7A.1 INTRODUCTION

Vitiligo is an acquired hypomelanotic pigmentary disorder characterized by the presence of circumscribed depigmented macules in the skin. It is caused due to the loss of functional melanocytes of unknown etiology. Studies have revealed a worldwide incidence ranging from 0.04-2.16 % (Krüger *et al.,* 2012). As per our earlier studies 21.93% of vitiligo patients exhibit family history and 13.68% patients have first degree relatives affected, implicating involvement of genetic predisposition (Shajil & Begum, 2006). The etiology of vitiligo remains obscure despite being in focused debate for several years (Laddha *et al.,* 2013 ; Mansuri *et al.,* 2014).Various hypotheses have been proposed to understand vitiligo pathomechanisms for e.g. autoimmune, neural and oxidative stress theories which alone or in various combinations contribute towards vitiligo precipitation. Increasing evidences including our previous studies propose that genetic polymorphisms of *TNFA* (Laddha *et al.,* 2012) , *IL4* (Imran *et al.,* 2012), *IFNG* and *ICAM1* (Dwivedi *et al.,* 2013), *IL1B* (Laddha *et al.,* 2014), *TNFB* (Laddha *et al.,* 2013; Laddha *et al.,* 2014), different isoforms of *SOD* (Laddha *et al.,* 2013), *CAT* (Shajil *et al.,* 2007; Mansuri *et al.,* 2017), *NLRP1* (Dwivedi *et al.,* 2013), *CTLA4* (Dwivedi *et al*., 2011), *MYG* (Dwivedi *et al*., 2013), *GPX1* (Mansuri *et al*., 2016) might be playing a crucial role in vitiligo susceptibility. Genetic studies on vitiligo have revealed that the genetic variants, which are potentially involved in the autoimmunity could be responsible for conferring susceptibility or protection towards vitiligo. A key component in the inflammatory response is the increased production of pro-inflammatory cytokines such as *IL1B* (Laddha *et al*., 2014), *IFNG* (Dwivedi *et al.,* 2013; Natarajan *et al*., 2014) and TNF-α (Laddha *et al*., 2012). Moreover recently, we have further substantiated our previous findings by demonstrating miRNA mediated increased expression of *IL1B* and *IL1R1* in vitiligo patients (Mansuri *et al*., 2016).

The importance of IL-1 regulation is evident by the presence of natural antagonist, decoy receptor and other IL-1 family members for its fine regulation. The IL-1 family consists of the cytokines IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RN), mapped on chromosome 2q14 (Smith *et al*., 2000; Patterson *et al.,* 1993). IL-1 has two

receptors IL-1RI is the functional receptor capable of mediating downstream signaling whereas IL-1RII acts as a decoy receptor (Arend and Guthridge, 2000). The IL-1RN is a unique naturally occurring cytokine that inhibits IL-1 activity by binding to the IL-1 receptors without signal transduction and acts as a negative regulator with anti-inflammatory effects (Granowitz *et al*., 1991). Apart from the presence of natural antagonist IL-1RN for IL1, various regulatory inhibitory molecules like IL-1RII, SIGIRR/TIR8, soluble IL-1RAcP, soluble IL-1RI or RII are present for the regulation of IL1 levels (Weber *et al*., 2010). Studies have shown that mice deficient in IL1RN exhibit reduced reproduction, stunted growth and develop spontaneous diseases in response to carcinogens (Zitvogel *et al*., 2012).

Aside from the nearness of normal rival IL-1RN for IL1, different administrative inhibitory particles like IL-1RII, SIGIRR/TIR8, solvent IL-1RAcP, dissolvable IL-1RI or RII are available for the direction of IL1 levels proposing significance of IL1 in keeping up resistant framework homeostasis

The *IL1RN* gene sequence shows 86-bp variable number tandem repeat (VNTR) in intron 2. This polymorphism has six alleles, comprising of 1-6 repeats of 86-bp sequence. The four-repeat (*IL1RN**1) and two-repeat (*IL1RN**2) alleles are most common, while the others occur at a combined frequency of lower than 5% (Table 3) (Tarlow *et al*., 1993; Vamvakopoulos *et al.,* 2002). The number of repeats may be of functional significance as these repeats contain putative binding sites for transcription factors which need further investigation (Tarlow *et al*., 1993). The role of *IL1RN* intron 2 VNTR (rs2234663) is well established with development of inflammatory disorders (Fischer *et al.,* 1992; McIntyre *et al.,* 1991; Xu *et al.,* 2011). The pro-inflammatory cytokine, IL1B and its antagonist, IL1RN are encoded by polymorphic genes (Wilkinson *et al*., 1991).

An association between polymorphism in the *IL1RN* intron 2 VNTR (rs2234663) and autoimmune disorders including vitiligo has been reported (Zaaber *et al*., 2014; Pehlivan *et al.,* 2009). Till date, there are no reports from India regarding rs2234663 and vitiligo. The aim of the present study was to investigate the association between *IL1RN* intron 2 VNTR polymorphism (rs2234663) with vitilgo; to assess *IL1RN*

transcript levels from PBMCs; and to perform possible genotype–phenotype correlation using case-control approach.

7A.2 MATERIALS AND METHODS

7A.2.1 Study Subjects

The study group included 307 vitiligo patients and 316 ethnically age and sex matched unaffected individuals who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). We used the clinical criteria to define the stability proposed by Falabella *et al*., (1995) discussed in the Vitiligo Global Issues Consensus Conference, 2012. (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active disease was defined as, appearance of new or spreading of existing lesions observed during past two years. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and lack of association with other autoimmune disorders. The importance of the study was explained to all participants along with written consent form. The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR).

Table 1: Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 307)	(n = 316)
Average age (mean age ± SD)	38.23 ± 19.15 yr	29.41 ± 12.16 yr
Sex: Male	156 (50.81%)	171(54.11%)
Female	151 (49.18%)	145 (45.88%)
Age of onset (mean age ± SD)	31.70±19.51 yr	NA
Duration of disease (mean ± SD)	7.723±9.154 yr	NA
Type of vitiligo		
Generalized	208(67.75%)	NA
Localized	99 (32.24%)	NA
Active vitiligo	242 (78.80%)	NA
Stable vitiligo	65 (21.19%)	NA

7A.2.2 Genotyping of *IL1RN* intron 2 VNTR (rs2234663) polymorphism

Polymerase chain reaction (PCR) was used to genotype rs2234663 polymorphism of *IL1RN*. The primers used for genotyping are mentioned in Table 2 and the product size for different alleles along with number of VNTR repeats has been described in Table 3.

The reaction mixture of the total volume of 20 μL included 5 μL (100 ng) of genomic DNA, 10 μL nuclease-free H_2O, 2.0 μL 10x PCR buffer, 2 μL 2 mMdNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 μL of 10 μM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 μL (5U/μL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a Mastercycler gradient (Eppendorf, Germany) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table S2) for 30 seconds, and 72°C for 30 seconds. The amplified products along with 50 bp DNA ladder (Invitrogen, USA) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. 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).

7A.2.3 Genomic DNA Preparation

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, USA). Genomic DNA was extracted from whole blood using QIAamp DNA Blood Kit (QIAGEN Inc., Valencia, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically. Quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analyses.

7A.2.4 RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambioninc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis while the RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambioninc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed

using the Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the CG Palm Cycler (Genetix, India).

7A.2.5 Real-time PCR

The expression of *IL1RN* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table S2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10s, 66°C and 65°C for 20 s for *IL1RN* and *GAPDH* respectively, 72°C for 20 s). 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 value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

Table 2: Primers used for genotyping and gene expression analysis.

Gene/Polymorphism Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing Temperature
IL1RN intron 2 VNTR F	CTCAGCAACACTCCTATTG	160+ n*86 (n=2-6)	62°C
IL1RN intron 2 VNTR R	TCATCTTCCTGGTCTGCA		
IL1RN expression F	GCTGGAGGCAGTTAACATC	165	66°C
IL1RN expression R	CTACTCGTCCTCCTGGAAG		

GAPDH expression F	CATCACCATCTTCCAGGAGCGAG	122	65°C
GAPDH expression R	CCTGCAAATGAGCCCCAGCCT		

Table 3: Different alleles of *IL1RN* intron 2 VNTR (rs2234663) polymorphism.

Allele	Size (bp)	No. of repeats
A1	418	4
A2	246	2
A3	332	3
A4	504	5
A5	590	6
A6	160	1

7A.2.6 Statistical analyses

The distribution of the genotypes and allele frequencies of rs2234663 for patients and control subjects were compared using chi-squared test with 2×2 contingency tables using Prism 3 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of *IL1RN* and genotype-phenotype correlation analysis in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 3 software. The A1/1 is considered as ancestral genotype, A2/2 is considered as mutant genotype while all the heterozygous genotypes grouped together with fewer repetitions genotypes. Finally, to compare the expected genotype frequencies against the observed frequencies, we used a chi-square test applied through the Hardy–Weinberg equilibrium.

7A.3 RESULTS

7A.3.1. Analysis of association between *IL1RN* intron 2 VNTR polymorphism (rs2234663) and susceptibility to vitiligo:

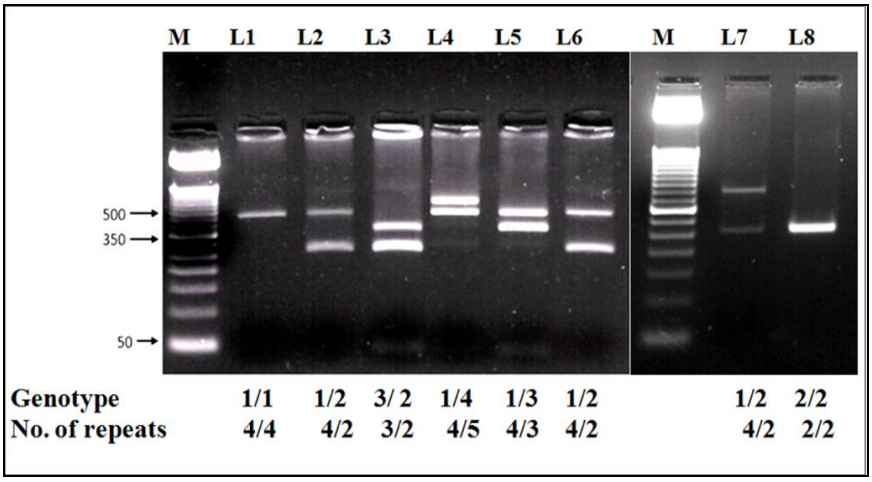

Figure 1: PCR amplification analysis of *IL1RN* VNTR polymorphism on 3.5% Agarose gel: M: DNA Marker; Lane 1: *ILRN**1/1; Lanes 2, 6 and 8: *ILRN**1/2; Lane 3: *ILRN**3/2; Lane 4: *ILRN**1/4; Lane 5: *ILRN**1/3; Lane 7: *ILRN**1/2.

Eight genotypes were identified in Gujarat population as shown in Figure1. Both patients and controls were under Hardy Weinberg equilibrium p = 0.6835 and p = 0.6003, respectively. Our studies suggested that there was no significant association of genotype or allele frequency among vitiligo patients and controls. When genotypes were compared between patients and controls we found: *IL1RN**1/2 (p=0.8946), *IL1RN**2/2 (p=0.8390), *IL1RN**3/2 (p=0.3144), *IL1RN**3/1 (p=0.9909), *IL1RN**4/2 (p=0.7250), *IL1RN**1/4 (p=0.5066), *IL1RN**5/2 (p=0.1623). Similarly when alleles were compared between patients and controls we found non-significant difference: *IL1RN**A2 (p =0.7177), *IL1RN**A3 (p=0.5554), *IL1RN**A4 (p =0.6805) and *IL1RN**A5 (p =0.1611) (Table 4). However, we observed *IL1RN**3/2 genotype to be present only in the vitiligo patients, whereas *IL1RN**5/2 genotype was observed only in the control group. Further stratification of results based on type, activity and gender of vitligo revealed non-significant difference both in genotype and allele frequencies among different groups. Intrestingly, we observed significant difference in *IL1RN**A1/2 genotype distribution between active vitiligo (AV)

(47.10%) and stable vitiligo (SV) pateints (33.84%) (p=0.0172). Significant difference was observed in genotype frequencies between SV (6.15%) and controls (14.87%) of *IL1RN**A2/2 (p=0.0246). Additionally, we found significant difference in allele frequency of *IL1RN**A2 between AV (35.95%) and SV (23.07%) (p=0.0060); and when SV (23.07%) were compared to controls (36.07%) (p=0.0046).

Further, when genotypes were compared between AV and SV we found: *IL1RN**1/2 (p=0.0172), *IL1RN**2/2 (p=0.0639), *IL1RN**3/2 (p=0.3740), *IL1RN**4/2 (p=0.3740), *IL1RN**1/4 (p=0.7219). Similarly upon allele comparison between AV and SV we found: *IL1RN**A2 (p =0.0060), *IL1RN**A3 (p =0.4225), *IL1RN**A4 (p =0.7192). When genotypes were compared between controls and AV we found: *IL1RN**1/2 (p=0.2146), *IL1RN**2/2 (p=0.5455), *IL1RN**3/2 (p=0.0940), *IL1RN**3/1 (p=0.4022), *IL1RN**4/2 (p=0.6952), *IL1RN**1/4 (p=0.6439), *IL1RN**5/2 (p=0.2369). Similarly when alleles were compared between controls and AV we found: *IL1RN**A2 (p =0.9599), *IL1RN**A3 (p =0.4182), *IL1RN**A4 (p =0.9971), *IL1RN**A5 (p =0.2157).

When genotypes were compared between controls and SV we found: *IL1RN**1/2 (p=0.1016), *IL1RN**2/2 (p=0.0246), *IL1RN**3/1 (p=0.5970), *IL1RN**4/2 (p=0.2916), *IL1RN**1/4 (p=0.4605), *IL1RN**5/2 (p=0.4550). Similarly when alleles were compared between controls and SV we found: *IL1RN**A2 to be significantely (p =0.0046), *IL1RN**A3 (p =0.6190), *IL1RN**A4 (p =0.7080), *IL1RN**A5 (p =0.4820) (Table 5).

Genotype and allele frequencies when compared among generalized vitiligo, localised vitilgo and controls; male and female; duration of disease and genotypes no association was found within different sub groups analysis (Tables 6 and 7; Figure 3).

