1 GENERAL ASPECTS OF VITILIGO

Vitiligo is a skin disorder characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches caused by loss of functioning epidermal melanocytes. The extent of involvement is highly variable, ranging from focal to generalized, and the onset can be abrupt or gradual (Glassman, 2011). The disease appears most commonly on hands, feet, arms, face and lips. The lesions may be progressive and may develop at any age (Nordlund *et al.,* 1998). Vitiligo generally leads to psychological turmoil, social embarrassment and cosmetic disfigurement particularly in brown and black people. Affected persons suffer from social stigma and girls in particular, are subjected to ostracization from the marital point of view (Mehta *et al.,* 1973). Patients with vitiligo struggle with low self-esteem. Many become socially isolated and experience clear indications of clinical depression (Silvan, 2004). The etiology of vitiligo remains obscure despite being in focused debate for the last six decades (Taieb, 2000; Le Poole, 1993; Ortonne, 1993; Shajil, *et al.,* 2006).

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 (Figure 1). It is most likely that loss of melanocytes in vitiligo occurs through a combination of pathogenic mechanisms that act in concert. However genetic factors, oxidative stress, autoimmunity, neurological factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people (Njoo and Westerhof, 2001).

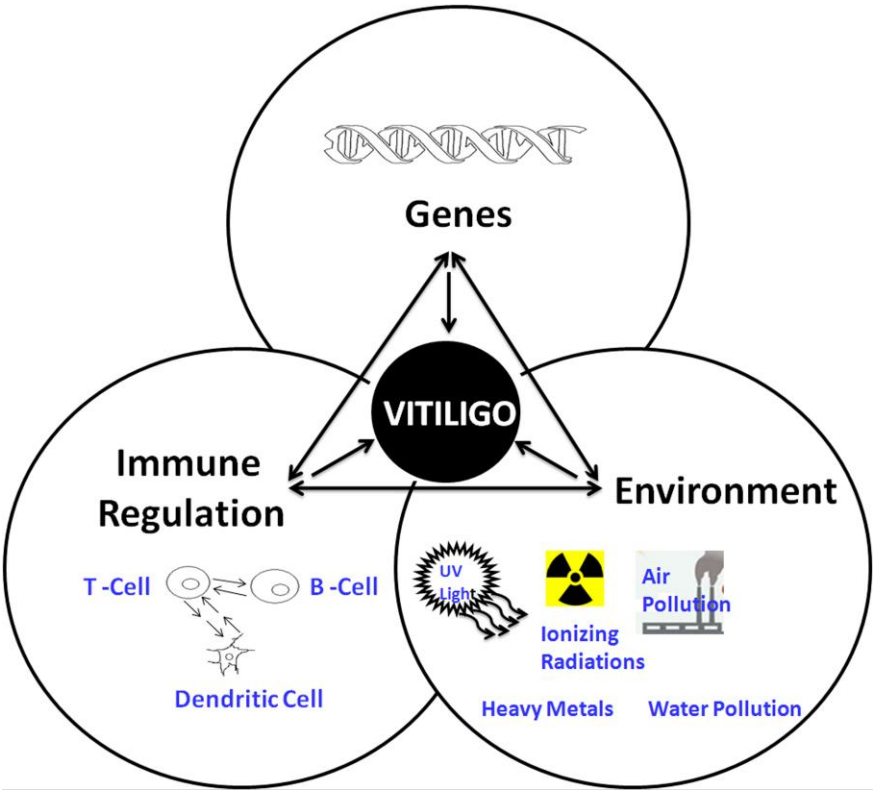


Figure 1. Interplay of genes, environment (ROS generation) and immune system in precipitation of Vitiligo.

1.1 HISTORICAL BACKGROUND

The earliest reports on patchy skin diseases that may be interpreted as today's vitiligo dates back to approximately 1500 BC. References on vitiligo can be found in the ancient Vedic scripture of India, *Atharva Veda* (Koranne and Sachdeva, 1988). The Indian *Manu Smriti* (200 BC) describes “Sweta Kushta", meaning 'white disease' skin lesions which probably was vitiligo (Koranne and Sachdeva, 1988).

1.2 PREVALENCE

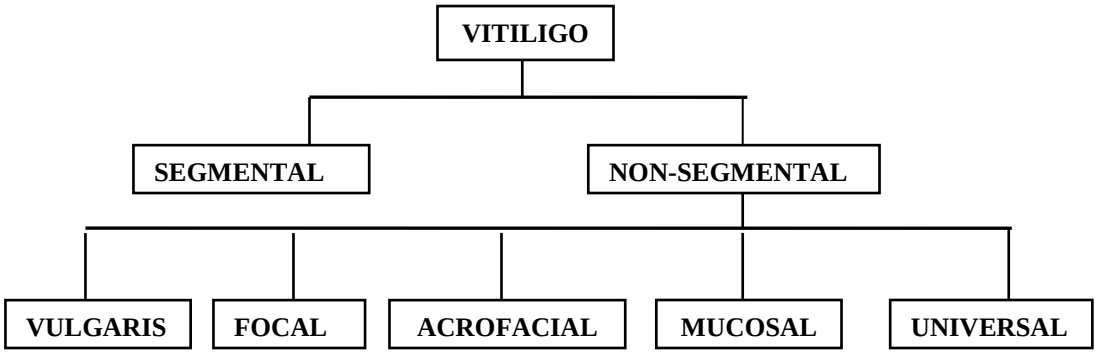
Vitiligo affects approximately 0.5 to 1% of the world population (Taieb *et al.*, 2007). The prevalence of the disease in United States has been estimated to be 1% (Lerner, 1971). In Denmark the prevalence is around 0.38% (Howitz *et al.*, 1977). The prevalence of vitiligo is estimated to be about 2% of the population in Japan and 1% in Egypt, 0.24% in UK, 0.14% in Russia (Majumder, 2001). Indian studies show a prevalence varying from 0.46 to 8.8%. (Levai, 1958; Behl and Bhatia, 1972; Sehgal,

1974; Koranne *et al.*, 1986; Dutta and Mandal, 1969; Mehta *et al.*, 1973; Das *et al.*, 1985; Handa and Kaur, 1999; Sehgal and Srivastava, 2007). The Gujarat and Rajasthan states have the highest prevalence of around 8.8% (Valia and Datta, 1996). Onset of the disease is before the age of 20 years in about half the cases, and three quarters have occurred by the age of 30 years. Both the sexes are equally affected, but there might be a female preponderance owing to reporting bias (Le Poole *et al.*, 1997).

1.3 TYPES OF VITILIGO:

Vitiligo is most often classified clinically according to the extent and distribution of depigmentation (Figure 2) (Gawkrodger et al., 1998). It has been proposed that the segmental and focal presentations of the disease constitute a separate subgroup to the non-segmental forms of vitiligo (Taieb et al., 2000) because, compared with focal and segmental vitiligo non-segmental forms show a later age of onset, a stronger association with autoimmunity and unstable results following autologous grafting.

Table 1. Classification of vitiligo (Dave *et al.*, 2002)



Segmental/unilateral- It is often present in childhood and occurs in dermatomal, asymmetric distribution with one or more macules localized to one area of the body. It is rarely associated with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

Focal vitiligo- It describes one or more depigmented patches localized in a discrete area with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

Symmetrical/bilateral vitiligo- This is the most common type of vitiligo and is often referred to as generalized. It is characterized by a bilateral, symmetrical depigmentation with a widespread distribution of many macules in a random pattern. Many parts of body can be affected, including the face (particularly periorificial areas), the neck, torso, hand and legs. A latter age of onset is normal for this clinical subclass and it is often associated with autoimmunity. Unstable results are evident following autologous grafting in patients with this type of vitiligo (Kemp *et al.*, 2001).

Acrofacial vitiligo- Vitiligo of this type is characterized by depigmentation of the distal fingers and facial orifices- the latter in a circumferential pattern. It often forms with symmetrical vitiligo (Kemp *et al.*, 2001).

Universal vitiligo- This type of vitiligo is characterized by loss of pigmentation over the entire body, but is rare (Kemp *et al.*, 2001).

Table 2. Clinical classification of vitiligo (Nordlund and Lerner, 1982).

Segmental Vitiligo (SV)	**Non-Segmental Vitiligo (NSV)**				
	Acrofacial	Vulgaris	Mixed	Focal	Universal
One or more macules in dermatomal, unilateral distribution.	Affects face and distal extremities	Symmetrical distribution of lesions in typical zones	Segmental along with vulgaris or acrofacial	One or more patches in one area but not in segmental pattern	Involves more than 80 % of the body

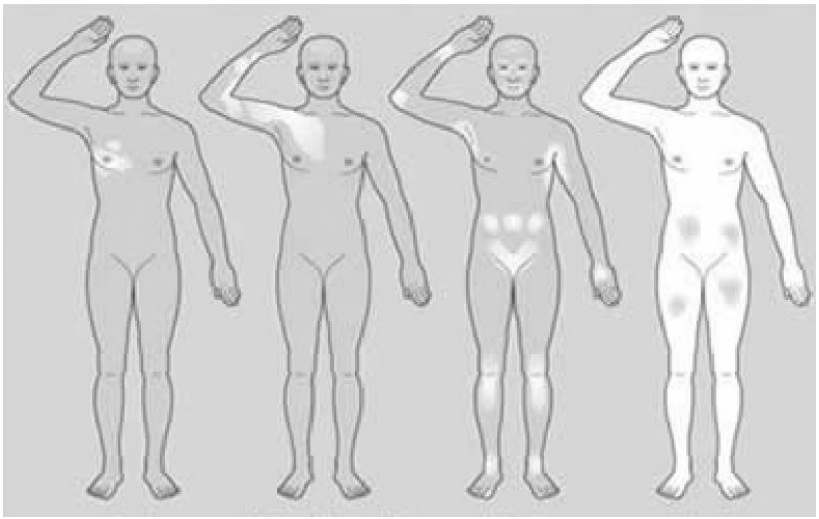


Figure 2. Clinical types of vitiligo

1.4 THE HUMAN SKIN

Human skin is a uniquely engineered organ that permits terrestrial life by regulating heat and water loss from the body whilst preventing the ingress of noxious chemicals or microorganisms. It is also the largest organ of the human body, providing around 10% of the body mass of an average person, and it covers an average area of 1.7 m^2. Whilst such a large and easily accessible organ apparently offers ideal and multiple sites to administer therapeutic agents for both local and systemic actions.

Skin comprises of two compartments: a stratified epithelium of 50-100 μm thickness which is composed predominantly of keratinocytes, and a relatively acellular dermis of approximately 1000 μm thickness which contains a complex extra cellular matrix comprising many types of collagen, fibroblasts, and a range of supporting structures, including blood vessels, inflammatory cells, nerves and ground substance (Figure 3). In addition to the keratinocytes, an estimated 10% of the cellular component of the epidermis is composed of neural crest derived melanocytes and Langerhans cells (Rees, 2003).

