CHAPTER III A

GENETIC ASSOCIATION OF PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR TYPE 22 *(PTPN22)* GENE POLYMORPHISM WITH GENERALIZED VITILIGO SUSCEPTIBILITY

3A. INTRODUCTION

Vitiligo is a common dermatological disorder of the epidermis and hair follicles, manifested clinically as expanding hypopigmented lesions of the skin. The clinical hallmark of vitiligo is loss of melanin pigment due to loss of melanocytes in the lesional skin. It affects 0.5-1% of the world population (Taieb *et al.,* 2007). The incidence of vitiligo is found to be 0.5-2.5% in India (Handa and Kaur, 1999) wherein Gujarat and Rajasthan states have the highest prevalence ~8.8% (Valia and Dutta, 1996). The Gujarat population may be susceptible to this disease due to multiple reasons. The three prevailing hypotheses for the pathogenesis of vitiligo are the neurochemical, autoimmune and oxidative stress hypotheses. However, none of them explains the entire spectrum of this disorder.

Although the etiology of vitiligo remains obscure, autoimmunity has been suggested to play a major role in the pathogenesis of generalized vitiligo (Nordlund *et al.,* 2006; Kemp *et al.,* 2001). Support for this theory arises from the frequent association of vitiligo with other autoimmune disorders (Alkhateeb *et al.,* 2003; Laberge *et al.,* 2005) and the demonstration of autoantibodies to melanocyte proteins in the serum of patients with the disease (Kemp *et al.,* 1997; Shajil *et al.,* 2006b). Moreover, autoreactive cytotoxic T lymphocytes (CTLs), which specifically recognize melanocyte differentiation antigens, have been detected in both the peripheral blood and perilesional skin of individuals with vitiligo (Palermo *et al.,* 2001).

The *PTPN22* gene, located on chromosome 1p13, encodes lymphoid protein tyrosine phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill *et al.,* 2002). Lymphoid protein tyrosine phosphatase is expressed in T lymphocytes and associates with C-terminal Src kinase (CSK) to form a complex that suppresses the T-cell receptor signaling kinases LCK and FYN (Palacios *et al.,* 2004). The *PTPN22* 1858T variant has been described to result in a gain-of-function form of the enzyme leading to stronger suppression of the early T cell activation process. T cells from individuals carrying the predisposing T allele produced less interleukin-2 and encoded a phosphatase that had higher catalytic activity and thus represents a gain of function polymorphism (Vang *et al.,* 2005).

The disease-associated LYP variant SNP lies within the first proline-rich domain of LYP and results in the substitution of tryptophan for arginine. Trp620 prevents the interaction of LYP with CSK (Bottini *et al.,* 2004). Consequently, the T-cell receptor-associated kinases might be able to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and may result in failure to delete autoreactive T cells during thymic selection or decreased activity of regulatory T cells, which in turn may predispose an individual to autoimmune disease.

Previously, an association between the minor allele (T) of a missense single-nucleotide polymorphism (SNP) 1858C/T in the protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*) and susceptibility to generalized vitiligo was described (Canton *et al.,* 2005). Besides its association with vitiligo this missense 1858C/T polymorphism is also reported to be associated with autoimmune diseases including type I diabetes mellitus, Graves' disease, systemic lupus erythematosus and rheumatoid arthritis (Bottini *et al.,* 2004; Orozco *et al.,* 2005) suggesting a genetic predisposition towards generalized T cell autoimmunity. Since generalized (nonsegmental) vitiligo is considered to have an autoimmune etiology, the present study investigated *PTPN22* 1858C/T (R620W; rs2476601) polymorphism in generalized vitiligo patients and ethnically age and sex matched controls.

3A.1 MATERIALS AND METHODS

3A.1.1 Study subjects:

The study group included 126 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 54 males and 72 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 140 ethnically age and sex-matched unaffected individuals (61 males and 79 females) were included as controls in the study. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and controls.

	Vitiligo Patients	**Controls**
	(n = 126)	(n = 140)
Average age (mean age ± SD)	30.34 ± 11.13 yrs	28.67 ± 12.37 yrs
Sex: Male	54 (42.86%)	61 (43.57%)
Female	72 (57.14%)	79 (56.43%)
Age of onset (mean age ± SD)	20.78 ± 12.71 yrs	NA
Duration of disease (mean ± SD)	7.32 ± 6.14 yrs	NA
Family history	17 (13.49%)	NA

3A.1.2 Genomic DNA preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analysis.

3A.1.3 Genotyping of *PTPN22* 1858C/T (R620W; rs2476601) polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype 1858C/T polymorphism of *PTPN*22 gene (Figure 1). The primers used for genotyping were: 5'-GCCTCAATGAACTCCTCAA-3' (forward) and 5'-CCTCCTGGGTTTGTACCTTA-3' (reverse). The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on 2.0% agarose gel stained with ethidium bromide.

Restriction enzyme: *Xcm*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of 1858 C/T polymorphism of *PTPN*22 gene. 5 µL of the amplified products were digested with 5 U of the corresponding restriction enzyme in a total reaction volume of 25 µL, according to the manufacturer's instructions. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.0% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

3A.1.4 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *PTPN22* 1858C/T SNP in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of 1858C/T polymorphism for patients and control subjects

were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). p-values less than 0.05 were considered as statistically significant.

3A.2 RESULTS

3A.2.1 Analysis of association between *PTPN22* 1858 C/T polymorphism and susceptibility to vitiligo:

PCR-RFLP for *PTPN22* 1858 C/T polymorphism yielded a 400 bp undigested product corresponding to C allele and 238 bp and 162 bp digested products corresponding to T allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for 1858 C/T polymorphism of *PTPN22* gene (Figure 1).

The genotype and allele frequencies of the 1858 C/T polymorphism in 126 vitiligo patients and 140 controls are summarized in Table 2. The *PTPN22* 1858 C/T polymorphism was not found to be in significant association with vitiligo patients (p=0.198) when genotypes were compared using chi-squared test-3x2 contingency table (Table 2). Also there was no significant difference in the allele frequencies for this polymorphism between patients and controls (p=0.560) (Table 2). The observed allele frequencies of C and T were 0.98 and 0.02 respectively in controls; 0.99 and 0.01 respectively in vitiligo patients (Table 2). The distribution of *PTPN22* 1858 C/T genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as patient groups (p=0.932 and p=0.994 respectively) (Table 2).

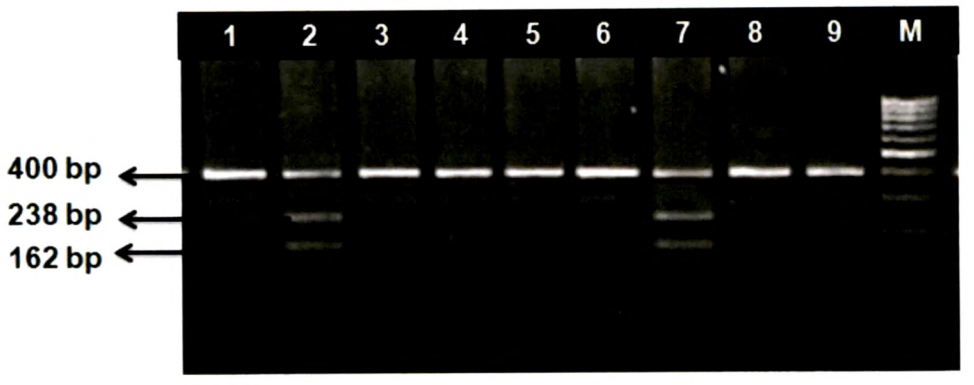

Figure 1. PCR-RFLP analysis of *PTPN22* 1858C/T SNP: 2.0 % agarose gel electrophoresis after overnight digestion of the PCR product with *Xcm*I. Lanes:1, 3, 4, 5, 6, 8 & 9 show subjects with homozygous CC genotypes; lanes: 2 & 7 show subjects with heterozygous CT genotypes. Lane: M shows a 100-bp ladder. No subject with homozygous TT genotypes was observed.

Table 2. Distribution of genotypes and alleles for the 1858C/T SNP of *PTPN22* in vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Associ-ation	*p* for HWE
	Genotype	(n = 126)	(n = 140)		
	CC	124 (0.98)	134 (0.96)		
PTPN22	CT	02 (0.02)	06 (0.04)	0.198	0.994[a]
1858 C/T	TT	00 (0.00)	00 (0.00)		(P)
(rs2476601)	Allele				0.932[b]
	C	250 (0.99)	274 (0.98)	0.560	(C)
	T	02 (0.01)	06 (0.02)		

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

(P) refers to Patients and (C) refers to Controls,

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

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table, Values are significant at $p \leq 0.05$.

