## INTRODUCTION

Vitiligo is an acquired, hypomelanotic skin disorder characterized by circumscribed depigmented macules on the skin due to loss of functional melanocytes. The worldwide prevalence of vitiligo is ~0.5–1% (Taieb and Picardo, 2007). In India, Gujarat has the highest prevalence of vitiligo i.e. ~8.8%; however, the underlying reason for the same is yet to be explored. The Gujarat population may be susceptible to vitiligo due to multiple reasons. Vitiligo develops at the age of 20 years in almost 50% of the patients and both males and females are affected equally. Affected persons suffer from social and family stigma and girls in particular, are subjected to ostracization from the marital point of view especially in India (Parsad et al., 2003). Vitiligo has been found to be associated with a number of other autoimmune diseases (Alkhateeb et al., 2003). Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis. In vitiligo patients, skin melanocytes are partially or completely lost, and several theories have been put forward to explain the etiology of the disease such as oxidative stress, autoimmune, neural and genetic hypotheses (Ongenae et al., 2003). Melanocyte death may occur due to intrinsic and/or extrinsic factors. Histological investigations have demonstrated presence of inflammatory infiltrate of mononuclear cells in the upper dermis and at the dermal-epidermal junction of peri-lesional skin of non-segmental vitiligo (NSV) patients (Picardo et al., 2010). The initiation mechanism of this micro-inflammatory reaction is still not clear, nevertheless local triggers are reported to signal the innate immune system of skin that trigger adaptive immune responses targeting melanocytes (van den Boorn et al., 2011). Impairment of humoral and cell-mediated immunity along with cytokine imbalance has been reported in vitiligo patients (Ongenae et al., 2003). Reactive oxygen species (ROS) are produced as byproducts of melanogenesis controlled by various antioxidant enzymes. Oxidative stress is considered to be the initial triggering event in the pathogenesis of melanocyte destruction. Vitiligo is accompanied by oxidative stress characterized by overproduction and accumulation of $H_2O_2$ leading to melanocyte destruction (Yildirim et al., 2004). Our previous studies on Gujarat vitiligo patients have shown altered antioxidant status with decreased catalase, glutathione peroxidase, glucose 6-phosphate dehydrogenase and increased superoxide dismutases as well as increased LPO levels (Agrawal et al., 2004;

Shajil and Begum, 2006), indicating oxidative stress as a major player in the pathogenesis of vitiligo.

Vitiligo susceptibility is a complex genetic trait that may involve genes important for melanin synthesis, response to oxidative stress and regulation to autoimmunity. Stromberg *et al.* (2007) studied transcriptional profiling in vitiligo and showed that melanocytes from vitiligo patients have differentially expressed genes compared with normal melanocytes and that altered expression pattern could play a role in the pathogenesis of vitiligo. MicroRNAs (miRNAs) are small conserved non-coding RNA molecules that post-transcriptionally regulate gene expression by targeting the 3' untranslated region (UTR) of specific messenger RNAs (mRNAs) for degradation or translational repression. Hence, microRNA-mediated gene regulation is critical for normal cellular functions.Emerging evidence has demonstrated that miRNAs play a vital role in the regulation of oxidative stress (Simone *et al.,* 2009), immunological functions and the prevention of autoimmunity. Recent studies in the last two years have made increasingly clear from cell culture and animal studies that proper miRNA regulation is critical for the prevention of autoimmunity and normal immune functions. However, it is not yet well understood whether miRNA dysregulation could play a role in autoimmune disease pathogenesis in humans. A few studies have indicated possible roles for miRNA regulation in autoimmune diseases, specifically rheumatoid arthritis (Nakasa *et al.,* 2008) and systemic lupus erythematosus (Yan et al., 2014). However, the role of miRNAs in pathogenesis of vitiligo has not yet been attempted.

Therefore, the aim of present study was to assess role of miRNAs and oxidative stress in pathogenesis of vitiligo. Based on our previous biochemical studies, the present study explores the role of glutathione peroxidase1 (*GPX1*), catalse (*CAT*) and glucose 6 phosphate dehydrogenase (*G6PD*) in oxidative stress mediated melanocyte death, through candidate gene approach as well as *in vitro* approach. The study aims to addressing various reported single nucleotide polymorphisms of these candidate genes, to establish linkage disequilibrium and genotype-phenotype correlation analyses for the same, in vitiligo patients and controls from Gujarat population. The present study also intends to reveal promising miRNA signatures and their targets, playing crucial role in pathogenesis of vitiligo.

