categories, Table-3.

The current study findings appeared to align data for peripheral blood T-cell subpopulations that have been also demonstrated in studies of [23,29,30].

Autoimmune diseases are often associated with an expansion of peripheral helper T cells. However, in relation to vitiligo, conflicting data on abnormalities in circulating helper T cells levels were reported, as several studies indicate to an increase in the number of helper T cells that was found in patients with stable vitiligo and their first-degree relatives [31-33], whereas it was found a decrease in the T helper cell population [34,35]. There are available evidences demonstrated an imbalance in the T-helper cell subsets in the peripheral circulation of individuals with non-segmental vitiligo [17,36,37].

There is no simple explanation to the results of present study and to discrepancies with other studies data concerning peripheral blood T-cells imbalances in general, but it may refer that T-cell subtypes predominate in the cutaneous site of autoimmune melanocyte loss, and it could be attributed to factors concerning the population of patients studied, such as ethnic variations in the research population and the possible received treatments in addition to the characteristics of the disease. The balance of cytotoxic/suppressor and helper/inducer T cells in peripheral blood is disrupted in the majority of vitiligo patients [12].

Lymphocytes include T cells, B cells and natural killer (NK) cells [38]. Immune responses in vitiligo can be mediated by cellular immunity and humoral antibody-mediated immunity. Humoral immunity provides support for autoimmunity's pathological role in vitiligo [14]. NK cells are reported to contribute in vitiligo etiopathogenesis [39,40], as a connection between the innate and adaptive immune systems. Innate immune cells contribute to the initiation and/or progression of the disease by activating adaptive immune response with the production of danger signals, cytokines, and chemokines [41], so based on statistically increased total lymphocytes count of the present study

findings in patients compared with controls ,that may refer there is an increase in the count of NK and/or B-cells in patients compared to controls, and it suggested to examine the incentive role of NK and B-cells in the pathogenesis of vitiligo.

5. Conclusion

The significant increasing total lymphocytes count in patients compared with controls support the findings of the other studies that point to the role of cellular immunity in disease development. More researches are needed to back up the studies regarding other peripheral lymphocytes subtypes such as B-lymphocytes and natural-killer cells in addition to T-lymphocytes populations to provide further precise correlative biomarkers for vitiligo disease progression assessment and it is preferable to research into the respective T-cells in the skin in addition to circulation, that will be critical to better understanding the disease's pathogenesis.

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