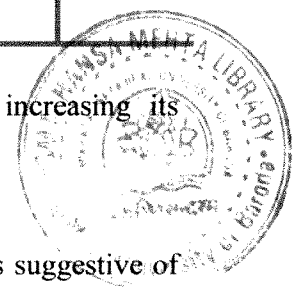
CONCLUDING REMARKS

Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis, linked with both genetic and non-genetic factors. The precise *modus operandi* for pathogenesis of vitiligo has remained elusive. Theories regarding destruction of melanocytes are based on autoimmune, cytotoxic, oxidant-antioxidant and neural mechanisms. Reactive oxygen species (ROS) in excess have been documented in active vitiligo skin. Numerous proteins and peptides, in addition to tyrosinase are affected. It is possible that oxidative stress is the principal cause of vitiligo. However, there also exists ample evidence for altered immunological processes in vitiligo, particularly in established chronic and progressive conditions. Both innate and adaptive arms of the immune system appear to be involved as a primary event or as a secondary promotive consequence. There is speculation on the interplay, if any, between ROS and the immune system in the pathogenesis of vitiligo.

The present study is an attempt to add some pieces in the jigsaw puzzle of vitiligo pathogenesis. Overall, this study shows that oxidative stress plays a major role in the precipitation of vitiligo in Gujarat population. The evaluation of oxidative stress and autoimmune hypotheses in patients at the onset of vitiligo (<3 months) showed a significant higher LPO levels, but exhibited significant lower antimelanocyte antibody levels. These results strongly suggest that oxidative stress plays a major role in the initiation of disease in susceptible vitiligo patients of Gujarat. Further, our results on LPO and antimelanocyte antibody levels in active versus stable patients suggest equal contribution of oxidative stress and autoimmunity in disease progression. Thus, this study shows that oxidative stress is the initial triggering event to precipitate vitiligo in Gujarat population which is then exacerbated by contribution of autoimmune factors together with oxidative stress. We speculate that oxidative stress may result in the formation of neo-antigens which might lead to autoimmunity in these patients.

Our SOD1 results suggest that the increased activity of SOD1 observed in vitiligo patients was not due to increased expression of *SOD1* mRNA or protein levels. Hence, our speculation was that presence of genetic variants of *SOD1* may be involved in increased SOD1 activity. However, we could not find any plausible answer as we did not find any variation in the *SOD1* exonic regions. Thus, post



translational modifications of SOD1 could be playing a role in increasing its efficiency.

We also found increased activity of SOD2 in vitiligo patients which is suggestive of mitochondrial impairment and high ROS in patients. The increased activity of SOD2 may in part be contributed by polymorphisms as well as increased *SOD2* mRNA levels. Further, we found association of Thr58Ile (C/T; rs35289490) and Leu84Phe (C/T; rs11575993) *SOD2* polymorphisms with vitiligo; however, Val16Ala (T/C; rs4880) was only associated with active cases of vitiligo suggesting genetic susceptibility towards oxidative stress in vitiligo patients.

The extracelluar SOD (SOD3) activity was also increased in vitiligo patients suggesting the increased ROS in extracellular compartments. Further, our results suggest that Arg213Gly (C/G; rs8192291) polymorphism of *SOD3* and increased levels of *SOD3* transcript may in combination are responsible for increased SOD3 activity in extracellular fluids. Our results on LPO substantiate that vitiligo patients exhibit high oxidative stress. Overall, the oxidative stress in vitiligo patients may be contributed by increased activity of all the three SODs. Our studies on the downstream systemic antioxidant system in vitiligo patients showed that activity of catalase and GPX were decreased, which results in accumulation of H_2O_2 and may finally lead to oxidative damage to the melanocytes.

