# Publication & Presentation

# *List of Publications*

1. **Vaishnav J,** Khan F, Yadav M, Parmar N, Buch H, Jadeja SD, Dwivedi M, Begum R. **(2022)** V-set domain containing T-cell activation inhibitor-1 (VTCN1): a potential target for the treatment of autoimmune diseases. **Immunobiology**. 2022 Sep 6:152274. **(IF:3.15)**
2. **Vaishnav J**, Jadeja SD, Singh M, Khan F, Yadav M, & Begum R. **(2022)**. Altered Levels of Negative Costimulatory Molecule V-Set Domain-Containing T-Cell Activation Inhibitor-1 (VTCN1) and Metalloprotease Nardilysin (NRD1) are Associated with Generalized Active Vitiligo. **Immunological Investigations**, 1-18. **(IF:3.65)**
3. Jadeja SD, **Vaishnav J,** Bharti AH & Begum R. **(2022)**. Elevated X-Box Binding Protein1 Splicing and Interleukin-17A Expression Are Associated with Active Generalized Vitiligo in Gujarat Population. **Frontiers in immunology**, 5676. **(IF:8.86)**
4. Singh M, **Vaishnav J**, Shah A, & Begum R. **(2021)**. Expression analysis of candidate genes in vitiligo patients & effect of oxidative stress on melanocytes. **Gene Reports**, 25, 101389. **(IF:0.72)**
5. Jadeja SD, Mayatra JM, **Vaishnav J**, Shukla N, & Begum, R **(2021)**. A Concise Review on the Role of Endoplasmic Reticulum Stress in the Development of Autoimmunity in Vitiligo Pathogenesis. **Frontiers in immunology**, 11, 3817. **(IF:6.42)**
6. Singh M, Jadeja SD, **Vaishnav J,** Mansuri MS, Shah C, Mayatra JM, Shah A, Begum R. **(2020)**. Investigation of the Role of Interleukin 6 in Vitiligo Pathogenesis. **Immunological Investigations**, 1-18. **(IF:2.56)**

# *Manuscript under communication*

**Vaishnav J,** Jadeja SD, Rajguru D, Giri P, Dwivedi M & Begum R. Decreased expression of V-set domain containing T-cell activation inhibitor-1 (VTCN1) on different subpopulation of lymphocytes in generalized active vitiligo patients.

# *List of Oral/Poster Presentations*

1. **Vaishnav J**, Jadeja SD, Khan F, Yadav M, Begum R. " Involvement of VTCN1 and its cleaving enzyme NRD1 in vitiligo pathogenesis" at **Vitiligo International Symposium - 2021 (VIS-2021)'** Organized by Global Vitiligo Foundation, Chicago, USA, held on 4th to 5th December 2021.

2. Jadeja SD, Mayatra JM, **Vaishnav J**, Harshe A, Begum R**.** "Two sides of the same coin: Association of Tyrosinase R402Q variant with Vitiligo and Melanoma" at the "2nd UK-India Cancer Informatics workshop on **Next- Generation Sequencing Data Analysis" at ACTREC, Tata Memorial Centre**, Navi Mumbai from 31st October - 2nd November 2019.
3. **Vaishnav J**, Jadeja SD, Khan F, Begum R. "VTCN1: A prospective immune check point inhibitor in vitiligo pathogenesis" at 9th International conference on **'Nextgen genomics, biology, bioinformatics and technologies (NGBT)-2019'** held at Mumbai, India from 30th September -2nd October, 2019.
4. **Vaishnav J**, Begum R. "Investigating the association of V-set domain containing T-cell activation inhibitor-1(VTCN1) rs12046117 and rs10923223 polymorphisms and its expression with vitiligo susceptibility in Gujarat population" at the Asian Advanced Course in Basic and Clinical Immunology organized by the **Federation of Clinical Immunology Societies (FOCIS)** at Jaipur, Rajasthan, India from 26th-29th March, 2019.
5. Jadeja SD, **Vaishnav J**, Vasava J, Khan F, Narayan P, Bharti A, **Begum R.** "Investigating the association of Interleukin-17A (IL17A) promoter polymorphisms and its expression with vitiligo susceptibility in Gujarat population" at the Asian Advanced Course in Basic and Clinical Immunology organized by the **Federation of Clinical Immunology Societies (FOCIS)** at Jaipur, Rajasthan, India from 26th-29th March, 2019.
6. Jadeja SD, **Vaishnav J**, Narayan P, Singh M, Mansuri MS, Begum R. "Investigating the association of IL17A -197 G/A and -737 C/T promoter polymorphisms and its transcripts with vitiligo susceptibility in Gujarat population" at International Conference on **'Proteins, miRNA and Exosomes in Health and Diseases'** held at The M. S. University of Baroda, Vadodara, Gujarat, India from 11th - 13th December, 2018.
7. **Vaishnav J**, Singh M, Shah C, Jadeja SD, Begum R. "Association of VTCN-1 intronic polymorphisms (rs10923223 and rs12046117) and its transcript levels with vitiligo susceptibility in Gujarat population" at International Conference on **'Proteins, miRNA and Exosomes in Health and Diseases'** held at The M. S. University of Baroda, Vadodara, Gujarat, India from 11th - 13th December, 2018.

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# Altered Levels of Negative Costimulatory Molecule V-Set Domain-Containing T-Cell Activation Inhibitor-1 (VTCN1) and Metalloprotease Nardilysin (NRD1) are Associated with Generalized Active Vitiligo

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# Altered Levels of Negative Costimulatory Molecule V-Set Domain-Containing T-Cell Activation Inhibitor-1 (VTCN1) and Metalloprotease Nardilysin (NRD1) are Associated with Generalized Active Vitiligo

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**ABSTRACT**

**Background:** Vitiligo is characterized by depigmented macules on the skin caused due to autoimmune destruction of melanocytes. V-set domain-containing *T*-cell activation inhibitor-1 (VTCN1) is a negative costimulatory molecule that plays a vital role in suppressing autoimmunity and tuning immune response. Nardilysin (NRD1), a metalloproteinase, cleaves membrane-tethered VTCN1 resulting in the shedding of soluble-VTCN1 (sVTCN1). However, the role of VTCN1 and NRD1 in vitiligo pathogenesis is unexplored.

**Objectives and methods:** This study was aimed to (i) Investigate the association of VTCN1 intronic polymorphisms (rs10923223 T/C and rs12046117 C/T) with vitiligo susceptibility in Gujarat population by using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) (ii) Estimate VTCN1 & NRD1 transcript levels from peripheral blood mononuclear cells (PBMCs) and skin samples of vitiligo patients by real-time PCR, (iii) Estimate sVTCN1 and NRD1 protein levels from plasma by ELISA and (iv) Estimate VTCN1 protein levels in the skin samples of vitiligo patients by immunofluorescence.

**Results:** The analysis revealed increased *VTCN1* and *NRD1* transcript levels in the skin ($p = .039$, $p = .021$ respectively), increased sVTCN1 and NRD1 levels ($p = .026$, $p = .015$ respectively) in the plasma, and decreased VTCN1 protein levels ($p = .0002$) in the skin of vitiligo patients as compared to healthy controls. The genetic analysis revealed no significant association of *VTCN1* intronic polymorphisms rs10923223 T/C and rs12046117 C/T with vitiligo susceptibility in Gujarat population ($p = .359$, $p = .937$, respectively).

**Conclusions:** The present study revealed altered VTCN1 and NRD1 expressions in the blood and skin of vitiligo patients, suggesting their potential role in the development and progression of Vitiligo.

**KEYWORDS**
Autoimmunity; Nardilysin (NRD1); polymorphisms; vitiligo; VTCN1 (B7-H4)

## Introduction

Vitiligo is a common skin disfiguring disease characterized by white-colored patches due to the erosion of functional melanocytes from the epidermal basal layer. The worldwide occurrence of Vitiligo from population and hospital-based studies was 0.2–1.8% (Zhang

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et al. 2016). In India, the prevalence of Vitiligo was reported 0.25–4% based on dermatology outpatients' studies across the country and up to 8.8% in Gujarat and Rajasthan (Jadeja et al. 2018; Krüger and Schallreuter 2012). Extensive research on understanding vitiligo pathogenesis emphasized the involvement of autoimmune response against melanocytes leading to the development and progression of vitiligo (Frisoli et al. 2020; Mohammed 2015). The involvement of both humoral and cellular arms of immunity has been substantiated by circulating melanocyte-specific autoantibodies in the blood and autoreactive anti-melanocyte CD8+ T cells in the skin of vitiligo patients and impaired inhibitory function of Treg cells (Giri et al. 2020; Laddha et al. 2014; van den Boorn et al. 2009). However, the exact modus operandi priming the induction and activation of autoreactive T cells and the loss of tolerance to melanocyte autoantigens in Vitiligo is unclear (Dwivedi et al. 2015).

Costimulatory pathways are known to influence the development and progression of autoimmunity (Xiao et al. 2017). The B7-CD28 family members are the key costimulatory molecules that play an essential role in the activation of T lymphocytes, loss of tolerance, and development of optimal T cell immunity (Wang and Chen 2004). VTCN1 (V-set domain-containing *T*-cell activation inhibitor 1), also named as B7-H4, B7S1, and B7x is a negative costimulatory molecule identified by three different research groups (Prasad et al. 2003; Sica et al. 2003; Zang et al. 2003). It belongs to the B7 family of immune-regulatory ligands. The human *VTCN1* gene is mapped on chromosome 1, comprising six exons and five introns, and the mature membrane-bound protein consists of 282 amino acids (Choi et al. 2003). Membrane-bound VTCN1 to the antigen-presenting cell interacts with an unidentified receptor on the T cell and inhibits T cell activation, clonal expansion, and cytokine production (John et al. 2019). A metalloproteinase, Nardilysin (NRD1), cleaves these membrane-bound VTCN1 molecules resulting in the shedding of the cleaved soluble-VTCN1 (sVTCN1) molecule (Radichev et al. 2014). The NRD1 (N-arginine dibasic (NRD) convertase 1) belongs to the M16 family, cleaving peptide substrates at the N-terminus of arginine in dibasic motifs (Hospital et al. 2000).

Recent studies suggest that negative co-stimulation mediated by VTCN1 offers a critical balance between abnormal T-cell activation and anergy (John et al. 2019). Experimental evidence indicated that dysregulated VTCN1 aggravates different autoimmune conditions such as Rheumatoid Arthritis (RA) (Azuma et al. 2009). Systemic Lupus Erythematosus (SLE) (Xiao et al. 2019), Type 1 Diabetes (T1D) (Wang et al. 2011), Primary Biliary Cirrhosis (PBC) (Chen et al. 2011). A GWAS study demonstrated that polymorphisms mapped to the *VTCN1* gene were significantly associated with Juvenile Idiopathic Arthritis (JIA) (Hinks et al. 2009). Given the key role of VTCN1 in other autoimmune diseases, we hypothesize that altered VTCN1 levels might result in an aggravated autoreactive T cell response resulting in melanocyte destruction in Vitiligo. Hence, the present study was aimed to investigate the association of *VTCN1* intronic polymorphisms (rs10923223 T/C and rs12046117 C/T) with susceptibility to Vitiligo in the Gujarat population, to assess *VTCN1* and *NRD1* transcript levels from peripheral blood mononuclear cells (PBMCs) as well as skin, to estimate sVTCN1 and NRD1 levels from plasma, and VTCN1 protein levels from the vitiliginous skin.

## Materials and methods

### *Study population*

A total of 411 patients with Vitiligo who visited the Dermatology department of S.S.G Hospital, Vadodara, Gujarat, India, from 2016 to 2019 were recruited (Table S1). Vitiligo patients from Gujarat aged between 5–60 years were considered for the study. Patients with other diseases or unwilling to participate in the study were excluded. The expert dermatologist made a diagnosis of Vitiligo using a woods lamp examination. Patients were categorized as generalized and localized Vitiligo as per clinical criteria proposed by Flabella et al (1995) and discussed in the Vitiligo Global Issues Consensus Conference 2012 (Ezzedine et al. 2012). Stable Vitiligo (SV) was defined as no progression of old lesions and no new lesions within the past two years, while active vitiligo (AV) was defined as the appearance of new lesions and spreading of existing lesions observed during the past two-year duration (Falabella et al. 1995; Ezzedine et al. 2012). The institutional ethical committee for human research (IECHR), Medical College Baroda, Faculty of Medicine, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (EC Reg No: ECR/85/Inst/GJ/2013/RR-16) approved the plan of study. Written consent was obtained, and the significance of the study was explained to all participants.

### Genotyping of VTCN1 *rs10923223 (T/C) and rs12046117 (C/T)* polymorphisms

Genomic DNA was isolated from the blood using QIAamp ™ DNA Blood Kit (QIAGEN Inc., Valencia, CA 91355, USA) as per the manufacturer's protocol. Genotyping of both the intronic polymorphisms was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described earlier (Jadeja et al. 2017). The primers used for PCR and annealing temperatures are mentioned in Table S3. The 5 µl amplified product was confirmed by 2.0% agarose gel electrophoresis, and the remaining 15 µl of the amplified product was digested with 1 U of *Sph I* and *Taq I* (Thermo Fischer Scientific, U.S.A.) for *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) polymorphisms respectively. Digested products were resolved on 3.5% agarose gel with a 50 bp DNA ladder (HiMedia™, 50 bp DNA ladder) and visualized under E-Gel Imager (Life Technologies ™, Carlsbad, CA). Representative gel images are shown in supplementary Figure S1.

### Estimation of *VTCN1* and *NRD1* transcript levels from PBMCs and the skin

RNA was isolated from PBMCs and skin tissue (20 mg) using the RNAiso Plus (TaKaRa Bio Inc) following the standardized protocol (Singh et al. 2020). Demographic details of vitiligo patients and controls for PBMCs and skin samples were given in Tables S1 and S2B, respectively. Skin samples were collected in RNA *later* Solution (Ambion®, USA) and stored at −80°C until use. Quality, quantity, and purity of the RNA were checked, and cDNA synthesis was performed as described earlier (Singh et al. 2020). Further *VTCN1*, *NRD1*, and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) expression were assessed by qPCR using gene-specific primers (Eurofins ™, Bangalore, India) at standardized annealing temperatures, as shown in Table S3. *GAPDH* was used as a reference. The fluorescence data was collected. The $\Delta C_P$ was calculated as the difference between the cycle threshold of

the target genes (*VTCN1/NRD1*) and the reference gene (GAPDH). The $\Delta\Delta C_P$ was considered as the difference between the two $\Delta C_P$ values ($\Delta C_P$ controls and $\Delta C_P$ patients) to get the value of fold change expression ($2^{-\Delta\Delta C_P}$).

