2.1 Introduction

Vitiligo, one of the most common pigmentory disorders, usually starts in childhood or young adulthood and the clinical manifestation begins before 20 years of age in 50% of cases, while in 25% of cases, the onset is before the age of 14 years (Kakourou, 2009). Our previous study revealed that 21.93% of Gujarat vitiligo patients exhibited positive family history and 13.68% of patients had at least one affected first-degree relative (Shajil *et al.,* 2006a). Large-scale epidemiological studies have reported that about ~15-20% of patients have one or more relatives with vitiligo and similar concordance has been observed in identical twins (Spritz, 2008). Though the exact mechanism underlying the loss of melanocyte in vitiligo is not clear, the advanced research in the past few years has added significantly to understand the disease pathology. Several theories have been put forward to explain the etiology of the disease such as oxidative stress, autoimmune, neurochemical and genetic hypotheses (Kemp *et al.,* 2001; Ongenae *et al.,* 2003; Shajil *et al.,* 2006b). Studies have revealed that vitiligo is a complex, multifactorial and polygenic disorder (Spritz, 2008; Strömberg *et al.,* 2008). The complex genetics of vitiligo involves multiple susceptibility loci, incomplete penetrance and genetic heterogeneity with gene-gene and gene-environment interactions (Zhang *et al.,* 2005). It has been reported that out of the total risk of vitiligo, 20% attributes to environmental factors, 57% to common genetic variants, and 23% to rare genetic variants (Roberts *et al.,* 2019). Currently, more than 50 vitiligo susceptibility loci have been identified in Caucasian population (Spritz and Andersen, 2017). Nevertheless, several vitiligo susceptibility loci in the genes involved in immunoregulation (*CTLA4, NLRP1, MYG1, ICAM1, HLA*), cytokines (*TNFA, TNFB, IL4, IFNG, IL1B, IL1RN*) and redox homeostasis (*SOD, CAT, GPX1, G6PD*) have been identified as susceptibility loci for vitiligo in Gujarat population (Singh *et al.,* 2012, 2018; Imran *et al.,* 2012b; Laddha *et al.,* 2012, 2013b, a, 2014b; Dwivedi *et al.,* 2013a, b; Mansuri *et al.,* 2016, 2017, 2019). Genetic polymorphisms might influence gene expression or protein function and thereby predispose individuals to dysregulation of the normal homeostasis leading to the onset of the disease. Hence, in the present study, we aimed to investigate the association of potential candidate genes involved in stress response and immunoregulatory mechanisms with vitiligo susceptibility in Gujarat population. Based on the literature study, role of the following selected candidate genes polymorphisms has been explored in the present study.

a) Proteasome Subunit Beta 8 (PSMB8) & Transporter associated with Antigen Processing 1 (TAP1)

Generation of antigenic peptides and their transport across the endoplasmic reticulum (ER) membrane for assembly with major histocompatibility complex (MHC) class I molecules are essential steps in antigen presentation to cytotoxic T lymphocytes. Genes within MHC class II loci along with genes involved in antigen processing and presentation, i.e. proteasome subunit beta 8 *(PSMB8)* and transporter associated with antigen processing 1 *(TAP1)* have been reported to be associated with several autoimmune diseases including vitiligo (Djilali-Saiah *et al.,* 1994; Casp *et al.,* 2003; Birlea *et al.,* 2011, 2013; Singh *et al.,* 2012). The *PSMB8*, often referred to as *LMP7,* encodes for interferon-gamma (IFN-γ) inducible subunit of immune proteasome, i.e. β5i involved in the degradation of ubiquitinated intracellular proteins into peptides that are especially suited for presentation by MHC class I molecules. Whereas, *TAP1* encodes the subunit of an IFN-γ inducible heterodimer, which binds with peptides cleaved by the proteasome and transports them to be loaded into nascent MHC class I molecules for its presentation to $CD8^+$ T cells (Song and Harding, 1996; Abele and Tampé, 1999). The genome-wide association study (GWAS) in generalized vitiligo patients revealed that the association of *TAP1-PSMB8* might have derived from linkage disequilibrium with major primary signals in the MHC class I and class II regions (Birlea *et al.,* 2013). Out of 8 different single nucleotide polymorphisms (SNPs) of *PSMB* and *TAP* genes studied, *PSMB8* intron 6 G/T and *TAP1* exon 10 A/G were found to be significantly associated with vitiligo in the Caucasian population (Casp *et al.,* 2003).

b) Methylenetetrahydrofolate reductase (MTHFR)

Several studies have reported increased homocysteine and reduced vitamin B_{12} and folic acid levels in vitiligo patients (Montes *et al.,* 1992; Kim *et al.,* 1999; Shaker and El-Tahlawi 2008; Silverberg and Silverberg 2011; Karadag *et al.,* 2012; Al-Ghamdi *et al.,* 2014). Methylenetetrahydrofolate reductase (MTHFR) is an essential regulatory enzyme involved in the conversion of homocysteine to methionine. It catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The human *MTHFR* gene is located at chromosome 1p363 and consists of 11 exons and 10 introns (Goyette *et al.,* 1994). Polymorphisms of *MTHFR,* i.e. exon 4 C/T and exon 7 A/C result in decreased activity of MTHFR enzyme and affect homocysteine levels (Hustad *et al.,* 2007; Misra *et al.,* 2010). These two polymorphisms of *MTHFR* were reported to be associated with several diseases including vitiligo (Kluijtmans *et al.,* 1996; Botto and Yang, 2000; Ergul *et al.,* 2012).

c) X-box binding protein 1 (XBP1)

X-box binding protein-1 is a transcription factor, encoded by the *XBP1* gene located on chromosome 22. The *XBP1* regulates the expression of genes necessary for the proper functioning of the immune system and in the cellular stress response (Yoshida *et al.,* 2006). XBP1 is involved in the downstream of Inositol Requiring Enzyme-1 (IRE1) activation in the unfolded protein response (UPR) mechanism (Malhotra and Kaufman, 2007). Apart from its known role in UPR, *XBP1* is also involved in the regulation of plasma cell differentiation and immunity (Kaufman and Cao, 2010). IRE1 oligomerizes and activates its ribonuclease domain through auto-phosphorylation. Activated IRE1 leads to the non-canonical splicing of a 26-nucleotide sequence from ubiquitously expressed *XBP1* mRNA (unspliced). Removal of this intron causes a frameshift in the *XBP1* mRNA coding sequence resulting in the translation of a 376 amino acid polypeptide i.e. spliced XBP1 (sXBP1) isoform rather than the 261 amino acid polypeptide, unspliced XBP1 (uXBP1) isoform (Lee *et al.,* 2002; Yoshida, 2007a). The sXBP1 is an active transcription factor that upregulates target genes via the ER stress-responsive element (ERSE) region. *XBP1* -116 G/C polymorphism is located in the promoter of the *XBP1* gene affecting its promoter activity (van Geel *et al.,* 2019). *XBP1 -116 G/C* promoter polymorphism was found to be associated with diabetes, inflammatory bowel disease and bipolar disorder (Chen *et al.,* 2004; Masui *et al.,* 2005; Kaser *et al.,* 2008).

d) Interleukin-17A (IL17A)

IL-17A is a disulfide-linked homodimeric pro-inflammatory cytokine produced by Th17 cells, which form a distinct subset of the $CD4^+$ T-cell lineage (Pappu *et al.,* 2011). It plays a major role in psoriasis and over recent years, has garnered interest of many researchers due to its association with several autoimmune disorders (Kamali *et al.,* 2019). The involvement of Th17 cells has also been reported in autoimmune skin inflammatory disorders such as psoriasis and atopic dermatitis (Asarch *et al.,* 2008; Lynde *et al.,* 2014). A previous study has reported elevated IL17 levels in lesional skin and serum of vitiligo patients (Bassiouny and Shaker, 2011). Another study showed a positive correlation between serum IL-17A levels and the extent of the depigmentation patch area in vitiligo, suggesting that Th17 cells are involved in the progression of vitiligo (Basak *et al.,* 2009). Interestingly, it was found that IL-17A can stimulate the keratinocytes and fibroblasts to secrete TNF-α (Kotobuki *et al.,* 2012) The *IL17A* gene is located on chromosome 6, spanning a region of 4252 bp. Two polymorphisms in the *IL17A* promoter region (-197 G/A and -737 C/T) are reported to be

associated with various disorders (Lew *et al.,* 2012; Omrane *et al.,* 2014; Shen *et al.,* 2015; Hu *et al.,* 2018; Kasamatsu *et al.,* 2018).

e) Tyrosinase (TYR)

Tyrosinase is present inside the melanosomes and it is a key enzyme involved in melanogenesis. The enzyme is mainly involved in two distinct reactions of melanin synthesis, i) the hydroxylation of a mono-phenol and, ii) the conversion of an o-diphenol to the corresponding o-quinone. The o-quinone undergoes several reactions to eventually form melanin pigment (Slominski *et al.,* 2004; Hearing 2007). Interestingly, tyrosinase is also identified as a principal autoantigen in vitiligo (Song *et al.,* 1994; Baharav *et al.,* 1996). Tyrosinase is encoded by the *TYR* gene present on the 11th chromosome (Barton *et al.,* 1988). Polymorphisms in the *TYR* gene have been associated with various pigmentary disorders and traits, including vitiligo (Sulem *et al.,* 2007; Han *et al.,* 2008; Nan *et al.,* 2009; Jin *et al.,* 2010). Two non-synonymous polymorphisms of the *TYR* gene, i.e. R402Q and S192Y were identified as susceptibility loci for generalized vitiligo in the Caucasian population (Jin *et al.,* 2010). Intriguingly, Jin *et al.,* (2012) have reported that *TYR* R402Q and S192Y haplotypes modulate the amount of presentation of tyrosinase peptides by HLA-A*02:01, thereby modulating recognition of melanocytes by autoreactive cytotoxic T lymphocytes (CTL).

2.2 Materials and methods

2.2.1 Ethics committee approval

The importance of the study was explained to all participants and written consent was obtained. Written consent was obtained from the next of kin, caretakers, or guardians on behalf of the minors/ children enrolled in the study. The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/RB/1).

2.2.2 Study Subjects

A total of 558 ethnically age and gender-matched controls and 520 patients with vitiligo were recruited in the present study from the Gujarat population. Controls with age between 5 to 60 years without any evidence of vitiligo or other disease were selected. Patients with vitiligo who referred to S.S.G. Hospital at Vadodara, Gujarat, India were selected for the study. The

inclusion criteria followed were outpatients of age between 5 to 60 years, and both the parents should be Gujarati by birth. Patients with other diseases and those unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white colour lesions on the skin, under Woods lamp. Generalized or non-segmental vitiligo (GV) was characterized by depigmented patches varying in size from a few to several centimetres in diameter, involving one or both sides of the body with a tendency towards symmetrical distribution. Whereas localized or segmental vitiligo (LV) typically has a rapidly progressive but limited course, depigmentation spreads within the segment during a period of 6–24 months and then stops; further extension is rare (Ezzedine *et al.,* 2012). Following clinical criteria proposed by Falabella *et al.,,* (1995) and discussed in the Vitiligo Global Issues Consensus Conference 2012, were used for characterizing stable vitiligo (SV): (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active vitiligo (AV) was defined as the appearance of new lesions and the spreading of existing lesions observed during the past two-year duration (Falabella *et al.,* 1995; Ezzedine *et al.,* 2012). The demographic characteristics of the patients are provided in Table 2.1.

2.2.3 Blood and skin sample collection

5ml blood was drawn from each participant and collected in EDTA tubes. Blood samples were centrifuged (3000g for 5 min at 4^{o}C) to separate the plasma and cells. The plasma fraction was used for ELISA assays and cell fraction was used for DNA and RNA extraction. Skin biopsy samples were collected from selected controls and vitiligo patients who had not received systemic immunosuppressive treatment or PUVA/UVB for at least 1 month, and topical therapy for at least 2 weeks. 4-mm punch biopsies were taken and snap-frozen from the lesional and non-lesional skin of patients with vitiligo, and non-inflamed, non-irritated skin of healthy individuals.

2.2.4 Genomic DNA extraction

Genomic DNA was extracted from PBMCs using 'QIAamp^{TM} DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. Concentration and purity of DNA were estimated spectrophotometrically, the quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses. Genotyping of the polymorphisms was carried out by polymerase chain reaction-

restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) methods as applicable.

Table 2.1. Demographic characteristics of patients with vitiligo and controls.

	Vitiligo Patients	**Controls**
	(n=520)	(n=558)
Average age (mean age ± SD)	34.97 ± 14.94 yrs	30.51 ± 11.55 yrs
Sex: male	248 (47.69%)	244 (43.73%)
female	272 (52.31%)	314 (56.27%)
Onset age (mean age ± SD)	23.54. ± 13.48 yrs	NA
Duration of disease (mean ± SD)	7.73 ± 6.29 yrs	NA
Family history	171 (31.92 %)	NA
Type of disease		
Generalized	396	NA
Localized	124	NA
Active	415	NA
Stable	105	NA

2.2.5 SNP genotyping by PCR-RFLP method

PCR-RFLP method was used to genotype *PSMB8* intron 6 C/T (rs2071627), *MTHFR* exon 4 C/T (rs1801133), *XBP1* -116 G/C (rs2269577), *IL17A* -197 G/A (rs2275913) and -737 C/T (rs8193036), *TYR* exon 1 A/C (rs1042602) and exon 4 G/A (rs1126809) polymorphisms. The details of specific primers and restriction enzymes used are provided in Table 2.2. The reaction mixture of the total volume of 20 µL included 3 µL (100ng) of genomic DNA, 11 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (PureGene™, India), 1 µL of 10 pM corresponding forward and reverse primers (Eurofins™, India), and 1 U of Taq Polymerase (PureGene™, India). Amplification was performed in Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany) according to the protocol: 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, annealing for 30 seconds (Table 2.2) and 72°C for 30 seconds, and 72°C for 10 minutes. The amplified products were checked by

electrophoresis on a 2.0% agarose gel stained with ethidium bromide. 15 µL of the amplified products were digested with 1U of respective restriction enzyme in a total reaction volume of 20 µL as per the manufacturer's instruction. The digested products were resolved with a 50 bp DNA ladder (HiMedia™, India) on 3.5% agarose gel stained with ethidium bromide and visualized under E-Gel Imager (Life Technologies™, Carlsbad, CA). More than 10% of the samples were randomly selected for reconfirmation and the results were 100% concordant.

2.2.6 SNP genotyping by ARMS-PCR method

TAP1 exon 10 A/G (rs1135216) and *MTHFR* exon 7 A/C (rs1801131) polymorphisms were genotyped by 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 for respective SNPs (Table 2.2). To assess the success of PCR amplification in both the reactions, a reaction control of 407 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (HGH) (Table 2.2). The reaction mixture of the total volume of 15 µL included 3 µL (100 ng) of genomic DNA, 4.7 µL nuclease-free H_2O, 1.5 µL 10x PCR buffer, 1.5 µL 2mM dNTPs (PureGene™, India), 1 µL of 10 pM allele-specific and common primers for respective SNP (Eurofins™, India), 1 µL of 10 pM control primers (HGH), and 1 U of Taq Polymerase (PureGene™, India). Amplification was performed using Mastercycler Gradient PCR (Eppendorf™, Germany) according to the protocol: 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, annealing for 30 seconds (Table 2), and 72°C for 30 seconds, and 72°C for 10 min. The PCR products were resolved on 3.5% agarose gel stained with ethidium bromide along with 50bp DNA ladder (HiMedia™, India) and visualized under E-Gel Imager (Life Technologies™, Carlsbad, CA). More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant.

Table 2.2. Details of primers and restriction enzymes used for genotyping of single nucleotide polymorphisms.

Gene/ SNP	Genomic context	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme	Digested Products (bp)
Genotyping by PCR- RFLP method							
PSMB8 rs2071464	Intron 6 C/T	FP	GCTATTCTGGAGGCGTTGTC	58	298	*Hha* I	257 + 41
		RP	AAGGAGTCTCACTCTGTGGC				
MTHFR rs1801133	Exon 4 C/T	FP	TGAAGGAGAAGGTGTCTGCGGGA	71	225	*Hinf* I	173 + 52
		RP	AGGACGGTGCGGTGAGAGTG				
XBP1 rs2269577	Promoter -116 G/C	FP	GTTTCAGGACCGTGGCTATG	60	309	*Tai* I	224 + 85
		RP	TCGGCTCCACTCGGATC				
IL17A rs2275913	Promoter -197 G/A	FP	GAGAAAAGAACCGCTAACTCC	68	361	*Xmn* I	259 + 102
		RP	TACTCAAGTTCCCATCATATAAG				
IL17A rs8193036	Promoter -737 C/T	FP	GAGAAAAGAACCGCTAACTCC	64	401	*Mbo* II	308 + 93
		RP	TACTCAAGTTCCCATCATATAAG				
TYR rs1042602	Exon 1 C/A	FP	GACCTCTTTGTCTGGATGC	60	281	*Sau3A* I	229 + 52
		RP	GATGCTGGGCTGAGT				
TYR rs1126809	Exon 4 G/A	FP	GACCTCTTTGTCTGGATGC	60	181	*Hpy*188 I	138 + 43
		RP	GATGCTGGGCTGAGT				
Genotyping by ARMS-PCR method							
TAP1 rs1135216	Exon 10 A/G	FP-A	TTGCTCTGCAGAGGTAGA	61	151	-	-
		FP-G	TTGCTCTGCAGAGGTAGG				
		RP	CACCTGTAACTGGCTGTTTG				
MTHFR rs1801131	Exon 7 A/C	FP-A	GGGAGGAGCTGACCAGTGAAGA	65	276	-	-
		FP-C	GGAGGAGCTGACCAGTGAAGC				
		RP	CGAAGACTTCAAAGACACTTT				
HGH	(Reaction control)	FP	CTATGCTCCGCGCCCATCGT	61 / 65	407	-	-
		RP	TGGGGAGAAGGCATCCACTCACG				

2.2.7 RNA isolation and cDNA synthesis from the blood and skin samples

Total RNA from whole blood was extracted using Ribopure™ blood kit (Ambion Inc. Texas, USA) following the manufacturer's protocol whereas total RNA from skin biopsies was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by 1.5% agarose gel electrophoresis. RNA yield and purity were determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion Inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. 1 µg of total RNA was used to prepare cDNA. cDNA synthesis was performed using the Verso cDNA synthesis kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf™, Germany).

2.2.8 Gene expression analysis using qPCR

Transcript level analyses of selected candidate genes were carried out from PBMCs and skin samples using qPCR. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* was used as a reference gene. The primer details for respective genes are provided in Table 2.3. Real-Time qPCR was performed in duplicates in 20 µl volume using Light Cycler® 480 SYBR Green I Master Mix (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's instructions 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 95°C for 20 seconds, annealing for 20 seconds (Table 2.3), and 72°C for 20 seconds. The fluorescence signals were captured 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. C_t values were determined by the first cycle number at which fluorescence was greater than the set threshold value. The ΔC_t value was determined as the difference between the cycle threshold of target genes and the reference gene (*GAPDH*). The difference between the two ΔC_t values (ΔC_t controls and ΔC_t patients) was considered as $\Delta\Delta C^t$ to obtain the value of fold expression ($2^{-\Delta\Delta Ct}$).

Table 2.3: Details of primers used for gene expression analysis.

Gene	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)
GAPDH	FP	CATCACCATCTTCCAGGAGCGAG	65	122
	RP	CCTGCAAATGAGCCCCAGCCT		
PSMB8	FP	TCCTACATTAGTGCCTTACGGG	65	135
	RP	CAGATAGTACAGCCTGCATTCC		
TAP1	FP	GGACCACTAGTATTTCAGGTATGC	65	149
	RP	GAGCAGTACCTCCACAGCC		
XBP1	FP-U (Unspliced)	TCCGCAGCACTCAGACTAC	60	Unspliced: 186 Spliced: 163
	FP-S (Spliced)	GAGTCCGCAGCAGGTGC		
	RP	TGGCAGGCTCTGGGGAAG		
IL17A	FP	ATCACAATCCCACGAAATCCAG	60	188
	RP	CTTTGCCTCCCAGATCACAGAG		

2.2.9 Western blot analysis for estimation of PSMB8 protein expression

Five ml of blood was drawn from healthy controls and patients with active generalized vitiligo and collected in EDTA vials. Red blood cells were lysed with RBC lysis buffer (0.17 M Tris/ 0.16 M NH4Cl pH 7.2) and the remaining leukocytes were washed in PBS, and lysed in lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) containing 1x proteinase inhibitors (Sigma, Bangalore, India). Protein concentration was determined by Bradford assay (HiMedia™ Laboratories, India) and 20µg protein was loaded on 12% SDS-PAGE along with Precision Plus Protein™ Dual Color Standards (Bio-Rad, Germany). Protein was electro-blotted on the PVDF membrane at 100 V for 1.5 hrs. Following the transfer, the membrane was blocked with 5% blocking buffer (5% BSA and 0.1% Tween-20 in PBS) for 1 hr at room temperature. The membrane was incubated overnight with the primary antibody against LMP7/PSMB8 (ab58094, Abcam, UK). After incubation, the membrane was washed four times with PBS-T (PBS containing 0.1% Tween 20) for 15 min. and incubated with a secondary anti-mouse IgG antibody (Bangalore Genei™, India) at room temperature for 1 hr. The membrane was similarly washed four times with PBS-T, and protein bands on the membrane were then visualized by using Bio-Rad

Clarity™ western ECL substrate (Bio-Rad, Germany), and the signal was scanned using the Chemidoc™ Touch Gel Imaging System (Bio-Rad, Germany). Intensities of target proteins were normalized with that of total protein loading by staining the membrane with Ponceau. The densitometric analysis of the protein bands was calculated by ImageJ software.