Oxidative stress is considered to be the initial pathogenic event in melanocyte destruction. The accumulated ROS cause DNA damage, lipid peroxidation and protein oxidation. Many proteins and peptides result in altered or even complete loss of functionality due to H_2O_2-mediated oxidation. For example, H_2O_2 can function as an inhibitor of tyrosinase as the presence of H_2O_2 and DOPA substrate can generate a secondary complex that can bind and inhibit tyrosinase suggesting a meaningful correlation between increased oxidative stress and decreased tyrosinase activity. Furthermore, increased levels of ROS in melanocytes may lead to its defective apoptosis resulting in the release of aberrated proteins. These proteins may serve as auto-antigens and are presented by MHC molecules to T-cells leading to autoimmunity. High ROS levels also increase the levels of cytokines such as TNFα,

TNFβ, IFNγ and IL2. IL2 further upregulates the expression of anti-apoptotic protein Bcl-2, thereby making T-cells resistant to apoptosis. Increased ROS also stimulates protein kinase C expression resulting in NF-kB activation which in turn activates JNK, ERK and p38 pathways in high ROS environment resulting in apoptosis. These events signify the importance of oxidative stress in precipitation of vitiligo.

The present study has also explored the autoimmune pathway leading to melanocyte death in vitiligo pathogenesis. Increased antimelanocyte antibody levels were observed in 75% of vitiligo patients. Moreover, active cases of vitiligo had higher antimelanocyte antibody levels compared to stable cases advocating the profound role of antimelanocyte antibodies in disease progression.

The exact pathway of destruction of melanocytes is not yet known, however, apoptotic death has been suggested in vitiligo. Cytokines such as IL1, IFNγ and TNFα that are released by lymphocytes and keratinocytes are paracrine inhibitors of melanocytes and can initiate apoptosis. Also an imbalance of cytokines in the epidermal microenvironment of lesional skin has been demonstrated which could impair melanocyte function. In addition, increased levels of TNFα cause maturation of dendritic cells and thus results in development of autoimmunity. The increased TNFα levels are significantly higher in lesional skin compared with the non-lesional skin in patients with vitiligo (Moretti *et al.*, 2002).

Our current study showed increased mRNA and protein levels of TNFα in vitiligo patients. The increased levels of TNFα were contributed by its promoter polymorphisms which are reported to increase the expression of *TNFA* gene. In our genetic association study we found significant association of *TNFA* promoter polymorphisms with vitiligo, in particular, -238 (G/A; rs361525) and -308(G/A; rs1800629) were found to have a profound effect on *TNFA* expression. Susceptible haplotypes generated for the polymorphisms showed significant increase in mRNA and protein levels of TNFα in vitiligo patients. Analysis based on the disease activity suggests both TNFα mRNA and protein levels were significantly increased in active cases of vitiligo as compared to stable vitiligo, indicating the role of TNFα in disease progression. The study also found gender biasness in the susceptibility to vitiligo.

Female patients had an early onset of vitiligo as compared to male patients, moreover the TNFα mRNA and protein levels were significantly higher in female patients as compared to male patients suggesting the inclination of females towards autoimmunity.

Further, *TNFB* polymorphisms: +252 (G/A; rs909253) and Thr26Asn (A/C; rs1041981) were found to be associated with vitiligo susceptibility. Both the polymorphisms were in 100% linkage disequilibrium. The expression analysis of *TNFB* suggests increased mRNA levels in vitiligo patients indicating its crucial role in vitiligo pathogenesis. Moreover, the genotype-phenotype correlation suggested that the two polymorphisms had a significant effect on *TNFB* expression as mRNA levels were high with the susceptible genotypes. Active cases of vitiligo had increased levels of *TNFB* transcripts as compared to stable cases suggesting the role of *TNFB* in disease progression. Females had increased *TNFB* expression as that of male patients suggesting their inclination towards vitiligo. Thus the study emphasizes the influence of *TNFB* on the disease progression and gender biasness for developing vitiligo.

Association of *TNFA* and *TNFB* with vitiligo susceptibility gains further importance due to their location in *MHC* region. Association of *MHC* alleles with the disease gains support because of the antigen-presenting function of the MHC. Our recent study has shown positive association of *HLA-A*33:01*, *HLA-B*44:03*, and *HLA-DRB1*07:01* with vitiligo patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts (Singh *et al.*, 2012). In our other study we identified the three most significant class II region SNPs: rs3096691 (just upstream of *NOTCH4*), rs3129859 (just upstream of *HLA-DRA*), and rs482044 (between *HLA-DRB1* and *HLA-DQA1*) (unpublished data) associated with generalized vitiligo suggesting an important link between vitiligo and *MHC* region in which *TNFA* and *TNFB* are also located.

