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Can Upregulation of CXCR3 By Phototherapy Improve Vitiligo? It's Role in Chronicity

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ABSTRACT

Background: Vitiligo is melanocyte death that is affected by oxidative stress which elevated levels of reactive oxygen species can elicit an immune response, and ultimately cause melanocytic death.

Objective: The current study aimed to investigate the impact of Narrowband Ultraviolet B (NBUVB) and excimer laser phototherapy on C-X-C motif chemokine receptor 3B expression in vitiligo patients and its impact on vitiligo improvement.

Patients and methods: CXCR3B mRNA expression was measured in skin of 25 patients suffering from non-segmental vitiligo before and after generalized 'NBUVB' and targeted phototherapy 'Excimer laser'. All biopsies were kept in -80C till RNA extraction that was performed utilizing QIAGEN RNeasy Little Pack, QuantiTect Switch Record Unit and QuantiNova SYBR Green PCR Unit.

Results: After phototherapy patients with vitiligo had higher CXCR3B mRNA levels, in addition, this was higher in excimer bunch treatment than NBUVB.

Conclusion: Such findings draw attention to CXCR3B as an inherited abnormality in vitiligo patients' skin and raise concerns about its potential contributions in maintaining vitiligo or triggering relapses & chronicity.

Keywords: Vitiligo, CXCR3, phototherapy, mRNA, PCR, Intervention study, Ain Shams University.

INTRODUCTION

Vitiligo is acquired skin condition described by the selective loss of melanocytes and typical chalky-white, non-scaly macules that appear. It is common, with a prevalence of about 0.5-2% worldwide [(1)]. A recent meta-analysis revealed that 23% of vitiligo patients have an anxiety disorder, with a significantly higher prevalence among females than males [(2)].

Clinically, vitiligo has been separated into segmental vitiligo and non-segmental vitiligo. There are acrofacial, mucosal, universal, mixed, and rare forms of non-segmental vitiligo [(3)]. Vitiligo's exact etiology is unknown. T cell-mediated autoimmune destruction, which may be triggered by oxidative stress, and an underlying genetic predisposition are theories regarding its pathogenesis [(4)].

After stopping treatment, vitiligo relapses occur at the same location, indicating an autoimmune memory of the skin cells that permits disease exacerbation. Vitiligo has been now referred to as memory skin disease, clearly demonstrates the presence of melanocyte-specific Tissue Resident Memory cell (TRM). In addition, as demonstrated for the chemokine receptors CXCR3 in vitiligo, signaling by chemokine receptors appears to be necessary for moving TRM into appropriate tissue environment for their formation and maintenance [(5)].

The gene encoding the chemokine receptor CXCR3is highly expressed on effector T cells and is crucial to T cell trafficking and function. The CXCR3 gene can be found in the region q13 of the long arm of chromosome X. CXCR3 can be alternatively spliced into 3 different isoforms: CXCR3-A, CXCR3-B, and chemokine receptor 3-alternative. CXCR3-A signals promote cell migration and proliferation, while CXCR3-B signals inhibit angiogenesis and migration but can promote apoptosis in tumor cells [(6)].

The 415-amino acid CXCR3B is produced through alternative splicing at 5′ end of exon 2 of CXCR3. CXCR3A is encoded by the gene; a 368-amino acid multi-pass membrane molecule with molecular mass of nearly 41 kDa, whereas CXCR3B has 415 amino acids (**Figure 1**) [(7)].