3A.3 DISCUSSION

The importance of genetic factors for vitiligo susceptibility is evident by reports of significant familial association (Bhatia *et al.,* 1992; Nordlund, 1997). About 20% of vitiligo patients have at least one first-degree relative affected (Bhatia et al, 1992; Nath *et al.,* 1994; Alkhateeb *et al.,* 2003) indicating that genetic predisposition may be involved in the vitiligo susceptibility. Our previous study also suggests that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.,* 2006b). The present study also includes 13.49% Gujarat vitiligo patients with positive family history (Table 1). The inheritance pattern of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. Vitiligo seems to be a complex hereditary disease governed by a set of recessive alleles situated at several unlinked autosomal loci, which may be involved in the antioxidant defense mechanism, melanin synthesis, autoimmunity etc. that could collectively confer the vitiligo phenotype (Nath *et al.,* 1994). Several candidate genes are reported for vitiligo (Fain *et al.,* 2003; Birlea *et al.,* 2011); however, the role of genetic factors in vitiligo pathogenesis is still not well understood.

PTPN22 gene has been found to play an important role in negative T cell regulation. The C/T 1858 polymorphism of *PTPN22* has been well documented as in association with several autoimmune diseases (Lee *et al.,* 2007). Previously, Canton *et al.,* (2005) showed an association of *PTPN22* C/T 1858 polymorphism with generalized vitiligo in Caucasian population (Canton *et al.,* 2005). In addition, two other studies from European population reported significant association of 1858C/T polymorphism with susceptibility to generalized vitiligo (Jin *et al.,* 2010; LaBerge, 2008). The present study shows that there is no association between *PTPN22* gene (C1858T) polymorphism with susceptibitlity to generalized vitiligo in Gujarat population (Table

2) and our results are in line with those of Alkhateeb *et al.,* (2010). There are several reports suggesting an association of C/T 1858 polymorphism with other autoimmune diseases (Bottini *et al.,* 2004; Orozco *et al.,* 2005; Begovich *et al.,* 2004; Zhernakova *et al.,* 2005). On the contrary, several reports also suggest a non association of *PTPN22* C/T 1858 polymorphism with autoimmune diseases (Hinks *et al.,* 2005; Martin *et al.,* 2005 Matesanz *et al.,* 2005). These contradictory reports may be because of the difference in ethnicity of the studied populations. However, in Indian population Ray *et al.* (2006) also reported a non association of *PTPN22* C/T 1858 polymorphism with rheumatoid arthritis suggesting that susceptible *PTPN22* 'T' allele is not prevalent in Indian population.

PTPN22 gene encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular protein tyrosine phosphatase (PTP) and physically bound through proline-rich motif to the SH3 domain of the Csk kinase, which is an important suppressor of kinases that mediate T-cell activation (Cohen and Dadi, 1999). The ability of Csk and Lyp to inhibit T-cell receptor signaling requires their physical association (Cloutier and Veillette, 1999). The C1858T substitution changes the amino acid from arginine (620R) to tryptophan (620W) at codon 620. This residue resides in the P1 proline-rich motif that is involved in binding to the SH3 domain of Csk (Cohen and Dadi, 1999). *In vitro* experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper responsive, and consequently, individuals carrying this allele may be prone to autoimmunity (Bottini *et al.,* 2004; Begovich *et al.,* 2004).

The exact genetic mechanism of autoimmune diseases is not well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors. The study could not attain statistical significance consequent to lower prevalence of 1858T allele in the studied population (Table 2). Interestingly, 1858T allele has been found to be absent in East Asians including Japanese (Ban *et al.,* 2005) Chinese (Begovich *et al.,* 2004) and South Asian Indians (Ray *et al.,* 2006). This was the first study to evaluate the association of the *PTPN22* 1858 C/T polymorphism with vitiligo in Gujarat population and this study has been published [Laddha NC,

Dwivedi M, Shajil EM, Prajapati H, Marfatia YS and Begum R (2008). Association of PTPN*22* 1858C/T polymorphism with vitiligo susceptibility in Gujarat population. J Dermatol Sci. 49: 260-262]. In conclusion, the present study suugests that C/T 1858 polymorphism of *PTPN22* is not a genetic risk factor for vitiligo susceptibility in Gujarat population.

3A.4 REFERENCES

Alkhateeb A, Fain PR, Thody A, Bennett DC, Spritz RA (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res* 16:208–214.

Alkhateeb A, Qarqaz F, Al-Sabah J, Al Rashaideh T (2010). Clinical characteristics and PTPN22 1858C/T variant analysis in Jordanian Arab vitiligo patients. *Mol Diagn Ther* 14:179-184.

Ban Y, Tozaki T, Taniyama M., et al. (2005). The codon 620 single nucleotide polymorphism of the protein tyrosine phosphatase-22 gene does not contribute to autoimmune thyroid disease susceptibility in the Japanese. *Thyroid* 15, 1115-1118.

Begovich AB, Carlton VE, Honigberg LA, et al. (2004). A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet.* 75(2): 330-7.

Bhatia PS, Mohan L, Pandey ON et al. (1992). Genetic nature of vitiligo. *J Dermatol Sci.* 4(3): 180-184.

Birlea S. A., Jin Y., Bennett D C., Herbstman D, Wallace M R,McCormack W T, Kemp EH , Gawkrodger D J, WeetmanA Y , Picardo M, Leonel G, Taieb A, Jouary T,Ezzedine K ,van Geel N, Lambert J, Overbeck A,Fain P R, Spritz R A (2011). Comprehensive Association Analysis of Candidate Genes for Generalized Vitiligo Supports XBP1,FOXP3, and TSLP. J Invest Dermatol. 1131: 371–381

Bottini N, Musumeci L, Alonso A et al. (2004). A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet* 36: 337–338.

Canton I, Akhtar S, Gavalas NG, et al. (2005). A single-nucleotide polymorphism in the gene encoding lymphoid protein tyrosine phosphatase (PTPN22) confers susceptibility to generalized vitiligo. *Genes Immun* 6:584–587.

Cloutier JF, Veillette A. (1999). Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase *Exp Med.* 189(1): 111-121.

Cohen S, Dadi H, Shaoul E, Sharfe N, Roifman M. (1999). Cloning and characterization of a lymphoid-specific, inducible human protein tyrosine phosphatase, *Lyp.Blood.* 93(6):2013-2024.

Fain PR, Gowan K, LaBerge GS et al. (2003). A genomewide screen for generalized vitiligo: confirmation of AIS1 on chromosome 1p31 and evidence for additional susceptibility loci. *Am J Hum Genet* 72: 1560–1564.

Handa S, Kaur I.(1999). Vitiligo: clinical findings in 1436 patients. *J Dermatol* 10: 653-657.

Hill RJ, Zozulya S, Lu YL, Ward K, Gishizky M, Jallal B. (2002). The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T cell activation. *Exp Hematol* 30: 237–244.

Hinks A, Barton A, John S et al. (2005). Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene. *Arthritis Rheum* 52:1694–1699.

Jin Y, Birlea SA, Fain PR, Gowan K, Riccardi SL, Holland PJ, Mailloux CM, Sufit AJ, Hutton SM, Amadi-Myers A, Bennett DC, Wallace MR, McCormack WT, Kemp EH, Gawkrodger DJ, Weetman AP, Picardo M, Leone G, Taieb A, Jouary T, Ezzedine K, van Geel N, Lambert J, Overbeck A, Spritz RA (2010). Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *N Engl J Med* 362:1686-1697.

Kemp EH, Gawkrodger DJ, MacNeil S, Watson PF, Weetman AP. (1997). Detection of tyrosinase autoantibodies in vitiligo patients using 35S-labelled recombinant human tyrosinase in a radioimmunoassay. *J Invest Dermatol* 109: 69–73.

Kemp EH, Waterman EA, Weetman AP. (2001). Autoimmune aspects of vitiligo. *Autoimmunity* 34: 65–77.

Laberge G, Mailloux CM, Gowan K, Holland P, Bennett DC, Fain PR, Spritz RA (2005). Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. *Pigment Cell Res* 18:300–305.

Laberge GS, Birlea SA, Fain PR, Spritz RA (2008). The PTPN22-1858C>T (R620W) functional polymorphism is associated with generalized vitiligo in the Romanian population. *Pigment Cell Melanoma Res* 21:206-208.

Lee YH, Rho YH, Choi SJ, et al. (2007). The PTPN22 C1858T functional polymorphism and autoimmune diseases-a meta-analysis. *Rheumatology* 46: 49-56.

Martin MC, Oliver J, Urcelay E et al. (2005). The functional genetic variation in the PTPN22 gene has a negligible effect on the susceptibility to develop inflammatory bowel disease. *Tissue Antigens* 66:314–7.

