Vitiligo, the most common hypopigmentary skin disorder, is an acquired disease characterized by progressive loss of melanocytes. However, the cause is yet unknown and there is no commonly used treatment. Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis (Shajil *et al*., 2006; Kostopoulou *et al*., 2009; Mashayekhi *et al*., 2010; Wang *et al*., 2011). Several theories have been put forward to explain the etiology of vitiligo such as oxidative stress, autoimmune, neural and genetic hypotheses (Kemp *et al*., 2001; Ongenae *et al*., 2003; Shajil *et al*., 2006). Vitiligo has been associated with concomitant occurrence of a number of other autoimmune diseases (Alkhateeb *et al*., 2003; Boelaert *et al*., 2010).

Melanocyte death may occur due to intrinsic and/or extrinsic factors. The onset of vitiligo is manifested by the crosstalk of the following three components: 1) a set of susceptibility genes that manipulate the autoimmune response; 2) genetically abnormal melanocytes; and 3) one or more physiological or environmental factors that induce oxidative stress and activate the genetic program for melanocyte destruction (Koshoffer and Boissy, 2014). Melanocytes from vitiligo patients are incapable of battling oxidative stress, thus sustaining cellular damage. This significant damage could trigger apoptosis in the melanocytes and create an environment rich with cellular fragments that could become autoimmune targets and turn on an immune response against melanocytes. Our lab studies have shown that oxidative stress may have a role in vitiligo onset, while autoimmunity contributes towards the disease progression (Laddha *et al*., 2013a; Laddha *et al*., 2014).

The evidences of oxidative stress have been demonstrated in both skin (Schallreuter *et al.*, 1999) and blood (Beazley *et al*., 1999) of patients with vitiligo. H_2O_2 accumulates in the epidermis of patients, concomitant with reduced levels of catalase (Schallreuter *et al.*, 1999). Altered antioxidant levels, including catalase, GSH, GPX, G6PD, SOD, and LPO, a hallmark of ROS-induced damage, were observed in vitiligo patients (Koca *et al*., 2004; Agrawal *et al*., 2004; Shajil and Begum, 2006; Shajil *et al*., 2007; Laddha *et al*., 2013; Laddha *et al*., 2014; Mansuri *et al*., 2016), suggesting oxidative stress in patients with vitiligo.

GPX1 is a major intracellular antioxidant enzyme of all cell types which protects cell from potential damaging effects of ROS. Several studies have demonstrated decreased GPX activity in lesional and non-lesional epidermal skin, PBMCs, whole blood and erythrocytes of vitiligo patients (Yildirim *et al*., 2003; Hasse *et al*., 2004; Zailaie *et al*., 2005; Ines *et al*., 2006; Jalel *et al*., 2009). The present study was aimed: to investigate *GPX1* Arg5Pro, Leu6Pro, Ala194Thr polymorphisms; to estimate erythrocyte GPX1 activity and LPO levels; to estimate *GPX1* transcript levels in PBMCs from vitiligo patients and controls from Gujarat population; and to analyze the structural and functional impacts of these variations on GPX1 protein using *in silico* tools. The *in silico* prediction results revealed decreased stability of Leu6Pro and Ala194Thr variants compared to native structure, which will in turn affect the function of the protein and Arg5Pro, Leu6Pro variants show disease like trait. The present study showed significant decrease in GPX1 activity and increase in LPO levels in vitiligo patients compared to controls (Figure 1). Both GV and AV patients showed decreased GPX1 activity and increased LPO levels compared to LV and SV patients respectively, suggesting the involvement of GPX1 in disease progression. However, no significant difference was found in *GPX1* transcript levels between patients and controls. Arg5Pro and Leu6Pro polymorphisms were found to be associated with vitiligo susceptibility, whereas Ala194Thr polymorphism was found to be monogenic in the population. Interestingly, genotype-phenotype correlation analyses for Arg5Pro and Leu6Pro polymorphisms revealed that individuals with susceptible genotypes had decreased GPX1 activity and increased LPO levels compared to the other genotypes, suggesting their crucial role in altered GPX activity. MD simulation analysis revealed altered backbone stability of GPX1 with Arg5Pro and Leu6Pro variants compared to wild type. The present study for the first time confirms the genetic association of vitiligo with variations in *GPX1*, which contains at least two independent risk signals, one tagged by the G/C (Arg5Pro) SNP and another tagged by the T/C (Leu6Pro) SNP. The study also emphasizes the influence of these SNPs on decreased GPX1 activity in patients which was also supported by our simulation studies on the mutation involved, suggests the crucial role of genetic factors and oxidative stress in vitiligo pathogenesis.

