Synopsis

Introduction:

Melanocytes are specialized skin cells, residing at the basal layer of epidermis, producing protective skin darkening pigment 'melanin' which provides protection against various kinds of genotoxic stress (Lin and Fisher, 2007). Melanocytes, the neural crest derived cells are present in various tissues other than epidermis for e.g. retinal epithelium, mucosa, cochlea (ear), iris (eye), and mesencephalon (brain), adipocytes etc. (Cichorek *et al.,* 2013; Yamaguchi *et al.,* 2014). Loss of functional melanocytes may lead to various kinds of disorders for e.g. vitiligo, albinism, oculocutaneous albinism etc. (Yamaguchi *et al.,* 2014).

Vitiligo is an acquired hypomelanotic pigmentary disorder characterized by the presence of circumscribed depigmented macules in the skin, caused due to loss of functional melanocytes. Studies have revealed a worldwide incidence ranging from 0.04-2.16 % (Kruger *et al.,* 2012). In India, it affects ~0.5 % of population and the states of Gujarat and Rajasthan have the highest incidence rate of ~8.8% (Sehgal and Srivastava, 2007). Earlier we have reported 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have first degree relative affected implicating importance for genetic predisposition for vitiligo susceptibility (Shajil *et al.,* 2006). The etiology of vitiligo remains obscure despite being in focused debate for more than six decades and hence, it is important to unravel the underlying pathomechanisms of vitiligo (Laddha *et al.,* 2013; Mansuri *et al.,* 2014). Various hypotheses have been put forward for explaining vitiligo pathomechanism i.e., autoimmune, neural and oxidative stress which alone or in various combinations contribute towards vitiligo precipitation. The inheritance pattern of vitiligo does not follow the simple Mendelian pattern of an autosomal dominant or autosomal recessive or X- linked inheritance. Therefore, it has been proposed that vitiligo is a polygenic disease influenced by a set of recessive alleles occurring at several unlinked autosomal loci that collectively confer the vitiligo phenotype (Nath *et al.,* 1994).

Increasing evidences including our previous studies propose that genetic polymorphisms of *TNFA, IL4, IFNG* and *ICAM1, IL1B, TNFB, SOD, CAT, NLRP1, CTLA4, MYG* and *GPX1* might be playing a crucial role in vitiligo susceptibility. Genetic studies on vitiligo suggest that the genetic variants of genes that are involved in oxidative stress and immune regulation could be responsible for conferring susceptibility or protection towards vitiligo (Laddha *et al.,* 2012; Imran *et al.,* 2012; Dwivedi *et al.,* 2013; Laddha *et al.,* 2014; Laddha *et al.,* 2013; Laddha *et al.,* 2013; Shajil *et al.,* 2007; Dwivedi *et al.,* 2013; Dwivedi *et al.,* 2011; Mansuri *et al.,* 2016). A key component in the inflammatory response is the increased production of pro-inflammatory

cytokines such as IL1β, IFNγ and TNFα which are found to be increased in vitiligo (Laddha *et al.,* 2014; Dwivedi *et al.,* 2013; Laddha *et al.,* 2012). 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 pathogenic mechanisms that act in concert.

To date the most accepted hypothesis is selective autoimmunity towards melanocytes therefore generalized vitiligo is also included in the list of autoimmune disorders (van den Boorn *et al.,* 2011, Mosenson *et al.,* 2013). Circulating autoantibodies that are present in the sera of vitiligo patients, and autoreactive T lymphocytes are found to recognize melanocyte antigens (Naughton *et al.,* 1983). Immunohistochemical studies have confirmed the presence of infiltrating T cells (Le Poole *et al.,* 1996). T cell infiltrates with a predominant presence of $CD8^+$ T cells are detected in generalized vitiligo (Abdel-Naser *et al.,* 1994; Badri *et al.,* 1993). Our studies also showed that ~75% of Gujarat vitiligo patients have antimelanocyte antibodies in their circulation and oxidative stress acts as an initial trigger for onset of vitiligo and autoimmunity is responsible for disease progression (Laddha *et al.,* 2014). Imbalance between pro and anti-inflammatory cytokines is well documented in vitiliginous skin and circulation. These immune-regulatory molecules are capable to initiate melanocyte apoptosis and are mainly produced by cells of immune system and/or surrounding keratinocytes. In recent years, a complex melanogenic cytokine network between skin cells which regulate melanocyte activity has been demonstrated. Epidermal melanin unit is a structural and functional unit in epidermis where single melanocyte is surrounded by ~36 keratinocytes. (Nordlund, 2007). Keratinocytes secrete additional cytokines, such as IL6 and TNFα, which function as paracrine inhibitors of growth and proliferation of melanocyte (Swope *et al.,* 1991). Altered levels of keratinocyte derived mediators have been recently described in vitiligo epidermis, suggesting an important role of epidermal cytokines in vitiligo pathogenesis (Moretti *et al.,* 2002; Lee *et al.,* 2005). Thus, cytokine imbalance has been well documented in vitiligo patients; however, their exact mode of action on melanocyte biology is not well explored.

PROPOSED OBJECTIVES:

(1) Isolation and culture establishment of primary normal human melanocytes from epidermal human skin.

(2) To study the transcript levels of the immune regulator genes: *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin.

(3) To study the dose dependent effect of the interleukins: TNFα, IL1α, IL6 and IL10 on *in vitro* cultured melanocyte cell death, expression of melanin synthesis genes and immunoregulatory genes.

(4) Effect of receptor activation of TNFR1, IL1R1, IL6R and IL10R upon treatment of TNFα, IL1α, IL6 and IL 10 respectively on *in vitro* cultured melanocytes.

(5) To study the role of PARP upon H_2O_2 stimulation of *in vitro* cultured melanocytes.

(6) Genotyping of candidate genes: *IL1RN* intron *2 VNTR, IL6* and *IL10* in vitiligo patients and controls from Gujarat population with possible genotype-phenotype correlation.