2.2.10 Collection of suction induced blister fluid samples

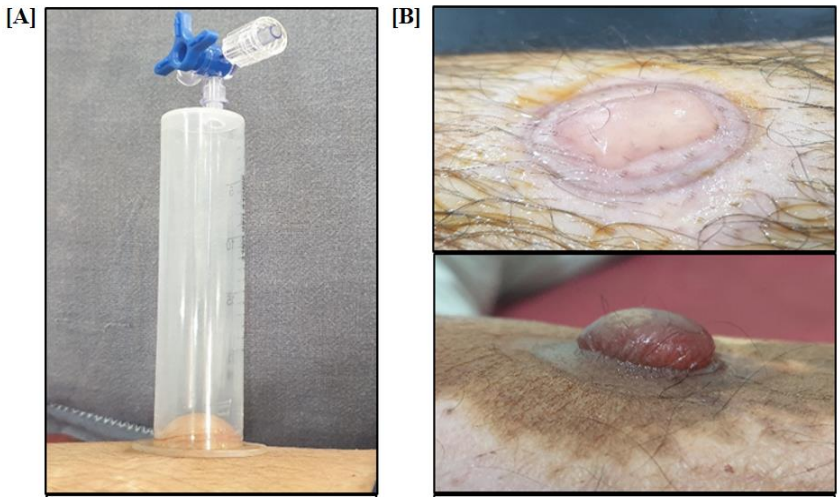

Figure 2.1: Representative images of [A] suction device and [B] suction induced blisters.

Suction induced blister fluid samples were collected from the skin of healthy controls (n=18) and patients with active generalized vitiligo (n=15) with the help of an expert dermatologist (Figure 2.1). The povidone-iodine solution was applied to the suction area, which was then locally anesthetized by the infiltration of 2% lignocaine. Suction blisters were induced by applying the bases of the sterile disposable 20 ml syringes whose piston was removed, and their nozzles were connected to other 50 ml syringes to generate suction, then the infusion set valve was closed to maintain a negative suction. After a unilocular blister was obtained, the fluid was collected by a 2ml syringe and centrifuged at 3000rpm for 5 min at 4^{o}C. The blister fluid samples were then stored at -80^{o} C until the time of analysis.

2.2.11 Estimation of IL-17A and anti-tyrosinase antibody levels by ELISA

IL-17A protein levels were measured from suction induced blister fluid samples of the skin by using the human IL-17A ELISA Kit (KB1079; KRISHGEN Biosystems, India) and anti-tyrosinase antibody levels were measured from plasma samples by using human anti-tyrosinase antibody ELISA kit (K12-2147, Kinesis Dx, USA) as per the manufacturer's protocol.

2.2.12 Statistical analyses

Hardy-Weinberg equilibrium (HWE) was evaluated for all the SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. Distribution of the genotypes and allele frequencies of polymorphisms in different groups were compared using the chi-square test with 2×2 contingency tables. The major genotype /allele was used as a reference. Multiple comparisons were controlled by Bonferroni's method. Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Haplotype and LD analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Yong and Lin 2005). For analyses of the transcript and protein levels, unpaired t-test and one-way ANOVA were performed as applicable. Tukey's multiple-correction was applied for multiple testing and the *p* values were adjusted. All the statistical tests were carried out using Prism 6 software (Graph Pad Software, USA).

2.2.13 Bioinformatics analyses

In silico prediction tools, HaploReg v4.1 (Ward and Kellis, 2012) and Regulome DB (Boyle *et al.,* 2012) were employed to predict the functional impact of non-coding polymorphisms. *In silico* prediction tools SIFT (Kumar *et al.,* 2009), PANTHER (Thomas *et al.,* 2003), I-MUTANT SUITE (Capriotti *et al.,* 2008), POLYPHEN (Adzhubei *et al.,* 2010), MUPRO (Cheng *et al.,* 2005) were employed to predict the sequence-based impact on the protein due to single amino acid variation. SNPs and GO (Capriotti *et al.,* 2006) predict the variation effect, which might lead to a disease like a trait. Further, structure-based *in silico* prediction was carried out to assess the effect of non-synonymous SNPs on MTHFR protein. The full-length amino acid sequence of human MTHFR (accession number: P42898) was retrieved from the Universal Protein Resource database (http:/www.uniprot.org/) with a predicted molecular mass of 74.6 kDa. The observed mutation was done at the sequence level and submitted for homology modeling to I-TASSER to generate a 3D model (Zhang, 2008; Roy *et al.,* 2010, 2012). The best model was selected from I-TASSER and refined using the ModRefiner tool (Xu and Zhang, 2011). The refined models were then aligned using the PyMOL viewer (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

2.3 Results:

2.3.1 Investigating the role of *PSMB8* intron 6 C/T (rs2071627) and *TAP1* exon 10 A/G (rs1135216) polymorphisms with vitiligo susceptibility

2.3.1.1 Analysis of *PSMB8* rs2071627 polymorphism

Three genotypes were identified for *PSMB8* rs2071627 polymorphism (Figure 2.2). Genotyping of *PSMB8* intron 6 rs2071464 SNP by PCR-RFLP using *Hha* I and subsequent sequencing results revealed that there is C>T nucleotide change instead of previously reported G>T change, which falls in *Hha* I recognition/ restriction site and was imputed to *PSMB8* rs2071464 SNP (Deng *et al.*, 1995; Jin *et al.*, 2016; Dani *et al.*, 2018).

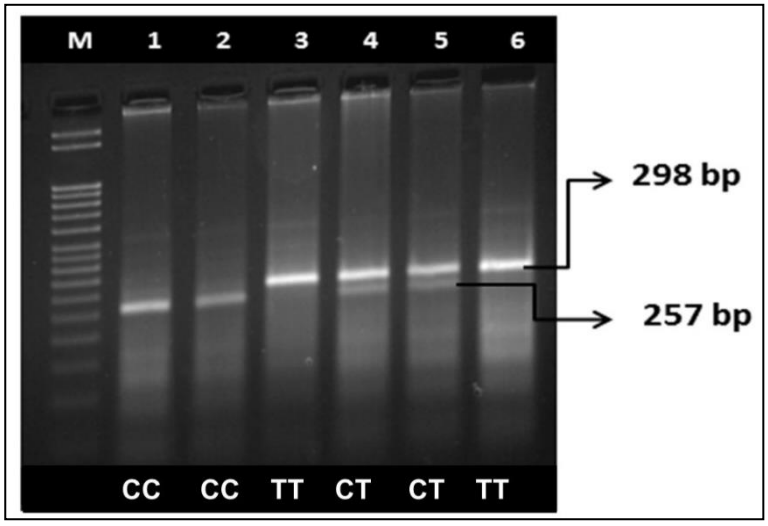

Figure 2.2: PCR-RFLP analysis of *PSMB8* rs2071464 polymorphism on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1 & 2 show homozygous (CC) genotype; lanes: 3 & 6 show homozygous (TT) genotype and lanes: 4 & 5 show heterozygous (CT) genotype.

The observed genotype frequencies of *PSMB8* rs2071464 SNP among the controls were in accordance with HWE (p=0.071), whereas genotype frequencies among the patients deviated (p=0.001) from HWE. When 'C' allele and CC genotype were used as the reference, the frequencies of the variant 'T' allele and homozygous 'TT' genotype were significantly lower in patients with vitiligo as compared to controls (49% vs. 54%, p=0.031; 19% vs. 27%, p=0.026 respectively) but it did not remain significant after Bonferroni's correction. The protective role of 'TT' genotype in patients was suggested by OR=0.629 (95% CI=0.41-0.94). The OR suggests that the minor allele 'T' might have a protective role in the disease pathogenesis (Table 2.4). Analysis based on types of vitiligo revealed a significantly low frequency of 'TT' genotype (18% vs. 27%, p=0.019) and 'T' allele (48% vs. 54%, p=0.024)

in patients with GV as compared to controls. No significant difference was observed in genotype and allele frequencies between patients with LV in comparison to patients with GV or controls (Table 2.5). Interestingly, a similar trend was observed upon an analysis based on the activity of the disease (Table 2.5). Predominantly, an increased frequency of the risk genotype 'CC' (24% vs.19%) and allele 'C' (53% vs. 46%) was observed in patients with AV as compared to controls. The frequency of the protective genotype 'TT' (18% vs. 27%, p=0.005) and allele 'T' (47% vs. 54%, p=0.007) was significantly lower in comparison to controls. However, no significant difference in allele and genotype frequencies was observed between patients with AV and SV.

2.3.1.2 Analysis of *TAP1* rs1135216 polymorphism

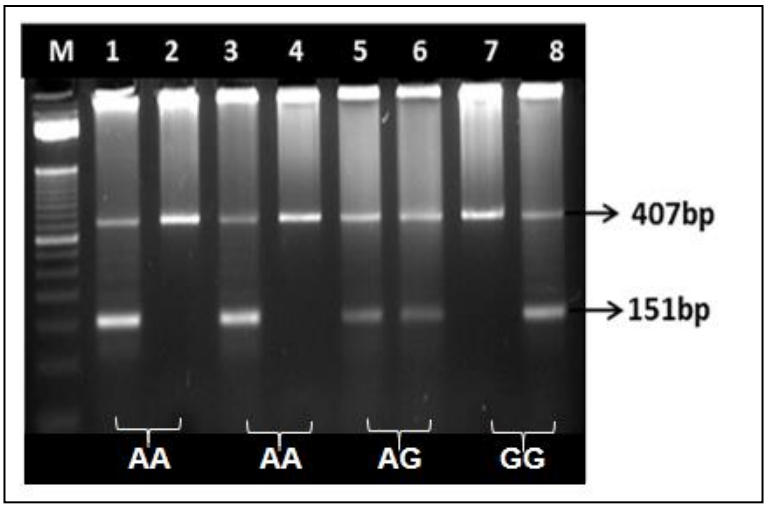

Figure 2.3: ARMS-PCR analysis of *TAP1* rs1135216 polymorphism on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1, 2 & 3, 4 show homozygous (AA) genotype; lanes: 5, 6 show heterozygous (AG) genotype and lanes: 7, 8 show homozygous (GG) genotype.

Three genotypes were identified for *TAP1* rs1135216 polymorphism (Figure 2.3). Both control and patient groups were following HWE (p=0.663 and p=0.167, respectively; Table 2.4). Major allele 'A' and 'AA' genotype were considered as the reference. There was no significant difference in the allele and genotype frequencies of controls and patients (Table 2.4). *TAP1* SNP, when analysed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed (Table 2.5). Analysis based on the activity of the disease also showed no significant difference among the genotypes as well as allele frequencies (Table 2.5).

Table 2.4. Distributions of genotype and allele frequencies of *PSMB8* rs2071464 and *TAP1* rs1135216 polymorphisms in vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=509	Patients (Freq) n=378	*p* for HWE	*p* value[#] for association	OR	95% CI
PSMB8 intron 6 C/T (rs2071464)	CC	97 (0.19)	82 (0.59)	0. 071 (C)	R	1	-
	CT	273 (0.54)	222 (0.59)		0.825[a]	0.961	0.68-1.35
	TT	139 (0.27)	74 (0.19)		0.026[a]	0.629	0.41-0.94
	C	467 (0.46)	386 (0.51)	0.001 (P)	R	1	-
	T	551 (0.54)	370 (0.49)		0.031[b]	0.812	0.67-1.98
TAP1 exon 10 A/G (rs1135216)	AA	341(0.67)	263(0.70)	0.663 (C)	R	1	-
	AG	153(0.30)	100(0.26)		0.278[a]	0.847	0.63-1.14
	GG	15(0.04)	15(0.04)		0.487[a]	1.297	0.62-2.70
	A	835(0.82)	626(0.83)	0.167 (P)	R	1	-
	G	183(0.18)	130(0.17)		0.670[b]	0.950	0.74-1.21

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
(P) refers to Patients and (C) refers to Controls,
[a]Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b]Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table,
[#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

Table 2.5 Distributions of genotype and allele frequencies of *PSMB8* rs2071464 and *TAP1* rs1135216 polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=509	GV n=292	LV n=86	*p* value[#]	AV n=350	SV n=73	*p* value[#]
PSMB8 intron 6 C/T (rs2071464)	CC	97 (0.19)	64 (0.22)	18 (0.21)	R	72 (0.24)	10 (0.16)	R
	CT	273 (0.54)	174 (0.60)	48 (0.56)	0.951[a] 0.854[b] 0.858[c]	178 (0.58)	44 (0.60)	0.123[x] 0.478[y] 0.224[z]
	TT	139 (0.27)	54 (0.18)	20 (0.23)	0.461[a] 0.019[b] 0.468[c]	55 (0.18)	19 (0.24)	0.031[x] 0.005[y] 0.493[z]
	C	467 (0.46)	302 (0.52)	84 (0.49)	R	322 (0.53)	64 (0.44)	R
	T	551 (0.54)	282 (0.48)	88 (0.51)	0.507[a] 0.024[b] 0.471[c]	288 (0.47)	82 (0.56)	0.052[x] 0.007[y] 0.644[z]
TAP1 exon 10 A/G (rs1135216)	AA	341 (0.67)	203 (0.69)	60 (0.70)	R	205 (0.67)	58 (0.80)	R
	AG	153 (0.30)	78 (0.27)	22 (0.26)	0.868[a] 0.347[b] 0.450[c]	86 (0.28)	14 (0.19)	0.086[x] 0.677[y] 0.045[z]
	GG	15 (0.04)	11 (0.04)	4 (0.04)	0.730[a] 0.608[b] 0.470[c]	14 (0.05)	01 (0.01)	0.156[x] 0.246[y] 0.352[z]
	A	835 (0.82)	484 (0.83)	142 (0.88)	R	496 (0.81)	130 (0.89)	R
	G	183 (0.18)	100 (0.17)	30 (0.12)	0.922[a] 0.666[b] 0.866[c]	114 (0.19)	16 (0.11)	0.026[x] 0.719[y] 0.035[z]

n: number of subjects; R: reference group; GV: Generalized vitiligo; LV: Localized vitiligo; AV: Active Vitiligo; SV: Stable Vitiligo; [a]Generalized vitiligo vs. Localized vitiligo; [b]Generalized vitiligo vs. Controls; [c]Localized vitiligo vs. Controls; [x]Active Vitiligo vs. Stable Vitiligo; [y]Active Vitiligo vs. Controls; [z]Stable Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table. [#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

2.3.1.3 Linkage disequilibrium and haplotype analyses

LD analysis revealed that two polymorphisms investigated i.e. *PSMB8* rs2071464 and *TAP1* rs1135216 were in low LD association (D'=0.432, r^2=0.044). Haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes were not significantly different between patients and controls (global *p*=0.278; Table 2.6).

Table 2.6. Distribution of haplotypes frequencies for *PSMB8* rs2071464 and *TAP1* rs1135216 polymorphisms in vitiligo patients and controls.

Haplotype [*PSMB8* (C/T): *TAP1* (A/G)]	**Patients (Freq)**	**Controls (Freq)**	***p* for association**	***p* (Global)**	**Odds Ratio [95%CI]**
C A	222 (0.38)	194 (0.31)	0.058	0.278	1.26 [0.99~1.60]
C G	72 (0.12)	75 (0.27)	0.904		0.98 [0.69~1.38]
T A	262 (0.45)	296 (0.15)	0.092		0.82 [0.65~1.03]
T G	30 (0.05)	31 (0.27)	0.908		0.97 [0.58~1.62]

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

2.3.1.4 Analysis of *PSMB8* transcript levels

Analysis of *PSMB8* transcript levels revealed a significant decrease in the expression of *PSMB8* transcripts in patients as compared to controls (*p*=0.002; Figure 2.4A) after normalization with *GAPDH* expression. The $2^{-\Delta\Delta Cp}$ analysis showed an approximate 0.52-fold decrease in the expression of *PSMB8* transcript levels in patients, as compared to controls (Figure 2.4A). Interestingly, analysis based on type and activity of the disease revealed that *PSMB8* transcript levels were significantly decreased in patients with GV as well as AV in comparison to controls (*p*=0.007 and *p*=0.006, respectively; Figure 2.4 B and C), suggesting a role in the autoimmune basis of the disease. However, there was no significant difference in patients with LV and SV as compared to controls (*p*=0.090 and *p*=0.112, respectively; Figure 2.4 B and C). Also, no significant difference in transcript levels was observed between GV as compared to LV and AV as compared to SV patients (Figure 2.4 B and C). When the expression of *PSMB8* transcripts was monitored in different groups of age at onset of patients, no significant difference was observed in any of the groups, i.e. 21–40, 41–60, and 61–80 years when compared with 1-20 years (Figure 2.4 D). The gender-based analysis also showed no significant difference in *PSMB8* transcripts in both the groups (*p*=0.396; Figure 2.4 E).

Further, the expression of *PSMB8* transcripts was analysed with respect to *PSMB8* rs2071464 genotypes. Interestingly, *PSMB8* transcript levels were significantly reduced in individuals with the susceptible CC genotype when compared with CT and TT genotypes (p=0.009 and p=0.003, respectively; Figure 2.4 F). However, no significant difference in *PSMB8* transcripts levels was observed between individuals with the CT and TT genotypes (Figure 2.4 F).

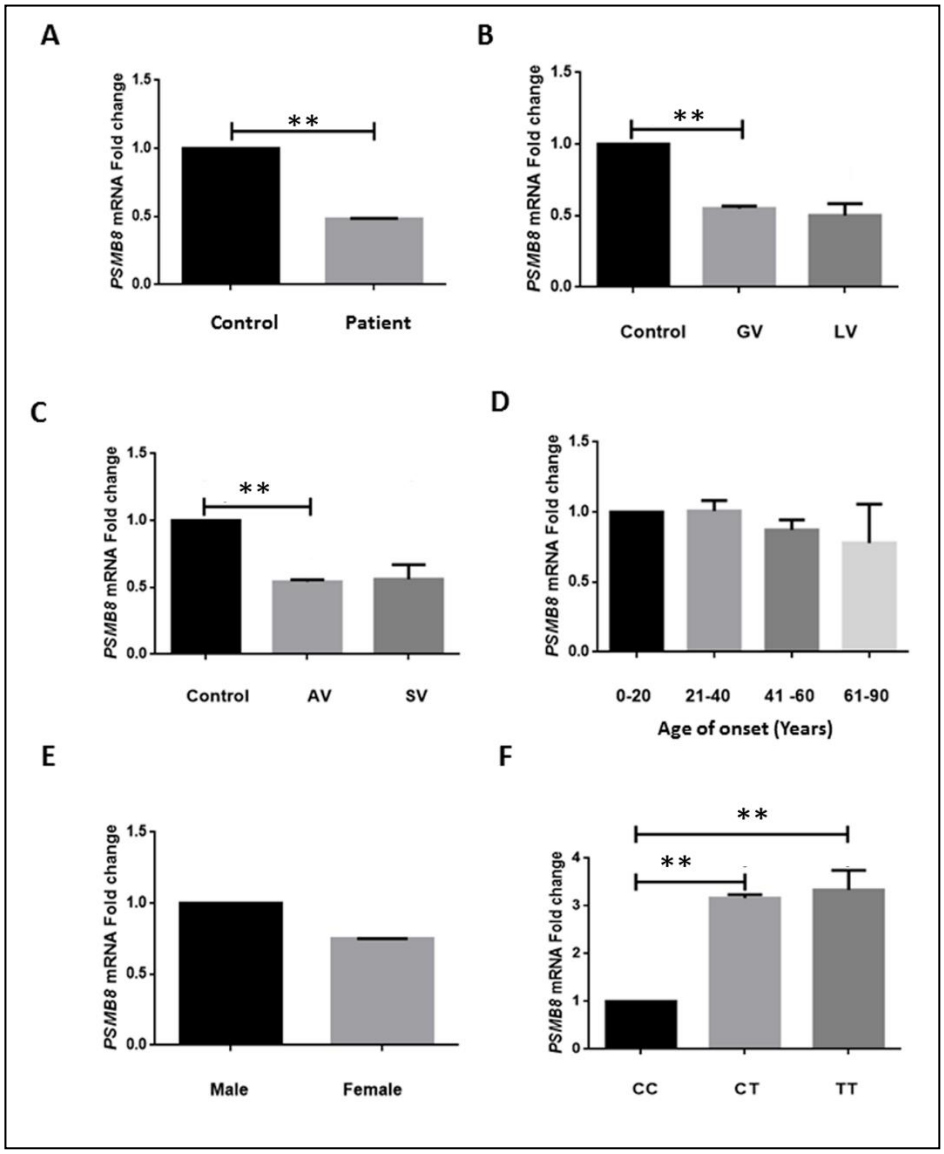

Figure 2.4. Relative gene expression of *PSMB8* in vitiligo patients and controls. Expression of *PSMB8* transcripts in 96 controls, 91 patients with vitiligo was analysed by applying unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups. [A] Patients showed a significant decrease in transcript levels of *PSMB8* compared to controls (mean ΔCt ± SEM: 8.958±0.239 vs. 10.01 ± 0.229; p=0.002). Expression of *PSMB8* transcripts in patients against controls showed 0.52 -fold decrease as determined by the $2^{-\Delta\Delta Cp}$ method. [B] Expression of *PSMB8* transcripts in 96 controls and 72 patients with GV and 19 patients with LV was analysed. Patients with GV showed significantly decreased *PSMB8* transcript levels as compared to controls (p=0.007).

However, there was no significant difference in *PSMB8* transcript levels between patients with GV and LV as well as in patients with LV as compared to controls (p=0.975 and p=0.090, respectively). [C] Analysis of *PSMB8* transcript levels in 96 controls and 69 patients with AV and 22 patients with SV revealed that patients with AV had significantly decreased *PSMB8* transcript levels as compared to controls (p=0.006). However, there was no significant difference in *PSMB8* transcript levels between patients with AV and SV as well as in patients with SV as compared to controls (p=0.999 and p=0.112, respectively). [D] Analysis of *PSMB8* transcript levels with respect to the different age of onset groups in 91 patients with vitiligo revealed no significant difference in *PSMB8* transcript levels in patients with respect to age at onset. [E] Expression of *PSMB8* transcripts with respect to sex differences in 48 male and 43 female patients revealed no significant difference in both groups (p=0.396). [F] Expression of PSMB8 transcripts with respect to the *PSMB8* rs2071464 SNP in 96 controls and 91 patients were further analysed. Individuals with the CC genotype showed a decreased *PSMB8* transcripts when compared with CT and TT genotypes (p=0.009 and p=0.003, respectively). No significant difference in *PSMB8* transcripts levels was observed in individuals with the CT and TT genotypes (p=0.448). [*p<0.05; **p<0.01].