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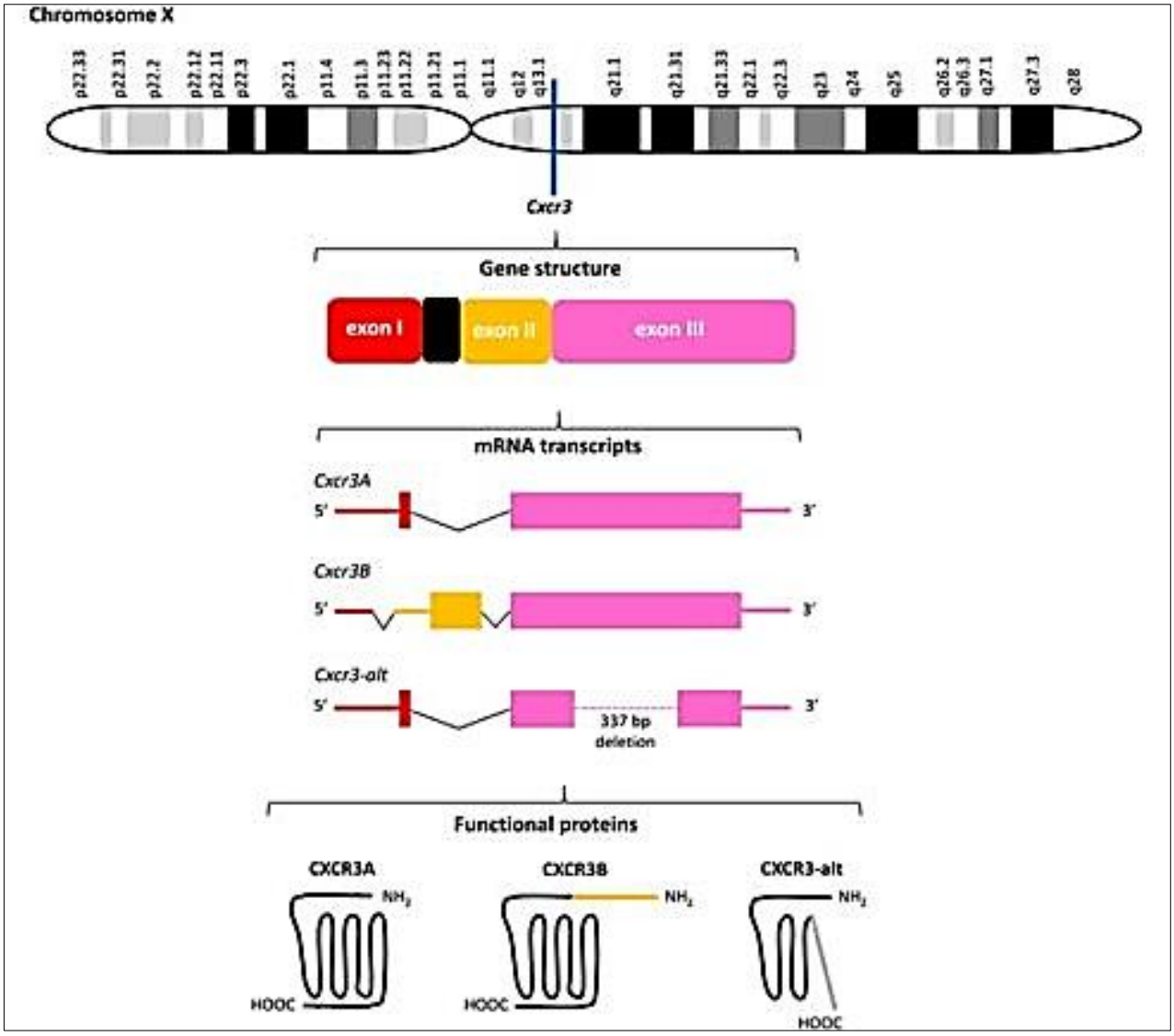

Figure 1: Alternative splicing of CXCR3.

According to previous studies, CXCR3 has been expressed on effector CD4+, CD8+ T cells, Natural killer cells, plasmacytoid dendritic cells, and subsets of B cells [8].

Early stages of melanocyte apoptosis are linked to CXCR3B expression in vitiligo patients' melanocytes. Additionally, endothelial cells may express CXCR3B. CXCR3 is a chemotactic receptor for a group of CXC chemokines that is involved in inflammation [9].

CXCR3B expression in human melanocytes has been greater in vitiligo melanocytes than in healthy melanocytes. Since CXCR3B mRNA silencing decreased CXCL10-induced apoptosis of melanocytes, it is now believed that this receptor is essential for vitiligo's anti-melanocyte immunity [9]. Many research studies showed that CXCL9 and CXCL10, especially CXCL10 generated by tumor or host cells, may attract CXCR3+ tumor infiltrating CD4+ T cells, CD8+ T cells, and NK cells that are linked to tumor suppression [10].

