CONCLUDING REMARKS

Vitiligo is a common skin disorder characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches caused by loss of functional epidermal melanocytes. Despite tremendous progress in molecular biology and genetics, there is still no universally accepted hypothesis. It could well be that vitiligo represents a 'syndrome' rather than a disease, with numerous different but not mutually exclusive pathways leading to melanocyte failure or disappearance. The exact etiology of vitiligo remains obscure, but autoimmunity has been strongly implicated in the development of disease, especially in generalized vitiligo. However, genetic factors probably determine which particular pathway predominates in a specific patient. Several immunogenetic factors predispose patients to autoimmune diseases and therefore the present study addresses the role of a few candidate genes involved in autoimmune pathogenesis of vitiligo.

Vitiligo is widely considered as an autoimmune disease, with involvement of humoral and cellular components of immune system. The evidences in support to this hypothesis are: association with other autoimmune disorders; chronic relapsing and remitting course typical of autoimmune disorders; possible response to immunosuppressive therapies; circulating anti-melanocyte antibodies; T-cell infiltrates in perilesional skin; anti-melanocyte cytotoxic T-cells in the skin and circulation and proinflammatory cytokine patterns of a Th-1 type response. Autoimmunity might not be the triggering event in vitiligo, but it could function instead as a promoter of disease progression and chronicity.

The present study evaluates the autoimmune hypothesis of vitiligo pathogenesis by addressing the levels of antimelanocyte antibodies and monitoring the T-cell subsets in vitiligo patients of Gujarat population. We found significantly higher levels of antimelanocyte antibodies in the circulation of patients and these antibodies correlate with disease activity and extent. Active vitiligo patients exhibited increased levels of antimelanocyte antibodies as compared to stable vitiligo patients. Moreover, patients with early age of onset had significantly higher levels of antimelanocyte antibodies suggesting the role of antimelanocyte antibodies in early onset of the disease. Gender based analysis for antimelanocyte antibodies revealed that female patients had higher levels of antimelanocyte antibodies as compared to male patients suggesting that

females are more susceptible towards autoimmune diseases. Targets of these antibodies include a variety of melanocyte and melanosomal antigens. Antibodies might trigger vitiligo as a primary event, but they could also arise secondary to melanocyte damage or serve to perpetuate the disease. Studies suggested that whatever may be their role in vitiligo pathogenesis; these antibodies have the capacity to injure melanocytes *in vivo* and *in vitro*.

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. 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 progression of disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells. $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes. Our results showed that $CD4^+/CD8^+$ ratio was significantly lowered in generalized vitiligo patients as compared to controls. The $CD4^+/CD8^+$ ratio was further lowered, as $CD8^+$ T-cell count was significantly higher in patients with active vitiligo as compared to stable vitiligo suggesting the role of $CD8^+$ T-cells in progression of the disease.

Association of Major Histocompatibility Complex (MHC) alleles with vitiligo gains importance because of the antigen-presenting function of the MHC. Our recent study suggests a consistent increase of A*33:01, B*44:03, and DRB1*07:01, implicating these alleles as possible markers of vitiligo in North India and Gujarat These data apparently suggest auto reactive CD4+ T-helper cells to be restricted by HLA-DRB1*07:01 and the auto-reactive CD8+ cytotoxic T cells by HLA-A*33:01, A*02:01, B*44:03, and B*57:01 in the Indian populations studied (Singh et al., 2012). In another recent 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 immune system.

Natural Treg cells play a key role in maintaining peripheral tolerance *in vivo* through the active suppression of self-reactive T cell activation and expansion, and dysfunction or deficiency of Tregs may result into autoimmune diseases including vitiligo. The most intriguing Tregs are those showing $CD4^+CD25^{hi}FoxP3^+$. However, very little is known about the function of Treg cells in generalized vitiligo (GV). The level of $CD4^+CD25^{hi}$ $FoxP3^+$ Tregs has been variably reported in different studies. In this study, we have found that GV patients exhibit a decreased prevalence of circulating Tregs and hence an increase in Teff cell population was observed in these patients which might be responsible for melanocyte destruction. Moreover, the FoxP3 expression in the $CD4^+CD25^{hi}$ Treg cells was found to be significantly decreased in cases of vitiligo as compared to controls suggesting that the Tregs may have functional defects which can affect their suppressive activity. In addition, our recent study suggests decreased level of IL10 in vitiligo patients, which is secreted by Tregs for supression of activated T-cells. We found that female patients showed significant low expression of FoxP3 in $CD4^+CD25^{hi}$ Treg cells as compared to male patients suggesting that females have increased susceptibility towards autoimmunity. Thus, a functional defect in Tregs might be involved in the pathogenesis of vitiligo. Early age group of patients (1-20 yrs) showed lower levels of Treg cells as compared to the late onset groups which suggests the crucial role of Treg cells in early onset of the disease.