The epidermis contains four histologically distinct layers which, from the inside to the outside, are the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (Figure 3).

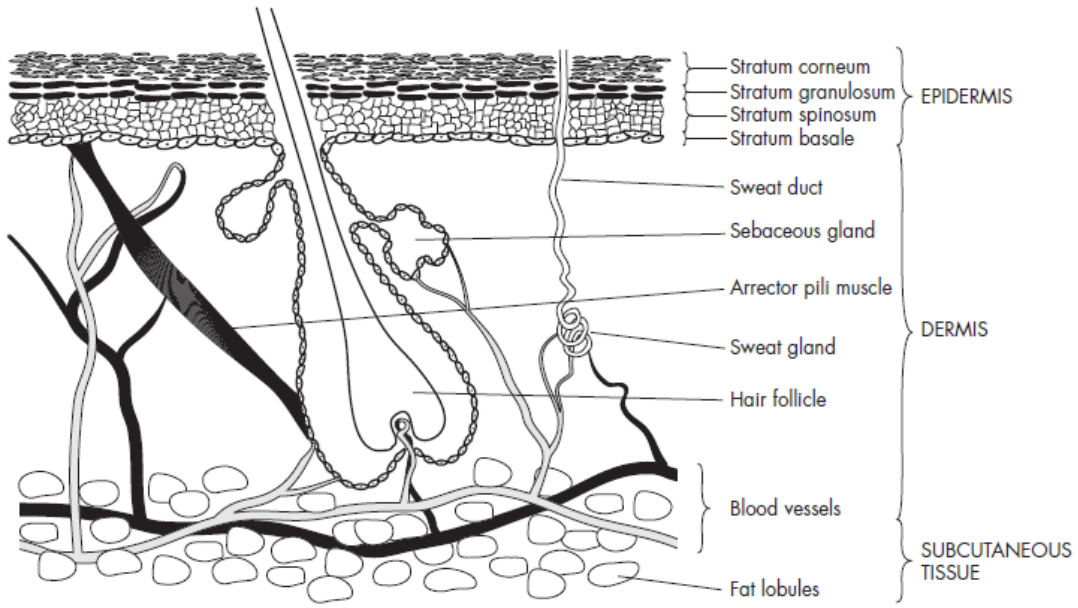


Figure 3. A diagrammatical cross-section of human skin

(El Maghraby *et al.*, 2008)

1.4.1 Melanocytes :

Melanocytes reside at the junction of the dermis and the epidermis and produce melanin that provides pigmentation for the skin and hair (Figure 4). The melanocyte is a neural crest-derived cell that migrates via the mesenchyme into the epidermis and hair follicles during embryogenesis. Additional sites of melanocyte migration include the uveal tract of the eye (choroid, ciliary body, and iris), the leptomeninges, and the inner ear (cochlea) (Figure 4). Presumably, the death of melanocytes within the leptomeninges, inner ear, and skin is responsible for the aseptic meningitis, auditory symptoms, and vitiligo, respectively.

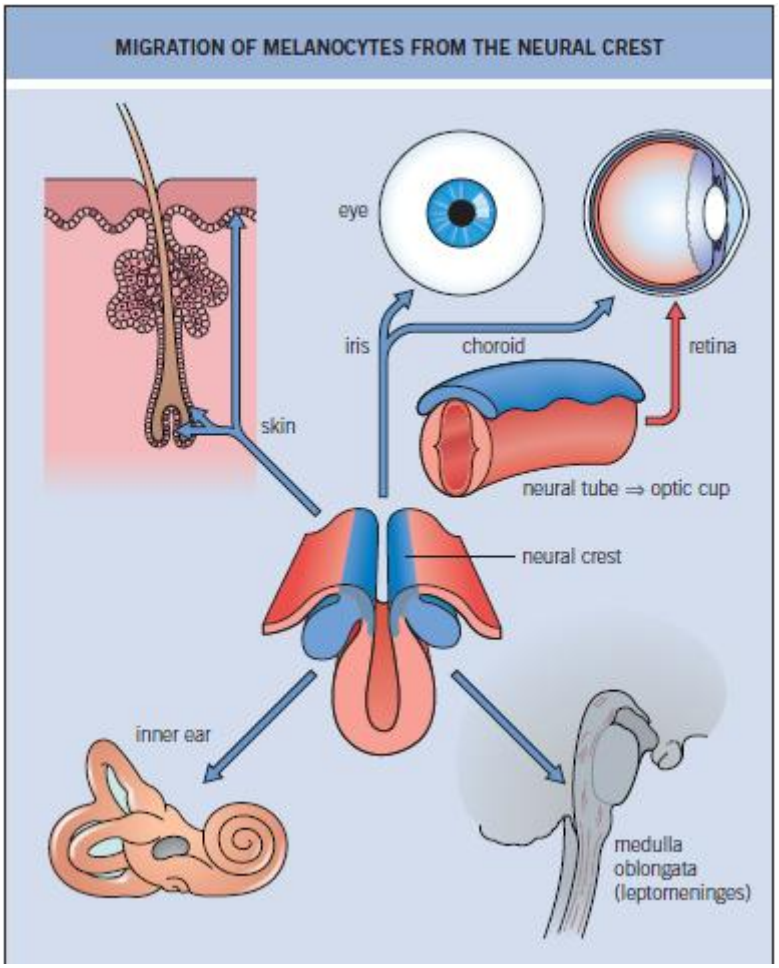


Figure 4. Migration of melanocytes from the neural crest: Melanocytes migrate to the uveal tract, the leptomeninges, and the cochlea, as well as the epidermis and hair follicle. The retina actually represents an outpouching of the neural tube (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

Melanocytes derive from the melanoblasts that originate from the neural crest from where they migrate during embryogenesis. Melanocytes are highly dendritic and these dendrites project into the malphigian layer of the epidermis where they transfer the melanosomes to keratinocytes (Jimbow *et al.*,1999). Each epidermal melanocyte secretes melanosomes into approximately 36 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit (Figure 5).

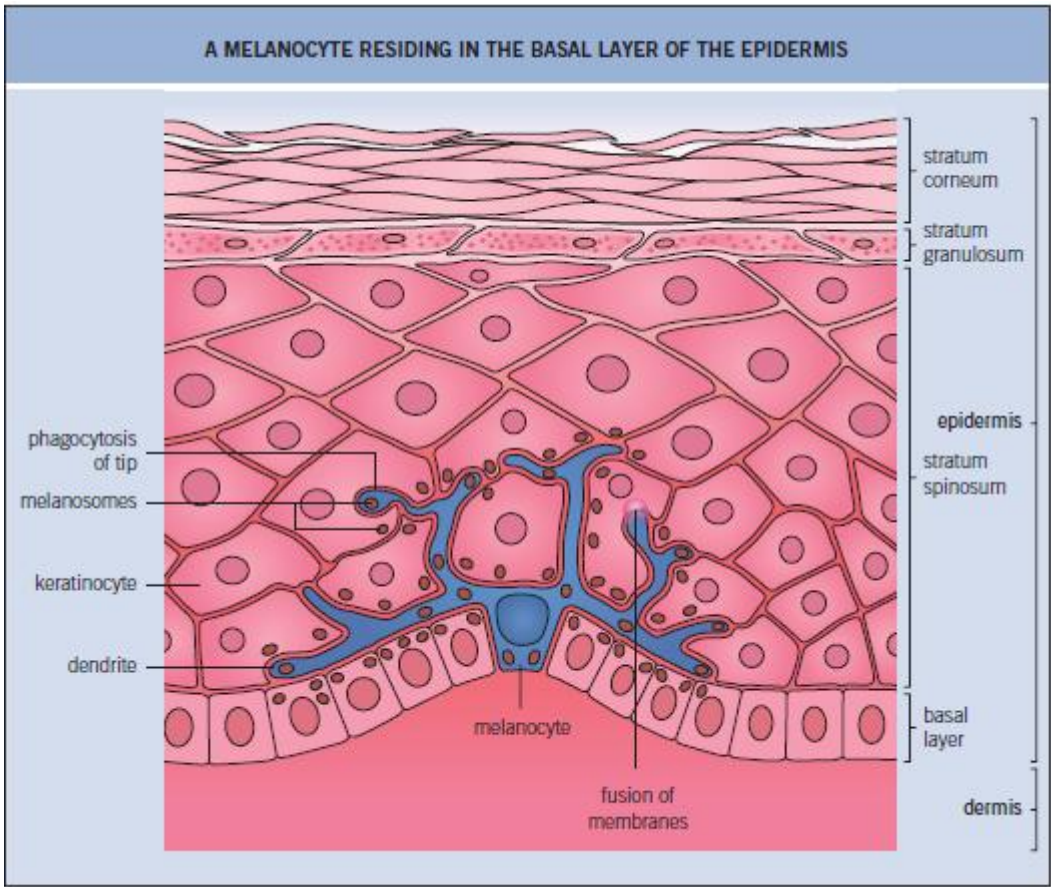


Figure 5. Epidermal Melanin unit showing a melanocyte residing in the basal layer of the epidermis: In normal skin, approximately every tenth cell in the basal layer is a melanocyte. Melanosomes are transferred from the dendrites of the melanocyte into neighboring keratinocytes of the epidermis, hair matrices and mucous membranes (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

1.4.2 Melanosomes

Melanosomes are specialized subcellular organelles in which melanin is synthesized and deposited (Orlow, 1995). There are four stages in the maturation of melanosome: stage I, the "premelanosome" a spherical organelle with ill defined matrix filaments is seen; stage II, in which the typical elliptical shape of the melanosome is filled with a well defined filamentous or laminar matrix; stage III, with deposition of electron opaque melanin occurs on this matrix; and stage IV, with complete opacification of melanosomal contents takes place by the melanin deposited therein (Orlow, 1995). The transition to stage II melanosomes involves elongation of the vesicle, and the appearance of distinct fibrillar structures. The production of internal matrix fibers and

the maturation from stages I to II melanosomes depend on the presence of a structural protein termed Pmel 17 or gp100. A melanosomal protein called MART 1 forms a complex with Pmel 17 and thus plays an important role in melanogenesis by regulating the expression, stability, trafficking and processing of Pmel 17, which in turn regulates the maturation of melanosomes (Hoashi *et al.*,2005). The structure of melanosome is shown in Figure 6.