Vitiligo can be treated with Psoralen + UVA (PUVA), broad-band ultraviolet B (BB-UVB), and NBUVB phototherapy, and finally with targeted phototherapy using Excimer lasers and lights [11]. NBUVB is preferred for widespread vitiligo (greater than five percent of body's surface area), excimer is a targeted treatment that is effective for localized disease [12].

UVB phototherapy uses artificial light instead of photosensitizers, which has immunosuppressive and immune-modulatory properties, UVB phototherapy typically results in direct regimentation of the affected skin and slows the progression of vitiligo [13].

Mechanisms of action of UVB phototherapy contain induction of apoptosis, reduction of T-cell numbers, decreased antigen presentation, and immunomodulation. It causes perifollicular regimentation by encouraging melanocyte migration to nearby amelanotic regions. It is believed that the amelanotic melanocytes in outer root sheaths of hair follicles have been activated by NBUVB to generate melanin. Additionally, it was discovered that NBUVB and the excimer laser increased keratinocyte endothelin-1 (ET-1) release, which may contribute to UVB-induced melanocytic synthesis and migration [14].

In vitiligo, the precise mechanism of action of Monochromatic Excimer Laser and Light (MEL) is unknown. Like NBUVB, MEL may cause apoptosis,

decrease in T-cell numbers, decreased antigen presentation, and modulation of inflammatory mediators and cytokines. Excimer, like NBUVB, causes keratinocytes to migrate from outer root sheath of hair follicles to vitiligous regions nearby [15].
The current study aimed to investigate the impact of Narrowband Ultraviolet B (NBUVB) and excimer laser phototherapy on C-X-C motif chemokine receptor 3B expression in vitiligo patients and its impact on vitiligo improvement.

PATIENTS AND METHODS

A prospective cohort study involved 25 participants recruited from Vitiligo Clinics of Department of Dermatology, Venereology and Andrology, Faculty of Medicine, Ain Shams University and Al-Haud Al-Marsoud, in collaboration with the Medical Biochemistry Department, Faculty of Medicine, Ain Shams University and Cairo University Research Park.

Inclusion criteria were studied cases with non-segmental vitiligo with various disease activity scores.

Exclusion criteria were studied cases who have been on systemic treatment throughout last 6 months for vitiligo, or topical treatment for 2 months, patients who have any other hypopigmented lesions: e.g. Pityriasis versicolor (hypopigmented), albinism, pityriasis alba…etc, also, all patients with severe or poorly controlled systemic diseases. For female subjects, pregnant women had been excluded.
The recruited studied cases had been submitted to full history, general and dermatological examination with evaluating vitiligo activity (VIDA) score based on 6-point scale according to ***Njoo et al.*** [16]:
+4: Activity of six weeks or less duration,
+3: Activity of six weeks to three months,
+2: Activity of three to six months,
+1: Activity of six to twelve months,
0: Stable for at least for one year and
-1: Stable for at least for one year with spontaneous re-pigmentation.

Vitiligo Extent Score plus (VES plus) was also calculated before and after treatment.
Research studied cases had been separated into two groups based on Treatment Schedule:13 patients had NBUVB, and 12 patients had excimer.
For each patient, the most recent lesions were selected. For duration of 12 weeks, treatments had been administered 2 times each week on days other than consecutive ones. Initial dose for both treatment modalities had been set at 70% of MED, which had been calculated prior to start of procedure. Dose had been increased by 40% for treatments 1 to 4, 30% for treatments 5 to 8, and 20% continuously for treatments 6 to 8, until faint erythema had been produced. Therapy had been discontinued (1 time or 2 times) and last dose had been 20% lower when therapy had been resumed if symptomatic erythema or blistering appeared. Unaffected skin inside irradiation field had been protected with template throughout 308-nm MEL treatment. Throughout trial, studied cases had been instructed to refrain from using any sort of topical medication.

Phototherapy sources: Conventional NBUVB was delivered, using a Waldmann ramp. A 308-nm MEL (Exciplex phototherapy device, designed and made in france) was used.

Specimen collection and processing: Skin Biopsy was taken from all participants, two punch skin biopsies (3 millimeter) were performed in each patient, using 2% lignocaine local anesthesia, one biopsy before and one biopsy after 12 weeks of starting phototherapy.
Biopsy was obtained from affected area and the edge of the most recent macule.
Biopsies had been preserved at -80 ˚C immersed in RNA later (Ambion, Thermo Fisher Scientific, Inc.) for protection, stabilization, and preservation of RNA, until they had been processed for expression analysis.