2.3.1.5 Analysis of PSMB8 protein levels

Protein expression analysis was carried out to validate the decreased transcript expression of *PSMB8* in PBMCs of healthy controls (n=6) and patients with active GV (n=7). A significant decrease (*p*=0.0460) in PSMB8 expression was observed in patients as compared to controls (Figure 2.5).

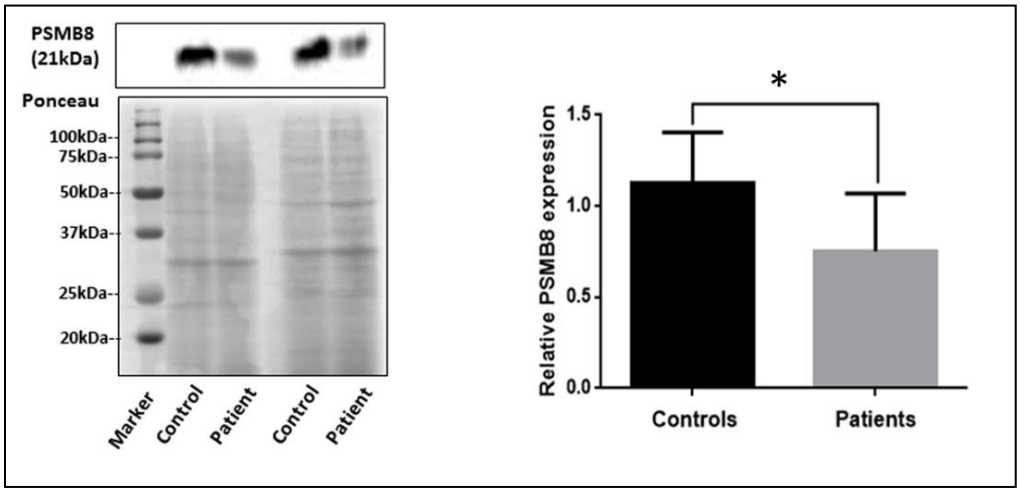

Figure 2.5. Analysis of PSMB8 protein expression: Western blot analysis in PBMCs of healthy controls (n=6) and patients with active GV (n=7) revealed a significant decrease (*p*=0.0460) in the expression of PSMB8 after normalization with Ponceau staining. [**p*<0.05].

2.3.1.6 Analysis of *TAP1* transcript levels

Analysis of *TAP1* transcript levels was carried out after normalization with *GAPDH* expression. No significant difference in expression of *TAP1* transcripts was observed (p=0.553) between patients and controls (Figure 2.6 A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 1.12- fold change in expression of *TAP1* transcript in patients as compared to controls (Figure 2.6 A). Analysis based on the type of the disease suggest no significant difference in *TAP1* transcript levels in patients with GV and LV in comparison to controls (p=0.090 and p=0.219, respectively; Figure 2.6 B). Moreover, there was no significant difference in patients with AV and SV as compared to controls (p=0.671 and p=0.291, respectively; Figure 2.6 C). When expression of *TAP1* transcripts was monitored in different age at onset groups of patients, no significant difference was observed in any of the age of onset groups, i.e. 21–40, 41–60, and 61–80 years when compared with 1-20 years (Figure 2.6 D). The gender-based analysis showed no significant difference in *TAP1* transcripts in both groups (Figure 2.6 F).

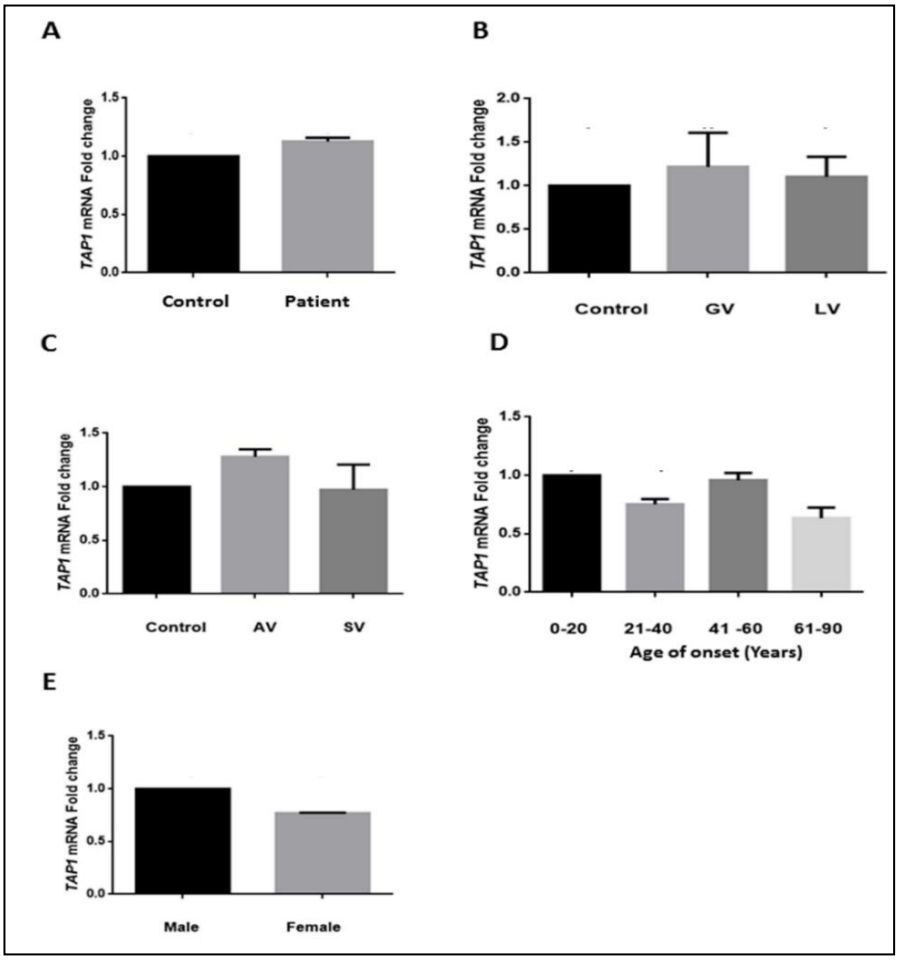

Figure 2.6. Relative gene expression of *TAP1* in patients and controls: Expression of *TAP1* transcripts in 96 controls and 91 patients with vitiligo was analysed by applying

unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups. [A] No significant difference in transcript levels of *TAP1* was observed as compared to controls (mean ΔCt ± SEM 5.59 ± 0.188 vs 5.421 ± 0.228; p=0.553). Expression of *TAP1* transcripts in controls and patients with vitiligo showed the approximately 1.12-fold change as determined by the $2^{-\Delta\Delta Cp}$ method. [B] Analysis of *TAP1* transcript levels in 96 controls and 72 patients with GV and 19 patients with LV revealed that patients with GV and LV had no significant difference in *TAP1* expression as compared to controls (p=0.856 and p=0.090, respectively). No significant difference in *TAP1* transcript levels was observed between GV and LV (p=0.219). [C] Expression of *TAP1* transcripts in 96 controls and 69 patients with AV and 22 patients with SV was further analysed. Patients with AV and SV showed no significant difference in *TAP1* transcripts levels as compared with controls (p=0.671 and p=0.291, respectively). No significant difference in *TAP1* transcript levels was observed among patients with AV and SV (p=0.634). [D] Analysis of *TAP1* transcripts with respect to the different age of onset groups in 91 patients with vitiligo did not show any significant difference with respect to the different age of onset groups. [E] Expression of *TAP1* transcripts with respect to sex differences in 48 male patients and 43 female patients revealed no significant difference was observed in both groups (p=0.444).

2.3.1.7 Bioinformatics analyses of *PSMB8* rs2071464 and *TAP1* rs1135216 polymorphisms

Table 2.7. *In silico* prediction results for *PSMB8* rs2071464 polymorphism.

SNP	SNP Location	Chromosomal Location	Regulome DB Score/ Prediction	HaploRedv4.1 Motifs changed by SNP	Tissue
PSMB8 rs2071464	Intron 6	chr6:32809075	6 / Minimal binding Evidence	7 altered motifs	Peripheral blood

Analysis of functional consequences of *PSMB8* rs2071464 polymorphism by RegulomeDB was scored 6 and classified as having minimal binding evidence (Table 2.7). HaploReg v4.1 predicted that *PSMB8* rs2071464 could alter 7 DNA motifs. RegulomeDB revealed that the SNP causes an altered chromatin state, which favours strong transcription and genic enhancer in peripheral blood cells (http://www.regulomedb.org/snp/chr6/32809075). Analysis by HaploReg v4.1 further confirmed the enhancer chromatin state in peripheral blood and T cells due to the polymorphism (http://archive.broadinstitute.org/mammals/haploreg/detail _v4.1.php ? query=&id=rs2071464).

Table 2.8. *In silico* prediction results for *TAP1* rs1135216 polymorphism.

Amino acid change	SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	I-MUTANT Score	MUPRO
Asp637Gly	Tolerated	0.34565	Neutral	Benign	Decrease	-1.00	Decrease

SIFT: Sorting Intolerant from Tolerant; PANTHER: Protein Analysis Through Evolutionary Relationships; SNPs and GO: Single Nucleotide Polymorphisms and Gene Ontology; PolyPhen: Polymorphism Phenotyping.

TAP1 rs1135216 polymorphism leads to variation in TAP1 protein from Asp to Gly at position 637 (Quadri and Singal 1998). PANTHER tool showed variation Asp to Gly at position 637 is not deleterious for TAP1 function, with a score of 0.3456 (Table 2.8). POLYPHEN tool showed that the substitution does not affect the phenotype or have damaging effects on the function of TAP1 protein. I-MUTANT and MUPRO predictions revealed decreased stability of Asp637Gly variants compared to native structure, which might affect the protein function. SNPs AND GO tool revealed that the variant does not show disease like trait. (Table 2.8).

2.3.2 Investigating the role of *MTHFR* exon 4 C/T (rs1801133) and exon 7 A/C (rs1801131) polymorphisms with vitiligo susceptibility

2.3.2.1 Analysis of *MTHFR* rs1801133 polymorphism

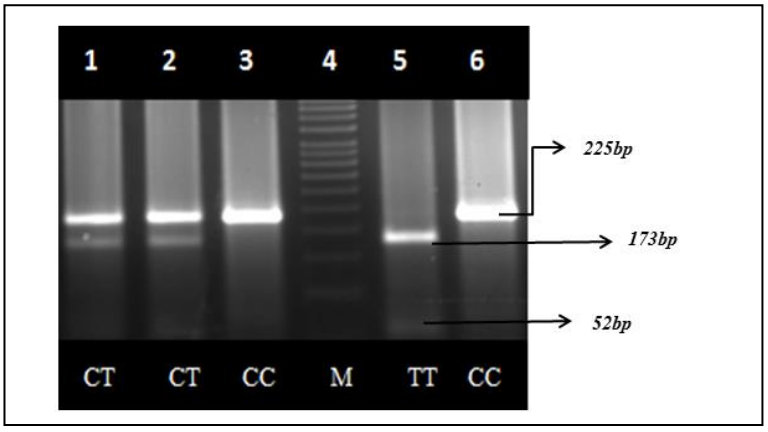

Figure 2.7. PCR-RFLP analysis of *MTHFR* rs1801133 polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 & 2 show heterozygous (CT) genotype; lanes 3 & 6 show homozygous (CC) genotype; lane 5 shows homozygous (TT) genotype; lane 4 shows 50 bp DNA ladder.

Three genotypes were identified for *MTHFR* rs1801133 polymorphism (Figure 2.7). Analysis of genotype distribution revealed that both control and patient populations were following HWE (p=0.267 and p=0.876, respectively). Wild type genotype 'CC' and allele 'C' were considered as the reference for further analysis. The allele and genotype frequencies did not differ in patient and control populations (Table 2.9). *MTHFR* rs1801133 polymorphism, when analysed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed between GV and LV patients with respect to controls (Table 2.10). Analysis based on the activity of the disease also showed no significant difference in genotype as well as allele frequencies (Table 2.10).

2.3.2.2 Analysis of *MTHFR* rs1801131 polymorphism

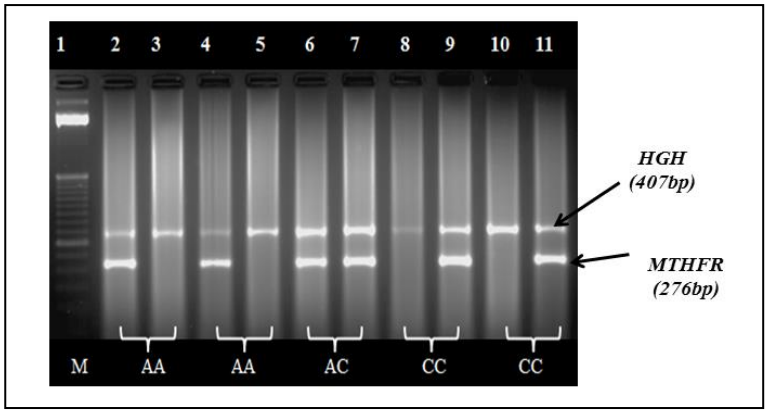

Figure 2.8. ARMS-PCR analysis of *MTHFR* rs1801131 polymorphism on 3.5% agarose gel electrophoresis: lanes: 2,3 & 4,5 show homozygous (AA) genotype; lanes 6 & 7 show heterozygous (AC) genotype; lanes: 8,9 & 10,11 show homozygous (CC) genotype; lane 1 shows 50 bp DNA ladder.

Three genotypes were identified for *MTHFR* rs1801131 polymorphism (Figure 2.8). The observed genotype frequencies of *MTHFR* rs1801131 polymorphism among the controls and patients were in accordance with HWE (p=0.2766 and p=0.2654, respectively). Wild type allele 'A' and genotype 'AA' were considered as the reference. 'CC' genotype was significantly increased in patients as compared to controls (13% vs. 19% respectively, p=0.015) and was identified as risk genotype (OR=1.56; CI=1.09-2.25). However, there was no significant difference in the distribution of other genotypes and alleles among patients and controls (Table 2.9). In analyses based on the type of vitiligo, a significant difference was observed in frequencies of 'CC' genotype and 'C' allele (p=0.035 and p=0.005 respectively)

among patients with GV and LV; however, the *p*-value in genotype frequency could not withstand Bonferroni's correction (Table 2.10). 'Significant increase in 'CC' genotype (21% vs. 13% respectively, $p<0.0001$) and 'C' allele (44% vs. 38% respectively, $p=0.003$) was observed in patients with GV as compared to controls. Analysis based on the activity of the disease revealed a predominant increase of 'CC' genotype (22% vs. 7% respectively, $p=0.003$) in patients with AV than with SV (Table 2.10). Significantly increased frequencies of 'CC' genotype (22% vs.13% respectively, $p=0.001$) and 'C' allele (43% vs. 38% respectively, $p=0.007$) were observed in patients with AV as compared to controls.

Table 2.9. Distributions of genotype and allele frequencies of *MTHFR* rs1801133 and rs1801131 polymorphisms in vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=558	Patients (Freq) n=520	*p* for HWE	*p* value[#] for association	OR	95% CI
MTHFR Exon 4 C/T (rs1801133)	CC	406 (0.73)	377 (0.73)	0.2667 (C) 0.8765 (P)	R	1	-
	CT	136 (0.24)	131 (0.25)		0.796[a]	1.04[a]	0.78-1.37
	TT	16 (0.03)	12 (0.02)		0.582[a]	0.81[a]	0.38-1.73
	C	948 (0.85)	885 (0.85)		R	1	-
	T	168 (0.15)	155 (0.15)		0.851[b]	1.02[a]	0.81-1.30
MTHFR Exon 7 A/C (rs1801131)	AA	211 (0.38)	181 (0.35)	0.2766 (C) 0.2654 (P)	R	1	-
	AC	274 (0.49)	241 (0.46)		0.852[a]	1.02	0.79–1.33
	CC	73 (0.13)	98 (0.19)		0.015[a]	1.56	1.09–2.25
	A	696 (0.62)	603 (0.58)		R	1	-
	C	420 (0.38)	437 (0.42)		0.037[b]	1.20	1.01–1.42

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
(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,
Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

Table 2.10. Distributions of genotype and allele frequencies of *MTHFR* rs1801133 and rs1801131 polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=558	GV n=396	LV n=124	*p* value[#]	AV n=415	SV n=105	*p* value[#]
MTHFR Exon 4 C/T (rs1801133)	CC	406 (0.73)	285 (0.72)	92 (0.74)	R	302 (0.73)	75 (0.71)	R
	CT	136 (0.24)	102 (0.26)	29 (0.23)	0.600[a] 0.663[b] 0.796[c]	104 (0.25)	27 (0.26)	0.860[x] 0.854[y] 0.769[z]
	TT	16 (0.03)	9 (0.02)	3 (0.03)	0.962[a] 0.600[b] 0.767[c]	9 (0.02)	3 (0.03)	0.664[x] 0.508[y] 0.981[z]
	C	948 (0.85)	672 (0.85)	213 (0.86)	R	708 (0.85)	177 (0.84)	R
	T	168 (0.15)	120 (0.15)	35 (0.14)	0.689[a] 0.953[b] 0.706[c]	122 (0.15)	33 (0.16)	0.712[x] 0.828[y] 0.807[z]
MTHFR Exon 7 A/C (rs1801131)	AA	211 (0.38)	129 (0.33)	52 (0.42)	R	143 (0.36)	38 (0.36)	R
	AC	274 (0.49)	182 (0.46)	59 (0.48)	0.327[a] 0.573[b] 0.522[c]	181 (0.42)	60 (0.57)	0.347[x] 0.859[y] 0.388[z]
	CC	73 (0.13)	85 (0.21)	13 (0.10)	0.035[a] <0.0001[b] 0.336[c]	91 (0.22)	7 (0.07)	0.003[x] 0.001[y] 0.140[z]
	A	696 (0.62)	440 (0.56)	163 (0.66)	R	467 (0.57)	136 (0.65)	R
	C	420 (0.38)	352 (0.44)	85 (0.34)	0.0005[a] 0.003[b] 0.322[c]	363 (0.43)	74 (0.35)	0.026[x] 0.007[y] 0.506[z]

n: number of subjects; R: reference group; GV: Generalized vitiligo; LV: Localized vitiligo; AV: Active Vitiligo; SV: Stable Vitiligo; [a]Generalized vitiligo vs. Localized vitiligo; [b]Generalized vitiligo vs. Controls; [c]Localized vitiligo vs. Controls; [x]Active Vitiligo vs. Stable Vitiligo; [y]Active Vitiligo vs. Controls; [z]Stable Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table. [#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

2.3.2.3 Linkage Disequilibrium and Haplotype analyses

LD analysis revealed that two polymorphisms investigated, i.e. *MTHFR* rs1801133 and rs1801131, were in low LD association (D'=0.468, r^2=0.028). Haplotype evaluation of the

two polymorphic sites was performed, and 'TC' haplotype, carrying variant alleles of both the SNPs, was found more frequently in patients as compared to controls and it increased the risk of vitiligo by 2.51 fold (p=0.008, OR=2.51; CI=1.24-5.07; Table 2.11).

Table 2.11. Distribution of haplotypes frequencies of *MTHFR* rs1801133 and rs1801131 polymorphisms in vitiligo patients and controls.

Haplotype [MTHFR (C/T): (A/C)]	Patients (Freq.)	Controls (Freq.)	*p* for association	*p* (Global)	Odds Ratio [95% CI]
CA	267.16 (0.45)	279.12 (0.49)	0.189	0.0110	0.86 [0.68–1.08]
CC	229.84 (0.39)	197.88 (0.35)	0.146		1.19 [0.94–1.51]
TA	66.84 (0.11)	81.88 (0.14)	0.117		0.76 [0.54–1.07]
TC	28.16 (0.05)	11.12 (0.02)	0.008		2.51 [1.24–5.07]

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

2.3.2.4 Bioinformatics analyses

We further investigated the impact of these polymorphisms on MTHFR protein using bioinformatics tools. *MTHFR* rs1801133 polymorphism results in an alanine to valine substitution at position 222 (Ala222Val) whereas, *MTHFR* rs1801131 polymorphism results in glutamate to alanine substitution at position 429 (Glu429Ala) (Lecler and Sibani, 2004).

Table 2.12. *In silico* prediction results for *MTHFR* rs1801133 and rs1801131 polymorphisms.

Amino acid change	SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	I-MUTANT Score	MUPRO
MTHFR rs1801133 Ala 222 Val	Damaging	Probably Damaging	Disease	Probably Damaging	Increase	0.11	Decrease
MTHFR rs1801131 Glu 429 Ala	Tolerated	Probably Damaging	Neutral	Benign	Neutral	-0.46	Decrease

PANTHER tool showed that both Ala222Val and Glu429Ala variations are probably damaging for MTHFR function (Table 2.12). POLYPHEN tool showed that Ala222Val substitution is probably damaging, whereas, Glu429Ala substitution does not affect the phenotype or have damaging effects on the function of MTHFR protein. I-MUTANT

predictions revealed increased stability of the Ala222Val variant as compared to native structure, whereas MUPRO predicted decreased stability for both variants. SNPs and GO tool revealed that the Ala222Val variant show disease like trait while Glu429Ala is neutral (Table 2.12).