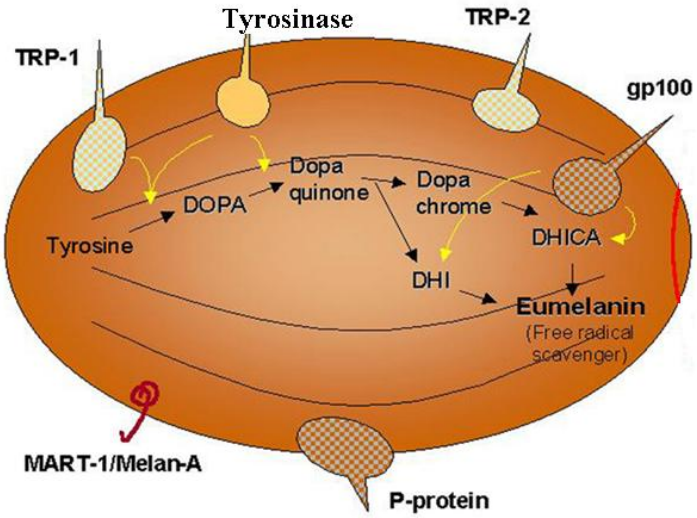


Figure 6. Structure of melanosome.

1.4.3 Melanin:

Melanin is transferred from melanocytes in the basal layer to associated keratinocytes via the epidermal melanin unit (Fitzpatrick *et al.,* 1967). Granules of melanin are transferred to keratinocytes via melanosomes by a possible phagocytic process (Mottaz *et al.*, 1967). Because melanocytes represent only 8–10% of all epidermal cells (Ivanova *et al.,* 2006), most of the skin colour is determined by melanin in the keratinocytes, and only at localized collections of melanocytes, as occurs in melanocytic nevi or 'moles', is the colour due to the melanocytes themselves. Two major forms of melanin are produced in melanocytes i.e. brown black eumelanin and yellow red yellow pheomelanin. Dark skin has a higher content of eumelanin, with larger melanosomes, and lighter skin has a higher proportion of phaeomelanin, with smaller melanosomes. Skin colour is not determined by the number or size of melanocytes. The supranuclear melanin cap structure in keratinocytes minimizes

photodamage to the nucleus (Kollias *et al.,* 1991; Kobayashi *et al.,* 1998). The major function of melanin is attributed to be photo protection to the skin from the UV and ionizing radiations (Hearing, 2000). Melanin also scavenges ROS, thereby limiting UV and ROS damage to other cutaneous cells. Melanocytes also play a role in the skin immune system, secreting a wide range of signal molecules and responding to growth factors and cytokines. Melanocytes can phagocytize and eliminate exogenous antigens, which have penetrated the skin barrier (Le Poole *et al.,* 1993), and they can process and present antigens in the form of peptides with HLA (human leucocyte antigen) class II molecules to T-cells, triggering an adaptive immune response. Melanosomal proteins are involved in this antigen processing (Le Poole and Luiten, 2008). Activation of T-cells by melanocytes is shown by the secretion of co-stimulatory molecules like ICAM (intercellular adhesion molecule)-1 and LFA (leucocyte fusion-associated molecule)-3 (Le Poole *et al.,* 1993; Das *et al.,* 2001). When melanocytes die, migrate or stop functioning, skin reverts to its unpigmented form.

Melanin synthesis, a multi step process takes place in melanosomes (Hearing, 1999). Tyrosinase is the key enzyme required for the melanin production and it catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA), which is the rate-limiting step for the melanin synthesis (Hearing, 1999). DOPA undergoes oxidation to dopaquinone, which is immediately converted into dopachrome. Dopachrome spontaneously converts into 5,6 hydroxyindole (DHI). Otherwise tyrosinase related protein 2 (TRP 2) converts dopachrome to dihydroxy indole carboxylic acid (DHICA). DHI and DHICA further polymerize to form eumelanin. The switch between eumelanogenesis and pheomelanogenesis occurs at the dopaquinone stage. Cysteine/glutathione reacts with the dopaquinone to produce cysteinyldopa which may undergo further cyclisation to benzothiazines and higher condensates giving rise to pheomelanins (Hearing, 1999). The different steps of melanin production are shown in Figure 7.

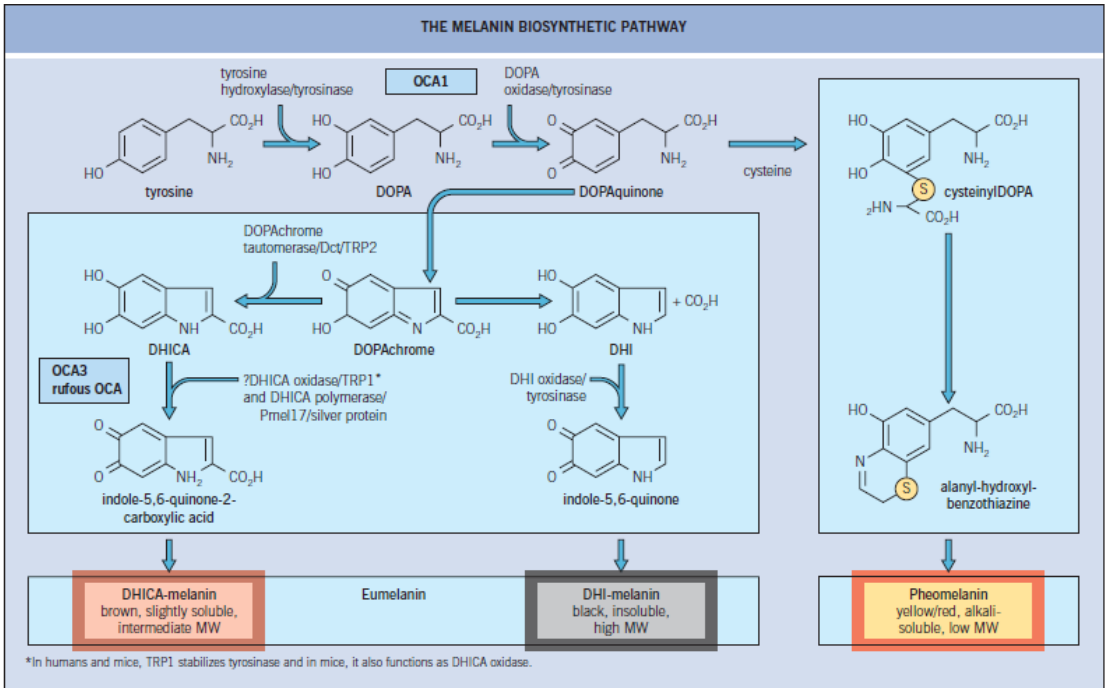


Figure 7. The melanin biosynthetic pathway: The pathway includes demonstration of the sites of dysfunction in OCA1 (tyrosinase), OCA3 (TRP1), and rufous OCA (TRP1). The two major forms of melanin in the skin and hair are brown–black eumelanin and yellow–red pheomelanin. DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DOPA, dihydroxyphenylalanine; MW, molecular weight; TRP, tyrosinase-related protein (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

1.5 VITILIGO ETIOPATHOGENESIS

Although essentially asymptomatic, the psychosocial impact of vitiligo can be devastating, and affected persons are often desperate for effective therapy (Linthorst Homan *et al.*, 2009). As of 2012, this goal has not yet been reached, as the underlying pathomechanisms in vitiligo are still incompletely understood, despite intense scrutiny since six decades.

The aetiopathogenesis of vitiligo has been reviewed by several groups recently (Passerson and Ortonne, 2005; Dell'Anna and Picardo, 2006; Westerhof and d'Ischia, 2007; Schallreuter *et al.*, 2008; Boissy and Spritz, 2009, Glassman, 2011), but 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. Three main recurring themes emanate from clinical and scientific analysis of melanocyte loss in vitiligo: neurochemical, oxidative stress (biochemical) and autoimmune mechanisms.

1.5.1 Neurochemical Hypothesis

Melanocytes are neural crest derived cells giving them an embryological link to the nervous system (Reedy *et al.,* 1998). According to this hypothesis neurochemical mediators like norepinephrine and acetylcholine secreted by the nerve endings are toxic to melanocytes leading to their destruction in vitiligo patients. Acetylcholine esterase activity is found to be lowered in vitiliginous skin during depigmentation (Iyengar, 1989).

There is evidence of a strong association between mental stress and the onset or progression of vitiligo. A case-control study on children afflicted with vitiligo and psoriasis showed that the onset of vitiligo was associated with psychological factors (Barisic-Drusko and Rucevic, 2004). Another case-control study done by Manolache and Benea demonstrated that vitiligo patients are much more likely (OR=6.81) to encounter stressful events in their life (Manolache and Benea, 2007). Their study also revealed that patients were much more likely to experience one stressful event before the onset of vitiligo. Furthermore, it has been suggested that patients with alexithymia (deficiency in the ability to express emotions) and those with poor social support are more susceptible to vitiligo (Picardi *et al.*, 2003).

An important consequence of mental stress is through its effect on the secretion of catecholamines via stimulation of the hypothalamic-pituitary-adrenal (HPA) axis, (Tolis and Stefanis, 1983; Stokes and Sikes, 1988) which consists of a set of complex interactions between the hypothalamus, pituitary and adrenal glands. Systemically, psychological and emotional disturbances can trigger the production and release of corticotropin releasing hormone (CRH) by the hypothalamus, which in turn stimulates the release of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH can act on the adrenal gland to produce various corticosteroids and catecholamines. More importantly, in addition to systemic effects, the HPA axis has been shown to play a

crucial regulatory role in the local microenvironment of the skin, where ACTH, CRH, and CRH receptors are involved (Slominski *et al.,* 2007). Moreover, melanocyte development and pigment production are directly regulated by local sympathetic adrenergic innervations, an increased activity of which has been shown in vitiligo lesions (McGuire, 1970; Bir and Aktan, 1999; Schallreuter *et al.,* 1993).

Different studies showed significantly higher levels of plasma and urinary catecholamines and their metabolites in vitiligo patients especially at the onset and in the active stage of the disease (Morrone *et al.,* 1992; Orecchia *et al.,* 1994; Cucchi *et al.,* 2000; Cucchi *et al.,* 2003). Increased catecholamine synthesis is observed with the disease activity suggesting their role in the process of depigmentation (Cucchi *et al.,* 2000). High levels of norepinephrine and its metabolites in vitiligo are related to decreased phenylethanolamine N-methyltransferase (PNMT) activity and increased activity of tyrosine hydroxylase (TH). TH produces L-dopa form L-tyrosine in the catecholamine biosynthesis pathway, (Schallreuter *et al.,* 1994) and the rate limiting cofactor/electron donor for TH is (6R)-5,6,7,8-tetrahydribiopterin (6-BH4), which is increased due to decreased 4a-hydroxy-6BH4 dehydratase (DH) activity (Schallreuter *et al.,* 2001) in vitiligo patients. There is a defective recycling of $6BH_4$ which leads to increased non-enzymatic production of $7BH_4$, an isomer, concomitant with an increased production of H_2O_2. The presence of this non-enzymatic by-product in epidermis may initiate the process of depigmentation by blocking the supply of L-tyrosine either to the melanocytes or to the surrounding keratinocytes. These alterations seem to cause melanocyte destruction in vitiligo (Schallreuter *et al.,* 1994). Increased norepinephrine appears to induce another catecholamine degrading enzyme, monoamine oxidase (MAO-A) (Bindoli *et al.,* 1992). The increased MAO-A activity favors the formation of hydrogen peroxide, which is toxic to melanocytes (Schallreuter *et al.,* 1996a). Also damage to the melanocytes is not buffered by the low catalase activity (Schallreuter *et al.,* 1991).

