Chapter 2

To investigate the association of VTCN1 polymorphisms, VTCN1 and NRD1 transcript and protein levels with vitiligo susceptibility in Gujarat population

2.1 Introduction

Vitiligo is a common skin disfiguring disease, characterized by white-colored patches on the skin due to the erosion of functional melanocytes from epidermal basal layer. The worldwide prevalence of vitiligo from population and hospital-based studies was found to be 0.2-1.8% [1]. In India, prevalence of vitiligo was reported to be 0.25-4% based on dermatology outpatients' studies across the country and up to 8.8% in Gujarat and Rajasthan states [2-3]. Extensive research on understanding vitiligo pathogenesis highlighted involvement of the immune system and strongly favors the autoimmune hypothesis for vitiligo development and progression [4-5]. The involvement of both humoral and cellular arms of immunity has been reported such as circulating melanocyte-specific autoantibodies in the blood, presence of autoreactive anti-melanocyte $CD8^+$ T cells in the skin of vitiligo patients and impaired suppressive function of Treg cells [6-8]. However, the exact modus operandi underlying the induction and activation of autoreactive T cells and the loss of tolerance to melanocyte autoantigens in vitiligo are not yet clear [9].

T cell co-stimulatory pathways are well known to influence the development and progression of autoimmunity [10]. The B7-CD28 family members are the crucial co-stimulatory molecules that play a central role in the activation of T lymphocytes, prevention of tolerance, and development of optimal T cell immunity [11]. VTCN1 (V-set domain-containing T-cell activation inhibitor 1) also named as B7-H4, B7S1 and B7x, is a negative co-stimulatory molecule identified by three different research groups [12-14]. It belongs to the B7 family of the immune-regulatory ligands. Human *VTCN1* gene is mapped on chromosome 1 comprising of 6 exons and 5 introns and the mature membrane-bound protein consists of 282 amino acids [15]. It binds to an unidentified receptor present on T cell and suppresses T cell activation, clonal expansion and cytokine production [16]. Besides membrane bound form of VTCN1, a soluble form is also present in plasma, resulting due to cleavage of VTCN1 by a metalloproteinase, Nardilysin (NRD1) [17]. NRD1 (N-arginine dibasic (NRD) convertase 1), belongs to the M16 family that cleaves peptide substrates at the N-terminus of arginine in dibasic motifs [18]. It has found to be involved in the processing of antigenic self-peptides [19].

Recent studies suggest that VTCN1 mediated negative co-stimulation provides a crucial balance between abnormal T cell activation and anergy [16]. Several studies showed its role

in different autoimmune diseases such as Rheumatoid Arthritis (RA) [20], Systemic Lupus Erythematosus (SLE) [21], Type 1 Diabetes (T1D) [22], and Primary Biliary Cirrhosis (PBC) [23]. One of the GWASs showed that SNPs mapped to the *VTCN1* gene are found to be strongly associated with juvenile idiopathic arthritis [24]. Given the role of VTCN1 in different autoimmune diseases, it becomes interesting to explore the role of VTCN1 in vitiligo pathogenesis. The present study was aimed to investigate the association of *VTCN1* intronic polymorphisms (rs10923223 T/C and rs12046117 C/T) with vitiligo susceptibility in Gujarat population; to assess *VTCN1* and *NRD1*transcript levels from PBMCs as well as skin; to estimate soluble VTCN1 (sVTCN1) and NRD1 levels from plasma, and VTCN1 protein levels from the skin of vitiligo patients.

2.2 Materials and methods:

2.2.1 Ethical Committee Approval:

The study plan and consent forms were approved by 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). Written consent was obtained from the next of kin, caretakers, or guardians on behalf of the minors/children enrolled in the study and significance of the study was also explained to all participants (Figure S4).

2.2.2 Study population:

The study has included 411 vitiligo patients and 450 age and sex-matched unaffected healthy controls. Vitiligo patients from S.S.G Hospital, Vadodara, Gujarat, India were recruited in the study. The inclusion criteria followed were: outpatients of age between 5 to 60 years and both the parents should be Gujarati by birth. Patients with ongoing treatments or other diseases, and pregnant/lactating women were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Generalized or non-segmental vitiligo (GV) was characterized by depigmented patches varying in size from a few to several centimeters in diameter, involving one or both sides of the body with a tendency towards symmetrical distribution (1). Whereas localized or segmental vitiligo (LV) typically has a rapidly progressive but limited course, depigmentation spreads within the segment during a period of

6 ± 24 months and then stops; further extension is rare (2,3). Following clinical criteria to proposed by Flabella et al., (3) and discussed in the Vitiligo Global Issues Consensus Conference 2012 (4), were used for characterizing stable vitiligo (SV): (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active vitiligo (AV) was defined as the appearance of new lesions and spreading of existing lesions observed during past two-years duration.

Table 2.1. Demographic characteristics of vitiligo patients and unaffected controls for genotyping study.

	Vitiligo Patients	**Controls**
	(n = 411)	(n = 450)
Average age (mean age ± SD)	34.23 ± 19.15 yr	29.41 ± 12.16 yr
Sex: Male	214 (52.10%)	244 (54.22%)
Female	197 (47.90%)	206 (45.77%)
Age of onset (mean age ± SD)	29.14±18.51 yr	NA
Duration of disease (mean ± SD)	9.262±10.60 yr	NA
Type of vitiligo		
Generalized	352(85.64%)	NA
Localized	59 (14.36%)	NA
Active vitiligo	350 (85.16%)	NA
Stable vitiligo	61 (14.84%)	NA

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

2.2.3.1 Genomic DNA extraction:

Genomic DNA was isolated from the whole blood using QIAamp™ DNA Blood Kit (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's protocol. After extraction, concentration and purity of DNA were estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was normalized to 50ng/µl. DNA was stored at -20˚ C until further analyses.