RNA extraction and RT-qPCR: Manufacturer's recommended RNeasy Mini Kit (QIAGEN, Germany) had been used to extract RNA. Extracted RNA absorbances at 260 and 280 nm wavelengths had been defined using spectrophotometric quantification in microvolume with Nanodrop: RNA concentration and purity were measured in a 2 µl of RNA.

RNA had been reversely transcribed into cDNA by QuantiTect® Reverse Transcription kit (QIAGEN, Germany) (Cat #:205,311). Real-time qPCR had been performed with QuantiNova SYBR Green PCR Kit (Cat #:208052) according to manufacturer's instructions. Instrument PCR express HyBaid thermal cycler (UK, Delta Scientific industries, INC.) was used. Data had been normalized to β -actin as reference gene, and 2 $^{-\Delta\Delta CT}$ method had been used for relative quantification of CXCR3 gene. Primers used for CXCR3and β-actin had been ready made QIAGEN® primers, sequence for CXCR3B gene was forward 5'-TGGTCCTTGAGGGGTCC-3', and 5'-TCCTATAACTGTCCCCGCCA-and β actin housekeeping gene was; forward 5'-CTCGCCTTTGCCGATCC-3', and reverse 5'- 5'-TCTCCATGTCGTCCCAGTTG-3'.

Ethical consideration:

Research design had been accepted by Ethical Committee Faculty of Medicine, Ain Shams University, and the Ethical committee of Egyptian Ministry of Health as well. (Ethical Approval Number, FWA 000017585: FMASU M S 382 / 2020). Each studied case signed written informed consent form to agree to participate in research. Declaration of Helsinki, World Medical Association's code of

ethics for studies including humans, guided conduct of this work.

Statistical analysis

Data had been collected, revised, coded, and entered to Statistical Package for Social Science version 20.0 (SPSS Inc., Chicago, Illinois, USA). Qualitative data were defined as numbers and percentages. Chi-Square test and Fisher's exact test were used for comparison between categorical variables as appropriate. Quantitative data were tested for normality by Kolmogorov-Smirnov test. Normal distribution of variables was described as mean and standard deviation (SD), and independent sample t-test/ ANOVA post hoc test was used for comparison between groups. P value ≤0.05 was considered to be statistically significant.

RESULTS

Studied case data: In this research, 25 studied cases with non-segmental vitiligo had been involved, 7 women (with average years old of 29 ± 7.7, minimum of 18 and maximum of 37) and 18 men (with average years old of 33.6 ± 13.6, minimum 18 years, maximum 64 years, and with skin phototypes II-V (Fitzpatrick classification [17], about 52% were type IV skin phototype, 32% type III, 12% type II and only 4% type V.

***Response to NBUVB phototherapy*:** Clinical assessment defined that all studied cases had some degree of re-pigmentation after NBUVB phototherapy or Excimer laser phototherapy. In this regard, 5 (20%) subjects confirmed low response to treatment with NBUVB **(Figure 2),** 12 (48%) displayed a medium response, 7 treated with NBUVB and 5 with excimer **(Figure 3)** and 8 (32%) exhibited a high response, 1 treated with NBUVB and 7 with excimer **(Figure 4).**

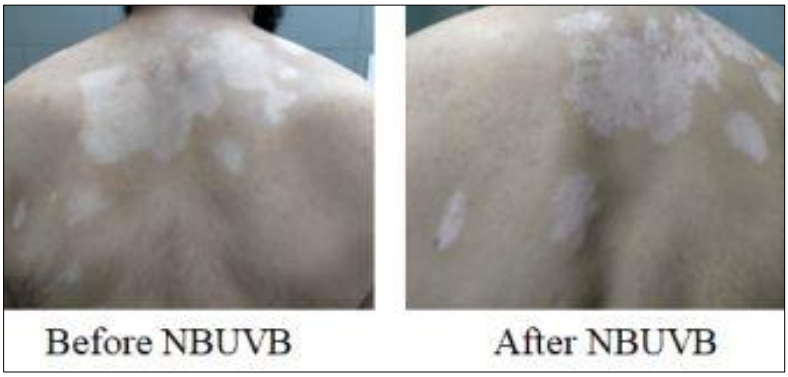

Figure (2): Low response to NBUVB.