### Estimation of plasma sVTCN1 and NRD1 levels

Soluble VTCN1 (sVTCN1) from the plasma of patients with Vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using CUSABIO® ELISA kit (CSB-E15013h) as per the manufacturer's protocol. Plasma NRD1 levels of patients with Vitiligo and controls were measured using a Fine Test ELISA kit (EH10653, Wuhan, Hubei, China) as per the manufacturer's protocol and optical density was measured at 450 nm.

### Monitoring expression of VTCN1 protein by immunofluorescence analysis of the skin

Expert dermatologist collected skin biopsies from 12 patients with Vitiligo and 13 controls using a punch probe (3 mm) under local anesthesia from lesional (L), perilesional (PL), and non-lesional (NL) skin. Demographical details of patients and controls were given in Table S2A. Each biopsy was immediately stored in NBF (Neutral buffered Formalin). Paraffin-embedded blocks were prepared, and 3 µm sections were placed on poly-L lysine-coated slides. Slides were deparaffinized, hydrated, permeabilized and antigen retrieved with 10 mM Sodium Citrate buffer (pH = 6.0). Slides were blocked with NDS (Normal Donkey Serum) and stained with PE-conjugated primary tagged VTCN1 Monoclonal Antibody (12-5949-42, eBioscience ™). After staining, slides were washed with washing buffer and mounted with Slow Fade™ Gold antifade reagent with DAPI (S36939, Life Technologies, Carlsbad, CA). A fluorescence microscope (Nikon eclipse Ti2-E, Tokyo, Japan) was used to detect fluorescence signals. Images were analyzed using NIS-Elements Advanced Research 5.20 software (Nikon, Tokyo, Japan) to calculate the mean fluorescence intensity, and graphs were plotted.

### Statistical analyses

Hardy-Weinberg Equilibrium (HWE) was calculated for both SNPs in controls and patients by comparing observed and expected frequencies of genotypes by chi-square analysis. Allele and genotype frequencies distribution of polymorphisms in different groups were compared using the chi-square test with $2 \times 2$ contingency tables. Bonferroni correction was used to control multiple comparisons. *VTCN1* and *NRD1* transcripts analysis in patients with Vitiligo and controls were analyzed by parametric unpaired *t*-test and one-way ANOVA. Comparison of sVTCN1, plasma NRD1, and mean fluorescence intensity of VTCN1 in skin tissue among the multiple groups were analyzed using one-way ANOVA with multiple comparisons. The correlation between sVTCN1 and NRD1 levels was analyzed using Spearman correlation analysis. The missing data were omitted for accurate analysis. Graph Pad Prism 8 software was used to conduct the statistical analysis (Graph Pad Software Inc; 2003).

### Bioinformatics analysis

The functional impact of non-coding polymorphism in *VTCN1* intron 1, i.e., rs12046117 and rs10923223, was assessed using *in silico* prediction tool RegulomeDB (Boyle et al. 2012).

## Results

### Association of *VTCN1* rs12046117 (C/T) with vitiligo susceptibility

Genotyping of intronic *VTCN1* rs12046117 SNP was done, and three genotypes were identified in both patient and control populations: CC, CT, and TT (Figure S1A). Both the populations followed Hardy-Weinberg equilibrium (HWE) ($p = .450$ and $p = .097$, respectively; Table 1). The observed frequency of the ancestral allele 'C' in patient and control groups was 79%. No remarkable difference was seen in the frequencies of variant allele 'T' between patient (21%) and control (21%) ($p = .937$; Table 1). VTCN1 SNP rs12046117, when analyzed according to the type of Vitiligo, no significant difference in genotype and allele frequencies was observed between patients with GV and LV to controls ($p > .05$; Table 2). Analysis based on the activity of the disease also showed no significant difference among the genotypes as well as allele frequencies between patients with AV and SV to controls ($p > .05$; Table 3).

### Association of *VTCN1* rs10923223 (T/C) with vitiligo susceptibility

Genotyping of intronic *VTCN1* rs10923223 SNP was done, and three genotypes were identified in both patient and control populations: TT, TC, and CC (Figure S1B). Both populations followed HWE ($p = .319$and $p = .458$ respectively; Table 1). The ancestral allele frequency in the patient and control groups was 67% and 69%, respectively. No remarkable difference was observed in the frequencies of variant allele 'T' between patient (33%) and control (31%) groups ($p = .359$; Table 1). VTCN1 SNP rs10923223, when analyzed according to the type of Vitiligo, no significant difference in genotype and allele frequencies was

**Table 1.** Distribution of genotypes and alleles for *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) in vitiligo patients and controls.

| SNP | Genotype/ allele | Control (Freq) | Vitiligo patients (Freq) | *p* value for HWE | *p* value | Odds ratio | CI (95%) |
|---|---|---|---|---|---|---|---|
| | Genotype | n = 450 | n = 411 | | | | |
| *VTCN1* rs12046117 (C/T) | CC | 275 (0.61) | 249 (0.61) | .450 | R | 1 | - |
| | CT | 157 (0.35) | 149 (0.36) | (C) | .744 | 1.04 | 0.79–1.39 |
| | TT | 18 (0.04) | 13 (0.03) | | .545 | 0.79 | 0.38–1.66 |
| | Allele | | | .097 | | | |
| | C | 707 (0.79) | 647 (0.79) | (P) | R | 1 | - |
| | T | 193 (0.21) | 175 (0.21) | | .937 | 0.99 | 0.78–1.24 |
| | Genotype | n = 382 | n = 310 | | | | |
| *VTCN1* rs10923223 (T/C) | TT | 179 (0.47) | 136 (0.44) | .319 | R | 1 | - |
| | TC | 171 (0.45) | 143 (0.46) | (C) | .550 | 1.10 | 0.80–1.50 |
| | CC | 32 (0.08) | 31 (0.10) | | .378 | 1.27 | 0.74–2.19 |
| | Allele | | | .458 | | | |
| | T | 529 (0.69) | 415 (0.67) | (P) | R | 1 | - |
| | C | 235 (0.31) | 205 (0.33) | | .359 | 1.11 | 0.88–1.39 |

n = Number of Patients/ Controls.
R= Reference group.
CI= Confidence Interval.
Bonferroni's correction has applied, so statistical significance was considered at $p < .02$.

**Table 2.** Association of *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) polymorphism with generalized and localized vitiligo.

| SNP | Genotype or allele | Generalized vitiligo (Freq.) | Localized Vitiligo (Freq.) | Controls (Freq.) | *p* for Association | Odds ratio | CI (95%) |
|---|---|---|---|---|---|---|---|
| *VTCN1* | Genotype | n = 352 | n = 59 | n = 450 | | | |
| rs12046117 | CC | 214 (0.61) | 35 (0.59) | 275 (0.61) | R | 1 | - |
| C/T | CT | 127 (0.36) | 22 (0.37) | 157 (0.35) | .84[a] | 1.05[a] | 0.59–1.88[a] |
| | | | | | .79[b] | 1.03[b] | 0.77–1.39[b] |
| | | | | | .73[c] | 1.10[c] | 0.62–1.94[c] |
| | TT | 11 (0.03) | 2 (0.04) | 18 (0.04) | .89[a] | 1.11[a] | 0.23–5.23[a] |
| | | | | | .53[b] | 0.78[b] | 0.36–1.69[b] |
| | | | | | .85[c] | 0.87[c] | 0.19–3.92[c] |
| | Allele | | | | | | |
| | C | 555 (0.79) | 92 (0.78) | 707 (0.79) | R | 1 | - |
| | T | 149 (0.21) | 26 (0.22) | 193 (0.21) | .83[a] | 1.05[a] | 0.65–1.68[a] |
| | | | | | .89[b] | 0.98[b] | 0.77–1.25[b] |
| | | | | | .88[c] | 1.03[c] | 0.65–1.64[c] |
| | Genotype | n = 257 | n = 53 | n = 382 | | | |
| *VTCN1* | | | | | | | |
| Rs10923223 | TT | 118 (0.46) | 19 (0.36) | 179 (0.47) | R | 1 | - |
| T/C | TC | 113 (0.43) | 32 (0.60) | 171 (0.45) | .069[a] | 0.56[a] | 0.31–1.06[a] |
| | | | | | .967[b] | 0.99[b] | 0.71–1.38[b] |
| | | | | | .063[c] | 1.76[c] | 0.97–3.29[c] |
| | CC | 29 (0.11) | 2 (0.04) | 32 (0.08) | .254[a] | 2.35[a] | 0.60–10.67[a] |
| | | | | | .246[b] | 1.38[b] | 0.80–2.43[b] |
| | | | | | .485[c] | 0.58[c] | 0.13–2.24[c] |
| | Allele | | | | | | |
| | T | 345 (0.67) | 70 (0.66) | 529 (0.69) | R | 1 | - |
| | C | 169 (0.33) | 36 (0.34) | 235 (0.31) | .829[a] | 0.95[a] | 0.61–1.46[a] |
| | | | | | .424[b] | 1.10[b] | 0.86–1.39[b] |
| | | | | | .504[c] | 1.15[c] | 0.74–1.79[c] |

'n' represents the number of Patients/ Controls.
'R' represents the reference group.
CI refers to Confidence Interval.
[a]Generalized Vitiligo vs. Localized Vitiligo.
[b]Generalized Vitiligo vs. Controls.
[c]Localized Vitiligo vs. Controls.
Statistical significance was considered at $p < .025$ due to Bonferroni's correction.

observed between patients with GV and LV to controls ($p > .05$; Table 2). Analysis based on the activity of the disease also showed no significant difference among the genotypes as well as allele frequencies between patients with AV and SV to controls ($p > .05$; Table 3).

## *Linkage Disequilibrium (LD) and haplotype analysis*

Linkage disequilibrium analysis divulged that both polymorphisms, i.e., *VTCN1* rs12046117 and rs10923223 were in moderate LD association (D' = 0.683, $r^2 = 0.260$). Haplotype analysis of the two polymorphic sites disclosed no remarkable difference in the estimated frequencies of haplotypes between patients with Vitiligo and controls (global $p = .170$; Table 4).

**Table 3.** Association of *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) polymorphism with active and stable vitiligo.

| SNP | Genotype/ allele | Active Vitiligo (Freq.) | Stable Vitiligo (Freq.) | Controls (Freq.) | *p* for Association | Odds ratio | CI (95%) |
|---|---|---|---|---|---|---|---|
| | Genotype | n = 350 | n = 61 | n = 450 | | | |
| *VTCN1* rs12046117 (C/T) | CC | 210 (0.60) | 39 (0.64) | 275 (0.61) | R | 1 | - |
| | CT | 128 (0.37) | 21 (0.34) | 157 (0.35) | .67[a] | 0.88[a] | 0.49–1.56[a] |
| | | | | | .66[b] | 1.06[b] | 0.79–1.43[b] |
| | | | | | .83[c] | 0.94[c] | 0.53–1.61[c] |
| | TT | 12 (0.03) | 1 (0.02) | 18 (0.04) | .43[a] | 0.44[a] | 0.05–3.55[a] |
| | | | | | .72[b] | 0.87[b] | 0.41–1.85[b] |
| | | | | | .35[c] | 0.39[c] | 0.05–3.01[c] |
| | Allele | | | | | | |
| | C | 548 (0.78) | 99 (0.81) | 707 (0.79) | R | 1 | - |
| | T | 152 (0.22) | 23 (0.19) | 193 (0.21) | .47[a] | 0.83[a] | 0.51–1.36[a] |
| | | | | | .89[b] | 1.01[b] | 0.79–1.29[b] |
| | | | | | .51[c] | 0.85[c] | 0.52–1.37[c] |
| *VTCN1* rs10923223 (T/C) | Genotype | n = 260 | n = 50 | n = 382 | | | |
| | TT | 118 (0.45) | 18 (0.36) | 179 (0.47) | R | 1 | - |
| | TC | 113 (0.44) | 30 (0.60) | 171 (0.45) | .086[a] | 0.57[a] | 0.30–1.06[a] |
| | | | | | .988[b] | 1.02[b] | 0.71–1.39[b] |
| | | | | | .076[c] | 1.74[c] | 0.93–3.17[c] |
| | CC | 29 (0.11) | 2 (0.04) | 32 (0.08) | .293[a] | 2.21[a] | 0.56–10.05[a] |
| | | | | | .258[b] | 1.37[b] | 0.79–2.41[a] |
| | | | | | .533[c] | 0.62[c] | 0.13–2.40[c] |
| | Allele | | | | | | |
| | T | 349 (0.67) | 66 (0.66) | 529 (0.69) | R | 1 | - |
| | C | 171 (0.33) | 34 (0.34) | 235 (0.31) | .828 [a] | 0.95[a] | 0.60–1.49[a] |
| | | | | | .421[b] | 1.10[b] | 0.86–1.39[b] |
| | | | | | .510[c] | 1.16[c] | 0.75–1.79[c] |

'n' represents the number of Patients/Controls.
'R' represents the reference group.
CI refers to Confidence Interval.
[a]Active vitiligo vs. Stable vitiligo.
[b]Active Vitiligo vs. Controls.
[c]Stable Vitiligo vs. Controls.
Statistical significance was considered at $p < .025$ due to Bonferroni's correction.

## Bioinformatics analysis of *VTCN1* rs12046117 and rs10923223 polymorphisms

To investigate the functional consequences of both *VTCN1* intronic polymorphisms (rs12046117 and rs10923223), RegulomeDB analysis was performed. Both SNPs scored five and had minimal binding evidence (Table 5). No ChIP, Motif, and QTL data were available for both SNPs.