Catalase (CAT) is an endogenous antioxidant enzyme that protects cells against ROS damage by detoxifying H_2O_2 into H_2O and O_2 (Rohrdanz and Kahl, 1998). Nevertheless, the molecular basis of low catalase activity in vitiligo patients has not been determined. It has been suggested that genetic variations in the *CAT* gene may have deleterious effects on the expression or function of *CAT* (Goth *et al*., 2004). In the present study we assessed the erythrocyte catalase activity and LPO levels, as well as *CAT* mRNA expression in skin and blood; investigated *CAT* 5'-UTR -20 T/C (rs1049982), promoter -89 A/T (rs7943316), -262 G/A (rs1001179) and exon 7 C/A (Ile242Ile; rs17886350); exon 10 C/T (Leu419Leu; rs11032709); exon 12 C/T (His492His; rs17880442); exon 13 G/A (Ala520Ala; rs35677492) polymorphisms; and performed genotype-phenotype correlation analyses for these polymorphisms in vitiligo patients and controls from Gujarat. Vitiligo patients showed significantly decreased *CAT* mRNA expression in lesional, non-lesional skin and in blood with reduced catalase activity compared to that of controls. *CAT* -89A/T and -20T/C polymorphisms were significantly associated with patients, especially with active and generalized vitiligo whereas, no association was observed for -262G/A and exon polymorphisms. The '$A_{-262}T_{-89}C_{-20}$' haplotype with variant alleles was found to be associated with 6.4 fold risk of vitiligo. Genotype/ haplotype-phenotype correlation analyses for *CAT* -262G/A, -89A/T and -20T/C polymorphisms revealed that individuals with susceptible genotypes/ haplotype showed significantly decreased catalase mRNA/ activity and increased lipid peroxidation levels compared to wild type genotypes/ haplotype. The present study proposes the crucial role of catalase and involvement of its allelic variants in oxidative stress mediated pathogenesis of vitiligo. Also, this is the first study suggesting that *CAT* promoter and 5'-UTR polymorphisms may decrease *CAT* mRNA expression and activity and affect the risk of vitiligo in the Gujarat population. The *CAT* -89A/T and -20T/C variant genotypes were associated with susceptibility to vitiligo and had interactions with -262G/A polymorphism in promoter. The Genotype-/ haplotype-phenotype correlation showed a relationship between increased risk and decreased *CAT* mRNA/ activity as well as increased LPO levels (Figure 1).