**OBJECTIVES:**

1. To study the role of glutathione peroxidase1 in vitiligo pathogenesis.
2. To study the role of catalase in vitiligo pathogenesis.
3. To study the role of glucose 6 phosphate dehydrogenase in vitiligo pathogenesis.
4. To study the role of miRNAs in vitiligo pathogenesis.
5. To study the *in vitro* effect of oxidative stress on menalocytes.

**Study Subjects:**

The present study focuses on Gujarat population and vitiligo patients included in the study refer to S.S.G. Hospital, Vadodara, Gujarat, India. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. Ethnically age and sex-matched unaffected individuals were also included 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 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.

**OBJECTIVE 1: TO STUDY THE ROLE OF GLUTATHIONE PEROXIDASE1 IN VITILIGO PATHOGENESIS**

Glutathione peroxidase 1 (GPX1; EC 1.11.1.9) is a major intracellular antioxidant enzyme of all cell types. *GPX1* gene is located on human chromosome 3 p21.3 and contains two exons and one intron (Kiss et al., 1997). GPX1 is a selenoenzyme that catalyses the breakdown of $H_2O_2$ and other organic peroxides into $H_2O$ and $O_2$ and the corresponding alcohols using reduced glutathione (GSH) as an essential co-substrate. *GPX1* Leu200Pro (rs1050450) SNP has been extensively reported in several diseases (Ratnasinghe et al., 2000; Wei et al., 2011), wherein it was reported to result in decreased GPX1 activity (Hu and Diamond, 2003). However, previous study from Gujarat on this SNP was found to be uninformative in vitiligo patients (Shajil et al., 2007). Therefore, we

hypothesized that other polymorphism/s might be responsible for the decreased GPX1 activity in vitiligo patients.

***Lipid peroxidation (LPO) levels:*** Oxidative stress has been suggested to be a primary cause of vitiligo and high lipid peroxidation (LPO) levels serve as marker for oxidative stress. Hence, erythrocyte LPO levels were estimated by the standard method in 521 vitiligo patients and 614 controls. Vitiligo patients showed significantly increased LPO levels as compared to controls ($p<0.0001$), suggesting increased oxidative stress in vitiligo patients. Analysis showed approximately 1.54 fold change increase in the LPO levels in patients compared to controls. Further, the LPO levels were analyzed with respect to type of vitiligo and progression of the disease. LPO levels were also increased significantly in generalized vitiligo (GV) patients compared to localized vitiligo (LV) ($p=0.001$). Interestingly, active vitiligo (AV) patients showed significant increased LPO levels compared to stable vitiligo (SV) ($p<0.0001$) suggesting the involvement of oxidative stress in disease progression.

***Glutathione peroxidase (GPX1) activity and transcript levels:*** Erythrocyte GPX1 was assayed according to the standard method in 521 vitiligo patients and 614 controls. Vitiligo patients showed significantly decreased GPX1 activity as compared to controls ($p<0.0001$). Analysis showed approximately 1.93 fold change in the GPX1 activity in patients compared to controls. Further, the GPX1 activity was analyzed with respect to type of vitiligo and progression of the disease. Interestingly, AV patients showed significant decrease in GPX1 activity compared to SV ($p=0.007$) suggesting the involvement of *GPX1* in disease progression. Moreover, GPX1 activity was significantly decreased in GV patients compared to LV ($p<0.0001$), which suggests accumulation of $H_2O_2$ in patients which in turn may be toxic to melanocytes.

*GPX1* transcripts levels were assessed in 102 patients and 105 controls by real time PCR. Comparison of the findings showed no significant difference in expression of *GPX1* transcripts between vitiligo patients compared to controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($p=0.378$). Expression of *GPX1* transcripts in vitiligo patients against controls showed 0.768 fold change as determined by the $2^{-\Delta\Delta Cp}$ method.

***Genotyping of GPX1 gene polymorphisms:*** *GPX1* polymorphisms were addressed in 521 vitiligo patients and 614 controls from Gujarat population. Genotyping of *GPX1* exon1 T/C SNP was done using PCR-RFLP, whereas ARMS-PCR method was used for exon1 G/C and exon2 C/T polymorphisms.