## Analysis of *VTCN1* and *NRD1* transcripts levels from PBMCs

To assess the possible involvement of VTCN1 in vitiligo, we have analyzed *VTCN1* transcripts from PBMCs of 188 patients with Vitiligo and 201 healthy controls. Curiously, a remarkable reduction of *VTCN1* transcripts in vitiligo patients ($p = .023$) was noticed. −2.38-fold decrease in the expression of *VTCN1* transcript levels in patients was revealed in comparison to controls (Figure 1a). Moreover, analysis of the type of Vitiligo revealed that *VTCN1* transcripts

**Table 4.** Distribution of haplotype frequencies for *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) polymorphisms in vitiligo patients and controls.

| Haplotype [VTCN1 (T/C) :(C/T)] | Patients (freq) | Controls (freq) | *p* for association | *P* (Global) | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| C T | 302.40(0.62) | 260.51(0.61) | .370 | .170 | 1.13 | 0.86–1.48 |
| C C | 67.60(0.14) | 74.49(0.17) | .193 | | 0.78 | 0.55–1.12 |
| T T | 16.60(0.03) | 24.49(0.05) | .109 | | 0.59 | 0.31–1.13 |
| T C | 83.40(0.17) | 64.51(0.15) | .309 | | 1.20 | 0.84–1.71 |

CI represents Confidence Interval.
(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

**Table 5.** *In silico* analysis results for *VTCN1* rs12046117 and rs10923223 polymorphisms.

| Sr. No | SNP ID | Gene Symbol | SNP Location | Chromosomal Location | Regulome DB score/Prediction |
|---|---|---|---|---|---|
| 1 | rs12046117 | VTCN1 | Intron 1 | chr1: 117751364– 117751365 | 5/Minimal Binding Evidences |
| 2 | rs10923223 | VTCN1 | Intron 1 | chr1: 117746572– 117746573 | 5/Minimal Binding Evidences |

were remarkably decreased in patients with GV with respect to controls ($p = .034$; Figure 1b), whereas patients with LV divulged no change in *VTCN1* transcript levels ($p = .664$). Also, no notable change was seen in transcripts between GV vs LV ($p = .896$). Besides, when we analyzed the results according to the activity of Vitiligo, AV patients displayed a remarkable decrease in *VTCN1* transcripts compared with controls ($p = .037$; Figure 1c). Moreover, SV patients revealed no change in *VTCN1* transcripts compared with controls ($p = .984$). Additionally, no change was seen in transcript levels between AV vs SV ($p = .521$).

We have also monitored the transcript levels of *NRD1*, involved in the cleavage of membrane-bound VTCN1, from the PBMCs of vitiligo patients and controls. Our findings revealed an increase in *NRD1* transcript levels of 175 patients with Vitiligo compared to 190 controls ($p = .034$; Figure 1e). The fold change analysis revealed a 1.26-fold higher expression of *NRD1* transcripts in patients with Vitiligo than in controls. Analysis according to the type of Vitiligo revealed that *NRD1* transcripts were notably increased in GV patients compared to controls ($p = .049$; Figure 1f), whereas patients with LV displayed no significant change compared with controls ($p = .701$). Additionally, no significant change was observed between patients with GV vs LV ($p = .896$). When the expression of transcript levels was monitored according to the activity of Vitiligo, we found a significant increase in *NRD1* transcripts in patients with AV in comparison to controls ($p = .047$; Figure 1g). Moreover, SV patients revealed no difference in *NRD1* transcripts compared with controls ($p = .659$). Also, no change was observed between patients with AV vs SV ($p = .912$).

## Analysis of *VTCN1* and *NRD1* transcript levels from the skin

To determine the status of *VTCN1* transcripts in the skin and its involvement in vitiligo disease, we compared *VTCN1* transcript levels from 12 lesional and non-lesional skin of patients with Vitiligo to 24 healthy skin samples. Interestingly, it revealed a significant increase in *VTCN1* transcripts in lesional skin of patients compared with control skin ($p = .039$; Figure 1d). The fold change analysis divulged a 2.70-fold higher expression of

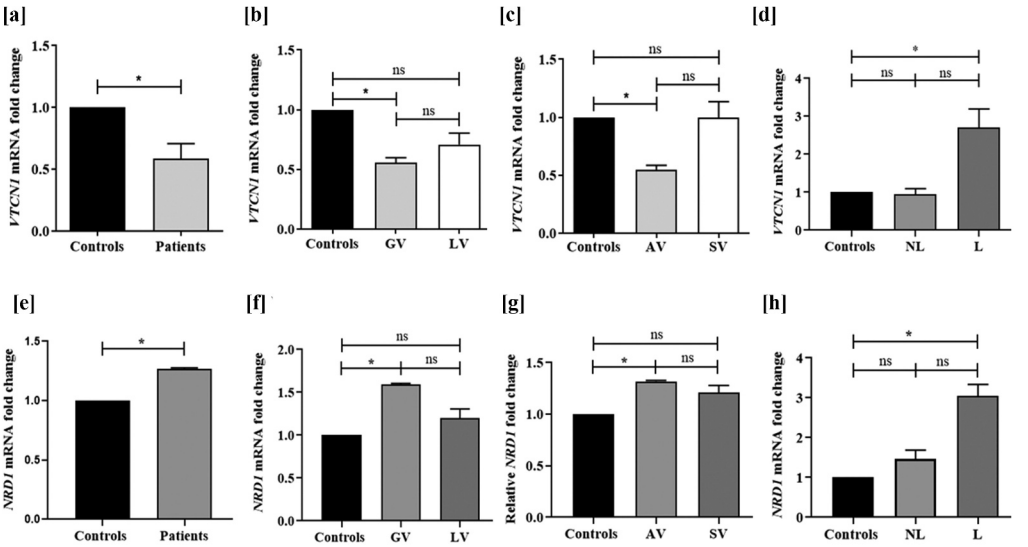


**Figure 1.** *VTCN1* and *NRD1* transcript levels in PBMCs and skin of patients with Vitiligo and controls. (a) VTCN1 expression in patients with Vitiligo (n = 188) and controls (201) were analyzed. Patients divulged a remarkable decrease in *VTCN1* transcripts with reference to controls (mean $\Delta C_P \pm$ SEM 4.883 ± 0.2408 vs. 4.082 ± 0.2530; $p$ = .0227). 0.42 -fold decrease was observed in vitiligo patients as calculated by the fold change ($2^{-\Delta\Delta cp}$) method. (b) *VTCN1* expression from 201 controls, 160 GV patients, and 28 LV patients was analyzed. Patients with GV disclosed a notable decrease in *VTCN1* transcript levels in comparison with controls ($p$ = .034). (c) Expression of *VTCN1* transcript levels in 201 controls and 161 AV and 27 SV patients was analyzed. Patients with AV revealed a significant decrease in *VTCN1* transcripts with reference to controls ($p$ = .037). (d) Expression of *VTCN1* transcript levels in 24 controls and 12 non-lesional and 12 lesional skin samples of vitiligo patients were analyzed. The lesional skin divulged a remarkable increase in *VTCN1* transcripts in comparison with control skin ($p$ = .039). Expression of *VTCN1* transcripts in lesional skin of patients in comparison with controls showed a 2.70 -fold increase as calculated by the fold change ($2^{-\Delta\Delta cp}$) method. (e) *NRD1* expression in vitiligo patients (n = 175) and controls (n = 190) was analyzed. Patients disclosed a remarkable increase in *NRD1* transcripts with reference to controls (mean $\Delta C_P \pm$ SEM 3.800 ± 0.1047 vs. 4.148 ± 0.1279; $p$ = .034). 1.26 -fold increase was observed in patients with vitiligo as calculated by the fold change ($2^{-\Delta\Delta cp}$) method. (f) *NRD1* transcript levels in 190 controls,150 GV, and 25 LV patients were analyzed. Patients with GV disclosed a notable increase in *NRD1* transcripts in comparison with controls ($p$ = .049). (g) *NRD1* transcripts analysis from 190 controls and 149 patients with AV, and 26 patients with SV were analyzed. Patients with AV revealed significantly increased *NRD1* transcripts with reference to controls ($p$ = .047). (h) Expression of *NRD1* transcript levels in 23 controls and 12 non-lesional and 12 lesional skin of vitiligo patients was analyzed. The lesional skin divulged a remarkable increase in *NRD1* transcripts in comparison with controls skin ($p$ = .021). Expression of *NRD1* transcripts in lesional skin of patients in comparison with controls showed a 3.05 -fold increase as calculated by the fold change ($2^{-\Delta\Delta cp}$) method.

*VTCN1* transcripts in lesional skin compared with healthy skin, but no change was seen in non-lesional skin of patients compared with control skin ($p$ = .903). Also, no change in transcript levels was seen between patients' lesional skin vs non-lesional skin ($p$ = .155).

Analysis of *NRD1* transcript levels from the same skin biopsies of 12 vitiligo patients and 24 controls found a notable increase in *NRD1* mRNA levels in lesional skin of patients in comparison to control skin ($p$ = .021; Figure 1h). The $2^{-\Delta\Delta Cp}$ analysis showed a 3.05-fold higher expression of *NRD1* transcripts in lesional skin with respect to control skin.

However, the non-lesional skin of patients divulged no remarkable changes in transcripts compared with control skin ($p = .664$). Also, no remarkable change was observed between patients' lesional skin vs. non-lesional skin ($p = .265$).

### *Analysis of plasma sVTCN1 and NRD1 levels*

To assess sVTCN1 levels in Vitiligo, we have analyzed plasma of 84 patients with Vitiligo and 81 healthy individuals. Surprisingly, a remarkable increase in sVTCN1 was noticed in patients with Vitiligo with respect to controls (Mean±SEM: 50.21 ± 3.80 ng/ml vs 39.22 ± 3.04 ng/ml, respectively; $p = .026$; Figure 2a). Interestingly, dividing the patient population according to the type and activity of Vitiligo revealed significantly higher sVTCN1 in patients with GV and AV compared with controls ($p = .030$ and $p = .041$, respectively; Figure 2b and 2c), but there were no remarkable changes observed in patients with LV and SV ($p = .856$ and $p = .987$, respectively). Also, no change in sVTCN1 levels was noticed between GV vs LV and AV vs SV patients ($p = .235$ and $p = .389$, respectively). Further,

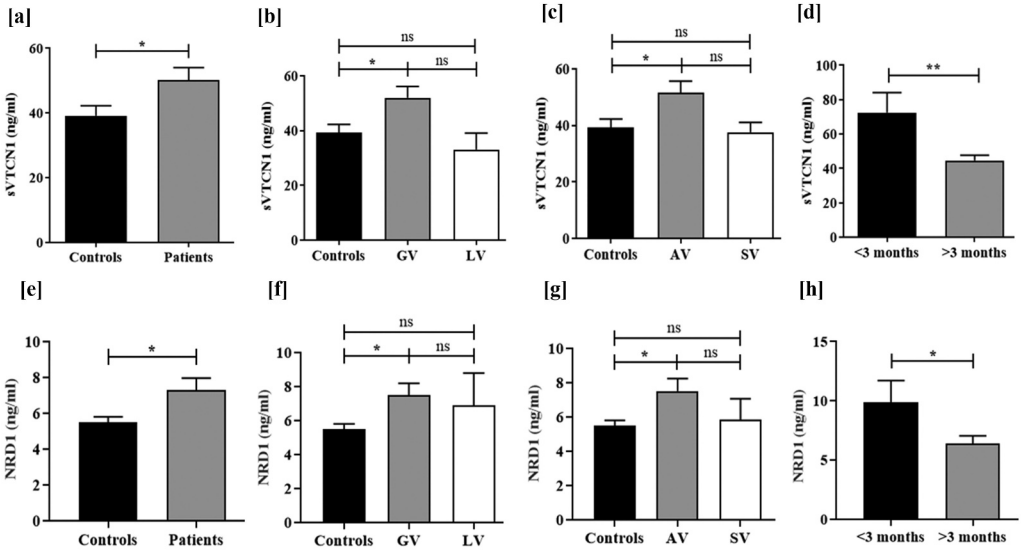


**Figure 2.** Estimation of sVTCN1 and NRD1 levels in the plasma of patients with vitiligo and controls. (a) Comparison of sVTCN1 (ng/ml) from the plasma of 84 patients with vitiligo and 81 healthy individuals showed a significantly higher sVTCN1 in patients compared with controls ($p = .026$). (b) sVTCN1 levels were considerably high in patients with GV in comparison to healthy individuals ($p = .030$) (Control n = 81, GV = 76, LV = 8). (c) Patients with AV showed remarkably increased sVTCN1 levels with reference to controls ($p = .041$) (Controls n = 81, AV = 74, SV = 10). (d) Patients with disease duration <3 months divulged remarkably high sVTCN1 compared to patients with disease duration >3 months ($p = .002$) (<3 months n = 17, >3 months n = 67). (e) Comparison of NRD1 (ng/ml) from the plasma of 82 patients with Vitiligo and 81 healthy individuals showed a significantly higher NRD1 in patients compared with controls ($p = .015$). (f) NRD1 levels were considerably high in patients with GV in comparison to healthy individuals ($p = .035$) (Control n = 81, GV = 75, LV = 7). (g) Patients with AV divulged remarkably higher *NRD1* levels with reference to controls ($p = .029$) (Controls n = 81, AV = 72, SV = 10). (h) Patients with disease duration <3 months divulged remarkably high NRD1 compared to patients with disease duration >3 months ($p = .030$) (<3 months n = 17, >3 months n = 65).

when sVTCN1 levels were estimated according to the duration of disease, we found notably higher sVTCN1 levels in patients with early onset of disease duration (<3 months) ($p = .002$; Figure 2d) in comparison to patients with disease duration of more than three months.

NRD1 levels from the plasma of the same vitiligo patients and healthy individuals revealed a remarkable increase in NRD1 levels in patients compared to controls (Mean ± SEM: 7.30 ± 0.66 ng/ml vs 5.50 ± 0.29 ng/ml, respectively; $p = .015$; Figure 2e). Also, dividing the patient population according to the type and activity of Vitiligo revealed a significant increase in NRD1 levels in patients with GV and AV in comparison with controls ($p = .027$ and $p = .023$, respectively; Figure 2f and 2g), but there was no significant difference found in patients with LV and SV ($p = .735$ and $p = .975$, respectively). Moreover, no difference was observed in NRD1 levels between GV vs LV and AV vs SV ($p = .949$ and $p = .538$, respectively). Further, when NRD1 levels were analyzed based on the duration of disease, it was found to be significantly higher in patients with disease duration <3 months ($p = .030$; Figure 2h) as compared to patients with disease duration >3 months.

### *Correlation of sVTCN1 with NRD1 from the patients with vitiligo*

Correlation analysis of sVTCN1 with NRD1 in 82 patients with Vitiligo was done. Interestingly, it revealed a positive correlation between elevated sVTCN1 and increased NRD1 protein levels from the plasma of patients with Vitiligo (n = 82, r = 0.59, $p = .00029$, Figure 3)

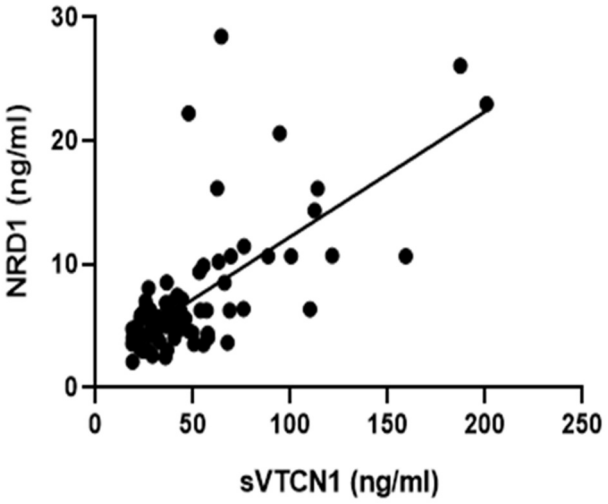


**Figure 3.** Correlation analysis of sVTCN1 with NRD1 levels in vitiligo individuals. Spearman correlation analysis of sVTCN1 with NRD1 levels in 82 vitiligo patients was analyzed. Increased sVTCN1 levels were positively correlated with increased NRD1 levels in the same vitiligo patients (r = 0.59, $p = .00029$).