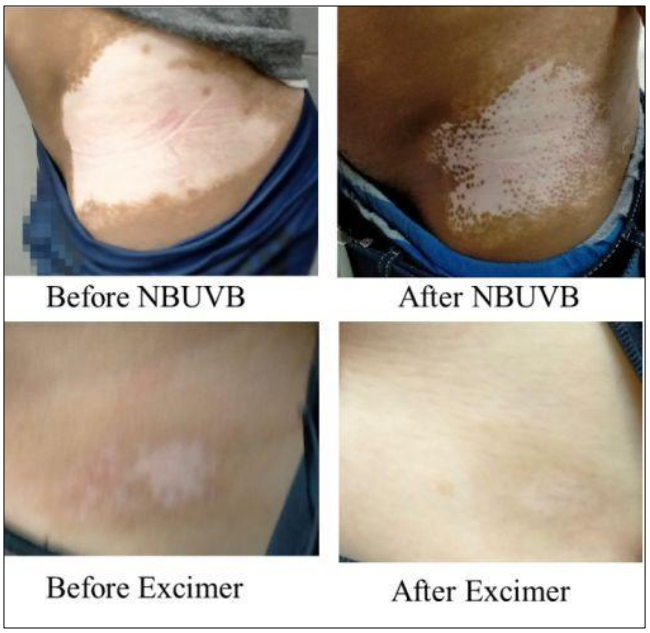

Figure (3): Medium response to NBUVB and Excimer.

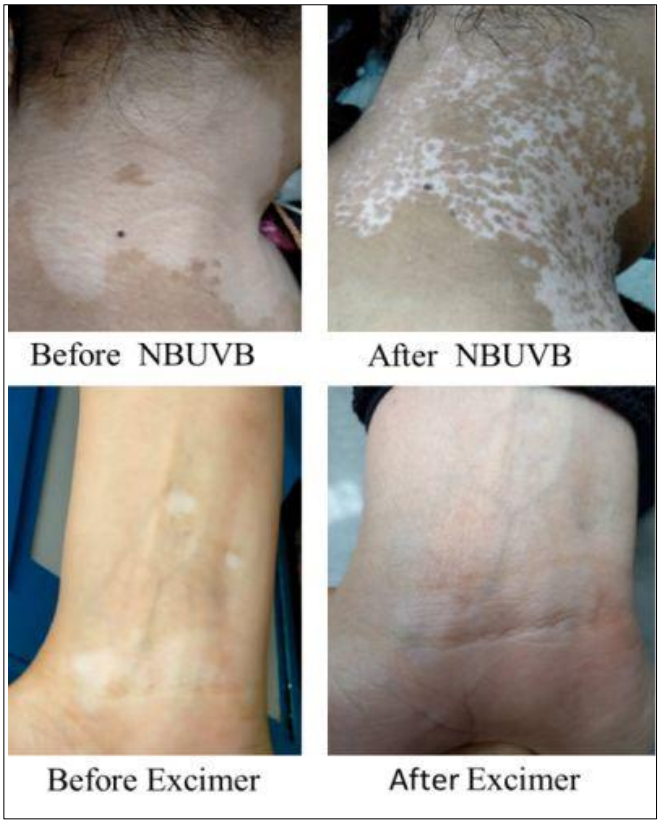

Figure (4): High response to NBUVB and excimer.

***Factors affecting response to treatment*:**
The Fisher's Exact Test analysis among degree of response to treatment and skin phenotype, VIDA sore and treatment modality allowed defining that there has been a significant relationship among response of treatment and these factors **(Table 1).**

Table (1) Factors may be affecting response to treatment.

Variable		No	Response to treatment			Fisher's Exact Test	P value	Significance
			Low	Medium	High			
Skin phototype	II	3	0%	0%	100%	10.951	0.029	<0.05*
	III	8	0%	75%	25%			
	IV	13	31%	46%	23%			
	V	1	100%	0%	0%			
VIDA score	+1	7	43%	29%	29%	11.201	0.036	<0.05* S
	+2	10	0%	40%	60%			
	+3	3	0%	100%	0%			
	+4	5	40%	60%	0%			
Treatment modality	NBUVB	13	38%	54%	8%	9.566	0.006	<0.01**
	EXCISMER	12	0%	42%	58%			

*: Significant difference. **: Highly significant difference.