For *GPX1* exon1 G/C (rs8179169; Arg5Pro) polymorphism, only two genotypes identified in both patients and controls were: GG and GC for the G/C polymorphism. 'GC' genotype was found to be significantly associated with vitiligo ($p$<0.0001, OR=1.726, CI=1.329-2.241). In particular, minor allele 'C' was more frequent in the patients compared to controls ($p$=0.0002, OR=1.577, CI=1.242-2.002). Analysis based on disease progression revealed that the increased frequency of the minor allele 'C' occurred predominantly in patients with AV ($p$<0.0001) compared to controls. However, there was no significant difference between SV patients and controls ($p$=0.546). Interestingly, the 'GC' genotype was predominant in AV patients compared to SV ($p$=0.013) suggesting the important role of 'C' allele in disease progression. Further, analysis based on type of vitiligo revealed that the increased frequency of the minor allele 'C' occurred predominantly in patients with GV ($p$<0.0001) compared to controls. However, there was no significant difference between LV patients and controls ($p$=0.578). Interestingly, the 'GC' genotype was predominant in GV patients compared to LV ($p$=0.015) suggesting the important role of 'C' allele in GV. Moreover, analysis based on gender of vitiligo patients revealed that the increased frequency of the 'GC' genotype occurred predominantly in male patients ($p$=0.014) compared to female patients.

Three genotypes were identified for *GPX1* exon1 T/C (rs4991448; Leu6Pro) polymorphism. 'TC' genotype was found to be significantly associated with vitiligo ($p$<0.0001, OR=1.766, CI=1.364-2.287). In particular, the minor allele 'C' was more frequent in patients as compared to controls ($p$<0.0001, OR=1.593, CI=1.280-1.984). Analysis based on disease progression revealed that the increased frequency of minor allele 'C' occurred predominantly in AV patients ($p$<0.0001) compared to controls. However, there was no significant difference between SV patients and controls ($p$=0.641). Interestingly, the 'C' allele was predominant in AV patients compared to SV

($p$=0.007) suggesting the important role of minor allele 'C' in disease progression. Further, analysis based on type of vitiligo revealed that the increased frequency of the minor allele 'C' occurred predominantly in patients with GV ($p$=0.001) compared to controls. Interestingly, the 'C' allele was predominant in LV patients compared to controls ($p$<0.0001) suggesting the important role of 'C' allele in LV. However, there was no significant difference between GV and LV patients ($p$=0.153).

Only single genotype was observed i.e. CC for *GPX1* exon2 C/T (rs6446261; Ala194Thr) polymorphism in both patient and control groups. The observed allele frequencies of C and T were 1.00 and 0 respectively in controls and vitiligo patients.

***Linkage disequilibrium (LD) and haplotype analyses:***

The LD analysis revealed that the two polymorphisms (G/C Arg5Pro and T/C Leu6Pro) investigated in the *GPX1* were in moderate LD association (D'=0.675, $r^2$=0.352). A haplotype evaluation of the two polymorphic sites was performed and estimated frequencies of the haplotypes differed significantly between patients and controls (global $p$-value= $3.53e^{-009}$). Interestingly, the frequency of susceptible haplotype 'CC' containing the minor alleles of both the polymorphisms were significantly higher in patients compared to controls ($p$=0.006) and increased the risk of vitiligo by 1.5-fold (OR=1.490; 95% CI= 1.117-1.986). Also, the other two haplotypes: 'CT' and 'GC' were significantly increased in vitiligo patients as compared to controls ($p$=0.0004, $p$=$5.09^{e-005}$). The wild type haplotype 'GT' was more frequently observed in control group as compared to the patient group ($p$=$4.34^{e-010}$).

***Bioinformatics analysis:***

*Sequence based in silico predictions:*

PANTHER tool showed GPX1 Arg5Pro, Leu6Pro and Ala194Thr variations to be deleterious for the GPX1 function, with highest score of -4.78994 and probability of ~86% for Leu6Pro variation. POLYPHEN tool showed none of the substitutions affecting phenotype or having damaging effects on the function of GPX1 protein. However, the score of mutant Leu6Pro was little higher than other two variations indicating that, this variant behaves differently than others. I-MUTANT and MUPRO predictions revealed decreased stability of Leu6Pro and Ala194Thr variants compared to native structure,

which will in turn affect the function of the protein. SNPs AND GO tool revealed Arg5Pro, Leu6Pro variants show disease like a trait. However, this tool could not return any output for Ala194Thr variation.

*Structure based in silico predictions:*

Molecular dynamics (MD) simulation was performed to investigated the structural differences as well as stability of GPX1 wild and Arg5Pro, Leu6Pro variants based on parameters such as energy variations, intra-molecular hydrogen bonds, inter-molecular hydrogen bonds with surrounding water molecules, radius of gyration (ROG), root mean square deviations (RMSD) and root mean square fluctuations (RMSF) of each amino acid residue, solvent accessible surface area and hydrophobic, hydrophilic interactions. Results will be discussed in thesis.