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:

Isolation and culture establishment of primary normal human melanocytes from epidermal human skin

Vitiligo is a skin disorder caused due to selective destruction of epidermal melanocytes of unknown etiology. Cytokine imbalance is documented in vitiligo patients, but their exact mode of action on melanocyte is not well explored. Therefore, to study the effect of candidate cytokines on melanocyte biology it is very essential to establish the *in vitro* system as the *in vivo* or animal models system is not apt to study the role of cytokines *per se*. Moreover, cytokines affect melanocyte viability, their respective receptors activation and to address other parameters to

explore melanocyte biology in relation to cytokine imbalance, required designing of an ideal *in vitro* system. In our preliminary standardization experiments we have used melanoma cell lines SKMel28 (low melanin producing Caucasian origin cell line), G361 (melanin producing cell line) as well as transformed primary melanocytes PIG1, PIG3V derived from healthy individuals and vitiligo patient respectively. Our results showed that primary cultured normal human melanocytes (NHM) showed increased sensitivity and resemblance as compared to other *in vitro* models. The difference can be attributed due to incorporation of mutations during the procedure of immortalization. Therefore, NHM is a better model for studying the effect of various immnoregulatory molecules on melanocytes. The NHM culture system used for our studies is free of phorbol 12-myristate 13-acetate (PMA) and serum which minimizes the artifact of cytokine mediated sensitive studies.

Culture establishment of primary normal human melanocytes (NHM): Melanocytes were isolated from human skin samples and cultured successfully using the standard protocol with slight modifications (Im and Park ,1992; Czajkowski *et al.,* 2007). Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease at 4°C. In order to separate epidermal cells, the epidermis was incubated in Trypsin Solution. Melanocyte-keratinocyte mixed population starts appearing around 4-9 days. Melanocytes were purified from keratinocytes by two rounds of differential trypsinisation, which is based on the sturdier and adherent property of keratinocytes as compared to melanocytes. Additionally, melanocytes were given G418 treatment for gradual removal of fibroblasts to obtain melanocyte and were further split in ratio of 1:3, when cell confluency reached ~80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to fifth passage were used for experiments. The pure culture of melanocytes was confirmed by L-DOPA staining (Iijima *et al.,* 1957).

Objective 2:

To study the transcript levels of the immune regulator genes: *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin

Skin samples stored in RNA preservative solution were used for RNA isolation and further converted to first strand cDNA for gene expression studies. Expression levels of *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* were monitored and analyzed between lesional vs. control, non- lesional vs. control and non- lesional vs. lesional skin (n=12) as stated in Table1.

Table 1: Transcripts levels analysis of candidate genes in vitiligo patients and controls (n=12)

Gene	Lesional skin vs. Control (*p* value)	Non lesional skin vs. Control (*p* value)	Non -Lesional vs. lesional skin (*p* value)
TNFA	0.0246 ↑	0.0467 ↑	0.6749 ns
IL1A	0.6000 ns	0.4004 ns	0.2886 ns
IL1B	0.5085 ns	0.0290 ↑	0.0021 ↑
IL4	0.2886 ns	0.2152 ns	0.9669 ns
IL6	0.5473 ns	0.5697 ns	0.9511 ns
IL10	0.0357 ↓	0.5565 ns	0.0980
IL1R1	0.8186 ns	0.2418 ns	0.8180 ns
IL1RN	0.2147 ns	0.8604 ns	0.4080 ns
IFNG	0.0231 ↑	0.0138 ↑	0.0808 ns

Increased levels of pro-inflammatory cytokines in the skin microenvironment of vitiligo patients were found as compared to healthy controls. Our results showed imbalance between pro and anti-inflammatory cytokines in the skin samples of vitiligo patients as compared to controls. *IL1B* expression level was higher in non lesional as compared to lesional skin suggesting its important role in disease progression. Expression levels of *TNFA, IFNG* was found to be increased in lesional as well as non lesional skin of vitiligo patients as compared to controls. Whereas, non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1*and *IL1RN* was observed between lesional and non lesional skin of vitiligo patients as well as in comparison to controls. Additionally, decreased level of *IL10* an anti inflammatory cytokine in lesional skin of vitiligo patients was observed. Overall, these skin based results provide evidence of cytokine imbalance in the microenvironment of skin.

Objective 3:

To study the dose dependent effect of the interleukins: TNFα, IL1α, IL6 and IL10 on *in vitro* cultured melanocyte cell death, expression of melanin synthesis genes and immunoregulatory genes

NHM between 3-5 passages were used for MTT assay (Mosmann, 1983) to monitor the dose dependent effect of various cytokines on *in vitro* cultured melanocytes.

Dose dependent effect of TNFα on melanocytes

NHM were treated with different doses of TNFα (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=4). The NHM did not show any difference in morphology and viability up to 24 hrs. After 48 hrs of treatment all doses ≥10ng/ml exhibited significant decrease in melanocyte viability (p<0.0001 for all treatments). TNFα inhibits significantly growth and proliferation of NHM in a dose and time dependent manner. Morphological observation revealed cellular vacuolization at higher magnification, tapering of cells and shedding of dendrites, cell distortion and clustering was observed upon exogenous TNFα. Additionally, we found significant synergism of TNFα with other pro inflammatory cytokines IL1α, IL6 and H_2O_2 (which is prevalent in the skin micro environment of vitiligo); on the other hand, IL10 an anti inflammatory cytokine was proficient to significantly rescue cytotoxicity caused due to TNFα.

Dose dependent effect of IL1α on melanocytes

NHM were treated with different doses of IL1α (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs. Also, there was no significant morphological change observed in NHM even after 48 hrs of IL1α treatment. However, 100 ng/ml of IL1α treated NHM showed significant decrease in viability (p=0.0210) after 48 hrs of treatment, as compared to control. However, 10 ng/ml & 50ng/ml doses of IL1α did not show any significant difference (p=0.6658 and p=0.9301 respectively) in cell viability. The effect of IL1α was found to be cytostatic on NHM (Singh *et al.*, 2016).

Dose dependent effect of IL6 on melanocytes

NHM were treated with different doses of IL6 (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs. Also, there was decrease in NHM confluency when observed after 48 hrs of IL6 exogenous stimulation and significant decrease in cell viability with 10, 50, 100 ng/ml IL6 stimulation (p<0.005 for all treatments).