G6PD plays an important role in protecting cells from oxidative damage by producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced glutathione (Alharbi and Khan, 2014). Various studies including ours have reported decreased G6PD levels in vitiligo patients (Agrawal *et al*., 2004; Shajil and Begum, 2006; Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014). However, there is no information about the molecular basis of G6PD deficiency in vitiligo. Amini and Ismail (2003) have reported the association of a SNP in 3'-UTR of *G6PD* (rs1050757) and its possible role in mRNA secondary structure with G6PD deficiency. hsa-miR-1 is a potent regulator of *G6PD* expression and polymorphisms in 3'UTR of *G6PD* mRNA affect the binding of hsa-miR-1. Therefore, the present study was aimed to investigate *G6PD* exon 2 G/C (Gln41His; rs1050827), exon 4 G/T (Val107Leu; rs11555344), exon 5 G/A (Asp143Asn; rs5030870), exon 5 I/D (C/-; Met155; rs34233392), exon 6 C/T (Ser218Phe; rs5030868); exon 9 G/C (Asp380His; rs34193178), exon 10 G/A (Gln402Gln; rs2230036), exon 11 C/T (Tyr467Tyr; rs2230037) and 3' UTR A/G (rs1050757) polymorphisms; to estimate G6PD activity and LPO levels in erythrocytes; to estimate *G6PD* and hsa-miR-1 expression levels in skin and blood of Gujarat vitiligo patients and controls. Vitiligo patients showed significantly decreased G6PD activity; decreased *G6PD* transcript in lesional skin as well as in the blood and increased miR-1 levels as compared to controls (Figure 1). Both genotype and allele frequencies differed significantly between patients and controls for 3'UTR polymorphism whereas; no significant difference was observed for rs5030868 and rs2230037. However, other exonic polymorphisms were found to be mono-allelic in both patient and control groups. Genotype-phenotype correlation analysis for *G6PD* 3' UTR polymorphism revealed that individuals with GG and GA genotypes showed significantly decreased *G6PD* mRNA, decreased activity and increased LPO levels in blood as compared to individuals with AA genotype. The present study for the first time confirm the genetic association of vitiligo with *G6PD* polymorphisms, which contains at least two independent risk factors, one tagged by 3'UTR rs1050757 polymorphism of *G6PD* and another tagged by miR-1 regulated *G6PD* expression in vitiligo patients. We have found these novel factors could contribute to oxidative stress mediated pathogenesis of vitiligo.

miRNAs have been shown to be promising biomarkers for different diseases (Weiland *et al*., 2012). The serum and PBMC's miRNA expression profiles have been shown in patients with NSV. Nevertheless, the role of miRNAs in skin micro-environment of vitiligo lesions is totally unknown. Hence, we hypothesized that miRNAs derived from melanocytes, keratinocytes and their neighboring cells during vitiligo development could be detected in the skin micro-environment and may serve as novel biomarkers for vitiligo.

The present study aimed to detect the differentially expressed miRNAs and to explore the potential role of miRNAs in vitiligo pathogenesis by miRNA expression profiling of lesional as well as non-lesional skin from non-segmental vitiligo patients compared to healthy skin from control individuals. We performed whole miRNA profiling of lesional and non-lesional skin from patients and controls. Our results suggest that 38 miRNAs were differentially expressed in the patients compared to controls. We identified 13 miRNAs which were significantly differentially expressed in lesional skin of patients. Further, 29 miRNAs were found to be significantly differentially expressed between non-lesional skin of patients and controls. Interestingly, three miRNAs were specifically down-regulated in the lesional skin compared to non-lesional skin from patients. We identified 38 differentially expressed miRNAs in the skin micro-enviornment of non-segmental vitiligo patients and their reciprocal interaction with the oxidative stress and immune regulatory genes, which might affect vitiligo pathogenesis and progression.

The subsequent study was performed to investigate the expression of previously identified 38 skin miRNA signatures in whole blood of vitiligo patients and controls; to predict the potential target genes of differentially expressed miRNAs; and to investigate transcript levels of potential miRNA targets in skin, whole blood and PBMCs from patients and controls. We predicted the potential targets of differentially expressed miRNAs and investigated their expression levels in skin, whole blood and PBMCs from patients and controls. Our results showed miR-1, miR-184, miR-328, miR-383 and miR-577 hold similar pattern of expression as of skin, suggesting their potent eminence for being putative markers for vitiligo. *In silico* target prediction for these key miRNAs revealed potential targets, which might be involved in melanocyte development,