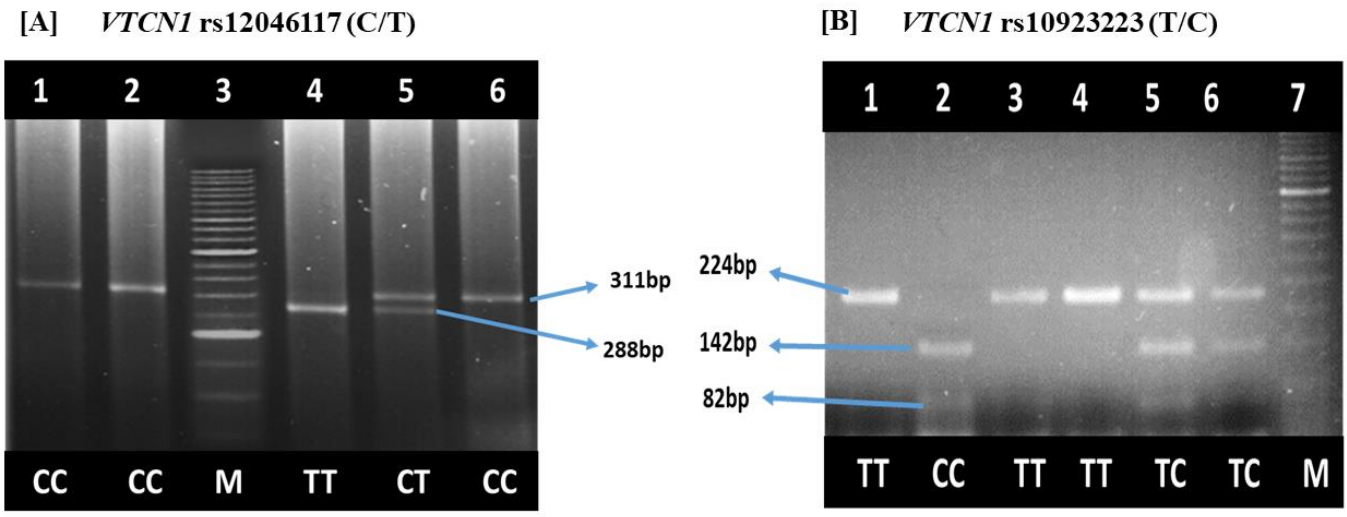
2.2.3.2 PCR-RFLP for genotyping of *VTCN1* rs10923223 (T/C) and rs12046117 (C/T) polymorphisms:

Genotyping of the intronic SNPs was performed using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). The reaction mixture of the total volume of 20 µL included 3 µL (150ng) of genomic DNA, 11 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (HiMedia™, Mumbai, India), 1 µL of 10 pM corresponding forward and reverse primers (Eurofins™, India; the primers used for PCR and annealing temperatures are mentioned in Table S3.), and 0.2 µL (2 U/µL) Taq Polymerase (HiMedia™, Mumbai, India). Amplification was performed Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany) according to the protocol: 95 ºC for 10 minutes followed by 45 cycles of 95 ºC for 30 seconds, 62 ºC for 30 seconds and 72 ºC for 30 seconds, and 72 ºC for 10 minutes for both the SNPs. The 5 µl amplified product was confirmed by 2.0% agarose gel electrophoresis and remaining 15 µl of the amplified product was digested with 1U of *Sph I* and *Taq1* (Thermo Fischer Scientific, U.S.A.) for *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) polymorphisms respectively (Table 2.2). The 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 Figure 2.1. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Gene (SNP)	Primer sequence	Amplicon size (bp)	RE	Digested product size (bp)
VTCN1 (rs12046117)	**FP:** 5'AGTTGTTATGTGGGAGTCTTTCTC3' **RP**: 5'GGAATGCTGATGTGCAGATTTTAA3'	311	Sph1	288 +23
VTCN1 (rs10923223)	**FP:** 5'GAGGTCTTGTTACGCCACAG3' **RP**: 5'TTGTTCTGTGAGGTGCGGG3'	224	Taq1	142 + 82

Table 2.2: Primer details used for polymorphism analysis along with amplicon size and RE

Figure: 2.1 [A] PCR-RFLP analysis of *VTCN1* (C/T) intronic polymorphisms on 3.5% agarose gel. PCR product was digested by restriction enzyme *SphI*. Digestion resulted in a 311 bp for C allele and 288 bp and 23 bp for T allele. **[B]** PCR-RFLP analysis of *VTCN1* intronic rs10923223 T/C polymorphism on 3.5% agarose gel: PCR product was digested by restriction enzyme Taq1. Digestion resulted in a 224-bp fragment for the T allele, and 142 and 82 bp fragments for the C allele.



2.2.4 Estimation of *VTCN1* and *NRD1* transcript levels from PBMCs and skin:

2.2.4.1 RNA extraction and cDNA synthesis:

RNA was isolated from PBMCs and skin tissue (~20 mg) using the RNAiso Plus (TaKaRa Bio Inc) following the standardized protocol [25]. Quality, quantity and purity of the RNA were checked, and cDNA synthesis was performed as described earlier [25]. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity were determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion™ inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. cDNA synthesis was performed using 1 µg of total RNA by High-capacity cDNA reverse Transcription Kit (Applied Biosystems™, Foster city, U.S.A.) according to the manufacturer's instructions in Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany).

2.2.4.2 Quantitative real-time PCR (qPCR):

Further *VTCN1*, NRD1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were measured by qPCR using gene-specific primers (Eurofins ™, Bangalore, India). qPCR was performed in duplicates in 20 µl volume using LightCycler1 480 SYBR Green I Master (Roche™ Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. The thermal cycling conditions included an initial activation step at 95 ºC for 10 min, followed by 45 cycles of denaturation, annealing, and extension (95 ºC for 10 sec, 64 ºC for 10 sec, 72 ºC for 12 sec for *VTCN1*, 95 ºC for 8 sec, 61 ºC for 8 sec, 72 ºC for 8 sec for *NRD1* and 95 ºC for 10 sec, 69 ºC for 10 sec, 72 ºC for 8 sec for *GAPDH*). The fluorescence data collection was performed during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to check the specificity of the products formed. The PCR cycle at which PCR amplification begins its exponential phase and product fluorescence intensity finally rises above the background and becomes visible was considered as the crossing point- PCR-cycle (CT) or cycle threshold (CT). The ΔCT value was determined as the difference between the cycle threshold of target genes (*VTCN1/NRD1*) and reference gene (GAPDH). The difference between the two ΔCT values (ΔCT Controls and ΔCT patients) was considered as ΔΔCT to obtain the value of fold expression ($2^{-\Delta\Delta CT}$).