Aberrations in beta-endorphin and met-enkephalin secretion are also reported in vitiligo patients (Mozzanica *et al.,* 1992) and the levels of met-enkephalin levels are found to be higher. It is suggested that this abnormality may be correlated with the emotional stress, which precipitates vitiligo in some patients.

Studies have demonstrated altered numbers and distribution of nerve fibers, including those that secrete neuropeptide Y (NPY) and calcitonin gene– related peptide (CGRP) (Al'Abadie *et al.,* 1994; Hristakieva *et al.,* 2000) and those that are immunoreactive for the low affinity (p75) nerve growth factor receptor (NGFr-IR) (Liu *et al.,*1996) in vitiligo lesional skin. Abnormalities of neuropeptides are observed in perilesional skin and blood of vitiligo patients (Al'Abadie *et al.,* 1994). The neuropeptide Y (NPY) is released by either exogenous stimulus like trauma (e.g. Koebner phenomenon) or by endogenous stimuli (e.g. stress) (Al' Abadie *et al.,*1994) and this altered balance of neuropeptides in vitiliginous skin supports nervous system's role in the pathogenesis of vitiligo (Liu *et al.,* 1996). Neuropeptides are also reported to have immunoregulatory effects (Covelli and Jirillo, 1988; Rameshwar *et al.,* 1992). Caixia *et al.,* (1999) showed that the levels of NPY in the plasma of vitiligo patients were found to be significantly higher than the normal controls. The levels of NPY from skin lesions were significantly higher than those from uninvolved skin in both the local type and segmental types of vitiligo. NPY could evoke the secretion of IFN-gamma and IL- 2 suggesting that NPY might be involved in the cell mediated immunological mechanism, and thus leading to melanocyte destruction in vitiligo (Caixia *et al.,* 1999). Keratinocytes and melanocytes in the depigmented skin are shown to have increased monoamine oxidase- A activity which causes keratinocytes to produce 4 -fold more norepinephrine, which is toxic to melanocytes and 6.5 -fold less epinephrine than control keratinocytes (Schallreuter *et al.,* 1996a).

A derangement of the enzymes involved in catabolism of adrenergic transmitters namely catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO-A) is reported. COMT normally prevents the formation of the toxic ortho-quinones during melanin synthesis. Epidermal homogenates form vitiligo patients showed higher COMT activity, probably induced by the elevated levels of catecholamines that were secreted by keratinocytes or by nerve endings. The events that support the neurochemical pathogenesis of vitiligo are shown in Figure 8.

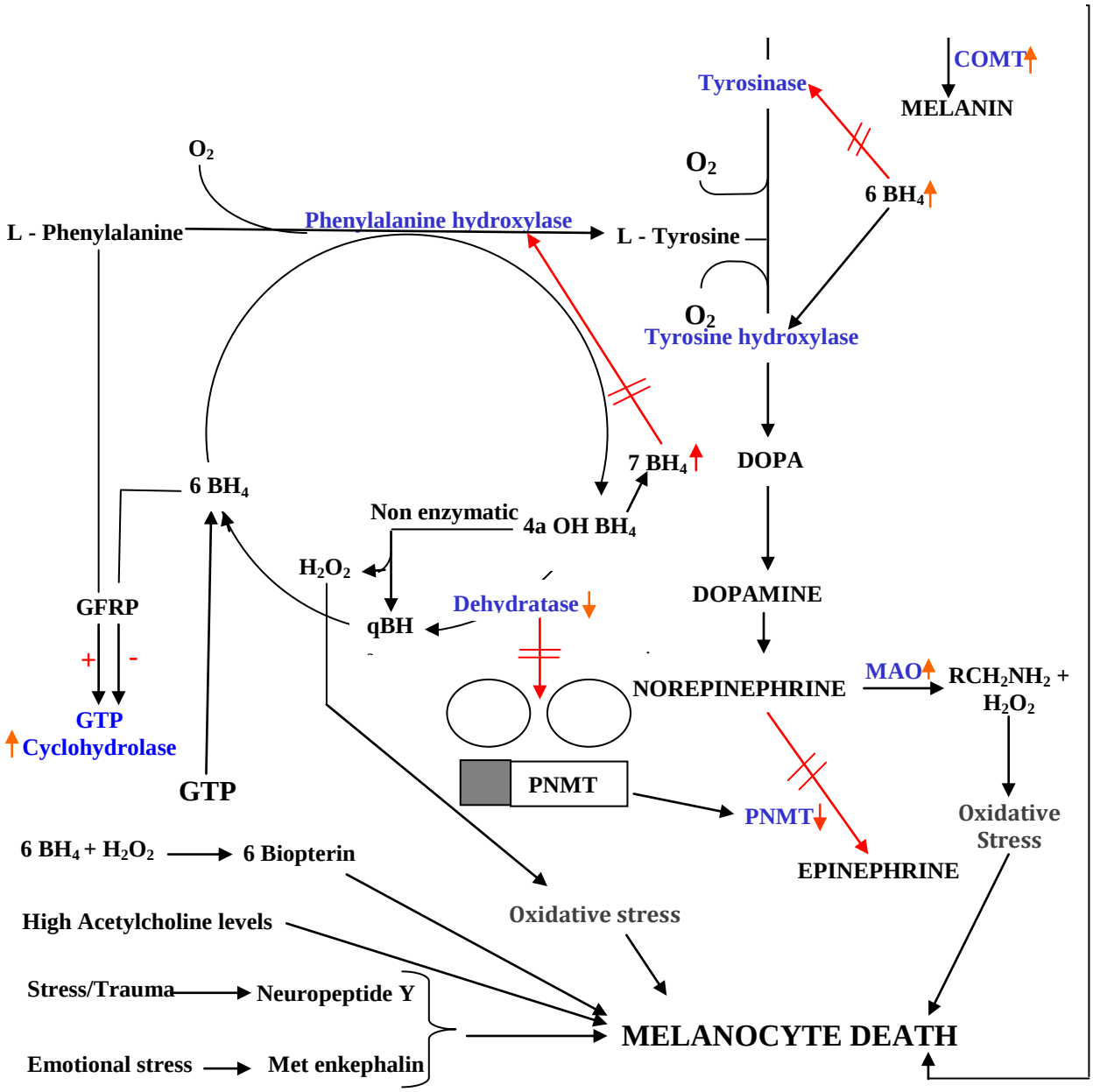


Figure 8. The events that support the neurochemical pathogenesis of vitiligo.

1.5.2 Oxidative Stress Hypothesis

Oxidative stress is a major form of assault on the skin. Human skin is exposed to many oxidative stressors daily through diet, our environment, by products of metabolism, and lifestyle factors such as smoking, alcohol and UV irradiation. Skin exposure to ionizing and UV radiation and/or xenobiotics/drugs generates ROS in excessive quantity. It overwhelms tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS and increased lipid peroxidation are seen in multiple skin disorders such as atopic dermatitis, psoriasis, acne vulgaris, pemphigus vulgaris (PV), lichen planus, alopecia areata and vitiligo (Yesilova *et al.,* 2012).

1.5.2.1 Free radicals

Free radicals can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule, either by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule (Ray and Husain, 2002). Free radicals have been implicated in the pathogenesis of several human diseases including vitiligo. The main free radicals formed in the body are reactive oxygen species (ROS) and reactive nitrogen species (RNS), and these radicals in excess result in oxidative stress, which has been implicated in the pathogenesis of several diseases.

1.5.2.2 Reactive Oxygen Species (ROS):

Oxygen is vital for the aerobic life process. However about 5% or more of the O_2 is converted to ROS (Harman, 1993). Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when the oxygen is partially reduced it becomes 'activated' and reacts readily with a variety of biomolecules. In all cell types, oxygen metabolism does lead to the production of oxygen free radicals that include superoxide anion radical ($\cdot O_2$), singlet oxygen ($^1O^-_2$), hydroxyl radical ($\cdot OH$) and perhydroxyl radical ($HOO^{\cdot}$), collectively termed ROS. The usual route of O_2 metabolism involves reduction of molecular O_2 by four electrons to form H_2O. However, with a single electron reduction several free radicals and hydrogen peroxide (H_2O_2) are formed (Figure 9).

ROS can be produced by both endogenous and exogenous sources. Potential endogenous sources include oxidative phosphorylation, cytochome P450 metabolism, peroxisomes and inflammation.

ROS are generated *in vivo* by oxidant enzymes such as NAD(P)H oxidase, xanthine oxidase, lipoxygenase and cytochome P450 monooxygenase besides by phagocytic cells, ionizing radiation etc. (Figure. 9). Superoxide anion is the first radical formed, when O_2 picks up a single electron from the electron transport chain. ·OH, HO_2· and H_2O_2 radicals are formed from O_2· (Grisham, 1992; Nappi and Vass, 1998) that undergoes a dismutation reaction catalyzed by the enzyme superoxide dismutase (SOD) to form H_2O_2. It is an important oxidant since it can cross biological membranes and form the highly reactive ·OH by interaction with transition metal ions such as Fe^{2+}or Cu^{+}. ·OH is the most potent damaging radical amongst the ROS, which can react with all biomolecules (lipids, proteins, nucleic acids). It is extremely reactive and can lead to the formation of DNA-protein cross-links, single- and double-strand breaks, base damage, lipid peroxidation and protein damage (Stohs and Bagchi, 1995; Lloyd *et al.*, 1997).

Neutrophils, eosinophils, and macrophages are additional endogenous sources and are the major contributors to the cellular reactive oxygen species. Activated macrophages, through "respiratory burst," elicit a rapid but transient increase in oxygen uptake that gives rise to several reactive oxygen species, including superoxide anion and hydrogen peroxide (Vuillaume, 1987; Witz, 1991).

Cellular H_2O_2 production is also due to the participation of peroxisomal oxidases, flavoproteins, D-amino acid oxidase, L-alpha-hydroxy acid oxidase and fatty acyl-CoA oxidase (Chance *et al.*, 1979; Bast *et al.*, 1991). The catalytic cycle of xanthine oxidase has emerged as an important source of O_2^- and H_2O_2 in a number of tissue injuries. Xanthine oxidase which is produced by the proteolytic cleavage of xanthine dehydrogenase during ischemia upon reperfusion in the presence of O_2 acts on xanthine or hypoxanthine to generate O_2^- or H_2O_2 (McCord *et al.*, 1987; Halliwell and Gutteridge, 1990).