melanogenesis, immune regulation, DNA repair, oxidative stress and endoplasmic reticulum (ER) stress response. *In silico* target prediction revealed miR-1 targets *EDN1, G6PD, HSP60, HSP70, SERP1, SIRT1 & TYR*; miR-184 targets *EZR & LAMP1*; miR-328 targets *IL1B, POLH & TRPM1*; miR-383 targets *EDN1 & TYRP1*; and miR-577 targets *PTPN22 & TYRP1* which were corroborated by our validation study. The present study for the first time provides new insights into the crucial role of miRNAs in the melanocyte death in skin micro-environment of vitiligo patients (Figure 1). We identified miR-1, miR-184, miR-328, miR-383 and miR-577 along with miR-211 as potential miRNAs, which might target key genes involved in melanocyte development, melanogenesis, immune response, oxidative stress response, ER stress response, DNA repair, etc. suggesting their crucial involvement in vitiligo pathogenesis and/ progression. These potential miRNAs can be further explored for therapeutic applications for vitiligo.

Oxidative stress plays a vital role in melanocyte destruction in vitiligo. Understanding the mechanism(s) by which H_2O_2 causes melanocyte death may elucidate the pathology underlying vitiligo in general. Thus, Melanocyte susceptibility to oxidative stress may play a role in the pathogenesis of vitiligo. G6PD plays an essential role in the regulation of oxidative stress by primarily regulating NADPH, the main intracellular reductant. Importantly, it has been shown that modest changes in G6PD activity itself have significant effects on cell growth and cell death in a variety of cell types (Tian *et al*., 1998 & 1999). Inhibition of G6PD by 6-aminonicotinamide (6-ANAD), showed increased cell death in bovine aortic endothelial cells (Zhang *et al*., 2000) and suppressed proliferation of prostate cancer cells (LNCaP) (Tsouko *et al*., 2014). G6PD per se was not evaluated in melanocytes, nor was the effect of G6PD inhibition reported in melanocytes. Thus, we hypothesize that under the condition of 6-ANAD with impaired G6PD activity, melanocytes would 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, EZR* and *LAMP1*) in NHM, PIG1 and PIG3V cells under H_2O_2 induced oxidative stress and G6PD inhibition. The NHM, PIG1 and PIG3V melanocytes

showed significantly decreased viability under H_2O_2 (10-100 μM) induced oxidative stress and G6PD inhibition (0.5-5 mM 6-ANAD). Further, PIG3V melanocytes showed significantly increased expressions of *PRDX3, HSP70, SERP1*, *POLH* as well as decreased expression of *CAT, MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* under H_2O_2 (10 & 20 μM) induced oxidative stress and/ G6PD inhibition (0.5 mM 6-ANAD) as compared to NHM and/ PIG1 melanocytes. The present study suggests that H_2O_2 and 6-ANAD induces oxidative stress in melanocytes and that melanocytes from individuals with vitiligo are more sensitive to H_2O_2 induced oxidative stress and G6PD inhibition compared with normal melanocytes (Figure 1). Therefore, vitiligo may result from an insufficient response to oxidative stress induced by H_2O_2 and 6-ANAD inhibition.

The present study has made an attempt to understand the pathogenesis of vitiligo in Gujarat vitiligo patients by assessing LPO levels, antioxidant enzyme (catalase, GPX1, G6PD) 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 GPX1, 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, CAT* 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. The present study for the first time provides new insights into the crucial role of miRNA regulated gene network involved in oxidative stress, autoimmunity and ER stress mediated pathogenesis of vitiligo (Figure 1).