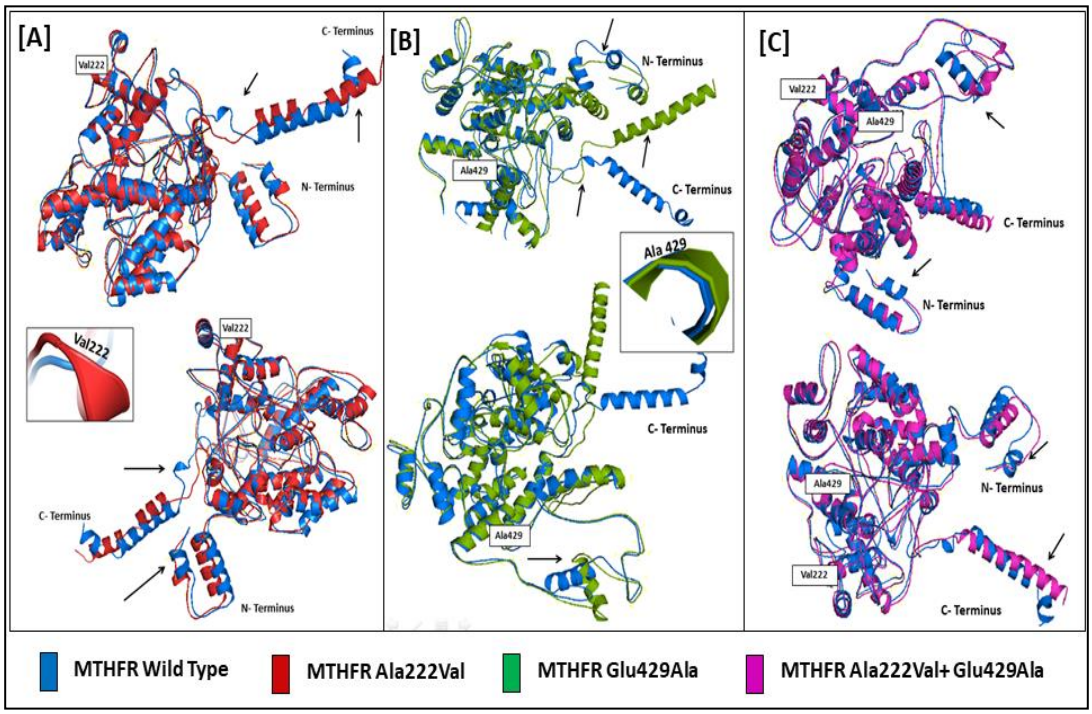

Figure 2.9: Structure-based *in silico* analysis of MTHFR variants: Structural superimposition of monomer MTHFR wild type (blue), Ala222Val (red), Glu429Ala (green) and Ala222Val Glu429Ala double mutant (magenta). The structural perturbations are pointed with arrows.

The superimposition of modelled structures revealed exciting findings. Ala222Val substitution resulted in the beginning of the helix at position 222, whereas in the Glu429Ala variant, there was no major alteration at position 429. Ala222Val variant showed structural alterations with the disappearance and shortening of the helix/s at the N-terminus as well as C-terminus (Figure 2.9A). Glu429Ala variant showed structural alterations, including the disappearance and shortening of the helix/s at the N-terminus as well as a change in orientation of helices towards C- terminus (Figure 2.9B). A double mutant model of Ala222Val and Glu429Ala substitutions also showed the disappearance of helix towards N-terminus (Figure 2.9C).

2.3.3 Investigating the role of *XBP1* -116 G/C (rs2269577) polymorphisms with vitiligo susceptibility

2.3.3.1 Analysis of *XBP1* rs2269577 polymorphism

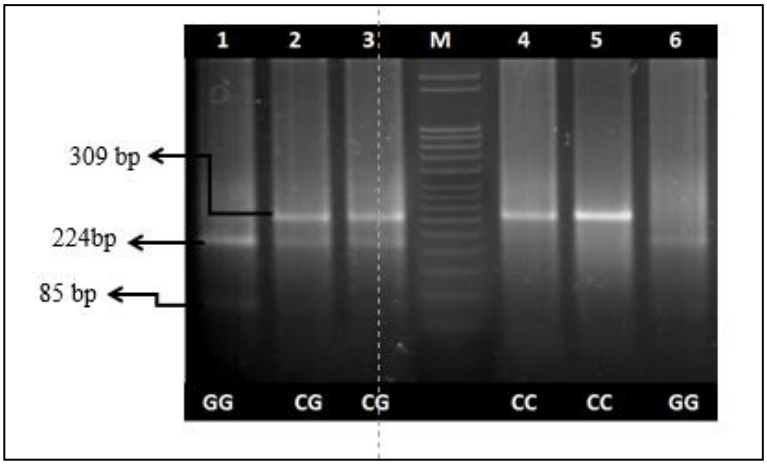

Figure 2.10. PCR-RFLP analysis of *XBP1* rs2269577 polymorphism on 3.5 % agarose gel electrophoresis: lanes 2 & 3 show heterozygous (CG) genotype; lanes 1 & 6 show homozygous (GG) genotype; lanes 4 & 5 show homozygous (CC) genotype; lane M shows 50 bp DNA ladder.

Three genotypes were identified for *XBP1* rs2269577 polymorphism (Figure 2.10). Genotyping and allele distribution for *XBP1* rs2269577 polymorphism revealed that both control and patient population were following HWE (p=0.979 and p=0.288, respectively). Genotype 'GG' and allele 'G' were considered as the reference. 'CC' genotype was significantly increased in patients as compared to controls (33% vs. 27% respectively, p=0.010) and was identified as the risk genotype (OR=1.88; CI=1.16-3.04; Table 2.13). The frequency of variant 'C' allele was also significantly higher in patients as compared to controls (59% vs. 52% respectively, p=0.014) and was identified as risk allele (OR=1.34; CI=1.06-1.68; Table 2.13). Upon analyses based on the type of vitiligo, a predominant increase in the frequency of 'CC' genotype was observed in patients with GV (p<0.0001) and LV (p<0.0001) as compared to controls. The analysis of allele frequency revealed a significant increase in the frequency of the 'C' allele in patients with LV (p=0.032) as compared to controls (Table 2.14). Further analysis based on the activity of the disease revealed a difference in the frequency of 'CC' genotype in patients with AV (p<0.0001) and SV (p<0.0001) as compared to controls. The analysis of allele frequency revealed a significant increase in the frequency of the 'C' allele in patients with AV (p=0.025) as compared to controls (Table 2.14).

Table 2.13. Distribution of genotype and allele frequencies of *XBP1* rs2269577 polymorphism in vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=312	Patients (Freq) n=276	*p* for HWE	*p* value for association	OR	95% CI
XBP1 -116 G/C (rs2269577)	GG	72 (0.23)	42 (0.15)	0. 979 (C) 0.288 (P)	R	1	-
	GC	156 (0.50)	142 (0.52)		0.048[a]	1.56	1.00-2.43
	CC	33 (0.27)	92 (0.33)		0.010[a]	1.88	1.16-3.04
	G	300 (0.48)	226 (0.41)		R	1	-
	C	324 (0.52)	326 (0.59)		0.014[b]	1.34	1.06-1.68

'n' represents number of Patients/ Controls, 'R' represents reference group, HWE refers to Hardy-Weinberg Equilibrium, CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution. (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

Table 2.14. Distributions of genotype and allele frequencies of *XBP1* rs2269577 polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=312	GV n=204	LV n=72	*p* value	AV n=198	SV n=78	*p* value
XBP1 -116 G/C (rs2269577)	GG	72 (0.23)	29 (0.14)	13 (0.18)	R	32 (0.16)	10 (0.13)	R
	GC	156 (0.50)	113 (0.55)	29 (0.40)	0.153[a] 0.019[b] 0.936[c]	98 (0.50)	44 (0.56)	0.369[x] 0.163[y] 0.057[z]
	CC	33 (0.27)	62 (0.31)	30 (0.42)	0.849[a] <0.0001[b] <0.0001[c]	68 (0.34)	24 (0.20)	0.779[x] <0.0001[y] <0.0001[z]
	G	300 (0.48)	171 (0.42)	55 (0.38)	R	162 (0.41)	64 (0.41)	R
	C	324 (0.52)	237 (0.58)	89 (0.62)	0.435[a] 0.052[b] 0.032[c]	234 (0.59)	92 (0.59)	0.980[x] 0.025[y] 0.114[z]

n: number of subjects; R: reference group; GV: Generalized vitiligo; LV: Localized vitiligo; AV: Active Vitiligo; SV: Stable Vitiligo; [a]Generalized vitiligo vs. Localized vitiligo; [b]Generalized vitiligo vs. Controls; [c]Localized vitiligo vs. Controls; [x]Active Vitiligo vs. Stable Vitiligo; [y]Active Vitiligo vs. Controls; [z]Stable Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table.

2.3.3.2 Analysis of unspliced and spliced *XBP1* transcript levels in PBMCs of vitiligo patients and controls.

We monitored the levels of unspliced *XBP1 (uXBP1)* and spliced *XBP1 (sXBP1)* transcript levels in 106 controls and 103 patients. The analysis revealed no significant difference in the *uXBP1* transcript levels (p=0.456; Figure 2.11A). However, there was a significant difference in the expression of *sXBP1* transcript levels in patients as compared to controls (p=0.026). The $2^{-\Delta\Delta Cp}$ analysis showed an approximate 1.76-fold increase in the expression of *sXBP1* transcript levels in patients, as compared to controls (Figure 2.11B). Further, the analysis based on type and activity of the disease revealed that *sXBP1* transcript levels were significantly increased in patients with GV as well as AV in comparison to controls (p=0.007 and p=0.006 respectively; Figure 2.11 D and F), suggesting a role of ER stress in the autoimmune basis of the disease. However, there was no significant difference in the expression of *uXBP1* transcript levels upon an analysis based on the type, or activity of the disease (Figure 2.11 C and E). The gender-based analysis also showed no significant difference in the expression of *uXBP1* and *sXBP1* transcript levels in both the groups (Figure 2.11 G and H). Also, the *uXBP1* and *sXBP1* transcripts levels were further analysed with respect to the *XBP1* rs2269577 polymorphism in controls and patients. The results suggest that the individuals with GC and CC genotypes had significantly higher *uXBP1* transcripts levels as compared to the GG genotype (p=0.033 and p=0.007, respectively). Whereas no significant difference in *uXBP1* transcripts levels was observed in individuals carrying GC and CC genotypes (p=0.741; Figure 2.11 I). However, *sXBP1* transcripts levels were not significantly different in individuals with GC and CC genotypes as compared to GG genotype (p=0.730 and p=0.969, respectively) and in individuals with the GC and CC genotypes (p=0.792; Figure 2.11 J).

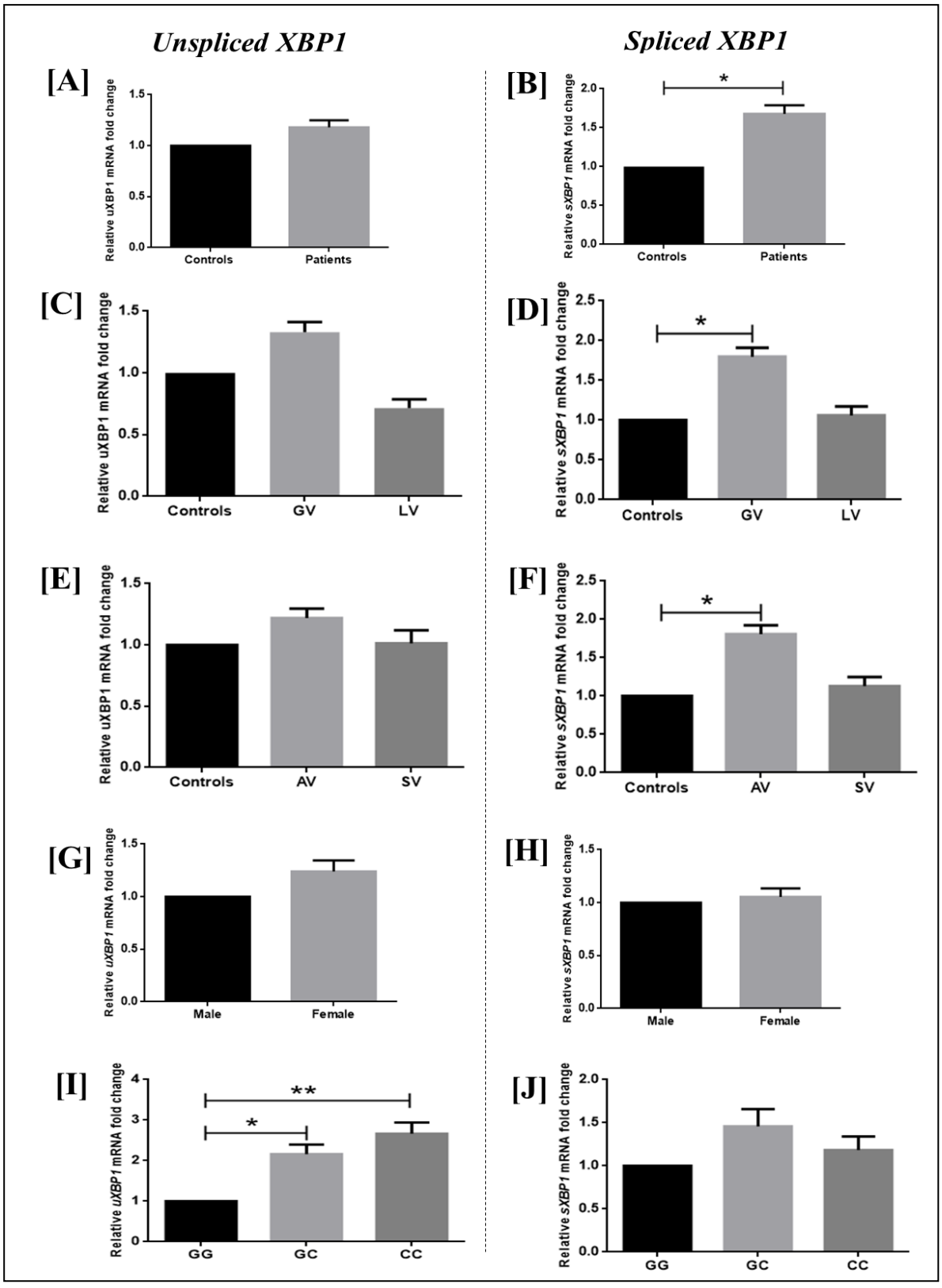

Figure 2.11. Relative gene expression analysis of *uXBP1* and *sXBP1* in PBMCs of vitiligo patients and controls. Expression of *uXBP1* and *sXBP1* transcript levels in 106 controls, 103 patients with vitiligo were analysed by applying unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups. [A] No significant

difference in *uXBP1* transcript levels was observed among patients and controls (mean ΔCt ± SEM: 5.76 ± 0.210 vs 5.98 ± 0.204 respectively; *p*=0.456). Expression fold change of *uXBP1* transcripts in patients against controls showed a 1.18-fold higher expression as determined by the $2^{-\Delta\Delta Cp}$ method. [B] Transcript levels of *sXBP1* were significantly different among patients and controls (mean ΔCt ± SEM: 4.61 ± 0.187 vs. 5.28 ± 0.229, respectively; *p*=0.026). Expression fold change of *sXBP1* transcripts in patients against controls showed 1.76-fold higher expression as determined by the $2^{-\Delta\Delta Cp}$ method. Expression of *uXBP1* and *sXBP1* transcripts were further analysed in 106 controls and 87 patients with GV and 16 patients with LV. [C] No significant difference in *uXBP1* transcripts was observed in patients with GV and LV as compared to controls (*p*=0.404 and *p*=0.610, respectively). Further, *uXBP1* transcript levels were not significantly different among patients with GV and LV (*p*=0.245). [D] Patients with GV showed significantly increased *sXBP1* transcript levels as compared to controls (*p*=0.030). However, there was no significant difference in *sXBP1* transcript levels between patients with GV and LV as well as in patients with LV as compared to controls (*p*=0.343 and *p*=0.999, respectively). Expression of *uXBP1* and *sXBP1* transcripts were also analysed in 106 controls and 83 patients with AV and 20 patients with SV. [E] No significant difference in *uXBP1* transcripts was observed in patients with AV and SV as compared to controls (*p*=0.683 and *p*=0.996, respectively). Further, *uXBP1* transcript levels were not significantly different among patients with GV and LV (*p*=0.843). [F] Patients with AV showed significantly increased *sXBP1* transcript levels as compared to controls (*p*=0.024). However, there was no significant difference in *sXBP1* transcript levels between patients with AV and SV as well as in patients with SV as compared to controls (*p*=0.408 and *p*=0.975, respectively). Expression of *uXBP1* and *sXBP1* transcripts were further analysed with respect to gender in 53 male and 50 female patients. [G & H] No significant difference was observed in the expression of *uXBP1*, and *sXBP1* transcripts was observed in both the groups (*p*=0.526 and *p*=0.914, respectively). Expression of *uXBP1* and *sXBP1* transcripts were further analysed with respect to the *XBP1* rs2269577 polymorphism in 106 controls and 103 patients. [I] Individuals with GC and CC genotypes showed significantly increased *uXBP1* transcripts as compared to GG genotype (*p*=0.033 and *p*=0.007, respectively). No significant difference in *uXBP1* transcripts levels was observed in individuals with GC and CC genotypes (*p*=0.741). [J] No significant difference in *sXBP1* transcripts levels was observed in individuals with GC and CC genotypes as compared to GG genotype (*p*=0.730 and *p*=0.969, respectively). Further, no significant difference was observed in *sXBP1* transcripts levels in individuals with the GC and CC genotypes (*p*=0.792). [*$p<0.05$; **$p<0.01$].

2.3.3.3 Analysis of unspliced and spliced *XBP1* transcript levels in skin samples of vitiligo patients and controls.

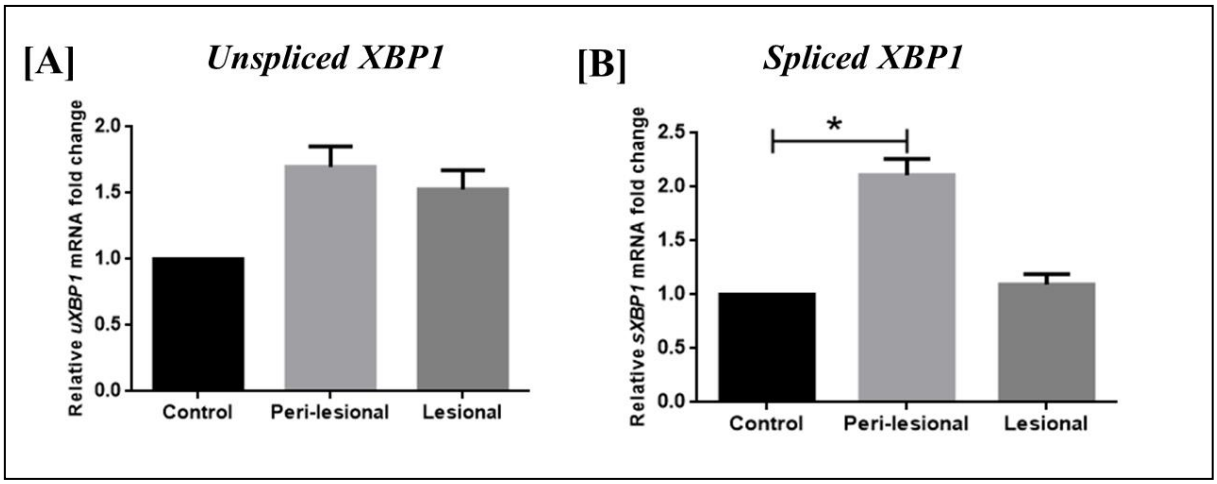

Figure 2.12. Analysis of *uXBP1* and *sXBP1* transcript levels in skin samples of 12 vitiligo patients and 15 controls was carried out by using one-way ANOVA [A] No significant difference in expression of *uXBP1* transcripts was observed in peri-lesional and lesional skin as compared to control skin (p=0.183 and p=0.496, respectively) as well as in peri-lesional skin as compared to lesional skin (p=0.805). [B] A significant increase in *sXBP1* transcript levels was observed in peri-lesional skin as compared to control skin (p=0.037; 2.26 fold) however, there was no significant difference in *sXBP1* transcript levels in lesional skin as compared to control skin (p=0.973) and among peri-lesional skin and lesional skin (p=0.071) [*p<0.05].

Analysis of *uXBP1* and *sXBP1* transcript levels in skin biopsies of 12 patients and 15 controls was carried out. The results suggest no significant difference in expression of *uXBP1* transcript levels among skin samples of patients and controls (p=0.037; Figure 2.12 A). Interestingly, a significant increase in *sXBP1* transcript levels was observed in the perilesional skin of vitiligo patients as compared to control skin (p=0.037). The $2^{-\Delta\Delta Cp}$ analysis showed about 2.26 fold increase in the expression of *sXBP1* transcript levels in peri-lesional skin as compared to control skin, whereas there was no significant difference in expression of *sXBP1* lesional skin as compared to control (p=0.973) and peri-lesional skin of vitiligo patients (p=0.071; Figure 2.12B).

2.3.4 Investigating the role of *IL17A* -197 G/A (rs2275913) and -737 C/T (rs8193036) polymorphisms with vitiligo susceptibility

2.3.4.1 Analysis of *IL17A* rs2275913 polymorphism

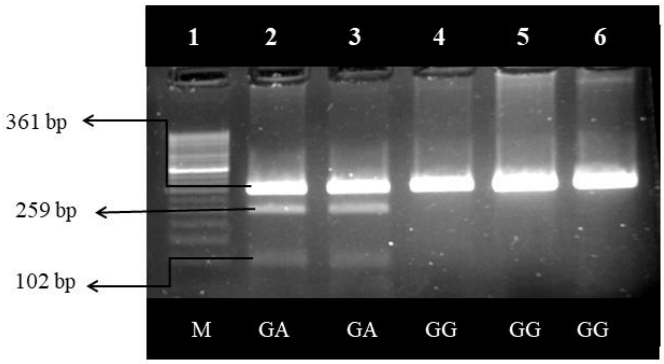

Figure 2.13. PCR-RFLP analysis of *IL17A* rs2275913 polymorphism on 3.5 % agarose gel electrophoresis: lanes: 2 & 3 show heterozygous (GA) genotype; lanes: 4, 5 & 6 show homozygous (GG) genotype; lane 1 shows 50 bp DNA ladder.

Three genotypes were identified for *IL17A* rs2275913 polymorphism (Figure 2.13). Analysis of genotype distribution revealed that the control population was following HWE (p=0.063) however patient population deviated from HWE (p=0.002). Wild type genotype 'GG' and allele 'G' were considered as the reference for further analysis. The allele and genotype frequencies were not significantly different in patient and control populations (Table 2.15). Further, *IL17A* rs2275913 polymorphism, when analysed based on the type of vitiligo, no significant difference in genotype and allele frequencies were observed between GV and LV patients with respect to controls (Table 2.16). Analysis based on the activity of the disease also showed no significant difference in genotype as well as allele frequencies (Table 2.16).