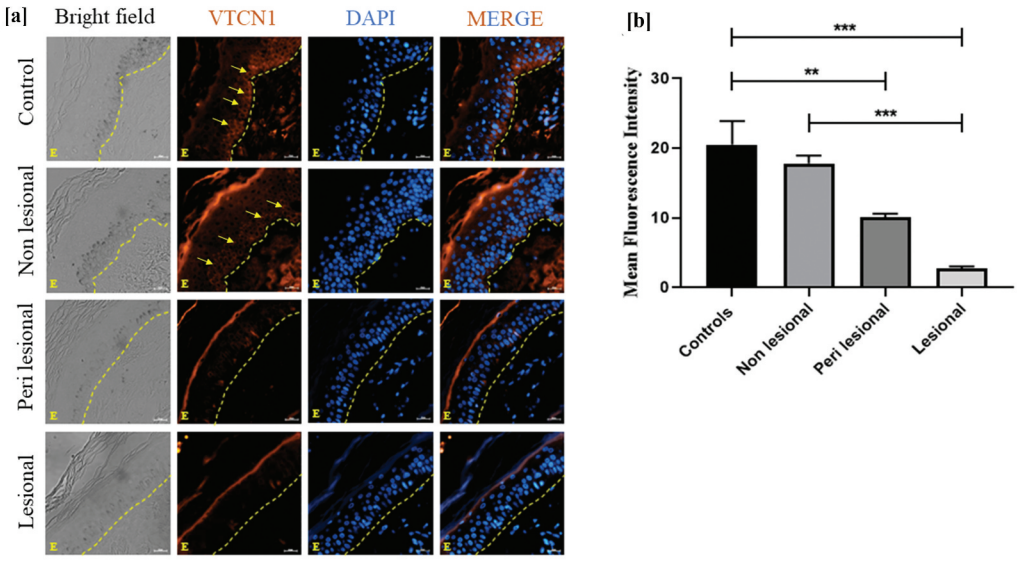


**Figure 4.** Immunofluorescence analysis of control skin, non-lesional, perilesional, and lesional skin of vitiligo patients. (a) the expression of VTCN1 was observed in the epidermal basal layer of control skin and non-lesional skin of vitiligo patients. VTCN1 expression is shown with yellow arrows. A dashed yellow color line distinguishes the basal layer. Nuclei were stained blue by DAPI. (E: Epidermis, Magnification: 60X, Scale bar: 20 µm). (b) the expression of VTCN1 was significantly lowered in lesional and perilesional skin of vitiligo patients in comparison with control skin ($p = .00025$, $p = .0036$) (Control n = 13, Non-lesional skin n = 12, Perilesional skin n = 12, and Lesional skin n = 12).

### *Decreased VTCN1 protein expression in vitiliginous skin*

To inspect the status of VTCN1 protein expression in vitiliginous skin, we analyzed 13 controls and 12 non-lesional, perilesional and lesional skin patients with Vitiligo by immunofluorescence analysis. Our results showed that VTCN1 expression was seen in cells located at the basal layer of the epidermis of healthy and non-lesional skin (Figure 4a). A remarkable decrease in VTCN1 protein levels was seen in lesional and perilesional vitiliginous skin compared to control skin ($p = .00025$, $p = .0036$; Figure 4b). Also, a significant decrease was noticed in the lesional skin in comparison with non-lesional skin ($p = .0002$; Figure 4b). However, no difference was detected in the VTCN1 expression between healthy and non-lesional skin as well as non-lesional and perilesional skin.

## Discussion

Vitiligo has a well-established link to other autoimmune diseases such as Addison's disease, Hashimoto's thyroiditis, Alopecia areata, etc. (Akay et al. 2010; Kemp et al. 2001). Infiltration of immune cells in the vitiliginous skin, decreased CD4+/CD8+ T cell ratio, decreased Treg cells in the blood of vitiligo patients, disrupted systemic and local cytokine balance, increased oxidative stress, and endoplasmic reticulum stress, involvement of tissue-resident memory T cells in the depigmentation, and several other studies have highlighted

autoimmunity as a key etiopathological factor in vitiligo pathogenesis (Dwivedi et al. 2013; Jadeja et al. 2021; Le Poole et al. 1996; Shah et al. 2021; Singh et al. 2019). Various costimulatory molecules such as CTLA4, PD-L1, CD80, etc., are essential for regulating *T*-cell activation (Dwivedi et al. 2011; Wang and Chen 2004). Various findings pointed out that dysregulated co-stimulation results in the initiation and maintenance of autoimmunity by activating the autoreactive *T*-cells (Kobata et al. 2000). VTCN1 is a negative costimulatory molecule that interacts with an unidentified receptor on T cells and inhibits its activation and proliferation. VTCN1 has been reported in various autoimmune diseases such as RA, T1D, and SLE (Azuma et al. 2009; Radichev et al. 2014; Xiao et al. 2019). The observation supported the immune regulatory function of VTCN1 that in the mouse model, VTCN1 deficiency worsens the pathophysiology of SLE (Xiao et al. 2017). Based on these studies, we explored VTCN1 in vitiligo pathogenesis.

A GWAS study revealed that polymorphisms mapped in the *VTCN1* gene were associated with JIA, and upon fine-mapping nominal association of 10 SNPs with JIA ($p < .05$) was revealed. Our selected SNPs rs10923223 and rs12046117 showed a strong association with JIA and moderate linkage disequilibrium (Hinks et al. 2009; Ramos et al. 2011). Association of the same SNPs was also reported in 272 Caucasian JIA patients (Albers et al. 2014). However, no association of these two SNPs was reported with RA in the Dutch population and with JIA in the Nordic and Northwest European population (Alberdi-Saugstrup et al. 2017; Daha et al. 1987; Reinards et al. 2015). Interestingly, genotyping of intronic *VTCN1* rs10923223 and rs12046117 SNPs in Gujarat vitiligo patients in our present study revealed no significant association of these polymorphisms with vitiligo susceptibility. The bioinformatics analysis also supported that both SNPs do not have significant functional consequences on gene expression. It is possible that other SNPs may be present in the *VTCN1* gene that might influence the gene expression.

Seven transcript variants are reported for VTCN1 gene, from which two, variant 4 and 5 are non-coding resulting in nonsense-mediated mRNA decay (NMD). From remaining five, Isoform 1 acts as precursor having 849 nucleotides that encodes 282 amino acids containing functional VTCN1 protein (Sica et al. 2003). Early reports in human and mice showed *VTCN1* mRNA expression in various organs, namely the brain, pancreas, heart, placenta, kidney, skin, liver, lung, spleen, etc. (Choi et al. 2003; Prasad et al. 2003; Sica et al. 2003). According to Choi *et al.*, and Sica *et al.*, most of the human tissue expressing two different VTCN1 transcripts having identical ORF and expression of these transcripts is not tissue specific (Choi et al. 2003; Sica et al. 2003). Further, cytokines are reported to alter VTCN1 expression (Kryczek et al. 2006). Our results suggested that *VTCN1* mRNA was notably reduced in PBMCs of patients with vitiligo, while there is a remarkable increase in *VTCN1* transcripts from the lesional skin of vitiligo patients. This dichotomy between transcript levels in skin and PBMCs might be due to the difference in local and systemic microenvironment expressing different levels of cytokines, which was well reported in vitiligo (Imran et al. 2012; Kryczek et al. 2006; Le Poole et al. 1996; Singh et al. 2019). Moreover, augmentation of *VTCN1* transcripts was also reported in islets of prediabetic NOD mice as a compensatory mechanism for the heavy loss of membrane-tethered VTCN1 (Radichev et al. 2014). Vitiligo and T1D are also considered organ-specific autoimmune diseases (Harris 2016). It may be one of the reasons for increased *VTCN1* transcripts in vitiliginous skin. We have also investigated the status of VTCN1 cleaving enzyme *NRD1* transcripts from blood and skin of vitiligo patients and found a remarkable increase. In support of our data, increased *NRD1* transcripts were also reported from PBMCs of RA patients using

transcriptome analysis (Teixeira et al. 2009). Reports pointed out the involvement of NRD1 in the regulating immune response via processing antigenic self-peptides (Kessler et al. 2011).

NRD1 cleaves membrane-tethered VTCN1, which enters the circulation as sVTCN1 (Radichev et al. 2016). Hence, we have also assessed sVTCN1 and NRD1 levels from sera of the vitiligo patients and found a significant increase. Furthermore, we found a positive correlation between sVTCN1 and NRD1 in the same vitiligo patients. Several studies reported raised soluble VTCN1 in sera of RA, type1 diabetes, and lupus nephritis patients and its positive association with disease severity (Azuma et al. 2009; Radichev et al. 2014; Xiao et al. 2019). To investigate the involvement of VTCN1 and NRD1 in the early stages of Vitiligo, we divided the patient population according to the disease duration: early-onset (<3 months) and longer duration of the disease (>3 months). Interestingly, elevated sVTCN1 and NRD1 were observed in early-onset patients compared to longer duration. Our observations indicated that increased *NRD1* transcripts in PBMCs and skin along with increased plasma NRD1 protein levels might lead to the cleavage of membrane-bound VTCN1 and ultimately result in increased sVTCN1 in vitiligo patients.

Previous reports suggested the restricted distribution of VTCN1 protein in both mice and humans, principally expressed in epithelial tissues of the lung, trachea, gynecologic tract (ovary, uterus), kidney, pancreas, breast, etc. (Hofmeyer et al. 2012; Lee et al. 2012). In addition, cytoplasmic VTCN1 was reported in the murine epidermal cells of haired skin (Smith et al. 2016). To determine the status of VTCN1 protein in the vitiligo-affected area,

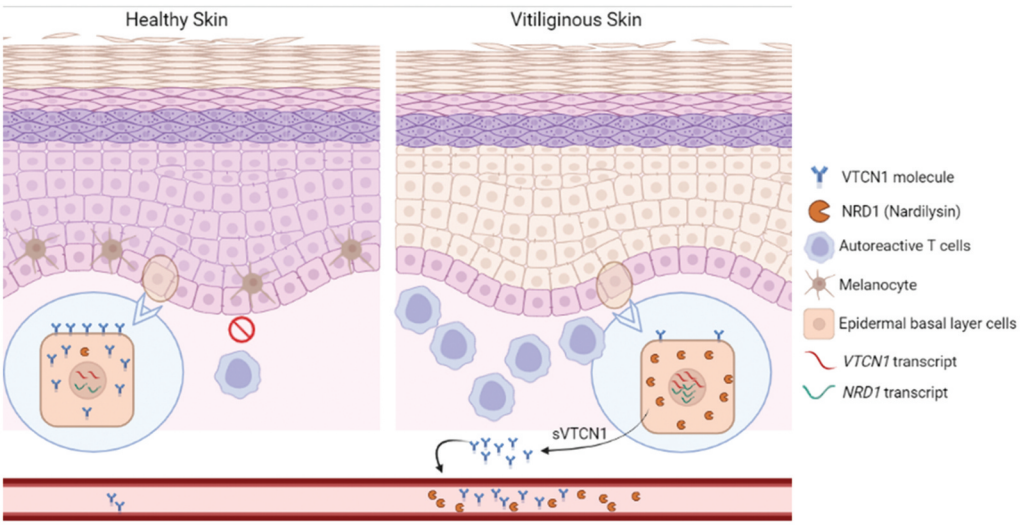


**Figure 5.** VTCN1 in vitiligo pathogenesis. The present study showed significantly higher sVTCN1 levels as well as its cleaving enzyme NRD1 levels in the blood of vitiligo patients. Moreover, we found a positive correlation between sVTCN1 and NRD1 from the same vitiligo patients suggesting a possible role of NRD1 in VTCN1 shedding from the membrane. Shedding of the negative costimulatory molecule VTCN1 might compromise T cell inhibition, resulting in an exaggerated T cell response against melanocytes of vitiligo. In line with that, we also observed decreased VTCN1 protein in the lesional vitiliginous skin. Decreased VTCN1 protein expression indicates the breakdown in tolerance, resulting in an exacerbated response of T cells to melanocytes that ultimately leads to melanocyte destruction and vitiligo progression.

we inspected its expression from the vitiliginous skin. Our study revealed VTCN1 expression in the epidermal basal layer cells in healthy individuals, while minimal expression of VTCN1 protein in lesional skin of vitiligo patients (Figure 2a). In support of our data, reduced VTCN1 expression was reported in salivary glands of patients with primary Sjogren's syndrome (pSS), indicating that deficiency of VTCN1 protein in the affected organ might lead to an aggravated response of autoreactive T cells and contribute to the disease development and progression (Yu et al. 2012).

In summary (Figure 5), our findings indicate that NRD1-mediated shedding of membrane-tethered VTCN1 from vitiliginous skin might result in higher sVTCN1 levels in the blood of vitiligo patients. Shedding of this negative costimulatory molecule VTCN1, might compromise T cell inhibition, resulting in an aggravated T cell response against melanocytes in Vitiligo. In line with that, we also observed a decrease in VTCN1 protein levels from the lesional vitiliginous skin. The findings of this study provide new insight into immune dysregulation reported in Vitiligo and may lead to the development of new therapeutic strategies using an immunomodulatory approach to cure Vitiligo.

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## Author contributions

RB and MS formulated the idea. JV, SJ, and RB designed the experiments. JV performed the experiments. FK and MY helped with experiments and data collection. JV and SJ analyzed the data. JV has written the original draft. SJ reviewed and edited the draft. RB helped with critical revision and approval of the manuscript. All the authors have read and agreed to the final version of the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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# V-set domain containing T-cell activation inhibitor-1 (VTCN1): A potential target for the treatment of autoimmune diseases

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ABSTRACT

Autoimmunity eventuates when the immune system attacks self-molecules as a result of the breakdown in immune tolerance. Targeting autoimmune diseases via immunomodulation has become an essential strategy in today's era. A B7 superfamily member immune checkpoint, the V-set domain containing T-cell activation inhibitor-1 (VTCN1), also known as B7-H4, B7S1, and B7x, is involved in negatively regulating T-cell activation. *VTCN1* transcript has been reported in various lymphoid and non-lymphoid tissues, but its protein expression is restricted, indicating its translational regulation. Dysregulation of VTCN1 has resulted in the exacerbation of various autoimmune diseases. Moreover, increased soluble form of VTCN1 in the patient's sera positively correlates with the disease progression and severity. The current review summarizes all the reports till date, unfolding the role of VTCN1 in various autoimmune diseases and its therapeutic potential.