Also, active cases of vitiligo showed significant increase in Teff cells which further signifies the importance of Tregs in progression of the disease. Thus, the present study indicates that an imbalance of $CD4^+/CD8^+$ ratio and natural Tregs in frequency and function might be involved in the T-cell mediated pathogenesis of vitiligo and its progression. The reduced levels of natural Treg cell population might lead to a global expansion and widespread activation of the $CD8^+$ population, which could result in the destruction of melanocytes in vitiligo patients.

CTLA4 is an important molecule in the down-regulation of T-cell activation and it is critical for the activity of regulatory T cells. The present study showed a significant decrease in *sCTLA4* and *flCTLA4* expression suggesting that lower levels of *s*CTLA4 and *fl*CTLA4 may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. We found that expression of *CTLA4* in vitiligo patients was

modulated by CT60AG dimorphism (rs3087243). The CT60A/G polymorphism was significantly associated with susceptibility to vitiligo in Gujarat population. Thus, our study suggests that the dysregulated *CTLA4* expression in vitiligo patients could result, at least in part, due to variations at the genetic level. For the first time, we showed that the 3' UTR CT60A/G polymorphism of the *CTLA4* gene influences both full length and soluble *CTLA4* mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA4* supports the autoimmune hypothesis of vitiligo pathogenesis (Dwivedi *et al.*, 2011).

The epidermal imbalance in skin of vitiligo patients suggests the role of cytokines in vitiligo skin autoimmunity. Cytokine studies of peripheral blood and skin in patients with vitiligo have yielded variable results, but with a trend to proinflammatory T-cell patterns. Studies suggested that topical tacrolimus, used successfully to treat vitiligo, increases tissue IL-10, which is an immunosuppressive Th-2 cytokine. This suggests that vitiligo might be a Th-1 type of autoimmune disease. IFNγ is one of the important Th-1 cytokine involved in the activation of antigen presenting cells (APCs). It promotes Th-1 differentiation by up regulating the transcription factor NF-kB and inhibits the development of Th-2 cells thereby playing role in maintaing the Th-1/Th-2 ratio which predominantly decides the development of autoimmunity. Our results suggest increased mRNA and protein levesl of IFNγ in vitiligo patients. The genetic polymorphisms of *IFNG* intron 1 +874A/T (rs2430561) with CA repeats (rs3138557) is reported to modulate its expression through NF-kB. We found significant association of high producer 12 CA repeats with vitiligo. Moreover, the intron 1 'T' allele as well as 12 CA repeat were correlated with higher mRNA and serum IFNγ levels in vitiligo patients. The study suggests that *IFNG* CA microsatellite but not +874 A/T may be a genetic risk factor for generalized vitiligo in Gujarat population; however, +874T allele plays a major role in increased expression of *IFNG* mRNA and protein levels which can affect the onset and progression of disease.

IFNγ stimulates the expression of intercellular adhesion molecule 1 (*ICAM1*), which is important for activating T cells and recruiting leukocytes. ICAM1 protein levels are upregulated in vitiligo skin and in melanocytes from perilesional vitiligo skin. The present study also showed increased expression of *ICAM1* in vitiligo patients

suggesting that increased IFNγ levels might be responsible for increased *ICAM1* expression in vitiligo patients. It has been reoprted that increased expression of this adhesion molecule on the melanocytes enhances T cell -melanocyte attachment in the skin and may lead to the destruction of melanocytes. Moreover, the *ICAM1* expression was increased in active cases of vitiligo as compared to stable vitiligo suggesting its role in progression of the disease. The *ICAM1* expression was increased with early age of onset of the disease further implicating the important role of *ICAM1* in early phase of the disease. Also, female patients showed an increased expression of *ICAM1* as compared to male patients suggesting that females have more susceptibility towards vitiligo. Thus, the study suggests that increased IFNγ levels in patients may lead to increased *ICAM1* expression which is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