2.3.4.2 Analysis of *IL17A* rs8193036 polymorphism

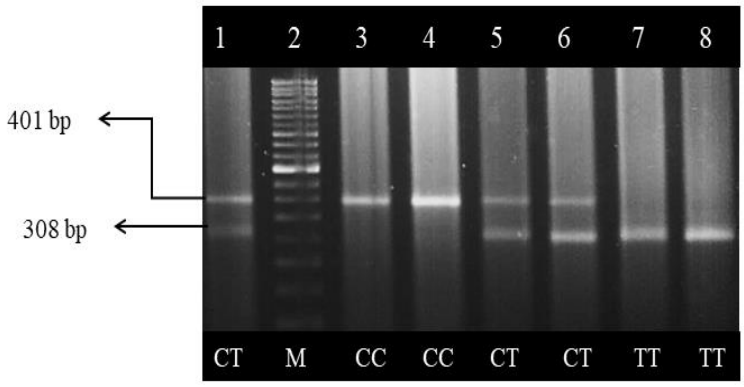

Figure 2.14. PCR-RFLP analysis of *IL17A* rs8193036 polymorphism on 3.5 % agarose gel electrophoresis: lanes: 1, 5 & 6 show heterozygous (CT) genotype; lanes: 3 & 4 show homozygous (CC) genotype; lane 7 & 8 show homozygous (TT) genotype; lane 2 shows 50 bp DNA ladder.

Three genotypes were identified for *IL17A* rs8193036 polymorphism (Figure 2.14). Analysis of genotype distribution revealed that the control population was following HWE (p=0.793) however, patient population deviated from HWE (p=0.041). Wild type genotype 'CC' and allele 'C' were considered as the reference for further analysis. The allele and genotype frequencies were not significantly different in patient and control populations (Table 2.15). *IL17A* rs8193036 polymorphism, when analysed based on the type of vitiligo and activity of the disease, no significant difference was observed in genotype and allele frequencies (Table 2.16).

Table 2.15. Distribution of genotype and allele frequencies of *IL17A* rs2275913 and rs8193036 polymorphisms in vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=312	Patients (Freq) n=276	*p* for HWE	*p* value[#] for association	OR	95% CI
IL17A -197 G/A (rs2275913)	GG	220 (0.70)	190 (0.69)	0.063 (C)	R	R	-
	GA	89 (0.29)	86 (0.31)		0.534[a]	1.12	0.78-1.59
	AA	3 (0.01)	0 (0.00)		0.109[a]	0.16	0.008-3.22
	G	529 (0.85)	466 (0.84)	0.002 (P)	R	R	-
	A	95 (0.15)	86 (0.16)		0.866[b]	1.03	0.75-1.41
IL17A -737 C/T (rs8193036)	CC	90 (0.29)	80 (0.29)	0.794 (C) 0.041 (P)	R	R	-
	CT	153 (0.49)	121 (0.44)		0.551[a]	1.89	0.60–1.31
	TT	69 (0.22)	75 (0.27)		0.375[a]	1.22	0.78–1.90
	C	333 (0.53)	281 (0.51)		R	R	-
	T	291 (0.47)	271 (0.49)		0.399[a]	1.10	0.88–1.39

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
(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,
[#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

Table 2.16. Distributions of genotype and allele frequencies of *IL17A* rs2275913 and rs8193036 polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=312	GV n=204	LV n=72	*p* value[#]	AV n=198	SV n=78	*p* value[#]
IL17A -197 G/A (rs2275913)	GG	220 (0.70)	130 (0.64)	52 (0.72)	R	127 (0.64)	48 (0.62)	R
	GA	89 (0.29)	74 (0.36)	20 (0.28)	0.191[a] 0.075[b] 0.862[c]	71 (0.36)	30 (0.38)	0.686[x] 0.095[y] 0.099[z]
	AA	03 (0.01)	0 (0.0)	0 (0.0)	-[a] 0.184[b] 0.400[c]	0 (0.0)	0 (0.0)	-[a] 0.189[b] 0.419[c]
	G	529 (0.58)	334 (0.82)	124 (0.86)	R	325 (0.82)	126 (0.81)	R
	A	95 (0.15)	74 (0.18)	20 (0.14)	0.243[a] 0.216[b] 0.686[c]	71 (0.18)	30 (0.19)	0.722[x] 0.254[y] 0.222[z]
IL17A -737 C/T (rs8193036)	CC	90 (0.29)	59 (0.29)	24 (0.33)	R	57 (0.29)	26 (0.33)	R
	CT	153 (0.49)	90 (0.44)	31 (0.43)	0.602[a] 0.612[b] 0.363[c]	81 (0.41)	40 (0.51)	0.795[x] 0.411[y] 0.726[z]
	TT	69 (0.22)	55 (0.27)	17 (0.24)	0.455[a] 0.427[b] 0.824[c]	60 (0.30)	12 (0.15)	0.034[x] 0.194[y] 0.183[z]
	C	333 (0.53)	208 (0.51)	79 (0.55)	R	195 (0.49)	92 (0.59)	R
	T	291 (0.47)	200 (0.49)	65 (0.45)	0.423[a] 0.453[b] 0.746[c]	201 (0.51)	64 (0.41)	0.039[x] 0.199[y] 0.208[z]

n: number of subjects; R: reference group; GV: Generalized vitiligo; LV: Localized vitiligo; AV: Active Vitiligo; SV: Stable Vitiligo; [a]Generalized vitiligo vs. Localized vitiligo; [b]Generalized vitiligo vs. Controls; [c]Localized vitiligo vs. Controls; [x]Active Vitiligo vs. Stable Vitiligo; [y]Active Vitiligo vs. Controls; [z]Stable Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table. [#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

2.3.4.3 Linkage disequilibrium and haplotype analyses

LD analysis of *IL17A* rs2275913 and rs8193036 polymorphisms revealed that both the SNPs were in low LD association (D'=0.068, r^2=0.001). Haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes were not significantly different between patients and controls (global *p*=0.697; Table 2.17).

Table 2.17 Distribution of haplotype frequencies for *IL17A* rs2275913 and rs8193036 polymorphisms in vitiligo patients and controls.

Haplotype *IL17A [G/A:C/T]*	**Patients (Freq.)**	**Controls (Freq.)**	***p* value**	***p* Global**	**Odds Ratio [95% CI]**
GC	146.59 (0.44)	154.06 (0.48)	0.380	0.697	0.87 [0.64~1.18]
GT	134.41 (0.41)	128.94 (0.40)	0.858		1.03 [0.75~1.41]
AC	23.41 (0.07)	19.94 (0.06)	0.643		1.16 [0.62~2.14]
AT	25.59 (0.08)	19.06 (0.06)	0.354		1.33 [0.72~2.47]

'CI' represents Confidence Interval. (Haplotype frequency <0.03 in has been dropped and was ignored in the analysis by the software).

2.3.4.4 Analysis of *IL17A* transcript levels in PBMCs of vitiligo patients and controls

Analysis of *IL17A* transcript levels revealed a significant increase in expression of *IL17A* transcripts in patients as compared to controls (p=0.007) after normalization with *GAPDH* expression. The $2^{-\Delta\Delta Cp}$ analysis showed an approximate 1.82-fold increase in the expression of *IL17A* transcript levels in patients as compared to controls (Figure 2.15A). Interestingly, analysis based on type and activity of the disease revealed that *IL17A* transcript levels were significantly increased in patients with GV as well as AV as compared to controls (p=0.039 and p=0.017, respectively; Figure 2.15 B and C), suggesting the association with autoimmune vitiligo. However, there was no significant difference in patients with GV vs. LV and AV vs. SV as compared to controls (p=0.975 and p=0.979, respectively; Figure 2.15 B and C). The gender-based analysis revealed no significant difference in *IL17A* expression among males and females (p=0.812; Figure 2.15 D). Expression of *IL17A* transcripts levels was further analysed with respect to *IL17A* rs2275913 and rs8193036 polymorphisms to assess the effect of SNPs on gene expression. The analysis with respect to *IL17A* rs2275913 polymorphism revealed no significant difference in *IL17A* transcript levels in individuals with GG as compared to those with GA+AA genotypes (p=0.944; Figure 2.15 E). Similarly, no significant difference in *IL17A* transcript levels was observed in individuals with the CC genotype as compared to those with CT and TT genotypes of *IL17A* rs8193036 polymorphism (p=0.549 and p=0.719, respectively Figure 2.15 F).

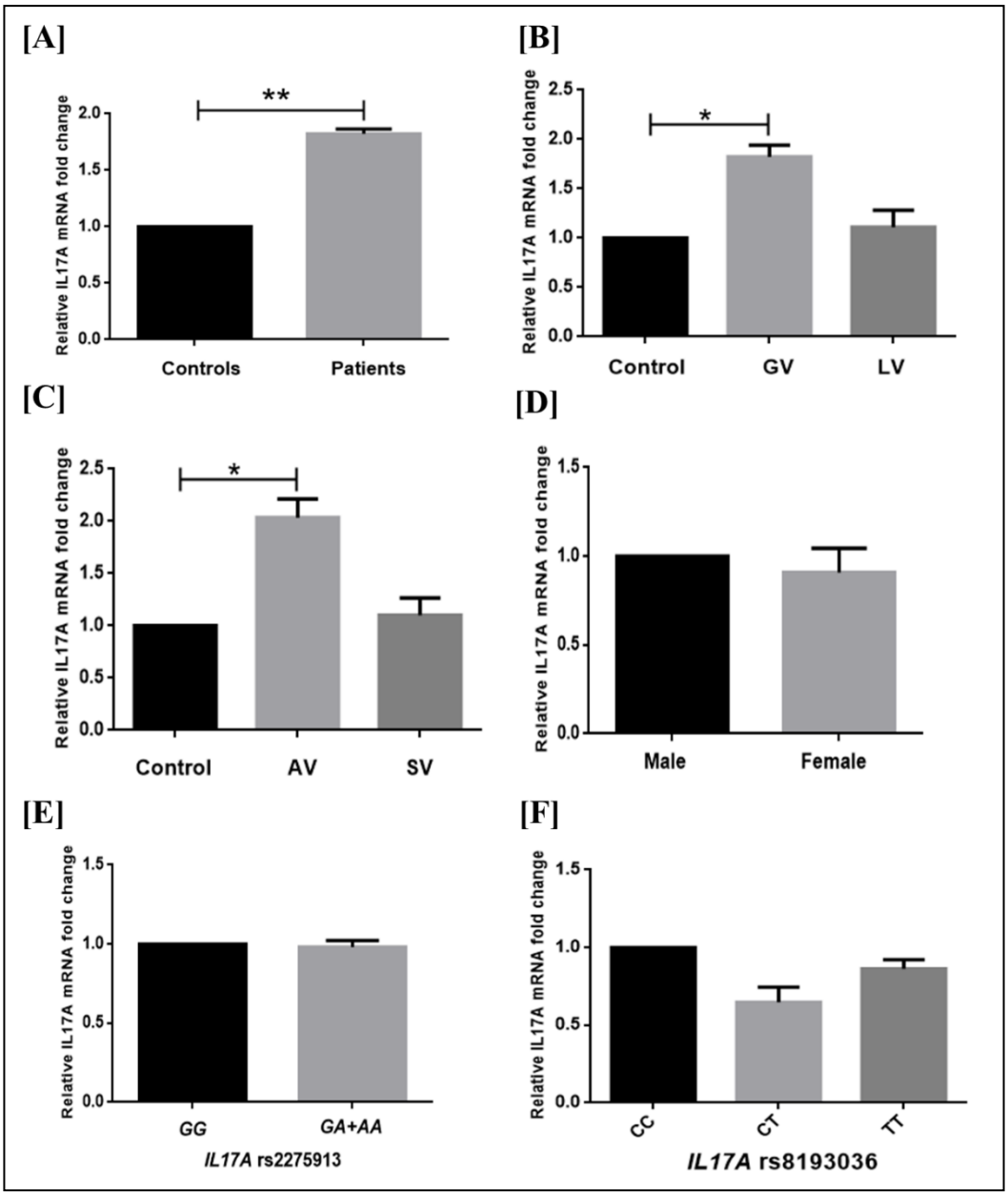

Figure 2.15. Relative gene expression of *IL17A* in PBMCs of vitiligo patients and controls. Expression of *IL17A* transcripts in 108 controls, 100 patients with vitiligo was analysed by applying unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups. [A] Patients showed a significant increase in transcript levels of *IL17A* compared to controls (mean ΔCt ± SEM: 3.29 ± 0.244 vs. 4.16 ± 0.210; p=0.007, respectively). Expression of *IL17A* transcripts in patients against controls showed 1.82 -fold increase as determined by the $2^{-\Delta\Delta Cp}$ method. [B] Expression of *IL17A* transcripts in 108 controls and 84 patients with GV and 16 patients with LV was further analysed. Patients with GV showed significantly increased *IL17A* transcript levels as compared to controls (p=0.039). However, there was no significant difference in *IL17A* transcript levels between patients with GV and LV as well as in patients with LV as compared to controls (p=0.508 and p=0.975, respectively). [C] Expression of *IL17A* transcripts in 108 controls and 81 patients with AV and 19 patients with SV was analysed. Patients with AV showed significantly increased *IL17A* transcript levels as compared to

controls (p=0.017). However, there was no significant difference in *IL17A* transcript levels between patients with AV and SV as well as in patients with SV as compared to controls (p=0.377 and p=0.979, respectively). [D] Expression of *IL17A* transcripts with respect to gender was analysed in 54 male and 46 female patients. No significant difference was observed in both groups (p=0.812). [E] No significant difference was observed in *IL17A* transcript levels in individuals with GG as compared to those with GA+AA genotypes of *IL17A* rs2275913 polymorphism (p=0.944). [F] Further, individuals with the CC genotype of *IL17A* rs8193036 polymorphism did not show any significant difference in IL17A expression as compared to those with CT and TT genotypes (p=0.549 and p=0.719, respectively). [*p<0.05; **p<0.01].

2.3.4.5 Analysis of IL-17A protein levels in suction induced blister fluid (SBF) samples of vitiligo patients and controls

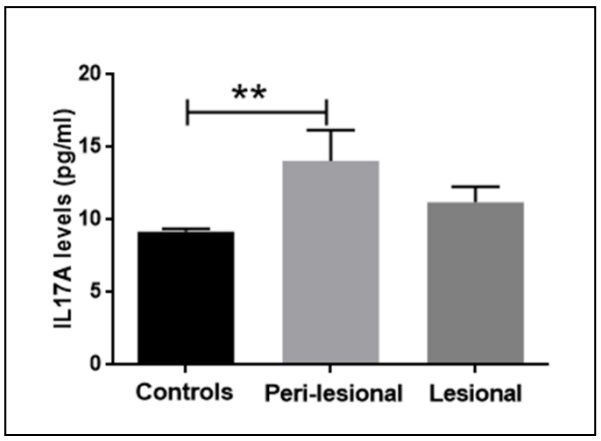

Figure 2.16. Estimation of IL-17A levels in SBF samples of patients with active generalized vitiligo (n=15) and controls (n=18). Significantly elevated IL-17A levels were observed in SBF samples from perilesional skin (p=0.009) of patients as compared to controls. No significant difference in IL-17A levels was observed in the lesional skin of patients as compared to peri-lesional (p=0.139) and control skin (p=0.266) [**p<0.01].

A significant increase in *IL17A* gene expression was observed in PBMCs of patients with generalized and active vitiligo. Hence, we have monitored IL-17A levels in the skin of patients with active generalized vitiligo by ELISA (Figure 2.16). The results suggest a significant increase in IL-17A levels in SBF samples from peri-lesional skin of active generalized vitiligo patients as compared to controls (mean ± SEM: 14.06 ± 2.139 pg/ml vs. 9.17 ± 0.201 pg/ml, respectively; p=0.009). However, there was no significant difference in IL-17A levels in SBF from lesional skin as compared to peri-lesional (mean ± SEM: 11.21 ± 1.058 pg/ml vs 14.06 ± 2.139 pg/ml, respectively; p=0.0430) and control skin (mean ± SEM: 11.21 ± 1.058 pg/ml vs 9.17 ± 0.201 pg/ml, respectively; p=0.0430).

2.3.5 Investigating the role of *TYR* exon 1 C/A (rs1042602) and exon 4 G/A (rs1126809) polymorphisms and anti-tyrosinase antibodies in vitiligo.

2.3.5.1 Analysis of *TYR* rs1042602 polymorphism

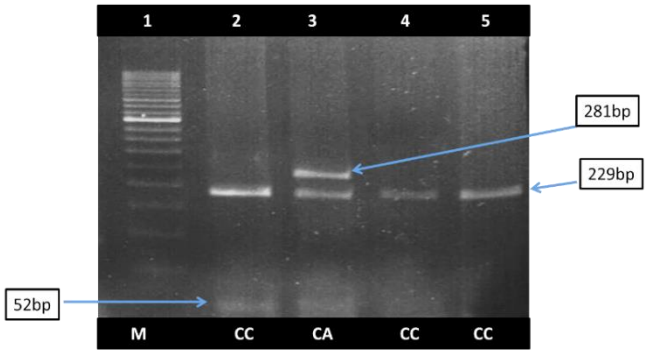

Figure 2.17. PCR-RFLP analysis of *TYR* rs1042602 polymorphism on 3.5 % agarose gel electrophoresis: lanes 2, 4 & 5 show homozygous (CC) genotype; lane 3 show heterozygous (CA) genotype; lane 1 shows 50 bp DNA ladder.

Three genotypes were identified for *TYR* rs1042602 polymorphism (Figure 2.17). Analysis of genotype distribution revealed that both control and patient populations were following HWE (p=0.856 and p=0.131, respectively). Wild type genotype 'CC' and allele 'C' were considered as the reference for further analysis. The allele and genotype frequencies were not significantly different in patient and control populations (Table 2.18). Further, *TYR* rs1042602 polymorphism, when analysed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed between GV and LV patients with respect to controls (Table 2.19). Analysis based on the activity of the disease also showed no significant difference in genotype as well as allele frequencies (Table 2.19).

2.3.5.2 Analysis of *TYR* rs1126809 polymorphism

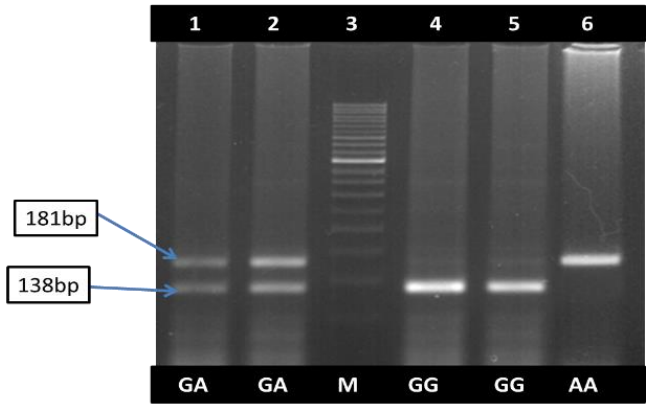

Figure 2.18. PCR-RFLP analysis of *TYR* rs1126809 polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 & 2 show heterozygous (GA) genotype; lanes 4 & 5 show homozygous (GG) genotype; lane 6 shows homozygous (AA) genotype; lane 3 shows 50 bp DNA ladder.

Three genotypes were identified for *TYR* rs1126809 polymorphism (Figure 2.18). The observed genotype frequencies of *TYR* rs1126809 polymorphism among the controls and patients were in accordance with HWE (p=0.130 and p=0.453, respectively). Wild type allele 'G' and genotype 'GG' were considered as the reference. The frequency of 'AA' genotype was significantly lower in patients as compared to controls (1% vs. 4% respectively, p=0.011) and was identified as a protective genotype (OR=0.22; CI=0.06-0.79). The frequency of 'A' allele was also significantly lower in patients as compared to controls (p=0.0124; Table 2.17). In analyses based on the type of vitiligo, a significant difference was observed in frequencies of 'AA' genotype and 'A' allele (p=0.011 and p=0.011 respectively) among patients with GV as compared to controls (Table 2.18). Furthermore, the analysis based on the activity of the disease revealed a reduction of 'AA' genotype (p=0.014) in patients with AV as compared to controls (Table 2.18). The frequency of 'A' allele was also significantly decreased (p=0.012) in patients with AV as compared to controls (Table 2.18).

Table 2.18. Distribution of genotype and allele frequencies of *TYR* rs1042602 and rs1126809 polymorphisms in vitiligo patients and controls.