Table 2.3. Demographic characteristics of vitiligo patients and unaffected controls of Gujarat recruited for skin biopsy (Gene Expression Study)

	Vitiligo Patients	Controls
	(n = 12)	(n = 24)
Average age (mean age ± SD)	29.38± 4.480	28.33± 3.240
Sex: Male	5	13
Female	7	11
Age of onset (Mean age ± SD)	27.83 ± 6.05 yr	NA
Duration of disease (Mean ± SD)	6.83 ± 2.36 yr	NA
Type of vitiligo		
Generalized vitiligo	8	NA
Localized vitiligo	4	NA

Table 2.4: Primer details used for gene expression analysis

Gene	Primer (5'-3')	Amplicon size (bp)	Annealing Temp. (ºC)
VTCN1	**FP:** GTGGTCTGGGCATCCCAAGTTG **RP:** CGCCTTTTGATCTCCGATTCTG	209	60
GAPDH	**FP:** GTCAAGCCTGTTTGTGAAGC **RP:** CTAATCTACTGCACAACAGTG	122	61
NRD1	**FP:** GCCTCAACTGATTGTGAACGC **RP:** GCGCCCATCTATCAAGAGCTT	82	60

2.2.5 Estimation of plasma sVTCN1 and NRD1 levels:

Soluble VTCN1 (sVTCN) levels from the plasma of vitiligo patients 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 vitiligo patients and controls were measured by ELISA using Fine Test ELISA kit (EH10653, Wuhan, Hubei, China) as per the manufacturer's protocol and optical density was measured at 450 nm.

2.2.6 Immunohistochemical analysis of vitiliginous skin:

Skin biopsies were collected from 12 vitiligo patients and 13 controls using a punch probe (3mm) under local anesthesia from lesional, perilesional and non-lesional skin. Demographical details of patients and controls were given in Table 2.5. Each biopsy was immediately stored in NBF (Neutral buffered Formalin). Paraffin-embedded blocks were prepared and three-micron sections were placed on Poly-L lysine coated slides. Slides were deparaffinized, hydrated, permeabilized and antigen retrieved with 10mM 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 FadeTM Gold antifade reagent with DAPI (S36939, Life Technologies, Carlsbad, CA). Fluorescence signals were detected with a fluorescence microscope (Nikon eclipse Ti2-E, Tokyo, Japan). Images were analyzed using NIS-Elements Advanced Research 5.20 software (Nikon, Tokyo, Japan) to calculate the mean intensity of fluorescence and graphs were plotted.

Table 2.5. Demographic characteristics of vitiligo patients and unaffected controls of Gujarat recruited for skin biopsy (Protein expression studies).

	Vitiligo Patients	**Controls**
	(n = 12)	(n = 13)
Average age (Mean age ± SD)	35.33± 4.758	29.08± 3.454
Sex: Male	6	7
Female	6	6
Age of onset (Mean age ± SD)	23.92 ± 3.13 yr	NA
Duration of disease (mean ± SD)	6.08 ± 3.63 yr	NA
Type of vitiligo		
Generalized vitiligo	7	NA
Localized vitiligo	5	NA

2.2.7 Statistical analyses:

For both SNPs, Hardy-Weinberg Equilibrium (HWE) was calculated in controls and patients via comparing observed and expected frequencies of genotypes by chi square analysis. Genotype and allele frequencies distribution of polymorphisms in different groups were

compared using chi-square test with 2×2 contingency tables. Relative gene expression of *VTCN1* in patients and controls were analysed by nonparametric unpaired t-test and one-way ANOVA. Comparison of sVTCN1 and mean fluorescence intensity among the multiple groups were analysed using one way ANOVA with multiple comparison. All the statistical tests were carried out using Prism 8 software (Graph Pad Software Inc; 2003). Correlation analysis between sVTCN1 and NRD1 was carried out using Spearman's correlation analysis among vitiligo patients using Prism 8 software.

2.3 Results

2.3.1 Analysis of association of *VTCN1* rs12046117 (C/T) polymorphism with vitiligo susceptibility:

Genotyping of intronic *VTCN1* SNP (rs12046117) was done in 411 patients and 450 controls and three genotypes were identified in both patients and controls: CC, CT and TT (Figure 2.1 A). Both control and patient population followed HWE (p=0.450 and p=0.097 respectively; Table 2.6). The observed frequency of the ancestral allele 'C' in patient and control groups was 79%. No significant difference in the frequencies of variant allele 'T' between patient (21%) and control (21%) groups was observed (p=0.937; Table 2.6). Further analysis based on disease type (Table 2.7) and activity (Table 2.8) did not show any significant difference in the distribution of genotype as well as allele frequencies in vitiligo patients.

2.3.2 Analysis of association of *VTCN1* rs10923223 (T/C) polymorphism with vitiligo susceptibility:

Genotyping of intronic *VTCN1* SNP (rs10923223) was done in 411 patients and 450 controls and three genotypes were identified in both patient and control groups: TT, TC and CC (Figure 2.1 B). Both control and patient population followed HWE (p=0.319 and p=0.458 respectively; Table 2.6). The observed frequency of the ancestral allele 'T' in the patient and control groups was 67% and 69% respectively. No significant difference in the frequencies of variant allele 'T' between patient (33%) and control (31%) groups was observed (p=0.359; Table 2.6). Further analysis based on disease type (Table 2.7) and activity (Table 2.8) did not show any significant difference in the distribution of genotype as well as allele frequencies in vitiligo patients.