TNFα and TNFβ can induce the expression of intercellular adhesion molecule 1 (ICAM1) on the cell-surface of melanocytes and the increased expression of *ICAM1* enhances T cell- melanocyte attachment in the skin and may play a role in the destruction of melanocytes (Al Badri, 1993). We found that *ICAM1* expression was increased in vitiligo patients. Our study also emphasizes the influence of ICAM1 on

the disease progression and gender biasness for developing vitiligo. Thus, ICAM1 is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

The high frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease (Wankowicz-Kalinska *et al.*, 2003). In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of distinct T-cell subtypes. Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells and $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes (Wankowicz-Kalinska *et al.*, 2003). Our recent studies also showed decreased $CD4^+/CD8^+$ ratio in vitiligo patients as compared to controls, indicating the prevalence of $CD8^+$ cells in patients.

Overall, the present study finds genetic predisposition in genes involved in oxidative stress and immune regulation i.e. *SOD2, SOD3, TNFA* and *TNFB* which can modulate the antioxidant enzyme system and immune response towards melanocytes. Moreover, the presence of increased antimelanocyte antibodies and the imbalace of T-cell subsets along with their functional defects might results into melanocyte destruction in vitiligo patients. However, a single dominant pathway appears unlikely to account for all cases of melanocytes loss in vitiligo and apparently, a complex interaction of genetic, environmental, biochemical and immunological events is likely to generate a permissive milieu. In the light of present study, possible molecular and cellular events leading to melanocyte death responsible for vitiligo manifestation are summarized in Figure 1.

The pathogenesis of vitiligo though partially understood still remains complex and enigmatic to a greater extent. However, the present study has yielded some interesting clues for vitiligo pathogenesis. Though the condition may be precipitated by multiple etiologies, the current study suggests that interplay of oxidative stress and immune system appears to be the key convergent pathway that initiates and/or amplifies the thus far enigmatic loss of melanocytes. Better understanding of trigerring factors for

generation of oxidative stress and autoimmunity in vitiligo patients could pave the way towards the development of preventive/ameliorative therapies.