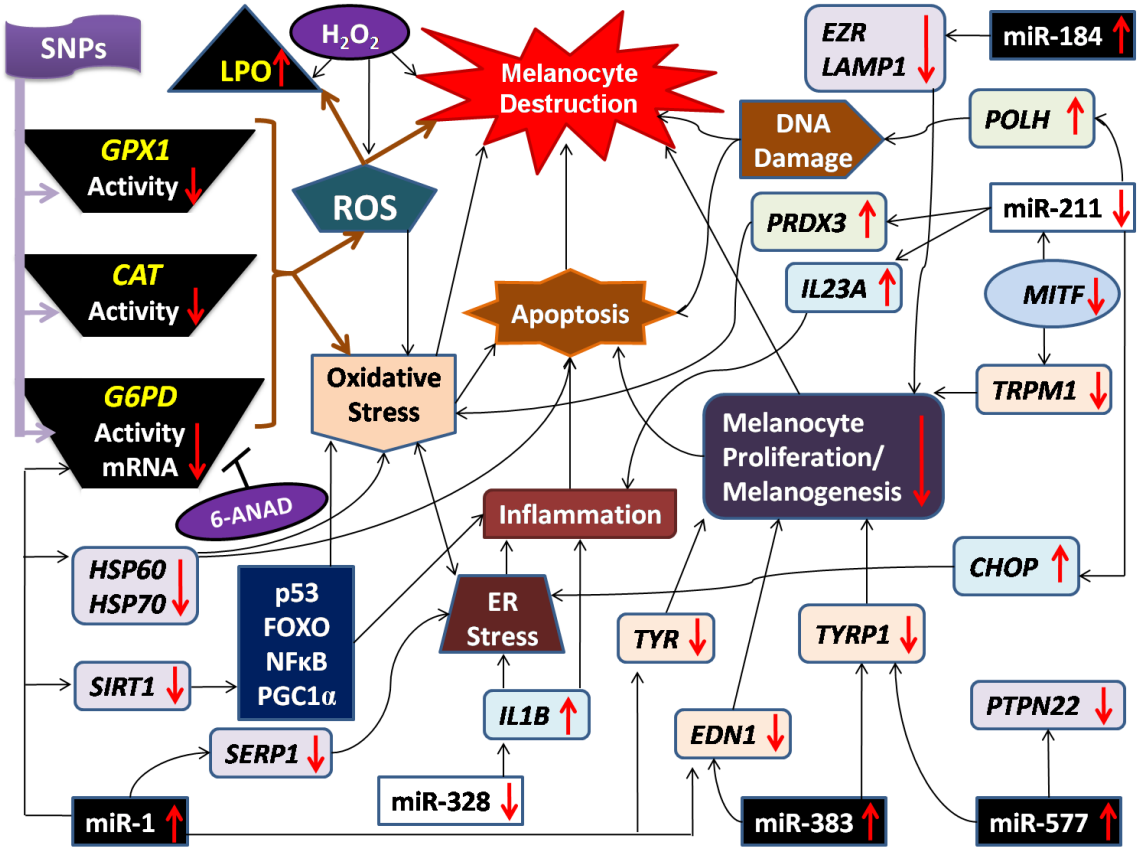

Figure 1: Possible mechanism of miRNA and oxidative stress mediated melanocyte destruction in vitiligo: The miRNAs which were found to be significantly altered and the possible interaction with their potential targets are elucidated in the proposed mechanism. We found up-regulation of miR-1, miR-184, miR-383 and miR-577 whereas miR-211 and miR-328 were down-regulated. Where miR-1 targets: *G6PD, EDN1, HSP60, HSP70, SERP1, SIRT1* and *TYR* genes; miR-328 targets: *IL1B, POLH* and *TRPM1*; miR-184 targets: *EZR* and *LAMP1*; miR-383 targets: *EDN1*, *TYRP1* and *PRDX3*; miR-577 targets: *PTPN22*, *TYRP1* and *POLH* genes; miR-211 targets: *IL1B, CHOP*, *IL1R1*, *IL23A*, *POLH* and *PRDX3* genes. miR-211 is co-expressed with TRPM1 by MITF dependent coupled transcriptional regulation, which is critical for active Ca^{2+} channel and melanocyte homeostasis. miR-1 targets *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.