7A.3.2 Relative gene expression analysis of *IL1RN* using case-control approach

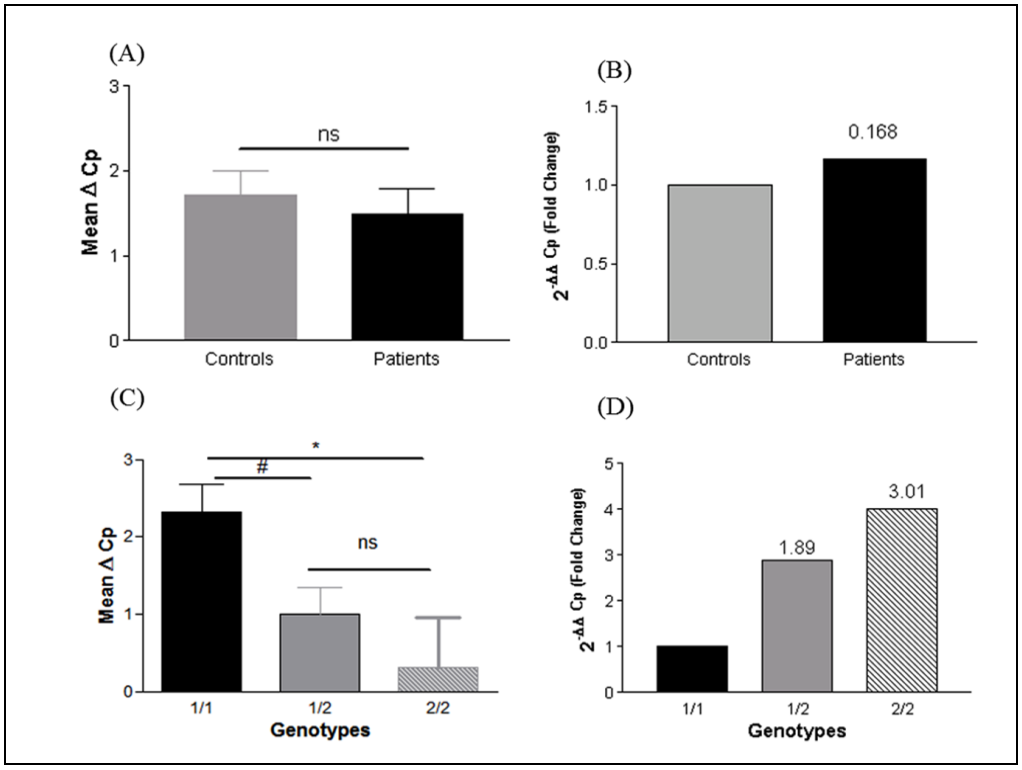

Figure 2: Relative gene expression analysis of *IL1RN* in vitiligo patients and controls.

(A) Expression of *IL1RN* transcripts in controls (n=36) and vitiligo patients (n=36), as suggested by Mean ΔCp. Vitiligo patients showed non-significant difference in mRNA levels of *IL1RN* as compared to controls ($p = 0.5962$). **(B)**Vitiligo patients showed 1.163 fold higher *IL1RN* expressions as compared to controls, as determined by $2^{-\Delta\,\Delta Cp}$ method. **(C)** Genotype-phenotype correlation of *IL1RN* transcript levels: When mean ΔCp for frequent genotypes *IL1RN**2/2 and *IL1RN**1/1 were compared we got significant increase in *IL1RN* expression in *ILRN* A2 carriers (**p* value= 0.03); additionally when mean ΔCp for frequent genotypes *IL1RN**1/1 and *IL1RN**2/1 were compared significant increase in *IL1RN* expression was observed ($^{\#}p$ value=0.01). However, when *IL1RN**2/2 and *IL1RN**1/2 mean ΔCp for genotypes were compared we obtained non-significant difference (^{ns}p value=0.45). **(D)** 1.89 increase in the fold change of *IL1RN* expression was observed when *IL1RN**1/1 and *IL1RN**2/2 genotype were compared; and 3.01 increase in

the fold change of *IL1RN* expression was observed when *IL1RN**1/1 and *IL1RN**1/2 genotype were compared.

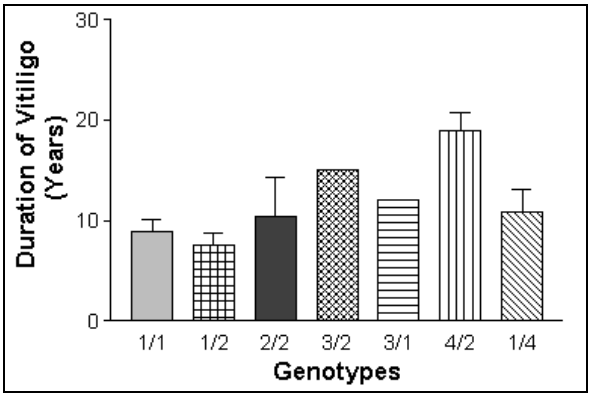

Figure 3: Correlation analysis for duration of disease and different genotypes of vitiligo patients. No significant difference was observed among different genotypes and duration of disease.

Relative gene expression analysis in 36 patients and 36 controls revealed a non-significant difference in expression of *IL1RN* transcripts in patients as compared to controls (Mean ΔCp ± SEM: 1.784±0.61659 vs 1.940±0.3340; p=0.5962), after normalization with *GAPDH*. The $2^{-\Delta\Delta Cp}$ analysis showed non-significant difference (0.168 fold increase) in the expression of *IL1RN* in patients, compared to controls (Figure 2 A, B). However, further stratification of data based on type, activity and gender of vitligo revealed no significant difference in expression level (data not shown).

7A.3.3 Genotype-phenotype correlation analysis of *IL1RN* levels:

Genotype-phenotype correlation of *IL1RN* levels showed significant increase in *IL1RN* expression in *IL1RN**A2 genotypes. Significant increase in transcript level was observed when mean ΔCp for frequent genotypes *IL1RN**2/2 and *IL1RN**1/1 were compared (p =0.03). Also, when mean ΔCp was compared for *IL1RN**1/1 and *IL1RN**2/1 genotypes, *IL1RN**A2 containing genotypes showed higher expression (p=0.01). However, non-significant difference in expression level of *IL1RN* was found for genotypes *IL1RN**2/2 and *IL1RN**2/1 (p=0.45) (Figure 2 C, D).

Table 4: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in vitiligo patients and controls from Gujarat population.

Genotype or allele	Vitiligo Patients (Freq. %)	Controls (Freq. %)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 307**	**n=316**			
*IL1RN** (A 1/1)	123 (40.06)	125(39.55)	R	1	-
*IL1RN** (A 1/2)	123 (40.06)	128(40.50)	0.8946	0.9766	0.6874-1.387
*IL1RN** (A 2/2)	44 (14.33)	47(14.87)	0.8390	0.9514	0.5883-1.539
*IL1RN** (A 3/2)	1 (0.32)	0(0)	0.3144	3.049	0.1229-75.62
*IL1RN** (A 3/1)	1 (0.32)	1(0.31)	0.9909	1.016	0.06282-16.44
*IL1RN** (A 4/2)	3 (0.97)	4(1.26)	0.7250	0.7622	0.1671-3.478
*IL1RN** (A 1/4)	12 (3.90)	9(2.84)	0.5066	1.355	0.5511-3.332
*IL1RN** (A 5/2)	0(0)	2(0.63)	0.1623	0.2032	0.009652-4.280
Allele					
*IL1RN**A1	382 (62.21)	388(61.39)	R	1	-
*IL1RN**A2	215 (35.01)	228(36.07)	0.7177	0.9578	0.7581-1.210
*IL1RN**A3	2 (0.32)	1 (0.15)	0.5554	2.031	0.1833-22.51
*IL1RN**A4	15 (2.44)	13 (2.05)	0.6805	1.172	0.5502-2.496
*IL1RN**A5	0 (0)	2 (0.31)	0.1611	0.2031	0.0097-4.248

'n' represents number of Patients/ Controls,
'R' represents reference group
CI refers to Confidence Interval,
(P) refers to Patients and (C) refers to Controls,
[a]Vitiligo Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Vitiligo Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table, A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 5: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in active and stable vitiligo patients & controls from Gujarat population.

Genotype or allele	Active Patients (Freq.%)	Stable Patients (Freq.%)	Controls (Freq.%)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 242 (78.80)**	**n=65 (21.19)**	**n=316**			
*IL1RN**(A 1/1)	88 (36.36)	35 (53.84)	125 (39.55)	R	1	-
*IL1RN**(A 1/2)	114 (47.10)	22 (33.84)	128 (40.50)	0.0172[a]	2.061[a]	1.129 - 3.761[a]
				0.2146[b]	1.265[b]	0.8724 – 1.835[b]
				0.1016[c]	0.6138[c]	0.3411- 1.105[c]
*IL1RN**(A 2/2)	28 (11.57)	4 (6.15)	47 (14.87)	0.0639[a]	2.787[a]	0.9095- 8.522[a]
				0.5455[b]	0.8462[b]	0.4923- 1.455[b]
				0.0246[c]	0.3040[c]	0.1024- 0.9020[c]
*IL1RN**(A 3/2)	2 (0.82)	0 (0.00)	0 (0)	0.3740[a]	2.006[a]	0.0938 - 42.86[a]
				0.0940[b]	7.090[b]	0.3360- 149.6[b]
				-	-	-
*IL1RN**(A 3/1)	0 (0.00)	0 (0.00)	1 (0.31)	-	-	-
				0.4022[b]	0.4727[b]	0.0190- 11.75[b]
				0.5970[c]	1.178[c]	0.0469 – 29.58[c]

*IL1RN**(A 4/2)	2 (0.82)	0 (0.00)	4 (1.26)	0.3740 [a]	2.006 [a]	0.0938-42.86 [a]
				0.6952[b]	0.7102[b]	0.1272-3.965[b]
				0.2916[c]	0.3928[c]	0.0206-7.476[c]
*IL1RN**(A 1/4)	8 (3.305)	4 (6.15)	9 (2.84)	0.7219 [a]	0.7955 [a]	0.2250-2.812 [a]
				0.6439[b]	1.263[b]	0.4687-3.401[b]
				0.4605[c]	1.587[c]	0.4610-5.465[c]
*IL1RN**(A 5/2)	0 (0.00)	0 (0.00)	2 (0.63)	0.2369[b]	0.2836[b]	0.0134-5.984[b]
				0.4550[c]	0.7070[c]	0.0331-15.08[c]

Allele						
*IL1RN**A1	298 (61.57)	96 (73.84)	388 (61.39)	R	1	-
*IL1RN** A2	174 (35.95)	30 (23.07)	228 (36.07)	0.0060 [a]	1.868 [a]	1.191 – 2.932 [a]
				0.9599[b]	0.9936[b]	0.7750-1.274[b]
				0.0046[c]	0.5318[c]	0.3420-0.8270[c]
*IL1RN** A3	2 (0.41)	0 (0.00)	1 (0.15)	0.4225 [a]	1.6160 [a]	0.07688-33.99 [a]
				0.4182[b]	2.604[b]	0.2349- 28.87[b]
				0.6190[c]	1.342[c]	0.0542-33.22[c]
*IL1RN**	10 (2.06)	4 (3.07)	13 (2.05)	0.7192	0.8054	0.2469-2.627 [a]

A4				[a]	[a]	
				0.9971[b]	1.002[b]	0.4331-2.316[b]
				0.7080[c]	1.244[c]	0.3965-3.900[c]
*IL1RN** A5	0 (0.00)	0 (0.00)	2 (0.31)	-	-	-
				0.2157[b]	0.2603[b]	0.0124 –5.446 [b]
				0.4820[c]	0.8052[c]	0.0383 –16.92[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Active Vitiligo vs. Stable Vitiligo,
[b]Active Vitiligo vs. Controls,
[c]Stable Vitiligo vs. Controls.
A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 6: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in generalised and localized vitiligo patients & controls from Gujarat population.

Genotype or allele	Generali-sed Patients (Freq.%)	Localised Patients (Freq.%)	Control	*p* value	Odds ratio	CI (95%)
Genotype	**n= 208**	**n=99**	**n=316**			
*IL1RN** (A 1/1)	92 (44.23)	37 (37.37)	125 (39.55)	R	1	-
*IL1RN** (A 1/2)	87 (41.82)	40 (40.40)	128 (40.50)	0.6235[a]	0.8747 [a]	0.5125-1.493 [a]
				0.6837[b]	0.9235 [b]	0.6296-1.355 [b]
				0.8350[c]	1.056 [c]	0.6336-1.759 [c]
*IL1RN**	21	17	47	0.0629[a]	0.4968[a]	0.2359-1.046[a]

(A 2/2)	(10.09)	(17.17)	(14.87)	0.0903[b]	0.6071 [b]	0.3396-1.085 [b]
				0.5543 [c]	1.222 [c]	0.6283-2.377[c]
*IL1RN** (A 3/2)	1 (0.48)	0 (0)	0 (0)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.2452 [b]	4.070 [b]	0.1638-101.1 [b]
				-	-	-
*IL1RN** (A 3/1)	1 (0.48)	0 (0)	1 (0.31)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.8286 [b]	1.359 [b]	0.0838-22.02 [b]
				0.5867 [c]	1.116 [c]	0.0444-27.98 [c]
*IL1RN** (A 4/2)	1 (0.48)	0 (0)	4 (1.26)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.3156 [b]	0.3397 [b]	0.0373 - 3.091 [b]
				0.2783 [c]	0.3719 [c]	0.0195 – 7.070 [c]
*IL1RN** (A 1/4)	5 (2.40)	5 (5.05)	9 (2.84)	0.1573[a]	0.4022[a]	0.1099-1.472[a]
				0.6234 [b]	0.7548 [b]	0.2448-2.328 [b]
				0.2782 [c]	1.877 [c]	0.5923-5.948 [c]
*IL1RN** (A 5/2)	0 (0)	0 (0)	2 (0.63)	-	-	-
				0.2266 [b]	0.2714 [b]	0.0129-5.724 [b]
				0.4425 [c]	0.6693 [c]	0.0314-14.26 [c]

Allele						
*IL1RN**A1	277 (66.58)	119 (60.10)	388 (61.39)	R	1	-
*IL1RN**A2	131 (31.49)	74 (37.37)	228 (36.07)	0.1323[a]	0.7605[a]	0.5322-1.087[a]
				0.1073[b]	0.8048[b]	0.6177-1.049[b]
				0.7391[c]	1.058[c]	0.7584-1.477[c]
*IL1RN**A3	2 (0.48)	0 (0)	1 (0.15)	0.3545[a]	2.153[a]	0.1025-45.22[a]
				0.3808[b]	2.801[b]	0.2526-31.07[b]
				0.5798[c]	1.084[c]	0.0438 - 26.80[c]
*IL1RN**A4	6 (1.44)	5 (2.52)	13 (2.05)	0.2736[a]	0.5155[a]	0.1543 - 1.723[a]
				0.3793[b]	0.6465[b]	0.2427-1.722[b]
				0.6725[c]	1.254[c]	0.4380 – 3.590[c]
*IL1RN**A5	0 (0)	0 (0)	2 (0.31)	-	-	-
				0.2326[b]	0.2800[b]	0.0134-5.859[b]
				0.4338[c]	0.6502[c]	0.0310-13.65[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Generalised vitiligo vs. Localised vitiligo,
[b]Generalised vitiligo vs. Controls
[c]Localised vitiligo vs. Controls.

A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 7: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in male and female vitiligo patients from Gujarat population.