## 1. Introduction

Over millions of years, the human immune system has evolved into an astounding defense mechanism offering protection against diseases or other potentially harmful invaders in the body. The protection is facilitated by both innate immunity and adaptive immunity. Innate immunity is a generalized, non-specific type of protection involving skin barrier, mucosa layers, and other chemical secretions. On the other hand, adaptive immunity imparts specificity by humoral and cell-mediated immune responses. The humoral immune system can recognize and neutralize foreign antigens by generating specific antibodies. In contrast, cell-mediated immunity can neutralize antigens by activating antigen-presenting cells (APCs), NK cells, $CD4^+$ and $CD8^+$ lymphocytes. Additionally, a variety of cytokines are involved in activating other immune cells to participate in immune response (Owen et al., 2013).

Dysregulation of this multifactorial process involving innate and adaptive immune systems leads the body to attack its own cells. This condition is known as autoimmunity. Usually, immune cells are tolerant to self-tissues; however, this self-tolerance is lost in the autoimmune condition (Dong et al., 2003). This condition activates self-reactive T or B cells, which suffer the loss of ability to recognize self-antigens by distinct mechanisms (Podojil et al., 2017; Waldner et al., 2009). Current research in autoimmune disease focuses on the development of new therapies that specifically target T-cell receptors (TCR) and/or co-stimulatory molecules to diminish the deleterious effect of the inflammatory immune response. Recently, Abatacept (CTLA-4–Ig human fusion protein) has been demonstrated as an effective treatment for different types of psoriatic arthritis (Noisette et al., 2018). Therefore, it is sensible to develop immune-modulatory therapies that would also be functional in the non-responder patient population. The 2018 Nobel Prize for Physiology or Medicine was awarded in recognition of these co-stimulatory molecules in cancer immunotherapy. This revolutionary concept has prompted the interest in immune-modulatory molecules relevant to various human diseases.

The current review focuses on the biological activity of one such immune modulatory molecule named V-set domain-containing T-cell activation inhibitor 1 (VTCN1, B7-H4, B7x, or B7S1) and its role in autoimmune diseases. Previous studies on this molecule focused on cancer immunity, but its role in autoimmune diseases was overlooked. So, this review comprises the summation of dysregulated VTCN1 in all reported autoimmune diseases. In addition, it discusses the clinical significance of VTCN1 as a therapeutic target for autoimmune diseases.

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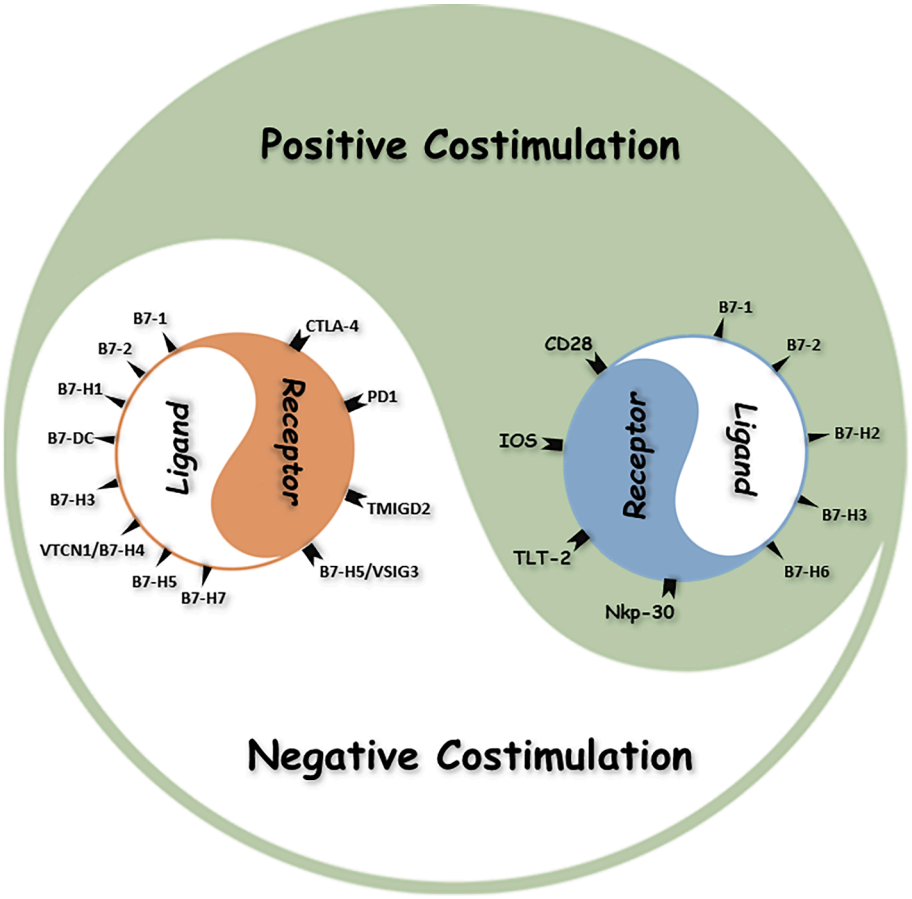


**Fig. 1.** B7 Family members (Receptors and Ligands) and Type of Co-stimulation.

## 2. T cell activation and B7 family proteins

Complete activation of Naïve T cells is achieved by two distinct signals from antigen-presenting cells (APCs): The first signal is triggered by the interaction of major histocompatibility complex (MHC)/ antigen on APCs with the antigen-specific T cell receptor (TCR) present on T cells. At the same time, the second signal (non-specific) is delivered by the interaction between co-stimulatory molecules present on the surface of the APCs and their corresponding receptors on T cells (Bretscher and Cohn, 1970). This co-stimulation is of two types, positive co-stimulation that promotes T-cell activation, proliferation, and its differentiation into effector T cells, while negative co-stimulation or co-inhibition leads to inhibition of T-cell activation via inactivating intrinsic signaling and transcriptional programs and thereby promoting the tolerance (Wang

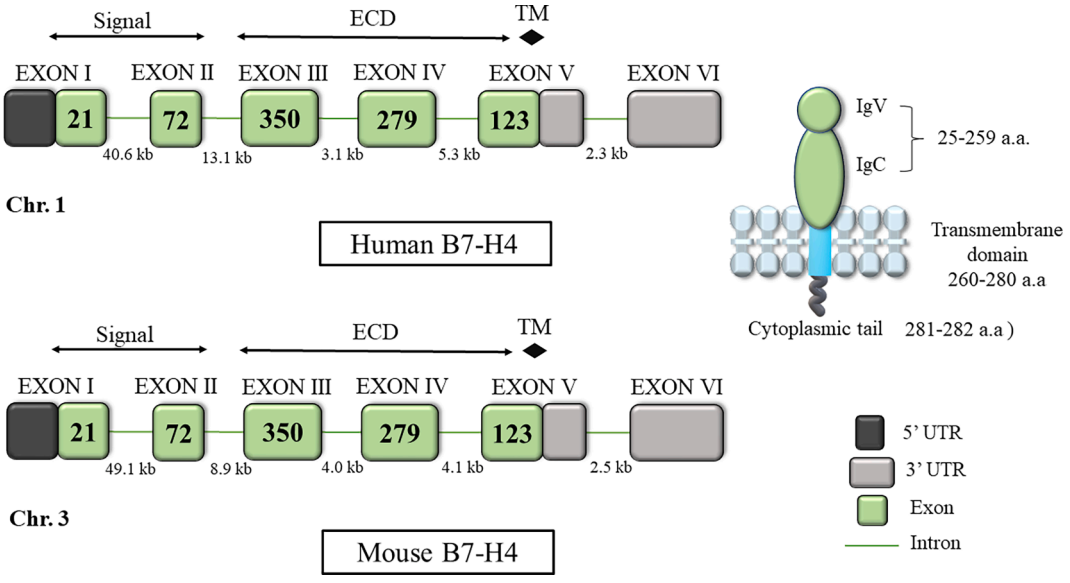


**Fig. 2.** Genomic and protein structure of VTCN1.

et al., 2004; Mcgrath et al., 2012). Immunological tolerance is the unresponsiveness of the immune system to self-antigens, which is of two types: central tolerance and peripheral tolerance. Exaggerated co-stimulation and/ or insufficient co-inhibition bring abnormal T cell activation that results in the breakdown of self-tolerance via activating and expanding autoreactive T cells. Likewise, other immune cells also require two signals for activation, maturation, and function. Thus, co-stimulatory and co-inhibitory molecules play a central role in shaping the immune response (Zhang and Vignali, 2016). This two-signal hypothesis laid the foundation for the development of many potential therapeutics for the treatment of autoimmune diseases. The B7/CD28 superfamily members serve as crucial co-stimulatory molecules. The B7 family ligands (present on APCs) bind to its counter receptor from the CD28 family (present on the T cell), which play a central role in fine-tuning the antigen-specific immune response.

The B7 family includes total eleven known members till date: B7-1 (CD80), B7-2 (CD86), B7-H1 (PD-L1, CD279), B7-DC (PD-L2, CD273), B7-H2 (ICOS, CD275), B7-H3 (CD276), B7-H4 (VTCN1), B7-H5 (VISTA), BTLN2 (BTL-II/BTN7), B7-H6 (NCR3LG1) and B7-H7 (HHLA-2) (Ceeraz et al., 2013; Zhao et al., 2020). All B7 family members are membrane-anchored proteins with extracellular immunoglobulin (Ig) like domains important for binding with their receptors of the CD28 family. All the B7 family members, their respective receptors, and the type of co-stimulation exerted by them are summarized in Fig. 1.

## 3. VTCN1: Genomic organization and protein structure

VTCN1 was identified by using DNA homology search in human and mouse EST (Expressed Sequence Tags) database with known B7 family members in 2003 by three separate laboratories and designated three different names: B7-H4 (B7 homolog 4), B7-S1 (B7 superfamily member 1), and B7x, respectively (Sica et al., 2003a; Prasad et al., 2003; Zang et al., 2003). Its gene name is *V-set domain containing T cell activation inhibitor 1 (VTCN1)* in literature, but all these terms indicate the same protein. The human *VTCN1* gene is located on 1p11.1 consisting of six exons (with only 849 bp) and five introns. Mature VTCN1 protein consists of one signal peptide, two extracellular immunoglobulins (IgV1 and IgV2) domains, one transmembrane domain, and a small cytoplasmic tail. Exon I and II encodes a signal peptide of the VTCN1 protein, and the extracellular portion is encoded by exon III, IV, and V. The transmembrane and intracellular portions encoded by exon V are mentioned in Fig. 2. A pseudogene of *VTCN1* is also reported on chromosome 20p11.1. It has been suggested to produce the truncated VTCN1 protein, but the exact function of this pseudogene remains to be elucidated (Choi et al., 2003).

Human VTCN1 protein belongs to the immunoglobulin superfamily showing structural similarity to other B7 family members. It consists of 282 amino acids comprising of N- terminal signal peptide (1–24 a.a), IgV1, and IgV2 containing an extracellular domain (25–259 amino acids), a hydrophobic transmembrane domain (260–280 amino acids), and a very short cytoplasmic tail (281–282 amino acids). It is a type-1 transmembrane protein. Initially, it was reported that VTCN1 is a GPI-linked protein (Prasad et al., 2003), but further experiments by Choi et al. (2003) did not reveal any GPI linkage. VTCN1 showed 25 % homology with other B7 family members and is evolutionarily conserved in lower vertebrates, including bony fish (Hansen et al., 2009). Human *VTCN1* has 87 % amino acid identity with mouse *VTCN1*; however, murine *VTCN1* encodes 283 amino acids containing protein while human *VTCN1* encodes 282 amino acids containing protein (Sica et al., 2003a). A recent report also found a functional nuclear localization sequence in human cells *in vitro* that is necessary to shuttle VTCN1 protein between the nucleus and cytoplasm (Zhang et al., 2013). Crystal structure of human VTCN1 containing IgV1 domain along with interacting ligands such as Beta-D-Mannose (BMA), *N*-acetyl glucosamine (NAG), and alpha-D-Mannose (MAN) has been reported (Jeon et al., 2014). No reports affirm the crystal structure of the whole extracellular domain of human VTCN1 protein, which includes both IgV1 and IgV2-like sub-domains in it. Hence, we sought to understand the structure of the extracellular domain of VTCN1 to enlighten upcoming studies on its interaction with ligands and receptors. The human VTCN1 sequence shares 100 % identity with the crystal structure of the human B7-H4 IgV-like domain (PDB id: 4GOS), as the structure covers only the IgV1 domain (amino acid residues 30–148) of human VTCN1 (Jeon et al., 2014). We predicted the human VTCN1 structure using SWISS-MODEL's 'user template' to perform homology modeling, which is shown in Fig. 3. The structure has 98.2 % amino acids residues in the favoured region of the Ramachandran plot, and it has also gained a 93.3 quality score from ERRAT, which makes this structure's validation significant.