Dose dependent effect of IL10 on melanocytes

IL10 an anti-inflammatory cytokine didn't have significant effect on melanocyte.NHM were treated with different doses of IL10 (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM showed non-significant difference in morphology and viability up to 48 hrs of IL10 stimulation (p>0.05 for all treatments).

Overall, among the studied cytokines, TNFα exhibits the most potent response. Further, we tried to monitor detailed response of TNFα on melanocytes. Exogenous stimulation of TNFα on melanocytes caused significantly decreased viability with significant alteration in following parameters: increased cellular and mitochondrial ROS levels; ~20% decrease in mitochondrial complex1 activity; decrease in melanin content via shedding of dendrites and down regulation of *MITF-M, TYR* and increased *TNFR1, IL6, ICAM1* expression while, *TNFR2* levels remained unaltered. Upon TNFα stimulation, LC3I-II conversion at 12 hrs and caspase-8 activation at 48 hrs were observed, which disappeared at 48 hrs and 24 hrs respectively. Pro inflammatory cytokines, TNFα, IL1α and IL6 caused a dose dependent decrease in cell viability of melanocytes. Anti- inflammatory cytokine IL10 did not reveal significant alteration in growth and viability of melanocytes. All cytokines in synergism with H_2O_2 (which is higher in skin microenvironment of vitiligo) can lead to enhanced compromised state of melanocytes, effecting overall survival and homeostasis of melanocytes.

Objective 4:

Effect of receptor activation of TNFR1, IL1R1, IL6R and IL10R upon treatment of TNFα, IL1α, IL6 and IL 10 respectively on *in vitro* cultured melanocytes

TNF receptors expression upon TNFα treatment to NHM

TNFα mediates its action *via* its two receptors TNFR1 and TNFR2, TNFR1 is present on most of the cells but the presence of TNFR2 is restricted to few cell lineages. TNFR1 has death domain and may be responsible for apoptosis, on the contrary TNFR2 lacks death domain. Melanocytes have both the receptors of TNFα i.e., TNFR1 as well as TNFR2. There was significant upregulation of *TNFR1* transcript levels (p=0.024; n=3) whereas there was non-significant difference in the transcript levels of *TNFR2* upon TNFα stimulation (10ng/ml). TNFR1 serves as the major mediator of TNFα induced signaling pathways. Also our confocal microscopy and flow cytometry results suggest that the total membrane expression of TNFR1 is increased upon TNFα stimulation (p=0.004 & 17.2% increased expression as compared to controls respectively; n=3).

IL1R1 membrane expression upon IL1α treatment to NHM

Cytokines mediate their action *via* interacting with their respective receptors and hence we monitored the effect of IL1α on the membrane expression of IL1R1 (n=3). IL1α stimulation showed significant increase in membrane expression of IL1R1 upon 10 ng/ml (~25%) and 100 ng/ ml (~22%) of IL1α treated NHM as compared to untreated controls. However, there was no significant difference in the transcript levels of *IL1R1* upon IL1α exogenous stimulation, suggesting involvement of post translational modifications in regulation of membrane expression of IL1R1 on NHM.

IL6R membrane expression upon IL6 treatment to NHM:

Significant upregulation of *IL6R* transcript levels upon exogenous IL6 (10ng/ml) stimulation was observed (n = 3; $p < 0.05$). Further, the membrane expression of IL6R upon exogenous IL6 (10ng/ml) stimulation was found to be increased (n =3; p=0.0262).

IL10R membrane expression upon IL10 treatment to NHM

The NHM cells were treated with IL10 (10ng/ml) and *IL10R* transcript levels were measured after 48 hrs of treatment and the results showed no significant difference in the transcript levels of *IL10R* at 10ng/ml IL10 (n = 3; p=0.6432). Further, effect of IL10 on expression of IL10R on NHM was observed and non significant difference i.e. ~0.9% increase in membrane expression was observed as compared to untreated controls (n =3). Overall, all receptor expression studies indicate positive feedback regulation of receptors upon treatment with their respective cytokines.

Objective 5:

To study the role of PARP upon H_2O_2 stimulation of *in vitro* cultured melanocytes

Poly (ADP-ribose) polymerase (PARP) is an enzyme involved in DNA repair, genomic stability, apoptosis, gene transcription, proliferation, and autoimmunity. One of the major players of ROS-induced cell death is hyper-activation of PARP1 for the recruitment of repair enzymes. PARP1 acts as a co-activator of nuclear factor-κB (NFκB) and regulates NFκB dependent gene expressions. However, the role of PARP1 activation upon oxidative stress induction in melanocytes has not been elucidated yet. Therefore, we aimed to monitor the effect of PARP inhibitor (PJ34) on H_2O_2 treated NHM. NHM with or without pretreatment with PJ34 were exposed to H_2O_2 to monitor their rescue from H_2O_2 induced cell death using PJ34, a potent

PARP1 inhibitor. Further, cell viabilities were measured by trypan blue exclusion assay. Activation of PARP and oxidative stress-induced PARylation were assessed by Western blotting. Our results showed significant NHM death upon H_2O_2 treatment (p<0.0001) in a dose dependent manner (0, 5, 10, 25 μM H_2O_2; 30 min exposure). PJ34 treatment showed no toxicity to NHM (10 μM; 48 hrs (p<0.0001)). Inhibition of PARP1 (PJ34 10 μM; 4 hrs) showed significant rescue of NHM from (p<0.0001) induced death (p=0.0002) and decreased levels PARP cleavage and PARylation pattern as compared to untreated controls. These results suggest that PJ34 attenuates H_2O_2 induced NHM death via oxidative stress generation and therefore could be used as a potential drug in treatment modalities of vitiligo. The present study indicates that PARP1 might be playing a crucial role in melanocyte biology under oxidative stress condition in vitiligo pathogenesis.