Genotype or allele	Male Patients (Freq.%)	Female Patients (Freq.%)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 156**	**n=151**			
*IL1RN**(A1/1)	52 (33.33)	64 (42.39)	R	1	-
*IL1RN**(A1/2)	76 (48.71)	61 (40.39)	0.0915	0.6521	0.3965 – 1.073
*IL1RN**(A2/2)	20 (12.82)	18 (11.92)	0.4027	0.7313	0.3508 – 1.524
*IL1RN**(A3/2)	2 (1.28)	0 (0)	0.1205	1.628	0.0076- 3.468
*IL1RN**(A 3/1)	-	-	-	-	-
*IL1RN**(A4/2)	3 (1.92)	0 (0)	0.0584	0.1163	0.0059-2.304
*IL1RN**(A1/4)	3 (1.92)	8 (5.29)	0.2614	2.167	0.5468-8.585
*IL1RN**(A5/2)	-	-	-	-	-
Allele					
*IL1RN**A1	183 (58.65)	197 (65.23)	R	1	-
*IL1RN**A2	121 (38.78)	97 (32.11)	0.0837	0.7447	0.5330 – 1.041
*IL1RN**A3	2 (0.64)	0 (0)	0.1434	0.1858	0.0088-3.899
*IL1RN**A4	6 (1.92)	8 (2.64)	0.6966	1.239	0.4216-3.696
*IL1RN**A5	-	-	-	-	-

'n' represents number of Male/Female Patients,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Male vs. Female Patients (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Male vs. Female Patients (allele) using chi-squared test with 2 × 2 contingency table.
A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

7A.4 DISCUSSION

Cytokine imbalance in the skin and systemic circulation in vitiligo is well reported. The balance between IL-1 and IL-1RN in local tissues plays an important role in the susceptibility to and severity of many diseases (Fischer *et al*., 1992; Arend, 2000). Polymorphisms in regulatory gene regions of cytokines that affect transcription may account for differences at individual levels for cytokine production (Danis *et al*., 1995). The rs2234663 polymorphism has been associated with multiple diseases primarily of epithelial and endothelial origin along with various autoimmune disorders. The *IL1RN* intron 2 86 bp VNTR involvement may be resulting in an imbalance in the IL-1 system with increased IL-1β and reduced production of the major intracellular isoform of IL-1RN (Arend, 2000). Altogether vitiligo is considered to be an autoimmune disorder due to cytokine imbalance (between pro- and anti- inflammatory) with increased levels of IL-1α and *IL1B* in skin and PBMCs in vitiligo patients respectively (Laddha *et al*., 2014; Birol *et al*., 2006).

The *IL1RN* intron 2 VNTR of 86 bp repeat may be of functional significance as these repeats contain putative binding sites for transcription factors. Number of repeats may affect the strength of enhancer binding sequence which in turn influences the transcription of *IL1RN* (Tarlow *et al*., 1993). Interestingly, the differences in circulating levels of IL-1RN have been reported with different alleles of *IL1RN* VNTR (Danis *et al*., 1995; Mandrup-Poulsen *et al*., 1994). The rs2234663 has been reported to be associated with Hashimoto thyroiditis (Zaaber *et al*., 2014), juvenile idiopathic inflammatory myopathies (Rider *et al*., 2000), systemic lupus erythematosus (SLE) (Blakemore *et al*., 1994), and ulcerative colitis (UC) (Mansfield JC, Holden *et al*., 1994) including vitiligo (Pehlivan *et al.,* 2009). Conversely, no association was found for rs2234663 with SLE in Italians (D'Alfonso *et al*., 2000) suggesting the importance of ethnicity in disease predisposition. We found lack of *IL1RN**A6 (single repeat of 86 bp) in the present study. Our results showed that genotype and allele frequencies for rs2234663 did not differ between vitiligo patients and controls. Exceptionally, we found significant difference in *IL1RN**A1/2 genotype distribution between AV and SV

(p=0.0172). Significant difference was observed in genotype frequencies between SV and controls for *IL1RN**A2/2 (p=0.0246). Additionally, we found significant difference in allele frequency of *IL1RN**A2 between AV and SV (p=0.0060); and when SV were compared to controls (p=0.0046). Also, we observed *IL1RN**3/2 genotype only in vitiligo patients confering suspectibility towards vitiligo while *IL1RN**5/2 genotype was observed only in controls. Also, the pro-inflammatory immune response of individuals homozygous for the *IL1RN**A2 allele was found to be more prominent compared to other genotypes (Witkin *et al.,* 2002). The influence of the *IL1RN**A2 allele has been widely studied in multiple diseases such as inflammatory bowel disease, SLE (Blakemore *et al*., 1994), UC (Mansfield *et al*., 1994), Graves' disease, nephropathy in diabetes mellitus, alopecia areata, chronic inflammatory diseases and psoriasis. *IL1RN**A2 was associated with increased production of IL-IRN, and also reduced production of IL-lα by monocytes (Danis *et al*., 1995). On the contrary, *IL1RN**A2 is associated with reduced levels of IL-1RN and *IL1RN* mRNA in the colonic mucosa (Carter *et al*., 2004). The association of low IL-lα production with *IL1RN**A2 polymorphism may be a consequence of higher IL-1RN production in individuals with *IL1RN**A2 genotype (Danis *et al*., 1995). It has also been demonstrated that *IL1RN**A2 is associated with significantly reduced levels of IL-1RN in human umbilical vein endothelial cells (Dewberry *et al*., 2000). It has been speculated that the impact of this polymorphism is different in cells synthesizing different mRNA splice variants; those synthesizing the intracellular form produce less IL1RN when carrying *IL1RN**A2, in contrast to monocytes that synthesize predominantly sIL-1RN and produce more protein when of genotype *IL1RN*A2* (Danis *et al*., 1995). Whereas it did not alter steady state levels of *IL1RN* mRNA in cultured keratinocytes (Clay *et al*., 1996).

Similarly, there are reports on vitiligo from Turkish population (n=48) and from Korean population (n=31), where they have reported the absence of *IL1RN**1/5 genotypes and *IL1RN**A5 in vitiligo patients and lack of association of rs2234663 polymorphism respectively (Pehlivan *et al*., 2009; Lee *et al*., 1995). In accordance with this we did not observe *IL1RN**5 genotype and allele in pateints, which was present

only in conrols implicating its possible protective role in vitiligo predisposition. Interestingly, genotype-phenotype correlation showed that *IL1RN**A2 of *IL1RN* VNTR was found to be associated with increased *IL1RN* transcript levels, suggesting important role of *IL1RN**A2 of *IL1RN* VNTR in *IL1RN* regulation.The IL1RN family of molecules include one secreted isoform (sIL1RN) and three intracellular isoforms (icIL1RN1, 2 and 3). Numerous studies indicate that the sole biological function of sIL1RN seems to competitively inhibit IL-1 binding to cell-surface receptors. Overall, suggesting the importance of gene regulation of various *IL1RN* isoforms which may exhibit tissue specific expression.

In the present study, non-significant difference was observed for *IL1RN* transcript levels and this can be attributed to the presence of various genotypes and fewer *IL1RN* VNTR 2/2 samples in expression analysis. TNF-α induces the production of IL-1α, IL-13, IL-lRN and IL-6 by monocytes (Santtila *et al*., 1998). Recently, we have reported up-regulation of *IL1RN* in normal human melanocytes upon IL-1α stimulation (Singh *et al*., 2016). There are reports suggesting involvement of various cytokines in vitiligo pathogenesis (Singh *et al*., 2014) and increased levels of IL-1α (Birol *et al*., 2006), TNF-α (Laddha *et al*., 2012; Birol *et al*., 2006), IFN-γ (Dwivedi *et al*., 2013; Natarajan *et al*., 2014) in vitiligo patients. IFN-γ down-regulates the production of IL-1RN while up regulating the production of IL-1α, IL-1β, IL-6 and TNF-α (Danis *et al*., 1995). Due to the importance of IL-1RN in regulation of IL-1 family, it has been used in human clinical trials for various autoimmune and inflammatory disorders. Variations in *IL1RN* can modulate the effectiveness of IL-1 signaling and thereby predispose to disease condition. The present study will be the second report of *IL1RN* VNTR polymorphism in vitiligo worldwide with sufficient sample size. Therefore, our study demonstrates that pateints with vitiligo carrying a less common allele (allele 2, containing two repeats) was associated with increased *ILIRN* transcript levels, and are associated with progressive vitiligo. Moreover, the allele 2 carrier *IL1RN**3/2 was observed only in vitiligo patients while *IL1RN**5/2 was seen exclusively in controls.

The present study demonstrates the association of allele and genotype of *IL1RN* intron 2 VNTR (A2) polymorphism with active vitiligo patients and

increased *IL1RN* expression (allele 2 carriers), suggesting *IL1RN**A2 as a risk factor for progressive vitiligo in Gujarat population. Larger studies with different ethnicities are required to find out the impact of *IL1RN* VNTR polymorphism as a risk factor for developing vitiligo.

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7B.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Boissy *et al.,* 2006). The clinical hallmark of vitiligo is loss of melanin pigment due to decreased number of functioning melanocytes or their complete absence in the lesional skin (Passeron and Ortonne, 2005). The inheritance of vitiligo does not follow the simple Mendelian pattern of an autosomal dominant or autosomal recessive or X- linked inheritance. Therefore, it has been proposed that vitiligo is a polygenic disease influenced by a set of recessive alleles occurring at several unlinked autosomal loci that collectively confer the vitiligo phenotype (Nath*et al.* 1994). IL-10 is a cytokine, which is expressed by cells of the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, CD4 & CD8 T cells and B cells. IL-10 has potent anti-inflammatory properties, repressing the expression of cytokines such as TNF-α, IL-6 and IL-1. The balance between TNF- α and IL-10 is important for the maintenance of immune homeostasis (Shmarina *et al.,* 2001). It has been reported that low levels of IL-10 are governed by its gene promoter polymorphisms (Abanmi *et al.,* 2008). The *IL10* gene is located on the chromosome 1 and IL-10 production appears to be genetically encoded, which has been reported to cause 75% of the variation in IL-10 levels. The highly polymorphic promoter region of human IL-10 gene has been found to be associated with numerous autoimmune diseases (Asadullah, 2003). Hence, it becomes pertinent to investigate *IL10* promoter polymorphisms and its levels in vitiligo.

In view of the above, we aimed to examine the association of *IL10* promoter polymorphisms: -819 (C/T; rs1800871),-592 (C/A; rs1800872) and -1082 (G/A; rs1800896) in Gujarat vitiligo patients and controls using a case control approach. Furthermore, IL-10 transcript and plasma levels were monitored in PBMCs and plasma samples respectively. Also, genotype-phenotype correlation, haplotype analysis and linkage disequilibrium were performed that may provide a better understanding of the role of *IL10* polymorphisms in vitiligo pathogenesis.

7B.2 MATERIALS AND METHODS

7B.2.1 Study Subjects

The study group included 404 vitiligo patients where 294 patients were having generalized vitiligo (including acrofacial vitiligo and vitiligo universalis) and 110 were with localized vitiligo; 195 males and 209 females; 313 patients were with active vitiligo and 91 with stable vitiligo who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). The mean age (years ± SD) of patients and healthy controls were 29.41 ± 12.09 and 32.22 ± 11.75 respectively (Table 1).

7B.2.2 Genomic DNA Preparation For details refer Chapter VIIA.

Table 1: Demographic characteristics of Gujarat vitiligo patients and controls.

	Vitiligo Patients	**Controls**
	(n = 404)	(n = 481)
Average age (mean age ± SD)	32.22 ± 11.75 yr	29.41 ± 12.09 yr
Sex: Male	195 (48.26%)	237 (49.27%)
Female	209 (51.73%)	244 (50.72%)
Age of onset (mean age ± SD)	22.07 ± 12.12 yr	NA
Duration of disease (mean ± SD)	9.11 ± 6.64 yr	NA
Type of vitiligo		
Active vitiligo	313 (77.47%)	NA
Stable vitiligo	91 (22.52%)	NA
Localized vitiligo	110 (27.22%)	NA
Generalized vitiligo	294 (72.77%)	NA

Table 2:Primers and restriction enzymes used for expression and genotyping of *IL10* promoter -819 (C/T; rs1800871); -592 (C/A; rs1800872) and -1082 (G/A; rs1800896) SNPs.

Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing Temperature (°C) & Restriction Enzyme (Digested Products) nn
IL10 -819 C Allele specific FP:	ACCCTTGTACAGGTGATGAAC	270	57°C
IL10 -819 T Allele specific FP:	ACCCTTGTACAGGTGATGTAAT		
IL10 -819 RP :	CTGTCGACTTCTCCACCTT		
HGH FP:	CCTTCCCAACCATTCCCTTA	428	62°C
HGH RP:	TCACGGATTTCTGGTGTTTC		
IL10 -592 FP:	TGGTGAGCACTACCTGACTAGC	413	58 °C & *RsaI* (235 & 178 bp)
IL10 -592 RP:	CCTAGGTCACAGTGACGTGGAC		
IL10-1082 FP:	CTCGCCGCAA CCCAACTGGC	180	64°C & *MnlI* (150 & 30bp)
*IL*10 -1082 RP:	GGTCCCTTACTTTCGTCTTACCTATCC		
IL10 FP	ACCTGGGTTGCCAAGCCTT	189	62^0C
IL10 RP	CCACGGCCTTGCTCTTGTT		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65^0C

GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

7B.2.3 Genotyping of *IL10* promoter -819 (C/T; rs1800871) polymorphism

IL10 promoter C/T genotyping was done using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele specific sense primers (Table 2). To assess the success of PCR amplification in both the reactions, an internal control of 428 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (*HGH*) (Table 2). The reaction mixture of the total volume of 15 µL included 3 µL (100 ng) of genomic DNA, 4.9 µL nuclease-free H_2O, 1.5 µL 10x PCR buffer, 1.5 µL 10 mM dNTPs , 1 µL of 10 µM allele-specific and common primers (MWG Biotech, India), 1 µL of 10 µM control primers (*HGH*) and 0.1 µL (10U/µL) Taq Polymerase. Amplification was performed using a Mastercycler Gradient PCR according to the protocol: 95°C for 10 minute followed by 15 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, then 24 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplified products were separated by electrophoresis on 3.5% agarose gel and stained with ethidium bromide. The gel was visualized under a UV transilluminator with a 100 base pair DNA ladder and photographed. Two amplicons were available for each sample (one each specific for T or C allele of the *IL10* polymorphism).

7B.2.4 Genotyping of *IL10* promoter -592 (C/A; rs1800872) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype C/A (rs1800872) polymorphism of *IL10* gene (Figure 1). The primers used for genotyping are mentioned in Table 2.

7B.2.5 Genotyping of *IL10* promoter -1082 (G/A; rs1800896) polymorphism

IL10 promoter -1082 (G/A; rs1800896) genotyping was done using PCR-RFLP method. Similar to above details regarding primers, annealing temperature, amplicon size, restriction enzyme etc. used for genotyping are mentioned in Table 2.