Table 2.6. 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%)
		n=450	n=411				
VTCN1	CC	275 (0.61)	249 (0.61)	0.450	R	1	-
rs12046117	CT	157 (0.35)	149 (0.36)	(C)	0.744	1.04	0.79-1.39
(C/T)	TT	18 (0.04)	13 (0.03)		0.545	0.79	0.38-1.66
				0.097			
	C	707 (0.79)	647 (0.79)	(P)	R	1	-
	T	193 (0.21)	175 (0.21)		0.937	0.99	0.78-1.24
		n=382	n=310				
VTCN1	TT	179 (0.47)	136 (0.44)	0.319	R	1	-
rs10923223	TC	171 (0.45)	143 (0.46)	(C)	0.550	1.10	0.80-1.50
(T/C)	CC	32 (0.08)	31 (0.10)		0.378	1.27	0.74-2.19
				0.458			
	T	529 (0.69)	415 (0.67)	(P)	R	1	-
	C	235 (0.31)	205 (0.33)		0.359	1.11	0.88-1.39

n = Number of Patients/ Controls
R= Reference group
CI= Confidence Interval.
Due to Bonferroni's correction statistical significance was considered at $p<0.025$

Table 2.7. 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%)
		n=352	n=59	n=450			
	Genotype						
	CC	214 (0.61)	35 (0.59)	275 (0.61)	R	1	-
***VTCN1* rs12046117 C/T**	CT	127 (0.36)	22 (0.37)	157 (0.35)	0.84[a]	1.05[a]	0.59-1.88[a]
					0.79[b]	1.03[b]	0.77-1.39[b]
					0.73[c]	1.10[c]	0.62-1.94[c]
	TT	11 (0.03)	2 (0.04)	18 (0.04)	0.89[a]	1.11[a]	0.23-5.23[a]
					0.53[b]	0.78[b]	0.36-1.69[b]
					0.85[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)	0.83[a]	1.05[a]	0.65-1.68[a]
					0.89[b]	0.98[b]	0.77-1.25[b]
					0.88[c]	1.03[c]	0.65-1.64[c]
	Genotype	n = 257	n= 53	n = 382			
	TT	118 (0.46)	19 (0.36)	179 (0.47)	R	1	-
	TC	113 (0.43)	32 (0.60)	171 (0.45)	0.07[a]	0.56[a]	0.31 – 1.06[a]
					0.96[b]	0.99[b]	0.71 – 1.38[b]
					0.06[c]	1.76[c]	0.97 – 3.29[c]
***VTCN1* Rs10923223 T/C**	CC	29 (0.11)	2 (0.04)	32 (0.08)	0.25[a]	2.35[a]	0.60 – 10.67[a]
					0.24[b]	1.38[b]	0.80 – 2.43[b]
					0.48[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)	0.83[a]	0.95[a]	0.61 – 1.46[a]
					0.42[b]	1.10[b]	0.86 – 1.39[b]
					0.50[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 $p < 0.025$ due to Bonferroni's correction.

Table 2.8. 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%)
		n=350	n=61	n=450			
	Genotype						
	CC	210 (0.60)	39 (0.64)	275 (0.61)	R	1	-
	CT	128 (0.37)	21 (0.34)	157 (0.35)	0.67[a]	0.88[a]	0.49-1.56[a]
					0.66[b]	1.06[b]	0.79-1.43[b]
VTCN1					0.83[c]	0.94[c]	0.53-1.61[c]
rs12046117	TT	12 (0.03)	1 (0.02)	18 (0.04)	0.43[a]	0.44[a]	0.05-3.55[a]
(C/T)					0.72[b]	0.87[b]	0.41-1.85[b]
					0.35[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)	0.47[a]	0.83[a]	0.51-1.36[a]
					0.89[b]	1.01[b]	0.79-1.29[b]
					0.51[c]	0.85[c]	0.52-1.37[c]
		n = 260	n= 50	n = 382			
	Genotype						
	TT	118 (0.45)	18 (0.36)	179 (0.47)	R	1	-
	TC	113 (0.44)	30 (0.60)	171 (0.45)	0.08[a]	0.57[a]	0.30–1.06[a]
					0.99[b]	1.02[b]	0.71–1.39[b]
					0.07[c]	1.74[c]	0.93–3.17[c]
	CC	29 (0.11)	2 (0.04)	32 (0.08)	0.29[a]	2.21[a]	0.56 – 10.05[a]
VTCN1					0.26[b]	1.37[b]	0.79 – 2.41[a]
rs10923223					0.53[c]	0.62[c]	0.13 – 2.40[c]
(T/C)	Allele						
	T	349 (0.67)	66 (0.66)	529 (0.69)	R	1	-
	C	171 (0.33)	34 (0.34)	235 (0.31)	0.83 [a]	0.95[a]	0.60 – 1.49[a]
					0.42[b]	1.10[b]	0.86 – 1.39[b]
					0.51[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 $p < 0.025$ due to Bonferroni's correction.

2.3.3 Linkage disequilibrium and haplotype analyses:

Linkage disequilibrium (LD) analysis revealed that two polymorphisms investigated i.e., *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) were in moderate LD association (D' =0.683, r^2 =0.260). Haplotype evaluation of the two polymorphic sites was also done and no significant difference was found in the estimated frequencies of haplotypes between patients and controls (global *p*=0.170; Table 2.9).

Table 2.9. 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)	0.370	0.170	1.13	0.86-1.48
C C	67.60(0.14)	74.49(0.17)	0.193		0.78	0.55-1.12
T T	16.60(0.03)	24.49(0.05)	0.109		0.59	0.31-1.13
T C	83.40(0.17)	64.51(0.15)	0.309		1.20	0.84-1.71

CI represents Confidence Interval

(Frequency <0.03 in both control & case was dropped and ignored in the analysis).

2.3.4 Bioinformatics analysis of *VTCN1* rs12046117 (C/T) and rs10923223 (T/C) 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 2.8). No ChIP, Motif, and QTL data were available for both SNPs.