Matesanz F, Rueda B, Orozco G et al. (2005). Protein tyrosine phosphatase gene (PTPN22) polymorphism in multiple sclerosis. *J Neurol* 252:994–995.

Moscher DB, Fitzpatrick TB, Ortonne JP, TB, Hori Y, Hypomelnosis and hypermelanosis (1999)In: Eisen AZ, Wolff K, Austen KF, Goldsmith LA, Kats SI, Fitzpatrick TB. *Dermatology in General Medicine*. New York: Mc Graw Hill.; 945-1017.

Nath SK, Majumder PP, Nordlund JJ.(1994). Genetic epidemiology of vitiligo: multilocus recessivity cross-validated.*Am J Hum Genet*.55(5):981-990.

Nordlund J J. (1997). The epidemiology and genetics of vitiligo. *Clin Dermatol*. 15, 875-878.

Nordlund JJ, Ortonne J-P, Le Poole IC (2006). Vitiligo vulgaris. In: Nordlund JJ, Boissy RE, Hearing VJ, King RA, Oetting WS, Ortonne J-P (eds) *The Pigmentary System*. 2nd edn Blackwell Publishing: Malden, MA, 551–98.

Orozco G, Sanchez E, Gonzalez-Gay MA, et al. (2005). Association of a functional single-nucleotide polymorphism of PTPN22, encoding lymphoid protein phosphatase, with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Rheum* 52: 219–224.

Palacios EH, Weiss A. (2004). Function of the Src-family kinases, Lck and Fyn in T-cell development and activation. *Oncogene* 23: 7990–8000.

Palermo B, Campanelli R, Garbelli S, et al. (2001). Specific cytotoxic T lymphocyte responses against MelanA/MART1, tyrosinase A PTPN22 gene polymorphism in vitiligo I Canton et al and Gp100 in vitiligo by the use of major histocompatibility complex/peptide tetramers: the role of cellular immunity in the etiopathogenesis of vitiligo. *J Invest Dermatol* 117: 326–332.

Ray D, Tomar N, Gupta N, Goswami R. (2006). Protein tyrosine phosphatase non-receptor type 22 (PTPN22) gene R620W variant and sporadic idiopathic hypoparathyroidism in Asian Indians. *Int J Immunogenet.* 33(4):237-40.

Shajil EM, Agrawal D, Vagadia K, Marfatia YS, Begum R. (2006). Vitiligo: Clinical profiles in Vadodara, Gujarat. *Ind J Dermatol* 51: 100-104.

Shajil EM, Chatterjee S, Agrawal D, Bagchi T, Begum R. (2006). Vitiligo: pathomechanisms and genetic polymorphism of susceptible genes. *Ind J Exp Biol* 44: 526-539.

Taieb A, Picardo M and VETF Members (2007). The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res* 20: 27-35.

Taieb A. (2000). Intrinsic and extrinsic pathomechanisms in vitiligo. *Pigment Cell Res* 8: 41-47.

Valia AK, Dutta PK. (1996). IADVL *Text book and Atlas of Dermatology*. Bombay: Bhalani Publishing House; 500 - 586.

Vang T, Congia M, Macis MD, Musumeci L, Orru V, Zavattari P, Nika K, Tautz L, Tasken K, Cucca F, et al. (2005). Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet* 37:1317-1319.

Zhernakova A, Eerligh P, Wijmenga C, Barrera P, Roep BO, Koeleman BP. (2005). Differential association of the PTPN22 coding variant with autoimmune diseases in a Dutch population. *Genes Immun* 6:459–461.

CHAPTER III B

GENETIC ASSOCIATION OF ANGIOTENSIN CONVERTING ENZYME (*ACE*) GENE POLYMORPHISM WITH GENERALIZED VITILIGO SUSCEPTIBILITY

3B.1 INTRODUCTION

Generalized vitiligo is a complex autoimmune disease in which patchy depigmentation of skin and hair results from loss of melanocytes from involved areas (Picardo and Taïeb, 2010) and is epidemiologically associated with several other autoimmune diseases (Alkhateeb *et al.*, 2003). Vitiligo, the most common pigmentary disorder, involves complex interaction of environmental and genetic factors that ultimately contribute to melanocyte destruction, resulting in the characteristic depigmented lesions. In the past few years, studies of the genetic epidemiology of vitiligo have led to the recognition that generalized vitiligo is part of a broader autoimmune disease diathesis.

Angiotensin converting enzyme (ACE), a key enzyme in the renin-angiotensin system, catalyzes the conversion of angiotensin I to angiotensin II in kidney through removal of two C-terminal residues. The ACE plays an important role in the physiology of the vasculature, blood pressure and inflammation, and it has also been found to be associated with several autoimmune disorders (Vuk-Pavlovic *et al.*, 1988, Scholzen *et al.*, 2003). The gene encoding *ACE* (or dipeptidyl carboxy peptidase 1: DCP1) is of 24 kb consisting of 26 exons, and is located on chromosome 17q23. An insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) has been associated with the development of vitiligo (Jin *et al.*, 2004; Deeba *et al.*, 2009; Tippisetty *et al.*, 2011). The insertion/deletion (I/D) polymorphism of the *ACE* gene accounts for a large proportion of the variability of serum ACE activity, D/D genotypes having the highest and I/I genotypes having the lowest ACE activity (Rigat *et al.*, 1990). In this study, the distribution of *ACE* I/D genotypes was investigated in generalized vitiligo patients and unaffected controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo.

3B.2 MATERIALS AND METHODS

3B.2.1 Study subjects:

The study group included 125 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 53 males and 72 females who referred

to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 156 ethnically, age and sex-matched unaffected individuals (68 males and 88 females) were included as controls in the study. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

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

	Vitiligo Patients	**Controls**
	(n = 125)	(n = 156)
Average age (mean age ± SD)	30.34 ± 11.13 yrs	29.78 ± 11.25 yrs
Sex: Male	53 (42.40%)	68 (43.59%)
Female	72 (57.60%)	88 (56.41%)
Age of onset (mean age ± SD)	20.89 ± 12.61 yrs	NA
Duration of disease (mean ± SD)	7.42 ± 6.11 yrs	NA
Family history	17 (13.49%)	NA

3B.2.2 Genomic DNA preparation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

3B.2.3 Genotyping of *ACE* I/D (AF118569) polymorphism:

Polymerase chain reaction (PCR) method was used to genotype I/D polymorphism of *ACE* gene (Figure 1). The primers used for genotyping were:
5'CTGGAGACCACTCCCATCCTTTCT-3'(forward) and
5'GATGTGGCCATCACATTCGTCAGAT-3' (reverse).
The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds.
The amplified products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.0% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

3B.2.4 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *ACE* gene I/D polymorphism in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of *ACE* I/D polymorphism for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. *p*-values less than 0.05 were considered as statistically significant.

3B.3 RESULTS

3B.3.1 Analysis of association between *ACE* I/D (AF118569) polymorphism and susceptibility to vitiligo:

PCR for *ACE* I/D polymorphism yielded a 480 bp product corresponding to I allele and 193 bp products corresponding to D allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: II homozygous, ID heterozygous and DD homozygous for I/D polymorphism of *ACE* gene (Figure 1).

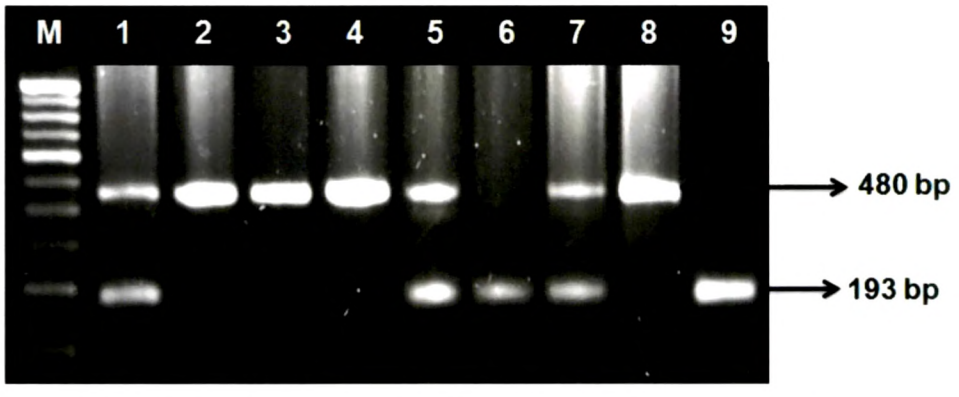

Figure 1. PCR analysis of *ACE* I/D polymorphism on 2.0% agarose gel electrophoresis: Lanes: 1, 5 & 7 show subjects with heterozygous ID genotypes; lanes: 2, 3, 4 & 8 show subjects with homozygous II genotypes; lanes: 6 &9 show subjects with homozygous DD genotypes; Lane: M shows a 100-bp ladder.