7B.2.6 Estimation of plasma IL-10 levels by enzyme-linked immunosorbent assay

Plasma levels of IL-10 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the eBioscience Human IL-10 ELISA kit (Human IL-10 ELISA Ready-SET-Go!®, eBioscience; Catalog Number: 88-7106) as per the manufacturer's protocol.

7B.2.7 Determination of *IL10* mRNA expression

The transcripts levels of *IL10* in whole blood of vitiligo patients and controls were estimated using gene specific primers (Eurofins, Bangalore, India) by SYBR green method, real-time PCR as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Details are described in Chapter III.

7B.2.8 Data analysis

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *IL10* promoter polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of polymorphisms in different groups, considering the major genotype / allele as a reference group and were compared using chi-square test with 2×2 contingency tables using Prism 4 software (Graph Pad Software, USA, 2003). Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Bonferroni's correction was applied for multiple testing of polymorphisms. Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi and He, 2005). The LD coefficients D' and r^2-values for the pair of most common alleles at each site were estimated using the Haploviewprograme version 4.1(Barrett *et al*., 2005). IL-10 mRNA and plasma level expression were compared between different groups and genotype/ haplotype-phenotype correlation was analyzed and plotted by non-parametric unpaired t-test using Prism 4 software to determine the statistical significance of data. *p*-values less than 0.05 were considered as statistically significant. Fold change in mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software.

7B.3 RESULTS

7B.3.1 Analysis of association between *IL10* promoter -819 (C/T; rs1800871) polymorphism and susceptibility to vitiligo:

Genotyping of *IL10* promoter -819 (C/T; rs1800871) SNP was performed by ARMS PCR method in 481 controls and 402 vitiligo patients. HGH was used as a PCR positive control. Three genotypes were identified in both patients and controls: CC, CT and TT for *IL10* -819 C/T promoter polymorphism (Figure.1). The genotype frequencies differed significantly between controls and patients population (p<0.0001). Also, significant difference in allele frequencies of T allele was observed in controls and vitiligo patients (p<0.0001) (Table 3). Hence, there is significant association of the *IL10* promoter -819 (C/T; rs1800871) with vitiligo in Gujarat population. The distribution of genotype frequencies for *IL10*-819 C/T SNP was not consistent with Hardy-Weinberg expectations in both patient and control groups (p=0.0468 and p<0.0001).

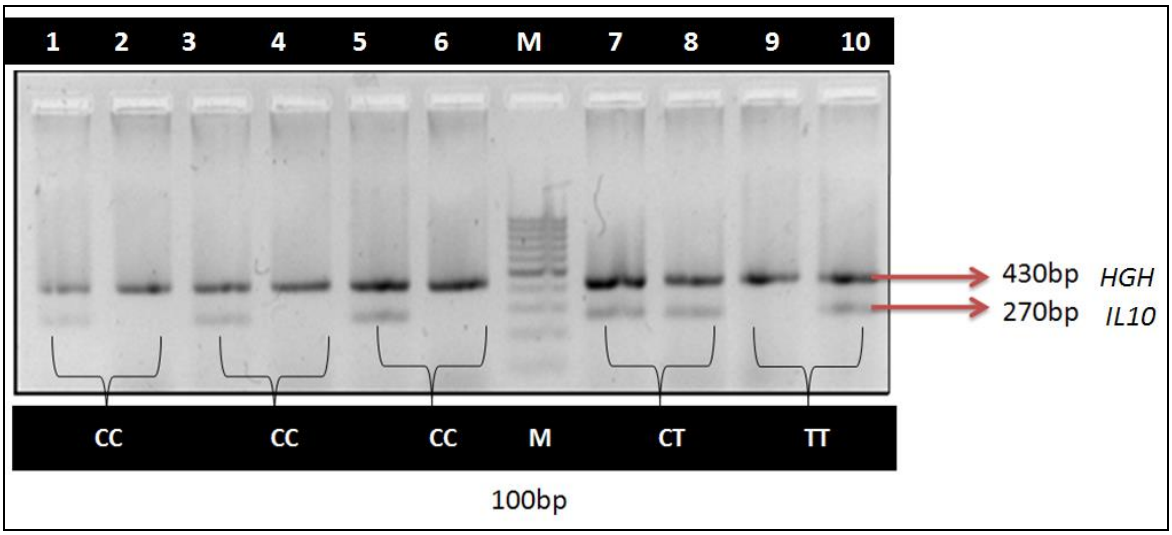


Figure 1: Representative gel image for the genotyping of *IL10*-819 C/T SNP using ARMS-PCR. Lanes 1-6 represent CC genotype, lanes 7, 8 represent CT genotype and lanes 9, 10 represent TT genotype. HGH: PCR positive control.

Table 3: Distribution of *IL10* -819 C/T (rs1800871) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 402)	(n = 481)			
IL10	CC	171 (42.54)	143 (29.73)	<0.0001[a]	0.0468	0.4880
-819 C/T (rs	CT	168 (41.79)	155 (32.22)		(P)	(0.4029 to
1800871)	TT	63 (15.67)	183 (38.05)		<0.0001	0.5910)
	Allele				(C)	
	C	510 (0.63)	441 (0.46)	<0.0001[b]		
	T	294 (0.37)	521 (0.54)			

[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b]Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

7B.3.2 Analysis of association between *IL10* promoter -592 (C/A; rs1800872) polymorphism and susceptibility to vitiligo:

We estimated the genotype frequency at this locus in 406 controls and 391 vitiligo patients. Three genotypes were identified in both patients and controls: CC, CA and AA for *IL10* -592 C/A promoter polymorphism (Figure. 2). *RsaI* was used for amplicon digestion and it cleaves if A allele is present in the amplicon and undigested product remains if C allele is present. Non-significant difference in genotype as well as allele frequencies of C/A allele was observed in controls and vitiligo patients (p=0.5796 and p=0.7096) (Table 4). Hence, there is no significant association of the *IL10* promoter -592 (C/A; rs1800872) with vitiligo. The distribution of genotype frequencies for *IL10* promoter -592 (C/T; rs1800872) were consistent with Hardy-Weinberg expectations in both patient and control groups (p=0.1861 and p=0.9594).

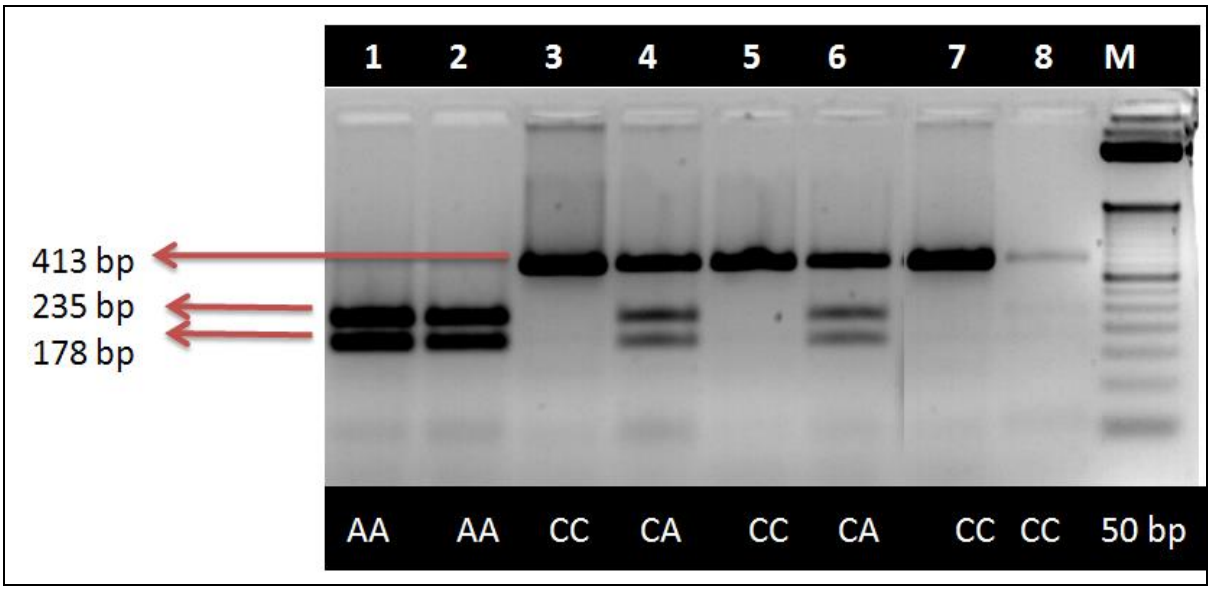


Figure 2: Representative gel image for the genotyping of *IL10* -592 C/A SNP using PCR-RFLP. Lanes 1and 2 represent AA genotype, Lanes 4 and 6 represent CA genotype and lanes 3,5,7 and 8 represent CC genotype.

Table 4: Distribution of *IL10* -592 C/A (rs1800872) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 391)	(n = 406)			
IL10	GG	182 (46.55)	178 (43.84)	0.5796[a]	0.1861	0.9612
-592 C/A (rs	GA	161 (41.18)	182 (44.83)		(P)	0.7803 to
1800872)	AA	48 (12.28)	46 (11.33)		0.9594	1.184
	Allele				(C)	
	G	525 (0.67)	538(0.66)	0.7096[b]		
	A	257 (0.33)	274 (0.34)			

[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

7B.3.3 Analysis of association between *IL10* promoter -1082 (G/A; rs1800896) polymorphism and susceptibility to vitiligo:

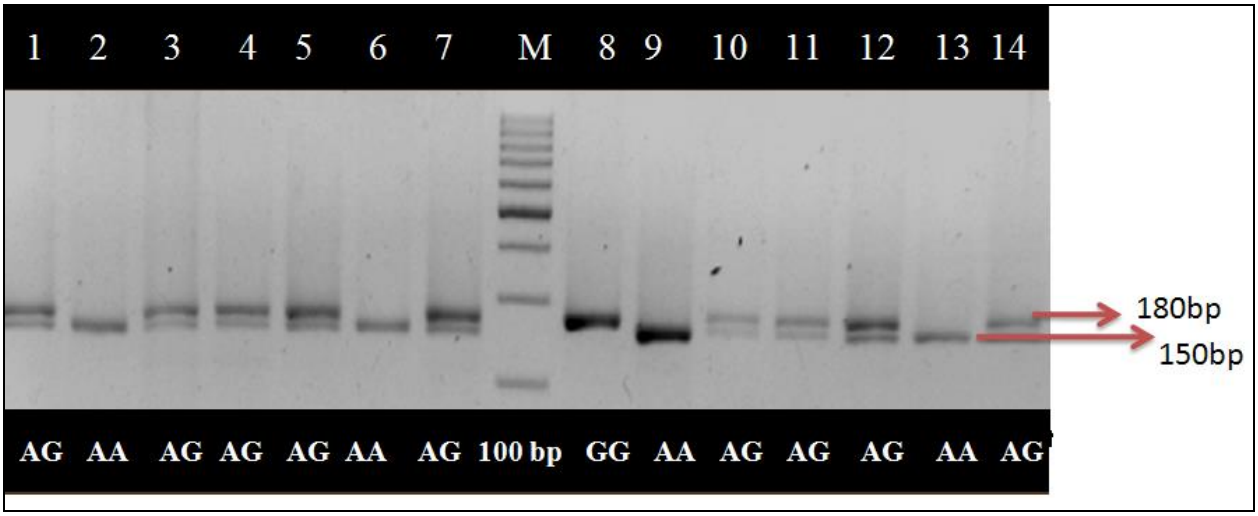


Figure 3: Representative gel image for the genotyping of *IL10* -1082 G/A using PCR-RFLP. Lanes 2, 6, 9 and 13 represent AA genotype, lanes 1, 3, 4, 5, 7, 10, 11, 12 & 14 represent AG genotype and lane 8 represents GG genotype.

We have estimated the genotype frequency at this locus in 446 controls and 404 vitiligo patients. Three genotypes were identified in both patients and controls: GG, GA and AA for *IL10* -1082 G/A promoter polymorphism as illustrated in representative figure (Figure. 3). *MnlI* restriction enzyme was used for amplicon digestion. *MnlI* cleaves if G allele is present in the amplicon and undigested product remains if 'A'allele is present. Significant difference in allele frequency of 'A' allele was observed in controls and vitiligo patients (p=0.0311) (Table 5). However, the genotype frequency did not differ significantly between patients and controls (p=0.1445). Also, the distribution of genotype frequencies for *IL10* promoter -1082 G/A SNP was not consistent with Hardy-Weinberg expectations in both patient and control population (p<0.0001 and p<0.0001 respectively).

Table 5: Distribution of *IL10* -1082 G/A (rs1800896) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 404)	(n = 446)			
IL10	GG	147 (36.39)	134 (30.04)	0.1445[a]	<0.0001	0.8107
-1082 G/A (rs	GA	106 (26.24)	127 (28.48)		(P)	(0.6697 to
1800896)	AA	151 (37.38)	185 (41.48)		<0.0001	0.9813)
	Allele				(C)	
	G	400 (0.50)	395 (0.44)	0.0311[b]		
	A	408 (0.50)	497 (0.56)			

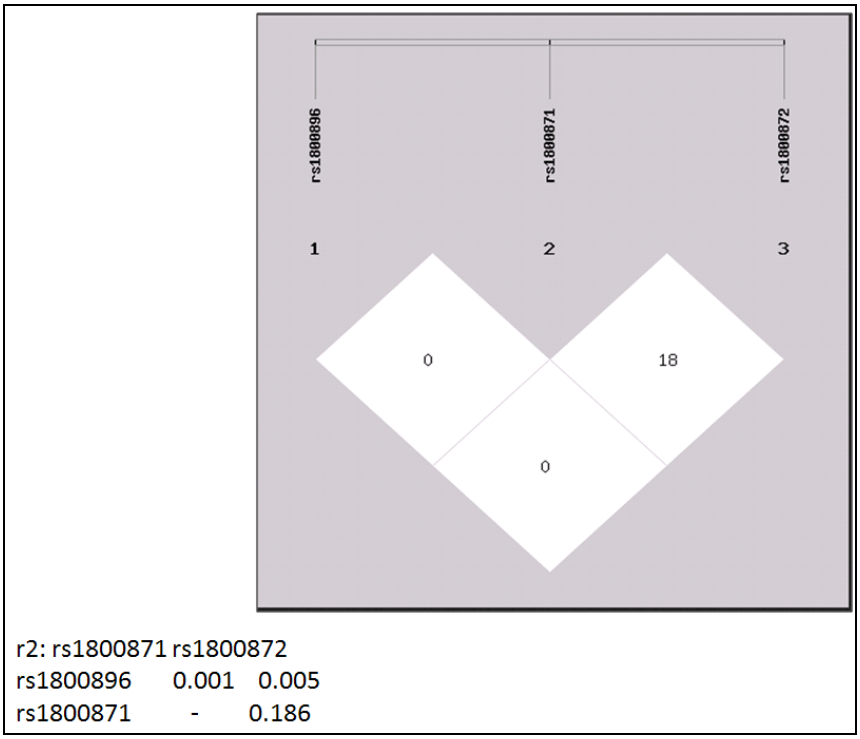
[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b]Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

Table 6: Haplotype analysis of *IL10* promoter -592 (C/A; rs 1800872), -819 (C/T; rs 1800871) and -1082 (G/A; rs 1800896) SNPs in Gujarat vitiligo patients and controls.