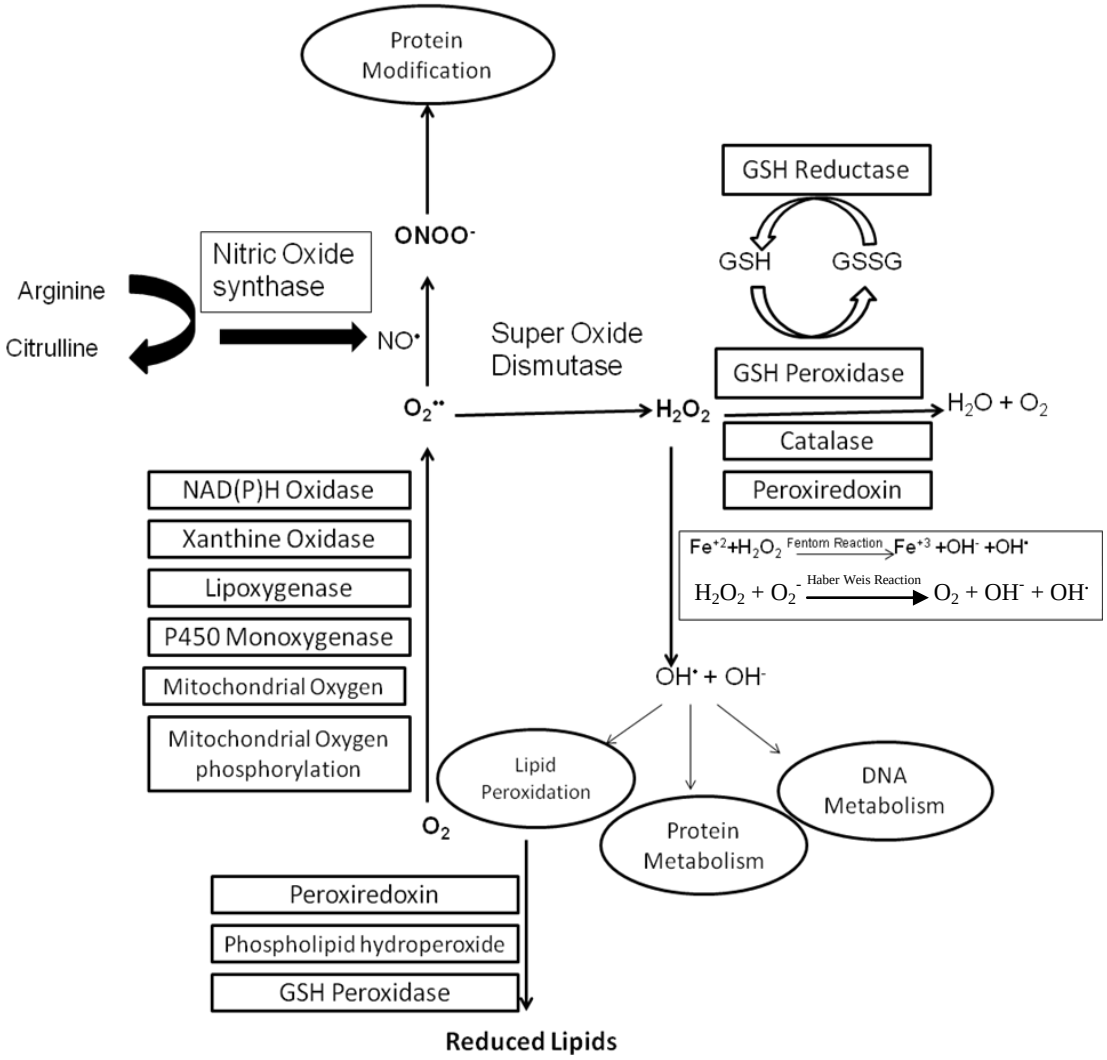


Figure 9. Intracellular generation of ROS.

ROScan be produced by exogenous sources also. Environmental agents including nongenotoxic carcinogens can directly generate or indirectly induce ROSin cells (Rice-Evans and Burdon 1993). The induction of oxidative stress and damage has been observed following exposure to UV, gamma rays, cigarette smoke and xenobiotics. Chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters, and some peroxisome proliferating compounds are among the classes of compounds that have been shown to induce oxidative stress and damage *in vitro* and *in vivo* (Klaunig and Kamendulis, 1997).

1.5.2.3 Reactive Nitrogen Species (RNS):

Reactive nitrogen species (RNS) are generated in a sequential reaction that begins with nitric oxide synthase mediated conversion of arginine to citrulline (Figure 9). In this reaction, nitric oxide free redical ($NO^{\cdot}$) is generated, which reacts with O_2^{-} to produce peroxynitryl ($ONOO^{\cdot}$). Significantly elevated levels of 8-oxoguanine reported recently in plasma and skin of patients with vitiligo, an indication of DNA damage. Due to DNA damage, there was up-regulation of epidermal p53 and enhanced short patch base-excision repair. In addition, high epidermal levels of iNOS (inducible nitric oxide synthase) were also demonstrated, with corresponding elevations in 3-nitrotyrosine and nitrated p53, implying that increased epidermal ONOO−• radicals are involved in the pathogenesis of vitiligo. H_2O_2 is also shown to enhance the DNA binding capacity of p53 while, ONOO−• completely inhibits this binding. Interestingly, H_2O_2 at a concentration of 10^{-3} M abolishes this deleterious effect of ONOO−•. Thus, H_2O_2 appears to be protective in the sense of improving DNA repair via enhanced p53. This could partly explain the relative absence of photoaging and skin cancer in chronic lesions of vitiligo (Salem *et al.,* 2009).

1.5.2.4 ROS in Vitiligo:

Human skin serves as an interface between the environment and the body. It is constantly exposed to a broad array of physical, chemical and biological agents, many of which are either inherent oxidants or catalyse the generation of ROS. ROS can react with proteins, alter apoptotic pathways, damage nuclear and mitochondrial DNA and mediate release of proinflammatory cytokines (Shigenaga *et al,*. 1994; Briganti and Picardo, 2003). ROS are believed to be involved in the pathogenesis of inflammatory skin diseases, carcinogenesis, photoaging and hair graying (Bickers and Athar, 2006; Wood *et al.,* 2009). Mitochondria are the most important endogenous source of ROS, but they are also a target of ROS-mediated damage. Thus, ROS can lead to mitochondrial dysfunction, reduced efficiency and more ROS in a vicious cycle of oxidant imbalance (Shigenaga *et al.,* 1994). Several lines of research have shown evidence of oxidative stress throughout the epidermis of patients with vitiligo, attributed to high levels of H_2O_2 in the 10^{-3} M range (Schallreuter *et al.,* 1999).

Impetus for this research came from the finding of low catalase levels in the epidermis of patients with vitiligo (Schallreuter *et al.,* 1991). Generation of H_2O_2 is a physiological reaction in all cells via several metabolic pathways. There are also numerous exogenous direct and indirect sources of epidermal H_2O_2. While low concentrations, of the order of 10^{-6} M, are necessary for cell signalling and transcription, high concentrations can have deleterious effects. Ultrastructural changes suggestive of lipid peroxidation have been demonstrated in melanocytes, keratinocytes and Langerhans cells in the skin of patients with vitiligo, both in affected and perilesional areas (Moellmann *et al.,* 1992; Bhawan and Bhutani, 1983; Boissy *et al.,* 1991; Tobin *et al.,* 2000). High levels of epidermal H_2O_2 as well as the methionine oxidation product, methionine sulfoxide, have been demonstrated *in vivo* in vitiligo using FT (Fourier Transform) Raman spectroscopy (Schallreuter *et al.,* 2008; Schallreuter *et al.,* 1999). This augmented previous findings of increased H_2O_2, which were *in vitro*, based on cell culture and skin biopsies (Schallreuter *et al.,* 1999). FT Raman spectroscopy also revealed oxidation of L-tryptophan in epidermal albumin, and HPLC showed the presence of allantoin in the epidermis, confirming the presence of oxidative stress in vitiligo (Rokos *et al.,* 2008; Shalbaf *et al.,* 2008). Oxidative destruction of polyunsaturated fatty acids of phospholipids is referred to as lipid peroxidation. It is one of the hallmarks of oxidative stress. MDA (malondialdehyde) is an end-product of lipid peroxidation, and elevated serum levels of MDA have been documented in patients with vitiligo (Agrawal *et al.,* 2004; Koca *et al.,* 2004; Jain *et al.,* 2008; Khan *et al.,* 2009).

1.5.2.4.1 Sources of epidermal H_2O_2:

There are numerous sources of H_2O_2 in the normal epidermis. NADPH oxidase activity in neutrophils and macrophages generate H_2O_2 (Darr and Fridovich, 1994). TNFα (tumour necrosis factor-α) may lead to the formation of H_2O_2 indirectly, by inducing manganese superoxide dismutase (Moretti *et al.,* 2002). Other cytokines such as TGF-β (transforming growth factor-β), EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) have also been reported to generate H_2O_2 (Thannickal *et al.,* 2000). Monoamine oxidase A in the epidermis also generates H_2O_2 (Schallreuter *et al.,* 1996). XO (xanthine oxidase) catalyses the conversion of purine bases into uric acid, and generates H_2O_2 as a by-product (Shalbaf *et al.,* 2008).

Table 3. Sources of ROS in Vitiligo.

H_2O_2	Increase	Schallreuter *et al.*, 1999
Peroxynitrite	Increase	Salem *et al*., 2009
NADPH oxidase	Increase	Darr *et al*., 1994
TNFα	Increase	Moretti *et al*., 2002
Oxidized pterins	Increase	Rokos *et al*., 2002
6BH4 recycling	Decrease	Schallreuter *et al*., 2006; Rokos *et al*., 2002
iNOS	Increase	Salem *et al*., 2009
Homocysteinea	Increase	Shaker *et al*., 2008
Monoamine oxidase A	Increase	Schallreuter *et al*., 1996
SOD	Increase	Agrawal *et al*., 2004; Picardo *et al*., 1994; Yildrim *et al*., 2004; Hazneci *et al*., 2005; Dammak *et al*., 2009; Koca *et al*., 2004
Thioredoxin reductase	Decrease	Gibbons *et al*., 2006
Xanthine oxidase	Increase	Shalbaf *et al*., 2008
Catecholamines	Increase	Westerhof *et al*., 2007
GTP-cyclohrdrolase-I	Increase	Schallreuter *et al*., 1994; Chavan *et al*., 2009
Catalase	Decrease	Schallreuter *et al*., 1999; Dammark *et al*., 2009
GSH-Px	Decrease	Khan *et al*., 2009; Yildrim *et al*., 2004; Hazneci *et al*., 2005; Dammak *et al*., 2009; Agrawal *et al*., 2004; Shajil and Begum, 2006
Vitamin E	Decrease	Jain *et al*., 2008; Khan *et al*., 2009

Oxidation of aromatic phenols like 17β-oestradiol to catechols by an NADPH-dependent CYP (cytochrome P450) yields superoxide anion, which disproportionates to H_2O_2 in the epidermis (Schallreuter *et al.*, 2006). Photo-oxidation of epidermal 6-biopterin and sepiapterin yields H_2O_2 (Rokos *et al.*, 2002). Several of these epidermal sources of H_2O_2 are shown to be augmented in vitiligo, providing the presumed source for the elevated levels, which have been documented, both in affected and

Table 4. Effects of ROS/ Biochemical changes in Vitiligo.