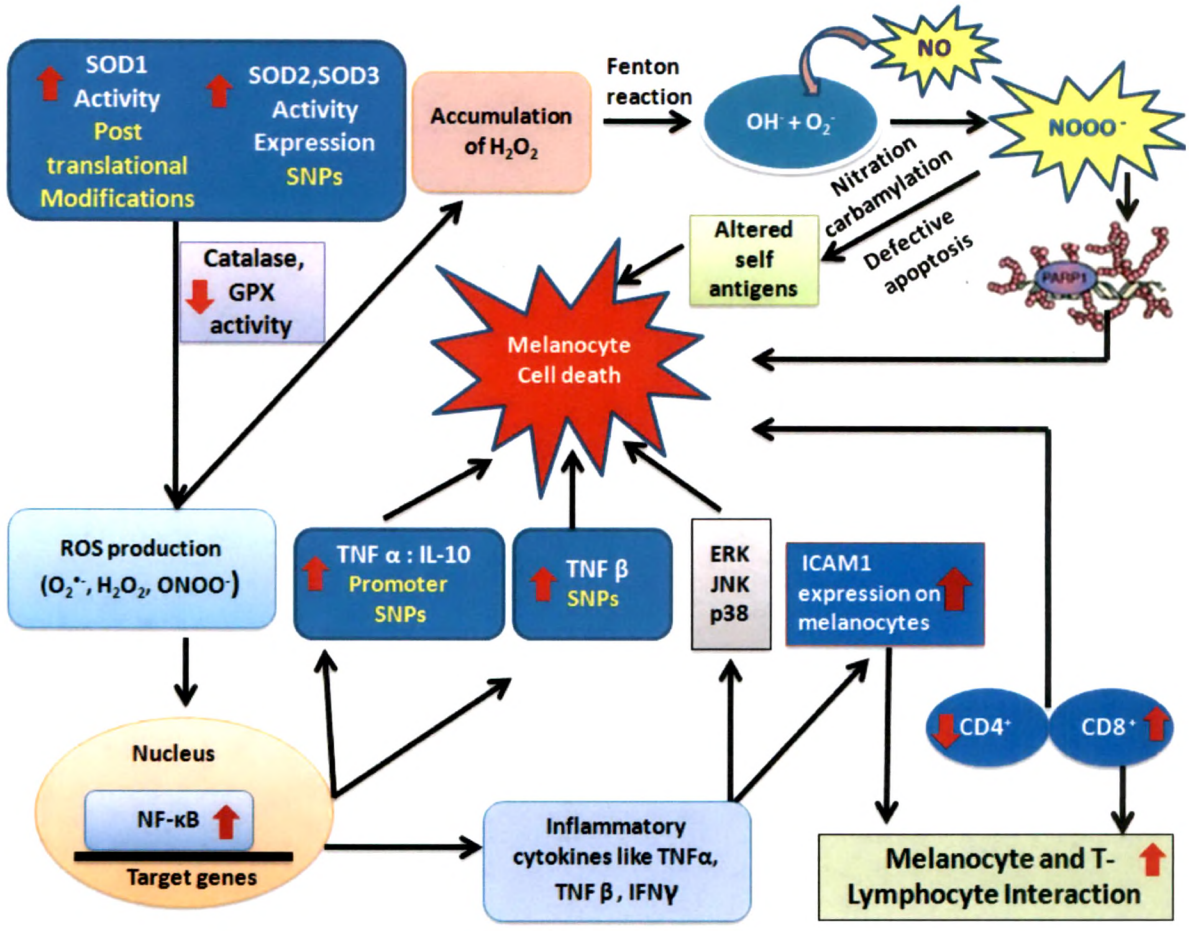


Figure 1. Possible molecular and cellular events responsible for melanocyte destruction in vitiligo:

Increased activity of SOD1, SOD2 and SOD3 generates H_2O_2. Increased activity of SOD1 may be attributed to post translational modification of SOD1 protein. The increased activity of SOD2 and SOD3 is due to increased expression and presence of SNPs in these genes. The subsequent enzymes imoprtant for the reduction of H_2O_2 i.e. catalase and GPx have decreased activity in vitiligo patients, ultimately leading to H_2O_2 accumulation. This leads to release of Fe^{2+} from iron-sulphur clusters of mitochondria. This Fe^{2+} is then available to carry out Fenton's reaction which generates hydroxyl radicals ($OH^{\bullet}$) and superoxide anions (O_2^-), which further reacts with nitric oxide (NO) and forms peroxynitrite ($NOOO^-$) which in turn can result in

PARP over activation and thereby cell death. ROS and RNS can also react with cellular constituents and can alter self proteins by nitration and carbamylation, generating neo-antigens, provoking the immune system which attacks the self cells generating autoimmunity. Increased ROS and RNS may also be responsible for defective execution of apoptosis due to defective apoptotic proteins. Increased ROS also activates PKC which alters NF-κB expression. NF-κB in a compromised state of melanocytes signals the secretion of various inflammatory cytokines like TNFα, TNFβ, IFNγ etc. NF-κB can also activate apoptotic pathways via JNK, p38 and ERK. Increased TNFα and TNFβ levels may result from genetic predisposition (SNPs) and high ROS. These cytokines are paracrine inhibitors of melanocytes and melanogenesis process. Increased level of TNFα can directly trigger apoptosis of melanocytes under ROS prevailing microenvironment and decreased IL10 levels. TNFα and TNFβ induce *ICAM1* expression on melanocytes which increases melanocyte and T-lymphocyte interaction. Increased $CD8^+$ T cells can lead to melanocyte destruction via ICAM1 induced melanocyte - T lymphocyte ($CD8^+$) interaction.