The genotype and allele frequencies of the *ACE* I/D polymorphism in 125 vitiligo patients and 156 controls are summarized in Table 2. No significant difference in the frequencies of I/I, I/D and D/D genotypes was detected between vitiligo patients and control subjects (p=0.459) using a 3×2 contingency table in a standard chi-squared test (Table 2), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. In addition, the results indicate that the D allele is not significantly over-represented in the group of patients with vitiligo compared with controls (p=0.252) by using 2×2 contingency table in a standard chi-squared test (Table 2).

The observed allele frequencies of I and D were 0.56 and 0.44 respectively in controls; 0.61 and 0.39 respectively in vitiligo patients (Table 2). The distribution of

ACE I/D genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as patient groups (p=0.905 and p=0.964 respectively) (Table 2).

Table 2. Distribution of genotypes and alleles for the *ACE* I/D polymorphism in vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 125)	(n = 156)			
	II	46 (0.37)	51 (0.33)		0.964[a]	
ACE	ID	61 (0.49)	74 (0.47)	0.459	(P)	
I/D	DD	18 (0.14)	31 (0.20)		0.905[b]	
(AF118569)	Allele				(C)	1.219
	I	153 (0.61)	176 (0.56)	0.252		(0.8685-
	D	97 (0.39)	136 (0.44)			1.710)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

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

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

Values are significant at $p \leq 0.05$.

3B.4 DISCUSSION

Autoimmunity plays a major role in the pathogenesis of generalized vitiligo (Nordlund *et al.*, 2006; Kemp *et al.*, 2001). The frequent association of vitiligo with autoimmune disorders, (Ochi and DeGroot, 1969), the demonstration of autoantibodies to melanosomal proteins in the serum of patients with the disease, (Baharav *et al.*, 1996; Cui *et al.*, 1995; Kemp *et al.*, 1997; Song *et al.*, 1994) and evidence that vitiligo autoantibodies can destroy melanocytes *in vitro*, (Norris, 1998) and *in vivo* (Gilhar *et al.*, 1995) support this theory.

ACE and its related substrates or products are known to have various functions in the immune system (Vuk-Pavlovic *et al.*, 1988) and in the inflammatory response.

However, its underlying pathogenic mechanism in autoimmune diseases is yet to be understood.

ACE is capable of degrading the substance P (SP) and other peptide mediators (Scholzen *et al.*, 2003). In the skin, neuropeptides such as SP are released from sensory nerves in response to noxious stimuli like chemical and mechanical injury. SP may induce or augment inflammatory responses such as plasma extravasation, leukocyte activation, cytokine production, and mast cell activation (Scholzen *et al.*, 1998).

ACE catalyzes the conversion of angiotensin I to angiotensin II which is a potent mediator of oxidative stress and stimulates the release of cytokines and the expression of leukocyte adhesion molecules that mediate vessel wall inflammation. Inflammatory cells release enzymes (including ACE) that generate angiotensin II which in turn can induce cytokines such as TNF-α and IFN-γ (Dzau, 2001). Our recent studies found increased transcript and protein levels of TNF-α and IFN-γ in vitiligo patients (unpublished data). These increased cytokines may modulate the microenvironment which in turn can signal for apoptosis of melanocytes as observed in vitiligo patients. Previous investigations have shown that elevated levels of ACE have been associated with autoimmune diseases (Czernobilsky *et al.*, 1985; Papadopoulos *et al.*, 2000) and that the D allele of *ACE* I/D polymorphism confers susceptibility to autoimmune disorders (Papadopoulos *et al.*, 2000) and vitiligo (Jin *et al.*, 2004).

Most of the studies reported significant association of *ACE* I/D polymorphism with vitiligo, although some yielded only marginal significance and some were not replicated by subsequent studies. Earlier, the D allele of I/D polymorphism of the *ACE* gene was reported to confer susceptibility to vitiligo in Korean population (Jin *et al.*, 2004). A recent study from South India revealed a significant association of DD genotype with vitiligo susceptibility in patients with a family history of vitiligo (Tippisetty *et al.*, 2011). Deeba *et al.* (2009) from South India also reported significant association of *ACE* I/D polymorphism with vitiligo (Deeba *et al.*, 2009). However, the current study suggests that the polymorphic variant D allele is not associated with vitiligo susceptibility (Table 2). This may be because of the differences in ethnicity of the studied populations. Our results are comparable to the

previous report in which genotype frequencies for *ACE* I/D polymorphism were not significantly different between controls and generalized vitiligo patient population (Akhtar *et al.*, 2005). Our results on the distribution rate of I/D > I/I > D/D in the control population are in accordance with the previously reported distribution rate of I/D for Chinese (Lee *et al.*, 1994), Japanese (Ishigami *et al.*, 1995) and Korean population (Jin *et al.*, 2004). Moreover, a study from Turkey did not find significant association of *ACE* I/D polymorphism with vitiligo susceptibility (Pehlivan *et al.*, 2009).

The molecular basis of autoimmune diseases is not yet well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors (Zhang *et al.*, 2004). The study could not attain statistical significance consequent to lower prevalence of D allele in the studied population. This was the first study to evaluate the association of the *ACE* I/D polymorphism with vitiligo in Gujarat population where prevalence of vitiligo is alarmingly high and we have published these results [Dwivedi M, Laddha NC, Shajil EM, Shah BJ and Begum R (2008). The ACE gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population. Pigment Cell Melanoma Res. 21: 407-408].

In conclusion, the *ACE* gene I/D polymorphism does not appear to play a role in the development of generalized vitiligo and hence it may not be a genetic risk factor for vitiligo susceptibility in Gujarat population.

3B.5 REFERENCES

Akhtar S, Gavalas NG, Gawkrodger DJ, Watson PF, Weetman AP, Kemp EH (2005). An insertion/deletion polymorphism in the gene encoding angiotensin converting enzyme is not associated with generalized vitiligo in an English population. *Arch Dermatol Res.* 297(2):94-98.

Alkhateeb A, Fain PR, Thody A, Bennett DC, Spritz RA (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res.* 16:208–214.

Baharav E, Merimski O, Shoenfeld Y, Zigelman R, Gilbrund B, Yecheskel G (1996). Tyrosinase as an autoantigen in patients with vitiligo. *Clin. Exp. Immunol.* 105:84-88.

Cui J, Arita Y, Bystryn JC (1995). Characterisation of vitiligo antigens. *Pigment Cell Res.* 8:53-59.

Czernobilsky H, Fiehn W, Ziegler R (1985). Comparison of serum angiotensin-converting enzyme in Graves' disease, toxic nodular goiter, and other thyroid conditions. *Klin Wochenschr.* 63:518–522.

Deeba F, Jamil K, Rabbani S, Waheed MA and Rao H (2009). Association of angiotensin converting enzyme gene I/D polymorphism with vitiligo in South Indian population. *Int J Med Med Sci.* 1:009-012.

Dzau VJ. Theodore (2001). Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension.* 37(4):1047-52.

Gilhar A, Zelickson B, Ulman Y, Etzioni A (1995). In vivo destruction of melanocytes by the IgG fraction of serum from patients with vitiligo. *J Invest Dermatol.* 105:683-686.

Ishigami T, Iwamoto T, Tamura K, Yamaguchi S, Iwasawa K, Uchino, K, Umemura S, Ishii M (1995). Angiotensin I converting enzyme (*ACE*) gene polymorphism and essential hypertension in Japan. Ethnic difference of *ACE* genotype. *Am J Hypertens.* 8:95-97.

Jin SY, Park HH, Li GZ, Lee HJ, Hong MS, Hong SJ, Park HK, Chung JH, Lee, MH (2004). Association of angiotensin converting enzyme gene I/D polymorphism of vitiligo in Korean population. *Pigment Cell Res.* 17:84-86.

Kemp EH, Gawkrodger DJ, MacNeil S, Watson PF, Weetman AP (1997). Detection of tyrosinase autoantibodies in vitiligo patients using 35 S-labelled recombinant human tyrosinase in a radioimmunoassay. *J Invest Dermatol.* 109: 69-73.

Lee, E.J. (1994). Population genetics of the angiotensin-converting enzyme gene in Chinese. *Br J Clin Pharmacol.* 37:212-214.

Nordlund JJ, Ortonne J-P, Le Poole IC (2006). Vitiligo vulgaris. In: Nordlund JJ, Boissy RE, Hearing VJ, King RA, Oetting WS, Ortonne J-P (eds) *The Pigmentary System.* 2nd edn Blackwell Publishing: Malden, MA, 551–98.