Tyrosinase	Decrease	Wood *et al*., 2004
TRP-1	Decrease	Jimbow *et al*., 2001
MSR	Decrease	Zhou *et al*., 2009
Catalase	Decrease	Schallreuter *et al*., 1991; Dammak *et al*., 2009
Thioredoxin reductase	Decrease	Gibbons *et al*., 2006
Tyrosine hydroxylase	Decrease	Schallreuter *et al*., 2001; Rokos *et al*., 2002
POMC peptides	Decrease	Griham *et al*., 1999; Spencer *et al*., 2005; Spencer *et al*., 2007
L-phenylalanine	Increase	Schallreuter *et al*., 1998
Acetylcholine	Increase	Gibbons *et al*., 2006; Schallreuter *et al*., 2004
6BH4 recycling	Decrease	Schallreuter *et al*., 2001; Hasse *et al*., 2004
Calmodulin, furin	Decrease	Schallreuter *et al.*, 1998; Schallreuter *et al*., 2007; Spencer *et al*., 2008
Albumin (epidermal)	Decrease	Rokos *et al*.,2004
Malondialdehyde	Increase	Koca *et al*., 2004; Jain *et al*., 2008; Khan *et al*., 2009
Methionine sulfoxide	Increase	Schallreuter *et al*.,2008; Zhou *et al*., 2009
Allantoin	Increase	Shalbaf *et al*., 2008

normal skin in patients with vitiligo (Table 3).Impaired recycling of the essential cofactor $6BH_4$ [(6R)-l-erythro-5,6,7,8,-tetrahydrobiopterin] by elevated H_2O_2 causes accumulation of H_2O_2 in the epidermis and affects all cofactor-dependent mechanisms. $6BH_4$ is an essential electron donor in the hydroxylation of the aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan. These amino acids are substrates for melanogenesis, and thus, $6BH_4$ is an essential component of the pigmentary system (Schallreuter *et al.,* 2001). Inducible nitric oxide synthase levels in vitiligo epidermis are elevated, producing both H_2O_2 and peroxynitrite (Salem *et al.,* 2009). Homocysteine oxidation also causes elaboration of ROS, and elevated serum homocysteine levels have been reported in vitiligo patients (Shaker and El-Tahlawi, 2008). Elevated SOD activity would seem a likely source of H_2O_2 in vitiligo, but results have been contradictory. Both normal and elevated serum and tissue SOD activity have been shown (Koca *et al.,* 2004; Picardo *et al.,* 2004; Yildirim *et al.,* 2004; Hazneci *et al.,* 2004; Dammack *et al.,* 2009). XO catalysase the oxidative hydroxylation of hypoxanthine to xanthine and xanthine to uric acid as part of purine degradation. These reactions generate H_2O_2, and XO is considered a major biological source of ROS, leading to oxidative stress in many organs. Because H_2O_2 also

oxidizes uric acid to allantoin, this metabolite is a useful marker of oxidative stress. XO activity has been shown in skin, and elevated plasma levels have been measured in patients with vitiligo. Recently, XO activity in melanocytes and keratinocytes was confirmed, with H_2O_2 regulating the enzyme activity in a concentration dependent fashion: low levels (10^{-6} M) up-regulated activity, whereas high levels were suppressive. Oxidation by H_2O_2 of tryptophan and methionine residues in XO is thought to be the mechanism for this effect. Allantoin was detected in the epidermis of acute vitiligo but not in control skin, further supporting a role for ROS in vitiligo (Shalbaf *et al.,* 2008).

1.5.2.4.2 Exogenous ROS :

The role of exogenous oxidants in vitiligo is highlighted by the conditions of chemical leukoderma and contact vitiligo, as shared mechanisms might elucidate trigger factors and reasons for progression and chronicity in idiopathic vitiligo. Chemical leukoderma refers to acquired depigmentation at sites of contact with certain chemicals; contact vitiligo starts in the same manner, but depigmentation then spreads to distant sites, in the same way as generalized idiopathic vitiligo. Depigmentation in both cases occurs from loss of melanocytes in the epidermis. Chemicals involved are mostly phenolic and catecholic derivatives, which resemble tyrosine and can occupy the catalytic centre of tyrosinase as surrogate substrates in the melanin synthesis pathway (Miyamoto and Taylor, 2000). They include hydroquinone, MBEH (monobenzyl ether of hydroquinone) and 4-TBP (4-tertiary butyl phenol). These are oxidized by tyrosinase or tyrosinase-related protein to more reactive o-quinones, with the generation of ROS, which contribute to oxidative stress (Thorneby-Andresson *et al.,* 2000). In the presence of excess H_2O_2, this process is further accelerated.

Melanin synthesis is reduced because the intermediate dopaquinone is not synthesized, and melanocyte viability can be compromised. There is marked variation in individuals' susceptibility to chemical leukoderma and contact vitiligo, emphasizing the key role of genetic factors in determining melanocyte sensitivity to these stimuli. Highly reactive o-quinones can react with nucleophilic groups on proteins to create neoantigens and stimulate an immune response. Phenols that are well known contact allergens, like poison ivy, might also be oxidized by similar

mechanisms to form antigens on keratinocytes. This could be the link between ROS and an altered immune response in vitiligo. Interestingly, the clinical picture of occupational contact vitiligo is similar to allergic contact dermatitis, with itching, redness and scaling. When MBEH is used to remove remaining pigment in patients with vitiligo universalis, a similar reaction is seen, but only in the pigmented areas, suggesting involvement of melanocytes rather than keratinocytes. Patients with established generalized idiopathic vitiligo are sensitive to exogenous phenols and catechols (Namazi, 2007; Jimbow *et al.*, 1974; Cummings and Nordlund, 1995; Taieb, 2000). Generation of a reactive o-quinone from MBEH via tyrosinase was confirmed *in vitro*, with isolation of several byproducts with potential relevance to melanocyte toxicity (Manini *et al.*, 2009). Cytotoxic experiments have also confirmed that both 4-TBP and MBEH induce melanocyte death, but by different pathways: 4-TBP activates the caspase cascade and causes DNA fragmentation with apoptosis, while MBEH induces release of Mobility Group Box-1 protein, which causes necrosis rather (Hariharan *et al.*, 2010). Many drugs are potential exogenous sources of ROS, especially when metabolized by CYP enzymes, as they produce reactive quinones and semiquinones (Namazi, 2009). Interestingly, proton pump inhibitors were recently shown to reactivate vitiligo, and the mechanism might also be through generation of free radicals (Namazi *et al.*, 2008) and pH changes relating to melanogenic enzymes could also play a role (Schallreuter and Rokos, 2007).

1.5.2.4.3 Endogenous ROS - sources and effects:

1.5.2.4.3.1 Catechols:

Endogenous catechols are a source of ROS. Elevated plasma and urine catecholamines like norepinephrine, epinephrine and dopamine and their metabolites have been documented in vitiligo patients (Moronne *et al.*, 1992). Keratinocytes possess β-2 adrenoceptors and synthesize and degrade catecholamines, and melanocytes synthesize norepinephrine. Patients with vitiligo have markedly elevated GTP cyclohydrolase I activity, which leads to excessive *de novo* production of $6BH_4$, leading to increased synthesis of catecholamines in the epidermis (Moronne *et al.*, 1992; Schallreuter *et al.*, 1993; Schallreuter *et al.*, 1994; Chavan *et al.*, 2009). Catecholamines also compete preferentially with tyrosine for tyrosinase active

binding sites, becoming hydrolysed in the process and generating H_2O_2 (Westerhof and d'Ischia, 2007). Norepinephrine-induced vasoconstriction in vitiligo skin could cause hypoxia and predispose to oxidative stress by this mechanism (Namazi, 2007). These phenomena, among others, are the basis for the 'neural' theory of vitiligo aetiopathogenesis. Patients often report increased emotional stress prior to onset of vitiligo or concurrent with a flare of disease activity (Papadopoulos *et al.,* 1998).

1.5.2.4.3.2 BH_4 (tetrahydrobiopterin):

BH_4 cofactor is essential for various enzyme activities and is present in all cells. Six and seven isoforms of BH_4 are synthesized *de novo* from GTP, but regeneration is crucial for adequate functioning, and requires two enzymes, pterin-4a-carbinolamine dehydratase and dihydropteridine reductase. The latter is deactivated by H_2O_2 by oxidation of active site tryptophan and methionine residues, and H_2O_2 also oxidizes both 6- and 7- BH_4 to 6- and 7-biopterin. This is the reason for fluorescence, which can be seen in vitiligo patches under Wood's UV light. Thus, the homoeostasis of this important cofactor is compromised (Schallreuter *et al.,* 2001; Thony *et al.,* 2000; Hasse *et al.,* 2004). BH_4 deficiency affects melanin, catecholamine, serotonin and NO synthesis. L-phenylalanine levels would be expected to rise in this setting, and in fact, increased epidermal phenylalanine levels have been documented in patients with vitiligo by *in vivo* FT Raman spectroscopy (Schallreuter *et al.,* 1998). Increased *de novo* synthesis of 6- BH_4 in vitiligo contributes to elevated norepinephrine levels and up-regulates monoamine oxidase A and catechol-O-methyl transferase in the epidermis. These processes result in increased epidermal H_2O_2 (Darr and Fridovich, 1994; Le Poole *et al.,* 1994; Namazi, 2005).

1.5.2.4.3.3 Acetylcholine:

High epidermal levels of acetylcholine have been reported in vitiligo, and this is attributed to low epidermal AchE (acetylcholinesterase) and BchE (butyrylcholinesterase) activities due to the effect of high levels of H_2O_2. While low levels of H_2O_2 (10^{-6} M) activate AchE, high concentrations (10^{-3} M) deactivate the enzyme. This regulation of enzyme activity by H_2O_2 is seen with several other enzymes. Molecular modelling of AchE suggests that the inhibition is due to H_2O_2-mediated oxidation of tryptophan and methionine residues in the protein, causing disorientation of the active-site histidine residue. The tetramerization domain and

calcium-binding domains in BchE are also affected by high levels of H_2O_2 (Gibbons *et al.,* 2006; Schallreuter *et al.,* 2004; Schallreuter *et al.,* 2007).