Objective 6:

Genotyping of candidate genes: *IL1RN* intron *2 VNTR, IL6* and *IL10* in vitiligo patients and controls from Gujarat population with possible genotype-phenotype correlation

a) Genotyping of Interleukin 1 Receptor Antagonist (*IL1RN*) intron 2 VNTR polymorphism and determining the expression levels of *IL1RN* in Gujarat vitiligo patients and controls

The IL1RN is an important immunologic regulator that competes with IL1α and IL1β for the IL1R1 and IL1 RII receptor in target cells and act as its negative regulator with anti-inflammatory effects (Granowitz *et al.,* 1991). The gene sequence shows an 86-bp variable number tandem repeat (VNTR) in intron 2 of the *IL1RN* gene. This polymorphism has six alleles, comprising of one to six repeats of an 86-bp sequence. The four-repeat (*IL1RN**1) and two-repeat (*IL1RN**2) alleles are most common, while the other alleles occur at a combined frequency of less than 5% (Tarlow *et al.,* 1993; Vamvakopoulos *et al.,* 2002). The number of repeats may be of functional significance as these repeats contain putative binding sites for transcription factors which needs further investigation (Tarlow *et al.,* 1993). The role of *IL1RN* intron 2 VNTR (rs2234663) is well established with development of inflammatory disorders for several years (Fischer *et al.,* 1992; McIntyre *et al.,* 1991; Xu *et al.,* 2011). The pro-inflammatory cytokine IL1β and its antagonist, IL1RN, are encoded by polymorphic genes (Wilkinson *et al.,* 1999). An association between polymorphism in the *IL1RN* intron 2 VNTR (rs2234663) and several other autoimmune disorders including vitiligo has been reported. To date, there is no report from India regarding the *IL1RN*

intron 2 VNTR (rs2234663) association with vitiligo. We have investigated the association between *IL1RN* intron 2 VNTR polymorphism and monitored *IL1RN* transcript levels from PBMCs, and performed possible genotype phenotype correlation in vitiligo patients and healthy controls from Gujarat population. 307 vitiligo patients and 316 controls were enrolled; genotyping was performed using polymerase chain reaction (PCR). Relative gene expression was measured in PBMCs (n=36) using real-time-PCR. Significant difference was observed in *IL1RN**1/2 genotype between active and stable vitiligo (p =0.0172). Also, *IL1RN**2/2 genotype and allele frequencies differed significantly between stable vitiligo and controls (p=0.0246) and (p=0.0046) respectively. Moreover, significant difference was observed for *IL1RN**A2 in active and stable vitiligo (p=0.0060).However, other camparisons showed non-significant association of genotype as well as allele frequencies. Also, non-significant difference for *IL1RN* expression (p=0.5962) was observed between pateints and controls. Interestingly, our genotype-phenotype correlation showed individuals with *IL1RN**2/2 showed higher *IL1RN* expression when compared to other major genotypes *IL1RN**1/2 (p=0.01) and *IL1RN**1/1 (p=0.03). Additionally, we observed *IL1RN**3/2 only in vitiligo patients while *IL1RN**5/2 was observed only in controls.

The present study demonstrates the association of allele as well as genotype of *IL1RN* intron 2 VNTR (A2) polymorphism with active vitiligo patients and increased *IL1RN* expression (allele 2 carriers), suggesting *IL1RN**A2 as a risk factor for progressive vitiligo in Gujarat population. Larger studies with different ethnicities are required to find out the impact of *IL1RN* VNTR polymorphism as a risk factor for developing vitiligo.

b) Genotyping of *IL6* -174 G/C (rs1800795); -572 G/C (rs1800796) promoter polymorphisms and determining the expression levels of *IL6* in Gujarat vitiligo patients and controls

IL6 is a multifunctional Th2 cytokine and increased levels of IL6 have been reported in the serum and lesional skin samples of vitiligo patients. The gene for human *IL6* has been mapped to chromosome 7p21. Increased levels of serum IL6 have been reported in vitiligo patients in Indian population (Singh *et al.*, 2012). IL6 secreted by neighboring keratinocytes in epidermal melanin unit is reported to be a paracrine inhibitor of growth and proliferation of melanocytes (Swope *et al.*, 1990). Several allelic variants have been identified in the *IL6* gene promoter region which regulates the expression of *IL6* (Fishman *et al.*, 1998; Terry *et al.*, 2000). IL6 induces and enhances ICAM-1 expression on melanocytes, which promotes melanaocyte-leukocyte attachment (Yohn *et al.*, 1990). IL6 stimulates the inflammatory and auto-immune processes in

many diseases such as diabetes, atherosclerosis, depression, Alzheimer's disease, systemic lupus erythematosus, multiple myeloma, prostate cancer, and rheumatoid arthritis.

Two promoter polymorphisms of IL6 -572G/C and -174 G/C have not been reported in vitiligo till date. But these polymorphisms have been found to be associated with various autoimmune disorders. Investigated polymorphisms are located adjacent to cis-acting regulatory elements involved in regulating *IL6* expression at the level of transcription, suggesting that they may influence the interaction of proteins with the DNA at these sites (Tanabe *et al.,* 1998). *IL6* -572 G/C (rs1800796) and -174 G/C (rs1800795) polymorphisms were genotyped in 322 vitiligo patients and 343 controls; 100 vitiligo patients and 100 controls respectively, using PCR-RFLP technique.

IL6 -174 G/C (rs1800795) promoter polymorphism was found to be monogenic for GG allele screened in 100 vitiligo patients and equal number of controls in Gujarat population. Due to occurrence of monogenic genotype the sample size was confined to 100 each for patients and controls. Three genotypes were identified in both patients and controls were: GG, GC and CC for *IL6* -572 G/C (rs1800796) promoter polymorphism. The heterozygous 'GC' genotype was found to be significantly higher in control group and is associated with vitiligo susceptibility (p=0.0336, OR=0.6416, CI=0.42-0.9678). In addition, other genotype CC frequencies did not differ significantly between vitiligo patients and controls for *IL6* -572 G/C SNP (p=0.4078, OR=0.8114, CI=0.49 – 1.331). Similarly, the allele frequency did not differ significantly between vitiligo patients and controls (p=0.5281, OR=0.9331, CI=0.75 – 1.157). Further, we analysed the vitiligo patient's data in subgroups based on gender, disease progression and type of vitiligo. Analysis based on male and female revealed that the increased frequency of the 'CC' genotype in male as compared to female vitiligo patients (22.0% versus 9.0%, p=0.0282). However, there was no significant difference between 'GC' genotype and minor allele frequency of 'C' (p=0.3322, OR=0.7781, CI=0.46-1.293 and p=0.1172, OR=1.286, CI=0.93-1.761 respectively).