Norris DA, Kissinger RM, Naughton GK, Bystryn JC (1998). Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocytes in vitro by complement-mediated damage and antibody-dependent cel ular cytotoxicity. *J. Invest Dermatol.* 90:783-789.

Ochi Y, DeGroot LJ (1969). Vitiligo in Graves' disease. *Ann. Intern. Med.* 71:935-940.

Papadopoulos K, Melander O, Orho-Melander M, Groop LC, Carlsson M, Hallengren B (2000). Angiotensin converting enzyme (*ACE*) gene polymorphism in sarcoidosis in relation to associated autoimmune disease. *J Int Med.* 247:71–77.

Pehlivan S,Ozkinay F, Alper S, Onay H, Yuksel E, Pehlivan M, Ozkinay C (2009). Association between IL4 (–590), *ACE* (I)/(D), CCR5 (Δ32), CTLA4 (+49) and IL1-RN (VNTR in intron 2) gene polymorphisms and vitiligo. *Eur J Dermatol.* 19(2):126-8.

Picardo M. & Taïeb A. (eds.) (2010).Vitiligo (Springer, Heidelberg & New York).

Rigat B, Hubert C, Alhenc-Getes F, Cambien F, Corvol P, Soubrier F (1990). An insertion/deletion polymorphism in the angiotensin I converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 86:1343-1346.

Scholzen T, Armstrong CA, Bunnett NW, Luger TA, Olerud JE, Ansel JC (1998). Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. *Exp Dermatol.* 7(2-3):81-96.

Scholzen TE, Stander S, Riemann H, Brzoska T, Luger TA (2003). Modulation of cutaneous inflammation by angiotensin converting enzyme. *J Immunol.* 170:3866-3873.

Song YH, Connor E, Li Y, Zorovich B, Balducci P, Maclaren N (1994). The role of tyrosinase in autoimmune vitiligo. *Lancet.* 344:1049-1052.

Tippisetty S, Ishaq M, Komaravalli PL, Jahan P (2011). Angiotensin converting enzyme (*ACE*) gene polymorphism in vitiligo: protective and predisposing effects of genotypes in disease susceptibility and progression. *Eur J Dermatol.* 21(2):173-7.

Vuk-Pavlovic Z, Rohrbach MS (1988). An in vitro model for the induction of angiotensin converting enzyme in sarcoidosis: possible parallels to the immune response. *Clin Exp Immunol.* 72:499-504.

Zhang XJ, Liu JB, Gui JP, Li M, Xiong QG, Wu HB, Li JX, Yang S, Wang HY, Gao M *et al.* (2004). Characteristics of genetic epidemiology and genetic models for vitiligo. *J Am Acad Dermatol.* 51:383-390.

CHAPTER III C

MANNAN BINDING LECTIN-2 (*MBL2*) GENE STRUCTURAL AND PROMOTER POLYMORPHISMS IN GENERALIZED VITILIGO

3C.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). It affects 0.5–1% of the world population (Taieb and Picardo, 2007). In India the incidence of vitiligo is found to be 0.5-2.5% (Handa and Kaur, 1999) where as Gujarat and Rajasthan states have the highest prevalence of ~8.8% (Valia and Dutta, 1996). The etiology of vitiligo is still unknown, but it is hypothesized to be of autoimmune origin due to its frequent association with several autoimmune diseases, the presence of circulating autoantibodies and autoreactive T-cells in the sera of vitiligo patients compared to unaffected individuals (Shajil *et al.*, 2006; Kemp *et al.*, 2001).

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people (Njoo and Westerhof, 2001). The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997). It has been found that about 20% of vitiligo patients have at least one first-degree relative affected (Nath *et al.*, 1994). Previous studies showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.*, 2006). The disease does not follow the simple Mendelian inheritance pattern and its mode of heredity suggests that it is a polygenic disease.

Furthermore, several genes are involved in regulating immunity have been associated with susceptibility to vitiligo including allelic variants in the cytotoxic T -lymphocyte antigen-4 gene (*CTLA4*) (Blomhoff *et al.*, 2005; Kemp *et al.*, 1999; McCormack *et al.*, 2001), the autoimmune susceptibility loci *AIS1, AIS2, AIS3* and *SLEV1* (Alkhateeb *et al.*, 2001; Fain *et al.*, 2003; Spritz *et al.*, 2004), the autoimmune regulator (*AIRE*) gene (Nagamine *et al.*, 1997), NACHT leucine-rich-repeat protein 1 (*NALP1*- maps at *SLEV1* sucsceptibility locus) (Jin *et al.*, 2007a,b), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) (Laberge *et al.*, 2008a,b ; Vang *et al.*,

2007) and certain human leukocyte antigen specificities of the major histocompatibility complex (Le Poole *et al.*, 2008, Fain *et al.*, 2006).

Mannan-binding lectin (MBL) [synonyms: mannose binding lectin (MBL), mannose binding protein (MBP), mannan binding protein (MBP)] is a liver-derived calcium dependent serum protein, which plays an important role in innate immune defense. MBL is a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q of the complement pathway. MBL binds to cell surface ligands such as mannose, N-acetylglucosamine residues, which are expressed on a wide range of microorganisms. MBL binds to carbohydrate moieties on microorganisms and thus leads to activation of the complement system via the lectin pathway (Neth *et al.*, 2000). In addition to complement activation, the protein has several distinct functions including promotion of complement-independent opsono-phagocytosis, modulation of inflammation and promotion of apoptosis (Turner, 2003). Role of the MBL pathway in complement activation and in the clearance of apoptotic cells suggests that genetic variability in *MBL* may be involved in the pathogenesis of autoimmune diseases (Turner and Hamvas, 2000). Possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells which may substantially contribute to the triggering of autoimmune responses (Werth *et al.*, 2002). Also, MBL is involved in inflammatory response, regulation of pro-inflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal. Thus these studies emphasize the importance of MBL in cutaneous autoimmunity (Jack *et al.*, 2001).

The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of *MBL2* gene have been reported: codon 54 (G**G**C→**GA**C; designated B allele), codon 57 (G**G**A→**GA**A; designated C allele), and codon 52 (**C**GT→**T**GT; designated D allele) (Sumiya *et al.*, 1991; Lipscombe *et al.*, 1992; Hansen and Holmskov, 1998). These variant alleles in the collagen like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (5 ng/ml to 5 μg/ml) (Garred *et al.*, 2003; Thiel, 2007). The promoter region of the *MBL2* gene

contains a number of regulatory elements, which affect transcription of the gene and hence polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these, exon 1 variant alleles, SNPs at promoters -550 (G/C; allele L) and -221 (G/C; allele X) have also been associated with low plasma levels of MBL (Madsen *et al.*, 1995). Previously, an association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in Turkish population (Onay *et al.*, 2007).

The aim of this study was to investigate the association of *MBL2* structural (codon 52, codon 54 and codon 57) and promoter (-221) polymorphisms with vitiligo susceptibility in Gujarat population.

3C.2 MATERIALS AND METHODS

3C.2.1 Study Subjects:

The study group included 92 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 29 males and 63 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). Of which 62 patients and 72 age matched controls were genotyped for codon 52 (allele D), 84 patients and 81 controls were genotyped for codon 54 (allele B), and 68 patients and 72 controls were genotyped for codon 57 (allele C). For genotyping of -221 promoter polymorphism, 92 patients and 93 control subjects were used. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 94 ethnically sex-matched unaffected individuals (61 males and 34 females) were included as controls in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

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

	Generalized Vitiligo (GV) Patients	**Controls**
	(n = 92)	(n = 94)
Average age (mean age ± SD)	28.37 ± 1.740 yrs	27.28 ± 0.841 yrs
Sex: Male	29 (31.52%)	61 (64.89%)
Female	63 (68.48%)	34 (36.17%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Family history	12 (13.04)	

3C.2.2 Genomic DNA preparation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

3C.2.3 Genotyping of *MBL2* structural and promoter polymorphisms:

The exon 1 structural and promoter polymorphisms were genotyped by polymerase chain reaction (PCR)-heteroduplex analysis technique. This technique involves the use of a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the target genomic sequence (Jack *et al.*, 1997). Genomic DNA-polymerase chain reaction (PCR) product was combined with UHG-PCR product and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These

heteroduplexes displayed different electrophoretic mobilities on a polyacrylamide gel enabling identification of a subject's genotype (Jack *et al.*, 1997).

3C.2.4 PCR amplification of genomic DNA and UHG:

Two separate polymerase chain reactions (PCRs) were performed on genomic DNA samples to amplify exon 1 and -221 (X/Y) promoter regions by using forward (5'CCAACACGTACCTGGTTCC3') and reverse (5'CTGTGACCTGTGAGGATG-C3') primers for exon 1 and forward (5'AGGCATAAGCCAGCTGGCAAT3') and (5'CTAAGGAGGGGTTCATCTG3') reverse primers for -221 (X/Y) promoter region.