***Genotype-phenotype correlation analyses for GPX1 polymorphisms:***

The activity of GPX1 was analyzed with respect to G/C (Arg5Pro) genotypes. Individuals with Arg/Pro (GC) genotype showed significantly decreased GPX1 activity ($p<0.0001$) compared to individuals with Arg/Arg (GG) genotype. In case of T/C (Leu6Pro) SNP, individuals with Pro/Pro (CC) genotype showed significantly decreased GPX1 activity ($p=0.0004$) compared to individuals with Leu/Leu (TT) genotype. Individuals with Leu/Pro (TC) genotype showed significantly decreased GPX1 activity ($p<0.0001$) compared to individuals with Leu/Leu (TT) genotype. Further, individuals with Pro/Pro (CC) genotype showed significantly decreased GPX1 activity ($p=0.0449$) compared to individuals with Leu/Pro (TC) genotype.

Further, LPO levels were also analyzed with respect to G/C (Arg5Pro) genotypes. Interestingly, individuals with Arg/Pro (GC) genotype showed significantly increased LPO levels ($p=0.006$) compared to individuals with Arg/Arg (GG) genotype. In case of T/C (Leu6Pro) SNP individuals with Pro/Pro (CC) genotype showed significantly increased LPO levels ($p=0.023$) compared to individuals with Leu/Leu (TT) genotype. Individuals with Leu/Pro (TC) genotype showed significantly increased LPO levels ($p=0.026$) compared to individuals with Leu/Leu (TT) genotype. However, individuals with Pro/Pro (CC) genotype did not show significant difference in LPO levels ($p=0.328$) compared to individuals with Leu/Pro (TC) genotype.

## OBJECTIVE 2: TO STUDY THE ROLE OF CATALASE IN VITILIGO PATHOGENESIS

Catalase (EC 1.11.1.6) is present in the peroxisomes. The *cat* gene is located on human chromosome 11p13. The *cat* gene is 34 kb in length and contains 12 introns and 13 exons and encodes for a protein of 526 amino acids.

***Catalase activity and transcript levels*:** Erythrocyte catalase activity was assayed by standard method in 344 patients and 497 controls. Vitiligo patients showed significant decrease in catalase activity as compared to controls ($p$=0.0007) suggesting accumulation of $H_2O_2$ in vitiligo patients leading to oxidative stress. The transcripts levels of *CAT* in PBMCs of vitiigo patients and controls will be estimated using real-time PCR and the results will be shown in the thesis.

***Genotyping of CAT gene polymorphisms:*** Genotyping of *CAT* exon7 C/A (Ile242Ile; rs17886350) and exon10 C/T (Leu419Leu; rs11032709) SNPs was performed by ARMS-PCR method whereas, PCR-RFLP method was used for genotyping of *CAT* exon12 C/T (His492His; rs17880442) and exon13 G/A (Ala520Ala; rs35677492) SNPs.

*CAT* exon7 C/A (rs17886350); exon10 C/T; exon12 C/T and exon13 G/A polymorphisms were addressed in 105 vitiligo patients and 107 controls. Only single genotype was observed (i.e. CC, CC, CC & GG respectively) for C/A (rs17886350); C/T (rs11032709); C/T (rs17880442) and G/A (rs35677492) polymorphisms in both patient and control groups, suggesting highly conserved loci of *CAT* in Gujarat population. Genotyping of *CAT* -20 5'UTR C/T (rs1049982); -89 A/T (rs7943316) and -262 A/G (rs1001179) promoter SNPs would be performed using PCR-RFLP method and results will be discussed in the thesis.

***Genotype -phenotype correlation analysis for CAT polymorphisms:***

To assess the effect of *CAT* SNPs, genotypes will be compared for the catalase activity and transcript levels between patients and controls and thus genotype-phenotype correlation will be established for vitiligo susceptibility and results will be discussed in the thesis.

## OBJECTIVE 3: TO STUDY THE ROLE OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE IN VITILIGO PATHOGENESIS

Glucose 6 phosphate dehydrogenase (EC 1.1.1.49) is a dimer and each subunit is of 59.3 kDa. It is the first enzyme of pentose phosphate pathway mainly involved in the defense against oxidizing agents through NADPH production. The human *G6PD* gene is located on human chromosome Xq28. It spans 16 kb and consists of 13 exons and 12 introns.

***G6PD activity and transcript levels:*** Erythrocyte G6PD was assayed by the standard method in 366 vitiligo patients and 449 controls. Vitiligo patients showed significantly decreased G6PD activity as compared to controls ($p<0.0001$). The transcripts levels of *G6PD* in PBMCs of vitiigo patients and controls were estimated using real-time PCR by SYBR green method. Vitiligo patients showed significantly decreased mRNA expression levels compared to controls ($p=0.038$) as determined by mean ΔCt. The $2^{-\Delta\Delta Ct}$ analysis showed an approximately 4.34 fold decrease in the expression of *G6PD* mRNA in patients as compared with controls.