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

Table 2.10. *In silico* analysis results for *VTCN1* rs12046117 and rs10923223 polymorphisms

2.3.5 Analysis of *VTCN1* and *NRD1* transcripts levels from PBMCs:

To assess the possible involvement of VTCN1 in vitiligo disease, we analyzed *VTCN1* transcripts from PBMCs of 188 patients with vitiligo and 201 healthy controls. Interestingly, a remarkable reduction of *VTCN1* transcripts in vitiligo patients (p=0.023) was noticed. A -2.38-fold decrease in the expression of *VTCN1* transcript levels in patients was revealed in comparison to controls (Fig. 2.2 A). Moreover, analysis of the type of Vitiligo revealed that *VTCN1* transcripts were remarkably decreased in patients with GV as compared to controls (p=0.034; Fig 2.2 B), whereas patients with LV divulged no change in *VTCN1* transcript levels (p=0.664). Also, no notable change was seen in the transcripts between GV vs LV (p=0.896). Besides, when we analyzed the results according to the activity of Vitiligo, AV patients displayed a remarkable decrease in *VTCN1* transcripts compared to controls (p=0.037; Fig. 2.2 C). Moreover, SV patients revealed no change in *VTCN1* transcripts compared to controls (p=0.984). Additionally, no change was seen in transcript levels between AV and SV patients (p=0.521).

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=0.034; Fig. 2.2 E). 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=0.049; Fig. 2.2 F), whereas patients with LV displayed no significant change compared to controls (p=0.701). Additionally, no significant change was observed between patients with GV and LV (p=0.896). 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=0.047; Fig. 2.2 G). Moreover, SV patients revealed no significant difference in *NRD1* transcripts compared to controls (p=0.659). Also, no change was observed between patients with AV and SV (p=0.912).

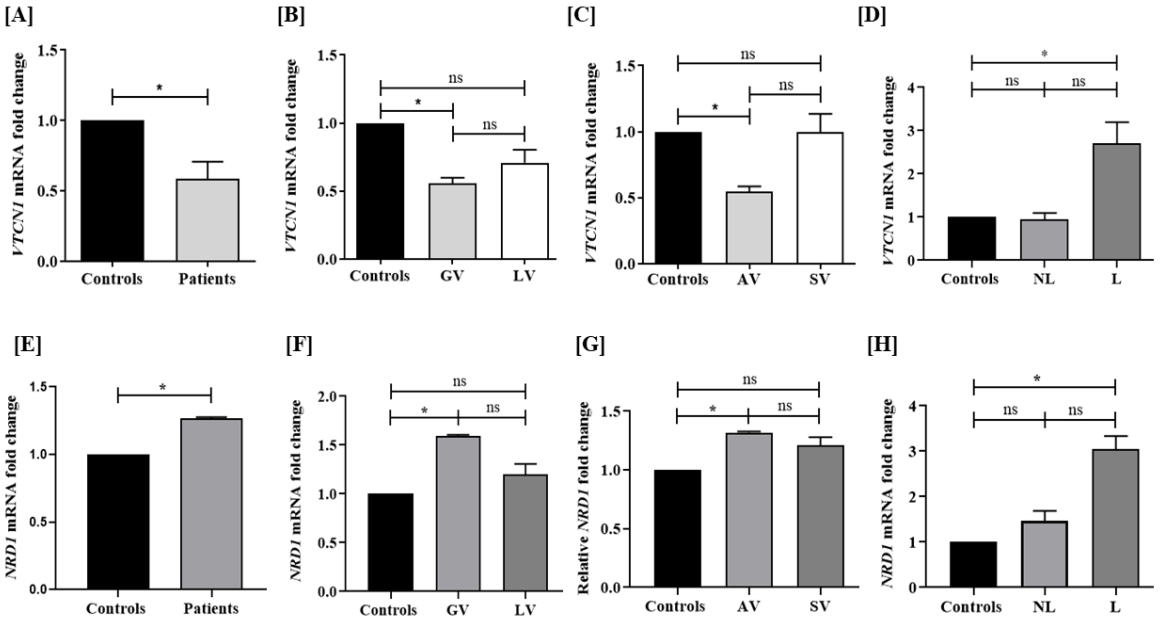


Figure 2.2: *VTCN1* and *NRD1* transcript levels in PBMCs and skin of patients with Vitiligo and controls [A] VTCN1 expression in patients with Vitiligo (n=188) and controls (201) were analyzed. Patients divulged a remarkable decrease in *VTCN1* transcripts with reference to controls (mean ΔCp ± SEM 4.883 ± 0.2408 vs. 4.082 ± 0.2530; p=0.0227). 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=0.034). **[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=0.037). **[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=0.039). 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 (190) was analyzed. Patients disclosed a remarkable increase in *NRD1* transcripts with reference to controls (mean ΔCp ± SEM 3.800 ± 0.1047 vs. 4.148 ± 0.1279; p=0.034). 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=0.049). **[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= 0.047). **[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=0.021). 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.

2.3.6 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=0.039; Fig. 2.2 D). The fold change analysis divulged a 2.70-fold higher expression of *VTCN1* transcripts in lesional skin compared with healthy skin, but no change was seen in non-lesional skin of patients compared to control skin (p=0.903). Also, no change in transcript levels was seen between patients' lesional skin and non-lesional skin (p=0.155).

Analysis of *NRD1* transcript levels from the same skin biopsies of 12 vitiligo patients and 24 controls revealed a notable increase in *NRD1* mRNA levels in lesional skin of patients in comparison to control skin (p=0.021; Fig. 2.2 H). 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 to control skin (p =0.664). Also, no remarkable change was observed between patients' lesional skin and non-lesional skin (p =0.265).