SNP	Genotype /Allele	Controls (Freq) n=341	Patients (Freq) n=296	*p* for HWE	*p* value[#] for association	OR	95% CI
TYR exon 1 C/A (rs1042602)	CC	283 (0.83)	251 (0.85)	0.856 (C) 0.131 (P)	R	R	-
	CA	55 (0.16)	41 (0.14)		0.437[a]	0.84	0.54 -1.30
	AA	3 (0.01)	4 (0.01)		0.593[a]	1.50	0.33 -6.78
	C	621 (0.91)	543 (0.92)		R	R	-
	A	61 (0.09)	49 (0.08)		0.672[b]	0.92	0.62 - 1.36
TYR exon 4 G/A (rs1126809)	GG	238 (0.70)	227 (0.77)	0.130 (C) 0.453 (P)	R	R	-
	GA	89 (0.26)	66 (0.22)		0.178[a]	0.77	0.54 -1.12
	AA	14 (0.04)	3 (0.01)		0.011[a]	0.22	0.06 – 0.79
	G	565 (0.83)	520 (0.88)		R	R	-
	A	117 (0.17)	72 (0.12)		0.0124[b]	0.66	0.49-0.92

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
(P) refers to Patients and (C) refers to Controls,
[a]Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b]Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table,
[#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

Table 2.19. Distributions of genotype and allele frequencies of *TYR* rs1042602 and rs1126809 polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (Freq) n=341	GV n=249	LV n=47	*p* value[#]	AV n=237	SV n=59	*p* value[#]
TYR exon 1 C/A (rs1042602)	CC	283 (0.83)	212 (0.85)	39 (0.83)	R	202 (0.85)	49 (0.83)	R
	CA	55 (0.16)	35 (0.14)	6 (0.13)	0.882[a] 0.696[b] 0.613[c]	33 (0.14)	8 (0.14)	0.998[x] 0.466[y] 0.669[z]
	AA	03 (0.01)	2 (0.01)	2 (0.04)	0.063[a] 0.899[b] 0.062	2 (0.01)	2 (0.03)	0.131[x] 0.941[y] 0.118[z]
	C	621 (0.91)	459 (0.92)	84 (0.89)	R	437 (0.92)	106 (0.90)	R
	A	61 (0.09)	39 (0.08)	10 (0.11)	0.365[a] 0.498[b] 0.593[c]	37 (0.08)	12 (0.10)	0.404[x] 0.494[y] 0.669[z]
TYR exon 4 G/A (rs1126809)	GG	238 (0.70)	192 (0.77)	35 (0.75)	R	183 (0.77)	44 (0.74)	R
	GA	89 (0.26)	55 (0.22)	11 (0.23)	0.806[a] 0.175[b] 0.636[c]	52 (0.22)	14 (0.24)	0.743[x] 0.170[y] 0.625[z]
	AA	14 (0.4)	2 (0.01)	1 (0.02)	0.396[a] 0.011[b] 0.483[c]	2 (0.01)	1 (0.02)	0.545[x] 0.014[y] 0.347[z]
	G	565 (0.83)	439 (0.88)	81 (0.86)	R	418 (0.88)	102 (0.86)	R
	A	117 (0.17)	59 (0.12)	13 (0.14)	0.589[a] 0.011[b] 0.418[c]	56 (0.12)	16 (0.14)	0.604[x] 0.012[y] 0.333[z]

n: number of subjects; R: reference group; GV: Generalized vitiligo; LV: Localized vitiligo; AV: Active Vitiligo; SV: Stable Vitiligo; [a]Generalized vitiligo vs. Localized vitiligo; [b]Generalized vitiligo vs. Controls; [c]Localized vitiligo vs. Controls; [x]Active Vitiligo vs. Stable Vitiligo; [y]Active Vitiligo vs. Controls; [z]Stable Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table. [#]Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

2.3.5.3 Linkage disequilibrium and haplotype analyses

LD analysis revealed that two polymorphisms investigated, i.e. *TYR* rs1042602 and rs1126809, were in low LD association (D'=0.017, r^2=0.00). Haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes were not significantly different between patients and controls (global *p*=0.682; Table 2.20).

Table 2.20 Distribution of haplotype frequencies of *TYR* rs1042602 and rs1126809 polymorphisms in vitiligo patients and controls.

Haplotype *[C/A:G/A]*	**Patients (Freq.)**	**Controls (Freq.)**	***p* value**	***p* Global**	**Odds Ratio [95% CI]**
CG	345.11 (0.80)	318.21 (0.79)	0.759	0.682	1.05 [0.75~1.49]
AG	37.89 (0.09)	31.79 (0.08)	0.648		1.12 [0.68~1.83]
CA	43.89 (0.10)	47.79 (0.12)	0.429		0.84 [0.54~1.29]
AA	5.11 (0.01)	6.21 (0.01)	-		-

'CI' represents Confidence Interval. (Haplotype frequency <0.03 in has been dropped and was ignored in analysis by the software).

2.3.5.4 Estimation of anti-tyrosinase antibodies in the plasma of vitiligo patients and controls

We have monitored the levels of anti-TYR antibodies levels in controls and vitiligo patients using ELISA. Interestingly, a significant increase in anti-TYR antibody levels was observed in vitiligo patients as compared to controls (mean ± SEM: 75.27 ± 7.629 ng/ml vs. 34.28 ± 3.240 ng/ml, respectively; p<0.0001). Further analysis revealed that patients with GV had significantly higher plasma anti-TYR antibody levels as compared to controls (mean ± SEM: 87.93 ± 10.870 ng/ml vs. 34.28 ± 3.240 ng/ml, respectively; p<0.0001) and patients with LV (mean ± SEM: 87.93 ± 10.870 ng/ml vs. 50.71 ± 2.887 ng/ml, respectively; p=0.008). Analysis based on the activity revealed significantly higher anti-TYR antibody levels in AV as compared to controls (mean ± SEM: 85.51 ± 10.390 ng/ml vs. 34.28 ± 3.240 ng/ml, respectively; p<0.0001) and SV (mean ± SEM: 85.51 ± 10.390 ng/ml vs. 53.17 ± 2.754 ng/ml, respectively; p=0.033). Analysis with respect to gender revealed no significant difference in male and female vitiligo patients (mean ± SEM: 64.47 ± 5.276 ng/ml vs. 85.21 ± 15.96 ng/ml, respectively; p=0.252). Interestingly, anti-TYR antibody levels were significantly increased in patients with disease duration of less than 6 months as compared to those with more than 6 months (mean ± SEM: 112.3 ± 22.91 ng/ml vs. 60.86 ± 4.018 ng/ml, respectively; p=0.002).

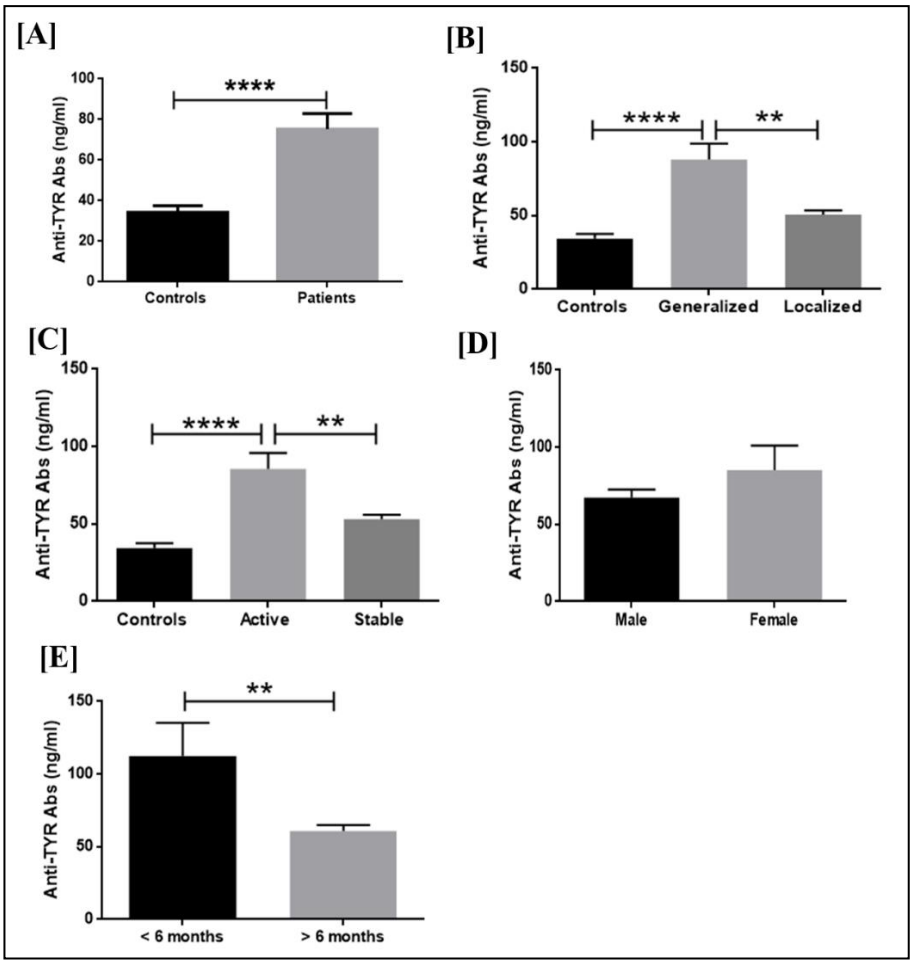

Figure 2.19. Estimation of plasma anti-tyrosinase (TYR) antibody levels in vitiligo patients and controls. Anti-TYR antibody levels in 40 controls, 50 patients with vitiligo were analysed by applying unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups. [A] Vitiligo patients showed a significant increase in anti-TYR antibody levels as compared to controls (p<0.0001). [B] Analysis of anti-TYR antibody levels in 40 controls and 33 patients with GV and 17 patients with LV revealed that patients with GV showed significantly increased in anti-TYR antibody levels as compared to controls (p<0.0001) and patients with LV (p=0.008). However, there was no significant difference in anti-TYR antibody levels between patients with LV as compared to controls (p=0.347). [C] Further anti-TYR antibody levels were analysed in 40 controls and 35 patients with AV and 15 patients with SV. Significantly increased anti-TYR antibody levels were observed in patients with AV as compared to controls (p<0.0001) and patients with SV (p=0.033). However, there was no significant difference in anti-TYR antibody levels between patients with SV as compared to controls (p=0.286). [D] Anti-TYR antibody levels upon analysis with respect to gender in 28 male and 22 female patients revealed no significant difference in both the groups (p=0.252). [E] A significant increase (p=0.002) in anti-TYR antibody levels was observed in patients with disease duration of less than 6 months (n=14) as compared to those with more than 6 months (n=36) [**p<0.01; ***p<0.001; ****p<0.0001].

2.4 Discussion

Vitiligo is an acquired hypo-pigmentary disorder characterized by the progressive loss of melanocytes. The etiopathogenesis of vitiligo remains obscure despite being in focused debate for several years (Laddha *et al.,* 2013b; Mansuri *et al.,* 2014). The melanocyte loss in vitiligo is a result of complex interactions of biochemical, environmental, and immunological events, in a permissive genetic milieu. Genetic polymorphisms in genes involved in the maintenance of redox homeostasis, immune response, and melanogenesis are associated with disease susceptibility and environmental triggers are involved in the manifestation of the disease (Shajil *et al.,* 2006b; Picardo *et al.,* 2015). Association of the MHC region has been implicated in several GWAS on vitiligo, including the Indian subcontinent (Fernando *et al.,* 2008; Singh *et al.,* 2012; Birlea *et al.,* 2013; Jin *et al.,* 2016). Association of MHC class II region with generalized vitiligo was reported in European-derived white population (Jin *et al.,* 2010). The strong link between autoimmune diseases and MHC class II genes suggests that abnormalities in MHC class II gene products may play a crucial role in vitiligo susceptibility. Interestingly, the association of GV with SNPs in the *PSMB8-TAP1* region of the MHC has been reported to derive from LD with primary association signals in the MHC class I and class II regions (Birlea *et al.,* 2013). Any alterations in function or expression of *PSMB8* or *TAP1* proteins could potentially affect the antigenic repertoire expressed on the cell surface and may alter peripheral tolerance (Groettrup *et al.,* 2001). Several studies have addressed the association of *PSMB8* and *TAP1* polymorphisms in patients with vitiligo (Casp *et al.,* 2003; Birlea *et al.,* 2013; Dani *et al.,* 2018); however, studies revealing the impact of these polymorphisms at transcript and protein levels are few. The present study suggests the association of *PSMB8* rs2071464 SNP with GV as well as with the disease activity (AV); however, *TAP1* rs1135216 SNP was not associated with vitiligo in Gujarat. Our results are in accordance with the previous study reported in the western population for *PSMB8* SNP (Casp *et al.,* 2003). In contrast, two studies have found *TAP1* exon 10 SNP to be associated with vitiligo in the Saudi population, and this may be due to differences in ethnicity (Seif-Eldin *et al.,* 2006; Babalghith, 2014). Birlea *et al.,* (2011) have addressed 34 SNPs spanning the *TAP1-PSMB8* region in GWAS and the meta-analysis study in GV patients; however, no association was observed for *TAP1* rs1135216 and *PSMB8* rs2071627 SNPs. The *PSMB8* encodes IFN-γ inducible subunit (b5i/LMP7) of the immunoproteasome, which degrades the ubiquitin-tagged cytoplasmic proteins into peptides that are especially suited for presentation by MHC class I molecules to $CD8^+$ cytotoxic T cells (Basler *et al.,* 2013). Further, a

significant association of *PSMB8* rs2071464 leads us to speculate on some functional consequences of this SNP in the disease pathogenesis. Intriguingly, the decreased expression of *PSMB8* was associated with the susceptible 'C' allele of *PSMB8* rs2071464; however, the mechanism is not yet clear. *In silico* prediction tools have predicted that *PSMB8* rs2071464 C>T variation might alter chromatin to enhancer state and result in induced gene expression in peripheral blood cells. Taudt *et al.,* have explored several *cis*-regulatory SNPs that may affect histone modifications and change chromatin state transition from a repressor to an enhancer state (Taudt *et al.,* 2016). Our results correlate with these findings as higher expression of *PSMB8* was observed in individuals having variant 'TT' genotype as compared to 'CC' genotype (Figure 2.4). A significant decrease in the transcript as well as protein expression of *PSMB8* in PBMCs of patients with GV and AV was obtained in the present study. Our findings have also been supported by the blood transcriptomic analysis of vitiligo patients, which revealed significant downregulation of *PSMB8* expression in patients (Dey-Rao and Sinha, 2017). In addition, another study has demonstrated the IFN-γ induced lower expression of *PSMB8* in PBMCs of vitiligo patients as compared to controls (Dani *et al.,* 2018). Moreover, it has been observed that the down-regulation of *PSMB8* expression leads to suppression of MHC class I molecule surface expression (Seliger *et al.,* 2000). In addition, the IFN-γ induced immunoproteasomes have been associated with the improved processing of MHC class I antigens (Seifert *et al.,* 2010). It has been reported that the presentation of a majority of MHC class I epitopes were strikingly reduced in immunoproteasome deficient mice (Kincaid *et al.,* 2012). Moreover, a significant decrease of 26S proteasome in lesions of vitiligo has been reported previously (Xu *et al.,* 2013). Thus, the decreased expression of *PSMB8* in the present study, in conjunction with the above discussed studies, advocates the possibility of reduced MHC class I molecules in the patients and indicates the crucial role of *PSMB8* in immunopathogenesis of vitiligo.

Transport of antigenic peptides across the ER membrane is mediated by TAP1 and TAP2 molecules (Monaco, 1992). We did not find significant association of *TAP1* rs1132516 SNP with vitiligo, and there was no difference in *TAP1* transcript levels between patients and controls. The 'G' allele occurred predominantly in AV patients compared to SV; however, it was considered non-significant due to Bonferroni's correction. The higher frequency of the 'G' allele in AV patients indicates its involvement in the autoimmune basis of vitiligo. Bioinformatics analysis substantiated that *TAP1* rs1135216 SNP (Asp637Gly) leads to a decrease in the stability of TAP1 protein. Moreover, it has been reported that the polymorphism in the *TAP1* gene did not show any measurable change in protein function but

has an influence on peptide selectivity (Quadri and Singal, 1998). The binding of antigenic peptides to class I molecules depends on both their length (usually 8-10 residues) and sequence (Bleek and Nathenson, 1990). The specificity of these reactions and their biological functions are affected by the 3D conformation of the peptide, HLA complexes, compatibility of the peptide sequence with its HLA class I binding pocket, etc. (Engelhard, 1994). Interestingly, significant differences in the amino acid signatures of the peptide-binding pockets of MHC class I α chains, as well as class II β chains, were observed between vitiligo patients and unaffected controls (Krämer *et al.,* 2007). Although *TAP1* SNP was not associated with vitiligo, the predominant presence of 'G' allele in combination with other SNPs in this region might affect the peptide selectivity in patients. *PSMB8* polymorphism, in addition to previously reported susceptibility loci such *TNFA, TNFB, IL1B, IFNG, NALP1, IL4*, etc. demonstrate immunogenetic predisposition in vitiligo patients from Gujarat (Imran *et al.,* 2012a; Laddha *et al.,* 2012, 2014a). Overall, these findings implicate a break in immunological tolerance in vitiligo.

Several studies have suggested decreased vitamin B_{12} and elevated homocysteine levels, which may be a cause for hyperhomocysteinemia in vitiligo patients (Lecler and Sibani, 2004; Park and Lee 2005; Shaker and El-Tahlawi, 2008; Agarwal *et al.,* 2015; Anbar *et al.,* 2016). Homocysteine levels can be elevated by various constitutive, genetic and lifestyle factors, by inadequate nutrient status (vitamin B_6, B_9 & B_{12}) and as a result of systemic disease and various medicines (Strain *et al.,* 2004). Among genetic factors, *MTHFR* rs1801133 and rs1801131 polymorphisms are widely associated with elevated homocysteine levels (Brustolin *et al.,* 2010). Earlier, we have speculated that *MTHFR* could be a small piece of the vitiligo jigsaw puzzle (Begum, 2014). The present study revealed a significant association of *MTHFR* rs1801131 polymorphism with generalized and active vitiligo in Gujarat population. Chen and colleagues have reported *MTHFR* rs1801133 polymorphism to be associated with vitiligo susceptibility in Chinese Han population (Chen *et al.,* 2014). In another study, no association of *MTHFR* rs1801133 and rs1801131 polymorphisms was observed with vitiligo in Turkish population (Yasar *et al.,* 2012). Kumar *et al.,* have reported that homocysteine levels are associated with *MTHFR* rs1801131 polymorphism in Indian population (Kumar *et al.,* 2005). *MTHFR* rs1801131 polymorphism has been identified as a risk factor for several diseases such as breast cancer, Alzheimer's disease, non-Hodgkin's lymphoma, ulcerative colitis, Rheumatoid arthritis, etc. (He *et al.,* 2014; Saad *et al.,* 2015; Awwad *et al.,* 2015; Varzari *et al.,* 2016; Wu *et al.,* 2017). *MTHFR* rs1801133 and rs1801131 polymorphisms lead to a significant reduction in MTHFR enzyme activity and influence the

homocysteine levels (Lecler and Sibani, 2004). *MTHFR* rs1801131 polymorphism leads to Glu429Ala substitution in the regulatory domain of the enzyme (Lecler and Sibani, 2004). Our structure-based *in silico* prediction revealed structural perturbations due to MTHFR Ala222Val and Glu429Ala substitutions (Figure 2.9). *In silico* prediction revealed a distortion in the S-Adenosyl methionine (SAM) binding site in Glu429Ala mutated structure of MTHFR enzyme (Shahzad *et al.,* 2013). Phosphorylation is crucial for the regulation of human MTHFR enzyme activity (Yamada *et al.,* 2005). A total of 21-serine phosphorylation sites have been predicted in Ala222Val and Glu429Ala mutants, which was one less than the total sites predicted in the wild type MTHFR protein. It was found that double mutants, containing both Ala222Val and Glu429Ala mutations, exhibit a lower number of serine phosphorylation sites as compared to the two single mutant structures, which might be responsible for decreased MTHFR activity (Shahzad *et al.,* 2013). Overall, our *in silico* analysis revealed that Ala222Val substitution is more deleterious for MTHFR activity than Glu429Ala substitution. The findings of our population based studies are also correlated with *in silico* analysis. The frequency of *MTHFR* rs1801131 polymorphism, which is relatively milder than *MTHFR* rs1801133 polymorphism, was higher in vitiligo patients. The frequency of 'TC' haplotype, carrying variant alleles of both the polymorphisms, was significantly higher in vitiligo patients (Table 2.11). Hence, the present study suggests that *MTHFR* rs1801131 polymorphism might influence the homocysteine levels and contribute to generalized and active vitiligo susceptibility. Correlating *MTHFR* polymorphisms with its enzyme activity in patients and controls would be interesting and further studies in this direction will throw light on the role of homocysteine in melanocyte biology and vitiligo pathogenesis. Homocysteine, a toxic by-product of methionine metabolism is reported to adversely affect via inducing ER stress and immune response (Desai *et al.,* 2001; Jakubowski 2004; Park *et al.,* 2012).

Perturbation in normal ER homeostasis due to internal or external stimuli that affect ER function leading to accumulation of misfolded proteins is known as ER stress. ER stress activates unfolded protein response via activating three transmembrane sensors viz. IRE-1, ATF-6 and PERK (Cao and Kaufman, 2014). Upon dissociation from GRP78, IRE1 is phosphorylated and its nuclease is activated, leading to splicing of the *XBP1* (X-box binding protein 1) mRNA. Spliced *XBP1* mRNA encodes a transcription factor that induces expression of genes containing an unfolded protein response element (UPRE). These genes include ER chaperones, heat shock proteins, immunoregulatory genes, and XBP1 itself (Calfon *et al.,* 2002; Yoshida, 2007b). The analysis of *XBP1* rs2269577 polymorphism

revealed a significant difference in allele as well as genotype frequencies among controls and patients (Table 2.13) suggesting its association with vitiligo susceptibility in Gujarat population. Interestingly, *XBP1* rs2269577 was found to be associated with vitiligo susceptibility in Caucasian as well as Chinese population (Ren *et al.,* 2009; Birlea *et al.,* 2011). Our results suggest a significant increase in 'CC' genotype in vitiligo patients as compared to controls, hence *XBP1* rs2269577 polymorphism leads to alteration in the motif 'AGGT' into 'ACGT' in the *XBP1* gene promoter (Kakiuchi *et al.,* 2003). Analysis of luciferase activity revealed higher promoter activity of 'C' allele as compared to 'G' allele (Kakiuchi *et al.,* 2003; Ren *et al.,* 2009). Our results on *XBP1* transcript levels also showed significantly increased transcript levels of *uXBP1* in individuals carrying 'CC' and 'CG' genotypes as compared to controls; however, no significant difference in s*XBP1* transcript levels was observed with respect to *XBP1* rs2269577 genotypes (Figure 2.11). Further, gene expression analysis revealed a significant increase in transcript levels of *sXBP1* in PBMCs of patients with generalized and active vitiligo (Figure 2.11), suggesting its involvement in immune-mediated pathogenesis. However, transcript levels of *uXBP1* were not significantly different in PBMCs of vitiligo patients and controls (Figure 2.11). In addition, there was a significant increase of *sXBP1* transcript levels in peri-lesional skin of vitiligo patients as compared to lesional and control skin (Figure 2.12). The peri-lesional skin of vitiligo patients is an immunologically active region having infiltration of cytotoxic T cells (Luiten *et al.,* 2009). Overall, the gene expression analysis in PBMCs and skin samples suggests the involvement of an active state of IRE1 in immune-mediated pathogenesis of vitiligo. IRE1/XBP1 is the most conserved branch of the UPR. It plays crucial roles in several important biological mechanisms such as development, metabolism, immunity, inflammation, and neurodegeneration (Kaufman and Cao, 2010). The active transcription factor XBP1 is involved in the regulation of several important immunological processes including MHC molecules, cytokine expression, development and differentiation of immune cells (Martinon *et al.,* 2010; Kaufman and Cao, 2010; Liu *et al.,* 2012; Bettigole and Glimcher, 2015; Pramanik *et al.,* 2018). These findings suggest a potential link between cellular stress and immunity in the pathogenesis of vitiligo.