Analysis based on disease progression revealed that the increased frequency of the minor allele 'C' occurred predominantly in patients with AV (43.0% versus 52.0%, p=0.0025) compared to controls suggesting the important role of 'C' allele in disease progression. However, there was no significant difference between SV patients and controls (p=0.0892). Interestingly, the 'GC' genotype was predominant in controls as compared to AV and SV patients (65.0% versus 57.0% and 50.0% respectively; p=0.0003 and 0.0020 respectively). However, we did not find significant association between other comparisons. Further, analysis based on type of vitiligo revealed that

the increased frequency of the minor allele 'C' occurred predominantly in patients with GV (43.0% versus 52.0%, p=0.0024) compared to controls. However, there was no significant difference between LV patients and controls (p=0.329). Interestingly, the 'GC' genotype was predominant in controls compared to GV patients (66.0% versus 54.0%, p<0.0001) suggesting the important role of 'C' allele in GV. The 'CC' genotype was predominant in controls compared to GV patients (19.0% versus 16.0%, p=0.0013). However, there was no significant difference in genotype and allele frequencies based on age of onset, family history of patients.

Relative gene expression analysis in 70 patients and 74 controls revealed a non-significant difference in expression of *IL6* transcripts in patients as compared to controls (Mean ΔCp – SEM: 12.29 ± 0.7436 versus 12.09 ± 0.6649; p =0.8423). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.147-fold increase in the expression of *IL6* transcript levels in patients compared to controls.

Gujarat population is monogenic (allele G) for *IL6* -174 G/C polymorphism; other genotype phenotype correlation studies have shown association of higher transcript levels of *IL6* with G allele in this polymorphism. The -572 G/C polymorphism minor allele 'C' has significant difference in distribution in stratification of vitiligo patients data, whereas there was no significant difference in *IL6* transcript levels between vitiligo patients and controls.

c) Genotyping of *IL10*: -819 C/T (rs1800871), -592C/A (rs1800872) and -1082G/A (rs 1800896) promoter polymorphisms and determining the expression levels of *IL10* in Gujarat vitiligo patients and controls

IL10 acts as an anti-inflammatory cytokine and it has been reported (Abanmi *et al.* 2008) that low levels of *IL10* is governed by the promoter polymorphisms. Hence, it becomes pertinent to investigate *IL10* promoter polymorphisms and its levels in vitiligo patients. Previously, Zhao *et al.,* showed significant decrease in the expression of *IL10* in vitiligo patients as compared to controls (Zhao *et al*. 2010). We have investigated three polymorphisms in promoter region of *IL10* gene for their role in susceptibility to vitiligo, by a case-control study involving around 400 patients and healthy age matched controls by PCR-RFLP and ARMS-PCR techniques. Our results showed that genotype and allele frequencies of *IL10* -819 C/T (p<0.0001; p<0.0001 respectively), were significantly different between vitiligo patients and unaffected controls suggesting the significant association of -819T allele with vitiligo susceptibility. Additionally, the allele frequency of -1082 G/A differed significantly between vitiligo patients and controls (p=0.0311). However, the genotype and allele frequencies for *IL10* -592C/A (p= 0.5796; p= 0.7096

respectively) and genotype frequencies of *IL10* -1082G/A (p = 0.1445) did not differ significantly between vitiligo patients and controls suggesting the non-association of these SNPs with vitiligo susceptibility. The LD analysis revealed that the three promoter polymorphisms investigated in the *IL10* gene were in low to moderate LD association; -819 C/T and -592 C/A polymorphisms were in moderate LD association (D'= 0.516 and r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection. Whereas, ACC (p=0.196 X 10^{-3}), GCC (p=0.021), GTA (p= 0.019) haplotypes were more found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546) haplotypes were not found to be associated with vitiligo. Further, transcript as well as protein levels of IL10 will be estimated from PBMCs and plasma respectively in vitiligo patients and controls, and the results will be discussed in thesis.

The present study supports the autoimmune hypothesis of vitiligo pathogenesis with strong association of *IL10* -819C/T polymorphism in vitiligo patients thus, suggesting the crucial role of *IL10* in vitiligo pathogenesis. The present study will pave the pathway for understanding the higher prevalence of vitiligo in Gujarat population.