1.5.2.4.3.4 Tryptophan:

Epidermal L-tryptophan is oxidized by H_2O_2 in vitiligo, as shown *in vivo* by FT Raman spectroscopy. Albumin contains a tryptophan residue in its sequence, and oxidation could explain low levels of epidermal albumin, which have been reported in vitiligo. Several other amino acid residues in albumin, such as methionine, are also prone to oxidation. Albumin plays a key role in calcium homoeostasis, and reduced albumin levels might account, in part, for impaired calcium uptake, which has been described in vitiligo. H_2O_2 also affects all four calcium EF-hand-binding domains of calmodulin, and calmodulin-ATPase activity is low in vitiligo skin. Epidermal furin is a calcium-dependent prohormone convertase, which plays a role in the cleavage of POMC. Loss of a calcium-binding site in furin because of oxidation from H_2O_2 has been shown in progressive vitiligo skin (Schallreuter *et al.,* 1998; Rokos *et al.,* 2004; Schallreuter *et al.,* 2007; Spencer *et al.,* 2008).

1.5.2.4.3.5 Cytokines:

SCF, a paracrine cytokine produced by keratinocytes, has a major role in promoting melanogenesis and in melanocyte survival. SCF interacts with its receptor on melanocytes, KIT protein which interacts with MITF-M. MITF-M serves as a transcription factor regulating expression of tyrosinase mRNA. MITF-M also interacts with Bcl-2 to prevent apoptosis of the melanocyte (Hachiya *et al.,* 2001). ET-1 is also an important regulator of melanin production, via its ETBR receptor (Imokawa *et al.,* 1996). SCF and ET-1 were not shown to be deficient in vitiligo lesions, suggesting that abnormal paracrine secretion by keratinocytes is not the cause of the hypopigmentation. At the edge of a vitiligo lesion, there are still melanocytes expressing tyrosinase, ETBR and S100α, albeit at slightly lower levels than unaffected skin, but KIT protein and MITF-M are markedly reduced. Reduced expression of KIT protein, and its downstream effectors like MITF-M, could explain melanocyte loss and/or dysfunction in vitiligo (Kitamura *et al.,* 2004). It is interesting that excessive H_2O_2 leads to down-regulation of MITF-M expression in cultured human melanocytes. Thus, ROS could be the cause of the cytokine abnormality seen in vitiligo (Jimenez-Cerventas *et al.,* 2001).

1.5.2.4.3.6 Trauma:

Trauma to the skin, UV radiation and other sources of inflammation probably contribute to the epidermal pool of H_2O_2 in a non-specific manner via NADPH oxidase stimulation. This could explain the prominent Koebner phenomenon seen in vitiligo, especially in active stages of the disease (Gauthier, 1996; Jezek and Hlavata, 2005).

1.5.2.4.3.7 Tyrosinase:

High levels of H_2O_2 ($0.5–5.0\times10^{-3}$ M) have been shown to deactivate tyrosinase, and this effect is compounded by increased $6BH_4$ (Wood *et al.,* 2004). Abnormal expression of TRP-1 (tyrosinase-related protein-1) has been reported following oxidative stress in cultured melanocytes taken from the advancing border of vitiligo lesions.This leads to early cell death, possibly through an interaction with calnexin (Jimbow *et al.,* 2001).

1.5.2.5 Antioxidant defense mechanisms:

The antioxidant system comprises of different types of functional components such as enzymatic and nonenzymatic antioxidants. The enzymatic antioxidants comprise of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase. The non-enzymatic antioxidants include reduced glutathione, vitamin C, vitamin E (α -tocopherol), uric acid, carotenoids, flavanoids, ubiquinol etc.

1.5.2.5.1 Catalase:

Catalase is present in the peroxisomes of nearly all aerobic cells and protects the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals and the overall reaction is as follows:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

This reaction is a first order reaction and depends entirely on the concentration of hydrogen peroxide. At high substrate concentrations the rate of reaction is usually

rapid. However, millimolar levels of H_2O_2 ($\geq 10^{-3}$ M) are reported to result in the inactivation of catalase (Schallreuter *et al.,* 1991).

1.5.2.5.2 Glutathione peroxidase :

Glutathione peroxidase catalyzes the reduction of various organic hydroperoxides, as well as hydrogen peroxide, with glutathione as hydrogen donor. The cytosolic from of GPX (GPX1) is the first and best characterized selenoprotein (Arthur, 2000). GPx1 is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. There are two locations of GPX in the cells, mitochondria and cytosol. The reaction catalyzed by GPX is given below.

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

Glutathione reductase then reduces the oxidized glutathione to GSH

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

1.5.2.5.3 Glutathione reductase:

Glutathione reductase (GR) is a ubiquitous enzyme associated with the hexose monophosphate shunt of glucose metabolism. It catalyses reduction of oxidized glutathione (GSSG) to reduced GSH, with the concomitant conversion of NADPH to $NADP^+$ (Beutler and Yeh, 1963). The reaction catalyzed by GR is given below:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

1.5.2.5.4 Glutathione S transferase :

Glutathione S-transferases (GSTs) are a super family of enzymes. It plays an important role in the detoxification and elimination of xenobiotics. This process involves conjugation of glutathione with electrophilic metabolites and extrusion of the conjugate out of the cell for further metabolism. GSTs also function as glutathione peroxidases by reducing organic hydroperoxides to the corresponding alcohols, of

importance for protection against oxidative stress and consequent lipid peroxidation. The reaction catalyzed by GST is given below

$$RX + \text{glutathione} \xrightarrow{GST} HX + \text{R-S-glutathione}$$

1.5.2.5.5 Glucose -6- phosphate dehydrogenase:

The intracellular redox potential is determined by the concentrations of oxidants and reductants. A critical modulator of the redox potential is NADPH, the principal intracellular reductant in all cell types. Glucose -6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway (PPP) determines the amount of NADPH by controlling the metabolism of glucose via PPP (Kletzien *et al.,* 1994). The amount of NADPH maintains an adequate level of reduced glutathione (GSH). G6PDH is present in all human cells but is particularly important to red blood cells.NADPH protects the sulfhydryl groups (-SH) of hemoglobin and the red cell membrane from oxidation by the reactive oxygen species. The reaction catalyzed by G6PD is given below:

$$\text{Glucose -6-phosphate} + NADP^{+} \longrightarrow \text{Glucono-1, 5-lactone 6-phosphate} + NADPH + H^{+}$$

1.5.2.5.6 Reduced glutathione :

Glutathione (γ-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the living system. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. Glutathione exists in two forms: the antioxidant "reduced glutathione" tripeptide is conventionally called glutathione (GSH) and the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Rahman *et al.,* 2005). Glutathione status is homeostatically controlled both inside and outside the cell, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH synthetase enzymes), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

The GSH can act as free radical scavenger and as an antioxidant enzyme cofactor. Glutathione is most concentrated in the liver (10 mM), where the "P450 Phase II" enzymes require it to convert fat soluble substances into water soluble GSH conjugates, in order to facilitate their excretion. GSH depletion leads to cell death, and has been documented in many degenerative conditions. Mitochondrial GSH depletion may be the ultimate factor determining vulnerability to oxidant attack.

1.5.2.5.7 Vitamin E:

Vitamin E refers to a group of antioxidants, which consists of tocopherols and tocotrienols, in which α-tocopherol has the highest biological activity. Alpha tocopherol is the major lipid soluble, chain breaking antioxidant, which protects mammalian membranes and lipoproteins from damage. Vitamin E is mainly found on membranes where they either interrupt the propagation step of lipid peroxidation by destroying peroxyl radicals ($ROO^{\cdot}$) or block the formation of hydroperoxides from singlet oxygen (Halliwel and Chirico, 1993).

Alpha tocopherols are efficient scavengers of peroxyl radicals in phospholipid bilayers. It scavenges lipid peroxyl radicals ($LOO^{\cdot}$) through hydrogen atom transfer. The α-tocopherol radical might also react with a further peroxyl radical to give a non radical product i.e. one molecule of α-tocopherol is capable of terminating two peroxidation chains (Chaudiere and Ferrari-Iliou, 1999).

$$\alpha\text{-TOH} + LOO^{\cdot} \longrightarrow \alpha\text{-TO} + LOOH$$

$$LOO^{\cdot} + \alpha\text{-}TO^{\cdot} \longrightarrow \alpha\text{-TocOOL}$$

1.5.2.5.8 Superoxide dismutase:

Superoxide dismutases (SODs) are metalloenzymes found widely distributed in prokaryotic and eukaryotic cells (Fridovich, 1995). They constitute an enzyme family that catalyzes the conversion of supeoxide anion to H_2O_2.

$$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$$

There are upto three different metal containing SOD enzymes present in different organisms depending upon the species. These SODs form the major superoxide scavenging system in the mitochondria, nucleus, cytoplasm and extracellular spaces. These SODs are the products of different genes and are designated by their primary

location as follows. SOD1 (Cu-Zn SOD, cytoplasmic), SOD 2 (Mn SOD, mitochondrial), SOD 3 (Cu-Zn SOD, extracellular) (Johnson and Giulivi, 2005).

In humans SOD family members are either dimeric- SOD 1; 32 kDa, (McCord and Frodovich, 1969) or tetrameric- SOD 2; 89 kDa, (McCord 1976), SOD 3; tetrameric, 135 kD, (Marklund, 1984). Part of cell's stress response is to increase the transcription of *SOD* genes, which in turn leads to increased SOD activity. This has been shown by gene exprssion profiles using a number of tissues, under different stress conditions (McMillan *et al.,* 2004; Nilakantan *et al.,* 2005). All mammalian SODs are nuclear encoded, being initially formed as inactive apo-enzymes. For fully functional mitochondrial MnSOD (SOD 2) the nascent polypeptide is targeted to the mitochondrial membrane, where it is folded and correctly receives its manganese prosthetic group (Luk *et al.,* 2005).

1.5.2.5.8.1 Superoxide dismutase 1 (SOD1)

1.5.2.5.8.1.1 Gene Structure:

The gene sequence for *SOD1* has been identified in the rat (Kim *et al.,* 1993; Hsu *et al.,* 1992), mouse (Benedetto *et al.,* 1991), and human (Levanon *et al.,* 1985). The organization of *SOD1* gene shows striking similarity among species and has five exons and four introns (Figure 10). The TATA and CCAAT boxes, as well as several highly conserved GC-rich regions, have been localized in all three species with a similar pattern in the proximal promoter region. Such a high level of homology in the 5' flanking sequence suggests that intense evolutionary factors have preserved key regulatory regions for this gene. The 3' end of *SOD1* gene possesses several poly(A) signal sequences that terminate the mRNA species with different lengths. The consensus sequences YGTGTTYY and a G/T cluster required for efficient formation of 3'-termini have also been located downstream from the polyadenylation signal in the rat *SOD1* gene. The promoter region of the human *SOD1* gene has been studied and several putative binding sites for NF1, Sp1, AP1, AP2, GRE, HSF, and NF-κB transcription factors have been found (Kim *et al.,* 1994). The role of Sp1 and Egr-1 transcription factors in basal and inducible expression of human *SOD1* has been confirmed (Minc *et al.,* 1999).