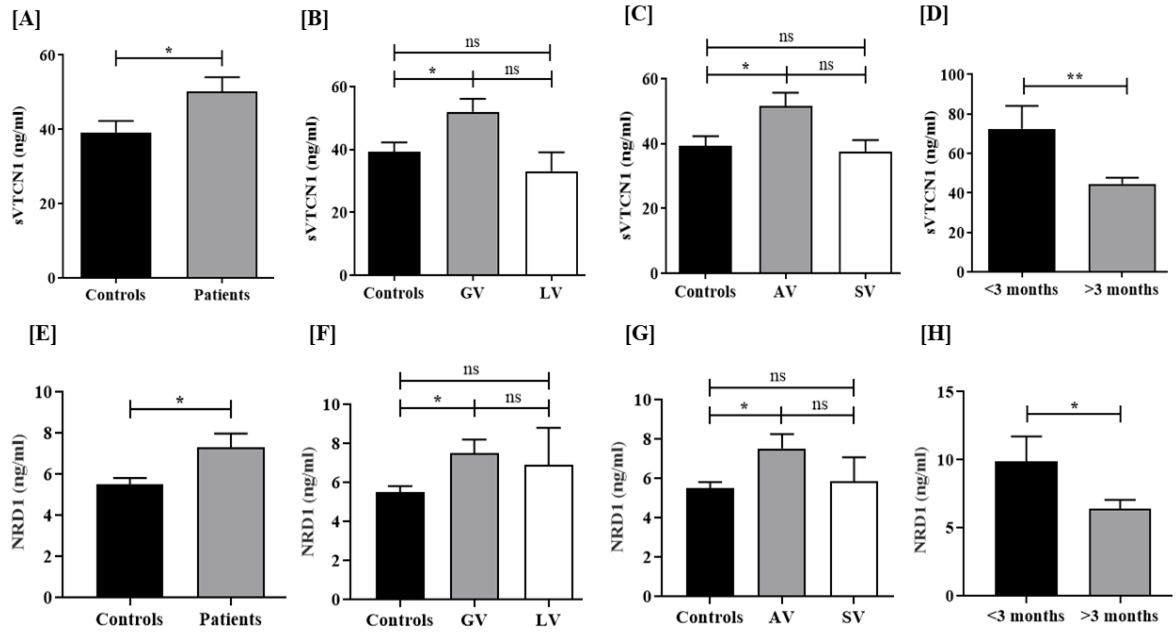
2.3.7 Analysis of plasma sVTCN1 and NRD1 levels:

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=0.026; Fig. 2.3 A). Interestingly, dividing the patient population according to the type and activity of Vitiligo revealed significantly higher sVTCN1 in patients with GV and AV compared to controls (p=0.030 and p=0.041, respectively; Fig. 2.3 B and 2.3 C), but there were no remarkable changes observed in patients with LV and SV for sVTCN1 levels (p=0.856 and p=0.987, respectively). Also, no change in sVTCN1 levels was noticed between GV vs LV and AV vs SV patients (p=0.235 and p=0.389, respectively). Further, 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=0.002; Fig. 2.3 D) as compared 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=0.015; Fig. 2.3 E). 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=0.027 and p=0.023, respectively; Fig. 2.3 F and 2.3 G), but there was no significant difference found in patients with LV and SV (p=0.735 and p=0.975, respectively). Moreover, no difference was observed in NRD1 levels between GV vs LV and AV vs SV patients (p=0.949 and p=0.538, 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=0.030; Fig. 2.3 H) as compared to patients with disease duration >3 months.

Figure 2.3: Estimation of sVTCN1 and NRD1 levels in the plasma of patients with Vitiligo and controls [A] Comparison of sVTCN1 level in the plasma of 84 patients with Vitiligo and 81 healthy individuals showed a significantly higher sVTCN1 in patients compared to controls (p=0.026) **[B]** sVTCN1 levels were considerably high in patients with GV compared to healthy individuals (p=0.030) (Control n=81, GV=76, LV=8) **[C]** Patients with AV showed remarkably increased sVTCN1 levels as compared to controls (p=0.041)

(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=0.002) (<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 to controls (p=0.015) **[F]** NRD1 levels were considerably high in patients with GV compared to healthy individuals (p=0.035) (Control n=81, GV=75, LV=7) **[G]** Patients with AV divulged remarkably higher *NRD1* levels compared to controls (p=0.029) (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=0.030) (<3 months n=17, >3 months n=65).



2.3.8 Correlation of sVTCN1 with NRD1 in 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 in plasma of patients with Vitiligo (n=82, r=0.59, p=0.00029, Fig. 3)

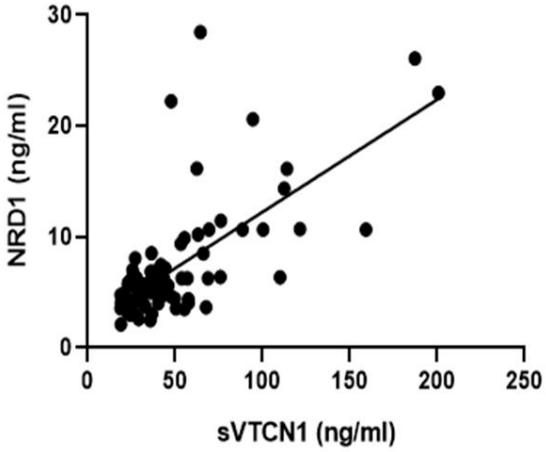


Figure 2.4: Correlation analysis of sVTCN1 with NRD1 levels in vitiligo individuals. Spearman's correlation analysis of sVTCN1 with NRD1 levels in 82 vitiligo patients was analyzed. Increased sVTCN1 levels were positively correlated with increased NRD1 levels in the same vitiligo patients (r=0.59, *p*=0.00029).

2.3.9 Decreased VTCN1 protein expression in vitiliginous skin:

To inspect the state of VTCN1 protein 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 (Fig. 4A). A remarkable decrease in VTCN1 protein levels was seen in lesional and perilesional vitiliginous skin compared to control skin (*p*=0.00025, *p*=0.0036; Fig. 4B). Also, a significant decrease was noticed in the lesional skin in comparison with non-lesional skin (*p*=0.0002; Fig. 4B). However, no difference was detected in the VTCN1 expression between healthy and non-lesional skin as well as non-lesional and perilesional skin.