Haplotypes (*IL 10*)	Patients Freq in% N=534	Control Freq in % N=592	P for association	P global	Odds ratio 95% CI
A C A*	21.34(0.040)	30.73(0.052)	0.340182		0.760 [0.432~1.338]
A C C*	168.98(0.316)	129.25(0.218)	0.000196		1.657 [1.269~2.165]
A T A*	49.95(0.094)	82.63(0.140)	0.016703		0.636 [0.438~0.923]
A T C*	28.73(0.054)	94.39(0.159)	$1.45e^{-008}$	$5.15e^{-011}$	0.300 [0.194~0.463]
G C A*	28.09(0.053)	26.56(0.045)	0.546307		1.182 [0.686~2.036]
G C C*	133.59(0.250)	114.46(0.193)	0.021619		1.392 [1.049~1.847]
G T A*	79.62(0.149)	61.08(0.103)	0.019947		1.523 [1.067~2.175]
G T C*	23.70(0.044)	52.90(0.089)	0.002765		0.473 [0.287~0.780]

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



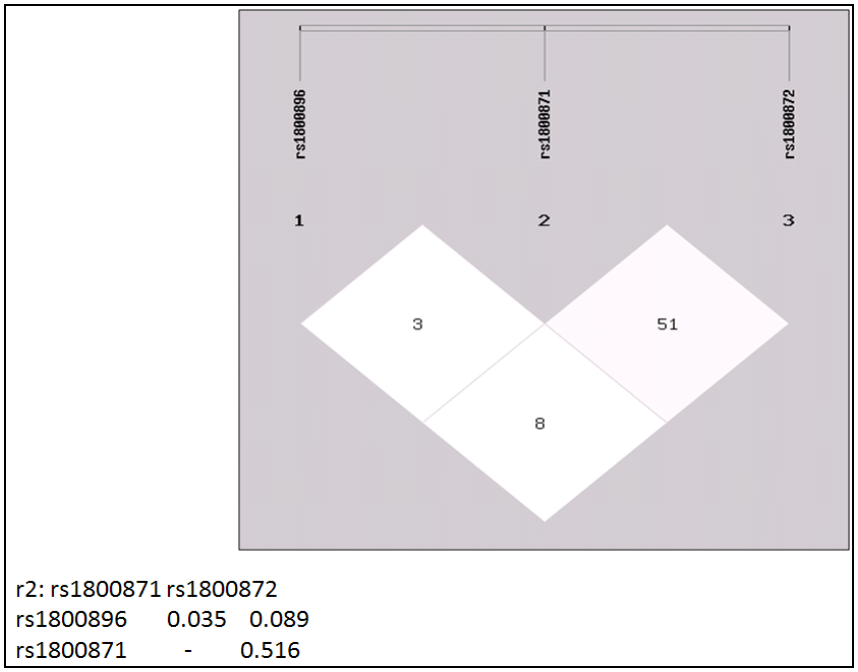


Figure 4: Haplotype analysis of *IL10* promoter -592 (C/A; rs 1800872), -819 (C/T; rs 1800871) and -1082 (G/A; rs 1800896) SNPs

The two polymorphisms investigated were in moderate LD association (*IL -819 C/T: IL 10 – 592 C/A;* D'= 0.516, r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection (Table 6) whereas, ACC (p=0.000196), GCC (p=0.021619), GTA (p= 0.019947) haplotypes were found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546307) haplotypes were not found to be associated with vitiligo.

7B.3.4 Relative gene expression of *IL10* in PBMCs of vitiligo patients:

The *IL10* transcript levels were compared in 80 PBMCs of vitiligo patients and 88 age matched unaffected controls after normalization with *GAPDH* transcript levels. The *IL10* transcript levels in vitiligo patients were significantly higher when compared to controls (p = 0.0150) as suggested by mean ΔCp values (Figure 5A). The $2^{\Delta\Delta Cp}$ analysis showed approximately 2.42 fold higher expression of *IL10* transcript in patients as compared to controls (Figure 5B).

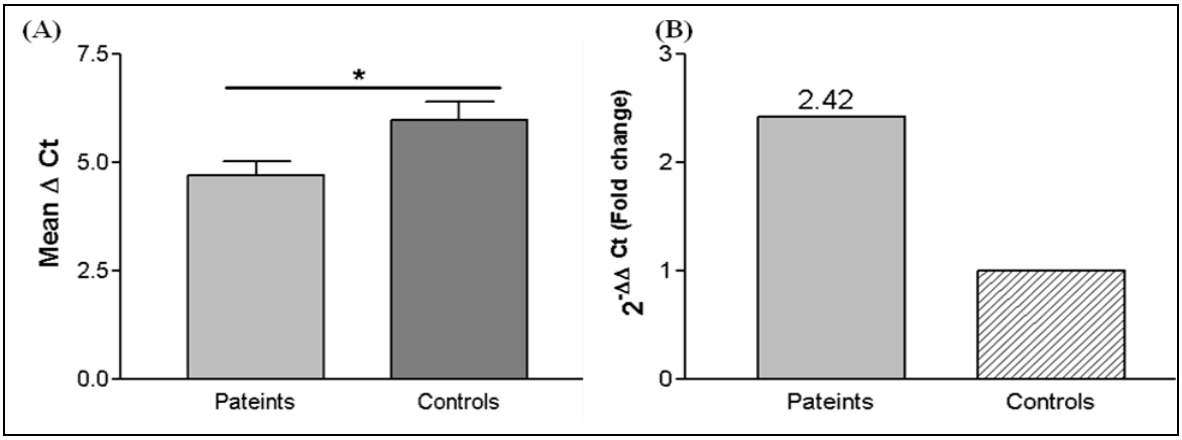


Figure 5: Relative gene expression of *IL10* in PBMCs of controls and vitiligo patients: (A) Expression of *IL10* transcripts in 88 controls and 80 vitiligo patients as suggested by mean ΔCp. Patients showed significantly increased *IL10* expression as compared to controls (p=0.0150). (B) Expression fold change of *IL10* transcripts in 80 vitiligo patients against 88 controls showed 2.42-fold change as determined by $2^{-\Delta\Delta Cp}$ method.

7B.3.5 Estimation of plasma levels of IL10 vitiligo patients and controls:

Plasma IL10 levels in 92 vitiligo patients and 72 controls were estimated by ELISA. IL10 levels were found to be significantly increased in patients with vitiligo when compared with controls [p=0.0041 Figure. 6; Mean ± SEM of patients 4.150 ± 0.2485 (N=92) and controls 3.214 ± 0.1783 (N=72)]. These results are in accordance with transcript analysis where we observed 2.42 fold increased expression of *IL10* in patients as compared to controls.

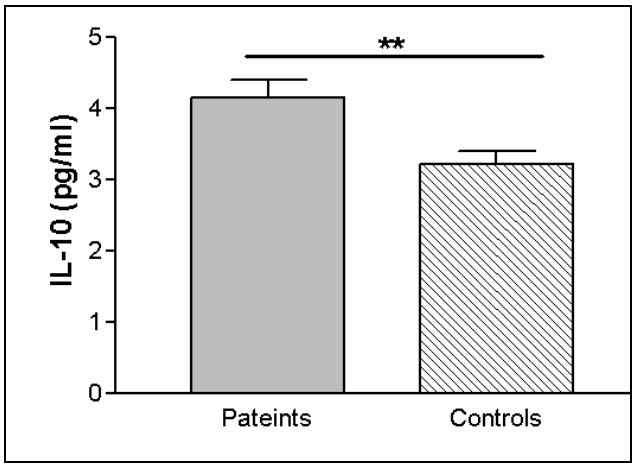


Figure 6: Plasma IL10 levels in controls and vitiligo patients: Comparison of plasma IL10 levels (pg/ml) in 72 controls and 92 vitiligo patients showed significantly increased IL10 levels in vitiligo patients (p=0.0041), as determined by ELISA.

7B.3.6 Genotype - phenotype correlation analysis:

In order to observe how SNPs alone and in combination are affecting the transcript and protein levels of IL10 in studied subjects, we carried out possible genotype - phenotype correlation with respect to different genotypes and haplotypes observed in the present study. Interestingly, among nine genotypes of three promoter polymorphisms of *IL10*, we observed *IL10*-819 C/C genotype was exhibiting increased expression of *IL10* as compared to TT genotype (p=0.0121). However, the ELISA results for the same showed no difference in the IL10 levels (p=0.9858). Moreover, there was no difference in the transcript as well as protein levels of IL10 for different observed genotypes (Figure. 7).

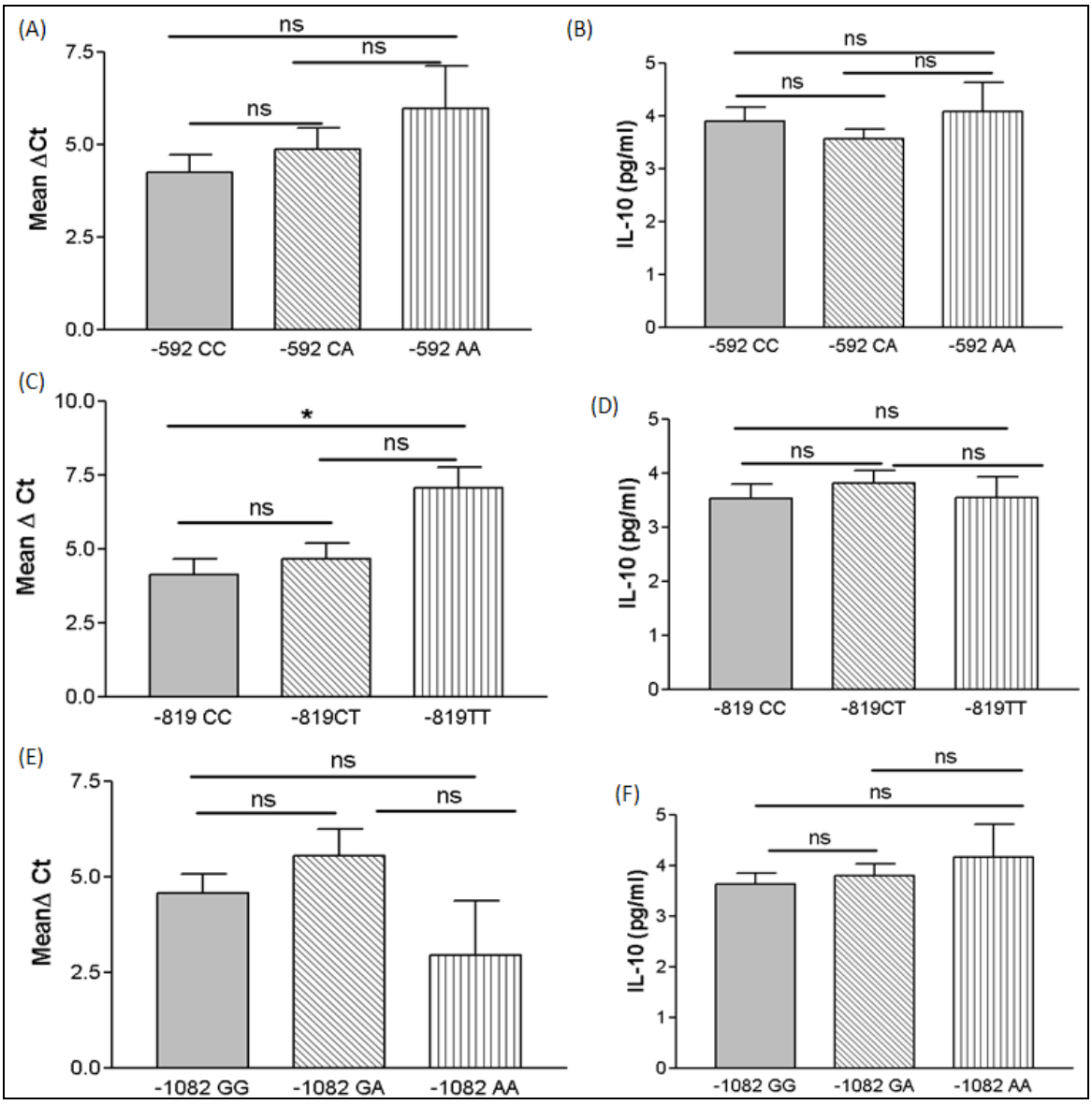


Figure 7: Genotype-phenotype correlation of -592 C/A, -819 C/T and -1082 G/A promoter polymorphisms of *IL10* transcript and plasma levels in controls and vitiligo patients: (A) Relative mRNA expression of *IL10* with respect to -592 C/A genotypes in vitiligo patients. None of the three genotypes CC vs CA (p=0.4142); CC vs AA (p=0.1535) and CA vs AA (p=0.4184) showed significant effect on *IL10* transcript levels as suggested by Mean ΔCp. (B) Similarly, the plasma levels among different genotypes CC vs CA (p=0.3013); CC vs AA (p=0.7514) and CA vs AA (p=0.2456) of -592 C/A also showed no significant difference (C) Relative mRNA expression of *IL10* with respect to -819 C/T genotypes in vitiligo patients. *IL10*-819 C/C genotype was exhibiting increased expression of *IL10* as compared to TT genotype (p=0.0121).

However, other genotypes CC vs CT (p=0.5045) and CT vs TT (p=0.0922) showed no difference for *IL10* transcript levels as suggested by Mean ΔCp. (D) Similarly, the plasma levels among different genotypes CC vs CT (p=0.4277); CC vs TT (p=0.9858) and CT vs TT (p=0.7016) of -819 C/T showed no significant effect on IL10 plasma levels. (E) Relative mRNA expression of *IL10* with respect to -1082 G/A genotypes in vitiligo patients. None of the three genotypes GG vs GA (p=0.2501); GG vs AA (p=0.2296) and GA vs AA (p=0.1012) showed significant effect on *IL10* transcript levels as suggested by Mean ΔCp. (F) Similarly, the plasma levels among different genotypes GG vs GA (p=0.5926); GG vs AA (p=0.3577) and GA vs AA (p=0.7951) of -1082 G/A showed no significant effect on IL10 plasma levels.

7B.3.7 Effect of IL10 expression on disease progression:

The levels of IL10 were analyzed with respect to progression of disease in 58 patients with active vitiligo (AV) and 22 patients with stable vitiligo (SV). As suggested by mean ΔCp and ELISA results analysis, there was no difference in transcript as well as plasma levels of IL10 p=0.8560 and p=0.0665 respectively between AV and SV (Figure 8).