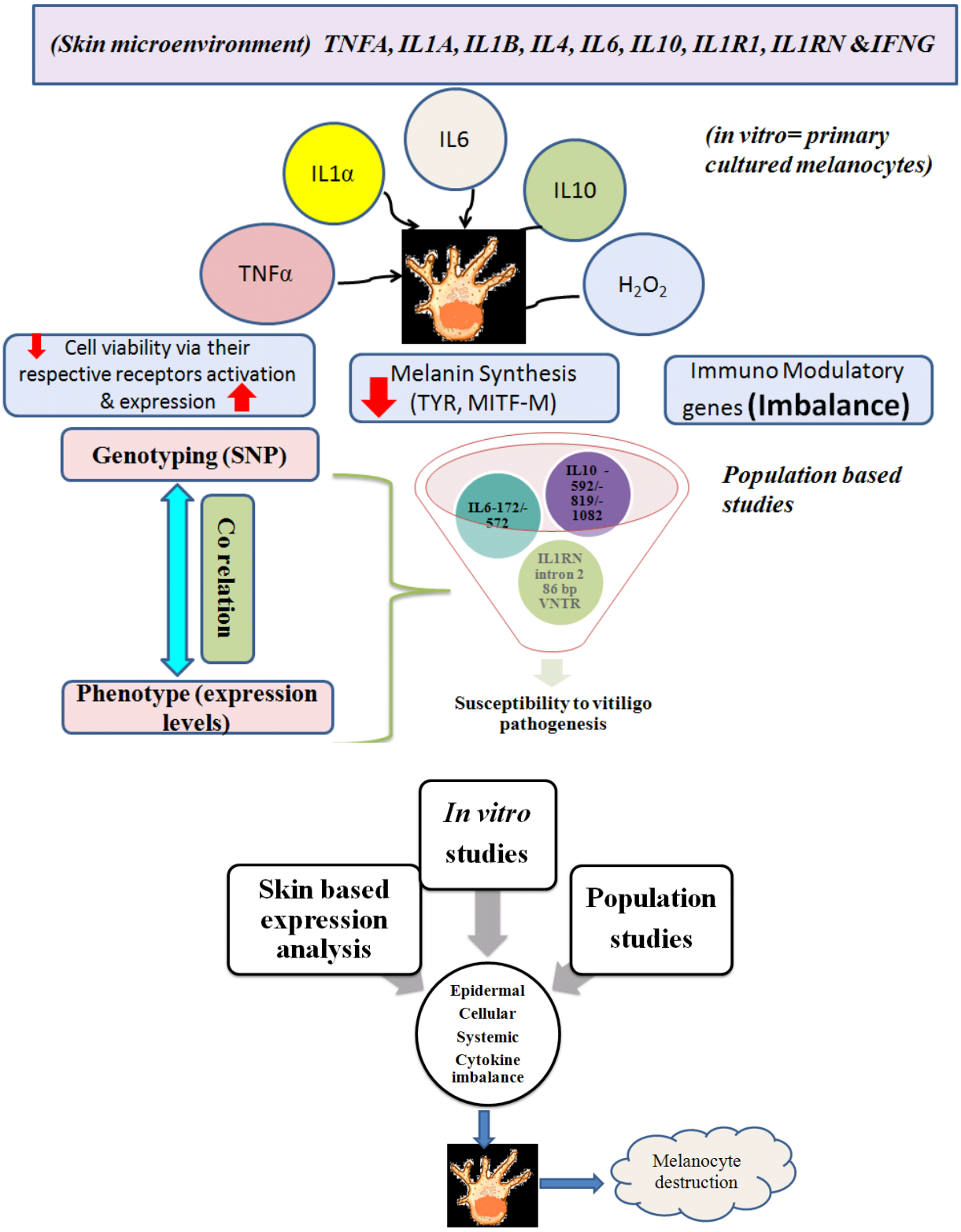

Figure 1: Role of cytokine imbalance in vitiligo pathogenesis: Increased levels of pro inflammatory cytokines *TNFA, IFNG* and *IL1B*; decreased levels of anti inflammatory cytokine

IL10 was observed in the skin microenvironment if vitiligo patients as compared to controls. Pro inflammatory cytokines: TNFα, IL1α, IL6 as well as H_2O_2 decreased melanocytes viability alone as well as in synergism. Additionally, the pro inflammatory cytokines altered expression of melanin synthesis genes (*TYR, MITF-M*), cell adhesion molecule (*ICAM-1*) as well as other immunoregulatory genes. On the contrary, IL10 was capable to rescue TNFα induced cytotoxicity in melanocytes. Population based study indicated the importance of genetic variants in vitiligo predisposition. Analysis of single nucleotide polymorphisms at promoter sites (*IL6, IL10*) as well as VNTR polymorphisms (*IL1RN)* of addressed cytokines revealed association of a few studied genetic variants with vitiligo susceptibility in Gujarat population. The genotype-phenotype analysis will be discussed in the thesis for holistic view of addressed polymorphisms. Overall, cytokine imbalance at tissue, cellular and genetic levels may aggravate the compromised state of melanocytes, advocating autoimmune mediated disease progression concept.

Conclusion: The present study suggests the cytokine imbalance and its involvement in vitiligo pathogenesis. Our skin based studies showed cytokine imbalance of pro- and anti inflammatory cytokines in the microenvironment of vitiligo. Expression levels of *TNFA, IFNG* were found to be increased in lesional and non lesional skin of vitiligo patients as compared to controls. Whereas, non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1*and *IL1RN* were observed in lesional skin compared to non lesional skin of vitiligo patients, as well as control skin. In addition, decreased levels of *IL10* (anti inflammatory cytokine) were observed in lesional skin of vitiligo patients compared to controls skin. Candidate cytokines TNFα, IL1α, IL6 and IL10 were further used for *in vitro* studies on primary normal human melanocytes (NHM). Pro inflammatory cytokines TNFα, IL1α, IL6 as well as H_2O_2 stress (which is higher in vitiligo skin) showed significant decrease in NHM viability. Interestingly, NHM upon H_2O_2 treatment showed PARP1 activation and increased PARylation of proteins which could be intercepted by PARP inhibitor (PJ34). The cytokines mediate their action *via* increased expression of the respective receptors on melanocytes. TNFα and IL1α also showed decreased expression of melanin synthesis genes *TYR, MITF* and melanin content upon exogenous stimulation of melanocytes. Increased expression of *ICAM* was observed which could act as a link between cytokines and T-cells; and T cells among others play a key role in the pathogenesis of generalized vitiligo. Further, to study genetic predisposition of vitiligo patients in Gujarat population, we investigated the promoter polymorphisms of *IL10* -819 C/T (rs1800871), -592C/A (rs1800872) and -1082G/A (rs 1800896); the promoter polymorphisms of *IL6* -174 G/C (rs1800795) -572 G/C (rs1800796) and *IL1RN*

intron 2 VNTR (rs2234663) polymorphism and performed possible genotype -phenotype correlation. Overall, our studies suggest compromised state of melanocytes due to cytokine imbalance that could lead to melanocyte loss in vitiligo pathogenesis (Figure 1).