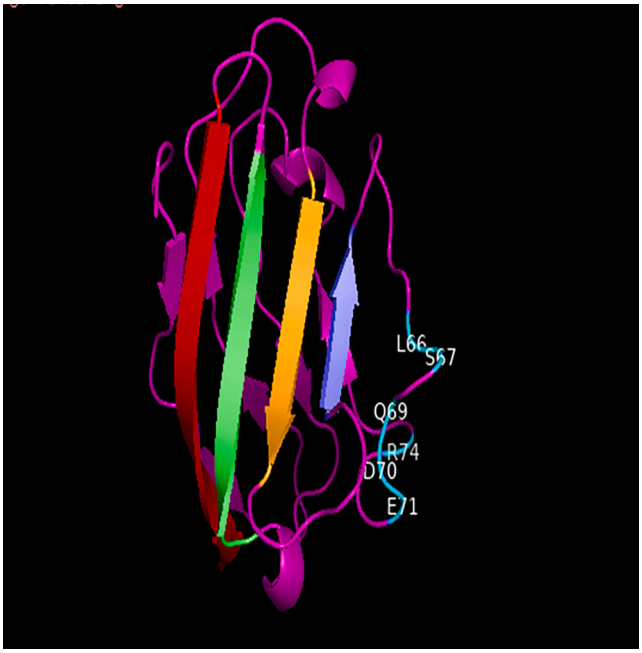


**Fig. 3.** Human VTCN1 structure predicted by SWISS-model software. The ribbon representation of human VTCN1 extracellular domain structure consisting of IgV1 and IgV2 domains (aa 25–259) predicted by SWISS-model software. Front beta-sheets are colored (red; light green; light orange; light blue), and the hot spot amino acid residues highlighted in cyan are LEU 66, SER 67, GLN 69, ASP 70, GLU 71, ARG 74, and the hot spot residues (Tian et al., 2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 4. The physiological function of VTCN1

*VTCN1* transcripts have been reported in various human lymphoid and non-lymphoid tissues, including the placenta, kidney, liver, spleen, ovary, testis, etc. (Sica et al., 2003a; Prasad et al., 2003; Zang et al., 2003). The immunohistochemistry (IHC) analyses of various healthy tissues showing positive *VTCN1* transcript did not reveal the expression of VTCN1 protein in muscle and intestine (Smith et al., 2016), suggesting tight regulation of VTCN1 protein expression at the transcription and translational levels (Choi et al., 2003). IHC data also showed VTCN1 expression mostly in epithelial cells of the lung, kidney, pancreas, haired skin, breast, and ovary (Hofmeyer et al., 2012; Lee et al., 2012; Smith et al., 2016). Its expression was also reported in human bone marrow-derived mesenchymal stem cells (hBMSCs) (Xue et al., 2010). VTCN1 is found as both intracellular and soluble VTCN1 (sVTCN1) forms. Though the exact function of these forms is not well explored in detail; elevated sVTCN1 levels were reported in various autoimmune diseases (Table 2). Radichev et al. (2014) reported that the structure of VTCN1 contains a cleavage site for metalloproteinase Nardilysin. They further confirmed that Nardilysin-mediated membrane-bound VTCN1

**Table 1**
VTCN1 protein expression in autoimmune diseases.

| Sr. No | Autoimmune disease | Organ/Cells | VTCN1 protein expression | Technique | Reference |
|---|---|---|---|---|---|
| 1 | Type 1 Diabetes | Pancreas | Decrease in VTCN1 levels in Langerhans' islets of diabetes-susceptible NOD | Immunofluorescence Staining of pancreatic section | Radicheva et al., 2016 |
| | | Pancreas | Decrease in VTCN1 levels in islets of T1D patients | Immunofluorescence Staining of pancreatic section | Radichev et al., 2016 |
| | | Macrophages | Decrease in surface localized VTCN1 in prediabetic NOD macrophages | Immunofluorescence analysis | Radichev et al., 2014 |
| | | PBMC derived macrophages | Decrease in surface localized VTCN1 in T1D patients | Immunofluorescence analysis | Radichev et al., 2014 |
| 2 | Primary Biliary Cirrhosis | Primary bile duct epithelial cells | Bile ducts within portal area was found positively stained for VTCN1 in sections from all PBC patients | Immunohistochemistry | Chen et al., 2011 |
| | | Human primary BEC cell lines (SS, HR, and 4.22.07) from PBC patients | B7-H4 in cytoplasm and membrane in primary BECs by FACs | Flow cytometry | Chen et al., 2011 |
| 3 | Rheumatoid arthritis | Synovium tissues | Weak staining of VTCN1 found on the membrane and in the cytoplasm of synoviocytes and $CD19^+$ B cells in rheumatoid synovium tissues | Immunofluorescence staining and confocal scanning | Chen et al., 2013 |
| | | PBMCs | Positive B7-H4 expression was found in $CD19^+$ B cells and $CD14^+$ monocytes | Flow cytometry | Chen et al., 2013 |
| 4 | Lupus Nephritis | Podocytes and Tubular cells | VTCN1 expression found up regulated upon stimulation of LPS or TNF-α + IFN-γ | Western blot, Flow cytometry | Pawar et al., 2015 |
| 5 | Primary Sjögren's syndrome (pSS) | Salivary gland epithelial cells (SGECs) of tubular epithelium | Lower expression of VTCN1 on SGECs in pSS patients | Immunohistological staining | Yu et al., 2012 |
| 6 | Vitiligo | Basal layer cells of Epidermis | Decreased expression of VTCN1 on basal layer epithelial cells | Immunofluorescence staining | Vaishnav et al., 2022 |

(mVTCN1) cleavage results in plasma sVTCN1in T1DM patients. Flow cytometric analysis revealed that VTCN1 is expressed on T-cells, B-cells, monocytes, and dendritic cells (DCs) upon stimulation (Sica et al., 2003a), while Wei et al. (2011) did not find VTCN1 expression on murine or human immune cells. However, the reports on VTCN1 expression on various immune cells were contradictory and needed further investigation. One study revealed higher VTCN1 expression in BDCA-$1^+$ and BDCA-$2^+$ DCs in peripheral blood of adults compared to umbilical cord blood (Serafin et al., 2010). Western blot analysis also reported VTCN1 expression in the kidney and placenta of human fetal tissues (Salceda et al., 2005). VTCN1 expression is mediated by STAT3 (Yao et al., 2016) and regulated by several cytokines. As per the report, IL-6 and IL-10 increase the VTCN1 expression (Yao et al., 2016), whereas IL-4 and GM-CSF decrease the expression of VTCN1 (Kryczek et al., 2006).

The general function of VTCN1 is to downregulate immune reactions by inhibiting T cell activation, proliferation, and cytokine production (Prasad et al., 2003; Sica et al., 2003b; Zang et al., 2003). It inhibits T cell proliferation by arresting the cell cycle at G0/G1 phase (Sica et al., 2003b). One FACS-based study revealed VTCN1 mediated reduction in expression of the eleven cytokines as listed: interferon γ (IFN- γ), Tumor necrosis factor α (TNF-α), and interleukin (IL)-5, IL-13, IL-2, IL-9, IL-10, IL-17A, IL-4, IL-21, and IL-22 (Cheng et al., 2018). VTCN1 was also reported to increase the number and activity of regulatory T cells (Tregs) resulting in an immunosuppressive environment (Podojil et al., 2018). Overall, VTCN1 suppresses the pro-inflammatory function of effector T cells. Additionally, VTCN1 also regulates neutrophil-mediated immunity against bacterial infections (Zhu et al., 2016).

## 5. VTCN1 and autoimmune diseases

Previous studies on VTCN1 were mainly focused on the different types of cancers. Increased VTCN1 expression was reported in cancers such as melanoma (Quandt et al., 2011), lung cancer (Zhang et al., 2013), breast cancer (Mugler et al., 2007), gastric cancer (Arigami et al., 2011), pancreatic cancer (Chen et al., 2014), prostate cancer (Zang et al., 2007), etc. Moreover, anti-VTCN1 antibody has demonstrated reduced metastatic capacity in mouse cancer models (Jeon et al., 2014). Nevertheless, dysregulated VTCN1 expression was also reported in different autoimmune diseases; however, its role in autoimmune diseases has been disregarded. Hence, the below sections discuss and summarize the role of VTCN1 in various autoimmune diseases.

### 5.1. VTCN1 in Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic, systemic inflammatory disorder that mainly attacks synovial joints and leads to bone deprivation because of excessive osteoclast activity (Schett and Gravallese, 2012). Dysregulated T cell mediated immune response plays an important role in the pathogenesis of RA (Cope et al., 2008). As a negative co-stimulatory molecule for T cell activation, VTCN1 would be an appealing target molecule to study in this disease. Juvenile Idiopathic Arthritis (JIA) is a common chronic inflammatory rheumatic disease in children. One of the GWAS studies identified novel JIA susceptibility loci that revealed the strongest association between JIA and polymorphisms present in the *VTCN1* gene. Upon fine-mapping the *VTCN1* gene, they found that 10 SNPs present in intron 1, 3, 4, and 3′ UTR showed association with JIA (Hinks et al., 2009). The same finding was also replicated in the Caucasian (Albers et al., 2014) and the Nordic JIA cohorts (Enevold et al., 2017).

In 2009, Azuma et al. reported increased sVTCN1 in RA patients showing a positive correlation with the severity of the disease (Table 2). Further, they confirmed the molecular weight of sVTCN1 to be 50 kDa by performing a western blot from the sera of RA patients. Further, the Collagen Induced Arthritis (CIA) mouse model study revealed that sVTCN1 acts as a decoy molecule, which blocks the normal physiological function of membrane bound VTCN1 and leads to enhanced autoimmune responses and exacerbation of CIA. The study also demonstrated that VTCN1 deficient mice developed much more severe CIA upon CII (Collagen type II) immunization, validating the VTCN1 role in RA development. They constructed VTCN1Ig fusion protein (agonist) to evaluate its therapeutic potential. Interestingly, they found that it significantly suppresses joint swelling and inhibits the infiltration of inflammatory cells, ultimately leading to the suppression of disease progression (Azuma et al., 2009). Though Azuma et al. (2009) established the possible role of VTCN1 in RA; its expression and localization in synovium tissue were unknown till Chen et al. in 2014 revealed VTCN1 positive staining in the cytoplasm and cell membrane of synoviocytes, $CD19^+$ B cells, $CD34^+$ endothelial cells of neovessels, and weak

**Table 2**
sVTCN1 levels in different autoimmune diseases.

| Sr. No | Autoimmune Disease | sVTCN1 level | | Reference |
|---|---|---|---|---|
| | | Patients | Controls | |
| 1 | Type 1 Diabetes | 57.7 ± 14.2 ng/ml | 8.9 ± 0.2.8 ng/ml | Radichev et al., 2014 |
| 2 | Rheumatoid Arthritis | 96.1 ng/ml | <5 ng/ml | Azuma et al., 2009 |
| 3 | Systemic Lupus Erythematosus (SLE) | 0.63 ± 0.26 ng/ml | 0.52 ± 0.11 ng/ml | Xiao et al., 2019 |
| | Lupus Nephritis | 0.66 ± 0.27 ng/ml | 0.52 ± 0.11 ng/ml | |
| 4 | Primary Sjögren's syndrome (pSS) | 49 ± 31 μg/L | 71 ± 27 μg/L | Yu et al., 2012 |
| 5 | Vitiligo | 50.21 ng/ml | 39.22 ng/ml | Vaishnav et al., 2022 |

expression of VTCN1 in infiltrating macrophages (Table 1). Flowcytometric analysis of VTCN1 expression in PBMCs of RA patients also revealed VTCN1 expression on $CD19^+$ B cells and $CD14^+$ monocytes (Chen et al., 2013). These results specify that the local abnormal expression of VTCN1 is involved in the pathogenesis of RA. Collectively, all these reports designate VTCN1 as a potential target for the treatment of RA.

### 5.2. VTCN1 in Type 1 diabetes

Type 1 Diabetes (T1D) is a complex autoimmune disease characterized by the continuous destruction of insulin-producing pancreatic β-cells, which is mediated by autoreactive islet-specific T lymphocytes (Haskins et al., 2011; Leung et al., 2010). So, it would be captivating to explore T cell regulatory VTCN1 molecule in T1D. The role of VTCN1 in T1D was first evaluated by Dawei Ou and his colleagues in 2006. They used VTCN1-Ig and demonstrated inhibition of active $CD4^+$ and $CD8^+$ T cell proliferation, cell cycle arrest, and apoptosis in T1D patients. Moreover, transfection of VTCN1-Ig constructs into human primary islet cells protected them from diabetogenic T cell clones isolated from T1D patients (Ou et al., 2006). Further, Xiaojie Wang and colleagues tried to explore the VTCN1 in allogeneic pancreatic islet transplantation and found inhibition of alloreactive T cell response with prolonged mouse islet allotransplant survival (Wang et al., 2009). Additionally, they assessed the mechanism behind it and found it to be associated with Treg cells. It has been suggested that local expression of VTCN1 also induces unresponsiveness to donor-specific alloantigen (Wang et al., 2012). The journey of exploring VTCN1 in T1D has been taken forward by Joyce Wei and James P. Allision. They detected VTCN1 transcript and protein expression in the pancreatic tissue of mice. To determine the role of VTCN1 in T1D, they administered diabetogenic T cells to mice and found severe disease development in VTCN1 deficient mice compared to control mice. Further, they have also checked whether the overexpression of VTCN1 could delay diabetes development. They found abrogated disease induction and inhibited cytokine production in overexpressed VTCN1 mice (Wei et al., 2011). Further, Lee and his colleagues explored a mechanism of VTCN1Ig mediated inhibition of T1D development. They observed a significant decrease in Th17 cells and associated cytokines in treated NOD (Non-obese diabetic) mice. Upon co-culture of splenocytes of NOD mice with Th17 polarizing cytokines, a substantial reduction in Th17 cells was observed when VTCN1Ig treatment was given, indicating that VTCN1 acts as a potential therapeutic target of autoimmune diabetes (Lee et al., 2013). Early treatment of VTCN1Ig to the NOD mice also reported the reduction of the incidence of T1D with an increasing number of Treg cells in the pancreas (Wang et al., 2011). Despite the growing number of functional studies utilizing genetically manipulated VTCN1 (overexpression and/or deletion) in mouse models, the state of natural VTCN1 on either APCs or islet cells in connection with T1D development in human is largely unknown.

Furthermore, the endogenous pathway of functional VTCN1 inactivation in APCs of NOD mice and T1D patients was recently identified. The study reveals a gradual loss of membrane bound VTCN1 due to a proteolytic cleavage mediated by metalloproteinase Nardilysin (NRD1; an enzyme with both intra and extracellular activities). This results in the release of soluble VTCN1 (sVTCN1) into the periphery, which further triggers hyper-proliferation of diabetogenic T-cells, leading to T1D progression. Moreover, high blood sVTCN1 concentrations in NOD mice and T1D patients were accompanied by almost complete loss of VTCN1 from the APCs' membranes (Table 1, Table 2). This mechanism is linked to T1D susceptibility and depends on two separate but synergistic processes. First is a result of an increased intracellular NRD1 expression, ultimately leading to enhanced intracellular VTCN1 shedding. The second process includes a systemic up-regulation of NRD1 in multiple tissues, which additionally potentiates VTCN1 proteolysis by extracellular NRD1 (Radichev et al., 2016;Radichev et al., 2014). Both recent studies suggest that disrupted co-stimulation mainly via VTCN1 loss in both APCs and pancreatic islets ultimately results in T1D development. Overall, the above-mentioned studies highlight VTCN1 as a potential therapeutic target for T1D.