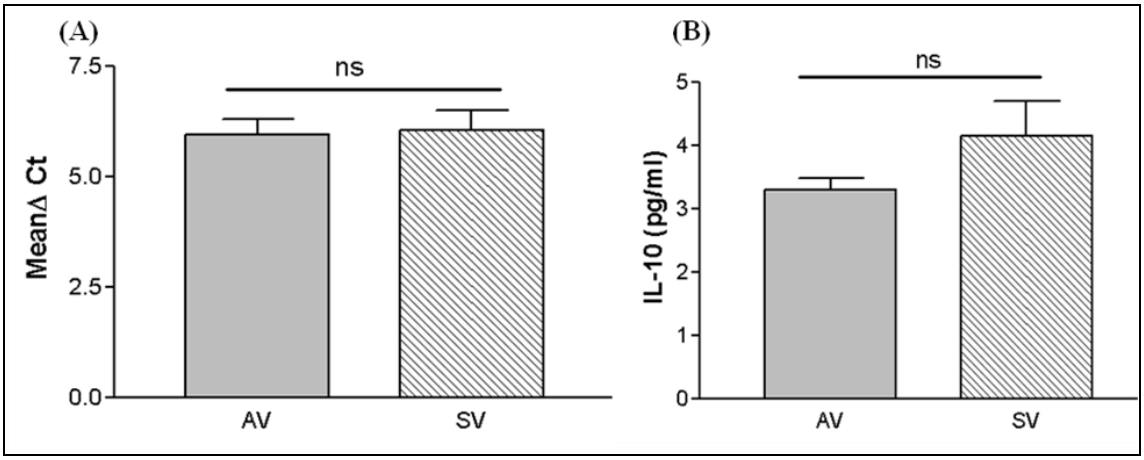


Figure 8: Effect of IL10 expression on disease progression showed no difference in IL10 transcript as well as protein levels between AV and SV (p=0.8560 and p=0.0665 respectively).

7B.3.8 Gender based analysis of IL10 expression:

Expression of IL10 was analyzed with respect to gender differences in 43 male patients and 37 female patients with vitiligo. The female patients showed significant increase in *IL10* mRNA levels as compared to male vitiligo patients (p = 0.0216, Figure 9A). Also,

the plasma levels of IL10 were significantly increased in female vitiligo patients compared to male vitiligo patients (p=0.0454; Figure 9B).

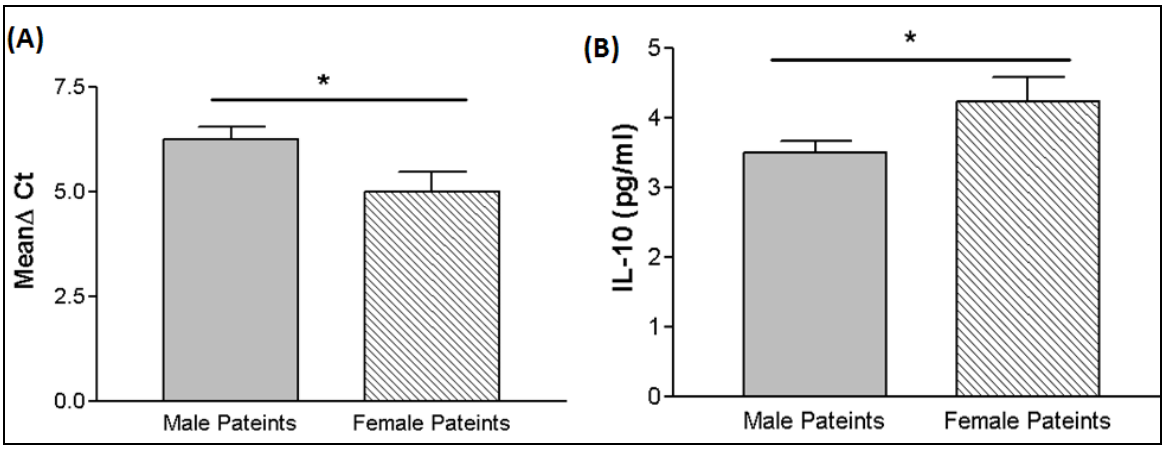


Figure 9: **Effect of IL10 expression on gender biasness showed significant difference in IL10 (A) transcript as well as (B) protein levels between male and female vitiligo patients p=0.0216 and p=0.0454 respectively**

7B.3.9 Effect of IL10 expression in different clinical types of vitiligo:

The levels of *IL10* were analyzed with respect to progression of disease in 50 patients with generalized vitiligo (GV) and 30 patients with localized vitiligo (LV). As suggested by mean ΔCp and ELISA, there was no difference in transcript as well as plasma levels of IL10 between GV and LV (p=0.8680 and p=0.6747) respectively (Figure 10).

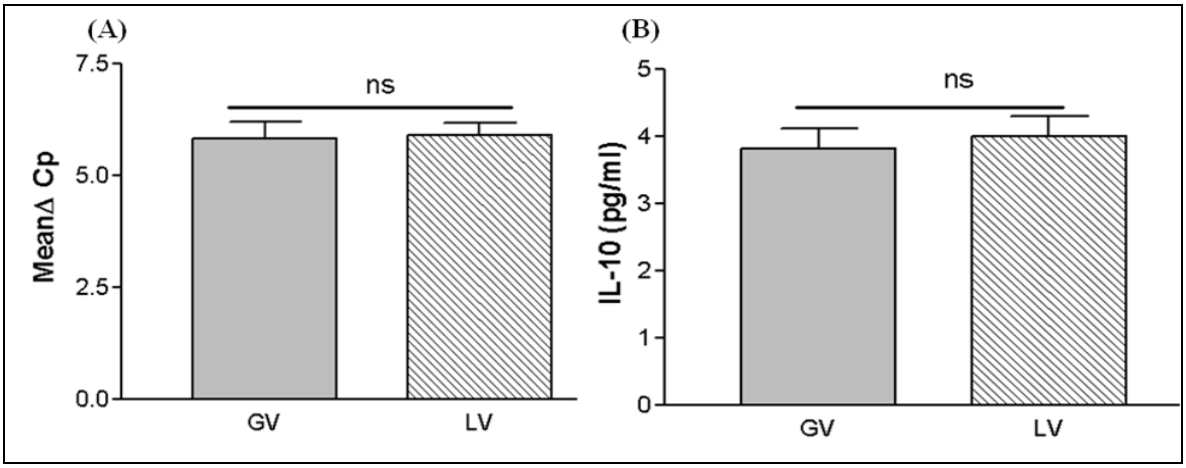


Figure 10: **Effect of IL10 expression on GV and LV showed no difference in IL10 (A) transcript as well as (B) protein levels between GV and LV (p=0.8680 and p=0.6747) respectively.**

7B.3.10 Haplotype based analysis:

Further, the transcript as well as plasma levels of IL10 were analyzed with respect to haplotypes generated from investigation of three promoter polymorphisms of *IL10*. However, there was no significant difference in transcript as well as plasma levels of IL10 among various haplotypes was observed (p>0.05; Figure 11).

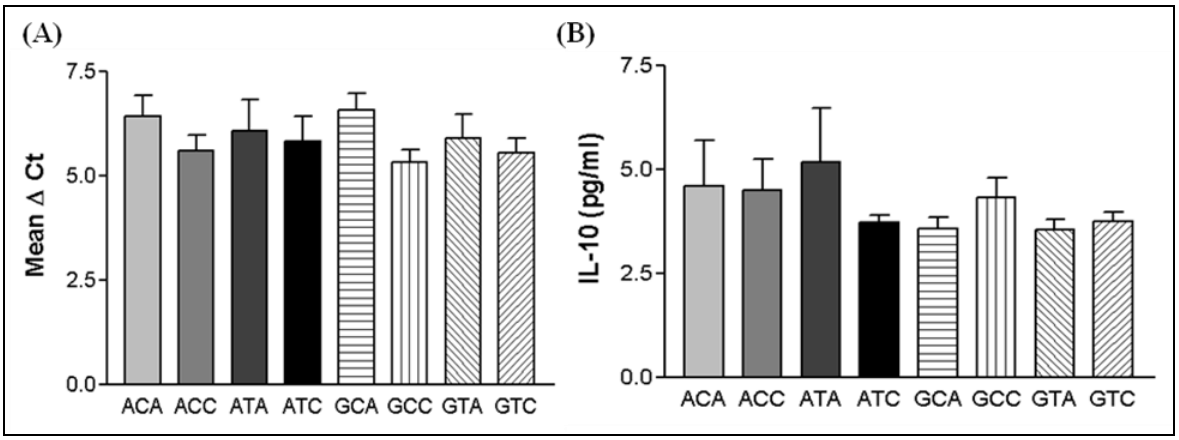


Figure 11: **Correlation of *IL10* transcript levels with -819 (C/T; rs1800871), -592 (C/A; rs1800872) and -1082 (G/A; rs1800896) promoter polymorphisms with different haplotypes showed no difference in the (A) transcript as well as (B) plasma levels of IL10 (p>0.05 for all comparisons).**

7B.4 DISCUSSION

Major hypotheses which could explain pathogenesis of vitiligo are oxidative stress, autoimmune and genetic hypotheses. IL10 acts as an anti-inflammatory cytokine wherein Zhao *et al.,* (year) and Ala *et al.,* (2015) showed significant decrease in the expression of IL10 in vitiligo. In addition to this, Taher *et al.,* (2009) have found increased levels of IL10 after application of topical tacrolimus treatment to patients with vitiligo. On the contrary, Grimes *et al.,* (2004) and Aydingoz *et al.,* (2014) have reported increased levels of IL10 in skin and serum samples of vitiligo patients respectively; and after topical application of tacrolimus, Grimes *et al.,* (2004) didn't find any significant difference in the IL10 levels. Polymorphism studies of *IL10* -1082, -592 and -819 promoter SNPs revealed association of *IL10* polymorphisms with vitiligo susceptibility (Aydingoz *et al.,* 2014, Abanmi *et al.,* 2008). Previous studies as mentioned in Table 7 have shown

significant difference in the expression of IL10 in vitiligo patients as compared to controls.

Table 7: **Reports on IL10 expression in vitiligo patients:**

Cytokine	Reference	No. of vitiligo patients & Sample type	Levels of IL 10 (Technique used)
IL10	Grimes *et al.*, 2004	19, tissue	significantly increased (real time -PCR)
	Basak*et al.*, 2009	40, serum	non-significant (ELISA)
	Taher *et al.*, 2009	20, tissue	significant increase post treatment (ELISA)
	Ala *et al.*, 2010	280, serum	significantly decreased (ELISA)
	Zhao *et al.*, 2010	20, PBMC	significantly decreased (real time -PCR)
	Tembhre*et al.*, 2012	80 (untreated), 25 (narrow band ultraviolet B treated vitiligo), serum	significantly increased (ELISA)

In the present study, we have investigated three polymorphisms in promoter region of *IL10* gene for their role in susceptibility to vitiligo, by using an approach of case-control study. Our results showed that genotype and allele frequencies of *IL10* -819 C/T (p<0.0001; p<0.0001 respectively) were significantly different between vitiligo patients and unaffected controls, suggesting the significant association of -819T allele with vitiligo susceptibility. Additionally, the allele frequency of -1082 G/A differed significantly between vitiligo patients and controls (p=0.0311). However, the genotype

and allele frequencies for *IL10* -592C/A (p= 0.5796; p= 0.7096 respectively) and genotype frequencies of *IL10* -1082G/A (p = 0.1445) did not differ significantly between vitiligo patients and controls, suggesting the non-association of these SNPs with vitiligo susceptibility. The two polymorphisms investigated were in moderate LD association (*IL -819 C/T: IL 10 – 592 C/A;* D'= 0.516, r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection. Whereas, ACC (p=0.000196), GCC (p=0.021619), GTA (p= 0.019947) haplotypes were more found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546307) haplotypes were not found to be associated with vitiligo.

Additionally, as mentioned in chapter III, we have estimated the transcript levels of *IL10* in skin samples from vitiligo patients and controls (n=12) and we observed siginificant decrease in *IL10* levels (p=0.0357) in lesional skin compared to control skin. However, this study further needs to be carried out in different populations with higher sample size. Moreover, we have estimated the transcript levels of *IL10* in PBMCs of vitiligo patients and controls and found significant increase in *IL10* transcript levels in vitiligo patients and gender biasness of females as compared to male vitiligo patients is also observed. However, there was no significant difference in *IL10* transcript levels with respect to disease progression (Av vs SV) and type of vitiligo (GV vs LV) in our studies. Also, to monitor the effect of polymorphism on the circulatory levels of IL10, we analyzed plasma levels of IL10 in vitiligo patients and controls and found significant increase in IL10 levels in vitiligo patients with gender biasness of females as compared to male vitiligo patients. However, there was no significant difference in IL10 levels with respect to disease progression (AVvs SV) and type of vitiligo (GV vs LV) in our studies.

Furthermore, in our genotype-phenotype corelation studies among nine genotypes of three promoter polymorphisms of *IL10*, we observed *IL10*-819 C/C genotype was exhibiting increased expression of IL10 as compared to TT genotype (p=0.0121). However, the ELISA results for the same showed no significant difference in the IL10 levels (p=0.9858). Moreover, there was no significant difference in the transcript as well as protein levels of IL10 when analyzed for different observed genotypes and haplotypes.

The present study supports the autoimmune hypothesis of vitiligo pathogenesis with significant association of *IL10* -819 C/T polymorphism in vitiligo patients thus, suggesting the crucial role of *IL10* in vitiligo pathogenesis. Also, the increased levels of transcript as well as plasma levels of lL10 in vitiligo patients indicate its potential role in vitiligo pathogenesis. The present study might pave the pathway for understanding the higher prevalence of vitiligo in Gujarat population.

7B.5 REFERENCES

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7C.1 INTRODUCTION

Vitiligo is a progressive depigmenting disorder affecting 0.45–2.16% of the population worldwide (Kruger *et al.*, 2012). There is an imperative need for investigating the genetic predisposition of candidate genes in vitiligo as it cannot be explained by simple Mendelian genetics; however, it is characterized by incomplete penetrance, multiple susceptibility loci, and genetic heterogeneity (Zhang *et al*., 2005).

We have earlier reported that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al*., 2006 a). The etiology of vitiligo remains obscure despite being in focused debate for more than six decades (Le Poole *et al*., 1993; Taieb, 2000; Shajil *et al*., 2006 b), hence, it is important to unravel the underlying pathomechanisms of vitiligo.

In addition to the cosmetic and psychosocial implications, there are evidences of association of vitiligo with other autoimmune diseases,, such as systemic lupus erythematosus, hypothyroidism, diabetes etc. (Laddha *et al*., 2014, Deretzi *et al*., 2010; Kocer *et al*., 2009; Nikiforidis *et al*., 1993; Ramagopalan *et al*., 2007; Rashtak and Pittelkow, 2008; Sabate *et al*., 1999; Varoglu *et al*., 2010; Yacubian *et al*., 2001).