***Genotyping of G6PD gene polymorphisms:*** Genotyping of *G6PD* exon2 G/C (Gln41His; rs1050827); exon4 G/T (Val107Leu; rs11555344); exon9 G/C (Asp380His; rs34193178); exon10 G/A (Gln402Gln; rs57052140) SNPs was carried out using PCR-RFLP whereas, ARMS-PCR method was used for genotyping of *G6PD* 3' UTR A/G (rs1050757); exon 5 I/D (C/-; Met155; rs34233392); exon5 G/A (Asp143Asn; rs5030870) and exon 11 C/T (Tyr467Tyr; rs2230037) SNPs. *G6PD* G/C (Gln41His); G/T (Val107Leu); G/A (Asp143Asn); C/- (Met155); G/C (Asp380His); G/A (Gln402Gln) polymorphisms were addressed in 105 vitiligo patients and 107 controls. Only single genotype was observed (i.e. GG, GG, GG, CC & GG respectively) for *G6PD* G/C (Gln41His); G/T (Val107Leu); G/A (Asp143Asn); I/D (C/-; Met155) G/C (Asp380His); G/A (Gln402Gln) polymorphisms in both patient and control groups, suggesting highly conserved loci of *G6PD* in Gujarat population.

*G6PD* exon11 C/T (Tyr467Tyr; rs2230037) polymorphism was addressed in 105 vitiligo patients and 107 controls. The observed allele frequencies of C and T were 0.99 and 0.005 in controls whereas 0.99 and 0.006 among vitiligo patients. The allele as well as

genotype frequencies of C/T SNP did not differ significantly between controls and patient population ($p$=0.159; $p$=0.270 respectively), suggesting no association of the *G6PD* exon 11 C/T SNP with vitiligo. *G6PD* 3' UTR A/G (rs1050757) polymorphism was addressed in 168 vitiligo patients and 170 controls. Genotype and allele frequencies differed significantly between patients and controls ($p$=0.0009; $p$<0.0001 respectively). In particular, the minor allele 'G' was more frequent in patients as compared to controls ($p$<0.0001, OR= 2.165, CI=1.492 to 3.140) suggesting the important role of minor allele 'G' in disease pathogenesis.

***Genotype -phenotype correlation analysis for G6PD 3' UTR A/G polymorphism:***

The activity of G6PD was analyzed with respect to 3' UTR A/G genotypes. Individuals with GG genotype showed significantly decreased G6PD activity ($p$= 0.012) compared to individuals with AA genotype. However, individuals with AG genotype did not show significant difference in G6PD activity ($p$=0.115) compared to individuals with AA genotype. In addition, *G6PD* expression was analyzed with respect to *G6PD* 3' UTR A/G genotypes: individuals with GG genotypes show significantly decreased ($p$=0.033) in *G6PD* transcript levels as compared to individuals with AA genotype suggesting important role of 3' UTR A/G SNP in decreased G6PD expression and activity in patients with vitiligo.

## OBJECTIVE 4: TO STUDY THE ROLE OF miRNAs IN VITILIGO PATHOGENESIS

At present, the expression and function of miRNAs in human skin is largely unknown. Four non-segmental vitiligo (NSV) patients and 4 healthy controls were recruited for skin biopsies (4mm) collection. Further, we recruited 45 vitiligo patients and 35 healthy controls for blood sample collection. Total RNA from skin biopsies and blood samples was extracted using mirVana miRNA isolation kit (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions and cDNA synthesis was performed using TaqManH MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems®, Foster City, California, USA). Whole miRNA profiling was

performed using TaqMan® Low Density Array (TLDA) cards Set v3.0 (Applied Biosystems®, Foster City, California, USA).

***Skin miRNA profiling:*** Analysis of the miRNA profiling data after global normalization revealed that 38 miRNAs were expressed in a non-random manner in vitiligo skin as compared to healthy skin. Based on further analysis of miRNA profiling data, we identified 13 miRNAs which were differentially expressed between lesional skin of vitiligo patients and healthy skin ($p<0.05$). Among the 13 miRNAs identified, 12 miRNAs were significantly up-regulated: miR-1 ($p=0.009$), miR-133b ($p=0.035$), miR-135a ($p=0.0001$), miR-183 ($p=0.007$), miR-190 ($p=0.04$), miR-214 ($p=0.022$), miR-301b ($p=0.027$), miR-30a-3p ($p=0.008$), miR-375 ($p=0.023$), miR-487a ($p=0.013$), miR-517c ($p=0.010$) and miR-616 ($p=0.023$) and interestingly one miRNA: miR-211 was significantly down-regulated ($p=0.014$) in lesional skin of vitiligo patients compared to healthy skin.