### 5.3. VTCN1 in Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory autoimmune disease that mainly affects the central nervous system, i.e., the brain and spinal cord (Sospedra and Martin, 2005). It is characterized by Th1 and Th17 $CD4^+$ Tcell mediated responses against epitopes on myelin basic protein (MBP), proteolipid protein (PLP), and/or myelin oligodendrocyte glycoprotein (MOG), leading to the progressive destruction of the myelin sheath surrounding exons (Bielekova et al., 2004; Iglesias et al., 2001). VTCN1 negatively regulates T cell response, making it fascinating to explore its function in MS. Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated MS model widely used for characterizing tolerance-based immunotherapies and their underlying mechanisms (Podojil et al., 2013). Initially, Prasad et al. reported accelerated and robust EAE in mice with greater $CD4^+$ T cells, $CD8^+$ T cells, and $CD11b^+$ macrophages infiltration in the brain upon treatment of VTCN1 blocking antibody (Prasad et al., 2003). This study pointed out the alleged role of VTCN1 in MS pathogenesis for the first time. In 2011, Wei et al. demonstrated that VTCN1 deficient mice developed exacerbated EAE upon treatment of MOG peptide (Wei et al., 2011). Further, Podojil et al. tried to explore the therapeutic potential of human and mouse VTCN1Ig (B7-H4 Ig fusion protein) to treat EAE. They found that VTCN1Ig treatment effectively improved both relapsing and chronic EAE (R-EAE/ C-EAE) by decreasing the numbers of activated $CD4^+$ T cells within the CNS and spleen, causing an increase in the number and functional capacity of Tregs. They also demonstrated that VTCN1Ig treatment produced a tolerogenic environment which was IL-10 and Treg dependent (Podojil et al., 2013). Overall, these studies suggest that VTCN1Ig can be a novel therapeutic agent for the treatment of MS.

### 5.4. VTCN1 in Primary Biliary Cirrhosis

Primary Biliary Cirrhosis (PBC) is a slow progressive organ-specific autoimmune disease characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts (Kakar et al., 2011). Autoreactive T cell infiltration in the intrahepatic bile ducts was reported to be responsible for organ-specific damage in PBC (Kita et al., 2002b, 2002a). As a negative regulator of T cell activation, expression of VTCN1 was for the first time reported in liver biopsies of PBC patients by Chen et al., 2011 (Table 1). Similarly, they also found intracellular VTCN1 expression on three primary BEC (Biliary Epithelial Cells) cell lines (SS, HR, and 4.22.07) isolated from three different PBC patients. For the first time, they demonstrated that knock-down of VTCN1 by RNAi resulted in increased BEC apoptosis mediated by FasL

upregulation (Fas/FasL pathway). Finally, they concluded VTCN1 as a possible target for therapeutic intervention of PBCs (Chen et al., 2011).

### 5.5. VTCN1 in Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease characterized by the breakdown of self-tolerance and the deposition of the circulatory immune complex (Marrack et al., 2001). It may involve many different organs and tissues, including skin, kidney, lungs, heart, and brain, among which Lupus Nephritis (LN) is one of the most common complications (Lau et al., 2006). It is supposed that SLE results from interactions between metabolic, hormonal, genetic, and environmental factors. Immune-mediated mechanisms involving cytokines and co-stimulatory molecules accentuate wide attention. VTCN1 is a known negative regulator of T cell response which makes it an astonishing target to be explored in SLE. For the first time, Ramos et al. reported that the *VTCN1* gene region showed the strongest novel association with SLE (Ramos et al., 2011). In 2017, Xiao et al. developed a murine model of SLE and demonstrated that VTCN1 KO-mice showed more splenomegaly and lymphadenopathy upon treatment with LPS-inducing agents. They also found swelling of lymph tissue and exacerbated kidney lesions in the VTCN1 deficient SLE model. Moreover, VTCN1 antagonist antibody treatment worsens the disease condition while treatment of VTCN1Ig improves the lupus manifestation (Xiao et al., 2017). Recently, Xiao et al., 2019 reported increased plasma sVTCN1 levels in SLE and LN patients compared to healthy individuals (Table 2). However, the exact mechanism and physiological role of sVTCN1 remains to be investigated (Xiao et al., 2019). Accumulatively, these reports insinuate VTCN1 as a chief molecule in developing the disease and a novel therapeutic target for SLE treatment.

### 5.6. VTCN1 in primary Sjögren's Syndrome:

Primary Sjögren's Syndrome (pSS) is a chronic systemic autoimmune disorder that mainly targets lacrimal and salivary glands resulting in loss of its secretory function (Huang et al., 2013). The exact pathogenesis of pSS is not fully understood, but massive lymphocytic infiltration in the exocrine gland is the hallmark of pSS (Katsifis et al., 2007). Due to the involvement of various epithelial cells, pSS has also been narrated as autoimmune epithelitis (Tzioufas and Voulgarelis, 2007). As a negative regulator of the immune response, VTCN1 has been an enchanting molecule to be studied in pSS. For the first time, Yu et al. reported VTCN1 expression in the epithelial cells of salivary glands (Table 1). They found lower VTCN1 expression in the salivary gland biopsies of pSS patients than in healthy controls. Moreover, they also reported a significant decrease in serum VTCN1 levels of pSS patients (Table 2), which was positively correlated with saliva and tear flow rates (Yu et al., 2012). To further investigate this, they co-cultured $CD4^+$ cells with SGECs (Salivary Gland Epithelial cells) from the patients and found reduced suppression of T cell proliferation. They validated the same by using the VTCN1 blockade antibody, which increased the $CD4^+$ T cell proliferation. Additionally, they have also shown VTCN1-mediated suppression of IL-13, IL-5, IL-17A, and IL-6 secretion (Li et al., 2017). Taken altogether, the study suggested that decreased VTCN1 expression in salivary glands of SS patients leads to faulty negative regulation of T cells causing inflammation. This study provided new insight into the role of VTCN1 in salivary gland inflammation in pSS.

### 5.7. VTCN1 in vitiligo

Vitiligo is a common skin disfiguring disease characterized by white-colored patches on the skin due to the loss of functional melanocytes from the basal layer of the epidermis (Singh et al., 2020). The involvement of inflammatory infiltrates in the vitiliginous skin of the patients, specifically decreased $CD4^+/CD8^+$ ratio and cytotoxic T cell infiltrates, are reported in the perilesional skin of vitiligo patients strongly suggest autoimmune destruction of melanocytes (Le Poole et al., 1996; van den Wijngaard et al., 2000). Our lab has also established altered levels of redox enzymes, such as the increase in Superoxide dismutase (SOD), Lipid peroxidase (LPO), and a decrease in Glutathione peroxidase (GPX) and Glucose 6-phosphate dehydrogenase (G6PD), along with its polymorphisms contribute to the vitiligo pathogenesis (Agrawal et al., 2004; Laddha et al., 2013b; Mansuri et al., 2019, 2017,2016; Shajil and Begum, 2006). Additionally, polymorphisms present in immune regulatory regions such as HLA, Interleukin 4 (IL4), Interleukin 1β (IL1β), Interleukin 1 receptor antagonist (IL1RN), IL17, TNFA, TNFB, IFNG, NLRP1, Neuropeptide Y (NPY), Proteosome subunit beta 8 (PSMB8), Transporter associated with antigen processing 1 (TAP1) also reported to be associated with vitiligo susceptibility in Gujarat population along with its altered transcripts and protein levels (Birlea et al., 2013; Dwivedi et al., 2013; Dwivedi et al., 2013b; Imran et al., 2012; Jadeja et al., 2022, 2017; Laddha et al., 2014, 2013a,2012; Singh et al., 2018, 2012). One of our lab studies also pointed out the association of polymorphism present in CTLA4 and its decreased transcript levels with Vitiligo susceptibility in Gujaratis (Dwivedi et al., 2011). Altered $CD4^+/CD8^+$ ratio in vitiligo patients from Gujarat and decreased Treg cells strongly support the autoimmune hypothesis for vitiligo development (Dwivedi et al., 2013a). Thus, our lab results draw the attention to explore the therapeutic potential of one of the co-inhibitory molecules, i.e., VTCN1.

Emerging vitiligo treatment involves molecules such as HSP70i, JAK-STAT inhibitors, and different immune checkpoints that regulate autoimmunity (Rashighi and Harris, 2017). Recently, the successful treatment of metastatic melanoma with the help of immune checkpoints has become the focus of interest. Furthermore, the therapy of immune checkpoint inhibitors to melanoma patients leads to development of vitiligo (Macdonald et al., 2015). Therefore, several people have hypothesized that activating these immune checkpoints could develop tolerance in vitiligo-affected patients (Speeckaert and van Geel, 2017). One of the successful examples of the immune checkpoint as a drug for autoimmune disease is Abatacept (fusion protein of CTLA4 and IgG1), which was approved by the FDA for the treatment of RA (Moreland et al., 2006). Recently, a clinical trial was initiated to check the efficacy of abatacept in vitiligo patients ("Open-label Pilot Study of Abatacept for the Treatment of Vitiligo," n.d.). In 2017, Miao et al. was reported that PD-L1 fusion protein reverses the depigmentation in Pmel-1 vitiligo mice. Further, they have found enrichment of Treg cells in the skin, spleen, and blood upon PD-L1 fusion protein treatment (Miao et al., 2018). Increased PD-1 and Tim-3 positive $CD8^+$ T cells were also reported in vitiligo patients by FACS analysis and positively correlated with disease severity (Rahimi et al., 2019). VTCN1 is also an immune checkpoint that negatively regulates the immune response. Our recent lab study has highlighted the role of VTCN1 in vitiligo pathogenesis (Vaishnav et al., 2022). The study reported an increased soluble VTCN1 due to Nardilysin-mediated cleavage in the blood of vitiligo patients and resulted in decreased VTCN1 protein expression in vitiliginous skin (Vaishnav et al., 2022). Thus, VTCN1 might have a role in vitiligo pathogenesis and can be an astonishing therapeutic target for the disease.

## 6. Therapeutic potential of VTCN1Ig (B7-H4 Ig) for various autoimmune diseases:

VTCN1 is reported to play an indispensable role in various autoimmune diseases suggesting it as a novel target for developing new therapies. Humann VTCN1Ig (hB7-H4Ig)/ mouse VTCN1Ig are immunoglobulin fusion proteins prepared by fusion of the extracellular domain of VTCN1 with IgG (Sica et al., 2003a). For the first time, mouse VTCN1Ig was reported by Sica et al. to prove VTCN1 mediated inhibition of T cell proliferation *in vivo*. Moreover, they have shown reduced alloreactive Cytotoxic T cells(CTL) activity in GVHD (Graft versus Host disease) upon mouse VTCN1Ig treatment (Sica et al., 2003b). Azuma et al. also developed a VTCN1 agonist by fusing mouse VTCN1

**Table 3**
VTCN1 in therapeutics of different autoimmune diseases.

| Sr. No | Autoimmune disease | Therapeutic usage of VTCN1 | Technical details | Reference |
|---|---|---|---|---|
| 1 | Type 1 Diabetes | Early treatment of NOD mice with VTCN1 Ig reduces the incidence of autoimmune diabetes | Intraperitoneal injections of B7-H4. Ig (AMP-110, AmplimmuneInc.,MD, USA) **Dosage:**7.5 mg/kg | Wang et al., 2011 |
| | | VTCN1 Ig inhibits the development of Type1 diabetes by regulating Th17 cells in NOD mice | Intraperitoneal injections of B7-H4.Ig (AMP-110, AmplimmuneInc.,MD, USA) **Dosage:**7.5 mg/kg | Lee et al., 2013 |
| | | Local expression of VTCN1 by recombinant adenovirus transduction in mouse islets prolongs allograft survival | Recombinant adenovirus expressing a B7-H4 complementary DNA(Ad-B7-H4) construct transduced in islets from donors | Wang et al., 2009 |
| 2 | Experimental autoimmune encephalomyelitis | mVTCN1 Ig (mouse VTCN1 Ig) treatment during disease remission significantly ameliorated disease progression of R-EAE and C-EAE mouse model | mVTCN1 Ig / h VTCN1 Ig **Dosage:**low dose (60 μg/dose) or high dose (300 μg/dose) | Podojil et al., 2013 |
| | | hVTCN1 Ig(human VTCN1 Ig) treatment during disease remission significantly ameliorated disease progression of R-EAE and C-EAE mouse model | mVTCN1 Ig / h VTCN1 Ig **Dosage:**low dose (60 μg/dose) or high dose (300 μg/dose) | Podojil et al., 2013 |
| 3 | Rheumatoid arthritis | VTCN1 Ig plasmid treatment significantly suppressedprogression of collagen induced arthritis (CIA) in mice model | 30 mg of VTCN1 Ig plasmid DNA in 3 mlof PBS was injected into the tail vein | Azuma et al., 2009 |
| 4 | Systemic Lupus Erythematosus (SLE) | VTCN1 Ig alleviated the lupus manifestations | hydrodynamically injected B7-H4IgV plasmid via tail vein to the mouse **Dosage:** 20 μg of plasmid DNA in 2 ml of PBS | Xiao et al., 2017 |
| 5 | Nephrotoxic serum nephritis | VTCN1 Ig attenuates renal damage in NTS (Nephrotoxic Serum)-challenged B6 mice | **Dosage:**200 μg of VTCN1 Ig in 200 μl PBS intra peritoneal injection to mice | Pawar et al., 2015 |

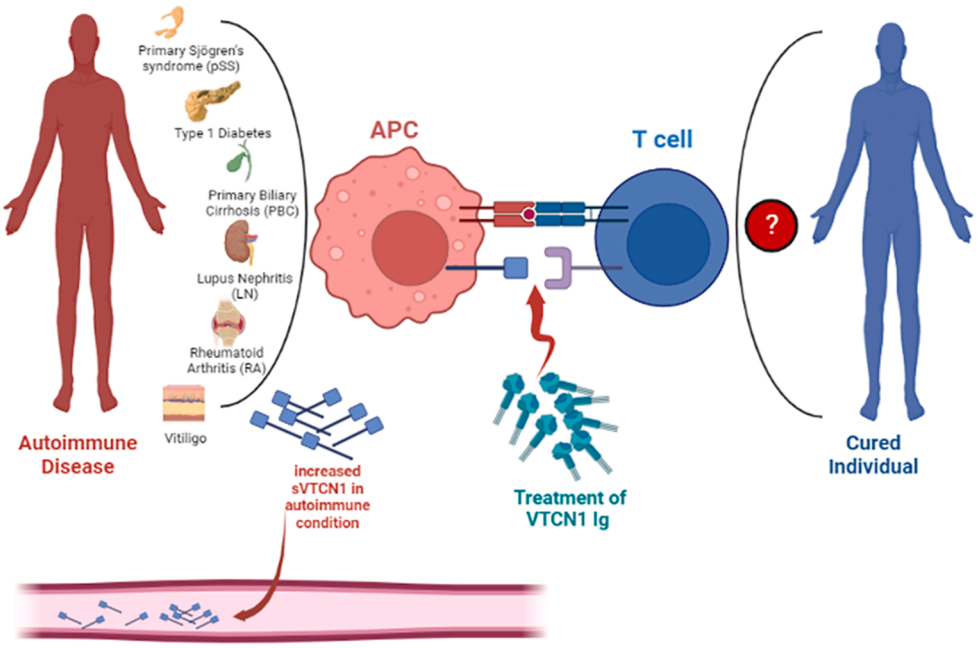


**Fig. 4.** VTCN1 in different autoimmune diseases and as a possible therapeutic target.

extracellular domain and mouse IgG2a Fc portion. Due to the Fc portion, it could bind to Fc receptor-positive cells to facilitate its agonistic effect. They have reported that VTCN1Ig significantly reduces the progression of CIA in mice (Azuma et al., 2009). VTCN1Ig is also known to reduce the incidence of autoimmune diabetes via increasing Treg cells (Wang et al., 2011), ultimately reducing the disease severity of T1D in NOD mice (Lee et al., 2013). Human and mouse VTCN1Igs were also evaluated as a therapeutic targets for the EAE (Prasad et al., 2003). Interestingly, the study found reduced disease severity in $PLP_{139–151}$-induced relapsing EAE (R-EAE) in SJL/J mice and $MOG_{35–55}$-induced chronic EAE (C-EAE) in C57BL/6 mice. Moreover, human and mouse VTCN1Igs modulated $CD4^+$ T cell activity along with a significant increase in IL-10 production. The study also demonstrated an increase in the number of Treg cells in the spleen and CNS of treated mice (Podojil et al., 2013). Table 3 summarizes the findings of various studies on the therapeutic potential of VTCN1Ig.