All primers were synthesized from MWG- Biotech, Germany. The PCR was performed in a 20 µl system containing 50 ng genomic DNA, 0.25mM dNTPs, 20 pmoles of each primer, 2mM $MgCl_2$, PCR gold buffer and 1.5U Amplitaq Gold DNA polymerase (Applied Biosystems, USA). PCR amplification was carried out in a thermal cycler (ABI 9700) with the following conditions: initial denaturation at 95^0C for 3 min followed by denaturation at 95^0C for 30 sec, annealing at 56^0C for 30 sec, extension at 72^0C for 30 sec and final extension at 72^0C for 7 min. For the synthesis of each UHG, same PCR conditions were used with each 50 ng UHG template (template size of 133 bp for exon 1 and 111 bp for -221 promoter region) which were commercially synthesized UHG (MWG- Biotech, Germany), specifically designed to detect the exon 1 and promoter regions of *MBL2*. PCR products were analyzed on a 2% agarose gel prior to heteroduplexing and confirmed with 100 bp DNA ladder (MBI Fermentas, Canada). Confirmation of specific amplification of genomic and UHG DNA using 2% agarose gel electrophoresis enabled comparison of the relative amounts of DNA obtained from each sample reaction against those from the UHG reaction.

3C.2.5 Heteroduplex analysis:

A mixture of approximately equal amounts of genomic and UHG-PCR products were combined prior to heating to 95^0C for 5 minutes and then allowed to cool at room temperature over 2 hours to enable greater specific annealing. The PCR mix was spun

briefly and the appropriate volume of 6x loading dye was added to each mix. Heteroduplexes thus formed were checked by 20% polyacrylamide gel electrophoresis followed by ethidium bromide staining (Figures 1 & 2).

On hetroduplexing homozygotes developed two heteroduplex bands and heterozygotes generated four heteroduplex bands (Figurel & 2). Exception was WT/R52C where only three bands were generated due to co-migration of two heteroduplexes as a single upper band (Jack *et al.*, 1997) (WT designating one wild-type allele and R52C, G54D, and G57E, indicating an allele with a mutation at codons: 52, 54 and 57 respectively) (Figure 1). Interpretation of band patterns was done manually with the aid of known samples used as reference grid. The genotype WT/G57E generating four bands spanning the whole gel was taken as reference, and was included in each run. The position of all the other bands could be judged using this sample as reference.

3C.2.6 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the *MBL2* genotypes and allele frequencies for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California, USA. The results were supported by odds ratios (OR) and 95% confidence intervals (CI) when appropriate. *p*-values less than 0.0125 were considered as statistically significant due to Bonferroni's correction for multiple testing.

3C.3 RESULTS

3C.3.1 Analysis of association between *MBL2* exon 1 structural polymorphisms and susceptibility to vitiligo:

Codon 52, codon 54 and codon 57 polymorphisms of *MBL2* exon 1 were not found to be associated with generalized vitiligo patients (p=0.019 for codon 52, p=0.373 for

codon 54, p=0.855 for codon 57) when genotypes were compared with chi-square test-3x2 contingency table (Table 2). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls when compared with 2x2 contingency table (p=0.020 for D allele, p=0.378 for B allele and p=0.858 for C allele) (Table 2). Both patients and control population were found to be in Hardy-Weinberg equilibrium for all the three exon 1 codon polymorphisms (p<1.000 and p<0.932 for codon 52 polymorphism respectively; p=0.869 and p=0.650 for codon 54 polymorphism respectively and p=0.932 and p=0.909 for codon 57 polymorphism respectively) (Table 2). The genotypes for *MBL2* structural polymorphisms were confirmed by running heteroduplexes on 20% polyacrylamide gel (Figure 1).

The association study for exon 1 polymorphisms was also done by A/O genotyping where all the three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. The A/O genotypes were compared between controls and patients using chi-square-3x2 contingency table. Interestingly, there was no significant association found between the A/O genotype and generalized vitiligo (p=0.999) (Table 1). Furthermore, 'O' allele was also not significantly associated with any of the population when compared using 2x2 contingency table (p=0.968) (Table 1).

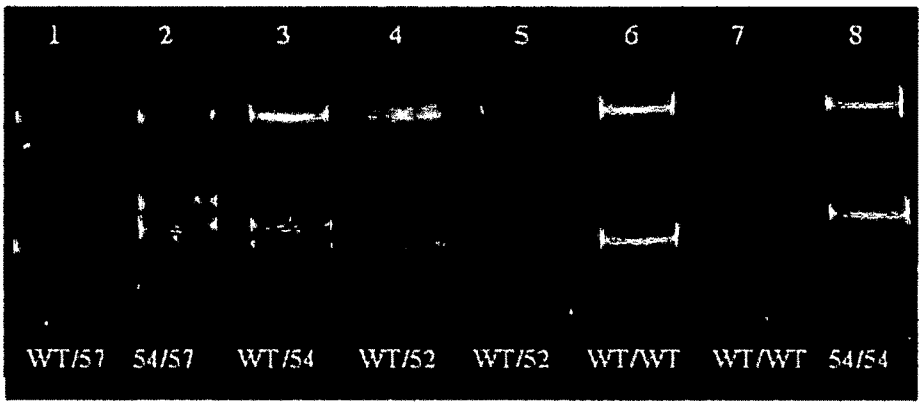

Figure 1. PCR-heteroduplex analysis of *MBL2* exon 1 (codon 52, codon 54 and codon 57) polymorphisms on 20% polyacrylamide gel electrophoresis: lane 1 shows heterozygous WT/57; lane 2 shows heterozygous 54/57; lane 3 shows heterozygous WT/54; lanes 4, 5 show heterozygous WT/52; lanes 6, 7 show homozygous WT/WT and lane 8 shows homozygous 54/54 individual.

Table 2. Association studies for mannan-binding lectin gene (*MBL2*) exon 1 polymorphisms in Gujarat patients with generalized vitiligo.

SNP	Genotype or allele	Vitiligo Patients	Controls	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n =62)	(n =72)			
	TT	00	00		1.0	
rs5030737	CT	00	06	0.0192	(P)	0.08
Codon 52	CC	62	65		0.932	(0.004-
(allele D)	Allele				(C)	1.462)
	T	00	08	0.007		
	C	124	136	(#0.02)		
	Genotype	(n = 84)	(n = 81)			
	AA	01	00			
rs1800450	AG	21	15	0.373	0.869	1.353
Codon 54	GG	62	65		(P)	(0.694-
(allele B)	Allele				0.650	2.639)
	A	23	17	0.378	(C)	
	G	145	145			
	Genotype	(n = 68)	(n = 72)			
	AA	00	00			
rs1800451	AG	06	07	0.855	0.932	0.899
Codon 57	GG	62	65		(P)	(0.286-
(allele C)	Allele				0.909	2.823)
	A	06	07	0.858	(C)	
	G	130	137			
	Genotype	(n =90)	(n = 94)			
	OO	01	01			
Genotype	AO	27	28	0.999	0.579	0.989
A/O†	AA	62	65		(P)	(0.566-
	Allele				0.563	1.726)
	A	151	158	0.968	(C)	
	O	29	30			

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
† A is the designation for wild-type alleles, and O is the common designation for the variant alleles B, C, and D;
(P) refers to Patients and (C) refers to Controls.
Yate's corrected *p* value.

3C.3.2 Analysis of association between *MBL2* -221 promoter polymorphism and susceptibility to vitiligo:

In addition, we have also genotyped the -221 (X/Y) promoter polymorphism of *MBL2* gene in 92 patients and 93 controls. The genotypes for -221 promoter polymorphism were confirmed by running heteroduplexes on 20% polyacrylamide gel (Figure 2). There was no significant association found between -221 promoter polymorphism and the risk of vitiligo when genotype frequencies of patients and controls were compared with chi-square test-3x2 contingency table (p=0.889) (Table 3). Also, the allele frequencies of patients and controls for -221 (X/Y) promoter polymorphism did not show significant difference when compared with 2x2 contingency table (p=0.765) (Table 3). However, both controls and patient population were in accordance with Hardy-Weinberg equilibrium (p=0.120 and p=0.370 respectively) (Table 3).