One of the emerging concepts favoured by autoimmune hypothesis is the role of epidermal cytokine imbalance in vitiligo pathogenesis. Cytokine imbalance between pro and anti-inflammatory cytokines is well reported in vitiligo. Complex cytokine network exists in skin cells which regulates melanocyte activity by paracrine mechanisms (Imokawa *et al*., 2004). Keratinocytes surround melanocytes and form a structural and functional unit called the epidermal melanin unit (Jimbow *et al.*, 1976). Keratinocytes also secretes cytokines, such as Interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which act as paracrine inhibitors of melanocyte growth and proliferation (Swope *et al*., 1991). Altered levels of keratinocyte-derived mediators have been recently described in vitiligo epidermis (Moretti *et al*., 2002 a,b ; Kitamura *et al*., 2004; Lee *et al*., 2005), suggesting a role of epidermal cytokines in vitiligo pathogenesis.

IL6 is a pleiotropic Th2 cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation etc. IL-6 induces and enhances Intercellular

Adhesion Molecule (ICAM)-1 expression on melanocytes, which promotes melanaocyte-leukocyte attachment (Yohn *et al*., 1990). IL6 stimulates the inflammatory and auto-immune processes in many diseases such as diabetes, atherosclerosis, depression, alzheimer's disease, systemic lupus erythematosus, multiple myeloma, prostate cancer, behçet's disease, and rheumatoid arthritis (RA) (Dubiński and Zdrojewicz 2007). Blockade of IL-6 activity with a soluble anti-IL-6 molecule 'tocilizumab' has been found to be promising for treatment of various autoimmune disorders like RA (Tanaka *et al*., 2014). Increased levels of *IL6* have been reported in vitiligo patients (Tu *et al*., 2003; Singh *et al*., 2012; Pichler *et al*., 2009). Human IL6 gene has been mapped to chromosome 7p21. There are two biallelic polymorphisms in the promoter region of *IL6* that may be associated with differences in IL-6 production: −174G/C (rs1800795) and −572G/C (rs1800796). These single nucleotide polymorphisms consist of a change from guanine (G) to cytosine (C) at positions −174 and −572 in the promoter region. Also, several allelic variants have been identified in the promoter region of *IL6*, responsible for its gene regulation at transcriptional level (Fishman *et al*., 1998; Terry *et al*., 2000). Polymorphisms solely do not exist in isolation, and it may be the combination of base changes at different loci, i.e. the haplotype, that may be influencing the function. Therefore, the present study aims to analyze the association of two promoter polymorphisms -174 G/C (rs1800795), -572 G/C (rs1800796) of *IL6* as well as its transcript levels in peripheral blood mononuclear cells (PBMCs) using a case-control approach.

7C.1 MATERIALS AND METHODS

7C.2.1 Study Subjects

The study group included 322 vitiligo patients (181 males and 141 females) wherein 239 patients were having generalized vitiligo (including acrofacial vitiligo and vitiligo universalis) and 83 were with localized vitiligo, who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of 343, ethnically age and sex

matched unaffected individuals (186 males and 157 females) were included in the study (Table 1).

Table 1: Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients		**Controls**
	(n = 322)		(n = 343)
Average age (mean age ± SD)	38.23 ± 19.15 yr		29.41 ± 12.16 yr
Sex: Male	181 (56.21%)		186 (54.2%)
Female	141 (43.7%)		157 (45.77%)
Age of onset (mean age ± SD)	31.70±19.51 yr		NA
Duration of disease (mean ± SD)	7.723±9.154 yr		NA
Type of vitiligo			
Generalized	239 (74.22%)		NA
Localized	83 (25.77%)		NA
Active vitiligo	235 (72.98%)		NA
Stable vitiligo	87 (27.01%)		NA

7C.2.2 Genomic DNA Preparation

As discussed in Chapter VII A.

Table 2: Primers used for gene expression and polymorphism analysis.

Gene Primer	Sequence	Annealing Temperature	Restriction Enzyme (Digested Products)
IL6 -174 F	5'-TCAAGACATGCCAAAGTGCTGAG-3'	62°C	Nsp1 (129&59 bp=188bp)
IL6 -174 R	5'- AGCGGGTGGGGCTGATTGG-3'		
IL6 -572 F	5'-GGAGACGCCTTGAAGTAACTGC -3'	58°C	Aci1 (366 & 76 bp=442bp)
IL6 -572 R	5'-GTTTCCTCTGACTCCATCGCAG-3'		
IL6 F	5'-AAATTCGGTACATCCTCGACGGCA-3'	64°C	
IL6 R	5'-AGTGCCTCTTTGCTGCTTTCACAC-3'		-
GAPDH F	5'- CATCACCATCTTCCAGGAGCGAG - 3'	65°C	-
GAPDH R	5'- CCTGCAAATGAGCCCCAGCCT- 3'		

7C.2.3 Genotyping of *IL6* promoter -174 G/C (rs1800795) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype G/C (rs1800795) polymorphism of *IL6* gene (Figure 1 A). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic- DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a Mastercycler gradient (Eppendorf, Germany) according to the

protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Restriction enzyme NspI (Fermentas, Lithuania) was used for digestion of PCR amplicons of *IL6* for genotyping of G/C (rs1800795) SNP (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp DNA ladder (Invitrogen) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. 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).

7C.2.4 Genotyping of *IL6* promoter -572 G/C (rs1800796) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype G/C (rs1800796) polymorphism of *IL6* gene (Figure 1B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Restriction enzyme AciI (Fermentas, Lithuania) was used for digestion of PCR amplicons of *IL6* for genotyping of G/C (rs1800796) SNP (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp DNA ladder (Invitrogen) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. 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).

7C.2.5 Determination of *IL6* and GAPDH mRNA expression As discussed in Chapter VII A.

7C.2.6 Real-time PCR

The expression of *IL6* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10s, 62°C and 65°C for 20 s for *IL6* and *GAPDH* respectively, 72°C for 20 s). 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 value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

7C.2.7 Statistical analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all four polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *IL6* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of *IL6* and genotype-phenotype correlation analysis of genotypes of *IL6* SNPs and *IL6* expression in patient and control groups was plotted and

analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

7C.3 RESULTS

7C.3.1 Analysis of association between *IL6* -174 G/C promoter polymorphism and susceptibility to vitiligo:

IL6 -174 G/C promoter polymorphism was found to be monogenic for 'G' allele and only 'GG' genotype was detected in 100 vitiligo patients and healthy age and gender matched controls in Gujarat population (Table 3; Figure 1A).

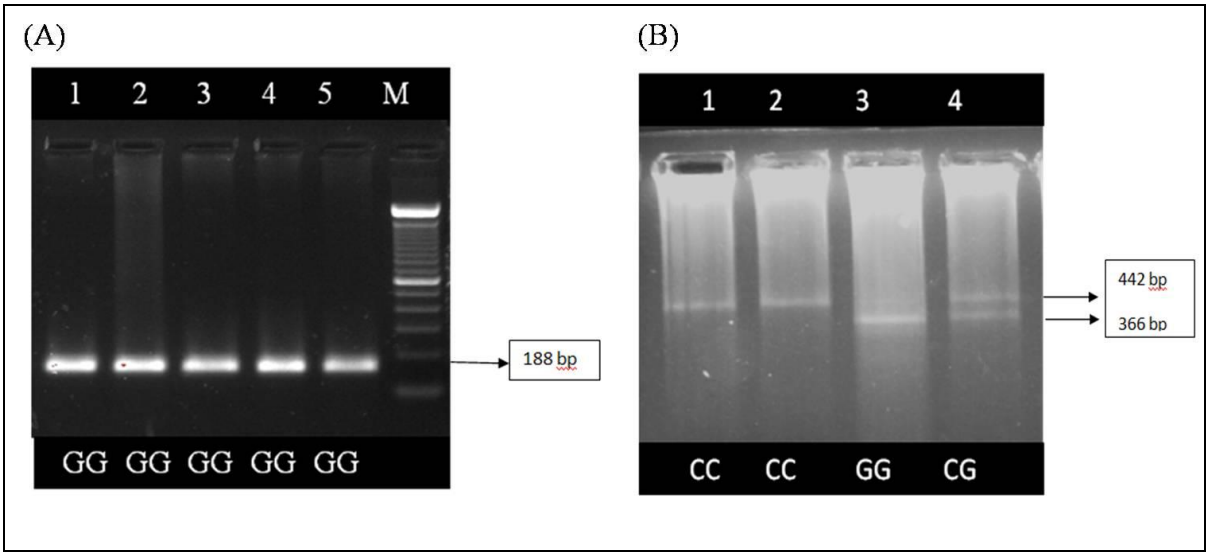

Figure 1: Genotyping of *IL6* gene polymorphisms: (A) PCR-RFLP analysis of *IL6* (-174 G/C) promoter polymorphism on 3.5 % agarose gel electrophoresis: lanes: All lanes exhibit homozygous (GG) genotypes. M shows 50 bp DNA ladder. (B) PCR-RFLP analysis of *IL6* (-572 G/C) promoter polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 & 2 show homozygous (CC) genotypes; lane 3 shows homozygous (GG) genotypes and lane 4 shows heterozygous (CG) genotype.

Table 3: Distribution of Genotypes and Alleles for *IL6* -572 G/C (rs1800796) and -174 G/C (rs1800795) in vitiligo patients and controls from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for HWE	*p* for Association	Odds ratio	CI (95%)
IL6 -572 G/C (rs1800796)	Genotype	n= 322	n = 343	<0. 0001 (C)			
	GG	67 (0.21)	52 (0.15)		R	1	-
	GC	186 (0.58)	225 (0.66)		**0.0336**[a]	0.6416[a]	0.42 -0.9678[a]
	CC	69 (0.21)	66 (0.19)		0.4078[a]	0.8114[a]	0.49 – 1.331[a]
	Allele			0.0053 (P)			
	G	320 (0.50)	329 (0.48)		R	1	-
	C	324 (0.50)	357 (0.52)		0.5281[b]	0.9331[b]	0.75- 1.157[b]
IL6 -174 G/C (rs1800795)	Genotype	n= 100	n = 100	-			
	GG	100 (1.00)	100 (1.00)		-	-	-
	GC	00 (0.00)	00 (0.00)		-	-	-
	CC	00 (0.00)	00 (0.00)	-	-	-	-
	Allele						
	G	100 (1.00)	100 (1.00)		-	-	-
	C	00 (0.00)	00 (0.00)		-	-	-

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(P) refers to Patients and (C) refers to Controls,
[a] Vitiligo Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Vitiligo Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table

7C.3.2 Analysis of association between *IL6* -572 G/C promoter polymorphism and susceptibility to vitiligo:

Three genotypes GG, GC and CC were identified in both patients and controls for *IL6* -572 G/C promoter polymorphism (Figure 1B). Ancestral 'GG' genotype was considered as reference genotype for the analyses. The heterozygous 'GC' genotype was found to be significantly higher in control group as compared to vitiligo patients (p=0.0336, OR=0.6416, CI=0.42-0.9678; Table 3). In addition, frequency of 'CC' genotype did not differ significantly between patients and controls for *IL6* -572 G/C SNP (p=0.4078, OR=0.8114, CI=0.49 – 1.331; Table 3). Similarly, the allele frequency also did not differ significantly between patients and controls (p=0.5281, OR=0.9331, CI=0.75 – 1.157; Table 3). Further, we analysed the vitiligo patient's data in subgroups based on gender, disease progression and type of vitiligo. Analysis based on gender revealed increased frequency of the 'CC' genotype in males as compared to females (22.0% versus 9.0%, p=0.0282). However, there was no significant difference between 'GC' genotype and minor allele frequency of 'C' (p=0.3322, OR=0.7781, CI=0.46-1.293 and p=0.1172, OR=1.286, CI=0.93-1.761 respectively; Table 4).

Analysis based on the disease progression revealed significantly decreased frequency of the minor allele 'C' in patients with active vitiligo (AV) compared to controls (43.0% versus 52.0%, p=0.0025; Table 5), suggesting the important role of 'C' allele in disease progression. However, there was no significant difference in the frequency of 'C' allele between stable vitiligo (SV) patients and controls (p=0.0892; Table 5). Interestingly, the 'GC' genotype was predominant in controls as compared to AV and SV patients (65.0% versus 57.0% and 50.0% respectively; p=0.0003 and 0.0020 respectively; Table 5). Further, analysis based on the type of vitiligo revealed significantly decreased frequency of the minor allele 'C' in patients with generalised vitiligo (GV) (43.0% versus 52.0%, p=0.0024) as compared to controls (Table 6). However, there was no significant difference between localised vitiligo (LV) and controls (p=0.329; Table 6). Interestingly, the 'GC' genotype was predominant in controls compared to GV (66.0% versus 54.0%, p<0.0001) The 'CC' genotype was predominant in controls as compared to GV (19.0% versus 16.0%, p=0.0013) suggesting the important protective role of 'C' allele in GV.

However, there was no significant difference in genotype and allele frequencies based onage of onset, family history of patients (data not shown).

Table 4: Association of *IL6* SNP in male and female vitiligo patients of Gujarat population.