Further, 29 miRNAs were found to be significantly differentially expressed between non-lesional skin of vitiligo patients and healthy skin. Among the 29 miRNAs identified in the analysis, 28 miRNAs were significantly up-regulated: let-7c ($p=0.039$), miR-100 ($p=0.005$), miR-10a ($p=0.043$), miR-1180 ($p=0.022$), miR-135a ($p<0.0001$), miR-135b ($p=0.04$), miR-136 ($p=0.023$), miR-145 ($p=0.012$), miR-183 ($p=0.019$), miR-184 ($p=0.014$), miR-194 ($p=0.043$), miR-25 ($p=0.023$), miR-296 ($p=0.041$), miR-30a-3p ($p=0.014$), miR-30a-5p ($p=0.002$), miR-30b ($p=0.002$), miR-30e-3p ($p=0.023$), miR-328 ($p=0.041$), miR-34b ($p=0.006$), miR-383 ($p=0.010$), miR-487a ($p=0.005$), miR-548d-5p ($p=0.007$), miR-577 ($p=0.031$), miR-581 ($p=0.009$), miR-642 ($p=0.004$), miR-654-3p ($p=0.031$), miR-9 ($p=0.016$) and miR-99b ($p=0.016$) and one miRNA: miR-141 was significantly down-regulated ($p=0.034$) in non-lesional skin of NSV patients compared to healthy skin.

In addition, among the differentially expressed 38 miRNAs identified, there were 4 miRNAs: miR-135a, miR-183, miR-30a-3p, miR-487a which showed significant increase in expression in both lesional skin ($p=0.0001$, $p=0.007$, $p=0.008$, $p=0.013$ respectively) as well as non-lesional skin ($p<0.0001$, $p=0.019$, $p=0.014$, $p=0.005$ respectively) of NSV patients as compared to healthy skin. Interestingly, comparison of findings between lesional and non-lesional skin from patients with NSV showed that three miRNAs: miR-

136, miR-296, miR-328 were specifically down-regulated in the lesional skin compared to non-lesional skin from patients with NSV ($p$=0.04, $p$=0.029, $p$=0.009 respectively) and specifically up-regulated in the non-lesional skin compared to healthy control skin ($p$=0.023, $p$=0.041, $p$=0.041 respectively).

***Expression levels of potential miRNAs in blood:*** To determine whether 38 miRNAs which were differentially expressed in skin of NSV patients exemplify similar pattern of expression in systemic blood or not, we performed a comprehensive analysis of these 38 miRNAs in whole blood from patients with vitiligo (n =45) and compared it to healthy controls (n= 35). Analysis of the miRNA expression data after normalization with U6 snRNA revealed that only 5 miRNAs: miR-1, miR-184, miR-328, miR-383 and miR-577 were expressed in similar manner as of skin in vitiligo patients as compared to healthy controls. miR-1, miR-184, miR-383 and miR-577 were found to be significantly up-regulated in vitiligo patients compared to controls ($p$=0.036, $p$=0.0002, $p$=0.016, $p$=0.010 respectively). Whereas, miR-328 was significantly down-regulated in patients with vitiligo compared to controls ($p$=0.03).

***Target gene prediction of potential microRNAs:*** Target genes of potential miRNAs were identified using *in silico* target prediction tools [1. TargetScan (http://www. targetscan.org); 2. miRNA.org (http://www.microrna.org/microrna/home.do); 3. PicTar WEB INTERFACE (http://miRNA/miRNA targets/pictaR/)] as well as from available literature. Our analysis revealed that miR-1 targets tyrosinase (*TYR*), endothelin 1 (*EDN1*), G6PD, heat shock protein 60 (HSP60), heat shock protein 70 (HSP70) stress-associated endoplasmic reticulum protein 1 (*SERP1*), sirtuin 1 (*SIRT1*) genes which are involved in melanocyte development and different stress responses. miR-184 targets ezrin (*EZR*), lysosome-associated membrane protein 1 (*LAMP1*), staphylococcal nuclease and tudor domain containing 1 (*SND1*) which mediate melanocyte proliferation, melanogenesis and migration. miR-328 targets *TYR*, which is a key molecule in melanogenesis pathway. miR-328 have poorly conserved sites on TYR. miR-328 was found to be upregulated in MITF knocked down melanocytes (Wang et al., 2012). Microphthalmia-associated transcription factor (MITF) is a master regulator in melanocyte proliferation, development, survival and melanoma formation. miR-383

targets peroxiredoxin 3 (PRDX3) gene, which encodes a mitochondrial protein with antioxidant function. miR-383 negatively regulated *PRDX3* by interaction between miR-383 and complementary sequences in the 3' UTR of PRDX3.