Recent genome-wide association studies (GWAS) revealed a highly significant association of familial cases of generalized vitiligo with polymorphic variants of the gene encoding *NALP1* - a key regulator of the innate immune system. Immunohistochemical studies have found high *NALP1* expression in epidermis within Langerhans cells and T cells suggesting involvement of *NALP1* in skin autoimmunity. *NALP1* plays a crucial role in the assembly of the apoptosome thereby it is involved in inflammation and apoptosis. *NALP1* may also contribute to modulate the response of cells towards proinflammatory stimuli like IL1β, IFNγ and TNFα. Overexpression of this gene induces apoptosis in cells. In the present study we found increased expression of *NALP1* in vitiligo patients. The promoter (rs2670660) and intronic (rs6502867) variants of *NALP1* were significantly associated with vitiligo susceptibility. The genotype-phenotype correlation of these SNPs revealed that susceptible genotypes confer increased expression of *NALP1* in vitiligo thereby implicating its crucial role in vitiligo pathogenesis. The study also emphasizes the influence of *NALP1* on the disease progression, onset of the disease and gender biasness for developing vitiligo.

MYG1 is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes. Studies showed that siRNA mediated knockdown of *MYG1* resulted in the altered levels of transcripts encoding transcription factors involved in development

and growth as well as immune-related processes. *MYG1* promoter and structural polymorphisms have a functional impact on the regulation of *MYG1* gene. Elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients has been reported. The present study showed that systemic expression of *MYG1* transcript is elevated in vitiligo patients as compared to controls. Also, patients with active vitiligo and early age of onset had elevated levels of *MYG1* transcript which further suggested the important role of MYG1 in progression of the disease. The *MYG1* mRNA levels in patients were also correlated with *MYG1* promoter polymorphism -119C/G (rs1465073). Thus, our results confirm that -119C/G polymorphism influences *MYG1* transcript levels and minor -119G allele is the risk-allele for the development of vitiligo. The study also emphasizes the influence of MYG1 on the disease progression, onset of the disease and gender biasness for developing vitiligo.

MBL2 plays an important role in innate immunity. It helps in the clearance of apoptotic cells and in complement activation. Genetic variability due to structural and promoter polymorphisms in *MBL2* gene has been reported to be associated with increased risk for several autoimmune diseases including vitiligo. *MBL2* exon variants (rs5030737, rs1800450, rs1800451) cause structural changes in the collagen like domain of MBL2, whereas *MBL2* promoter variants (rs7096206) lead to its low plasma levels. However, the present study could not find association of these variants with vitiligo susceptibility (Dwivedi *et al.,* 2009).

Moreover, *PTPN22* which is a T-cell down regulator gene has a geneitc variant (1858C/T) (rs2476601) which leads to alteration in T-cell suppressive capacity; however, this SNP was not associated with vitiligo susceptibility in Gujarat population (Laddha *et al.,* 2008). In addition, *ACE* gene which catalyzes the conversion of angiotensin I to angiotensin II 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 ACE that generates angiotensin II which in turn can induce cytokines such as TNFα and IFNγ. Our recent studies found increased transcript and protein levels of TNFα in vitiligo patients. These increased cytokines may modulate the microenvironment which in turn can signal for apoptosis of melanocytes as observed in vitiligo patients. The *ACE* I/D polymorphism (AF118569) is reported to alter the ACE levels;

however, the present study could not find significant association of this polymorphism (Dwivedi *et al.,* 2007). Thus, our study proposes that the genetic variants in *MBL2, PTPN22* and *ACE* genes may not be a genetic risk factor for generalized vitiligo susceptibility in Gujarat population.