SNP	Genotype or allele	Male (Freq.)	Female (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n= 181	n = 141			
IL6 -572 G/C (rs1800796)	GG	52(0.28)	40 (0.28)	R	1	-
	GC	88(0.48)	87 (0.61)	0.3322[a]	0.7781[a]	0.46 – 1.293[a]
	CC	41(0.22)	14 (0.09)	**0.0283[a]**	2.253[a]	1.08 – 4.69[a]
	Allele G	192 (0.53)	167(0.59)	R	1	-
	C	170(0.47)	115(0.41)	0.1172[b]	1.286[b]	0.93 – 1.761[b]

'n' represents number of Male/Female Patients,

'R' represents reference group,

CI refers to Confidence Interval,

[a] Male vs. Female Patients (genotype) using chi-squared test with 2 × 2 contingency table,

[b] Male vs. Female Patients (allele) using chi-squared test with 2 × 2 contingency table

Table 5: Distribution of Genotypes and Alleles for *IL6* -572 G/C (rs1800796) in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n = 235	n= 87	n = 343			
IL6 -572 G/C (rs1800796)	GG	67 (0.28)	26 (0.29)	52 (0.15)	R	1	-
					0.5632[a]	1.182[a]	0.67-2.08[a]
	GC	134 (0.57)	44 (0.50)	225 (0.65)	**0.0003[b]**	0.462[b]	0.30-0.70[b]
					0.0020[c]	0.391[c]	0.22-0.69[c]
	CC	34 (0.14)	17 (0.19)	66 (0.19)	0.5691[a]	0.776[a]	0.37 – 1.62[a]
					0.0010[b]	0.399[b]	0.23 – 0.69[a]
					0.0655[c]	0.515[c]	0.25 – 1.04[c]
	Allele						
	G	268 (0.57)	96 (0.55)	329(0.48)	R	1	-
					0.6743 [a]	0.927[a]	0.65 – 1.31[a]
	C	202 (0.43)	78 (0.45)	357(0.52)	0.0025[b]	0.694[b]	0.62 – 0.87[b]
					0.0892[c]	0.748[c]	0.76 -1.04[c]

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Table 6: Association of *IL6* SNPs in patients, with generalized and localized vitiligo from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo (Freq.)	Localized Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n = 299	n= 44	n = 343			
IL6 -572 G/C (rs1800796)	GG	82(0.29)	9 (0.21)	52 (0.15)	R	1	-
					0.2911[a]	0.650[a]	0.29 – 1.45[a]
	GC	154(0.54)	26 (0.63)	225 (0.66)	**<0.0001[b]**	0.434[b]	0.28 – 0.64[b]
					0.3292[c]	0.667[c]	0.29 – 1.51[c]
	CC	45 (0.16)	6 (0.14)	66 (0.19)	0.7273[a]	0.823[a]	0.27 – 2.46[a]
					0.0013[b]	0.432[b]	0.25 – 0.72[b]
					0.2434[c]	0.525[c]	0.17 – 1.57[c]
	Allele						
	G	318 (0.57)	44(0.54)	329(0.48)	R	1	-
					0.6180[a]	0.888[a]	0.55 - 1.41[a]
	C	244 (0.43)	38 (0.46)	357(0.52)	**0.0024[b]**	0.707[b]	0.56 – 0.88[b]
					0.32910[c]	0.795[c]	0.50 - 1.26[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Generalized vitiligo vs. Localized vitiligo,
[b]Generalized vitiligo vs. Controls
[c]Localized vitiligo vs. Controls,

7.C.3.3 Relative gene expression analysis of *IL6* in PBMCs of vitiligo patients and controls:

Relative gene expression analysis in PBMCs of 70 patients and 74 controls revealed no significant difference in transcript levels of *IL6* in patients as compared to controls (Mean ΔCp ± SEM: 11.89 ± 0.7297 versus 12.09 ± 0.6649; *p* =0.8354) after normalization with

GAPDH. The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.147-fold increase in the expression of *IL6* transcript levels in patients as compared to controls (Figure 2). Moreover, analysis based on type of the disease revealed non-significant difference in *IL6* transcript levels between GV and LV (p=0.8744); furthermore, GV as well as LV also showed no significant difference in *IL6* transcript levels compared to controls (p=0.1660 and p=0.6135 respectively; Figure 3A). Similarly, analysis based on activity of the disease revealed no significant difference in *IL6* transcript levels between AV and SV (p=0.6041); furthermore, AV as well as SV also exhibited no significant difference in *IL6* transcript levels compared to controls (p=0.1343 and p=0.7148 respectively; Figure 3B). Gender based analysis showed no significant difference in *IL6* transcripts in both the groups (p=0.8995; Figure 3C).

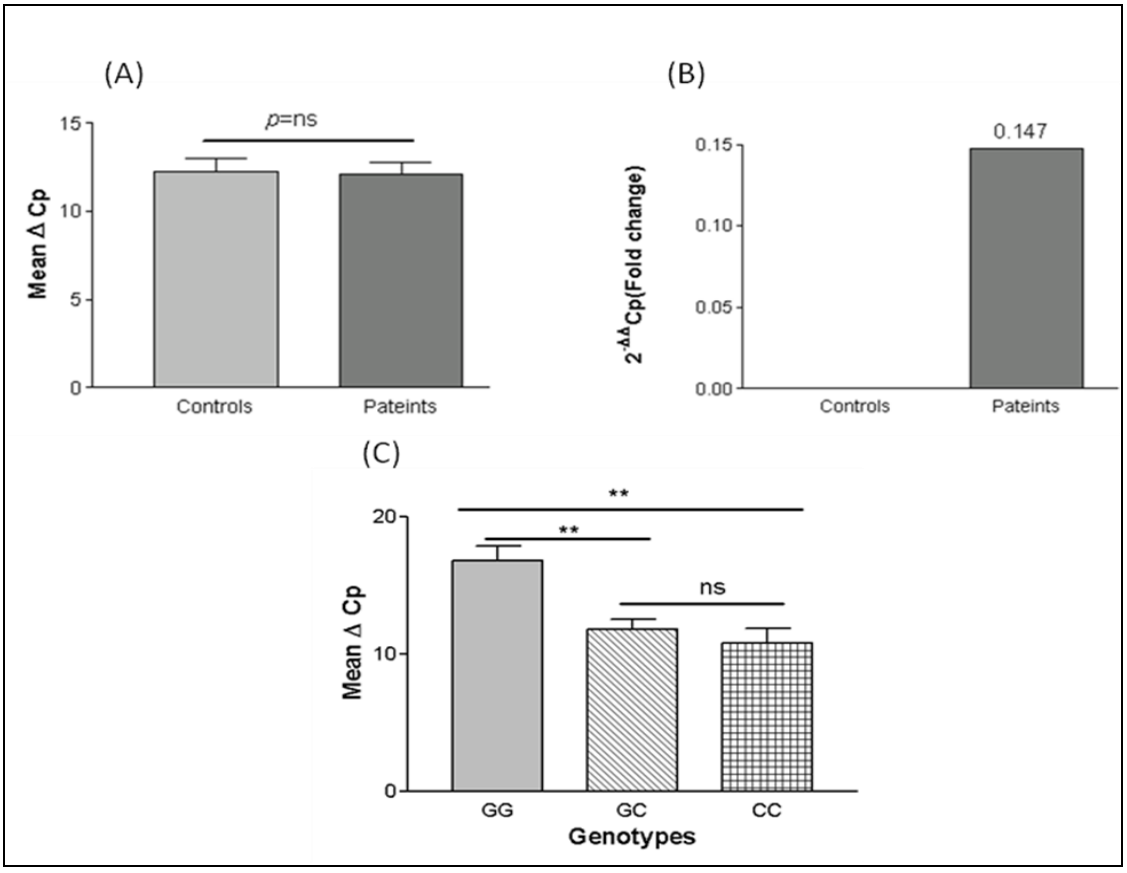

Figure 2: Analysis of *IL-6* transcript levels in controls and vitiligo patients:

(A) Expression of *IL6* transcripts in 74 controls and 70 vitiligo patients as suggested by mean ΔCp. Patients showed no significant difference in the mRNA levels of *IL6* as compared to controls (Mean ΔCp ± SEM: 12.29 ± 0.7436 versus 12.09 ± 0.6649 ; p =0.8423).

(B) Expression fold change of *IL6* transcripts in 70 vitiligo patients against 74 controls showed 0.147-fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Genotype-phenotype correlation analysis showed increased *IL6* expression in carriers of GC genotype as well as CC genotype as compared to carriers of GG genotype (p=0.0060 and p=0.0014, respectively) as suggested by mean ΔCp.

There was non-significant difference in the *IL6* transcript between GC genotype carriers and CC genotype carriers (p=0.4635).

7.C.3.4 Genotype-phenotype correlation analysis:

The expression of *IL6* was analyzed with respect to G/C (-572) genotypes (Figure 2C). Individuals with GC genotype showed significantly increased *IL6* expression levels (p=0.0060) as compared to individuals with GG genotype. Similarly, increased *IL6* expression levels were found in CC genotype as compared to GG genotype (p=0.0014). There was non-significant difference in the *IL6* transcript levels between GC and CC genotype (p=0.4635) (Figure 2 C). The results indicate increased *IL-6* expression is associated with individuals carrying 'C' allele as compared to 'G' allele.

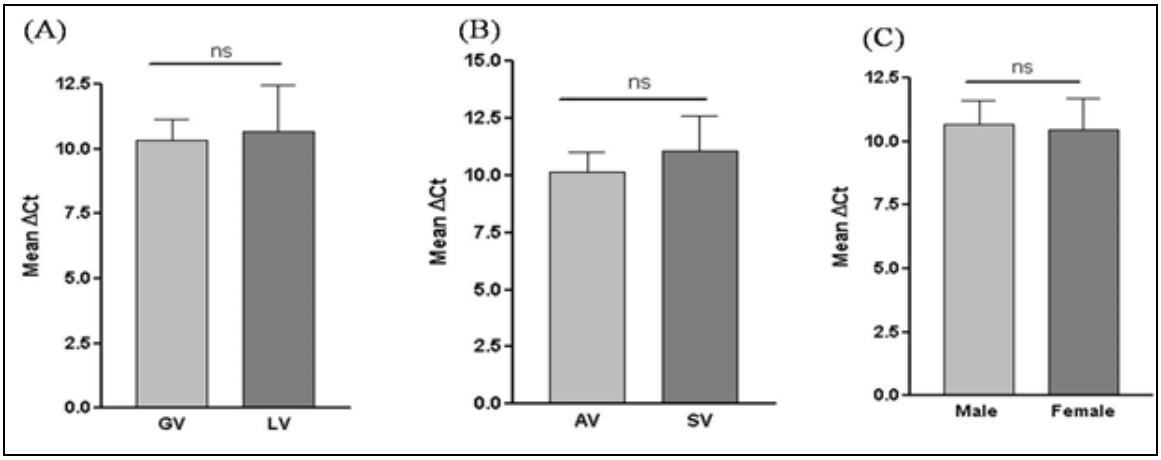

Figure 3 Analysis based on type of the disease revealed no significant difference in *IL6* transcript levels (A) between GV and LV patients (p=0.8744); furthermore GV as well as LV patients showed non-significant difference in *IL6* transcript levels compared to controls (p=0.1660 and p=0.6135 respectively.

(B) analysis based on activity of the disease revealed non-significant difference in *IL6* transcript levels between AV and SV patients (p=0.6041); furthermore AV as well as SV patients showed non-significant difference in *IL6* transcript levels compared to controls (p=0.1343 and p=0.7148 respectively).

(C) Gender based analysis showed no significant difference in *IL6* transcripts in both the groups (p=0.8995).

7C.4 DISCUSSION

Genetic predisposition of key molecules involving cytokines, their receptors and other immune regulatory molecules in addition to epidermal cytokine imbalance advocates autoimmune component for vitiligo precipitation IL-6 plays an important role during the transition from innate to acquired immunity. Also, IL-6 is capable of creating an immunological imbalance between Th-17 cells and Treg cells, resulting in an autoimmune pathology (Kimura *et al*., 2010). Increased levels of IL-6 in skin (Moretti *et al*., 2009) as well as in serum (Farhan *et al*., 2014; Singh *et al*., 2012; Yu *et al*., 1997; Tu *et al*., 2003) of vitiligo patients are well documented. However, a few studies concerning IL6 expression in vitiligo patients are summarized in Table 7.

Table 7: **Reports on IL6 levels in vitiligo:**

Population	IL6 Expression Levels (No. of patients and controls)	Reference
Austrian	Increased levels in Serum (40 patients and 40 controls)	Pichler *et al*., 2009
Chinese	Increased levels in Serum	Tu *et al*., 2003

	(50 patients and 20 controls)	
Indian	Increased levels in Serum (80 patients and 50 controls)	Singh *et al*., 2012
Turkish	No difference in serum levels (40 patients and 40 controls)	Basak *et al*., 2009
Turkish *IL6* promoter -174 C/G (rs1800795) Turkish	No association of *IL6* SNP and vitiligo (105 patients and 211 controls)	Aydıngoz *et al*., 2015
	No difference in serum levels (105 patients and 211 controls)	Aydıngoz *et al*., 2015
Caucasian population	No difference in skin samples as well as serum (15 patients and 14 controls)	Miniati *et al*., 2014
Caucasian	Increased expression levels in skin biopsies (15 patients and 5 controls)	Moretti *et al*., 2009
Taiwanese	MNCs increased (12 patients and 12 controls)	Yu *et al*., 1997
	Increased levels in Serum (39 patients and 42 controls)	Farhan *et al*., 2014
Egypt	Increased levels in Serum (74 patients and 75 controls)	Rashed *et al*., 2015

Saudi Arabia	Increased expression in PBMCs, (32 patients and 32 controls)	Zailaie, 2005

IL6 -174 G/C and -572 G/C promoter polymorphisms are located adjacent to cis-acting regulatory elements involved in controlling *IL6* transcript levels, suggesting that they may influence the interaction of proteins with DNA at these sites (Tanabe *et al*., 1988). Additionally, various findings suggest that promoter polymorphism of *IL6* have regulatory effect on its transcript as well as protein levels, implicating the importance of investigation of *IL6* genetic variants in an autoimmune disorders like vitiligo (Fishman *et al*., 1998). There is a paucity of data on the study of *IL6* genetic variants and vitiligo susceptibility. However, Aydingoz *et al.,* (2015) observed lack of association between *IL6* polymorphism (-174 G/C) and vitiligo with a sample size of 105 vitiligo patients and 211 controls. In the present study, for the first time -572 G/C promoter polymorphism has been addressed in the context of vitiligo. *IL6* -174 G/C and -572 G/C promoter polymorphisms have already been reported in various autoimmune diseases like Systemic Lupus Erythematosus, Multiple Sclerosis, Rheumatoid arthritis. However, Psoriasis vulgaris, Primary Sjogren Syndrome, Dermatomyositis etc. exhibited no association with *IL6* polymorphisms (Table 8).

Table 8: *IL6* promoter polymorphisms and correlation with other diseases.

Disease	**SNP position**	**Result**	**Reference**
Rheumatoid arthritis	-572G/C -174G/C	Associated	Pawlik *et al.*,2005
Kaposi sarcoma	-174G/C	Associated	Foster *et al*.,2000
Type 2 Diabetes	-174G/C	Associated	Huth *et al.*, 2006
Celiac Disease	-174G/C	Associated	Dema *et al*.,2009
Coronary Heart	-174G/C	Associated	Humphries *et al*.,

Disease			2001
Psoriasis Vulgaris	-174G/C	Not Associated	Baran *et al.*, 2008
Primary Sjogren Syndrome	-174G/C	Not Associated	Hulkkonen *et al.*, 2001

Our results suggest that *IL6* -174 G/C is monogenic in Gujarat population which is in accordance with SNPedia report (https://www.snpedia.com/index.php/Rs1800795), stating monomorphic G allele in the promoter region of the *IL6* at position -174 for Asian population. Interestingly, our analysis for -572G/C SNP indicated that the 'GC' genotype was predominant in controls compared to vitiligo patients. The 'CC' genotype was predominant in controls compared to GV patients. Also, our results demonstrated no significant difference in *IL6* transcript levels between lesional and non-lesional skin of patients, or between patient and control skin which are in accordance with the results obtained by Miniati *et al*., (2014). Contrary to this, Moretti *et al*., (2009) have demonstrated increased levels of IL6 in vitiligo skin. Similar to skin *IL6* levels, our PBMC expression analysis also revealed no difference in the expression levels of *IL6* among vitiligo patients and controls as well as upon further analysis based on type of disease, activity and gender. Aydıngoz *et al*., (2014) and Basak *et al*., (2009) reports are in accordance with our results where no-significant difference was found in IL6 levels of vitiligo patients. However, as mentioned in Table 7, Yu *et al*., (1997), Farhan *et al*., (2014), Singh *et al*., (2012); Zailaie, (2005); Tu *et al*., (2003) have reported increased levels of IL6 in vitiligo.

Overall, present study suggests that the -572 G/C promoter polymorphism of *IL6* maybe a genetic risk factor for vitiligo susceptibility in Gujarat population.

7C.5 REFERENCES

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