miR-577 targets polymerase eta (*POLH*) gene which is a member of nucleotide excision repair (NER) family genes, which encodes a member of the Y family of specialized DNA polymerases that is able to bypass UV lesions (Yu et al., 2013). When POLH is defective, UV-induced DNA lesions are replicated by a more error-prone polymerase that produces more mutations (Kannouche et al., 2001).

miR-211 was specifically down-regulated in lesional skin, which is co-expressed with TRPM1 by MITF dependent coupled transcriptional regulation, which is critical for active $Ca^{2+}$ channel and melanocyte homeostasis. Increased expression of let-7c in non-lesional skin may induce inflammation by targeting anti-inflammatory and immunosuppressive IL10. The up-regulated miRNAs: miR-135a, miR-9 and miR-1 are known to target *SIRT1,* which protects against stress by deacetylating targets such as p53, FOXO, NFκB and PGC-1α which regulate diverse cellular processes, including stress response, replicative senescence, inflammation and metabolism. As a whole, differentially expressed miRNAs in the skin micro-environment of NSV patients and their reciprocal interaction with the oxidative stress and immune regulatory genes, might play an important role in vitiligo pathogenesis and progression.

***Expression levels of potential miRNA target genes:*** The transcripts levels of potential miRNA target genes in skin and/ blood samples from vitiligo patients and controls will be estimated using real-time PCR and the results will be shown in the thesis.

## OBJECTIVE 5: TO STUDY THE *IN VITRO* EFFECT OF OXIDATIVE STRESS ON MELANOCYTES

ROS attack almost all essential cell components, including DNA, protein and lipids (Moradas-Ferreira and Costa, 2000). To mitigate and repair the damage caused by ROS, cells have developed a complex antioxidant response, which usually encompasses well-characterized enzymes, e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase

(G6PD) (Moradas-Ferreira and Costa, 2000) and also a number of small molecules such as ascorbate, glutathione or tocopherols. G6PD plays an essential role in the regulation of oxidative stress by primarily regulating NADPH, the main intracellular reductant (Tian et al., 1998 & 1999). Thus, proper activity of G6PD is required for adequate defense against oxidative stress and prevention of cell damage/death. Inhibition of G6PD by 6-aminonicotinamide (6-ANAD) showed increased cell death in bovine aortic endothelial cells (Zhang et al., 2000). Thus, we hypothesized that under the condition of 6-ANAD with impaired G6PD activity, melanocytes would be predispose to oxidant damage and cell death. Therefore, the present study aimed to evaluate melanocyte viability and to investigate the mRNA expression levels of anti-oxidant genes (*CAT, GPX1, G6PD* and *PRDX3*), stress related genes (*HSP60, HSP70, SERP1, SIRT1* and *POLH*) and melanocyte specific genes (*MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1*) in NHM, PIG1 and PIG3V cells under $H_2O_2$ induced oxidative stress and G6PD inhibition. Cell viability will be measured by MTT assay. Gene expression will be estimated by SYBR Green method using Real-time PCR and the results will be shown in the thesis.

**CONCLUSION:** The present study has made an attempt to understand the pathogenesis of vitiligo in Gujarat vitiligo patients by assessing LPO levels, antioxidant enzyme levels as well as their genetic polymorphisms along with role of miRNAs and their target genes possibly involved in pathogenesis of vitiligo. Significant increase in LPO levels and significantly decreased GPX, catalase and G6PD levels in vitiligo patients supports the involvement of oxidative stress in vitiligo pathogenesis. Our results on genetic study suggests that significant association of polymorphisms of *GPX1* and *G6PD* genes and their altered mRNA expression suggest their crucial role in vitiligo susceptibility. In addition, we identified 38 differentially expressed miRNAs in the skin micro-environment of vitiligo patients and their reciprocal interaction with the oxidative stress and immune regulatory genes, which might affect vitiligo pathogenesis and progression. Validation of these 38 miRNAs in blood from vitiligo patients revealed that five miRNAs: miR-1, miR-184, miR-328, miR-383 and miR-577 exhibit similar pattern of expression as of the skin, suggesting their importance as putative markers for vitiligo. These five miRNAs can be further explored for therapeutic applications for vitiligo.