Another interesting candidate gene in the present study is a pro-inflammatory cytokine *IL17A.* The cytokine IL-17A has been widely implicated in the pathogenesis of several immune-mediated disorders. The results of genetic analysis in the present study suggest no significant association of *IL17A* rs2275913 and rs8193036 polymorphisms with vitiligo susceptibility in the Gujarat population (Table 2.15). Several studies have reported a significant association of

IL17A rs2275913 and rs8193036 polymorphisms with inflammatory disorders such as inflammatory bowel disease, autoimmune thyroid disease, ulcerative colitis, rheumatoid arthritis, Behcet's disease, etc. (Arisawa *et al.,* 2008; Kim *et al.,* 2012; Yan *et al.,* 2012; Zhang *et al.,* 2013; Shen *et al.,* 2015). However, Mohammed *et al.,* (2017) have reported no significant association of *IL17A* rs2275913 with vitiligo susceptibility in the Egyptian population. The gene expression analysis revealed a significant increase in *IL17A* transcript levels in PBMCs of vitiligo patients as compared to controls (Figure 2.15). The analysis based on type and activity of vitiligo revealed a significant increase in *IL17A* transcript levels in PBMCs of patients with generalized and active vitiligo (Figure 2.15). However, there was no significant difference in *IL17A* transcript levels with respect to *IL17A* rs2275913 and rs8193036 polymorphisms suggesting no significant effect of both the polymorphisms on *IL17A* expression. A significant increase in *IL17A* transcript levels in PBMCs of generalized and active vitiligo led us to monitor the expression of IL-17A in skin of vitiligo patients and controls. Hence, we estimated the IL-17A protein levels in suction induced blister fluid (SBF) samples of vitiligo patients and controls. Interestingly, there was a significant increase in IL-17A levels in peri-lesional skin SBF samples as compared to controls (Figure 2.16). Several studies have reported a significant increase in IL-17A levels in the skin as well as blood samples of vitiligo patients (Zhen *et al.,* 2016; Bhardwaj *et al.,* 2017; Mohammed *et al.,* 2017; Sushama *et al.,* 2019; Acharya and Mathur, 2020). There are several reports demonstrating the infiltration of Th17 cells in vitiliginous skin of vitiligo patients (Wang *et al.,* 2011; Kotobuki *et al.,* 2012; Bhardwaj *et al.,* 2017). In addition, a positive correlation between IL-17A levels and the extent of depigmentation has also been reported, suggesting its involvement in the progression of the disease (Basak *et al.,* 2009). Kotobuki *et al.,* (2012) have demonstrated that IL-17A can induce the neighbouring keratinocytes and fibroblasts to secrete other pro-inflammatory cytokines such as TNFα, IL-6, and IL-1β. They further demonstrated the adverse effect of IL-17A on melanocyte homeostasis. A significant decrease in genes involved in melanogenesis such as *MITF, TYR, TYRP1* and *DCT* was observed in primary human melanocytes treated with IL-17A affecting normal melanogenesis (Kotobuki *et al.,* 2012). Overall, the finding of the present study suggests a vital role of IL-17A in immune-mediated pathogenesis of vitiligo.

Tyrosinase (TYR) is a crucial enzyme involved in the melanogenesis pathway (Hearing, 2007). Two non-synonymous polymorphisms of the *TYR* gene, rs1042602 and rs1126809 were found to be significantly associated with generalized vitiligo in Caucasian population (Jin *et al.,* 2010). Our results suggest a significant association of *TYR* rs1126809

polymorphism with vitiligo susceptibility in Gujarat population and the minor allele 'A' was identified as a protective allele (Table 2.18). Interestingly, *TYR* rs1126809 polymorphism was associated with susceptibility to generalized and active vitiligo (Table 2.19). *TYR* S192Y (rs1042602) polymorphism has been reported to cause steric hindrance at copper-binding site and affect the enzyme activity whereas, *TYR* R402Q (rs1126809) polymorphism is reported to reduce N-glycosylation leading to retention of tyrosinase in ER (Tripathi *et al.,* 1992; Spritz *et al.,* 1997; Toyofuku *et al.,* 2001; Chaki *et al.,* 2011). Interestingly, the minor allele 'A' of *TYR* R402Q polymorphism was found to be associated with susceptibility to malignant melanoma (Gudbjartsson *et al.,* 2008; Bishop *et al.,* 2009; Duffy *et al.,* 2010). It was reported that *TYR* rs1126809 might protect from GV by reducing the availability of TYR peptide for antigen presentation by HLA*02:01 and increase the risk of melanoma by conferring efficient epitope presentation (Spritz 2010; Jin *et al.,* 2012). Tyrosinase is also recognized as a major melanocyte autoantigen involved in autoimmune vitiligo (Song *et al.,* 1994; Baharav *et al.,* 1996; Kemp *et al.,* 1997, 2007). In accordance, the present study also revealed significantly higher levels of anti-TYR antibodies in the plasma of vitiligo patients as compared to controls. Further analysis revealed that the elevated anti-TYR antibody levels are associated with generalized and active vitiligo. Additionally, we observed significantly higher anti-TYR antibodies in patients with the disease duration of less than 6 months (Figure 2.16). These results suggest the potential role of Tyrosinase in the progression of the disease. Intriguingly, significantly increased levels of auto-antibodies against oxidized-tyrosinase are observed in vitiligo patients (Al-shobaili and Rasheed, 2015). These findings led us to speculate that oxidative stress might be involved in generating the tyrosinase as a neo-auto antigen and thereby inducing the anti-melanocyte autoimmune response.

In conclusion, the present study identifies *PSMB8* rs2071627, *MTHFR* rs1801131, *XBP1* rs2269577 and *TYR* rs1126809 polymorphisms as genetic susceptibility loci for vitiligo in Gujarat population. In addition, we report altered *PSMB8, XBP1, IL17A* expression and elevated anti-tyrosinase autoantibodies in vitiligo patients. Overall, the findings suggest an involvement of genetic components in the dysregulation of antigen processing, methionine metabolism, ER stress mechanism, and melanogenesis in vitiligo patients.

2.5 References

1. Abele R, Tampé R (1999) Function of the transport complex TAP in cellular immune recognition. Biochim. Biophys. Acta - Biomembr. 1999;1461(2):405-419. doi:10.1016/s0005-2736(99)00171-6
2. Acharya P, Mathur M (2020) Interleukin-17 level in patients with vitiligo: A systematic review and meta-analysis. Australas. J. Dermatol. 2020;61(2):e208-e212. doi:10.1111/ajd.13233
3. Adzhubei IA, Schmidt S, Peshkin L, et al (2010) A method and server for predicting damaging missense mutations. Nat Methods 2010;7(4):248-249. doi:10.1038/nmeth0410-248
4. Agarwal S, Mendiratta V, Chander R, et al (2015) Study of serum levels of Vitamin B 12, folic acid, and homocysteine in vitiligo. Pigment Int 2:76-80**.** doi: 10.4103/2349-5847.172777
5. AlGhamdi KM, Khurrum H, Moussa NA (2014) Is there a real relationship between serum level of homocysteine and vitiligo? A controlled study on 306 subjects. J. Cutan. Med. Surg. 2014;18(1):5-7. doi:10.2310/7750.2013.13050
6. Al-shobaili HA, Rasheed Z (2015) Oxidized tyrosinase : A possible antigenic stimulus for non-segmental vitiligo autoantibodies. J Dermatol Sci. 2015;79(3):203-213. doi:10.1016/j.jdermsci.2015.06.009
7. Anbar T, Zuel-Fakkar NM, Matta MF, Arbab MMI (2016) Elevated homocysteine levels in suction-induced blister fluid of active vitiligo lesions. Eur J Dermatology. 2016;26(1):64-67. https://doi.org/10.1684/ejd.2015.2682
8. Arisawa T, Tahara T, Shibata T, et al (2008) The Influence of Polymorphisms of Interleukin-17A and Interleukin-17F Genes on the Susceptibility to Ulcerative Colitis. J Clin Immunol 28:44–49. https://doi.org/10.1007/s10875-007-9125-8
9. Asarch A, Barak O, Loo DS, Gottlieb AB (2008) Th17 cells: A new therapeutic target in inflammatory dermatoses. J. Dermatolog. Treat. 2008;19(6):318-326. doi:10.1080/09546630802206660
10. Awwad N, Yousef A-M, Abuhaliema A, et al (2015) Relationship between Genetic Polymorphisms in MTHFR (C677T, A1298C and their Haplotypes) and the Incidence Of Breast Cancer among Jordanian Females - Case-Control Study. Asian Pacific J Cancer Prev 16:5007–5011. https://doi.org/10.7314/APJCP.2015.16.12.5007
11. Baharav E, Merimski O, Shoenfeld Y, et al (1996) Tyrosinase as an autoantigen in

patients with vitiligo. Clin Exp Immunol 105:84–88. https://doi.org/10.1046/j.1365-2249.1996.d01-727.x

12. Barton DE, Kwon BS, Francke U (1988) Human tyrosinase gene, mapped to chromosome 11 (q14 → q21), defines second region of homology with mouse chromosome 7. Genomics 3:17–24. https://doi.org/10.1016/0888-7543(88)90153-X
13. Basak PY, Adiloglu AK, Ceyhan AM, et al (2009) The role of helper and regulatory T cells in the pathogenesis of vitiligo. J Am Acad Dermatol 60:256–260. https://doi.org/10.1016/j.jaad.2008.09.048
14. Basler M, Kirk CJ, Groettrup M (2013) The immunoproteasome in antigen processing and other immunological functions. Curr Opin Immunol 25:74–80. https://doi.org/10.1016/j.coi.2012.11.004
15. Bassiouny D, Shaker O (2011) Role of interleukin-17 in the pathogenesis of vitiligo. Clin Exp Dermatology Exp dermatology 36:292–297.doi:10.1111/j.1365-2230.2010.03972.x
16. Begum R (2014) Methylenetetrahydrofolate reductase (MTHFR): Could it be a small piece in vitiligo jigsaw puzzle? Br. J. Dermatol. 170(5):1009-1010. doi:10.1111/bjd.13017
17. Bettigole SE, Glimcher LH (2015) Endoplasmic reticulum stress in immunity. Annu Rev Immunol 33:107–38. https://doi.org/10.1146/annurev-immunol-032414-112116
18. Bhardwaj S, Rani S, Srivastava N, et al (2017) Cytokine Increased systemic and epidermal levels of IL-17A and IL-1 b promotes progression of non-segmental vitiligo. Cytokine 91:153–161. https://doi.org/10.1016/j.cyto.2016.12.014
19. Birlea SA, Ahmad FJ, Uddin RM, et al (2013) Association of Generalized Vitiligo with MHC Class II Loci in Patients from the Indian Subcontinent. J Invest Dermatol 133:1369–1372. https://doi.org/10.1038/jid.2012.501
20. Birlea SA, Jin Y, Bennett DC, et al (2011) Comprehensive association analysis of candidate genes for generalized vitiligo supports XBP1, FOXP3, and TSLP. J Invest Dermatol. 2011;131(2):371-381. https://doi.org/10.1038/jid.2010.337
21. Bishop DT, Demenais F, Iles MM, et al (2009) Genome-wide association study identifies three loci associated with melanoma risk. Nat Genet. 2009;41(8):920-925 https://doi.org/10.1038/ng.411
22. Bleek GM Van, Nathenson SG (1990) Isolation of an endogenously processed immunodominant viral peptide from the class I H–2Kb molecule. Nature 348:213–216. https://doi.org/10.1038/348213a0

23. Botto LD, Yang Q (2000) 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: A HuGE review. Am J Epidemiol. 2000;151(9):862-877. https://doi.org/10.1093/oxfordjournals.aje.a010290
24. Boyle AP, Hong EL, Hariharan M, et al (2012) Annotation of functional variation in personal genomes using RegulomeDB. Genome Res 22:1790–1797. https://doi.org/10.1101/gr.137323.112
25. Brustolin S, Giugliani R, Félix TM (2010) Genetics of homocysteine metabolism and associated disorders. Brazilian J. Med. Biol. Res. 2010;43(1):1-7. doi:10.1590/s0100-879x2009007500021
26. Calfon M, Zeng H, Urano F, et al (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature. 2002;415(6867):92-96 https://doi.org/10.1038/415092a
27. Cao SS, Kaufman RJ (2014) Endoplasmic Reticulum Stress and Oxidative Stress in Cell Fate Decision and Human Disease. Antioxid Redox Signal 21:396–413. https://doi.org/10.1089/ars.2014.5851
28. Capriotti E, Calabrese R, Casadio R (2006) Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. Bioinformatics 22:2729–2734. https://doi.org/10.1093/bioinformatics/btl423
29. Capriotti E, Fariselli P, Rossi I, Casadio R (2008) A three-state prediction of single point mutations on protein stability changes. BMC Bioinformatics 9:S6. https://doi.org/10.1186/1471-2105-9-S2-S6
30. Casp CB, She JX, McCormack WT (2003) Genes of the LMP/TAP cluster are associated with the human autoimmune disease vitiligo. Genes Immun. 2003;4(7):492-499.https://doi.org/10.1038/sj.gene.6364016
31. Chaki M, Sengupta M, Mondal M, et al (2011) Molecular and functional studies of tyrosinase variants among indian oculocutaneous albinism type 1 patients. J. Invest. Dermatol. 2011;131(1):260-262. doi:10.1038/jid.2010.274
32. Chen JX, Shi Q, Wang XW, et al (2014) Genetic polymorphisms in the methylenetetrahydrofolate reductase gene (MTHFR) and risk of vitiligo in Han Chinese populations: A genotype-phenotype correlation study. Br J Dermatol. 2014;170(5):1092-1099. https://doi.org/10.1111/bjd.12845
33. Chen W, Duan S, Zhou J, et al (2004) A case–control study provides evidence of association for a functional polymorphism −197C/G in XBP1 to schizophrenia and

suggests a sex-dependent effect. Biochem Biophys Res Commun 319:866–870. https://doi.org/10.1016/j.bbrc.2004.05.060

34. Cheng J, Randall A, Baldi P (2005) Prediction of protein stability changes for single-site mutations using support vector machines. Proteins Struct Funct Bioinforma 62:1125–1132. https://doi.org/10.1002/prot.20810
35. Dani P, Patnaik N, Singh A, et al (2018) Association and expression of the antigen-processing gene PSMB8, coding for low-molecular-mass protease 7, with vitiligo in North India: case–control study. Br J Dermatol. 2018;178(2):482-491. https://doi.org/10.1111/bjd.15391
36. Deng GY, Muir A, Maclaren NK, She JX (1995) Association of LMP2 and LMP7 genes within the major histocompatibility complex with insulin-dependent diabetes mellitus: Population and family studies. Am J Hum Genet 1995;56(2):528-534.
37. Desai A, Lankford HA, Warren JS (2001) Homocysteine augments cytokine-induced chemokine expression in human vascular smooth muscle cells: Implications for atherogenesis. Inflammation 25:179–186. https://doi.org/10.1023/A:1011088431191
38. Dey-Rao R, Sinha AA (2017) Vitiligo blood transcriptomics provides new insights into disease mechanisms and identifies potential novel therapeutic targets. BMC Genomics 18:109. https://doi.org/10.1186/s12864-017-3510-3
39. Djilali-Saiah I, Caillat-Zucman S, Schmitz J, et al (1994) Polymorphism of antigen processing (TAP, LMP) and HLA class II genes in celiac disease. Hum Immunol 40:8–16. https://doi.org/10.1016/0198-8859(94)90015-9
40. Duffy DL, Zhao ZZ, Sturm RA, et al (2010) Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma.JInvestDermatol. 2010;130(2):520-528 https://doi.org/10.1038/jid.2009.258
41. Dwivedi M, Laddha NC, Begum R (2013a) Correlation of increased MYG1 expression and its promoter polymorphism with disease progression and higher susceptibility in vitiligo patients. J Dermatol Sci 2013;71(3):195-202. doi:10.1016/j.jdermsci.2013.04.026
42. Dwivedi M, Laddha NC, Mansuri MS, et al (2013b) Association of NLRP1 genetic variants and mRNA overexpression with generalized vitiligo and disease activity in a Gujarat population. Br J Dermatol 169:1114–1125. https://doi.org/10.1111/bjd.12467
43. Engelhard VH (1994) Structure of peptides associated with class I and class II MHC molecules. Annu Rev Immunol 1994;12:181-207.

doi:10.1146/annurev.iy.12.040194.001145

44. Ergul E, Sazci A, Kara I (2012) Methylenetetrahydrofolate Reductase Gene Polymorphisms in Turkish Children with Attention-Deficit/Hyperactivity Disorder. Genet Test Mol Biomarkers 16:67–69. https://doi.org/10.1089/gtmb.2011.0062
45. Ezzedine K, Lim HW, Suzuki T, et al (2012) Revised classification/nomenclature of vitiligo and related issues: the Vitiligo Global Issues Consensus Conference. Pigment Cell Melanoma Res 2012;25(3):E1-E13. doi:10.1111/j.1755-148X.2012.00997.x
46. Falabella R, Arrunategui A, Barona MI, Alzate A (1995) The minigrafting test for vitiligo: detection of stable lesions for melanocyte transplantation. J Am Acad Dermatol 1995;32(2 Pt 1):228-232. doi:10.1016/0190-9622(95)90131-0
47. Fernando MMA, Stevens CR, Walsh EC, et al (2008) Defining the role of the MHC in autoimmunity: A review and pooled analysis. PLoS Genet 2008;4(4):e1000024.https://doi.org/10.1371/journal.pgen.1000024
48. Goyette P, Sumner JS, Milos R, et al (1994) Human methylenetetrahydrofolate reductase: Isolation of cDNA, mapping and mutation identification. Nat Genet. 1994 Aug;7(4):551]. *Nat Genet*. 1994;7(2):195-200. doi:10.1038/ng0694-195
49. Groettrup M, Khan S, Schwarz K, Schmidtke G (2001) Interferon-γ inducible exchanges of 20S proteasome active site subunits: Why? In: Biochimie 2001;83(3-4):367-372. doi:10.1016/s0300-9084(01)01251-2
50. Gudbjartsson DF, Sulem P, Stacey SN, et al (2008) ASIP and TYR pigmentation variants associate with cutaneous melanoma and basal cell carcinoma. Nat Genet. 2008;40(7):886-891. doi:10.1038/ng.161https://doi.org/10.1038/ng.161
51. Han J, Kraft P, Nan H, et al (2008) A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. PLoS Genet 4:e1000074. https://doi.org/10.1371/journal.pgen.1000074
52. He J, Liao XY, Zhu JH, et al (2014) Association of MTHFR C677T and A1298C polymorphisms with non-Hodgkin lymphoma susceptibility: Evidence from a meta-analysis. Sci Rep. 2014;4:6159. https://doi.org/10.1038/srep06159
53. Hearing VJ (2007) The Regulation of Melanin Formation. In: The Pigmentary System. Blackwell Publishing Ltd, Oxford, UK, pp 191–212 https://doi.org/10.1002/9780470987100.ch10
54. Hu L, Kong F, Pan Y (2018) Association between IL-17A G197A polymorphism and gastric cancer risk: an updated meta-analysis based on 6,624 cases and 7,631 controls. Onco Targets Ther 2018;11:703-710. Published 2018 Feb 5.

doi:10.2147/OTT.S151129

55. Hustad S, Midttun Ø, Schneede J, et al (2007) The Methylenetetrahydrofolate Reductase 677C→T Polymorphism as a Modulator of a B Vitamin Network with Major Effects on Homocysteine Metabolism. Am J Hum Genet 80:846–855. https://doi.org/10.1086/513520
56. Imran M, Laddha NC, Dwivedi M, et al (2012a) Interleukin-4 genetic variants correlate with its transcript and protein levels in patients with vitiligo. Br J Dermatol. https://doi.org/10.1111/j.1365-2133.2012.11000.x
57. Imran M, Laddha NC, Dwivedi M, et al (2012b) Interleukin- 4 genetic variants correlate with its transcript and protein levels in patients with vitiligo. 1:314–323. https://doi.org/10.1111/j.1365-2133.2012.11000.x
58. Jakubowski H (2004) Molecular basis of homocysteine toxicity in humans. Cell. Mol. Life Sci. 2004;61(4):470-487. doi:10.1007/s00018-003-3204-7
59. Jin Y, Andersen G, Yorgov D, et al (2016) Genome-wide association studies of autoimmune vitiligo identify 23 new risk loci and highlight key pathways and regulatory variants. Nat Genet. 2016;48(11):1418-1424 https://doi.org/10.1038/ng.3680
60. Jin Y, Birlea SA, Fain PR, et al (2010) Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. N Engl J Med 2010;362(18):1686-1697. doi:10.1056/NEJMoa0908547
61. Jin Y, Ferrara T, Gowan K, et al (2012) Next-generation DNA re-sequencing identifies common variants of TYR and HLA-A that modulate the risk of generalized vitiligo via antigen presentation. J Invest Dermatol 2012;132(6):1730-1733. doi:10.1038/jid.2012.37
62. Kakiuchi C, Iwamoto K, Ishiwata M, et al (2003) Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder. Nat Genet. 2003;35(2):171-175 https://doi.org/10.1038/ng1235
63. Kakourou T (2009) Vitiligo in children. 5:265–268. https://doi.org/10.1007/s12519-009-0050-1
64. Kamali AN, Noorbakhsh SM, Hamedifar H, et al (2019) A role for Th1-like Th17 cells in the pathogenesis of inflammatory and autoimmune disorders. Mol Immunol. 2019;105:107-115.https://doi.org/10.1016/j.molimm.2018.11.015
65. Karadag AS, Tutal E, Ertugrul DT, et al (2012) Serum holotranscobalamine, vitamin B12, folic acid and homocysteine levels in patients with vitiligo. Clin Exp Dermatol.