Duration of vitiligo affected the response of treatment in a significant manner (P<0.05). Patients with longer durations showed lower response indicating a reverse relation between duration of vitiligo and response to treatment. However, age didn't affect the response of treatment (P<0.05) **(Table 2).**

Table (2) ANOVA analysis of age, duration, and response to treatment.

Parameter		Response to treatment			P value
		Low	Medium	High	
Age	**Mean**	27	29	38	0.166
	SD	9	12	13	NS
Duration	**Mean**	11.2	5.5	3.9	0.001**
	SD	2.4	2.8	3.2	

NS: Nonsignificant. **: highly significant difference.

Expression analysis:

Using z- Wilcoxon test, the fold change of CXCR3B with before treatment with (mean 1.00±0.5), compared to after treatment with (mean 0.73±0.65), there was a statistically significant higher fold change in after treatment compared to before treatment(P<0.05) **(Figure 5).**

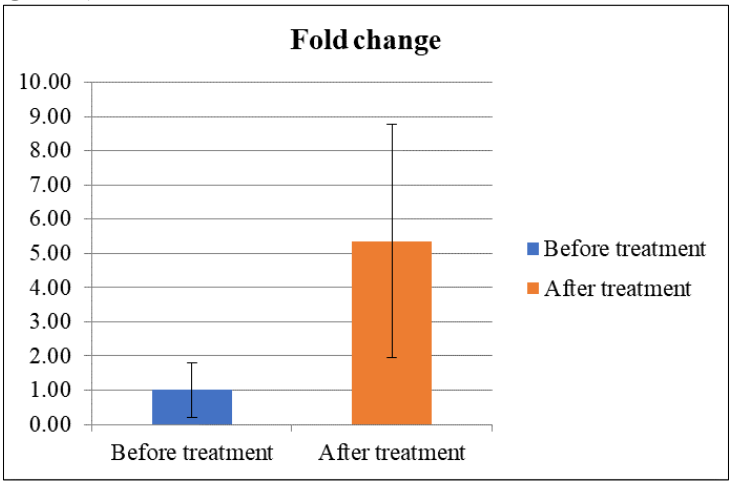

Figure (5): Comparison between before treatment and after treatment according to fold change about CXCR3B.

Using U Mann-Whitney test, there was significant average fold change of CXCR3B for the Excimer (7.13±3.82) compared to the NBUVB (3.57±1.93) **(Figure 6).**

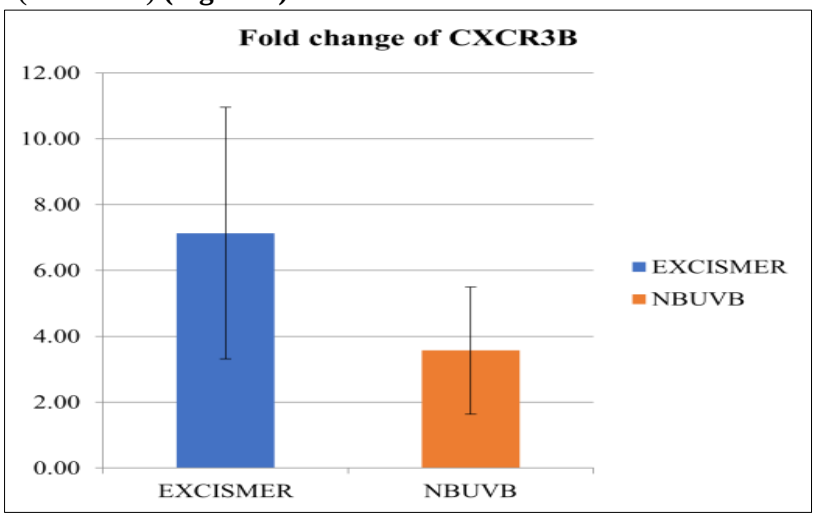

Figure (6): Comparison between EXCISMER and NBUVB according to fold change of CXCR3B.