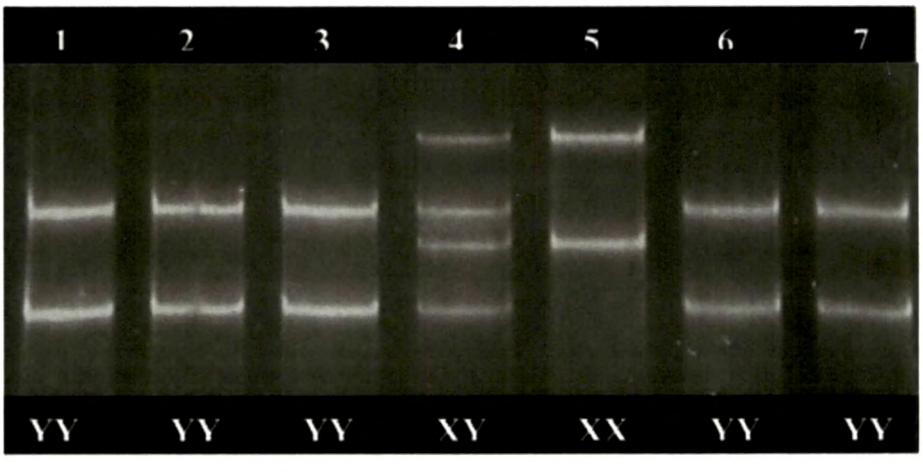

Figure 2. PCR-heteroduplex analysis of *MBL2* -221 (X/Y) promoter polymorphism on 20% polyacrylamide gel electrophoresis: lanes 1, 2, 3, 6, 7 show homozygous YY; lane 4 shows heterozygous XY and lane 5 shows homozygous XX individual.

Table 3. Distribution of alleles and genotypes for the -221 (X/Y; rs7096206) promoter polymorphism of *MBL2* gene in generalized vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 92)	(n = 93)			
	XX	08 (0.09)	10 (0.11)			
MBL2	XY	29 (0.31)	28 (0.30)	0.889	0.370[a]	
-221	YY	55 (0.60)	55 (0.59)		(P)	0.9308
(X/Y;	Allele					(0.5817-
rs7096206)	X	45 (0.24)	48 (0.26)	0.765	0.120[b]	1.489)
	Y	139 (0.76)	138(0.74)		(C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

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

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

Values are significant at $p \leq 0.05$.

3C.3.3 Haplotype analysis:

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be associated with generalized vitiligo when compared with chi-square test (p=0.962) (Table 4).

Moreover, the haplotypes were distributed to represent MBL levels as: high (YA/YA, YA/XA), medium (XA/XA, YA/YO) and low (XA/YO, YO/YO) in both controls and patients. However, no significant difference was found in the distribution of high, medium and low haplotypes when compared with 2x2 contingency table (p=0.838) (Table 4).

Table 4. Distribution of mannan-binding lectin (*MBL2*) gene combined (structural & promoter) genotypes among generalized vitiligo patients and controls.

Combined Genotype (Structural + Promoter)	Vitiligo Patients (n=90)	Controls (n=88)	*p* for Association
YA/YA	33 (36.67%)	34 (38.63%)	0.962
YA/XA	21 (23.34%)	16 (18.18%)	
YA/YO	20 (22.22%)	19 (19.19%)	
XA/XA	08 (8.89%)	09 (10.22%)	
XA/YO	07 (7.78%)	09 (10.10%)	
YO/YO	01 (1.11%)	01 (1.10%)	
MBL High (YA/YA, YA/XA)	54 (60.00%)	50 (56.80%)	0.838
MBL Medium (XA/XA, YA/YO)	28 (32.20%)	28 (31.20)	
MBL Low (XA/YO, YO/YO)	08 (8.89%)	10 (11.36%)	

3C.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease etc. (Ochi *et al.*, 1969). One of the important regulators of innate immunity is *MBL2* which is involved in complement activation and clearance of apoptotic cells suggesting its importance in pathogenesis of autoimmune diseases. We therefore, selected *MBL2* as a candidate gene to study vitiligo susceptibility in Gujarat population and our results suggest that there is no association of *MBL2* structural and promoter polymorphisms with vitiligo susceptibility.

MBL is a pattern-recognition molecule of the innate immune system and primarily recognizes specific sugar groups on the surface of microorganisms, enabling it to distinguish self from non-self. The three structural variants in exon 1 of *MBL2* gene are associated with decreased plasma levels of MBL compared with homozygotes of the wild type gene (Wallis *et al.*, 2002). The structural alterations in the MBL due to three coding polymorphisms disrupt interactions of MBL with the associated serine proteases, there by greatly diminishing complement-activating ability (Wallis *et al.*, 2002; Yokota *et al.*, 1995).

The MBL concentration is also regulated by two additional promoter polymorphisms namely: -550 (G → C; allele L) and -221 (G → C; allele X) which directly affect the expression of the *MBL* gene. Since MBL is one of the proteins shown to be involved in the clearance of apoptotic cells, Werth *et al*, suggested that any defect in the clearance of apoptotic cells may result in aggravation of autoimmune responses (Werth *et al.*, 2002). In addition, MBL is also involved in inflammatory response, regulation of pro-inflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal; thus emphasizing its importance in cutaneous autoimmunity (Jack *et al.*, 2001). Reports from Schallreuter *et al.*, showed a perturbed calcium homeostasis in vitiliginous melanocyte and keratinocyte cell cultures (Schallreuter *et al.*, 1996; Schallreuter *et al.*, 1988) suggesting that vitiligo

patients carrying *MBL* functional and promoter polymorphisms may have defective calcium uptake resulting in low MBL concentration.

MBL2 gene polymorphisms have been associated with increased risk of autoimmune diseases such as systemic lupus erythematosus (SLE) in both Caucasoid and Chinese populations (Davies *et al.*, 1995; Ip *et al.*, 1998) cutaneous lupus erythematosus and dermatomyositis (Werth *et al.*, 2002). Senaldi *et al.* (1995) suggested that patients with SLE had a higher frequency of low levels of MBL than controls. Wang *et al.* (2001) reported that a codon 54 (allele B) of the *MBL2* gene might be associated with genetic susceptibility to Sjögren's syndrome (SJS). Thus most of the studies show that MBL deficiency is associated with poorer outcome as judged by clinical, inflammatory and radiographic indices (Graudal *et al.*, 1998; Graudal *et al.*, 2000). However, evaluation of *MBL2* coding mutations in Japanese population found no association with SLE and rheumatoid arthritis (RA) (Horiuchi *et al.*, 2000). Graudal *et al.* (1998) suggested that there is a greater prevalence of MBL deficiency in patients with RA but other studies refute this assertion (Kilpatrick *et al.*, 1997). Rector *et al.* (2001) found no association between structural polymorphisms in exon 1 of *MBL2* gene and susceptibility to Crohn's disease.

Since vitiligo is hypothesized to be of autoimmune origin, it becomes relevant to screen vitiligo patients for the presence of *MBL2* structural and promoter variants which may predispose an individual for vitiligo. Earlier, an association of codon 54 (allele B) with vitiligo was observed in Turkish population where only two codons 54 and 57 were genotyped with smaller sample size (Onay *et al.*, 2007). Hence, it was pertinent to study all such important polymorphisms of *MBL2* with an adequate sample size and we found there was no association between vitiligo and *MBL2* structural and promoter polymorphisms. The positive association of codon 54 polymorphism and susceptibility to vitiligo described by Onay *et al.* (2007) may be due to differences in the ethnicity of the studied populations.

This is the first report that shows non-association of structural and promoter polymorphisms of *MBL2* gene with generalized vitiligo and our results are in line with those of Birlea *et al.* (2011) suggesting the non-association of generalized vitiligo with codon 54 (rs1800450) or with any of the studied 15 SNPs spanning the *MBL2* region. This study has been published [Dwivedi M, Gupta K, Gulla KC,

Laddha NC, Hajela K and Begum R; Lack of genetic association of promoter and structural variants of Mannan-binding lectin (*MBL2)* gene with susceptibility to generalized vitiligo. *Brit. J Dermatol.* (2009) **161,** 63-69].

In conclusion, it can be considered that the structural and promoter polymorphisms in the *MBL2* gene may not confer a role in generalized vitiligo susceptibility of Gujarat population.

3C.5 REFERENCES

Alkhateeb A, Stetler GL, Old W *et al.* (2001). Mapping of an autoimmunity susceptibility locus (AIS1) to chromosome 1p313-p322. *Hum Mol Genet.* 11:661-667.

Birlea SA, Jin Y, Bennett DC, Herbstman DM, Wallace MR, McCormack WT, Kemp EH, Gawkrodger DJ, Weetman AP, Picardo M, Leone G, Taïeb A, Jouary T, Ezzedine K, van Geel N, Lambert J, Overbeck A, Fain PR, Spritz RA (2011). Comprehensive association analysis of candidate genes for generalized vitiligo supports XBP1, FOXP3, and TSLP. *J Invest Dermatol.* 131(2):371-81.