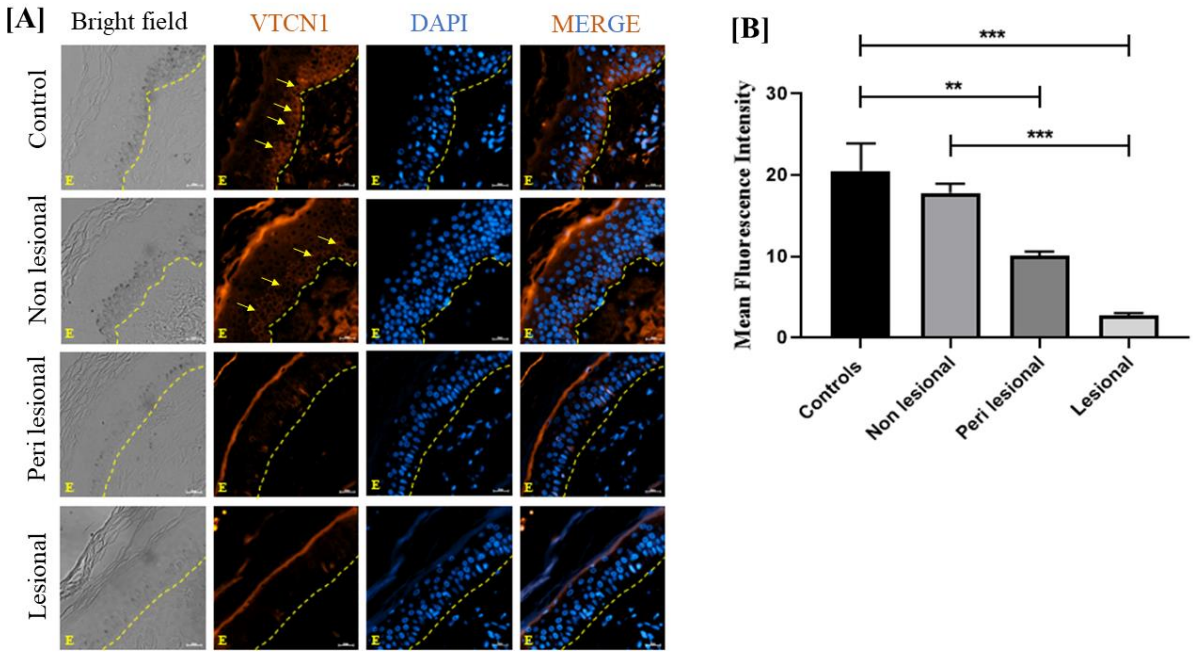


Figure 2.5: Immunofluorescence analysis of control skin, non-lesional, perilesional, and lesional skin of vitiligo patients [A] The expression of VTCN1 was observed in the epidermal basal layer of control skin and non-lesional skin of vitiligo patients. VTCN1 expression is shown with yellow arrows. A dashed yellow color line distinguishes the basal layer. Nuclei were stained blue by DAPI. (E: Epidermis, Magnification: 60X, Scale bar: 20µm) **[B]** The expression of VTCN1 was significantly lowered in lesional and perilesional skin of vitiligo patients compared to control skin (p=0.00025, p= 0.0036) (Control n=13, non-lesional skin n=12, Perilesional skin n=12, and Lesional skin n=12)

2.4 Discussion

Vitiligo has a well-established association with other autoimmune diseases such as Hashimoto's thyroiditis, Alopecia areata, Addison's disease etc. [27-28]. Infiltration of immune cells in the skin of vitiligo patients [29], decreased CD4/CD8 ratio, decreased Treg cells in the blood of vitiligo patients [30], disrupted systemic and local cytokine balance [31], higher local and peripheral endoplasmic reticulum stress in vitiligo patients [46], involvement of tissue resident memory T cells in the depigmentation [45] and several other studies have highlighted autoimmunity as a key etiopathological factor in vitiligo pathogenesis. Dysregulated co-stimulation contributes to the initiation and maintenance of autoimmunity due to the activation of autoreactive T-cells [32]. Various costimulatory molecules found to be essential for T cell activation such as CTLA4 which is a protein receptor that

downregulates the immune system and is found to be associated with Autoimmune Thyroid Disease (ATD) [33]. Several investigations also showed that CTLA4-Ig blocks the secondary signal required for T cell activation and it prevents structural damage in RA, thus may be used in RA therapeutics [34]. Decreased *CTLA4* transcripts were also reported in vitiligo patients [35]. VTCN1 is also a negative costimulatory molecule that binds to an unidentified receptor on T cell and inhibits T cell activation and its proliferation. VTCN1 was also associated with various autoimmune diseases such as RA [19], T1D [17] and SLE [21]. The immune regulatory function of VTCN1 was supported by the observation that in mouse model, VTCN1 deficiency worsens the pathophysiology of SLE [10]. Based on these studies, we aimed to investigate the role of VTCN1 in vitiligo pathogenesis.

One GWAS study revealed that SNPs mapped to the *VTCN1* gene found to be strongly associated with Juvenile Idiopathic Arthritis (JIA) and upon fine-mapping the intronic polymorphisms rs10923223 and rs12046117 showed the strongest association [24]. Association of the same SNPs also reported in 272 Caucasian JIA patients [36]. *VTCN1* rs12046117 also showed a novel association with SLE [37]. However, no association of these two SNPs was reported with RA in Dutch population [38]. Interestingly, genotyping of intronic *VTCN1* rs10923223 and rs12046117 SNPs in Gujarat vitiligo patients in the present study revealed no significant association of these polymorphisms with vitiligo susceptibility suggesting that other SNPs might be associated.

VTCN1 mRNA expression was reported in various organs such as brain, heart, kidney, liver, skin, lung, pancreas, placenta, spleen etc. [14]. However, despite the widespread expression of *VTCN1* mRNA, the presence of VTCN1 protein on the surface of normal cells is limited [16]. Our results suggest that *VTCN1* mRNA was significantly reduced in PBMCs of vitiligo patients as compared to controls (p=0.0346) which might lead to its decreased expression and might result in an overwhelming T cell response. Cytokines have been reported to affect the expression of the *VTCN1* transcript. IL-4 and GM-CSF are involved in the downregulation of VTCN1 expression in macrophages [39]. Elevated IL-4 levels were found in the blood of vitiligo patients [40], which might be one of the reasons for decreased *VTCN1* transcript levels observed in vitiligo patients. However, we found a significant increase in *VTCN1* expression in the skin of vitiligo patients as compared to controls. This might be due to differences in the local microenvironment of the vitiliginous skin and blood. Augmentation of *VTCN1* mRNA also reported in islets of prediabetic NOD mice compared to the diabetic

resistant strain [17]. Vitiligo and T1D are also considered as organ specific autoimmune diseases [42]. So, the observation supports discrepancy between transcript levels of *VTCN1* between blood and skin of vitiligo patients as the result of difference in local and systemic microenvironment.