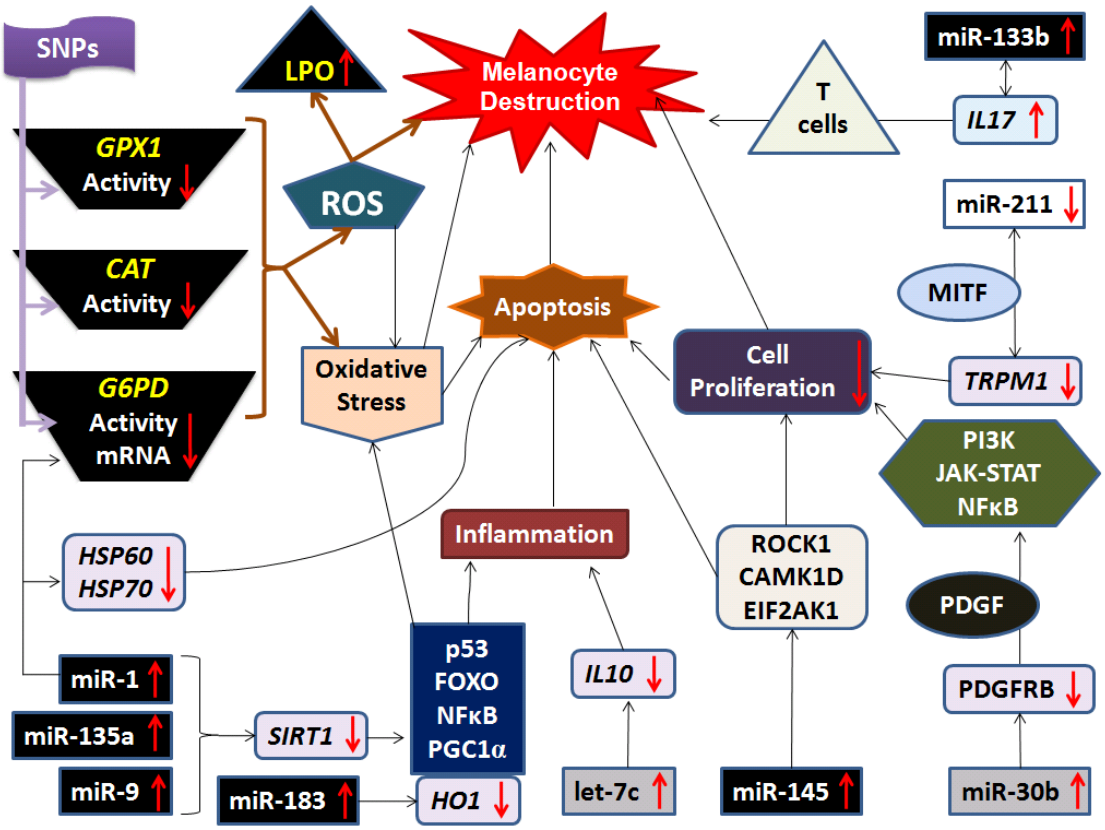


**Figure: Possible mechanism of miRNA and oxidative stress mediated melanocyte destruction in vitiligo:** The miRNAs which were found to be significantly altered and the possible interaction with their potential targets are elucidated in the proposed mechanism. We found up-regulation of miR-1 which targets *G6PD, HSP60, HSP70* and miR-183 which targets *HO1* resulting in increased oxidative stress. miR-133b is co-regulated with *IL17* which skews the immune response towards Th1 or Th17 and away from Tregs and Th2 cells. miR-211 was specifically down-regulated in lesional skin, which is co-expressed with TRPM1 by MITF dependent coupled transcriptional regulation, which is critical for active $Ca^{2+}$ channel and melanocyte homeostasis. miR-30b which was upregulated in non-lesional skin, represses the *PDGFRB* expression which binds to PDGF and plays an important role in regulating cell proliferation, cell division and cell migration. Increased expression of miR-145 in non-lesional skin may lead to reduction in cell proliferation and induction of apoptosis via caspase-3 and -7 activation by targeting *ROCK1, CAMK1D* and *EIF2AK1*. Increased expression of let-7c in non-lesional skin may induce inflammation by targeting anti-inflammatory and immunosuppressive IL10. The up-regulated miRNAs: miR-135a, miR-9 and miR-1 are

known to target *SIRT1,* which protects against stress by deacetylating targets such as p53, FOXO, NFκB and PGC-1α which regulate diverse cellular processes, including stress response, replicative senescence, inflammation and metabolism. Along with miRNAs, genetic variations in *GPX1, G6PD* and *CAT* genes lead to decreased transcript levels and/ enzyme activities resulting in increased LPO levels leading to oxidative stress in patients. As a whole, differentially expressed miRNAs in the skin micro-enviornment of vitiligo patients and their reciprocal interaction with the oxidative stress and immune regulatory genes, might play an important role in vitiligo pathogenesis and progression.

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