Furthermore, published preclinical data demonstrated the potency of VTCN1Ig to modulate autoimmune disease in several animal disease models, so clinical trials have also been started to evaluate the therapeutic potential of the VTCN1 fusion protein, AMP-110 in RA patients. In collaboration with Daiichi Sankyo Co., ltd. MedImmune LLC has started phase1 (NCT01878123) and phase 1b (NCT02277574) placebo-controlled clinical trials to assess the safety, tolerability, and pharmacokinetics of AMP-110 in patients with RA, but results have not been published yet. Fig. 4 summarizes VTCN1 as a possible therapeutic target for autoimmune disease.

## 7. Conclusions and future prospects

The published data manifests VTCN1 as an essential player in fine-tuning the immune response. Its expression pattern and functions are pretty different from the other members of the B7 family. It exerts its immunosuppressive effect by binding with its receptor present on T cells. Unfortunately, its receptor is unknown to date. Different autoimmune disease models show that VTCN1 plays a critical role in regulating peripheral tolerance. The present review highlights the role of VTCN1 in various autoimmune diseases. VTCN1 deficiency in mice results in exacerbated autoimmune conditions upon treatment of disease-inducing agents. The autoimmune hypothesis is widely accepted for vitiligo development. Our lab data also revealed increased soluble VTCN1 due to Nardilysin mediated cleavage in the blood of vitiligo patients resulting in decreased VTCN1 protein expression in vitiliginous skin, indicating its alleged role in the disease pathogenesis. Overexpression of VTCN1/ treatment with agonist VTCN1Ig was reported to reduce the progress of autoimmune diseases. Clinical trials in humans have also been started to develop VTCN1Ig as a novel therapy for autoimmune diseases. However, further research is required to understand the underlying molecular mechanism and to identify its receptor.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### *Author contributions*

RB and JV formulated the idea. JV, FK, MY, NP, and HB helped with the literature review and writing. JV has compiled the original draft. RB, MD and SJ reviewed and edited the draft. All the authors have read and agreed to the final version of the manuscript.

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# Elevated X-Box Binding Protein1 Splicing and Interleukin-17A Expression Are Associated With Active Generalized Vitiligo in Gujarat Population

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Vitiligo is an autoimmune skin disorder defined by the destruction of functional epidermal melanocytes. It is a multifactorial and polygenic disorder caused due to oxidative stress, endoplasmic reticulum (ER) stress, and autoimmunity, among other factors. In the present study, we aimed to investigate the association of X-box Binding Protein 1 (XBP1) and Interleukin-17A (IL-17A) polymorphisms and monitor their systemic as well as skin expression levels in vitiligo patients from Gujarat population in India. *XBP1* rs2269577 G/C, *IL17A* rs2275913 G/A and *IL17A* rs8193036 C/T polymorphisms were genotyped by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method in 312 controls and 276 vitiligo patients. Transcript levels of spliced (*sXBP1*), unspliced *XBP1* (*uXBP1*) and *IL17A* from peripheral blood mononuclear cells (PBMCs) as well as spliced and unspliced *XBP1* from skin samples were analyzed by qPCR. IL-17A protein levels in suction-induced blister fluid (SBF) from the skin of study subjects were estimated by ELISA. The results revealed that genotype ($p$=0.010) and allele ($p$=0.014) frequencies of *XBP1* rs2269577 G/C polymorphism were significantly different, however, no significant difference was observed in frequencies of *IL17A* rs2275913 G/A and *IL17A* rs8193036 C/T polymorphisms in control and patient population. Gene expression analysis revealed that *sXBP1* and *IL17A* levels were significantly higher in PBMCs of generalized ($p$=0.030 and $p$=0.039, respectively) and active ($p$=0.024 and $p$=0.017, respectively) vitiligo patients. Moreover, we observed a significantly elevated *sXBP1* expression ($p$=0.037) as well as IL-17A protein levels ($p$=0.009) in perilesional skin of vitiligo patients as compared to controls. Overall, these findings suggest XBP1 and IL17A play an important role in vitiligo and further substantiate the involvement of ER stress in exacerbating immune-mediated vitiligo pathogenesis.

**Keywords: vitiligo, interleukin, endoplasmic reticulum stress (ER stress), genetic polymorphisms and disease association, autoimmunity, cytokines**

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# Investigation of the Role of Interleukin 6 in Vitiligo Pathogenesis

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**ABSTRACT**

Interleukin-6 (IL6) is involved in pathogenesis of several autoimmune disorders including vitiligo. Hence, we aimed to investigate the association of IL6 −174 G/C and −572 G/C polymorphisms and its transcript levels with vitiligo; to evaluate the effect of IL-6 on normal human melanocyte (NHM) viability and expression of IL6R, MITF and TYR. IL6 -174 G/C and −572 G/C polymorphisms were genotyped by ARMS-PCR and PCR-RFLP respectively in 343 controls and 322 vitiligo patients. IL6 transcript levels were estimated from PBMCs (96 controls and 77 patients) and skin samples (15 controls and 15 patients) by qPCR. NHM viability was assessed by MTT; IL6R, MITF and TYR transcript and protein levels were monitored by qPCR and ICC respectively. Genetic analyses revealed no association of IL6 −174 G/C polymorphism ($p > .05$) with vitiligo. Analysis of IL6 −572 G/C revealed reduced risk of vitiligo in individuals with GC/CC genotypes compared to GG genotype ($p = .010$). IL6 expression was significantly increased ($p = .0197$) in PBMCs of patients. Further, IL6 expression was significantly higher in non-lesional skin compared to controls ($p = .009$). In-vitro NHM viability was decreased upon IL-6 exposure (10-50 ng/ml; $p < .05$), with significantly increased IL6R transcript ($p = .042$) and protein levels ($p = .003$) however, MITF transcript ($p = .0003$) and protein levels ($p = .016$), and TYR transcript levels ($p = .001$) were significantly decreased. The results suggest that IL6 −572 G/C polymorphism might be associated with vitiligo susceptibility in Gujarat population. Moreover, increased IL6 expression in vitiligo patients and its effect on NHM suggest a potential role in melanocyte biology.

**Conclusion:** The results suggest that *IL6 − 572* G/C polymorphism might be associated with vitiligo susceptibility in Gujarat population. Moreover, increased *IL6* expression in vitiligo patients and its effect on NHM suggest a potential role in melanocyte biology.

**KEYWORDS**

Vitiligo; melanocyte; cytokine; autoimmunity; polymorphisms

## Introduction

Vitiligo is an acquired skin disorder caused due to the destruction of epidermal melanocytes. Its occurrence ranges from 0.4% to 2.0%, worldwide, affecting about 0.5% to 2.5% of the population in India (Sehgal and Srivastava 2007; Silverberg 2015). To understand vitiligo pathogenesis, it is crucial to investigate the genetic predisposition of candidate

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# Expression analysis of candidate genes in vitiligo patients & effect of oxidative stress on melanocytes

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ABSTRACT

*Background:* Vitiligo is an acquired hypomelanotic autoimmune disorder characterized by depigmented macules on the skin. Oxidative stress acts as an initial trigger and autoimmunity leads to vitiligo progression. Cytokines are key mediators of immune system and imbalance between pro- and anti-inflammatory cytokines play a vital role in the pathogenesis of autoimmune diseases. Also, relationship between oxidative stress and oxidative stress induced cytokine secretion is well established. Therefore, the present study aimed to monitor the expression levels of selected cytokine genes (*IL1A*, *IL4*, *IL1R1*, *IL1RN* and *IFNG*) from vitiliginous skin along with the effect of oxidative stress on *in vitro* cultured normal human melanocyte (NHM).

*Methods:* The gene expression of *IL1A*, *IL1R1*, *IL1RN*, *IL4* and *IFNG* was performed by real-time PCR. Effect of oxidative stress on NHM was measured by MTT assay upon $H_2O_2$ treatment.

*Results:* Outcome of the study revealed significant increase in *IFNG* transcript levels from the non lesional and lesional skin of vitiligo patients as compared to controls ($p = 0.0045$ and $p = 0.0198$, respectively). However, no significant differrence was observed in the transcript levels of *IL1A*, *IL4*, *IL1R* and *IL1RN* from non lesional and lesional skin of vitiligo patients as compared to controls ($p > 0.05$). A significant decrease in melanocyte viability was observed upon $H_2O_2$ treatment in a dose dependent manner ($p < 0.0001$).

*Conclusion:* The increased *IFNG* expression in vitiliginous skin advocates IFN-γ associated melanocyte destruction in vitiligo and decreased melanocyte viability upon $H_2O_2$ treatment suggests the vulnerability of melanocytes to oxidative stress which persists in vitiligo microenvironment.

## 1. Introduction

Vitiligo is the most abundant skin pigmentary disorder showing a worldwide prevalence of 0.2–2% (Picardo et al., 2015). The presence of white coloured macules on the skin is a pathological hallmark of vitiligo, which is due to the destruction of melanocytes from the skin's epidermal layer. Even if the exact cause or initial trigger of the disease is mostly unknown, multiple mechanisms such as autoimmunity, oxidative stress and the production of inflammatory mediators are reported to be involved in the development of vitiligo (Laddha et al., 2013a; Harris, 2016). The presence of antimelanocyte antibodies and percolation of autoreactive T cells in the vitiliginous skin strengthen the autoimmune hypothesis for melanocyte destruction (Laddha et al., 2014; van den Boorn et al., 2009). A growing body of evidence suggests that epidermal melanocytes are a vital part of the skin immune system and can be considered immunologically active cells with the ability to process and present antigens to T cells (Tam and Stepień, 2007). Besides the photoprotective function of melanocytes, they also have neuroendocrine activity and produce stress molecules such as neurotransmitters, neuropeptides, cytokines and hormones (Slominski et al., 2009). For the development, differentiation and regulation of immunological cells, cytokines play a pivotal role. The abnormal regulation of cytokine production or their action is believed to play a central role during the development of autoimmune condition, inflammatory diseases and cancer (O'Shea et al., 2002; Kany et al., 2019; Germano et al., 2008).

Within the skin epidermal melanin unit, the surrounding keratinocytes produce cytokines such as IL-6 and IL-1α, which serve as paracrine inhibitors for melanocyte division and melanogenesis. Keratinocyte-

*Abbreviations:* *IL1A*, interleukin-1α; *IL1R1*, interleukin 1 receptor 1; *IL1RN*, interleukin-1 receptor antagonist; *IL4*, interleukin-4; *IFNG*, interferon-γ; SOD, superoxide dismutase; TNFB, tumor necrosis factor B; NHM, normal human melanocytes.

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# A Concise Review on the Role of Endoplasmic Reticulum Stress in the Development of Autoimmunity in Vitiligo Pathogenesis

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Vitiligo is characterized by circumscribed depigmented macules in the skin resulting due to the autoimmune destruction of melanocytes from the epidermis. Both humoral as well as cell-mediated autoimmune responses are involved in melanocyte destruction. Several studies including ours have established that oxidative stress is involved in vitiligo onset, while autoimmunity contributes to the disease progression. However, the underlying mechanism involved in programing the onset and progression of the disease remains a conundrum. Based on several direct and indirect evidences, we suggested that endoplasmic reticulum (ER) stress might act as a connecting link between oxidative stress and autoimmunity in vitiligo pathogenesis. Oxidative stress disrupts cellular redox potential that extends to the ER causing the accumulation of misfolded proteins, which activates the unfolded protein response (UPR). The primary aim of UPR is to resolve the stress and restore cellular homeostasis for cell survival. Growing evidences suggest a vital role of UPR in immune regulation. Moreover, defective UPR has been implicated in the development of autoimmunity in several autoimmune disorders. ER stress-activated UPR plays an essential role in the regulation and maintenance of innate as well as adaptive immunity, and a defective UPR may result in systemic/tissue level/organ-specific autoimmunity. This review emphasizes on understanding the role of ER stress-induced UPR in the development of systemic and tissue level autoimmunity in vitiligo pathogenesis and its therapeutics.

**Keywords: endoplasmic reticulum, unfolded protein response, vitiligo, melanocytes, autoimmunity**

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## INTRODUCTION

Extensive research over the years established that a complex interaction between genetic, environmental, biochemical, and immunological factors collectively generate a microenvironment favoring melanocyte loss in vitiligo (1–3). The complex genetics of vitiligo involves multiple susceptibility loci, incomplete penetrance, and genetic heterogeneity with gene-gene and gene-environment interactions and altered miRNA expression (**Table S1**) (4–6). Accumulation of oxidative stress due to defective recycling of tetrahydrobiopterin, mitochondrial impairment