Receiver operating characteristics (ROC) curve was performed for fold change of CXCR3B and demonstrated a significant increase (P<0.05) of fold change after phototherapy treatment; **(Table 3, Figure 7).**

Table (3): Analysis of (ROC) curve for prediction of exposure to phototherapy.

Cut-off	Sensitivity	specificity	PPV	NPV	AUC [95% CI]	P-value
>1.74	93%	66%	80.40%	86.27%	0.844 [0.599-0.969]	0.004

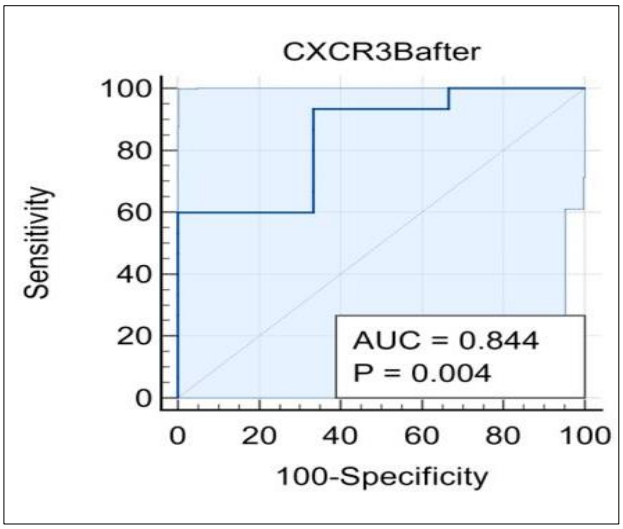

Figure (7): Receiver-operating characteristic curve to predict exposure to phototherapy using the fold change of CXCR3B.

DISCUSSION

The autoimmune destruction of melanocytes causes vitiligo, a chronic skin disease that causes the skin to become less pigmented. In the US, 1.9 to 2.8 million people have been affected with estimated prevalence among 0.76% and 1.11%. Males and females alike, as well as people of all races, ethnicities and socioeconomic status, have been influenced by the condition [(18)].

Stressed melanocytes and keratinocytes may generate more proinflammatory cytokines chemokines, damaged melanocytes release self-antigens and immunostimulatory signals, all of which contribute to the aberrant epidermal microenvironment. Antigen-presenting cells, like DCs, can process the induced antigen release in the presence of ROS. This, in turn, leads to rising in the infiltration of melanocyte-specific T cells in the perilesional and lesional skin, as well as production of antibodies against melanocytes to initiate adaptive immune response in vitiligo epidermis [(19)].

Since approximately 85% of vitiligo susceptibility genes are found to encode proteins included in immunity and apoptosis [(20)], this autoimmune hypothesis is now clearer. The researchers showed that Interferon levels had been connected to progression and maintenance of vitiligo. Further research confirmed elevated levels of IFN-inducible chemokines CXCL9 and CXCL10 in vitiligo patients' serum or skin samples [(21)]. CXCR3 receptor is shared by these two chemokines. Studied cases with progressive vitiligo had greater frequencies of CXCR3+ CD8+ and CXCR3+ CD4+ T cells than those with stable vitiligo [(22)]. This suggests that CXCR3 can play role in activity of the disease. DCs produce type I interferon IFN-. Together with IFN-, IFN- increases keratinocyte secretion of CXCL9 and CXCL10 to increase infiltration of CXCR3-expressing immune cells in lesional skin [(9)].

After successful repigmentation, vitiligo frequently recurs, with a 40% chance occurring in 1st year. Remarkably, relapse almost always appears in same areas that were previously contained, suggesting that autoimmune memory has been formed in these areas [(23)]. Both active and stable vitiligo contained functional CD8 TRM, indicating that TRM in stable vitiligo may be responsible for reactivation. After being stimulated, TRM produces IFN-, CXCL9, CXCL10 and TNF-, as well as characteristic markers CD69, CD103, CD49a and CXCR3 [(9)].

On the other hand, it was reported that blocking their receptor CXCR3 with CXCR3 antibodies caused mice with established vitiligo to re-pigment and prevented CXCL10-induced melanocyte apoptosis and T cell infiltration in skin [(22)].