Several studies reported high levels of soluble VTCN1 in sera of RA patients and also showed association with the disease severity [20]. An increase in sVTCN1 was also reported in SLE and LN (Lupus Nephritis) [21]. Elevated sVTCN1 levels are reported in T1D which may be used as a potential biomarker for T1D [17]. In the present study, we observed significantly higher sVTCN1 level in vitiligo patients as compared to controls (p=0.026). Significantly higher sVTCN1 levels were also observed in vitiligo patients with early-onset (< 3 months) as compared to longer duration of the disease (> 3 months) suggesting its role in development of the disease. Increased transcript levels of *VTCN1* in the skin of vitiligo patients might be due to the compensatory mechanism for the loss of membrane-tethered VTCN1 protein. Cytoplasmic VTCN1 was reported in the murine epidermal cells of haired skin [44]. We found VTCN1 expression in the basal layer of epidermal cells in healthy individual, while very low expression of VTCN1 protein in lesional skin of vitiligo patients.

Proteolytic cleavage of membrane-tethered VTCN1 by NRD1 metalloproteinase was firstly demonstrated in T1D. It is also responsible for elevated sVTCN1 levels in T1D patients [17]. Nardilysin is also reported to be involved in regulating immune response via processing antigenic self-peptides [19]. Reports also showed elevated sVTCN1 and increased NRD1 expression in RA patients [20,43]. So, we also investigated *NRD1* transcript levels from blood and skin of vitiligo patients and found a significant increase in *NRD1* transcripts in PBMCs and skin of vitiligo patients as compared to controls. We have also found increased plasma NRD1 levels in vitiligo patients as compared to controls. Our observations indicate that increased *NRD1* transcript levels in PBMCs and skin, and increased plasma NRD1 protein levels might lead to the cleavage of membrane tethered VTCN1 and ultimately result in increased sVTCN1. Correlation analysis of plasma sVTCN1 and NRD1 from the same vitiligo patients also revealed positive correlation.

Overall, the present study suggested no significant association of the intronic polymorphisms of *VTCN1* (rs12046117 & rs10923223) with vitiligo susceptibility in Gujarat population. However, elevated sVTCN1 protein levels and increased NRD1 protein levels were observed

in the plasma of vitiligo patients. Moreover, increased *NRD1* transcript levels were observed in the skin as well as blood of vitiligo patients as compared to controls. Interestingly, elevated sVTCN1 protein levels were positively correlated with increased NRD1 protein levels in plasma suggesting a possible role of NRD1 in VTCN1 shedding from the membrane. Shedding of negative costimulatory molecule, VTCN1 will compromise T cell inhibition and this may result in an exaggerated T cell response against melanocytes in vitiligo. In line with that, we also observed decreased VTCN1 protein in the lesional skin of vitiligo patients (Figure 2.6). The findings of this study provide new insight into immune dysregulation reported in vitiligo and may lead for developing therapeutic strategies using an immunomodulatory approach to combat vitiligo.

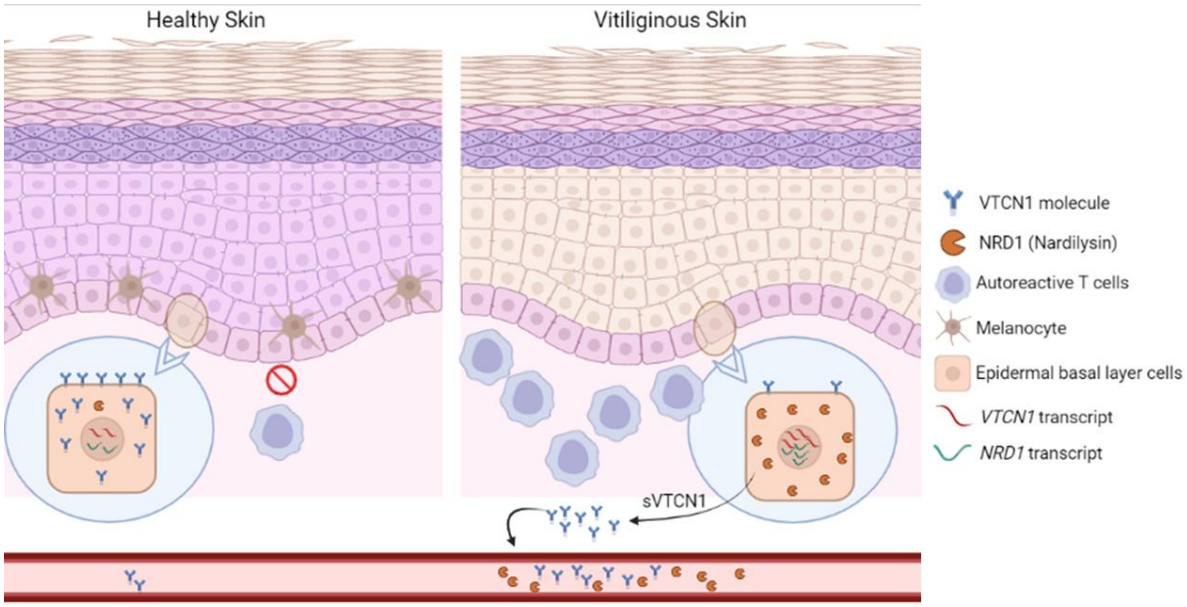


Figure 2.6: VTCN1 in vitiligo pathogenesis. The present study showed significantly higher sVTCN1 levels as well as its cleaving enzyme NRD1 levels in the blood of vitiligo patients. Moreover, we found a positive correlation between sVTCN1 and NRD1 from the same vitiligo patients suggesting a possible 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.

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