Overall, our studies find genetic predisposition in immune regulator genes including *CTLA4, IFNG, NALP1* and *MYG1* which can modulate the immune response towards melanocytes. Moreover, the presence of increased antimelanocyte antibodies and the imbalace of T-cell subsets along with their functional defects might result in melanocyte destruction in vitiligo patients. However, a single dominant pathway appears unlikely to account for all cases of melanocyte loss in vitiligo and apparently, a complex interaction of genetic, environmental, biochemical and immunological events is likely to generate a permissive milieu. It is most likely that loss of melanocytes in vitiligo occurs through a combination of several pathogenic mechanisms at molecular and cellular levels that act in concert (Figure 1).

Further studies on melanocyte antibodies and target antigens along with possible trigerring factors for generation of autoimmunity in vitiligo patients might refine the diagnostic and prognostic testing of vitiligo and also reveals putative T-cell targets which add to the therapeutic armamentarium.

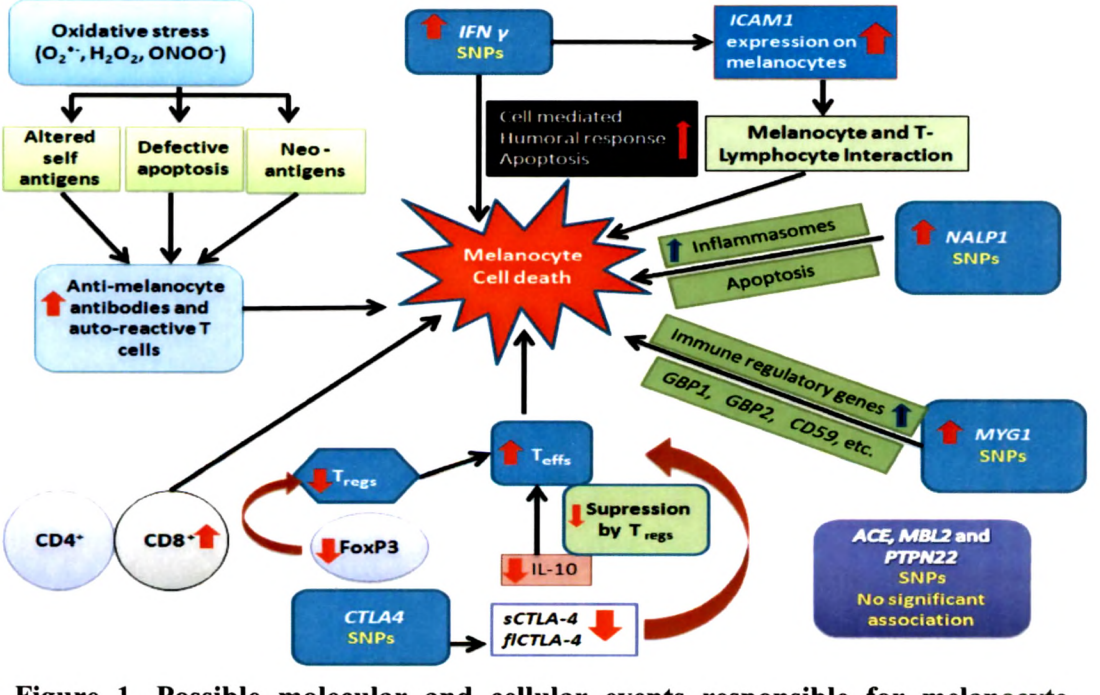

Figure 1. Possible molecular and cellular events responsible for melanocyte destruction in autoimmune pathogenesis of vitiligo:

Melanocyte cell death in vitiligo is a complex process triggered by several different pathways. One of the normal pathways is disturbed i.e., the regulation of T-cells as $CD8^+$ T-cell counts are increased in patients resulting in melanocyte death. Tregs which are important in suppression of activated T-cells are decreased in vitiligo patients. The lowered Treg levels may be due to decreased FoxP3 expression thereby increasing the levels of Teff cells. Moreover, increased Teff cells may be due to low *IL10* expression and thereby conferring low suppressive capacity of Tregs as observed in vitiligo patients. Another important regulator of T-cells is *CTLA4*. Both *sCTLA4* and *flCTLA4* levels were decreased in vitiligo patients and correlated with the presence of SNPs in *CTLA4*. Decreased *CTLA4* expression increases the Teff cells resulting in melanocyte death. A Th-1 cytokine IFNγ is increased in vitilgio patients and its expression is correlated with its polymorphisms. IFNγ is a paracrine inhibitor of melanocyte and hence can result in melanocyte destruction via enhancing humoral, cell mediated immunity and apoptosis. In turn IFNγ induces the expression of *ICAM1* on melanocytes. Increased *ICAM1* expression as observed in vitiligo enhances the melanocyte and T-lymphocyte interaction and thereby leading to melanocyte death. Expression of *NALP1* is increased in vitiligo patients and correlated with the presence of SNPs. Increased *NALP1* expression results in increased inflammasomes formation leading to apoptosis of melanocytes. MYG1, a stress induced molecule is increased in vitiligo patients. Increased *MYG1* levels result in overexpression of several immune regulator genes thereby affecting melanocyte death.