References:

1. Abanmi, A., Al Harthi, F., Zouman, A., Kudwah, A., Jamal, M.A., Arfin, M. and Tariq, M., 2008. Association of Interleukin-10 gene promoter polymorphisms in Saudi patients with vitiligo. *Disease markers*, *24*(1), pp.51-57.
2. Abdel-Naser, M.B., Krüger-Krasagakes, S., Krasagakis, K., Gollnick, H. and Orfanos, C.E., 1994. Further evidence for involvement of both cell mediated and humoral immunity in generalized vitiligo. *Pigment Cell & Melanoma Research*, *7*(1), pp.1-8.
3. Al Badri, A.M., Foulis, A.K., Todd, P.M., Garioch, J.J., Gudgeon, J.E., Stewart, D.G., Gracie, J.A. and Goudie, R.B., 1993. Abnormal expression of MHC class II and ICAM-1 by melanocytes in vitiligo. *The Journal of pathology*, *169*(2), pp.203-206.
4. Cichorek, M., Wachulska, M., Stasiewicz, A. and Tymińska, A., 2013. Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii I Alergologii*, *30*(1), p.30.
5. Czajkowski, R., Placek, W., Drewa, T., Kowaliszyn, B., Sir, J. and Weiss, W., 2007. Autologous cultured melanocytes in vitiligo treatment. *Dermatologic Surgery*, *33*(9), pp.1027-1036.
6. Dwivedi, M., Laddha, N.C. and Begum, R., 2013. Correlation of increased MYG1 expression and its promoter polymorphism with disease progression and higher susceptibility in vitiligo patients. *Journal of dermatological science*, *71*(3), pp.195-202.
7. Dwivedi, M., Laddha, N.C., Imran, M., Shah, B.J. and Begum, R., 2011. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) in isolated vitiligo: a genotype-phenotype correlation. *Pigment cell & melanoma research*, *24*(4), pp.737-740.
8. Dwivedi, M., Laddha, N.C., Mansuri, M.S., Marfatia, Y.S. and Begum, R., 2013. Association of NLRP1 genetic variants and mRNA overexpression with generalized vitiligo and disease activity in a Gujarat population. *British Journal of Dermatology*, *169*(5), pp.1114-1125.
9. Dwivedi, M., Laddha, N.C., Shah, K., Shah, B.J. and Begum, R., 2013. Involvement of interferon-gamma genetic variants and intercellular adhesion molecule-1 in onset and

progression of generalized vitiligo. *Journal of Interferon & Cytokine Research*, *33*(11), pp.646-659.

10. Fischer, E., Van Zee, K.J., Marano, M.A., Rock, C.S., Kenney, J.S., Poutsiaka, D.D., Dinarello, C.A., Lowry, S.F. and Moldawer, L.L., 1992. Interleukin-1 receptor antagonist circulates in experimental inflammation and in human disease. *Blood*, *79*(9), pp.2196-2200.
11. Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J.S., Humphries, S. and Woo, P., 1998. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *Journal of Clinical Investigation*, *102*(7), p.1369.
12. Granowitz, E.V., Clark, B.D., Mancilla, J. and Dinarello, C.A., 1991. Interleukin-1 receptor antagonist competitively inhibits the binding of interleukin-1 to the type II interleukin-1 receptor. *Journal of Biological Chemistry*, *266*(22), pp.14147-14150.
13. Iijima, S. and Watanabe, K., 1957. Studies on DOPA reaction. II. Effect of chemicals on the reaction. *The Journal of investigative dermatology*, *28*(1), p.1.
14. Sungbin Im, S.K.H., Park, Y.K. and Kim, H.I., 1992. Culture of melanocytes obtained from normal and vitiligo subjects. *Yonsei Medical Journal*, *33*(4).
15. Imran, M., Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, J., Rani, R., Gokhale, R.S., Sharma, V.K., Marfatia, Y.S. and Begum, R., 2012. Interleukin-4 genetic variants correlate with its transcript and protein levels in patients with vitiligo. *British Journal of Dermatology*, *167*(2), pp.314-323.
16. Krüger, C. and Schallreuter, K.U., 2012. A review of the worldwide prevalence of vitiligo in children/adolescents and adults. *International journal of dermatology*, *51*(10), pp.1206-1212.
17. Laddha, N.C., Dwivedi, M. and Begum, R., 2012. Increased Tumor Necrosis Factor (TNF)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo. *PLoS One*, *7*(12), p.e52298.
18. Laddha, N.C., Dwivedi, M., Gani, A.R., Mansuri, M.S. and Begum, R., 2013. Tumor Necrosis Factor B (TNFB) genetic variants and its increased expression are associated with vitiligo susceptibility. *PloS one*, *8*(11), p.e81736.

19. Laddha, N.C., Dwivedi, M., Gani, A.R., Shajil, E.M. and Begum, R., 2013. Involvement of superoxide dismutase isoenzymes and their genetic variants in progression of and higher susceptibility to vitiligo. *Free Radical Biology and Medicine*, *65*, pp.1110-1125.
20. Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, M., Patel, H.H., Agarwal, N., Shah, A.M. and Begum, R., 2014. Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility. *PloS one*, *9*(9), p.e107020.
21. Laddha, N.C., Dwivedi, M., Mansuri, M.S., Gani, A.R., Ansarullah, M., Ramachandran, A.V., Dalai, S. and Begum, R., 2013. Vitiligo: interplay between oxidative stress and immune system. *Experimental dermatology*, *22*(4), pp.245-250.
22. Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, M., Gani, A.R., Yeola, A.P., Panchal, V.N., Khan, F., Dave, D.J., Patel, A. and Madhavan, S.E., 2014. Role of oxidative stress and autoimmunity in onset and progression of vitiligo. *Experimental dermatology*, *23*(5), pp.352-353.
23. Le Poole, I.C., Van den Wijngaard, R.M., Westerhof, W. and Das, P.K., 1996. Presence of T cells and macrophages in inflammatory vitiligo skin parallels melanocyte disappearance. *The American journal of pathology*, *148*(4), p.1219.
24. Lee, A.Y., 2012. Role of keratinocytes in the development of vitiligo. *Annals of dermatology*, *24*(2), pp.115-125.
25. Lin, J.Y. and Fisher, D.E., 2007. Melanocyte biology and skin pigmentation. *Nature*, *445*(7130), pp.843-850.
26. Mansuri, M.S., Laddha, N.C., Dwivedi, M., Patel, D., Alex, T., Singh, M., Singh, D.D. and Begum, R., 2016. Genetic variations (Arg5Pro and Leu6Pro) modulate the structure and activity of GPX1 and genetic risk for vitiligo. *Experimental dermatology*, *25*(8), pp.654-657.
27. Singh, M., Mansuri, M.S., Parasrampuria, M.A. and Begum, R., 2016. Interleukin 1-α: A modulator of melanocyte homeostasis in vitiligo. *Biochem Anal Biochem*, *5*(273), pp.2161-1009.
28. McIntyre, K.W., Stepan, G.J., Kolinsky, K.D., Benjamin, W.R., Plocinski, J.M., Kaffka, K.L., Campen, C.A., Chizzonite, R.A. and Kilian, P.L., 1991. Inhibition of interleukin 1 (IL-1) binding and bioactivity in vitro and modulation of acute inflammation in vivo by

IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody. *Journal of Experimental Medicine*, *173*(4), pp.931-939.

29. Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M. and Fabbri, P., 2002. New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell & Melanoma Research*, *15*(2), pp.87-92.
30. Mosenson, J.A., Eby, J.M., Hernandez, C. and Poole, I.C., 2013. A central role for inducible heat-shock protein 70 in autoimmune vitiligo. *Experimental dermatology*, *22*(9), pp.566-569.
31. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), pp.55-63.
32. Natarajan, V.T., Ganju, P., Ramkumar, A., Grover, R. and Gokhale, R.S., 2014. Multifaceted pathways protect human skin from UV radiation. *Nature chemical biology*, *10*(7), pp.542-551.
33. Nath, S.K., Majumder, P.P. and Nordlund, J.J., 1994. Genetic epidemiology of vitiligo: multilocus recessivity cross-validated. *American journal of human genetics*, *55*(5), p.981.
34. Naughton, G.K., Reggiardo, D. and Bystryn, J.C., 1986. Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. *Journal of the American Academy of Dermatology*, *15*(5), pp.978-981.
35. Paravar, T. and Lee, D.J., 2010. Vitiligo in an urban academic setting. *International journal of dermatology*, *49*(1), pp.39-43.
36. Shajil, E.M. and Begum, R., 2006. Antioxidant status of segmental and non-segmental vitiligo. *Pigment Cell & Melanoma Research*, *19*(2), pp.179-180.
37. Laddha, N.C., Chatterjee, S., Gani, A.R., Malek, R.A., Shah, B.J. and Begum, R., 2007. Association of catalase T/C exon 9 and glutathione peroxidase codon 200 polymorphisms in relation to their activities and oxidative stress with vitiligo susceptibility in Gujarat population. *Pigment Cell & Melanoma Research*, *20*(5), pp.405-407.
38. Singh, S., Singh, U. and Pandey, S.S., 2012. Serum concentration of IL-6, IL-2, TNF-α, and IFNγ in Vitiligo patients. *Indian journal of dermatology*, *57*(1), p.12.
39. Swope, V.B., Abdel-Malek, Z., Kassem, L.M. and Nordlund, J.J., 1991. Interleukins 1α and 6 and tumor necrosis factor-α are paracrine inhibitors of human melanocyte proliferation and melanogenesis. *Journal of investigative dermatology*, *96*(2), pp.180-185.

40. Joensuu, H., Roberts, P.J., Sarlomo-Rikala, M., Andersson, L.C., Tervahartiala, P., Tuveson, D., Silberman, S.L., Capdeville, R., Dimitrijevic, S., Druker, B. and Demetri, G.D., 2001. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *New England Journal of Medicine*, *344*(14), pp.1052-1056.
41. Tarlow, J.K., Blakemore, A.I., Lennard, A., Solari, R., Hughes, H.N., Steinkasserer, A. and Duff, G.W., 1993. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Human genetics*, *91*(4), pp.403-404.
42. Terry, C.F., Loukaci, V. and Green, F.R., 2000. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *Journal of Biological Chemistry*, *275*(24), pp.18138-18144.
43. Vamvakopoulos, J., Green, C. and Metcalfe, S., 2002. Genetic control of IL-1β bioactivity through differential regulation of the IL-1 receptor antagonist. *European journal of immunology*, *32*(10), pp.2988-2996.
44. Van Den Boorn, J.G., Picavet, D.I., Van Swieten, P.F., Van Veen, H.A., Konijnenberg, D., Van Veelen, P.A., Van Capel, T., De Jong, E.C., Reits, E.A., Drijfhout, J.W. and Bos, J.D., 2011. Skin-depigmenting agent monobenzone induces potent T-cell autoimmunity toward pigmented cells by tyrosinase haptenation and melanosome autophagy. *Journal of Investigative Dermatology*, *131*(6), pp.1240-1251.
45. Wilkinson, R.J., Patel, P., Llewelyn, M., Hirsch, C.S., Pasvol, G., Snounou, G., Davidson, R.N. and Toossi, Z., 1999. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1β on tuberculosis. *Journal of Experimental Medicine*, *189*(12), pp.1863-1874.
46. Xu, D.P., Ruan, Y.Y., Pan, Y.Q., Lin, A., Li, M. and Yan, W.H., 2011. VNTR polymorphism of human IL1RN in Chinese Han and She ethnic populations. *International journal of immunogenetics*, *38*(1), pp.13-16.
47. Bhargava, P., Prakash, C., Tiwari, S. and Lakhani, R., 2016. Correlating melanin index to repigmentation potential: A novel prognostic tool in vitiligo. *Pigment International*, *3*(2), p.72.
48. Yohn, J.J., Critelli, M., Lyons, M.B. and Norris, D.A., 1990. Modulation of melanocyte intercellular adhesion molecule-1 by immune cytokines. *Journal of investigative dermatology*, *95*(2), pp.233-237.

49. Zhao, M., Gao, F., Wu, X., Tang, J. and Lu, Q., 2010. Abnormal DNA methylation in peripheral blood mononuclear cells from patients with vitiligo. *British Journal of Dermatology*, *163*(4), pp.736-742.