2012;37(1):62-64. https://doi.org/10.1111/j.1365-2230.2011.04142.x

66. Kasamatsu T, Kimoto M, Takahashi N, et al (2018) IL17A and IL23R gene polymorphisms affect the clinical features and prognosis of patients with multiple myeloma. Hematol Oncol. 2018;36(1):196-201. https://doi.org/10.1002/hon.2469
67. Kaser A, Lee A-H, Franke A, et al (2008) XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 2008;134(5):743-756. doi:10.1016/j.cell.2008.07.021
68. Kaufman RJ, Cao S (2010) Inositol-requiring 1/X-box-binding protein 1 is a regulatory hub that links endoplasmic reticulum homeostasis with innate immunity and metabolism. EMBO Mol Med 2:189–192. https://doi.org/10.1002/emmm.201000076
69. Kemp EH, Gavalas NG, Gawkrodger DJ, Weetman AP (2007) Autoantibody responses to melanocytes in the depigmenting skin disease vitiligo. Autoimmun. Rev. 2007;6(3):138-142. doi:10.1016/j.autrev.2006.09.010
70. Kemp EH, Gawkrodger DJ, MacNeil S, et al (1997) Detection of tyrosinase autoantibodies in patients with vitiligo using 35S-labeled recombinant human tyrosinase in a radioimmunoassay. J Invest Dermatol. 1997;109(1):69-73. https://doi.org/10.1111/1523-1747.ep12276556
71. Kemp EH, Waterman EA, Weetman AP (2001) Autoimmune Aspects of Vitiligo. 2001;34(1):65-77. doi:10.3109/08916930108994127
72. Kim ES, Kim SW, Moon CM, et al (2012) Interactions between IL17A, IL23R, and STAT4 polymorphisms confer susceptibility to intestinal Behcet's disease in Korean population. Life Sci. 2012;90(19-20):740-746. https://doi.org/10.1016/j.lfs.2012.03.017
73. Kim SM, Kim YK, Hann SK (1999) Serum levels of folic acid and vitamin B12 in Korean patients with vitiligo. Yonsei Med J 1999;40(3):195-198. doi:10.3349/ymj.1999.40.3.195
74. Kincaid EZ, Che JW, York I, et al (2012) Mice completely lacking immunoproteasomes show major changes in antigen presentation. Nat Immunol. 2011;13(2):129-135 https://doi.org/10.1038/ni.2203
75. Kluijtmans LAJ, Van Den Heuvel LPWJ, Boers GHJ, et al (1996) Molecular genetic analysis in mild hyperhomocysteinemia: A common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58(1):35-41.

76. Kotobuki Y, Tanemura A, Yang L, et al (2012) Dysregulation of melanocyte function by Th17-related cytokines: Significance of Th17 cell infiltration in autoimmune vitiligo vulgaris. Pigment Cell Melanoma Res 25:219–230. https://doi.org/10.1111/j.1755-148X.2011.00945.x
77. Krämer U, Illig T, Grune T, et al (2007) Strong associations of psoriasis with antigen processing LMP and transport genes TAP differ by gender and phenotype. Genes Immun. 2007;8(6):513-517. https://doi.org/10.1038/sj.gene.6364404
78. Kumar J, Das SK, Sharma P, et al (2005) Homocysteine levels are associated with MTHFR A1298C polymorphism in Indian population. J Hum Genet 50:655–663. https://doi.org/10.1007/s10038-005-0313-1
79. Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-1081. https://doi.org/10.1038/nprot.2009.86
80. Laddha NC, Dwivedi M, Begum R (2012) Increased Tumor Necrosis Factor (TNF)-α and Its Promoter Polymorphisms Correlate with Disease Progression and Higher Susceptibility towards Vitiligo. PLoS One 7:e52298. https://doi.org/10.1371/journal.pone.0052298
81. Laddha NC, Dwivedi M, Gani AR, et al (2013a) Involvement of superoxide dismutase isoenzymes and their genetic variants in progression of and higher susceptibility to vitiligo. Free Radic Biol Med. 2013;65:1110-1125. https://doi.org/10.1016/j.freeradbiomed.2013.08.189
82. Laddha NC, Dwivedi M, Mansuri MS, et al (2013b) Vitiligo: Interplay between oxidative stress and immune system. Exp Dermatol 22:245–250. https://doi.org/10.1111/exd.12103
83. Laddha NC, Dwivedi M, Mansuri MS, et al (2014a) Association of Neuropeptide Y (NPY), Interleukin-1B (IL1B) Genetic Variants and Correlation of IL1B Transcript Levels with Vitiligo Susceptibility. PLoS One 9:e107020. https://doi.org/10.1371/journal.pone.0107020
84. Laddha NC, Dwivedi M, Mansuri MS, et al (2014b) Association of Neuropeptide Y (NPY), Interleukin-1B (IL1B) Genetic Variants and Correlation of IL1B Transcript Levels with Vitiligo Susceptibility 2014;9(9):e107020 https://doi.org/10.1371/journal.pone.0107020
85. Lecler D, Sibani S RR (2004) Molecular biology of methylenetetrahydrofolate reductase (MTHFR) and overview of mutations/polymorphisms. Madame Curie

Biosci Database 271-289 https://doi.org/10.1007/978-94-017-1789-2_16

86. Lee K, Tirasophon W, Shen X, et al (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev. 2002;16(4):452-466. https://doi.org/10.1101/gad.964702
87. Lew B-L, Cho H-R, Haw S, et al (2012) Association between IL17A/IL17RA Gene Polymorphisms and Susceptibility to Alopecia Areata in the Korean Population. Ann Dermatol 24:61. https://doi.org/10.5021/ad.2012.24.1.61
88. Liu Y-P, Zeng L, Tian A, et al (2012) Endoplasmic Reticulum Stress Regulates the Innate Immunity Critical Transcription Factor IRF3. J Immunol. 2012;189(9):4630-4639. https://doi.org/10.4049/jimmunol.1102737
89. Luiten RM, Van Den Boorn JG, Konijnenberg D, et al (2009) Autoimmune destruction of skin melanocytes by perilesional T cells from vitiligo patients. J Invest Dermatol 129:2220–2232. https://doi.org/10.1038/jid.2009.32
90. Lynde CW, Poulin Y, Vender R, et al (2014) Interleukin 17A: Toward a new understanding of psoriasis pathogenesis. J Am Acad Dermatol 71:141–150. https://doi.org/10.1016/j.jaad.2013.12.036
91. Malhotra JD, Kaufman RJ (2007) The endoplasmic reticulum and the unfolded protein response. In: Seminars in cell & developmental biology. Elsevier, pp 716–731
92. Mansuri MS, Jadeja SD, Singh M, et al (2017) Catalase (*CAT*) promoter and 5′-UTR genetic variants lead to its altered expression and activity in vitiligo. Br J Dermatol. 2017;177(6):1590-1600. https://doi.org/10.1111/bjd.15681
93. Mansuri MS, Laddha NC, Dwivedi M, et al (2016) Genetic variations (Arg5Pro and Leu6Pro) modulate the structure and activity of GPX1 and genetic risk for vitiligo. Exp. Dermatol. 2016;25(8):654-657. doi:10.1111/exd.13007
94. Mansuri MS, Singh M, Jadeja SD, Begum R (2019) Association of glucose 6-phosphate dehydrogenase (G6PD) 3'UTR polymorphism with vitiligo and in vitro studies on G6PD inhibition in melanocytes. J Dermatol Sci 2019;93(2):133-135. doi:10.1016/j.jdermsci.2018.12.001
95. Martinon F, Chen X, Lee AH, Glimcher LH (2010) TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. Nat Immunol. 2010;11(5):411-418. https://doi.org/10.1038/ni.1857
96. Masui T, Hashimoto R, Kusumi I, et al (2005) A possible association between the [minus sign]116C/G single nucleotide polymorphism of the XBP1 gene and lithium

prophylaxis in bipolar disorder. Int J Neuropsychopharmacol 2006;9(1):83-88. https://doi.org/10.1017/S1461145705005523

97. Misra UK, Kalita J, Srivastava AK, Agarwal S (2010) MTHFR Gene Polymorphism and Its Relationship with Plasma Homocysteine and Folate in a North Indian Population. Biochem Genet 48:229–235. https://doi.org/10.1007/s10528-009-9312-9
98. Mohammed FN, Sayed Amr K, Abdel Raheem HM, et al (2017) Study of interleukin-17 gene polymorphism and susceptibility to vitiligo in a sample of the Egyptian population. J Egypt Women's Dermatologic Soc 14:45–48 doi: 10.1097/01.EWX.0000503399.60863.9f
99. Monaco JJ (1992) A molecular model of MHC class-I-restricted antigen processing. Immunol. Today 1992;13(5):173-179. doi:10.1016/0167-5699(92)90122-N
100. Montes LF, Diaz ML, Lajous J, Garcia NJ (1992) Folic acid and vitamin B12 in vitiligo: a nutritional approach. Cutis 50:39–42
101. Nan H, Kraft P, Hunter DJ, Han J (2009) Genetic variants in pigmentation genes, pigmentary phenotypes, and risk of skin cancer in Caucasians. Int J cancer 125:909–917. https://doi.org/10.1002/ijc.24327
102. Omrane I, Marrakchi R, Baroudi O, et al (2014) Significant association between interleukin-17A polymorphism and colorectal cancer. Tumor Biol 2014;35(7):6627-6632. doi:10.1007/s13277-014-1890-4
103. Ongenae K, Geel NVAN, Naeyaert J (2003) Evidence for an Autoimmune Pathogenesis of Vitiligo. 2003;16(2):90-100. doi:10.1034/j.1600-0749.2003.00023.x
104. Pappu R, Ramirez-Carrozzi V, Sambandam A (2011) The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. Immunology 134:8–16. https://doi.org/10.1111/j.1365-2567.2011.03465.x
105. Park HH, Lee MH (2005) Serum levels of vitamin B12 and folate in Korean patients with vitiligo [3]. Acta Derm. Venereol. 2005;85(1):66-67. doi:10.1080/00015550410021565
106. Park SJ, Kim KJ, Kim WU, et al (2012) Involvement of endoplasmic reticulum stress in homocysteine-induced apoptosis of osteoblastic cells. J Bone Miner Metab. 2012;30(4):474-484. https://doi.org/10.1007/s00774-011-0346-9
107. Picardo M, Dell'Anna ML, Ezzedine K, et al (2015) Vitiligo. Nat Rev Dis Prim 2015;1:15011. https://doi.org/10.1038/nrdp.2015.11
108. Pramanik J, Chen X, Kar G, et al (2018) Genome-wide analyses reveal the IRE1a-XBP1 pathway promotes T helper cell differentiation by resolving secretory stress and

accelerating proliferation. Genome Med. 2018;10(1):76. Published 2018 Oct 24. https://doi.org/10.1186/s13073-018-0589-3

109. Quadri SA, Singal DP (1998) Peptide transport in human lymphoblastoid and tumor cells: effect of transporter associated with antigen presentation (TAP) polymorphism. Immunol Lett 61:25–31. https://doi.org/10.1016/s0165-2478(97)00157-0
110. Ren Y, Yang S, Xu S, et al (2009) Genetic variation of promoter sequence modulates XBP1 expression and genetic risk for vitiligo. PLoS Genet 2009;5(6):e1000523. https://doi.org/10.1371/journal.pgen.1000523
111. Roberts GHL, Santorico SA, Spritz RA (2019) The Genetic Architecture of Vitiligo. Pigment Cell Melanoma Res 0–2. https://doi.org/10.1111/pcmr.12848
112. Roy A, Kucukural A, Zhang Y (2010) I-TASSER: A unified platform for automated protein structure and function prediction. Nat Protoc. 2010;5(4):725-738 https://doi.org/10.1038/nprot.2010.5
113. Roy A, Yang J, Zhang Y (2012) COFACTOR: An accurate comparative algorithm for structure-based protein function annotation. Nucleic Acids Res. 2012;40:W471-W477https://doi.org/10.1093/nar/gks372
114. Saad MN, Mabrouk MS, Eldeib AM, Shaker OG (2015) Genetic case-control study for eight polymorphisms associated with rheumatoid arthritis. PLoS One 2015;10(7):e0131960. https://doi.org/10.1371/journal.pone.0131960
115. Seifert U, Bialy LP, Ebstein F, et al (2010) Immunoproteasomes Preserve Protein Homeostasis upon Interferon-Induced Oxidative Stress. Cell 142:613–624. https://doi.org/10.1016/j.cell.2010.07.036
116. Seliger B, Maeurer MJ, Ferrone S (2000) Antigen-processing machinery breakdown and tumor growth. Immunol Today 21:455–464. https://doi.org/10.1016/S0167-5699(00)01692-3
117. Shahzad K, Hai A, Ahmed A, et al (2013) A Structured-based Model for the Decreased Activity of Ala222Val and Glu429Ala Methylenetetrahydrofolate Reductase (MTHFR) Mutants. Bioinformation 9:929–936. https://doi.org/10.6026/97320630009929
118. Shajil E, Agrawal D, Vagadia K, et al (2006a) Vitiligo: Clinical profiles in Vadodara, Gujarat. Indian J Dermatol 51:100–104
119. Shajil EM, Chatterjee S, Agrawal D, et al (2006b) Vitiligo: Pathomechanisms and genetic polymorphism of susceptible genes. Indian J. Exp. Biol. 2006;44(7):526-539.
120. Shaker OG, El-Tahlawi SMR (2008) Is there a relationship between homocysteine and

vitiligo? A pilot study. Br J Dermatol 159:720–724. https://doi.org/10.1111/j.1365-2133.2008.08712.x

121. Shen L, Zhang H, Yan T, et al (2015) Association between interleukin 17A polymorphisms and susceptibility to rheumatoid arthritis in a Chinese population. Gene 566:18–22. https://doi.org/10.1016/j.gene.2015.04.028
122. Shoab Mansuri M, Singh M, Jadeja SD, et al (2014) Could ER Stress Be A Major Link Between Oxidative Stress And Autoimmunity In Vitiligo? J Pigment Disord 01: https://doi.org/10.4172/2376-0427.1000123
123. Silverberg JI, Silverberg NB (2011) Serum homocysteine as a biomarker of vitiligo vulgaris severity: A pilot study. J Am Acad Dermatol 64:445–447. https://doi.org/10.1016/j.jaad.2010.08.025
124. Singh A, Sharma P, Kar HK, et al (2012) HLA alleles and amino-acid signatures of the peptide-binding pockets of HLA molecules in vitiligo. J Invest Dermatol 132:124–134. https://doi.org/10.1038/jid.2011.240
125. Singh M, Mansuri M, Jadeja S, et al (2018) Association of interleukin 1 receptor antagonist intron 2 variable number of tandem repeats polymorphism with vitiligo susceptibility in Gujarat population. Indian J Dermatol Venereol Leprol. https://doi.org/10.4103/ijdvl.IJDVL_1_17
126. Slominski A, Tobin DJ, Shibahara S, Wortsman J (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol. Rev. 2004;84(4):1155-1228. doi:10.1152/physrev.00044.2003
127. Song R, Harding C V (1996) Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. J Immunol 1996;156(11):4182-4190.
128. Song Y-H, Connor E, Li Y, et al (1994) The role of tyrosinase in autoimmune vitiligo. Lancet 344:1049–1052. https://doi.org/10.1016/S0140-6736(94)91709-4
129. Spritz RA (2008) The genetics of generalized vitiligo. In: Dermatologic Immunity. Karger Publishers, pp 244–257 https://doi.org/10.1159/000131501
130. Spritz RA (2010) The genetics of generalized vitiligo: Autoimmune pathways and an inverse relationship with malignant melanoma. Genome Med. 2010;2(10):78. Published 2010 Oct 19. doi:10.1186/gm199
131. Spritz RA, Andersen GHL (2017) Genetics of Vitiligo. Dermatol Clin 35:245–255. https://doi.org/10.1016/j.det.2016.11.013

132. Spritz RA, Ho L, Furumura M, Hearing VJ (1997) Mutational analysis of copper binding by human tyrosinase. J Invest Dermatol. 1997;109(2):207-212. https://doi.org/10.1111/1523-1747.ep12319351
133. Strain JJ, Dowey L, Ward M, et al (2004) B-vitamins, homocysteine metabolism and CVD. Proc Nutr Soc 63:597–603. https://doi.org/10.1079/PNS2004390
134. Strömberg S, Björklund MG, Asplund A, et al (2008) Transcriptional profiling of melanocytes from patients with vitiligo vulgaris. Pigment Cell Melanoma Res. 2008;21(2):162-171. https://doi.org/10.1111/j.1755-148X.2007.00429.x
135. Sulem P, Gudbjartsson DF, Stacey SN, et al (2007) Genetic determinants of hair, eye and skin pigmentation in Europeans. Nat Genet 39:1443–1452. https://doi.org/10.1038/ng.2007.13
136. Sushama S, Dixit N, Gautam RK, et al (2019) Cytokine profile (IL-2, IL-6, IL-17, IL-22, and TNF-α) in vitiligo—New insight into pathogenesis of disease. J Cosmet Dermatol 2019;18(1):337-341.https://doi.org/10.1111/jocd.12517
137. Taudt A, Colomé-Tatché M, Johannes F (2016) Genetic sources of population epigenomic variation. Nat. Rev. Genet. 2016;17(6):319-332. doi:10.1038/nrg.2016.45
138. Thomas PD, Campbell MJ, Kejariwal A, et al (2003) PANTHER: A library of protein families and subfamilies indexed by function. Genome Res. 2003;13(9):2129-2141.https://doi.org/10.1101/gr.772403
139. Toyofuku K, Wada I, Valencia JC, et al (2001) Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins. FASEB J 15:2149–2161. https://doi.org/10.1096/fj.01-0216com
140. Tripathi RK, Hearing VJ, Urabe K, et al (1992) Mutational mapping of the catalytic activities of human tyrosinase. J Biol Chem 1992;267(33):23707-23712.
141. van Geel N, Wolkerstorfer A, Ezzedine K, et al (2019) Validation of a physician global assessment tool for vitiligo extent: Results of an international vitiligo expert meeting. Pigment Cell Melanoma Res 32:728–733. https://doi.org/10.1111/pcmr.12784
142. Varzari A, Deyneko I V., Tudor E, Turcan S (2016) Polymorphisms of glutathione S-transferase and methylenetetrahydrofolate reductase genes in Moldavian patients with ulcerative colitis: Genotype-phenotype correlation. Meta Gene 7:76–82. https://doi.org/10.1016/j.mgene.2015.12.002
143. Wang CQF, Cruz-Inigo AE, Fuentes-Duculan J, et al (2011) Th17 cells and activated

dendritic cells are increased in vitiligo lesions. PLoS One. 2011;6(4):e18907. https://doi.org/10.1371/journal.pone.0018907

144. Ward LD, Kellis M (2012) HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Res 40:D930–D934. doi:10.1093/nar/gkr917
145. Wu S-M, Chen Z-F, Young L, Shiao S (2017) Meta-Prediction of the Effect of Methylenetetrahydrofolate Reductase Polymorphisms and Air Pollution on Alzheimer's Disease Risk. Int J Environ Res Public Health 2017;14(1):63. https://doi.org/10.3390/ijerph14010063
146. Xu D, Zhang Y (2011) Improving the Physical Realism and Structural Accuracy of Protein Models by a Two-Step Atomic-Level Energy Minimization. Biophys J 101:2525–2534. https://doi.org/10.1016/j.bpj.2011.10.024
147. Xu W, Lin FQ, Liu JF, et al (2013) Impact on tyrosinase expression and export from endoplasmic reticulum by inhibition of 26S proteasome. Natl Med J China. 2013;93(2):123-127. https://doi.org/10.3760/cma.j.issn.0376-2491.2013.02.011
148. Yamada K, Strahler JR, Andrews PC, Matthews RG (2005) Regulation of human methylenetetrahydrofolate reductase by phosphorylation. Proc Natl Acad Sci U S A 102:10454–10459. https://doi.org/10.1073/pnas.0504786102
149. Yan N, Yu Y-L, Yang J, et al (2012) Association of interleukin-17A and -17F gene single-nucleotide polymorphisms with autoimmune thyroid diseases. Autoimmunity 45:533–539. https://doi.org/10.3109/08916934.2012.702814
150. Yasar A, Gunduz K, Onur E, Calkan M (2012) Serum homocysteine, vitamin B12, folic acid levels and methylenetetrahydrofolate reductase (MTHFR) gene polymorphism in vitiligo. Dis Markers. 2012;33(2):85-89. https://doi.org/10.3233/DMA-2012-0908
151. Yong Y, Lin HE (2005) SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 2005;15(2):97-98. doi:10.1038/sj.cr.7290272
152. Yoshida H (2007a) ER stress and diseases. FEBS J. 2007;274(3):630-658. doi:10.1111/j.1742-4658.2007.05639.x
153. Yoshida H (2007b) Unconventional splicing of XBP-1 mRNA in the unfolded protein response. Antioxidants Redox Signal. 2007;9(12):2323-2333. doi:10.1089/ars.2007.1800
154. Yoshida H, Oku M, Suzuki M, Mori K (2006) pXBP1(U) encoded in XBP1 pre-

mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. J Cell Biol 172:565–575. https://doi.org/10.1083/jcb.200508145

155. Zhang X, Yu P, Wang Y, et al (2013) Genetic polymorphisms of interleukin 17A and interleukin 17F and their association with inflammatory bowel disease in a Chinese Han population. Inflamm Res. 2013;62(8):743-750. https://doi.org/10.1007/s00011-013-0629-9
156. Zhang XJ, Chen JJ, Liu JB (2005) The genetic concept of vitiligo. J Dermatol Sci 39:137–146. https://doi.org/10.1016/j.jdermsci.2005.06.004
157. Zhang Y (2008) I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9:40. https://doi.org/10.1186/1471-2105-9-40
158. Zhen Y, Yao L, Zhong S, et al (2016) Enhanced Th1 and Th17 responses in peripheral blood in active non-segmental vitiligo. Arch Dermatol Res 308:703–710. https://doi.org/10.1007/s00403-016-1690-3.