Blomhoff A, Kemp EH, Gawkrodger DJ *et al.* (2005). CTLA4 polymorphisms are associated with vitiligo in patients with concomitant autoimmune diseases. *Pigment Cell Res.*18:55-58.

Davies EJ, Snowden N, Hillarby MC *et al.* (1995). Mannose-binding protein gene polymorphism in systemic lupus erythematosus. *Arthritis Rheum.* 38:110-114.

Fain PR, Babu SR, Bennett DC, Spritz RA. (2006). HLA class II haplotype DRB1*04-DQB1*0301 contributes to risk of familial generalized vitiligo and early disease onset. *Pigment Cell Res.* 19:51-57.

Fain PR, Gowan K, LaBerge GS *et al.* (2003). A genomewide screen for generalized vitiligo: confirmation of AIS1 on chromosome 1p31 and evidence for additional susceptibility loci. *Am J Hum Genet.* 72:1560-1564.

Garred P, Larsen F, Madsen HO, Koch C (2003). Mannose-binding lectin deficiency: revisited. *Mol Immunol.* 40:73-84.

Graudal NA, Homann C, Madsen HO *et al.* (1998). Mannan binding lectin in rheumatoid arthritis. A longitudinal study. *J Rheumatol.* 25:629-635.

Graudal NA, Madsen HO, Tarp U *et al.* (2000). The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. *Arthritis Rheum.* 43:515-521.

Handa S and Kaur I (1996). Vitiligo: clinical findings in 1436 patients. *J Dermatol.* 10:653-657.

Hansen S and Holmskov U (1998). Structural aspects of collectins and receptors for collectins. *Immunobiology.* 199:165-189.

Horiuchi T, Tsukamoto H, Morita C, Sawabe T *et al.* (2000). Mannose binding lectin (*MBL*) gene mutation is not a risk factor for systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in Japanese. *Genes Immun.*1:464-466.

Ip WK, Chan SY, Lau CS, Lau YL (1998). Association of systemic lupus erythematosus with promoter polymorphisms of the mannosebinding lectin gene. *Arthritis Rheum.* 41:1663-1668.

Jack D, Bidwell J, Turner M and Wood N (1997).Simultaneous genotyping for all three known structural mutations in the human mannose-binding lectin gene. *Hum Mutat.* 9:41-46.

Jack DL, Read RC, Tenner AJ *et al.* (2001). Mannose-binding lectin regulates the inflammatory response of human professional phagocytes to Neisseria meningitidis serogroup B. *J Infect Dis.* 184:1152-1162.

Jin Y, Birlea SA, Fain PR, Spritz RA (2007a). Genetic variations in NALP1 are associated with generalized vitiligo in a Romanian population. *J Invest Dermatol.* 127:2558-2562.

Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, Fain PR, Spritz RA (2007b). NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med.* 356:1216-1225.

Kemp EH, Ajjan RA, Waterman EA *et al.* (1999). Analysis of a cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism in patients with vitiligo. *Br J Dermatol.* 140:73-78.

Kemp EH, Waterman EA, and Weetman AP (2001). Immunological pathomechanisms in vitiligo. *Expert Rev Mol Med.* 23:1.

Kilpatrick DC (1997). Mannan binding protein in sera positive for rheumatoid factor. *Br J Rheumatol.* 36:207-209.

Laberge GS, Bennett DC, Fain PR, Spritz RA (2008a). PTPN22 is genetically associated with risk of generalized vitiligo, but CTLA4 is not. *J Invest Dermatol.* 128:1757-1762.

Laberge GS, Birlea SA, Fain PR, Spritz RA (2008b). The PTPN22-1858C>T (R620W) functional polymorphism is associated with generalized vitiligo in the Romanian population. *Pigment Cell Melanoma Res.* 21:206-208.

Le Poole IC, Luiten RM (2008). Autoimmune etiology of generalized vitiligo. *Curr Dir Autoimmun.* 10:227-243.

Lipscombe RJ, Sumiya M, Hill AV *et al.* (1992). High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. *Hum Mol Genet.* 1:709-715.

Madsen HO, Garred P, Thiel S *et al.* (1995). Interplay between promoter and structural gene variants controls basal serum level of mannan-binding protein. *J Immunol.* 155:3013-3020.

McCormack WT, Bradley CA, Kristensen DL, She JX (2001). Genetics of vitiligo susceptibility: association studies of immune response genes. *Pigment Cell Res.*14:231.

Nagamine K, Peterson P, Scott HS *et al.* (1997). Positional cloning of the APECED gene. *Nat Genet.* 17:393-398.

Nath SK, Majumder PP, and Nordlund JJ (1994). Genetic epidemiology of vitiligo: multilocus recessivity cross-validated. *Am J of Hum Genet.* 55:981-990.

Neth O, Jack DL, Dodds AW *et al.* (2000). Mannose binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect Immun.* 68:688-693.

Njoo MD, and Westerhof W (2001). Vitiligo: Pathogenesis and treatment. *Am J Clin Dermatol* 2:167-181.

Nordlund J J (1997). The epidemiology and genetics of vitiligo. *Clin Dermatol.* 15:875-878.

Ochi Y, DeGroot LJ (1969).Vitiligo in Graves' disease. *Ann Intern Med.* 71:935-940.

Nordlund JJ, Ortonne J-P, Le Poole IC (2006). Vitiligo vulgaris. In: Nordlund JJ, Boissy RE, Hearing VJ, King RA, Oetting WS, Ortonne J-P (eds) The Pigmentary System. 2nd edn Blackwell Publishing: Malden, MA, 551–98.

Onay H, Pehlivan M, Alper S *et al.* (2007). Might there be a link between mannose binding lectin and vitiligo? *Eur J Dermatol.* 17:146-148.

Rector A, Lemey P, Laffut W, *et al.* (2001). Mannanbinding lectin (*MBL*) gene polymorphisms in ulcerative colitis and Crohn's disease. *Genes Immun* 2:323-328.

Schallreuter KU, Pittelkow MR, Swanson NN (1996). Defective calcium transport in vitiliginous melanocytes. *Arch Dermatol Res.* 228:11-13.

Schallreuter KU, Pittelkow MR. (1988). Defective calcium uptake in keratinocyte cell culture from vitiliginous skin. *Arch Dermatol Res.* 280:137-139.

Senaldi G, Davies ET, Peakman M *et al.* (1995). Frequency of mannose-binding protein deficiency in patients with systemic lupus erythematosus. *Arthritis Rheum.* 38:1713-1714.

Shajil EM, Agrawal D, Vagadia K *et al.* (2006). Vitiligo: Clinical profiles in Vadodara, Gujarat. *Ind J Dermatol.* 51:100-104.

Spritz RA, Gowan K, Bennett DC, Fain PR (2004). Novel vitiligo susceptibility loci on chromosomes 7 (ASI2) and 8 (ASI3), confirmation of SLEV1 on chromosome 17, and their roles in an autoimmune diathesis. *Am J Hum Genet.* 74:188-191.

Sumiya M, Super M, Tabona P *et al.* (1991). Molecular basis of opsonic defect in immunodeficient children. *Lancet.* 337:1569-1570.

Taieb A, Picardo M, VETF Members (2007). The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res* 20:27-35.

Thiel S. (2007). Complement activating soluble pattern recognition molecules with collagen like regions, mannan-binding lectin, ficolins and associated proteins. *Mol Immunol.* 44:3875-3888.

Turner MW, Hamvas RM (2000). Mannose-binding lectin: structure, function, genetics and disease associations. *Rev Immunogenet.* 2:305-322.

Turner MW (2003). The role of mannose-binding lectin in health and disease. *Mol Immunol.* 40:423-429.

Valia AK, Dutta PK (1996). IADVL. Text book and Atlas of Dermatology *Bombay: Bhalani Publishing House.* 500-586.

Vang T, Miletic AV, Bottini N, Mustelin T (2007). Protein tyrosine phosphatase PTPN22 in human autoimmunity. *Autoimmunity.* 40:453-461.

Wallis R. (2002). Dominant effects of mutations in the collagenous domain of mannose-binding protein. *J Immunol.* 168:4553-4558.

Wang ZY, Morinobu A, Kanagawa S, Kumagai S (2001). Polymorphisms of the mannose binding lectin gene in patients with Sjögren's syndrome. *Ann Rheum Dis.* 60:483-486.

Werth VP, Berlin JA, Callen JP *et al.* (2002). Mannose binding lectin (*MBL*) polymorphisms associated with low MBL production in patients with dermatomyositis. *J Invest Dermatol.* 119:1394-1399.

Yokota Y, Arai T, Kawasaki T (1995). Oligomeric structures required for complement activation of serum mannan-binding proteins. *J Biochem.* 117:414-419.