High levels of ROS and RNS are observed in vitiligo patients, which can directly cause melanocyte death. Moreover, the free radicals can react with cellular constituents resulting in altered antigenicity of the self antigens, formation of neo-antigens and cause defects in the apoptotic machinery of the cell, ultimately provoking humoral and cell mediated immunity by producing antimelanocyte antibodies and auto-reactive T-cells leading to melanocyte destruction. Certain other molecules like ACE, PTPN22 and MBL2 with their mutant proteins may directly or indirectly participate in melanocyte destruction.

Vitiligo Clinical Proforma

Department of Biochemistry, The Maharaja Sayajirao University of Baroda,
Faculty of Science, Vadodara, Gujarat - 390 002

Dr. _____ Date: _____
Name: _____ Age: _____ Sex: _____

Address_____
_____

Marital status: Married/Single Religion: _____

Occupation: _____ Income: _____

Education: _____ Native Place: _____

History of illness

1. Age of onset: _____
2. Site of onset: _____
3. Duration: _____
4. Lesions: Number_____ Size: _____ Shape_____
5. Condition of hair: no/ black/ gray
6. Any associated symptoms: Itching/ burning/ pain
7. Mode of spread: Static/ growing/ receding
8. Use of any drugs before onset of illness
   _____
   _____
   _____
   _____
9. Aggravating factors: occupational/ hobbies/ trauma/ drug/ work/sunlight/ emotional factors/ menstruation/ pregnancy/ food/ cosmetics/ chemicals/ any other:_____
10. What does the patient associate it with as cause_____
    _____
11. Treatment: yes/ no Regular/ Irregular
12. Recovery: Some/ good/ poor/ no response
13. Sudden repigmentation: yes/ no
14. Local sensitivity (photo):
    _____

Appendix I

15. Associated diseases:

_____

_____

16. Family history
    A. I[st] degree relatives: father/mother/sister/brother/daughter/son
    B. 2[nd] degree relatives: paternal grandmother/paternal grandfather/ maternal grandmother/maternal grandfather/maternal or paternal uncles or aunts
    C. 3[rd] degree relatives: cousins/nephews/nieces

17. Personal history
    Diet: veg/ nonveg/ ovoveg/ mixed

    Routine food: _____

18. Habits: smoking/ tobacco chewing/ alcoholism

19. Types of vitiligo
    a. Generalized/ Localized
    b. Unilateral/ Bilateral
    c. Symmetrical/ Symmetrical
    d. Universal
    e. Acrofacial
    f. Segmental
    g. Focal
    h. Liptip vitiligo
    i. Trichrome
    j. Quadrichrome

20. Treatment:

_____

_____

21. Koebners phenomenon: Yes/ No

The purpose of the study has been explained to me. I..... hereby agree to donate 5 ml of blood sample for the research purpose.

Signature of Patient

Date:

Appendix I

CONTROL CLINICAL PROFORMA

Department of Biochemistry

Faculty of Science

The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat- 390 002

Date: _____

Name: _____ Age: _____ Sex: _____

Address_____

Marital status: Married/Single Religion:

_____

Blood Group:

Occupation: _____ Income: _____

Education: _____ Native place: _____

1. Any Disease (including Vitiligo):

   _____

---

2. Personal history
   Diet: Veg/ nonveg/ ovoveg/ mixed

   Routine food: _____

3. Habits: Smoking/ tobacco chewing/ alcoholism

I, _____ have understood the aim of this study and willing to donate 5 ml blood sample for this purpose.

Signature