Furthermore, CXCR3 isoform B that has been directly regulated by IFN was found to be strongly expressed in the basal melanocytes of vitiligo patients; and human melanocytes in culture were killed when CXCL10 activated CXCR3B on their surface. In contrast, inhibiting CXCR3B activation prevented T cell apoptosis and subsequent activation [(9)].

Passeron et al. [(24)], found a huge expansion in CXCR3B mRNA levels in segmental vitiligo skin and an essentially expanded number of CXCR3B-positive melanocytes in studied cases contrasted and those of the solid controls.

In this study we compared the mRNA levels of CXCR3B in skin biopsy of 25 patients taken from perilesional areas before and after localized and generalized phototherapy treatment. Evaluation of response to treatment was done as in other studies, authors chose % regimentation as their primary result measure with ranges of: <25, 25 to 50, 50 to 75, and >75% [(25)]. Phototherapy is used because it can regulate and control the immune system.

The induction of local immunosuppression and stimulation of melanocytes in skin are the mechanisms by which NBUVB works [(26)]. Perifollicular melanocytes are stimulated by the 308-nm MEL [(27)]. Several molecular responses in the skin are triggered when skin has been exposed to UV radiation. In addition, NBUVB increases the expression of the tumor suppressor gene p53, which causes either cell cycle arrest to give DNA repair more time or apoptosis when DNA damage is irreparable. After exposure to UV radiation, DNA repair fails, resulting in the accumulation of CPDs, which suppress the immune system [(28)].

Induction and activation of immunosuppressive Tregs, as well as improved release of inhibitory cytokines like tumor necrosis factor (TNF)-a and Interleukin-10, which promotes Treg differentiation and suppresses autoreactive T cells, are the predominant mechanisms of UVB-induced immunosuppression in the skin [(29)].

Since NBUVB treatment can significantly reduce Interleukin-17 levels in lesional and perilesional skin of vitiligo studied cases, it is proposed that 1

potential pathway for clinical enhancement seen in vitiligo studied cases following NBUVB is the restoration of balance among TH17 cells and Tregs. On the other hand, because vitiligo lesions have lower levels of as basic fibroblast growth factor, phototherapy induces other mechanisms to stimulate regimentation. Proliferation of melanocytes has been prompted by expanded arrival of melanocyte development factors, for example, fundamental bFGF and ET-1 from keratinocytes after NBUVB treatment. Expression of phosphorylated focal adhesion kinase and matrix metalloproteinase 2 also stimulates melanocyte migration. Micropthalmia-associated transcription factor is used to trigger tyrosinase transcription by NBUVB. Keratinocyte and melanocyte expression of proopiomelanocortin and its derivative peptides is also increased, as is melanocyte dendricity and melanosome transport to keratinocytes [(23)].

We set out to examine their CXCR3B expression after treatment in comparison to before treatment to determine whether there has been inherent default in melanocyte function from prelesional locations of studied cases with vitiligo, which can explain their increased responses to chemokines. These immune suppressive effects were combined with the melanogenesis potentials of phototherapy. We showed significant rise in CXCR3B mRNA levels after phototherapy treatment. These data may emphasize that vitiligo studied cases have an inherent defect in their melanocytes that persists after phototherapy, whatever its modality. The lack of immunofluorescence analysis of CXCR3B +ve melanocytes and TRM cells was one of our study's limitations.

With prior research in vitiligo demonstrating elevated CXCR3B expression in vitiligo melanocytes, this information raises possibility that CXCR3B may be 1 of suspected melanocytic abnormalities in vitiligo studied cases [(9)]. In addition to the CD8 TRM, CXCR3B persistent and increased expression after treatment may be a cause of vitiligo patient's relapses; suggesting that targeting more specifically CXCR3B activation could offer effective approaches for not only halting progression of disease but may also prevent relapses in these patients.

CONCLUSION

When compared to levels prior to therapy, CXCR3B mRNA levels significantly increased following either NBUVB or Excimer laser treatment, resulting in improved pigmentation in these patients. Therefore, elevated CXCR3B mRNA levels are characteristic of vitiligo patients and suggest a persistent melanocyte defect that is inherited. It may also be responsible for vitiligo's recurrence and persistence.

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Author contribution: Authors contributed equally to research.

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