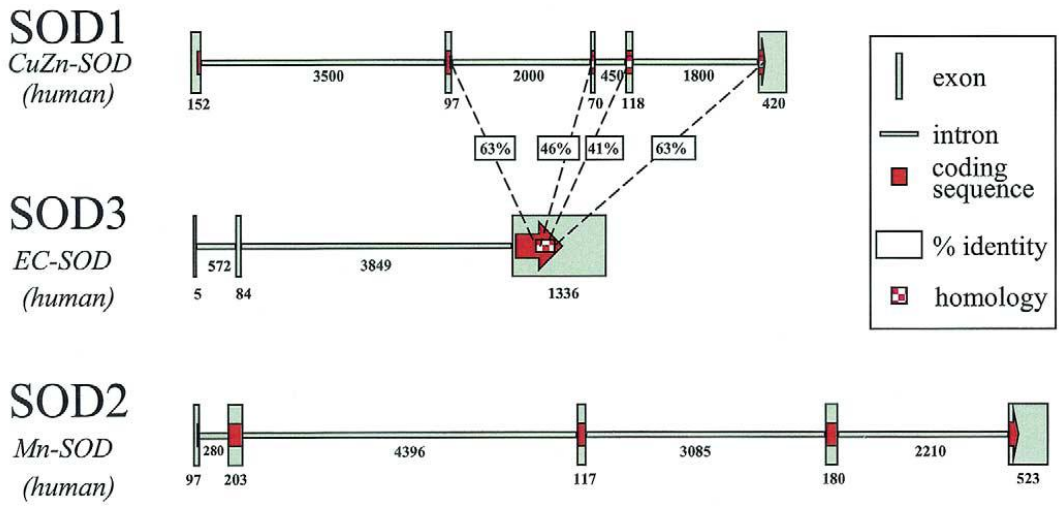


Figure 10. Genomic organization of the three known members of the human *SOD* family. *SOD3* was placed in the middle in order to demonstrate areas of amino acid sequence homology between *SOD1* and *SOD3*. *SOD2* has no significant amino acid sequence homology with either *SOD1* or *SOD3*. The size of each exon and intron, in base pairs, is shown in association with that fragment. Data for this figure was taken from the following sources: *SOD1* (Levanon *et al.*, 1985), *SOD2* (Ho *et al.*, 1991), and *SOD3* (Folz *et al.*, 1994).

1.5.2.5.8.1.2 Chromosomal localization and polymorphisms:

The *SOD1* gene has been localized to chromosome 21 (region 21q22) in humans (Levenon *et al.*, 1985), chromosome 1 (1q12 →14) in bovine species (Schmutz *et al.*, 1996), and chromosome 16 (region 16B4→3 ter) in the mouse (Francke *et al.*, 1979). Human chromosome 21 has been studied intensely because of the association between Down Syndrome and trisomy 21. Although patients with Down Syndrome show a 50% increase in SOD1 activity due to higher levels of SOD1 protein, the role of this enzyme in pathology associated with this disease is not known. The increased dosage of *SOD1* gene associates with some symptoms of Down Syndrome, such as the pathological abnormalities of tongue neuromuscular junctions (Avraham *et al.*, 1998; Groner *et al.*, 1994) but has no obvious implication in the development of the major symptoms (Torre *et al.*, 1996). On the other hand, more than 90 different mutations in the . gene have been associated with Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease. This fatal disorder causes degradation of motor

neurons in the corticospinal tracts and brain stem. Although only 2% of patients with ALS and 10–15% with familial ALS have mutations in the *SOD1* gene, the discovery of these mutations by Rosen *et al.* (1993) provided the first molecular insight into the pathogenesis of this disease. Since this discovery, several theories have been proposed to explain the mechanism of motor neuron damage caused by mutations in *SOD1*. One hypothesis is that mutations in the *SOD1* gene may impair antioxidant enzyme activity that in turn could lead to accumulation of toxic superoxide anions. This theory was dismissed experimentally when *SOD1* bearing the G93A mutation was overexpressed in mice, resulting in motor neuron disease despite the elevated SOD1 activity (Gurney *et al.,* 1994). Moreover, complete inactivation of *SOD1* in "knock-out" mice did not cause any motor neuron abnormalities (Reaume *et al.,* 1996), although they exhibit increasing embryonic lethality and reduced fertility in females (Ho *et al.,* 1998). The opposite gain-of-function theory has been proposed based on the assumption that mutations in the *SOD1* gene change affinity of the enzyme to the natural and abnormal substrates (Wiedau-Pazos *et al.,* 1996), impair ability of enzyme to bind zinc (Estevez *et al.,* 1999) or increase the enzyme aggregation in neurons (Bruijn *et al.,* 1998; Chou *et al.,* 1996). Either way, the dominant mutations in *SOD1* play a key role in the pathogenesis of familial ALS.

1.5.2.5.8.1.3 Transcriptional regulation:

SOD1 was found to have a widespread distribution in a variety of cells (Crapo *et al.,* 1992). The expression of cytoplasmic *SOD1* is constitutive and its activity is often considered as an internal control for *SOD2* gene expression.

1.5.2.5.8.1.4 Stimuli upregulating *SOD1* expression:

Despite the fact that *SOD1* is considered to be constitutively expressed, its mRNA levels can be dramatically regulated by various physiological conditions. *SOD1* mRNA levels elevate in response to a wide array of mechanical, chemical, and biological messengers such as heat shock (Hass *et al.,* 1988; Yoo *et al.,* 1999a), shear stress (Inoue *et al.,* 1996; Dimmeler *et al.,* 1999), UVB- and X-irradiation (Isoherranen *et al.,* 1997; Leccia *et al.,* 2001; Yamaoka *et al.,* 1994), heavy metals (Yoo *et al.,* 1999b), hydrogen peroxide (Yoo *et al.,* 1999c), ozone (Rahman *et al.,* 1991), nitric oxide (Frank *et al.,* 2000), arachidonic acid (Yoo *et al.,* 1999d), and

xenochemicals such as α-naphthoflavone, t-butyl-hydroquinone, iodoacetamide (Yoo *et al.,* 1999e), 2,3,7,8-tetrachlorodibenzo-p-dioxin (Cho *et al.,* 2001), and phenobarbital (Ueda *et al.,* 2002). Analysis of the proximal promoter region reveals Sp1/Egr-1/WT-1 binding sites that are involved in basal and TPA inducible expression of *SOD1* (Minc *et al.,* 1999) as well as C/EBP cis-acting elements (Seo *et al.,* 1997; Seo *et al.,* 1996), which are also important for high level expression in rat liver cells (Kim *et al.,* 1997). *SOD1* expression can also be triggered by ginseng saponins through activation of the AP2 transcription factor (Kim *et al.,* 1996). Metal ions are a potent source for the large scale catalysis and production of intracellular ROS. In order to neutralize ROS mediated harmful effects, transcriptional upregulation of *SOD1* is seen through the metal responsive element located in the 5'-flanking region (Yoo *et al.,* 1999b).

1.5.2.5.8.1.5 Stimuli downregulating *SOD1* expression:

Downregulation of *SOD1* has been shown in alveolar type II epithelial cells and lung fibroblasts after exposure to hypoxia (Jackson *et al.,*1996). The anticancer drug, mitomycin C also represses the transcription of *SOD1* gene in Hep G2 cells (Cho *et al.,* 1997). *SOD1* mRNA levels and enzymatic activity slightly increase from birth to adulthood in lung of the guinea pig (Yuan *et al.,* 1996), rat (Hass *et al.,* 1984; Clerch *et al*., 1992; Hayashibe *et al*., 1990), and rabbit (Frank *et al*., 1984). This rise in SOD1 activity is due mostly to an increased rate of mRNA synthesis. SOD1 is also developmentally regulated in the rat kidney where its activity increases 1.7- fold from gestational day 18 to day 22, while in heart its activity remains unchanged (Hayashibe *et al*., 1990). In mouse, the expression pattern for SOD1 is highly variable among different strains, but shows an increased level in lung and brain during aging, but no difference is seen in mRNA levels in both heart and kidney (Schisler *et al*., 1985; de Haan *et al.,* 1985).

1.5.2.5.8.2 Superoxide dismutase 2 (SOD2)

1.5.2.5.8.2.1 Gene structure:

The complete gene structure for *SOD2* has been determined for the human (Church *et al.,* 1992; Wan *et al*., 1994), rat (Ho *et al*., 1991), and mouse (Di Silvestre *et al*.,

1995; Jones *et al*., 1995). Partial identification and characterization of a bovine *SOD2* gene has been described (Meyrick *et al*., 1994). All of these species show marked conservation of structure and sequence. *SOD2* gene is composed of 5 exons and 4 introns (Figure 10). Southern blotting supports the existence of one *SOD2* gene in human (Wan *et al*., 1994) murine (Jones *et al*., 1995), and bovine species (Meyrick *et al*., 1994), whereas two genes per haploid genome have been described in the rat (Ho *et al*., 1991). The promoter regions in all four species share common features. There are no upstream TATA or CAAT box elements identified, however, GC-rich regions are present in all four species. Such features can be typical of housekeeping genes (Jones *et al*., 1988; Dynan, 1986). The human and mouse genes each contain putative NF-kB transcription regulatory element, for humans, it is located in the 3'-flanking region of the gene (Wan *et al*., 1994) while the mouse contains two potential elements in the 5'-flanking region (Jones *et al*., 1995). Multiple copies of Sp-1 and AP-2 consensus sequences are also present in the promoter region of all four species.

1.5.2.5.8.2.2 Chromosomal localization and polymorphisms:

Using enzymatic analysis of mouse/human hybrids, the *SOD2* gene was initially localized to chromosome 6 (Creagan *et al*., 1973). Later, the *SOD2* gene was sublocalized to region 6q25 by fluorescence *in situ* hybridization and somatic cell hybrid mapping (Church *et al*., 1992). The importance of SOD2 function in mammals were confirmed by disruption of the *SOD2* gene, which turns out to be lethal for mice due to neurodegeneration and heart damage (Lebovitz *et al*., 1996). Several genetic variations have been described for the human *SOD2* gene. The substitution of Ala-9 to Val in the mitochondrial targeting sequence causes premature aging or progeria (Rosenblum *et al*., 1996) and is associated with an increased risk of sporadic motor neuron disease, especially in females (Van Landeghem *et al*., 1996) and with nonfamilial idiopathic cardiomyopathy (Hiroi *et al*., 1999) but has no effect on the occurrence of Parkinson's disease (Farin *et al*., 2001) or ALS (Parboosingh *et al*., 1995; Tomkins *et al*., 2001). At least three heterozygous mutations in the proximal promoter of human *SOD2* have been identified and linked to the reduced transcriptional activity in transient transfection experiments (Xu *et al*., 1999).

1.5.2.5.8.2.3 Transcriptional regulation:

Despite the fact that *SOD2* is expressed in many cell types and tissues at relatively high levels, it is also highly regulated by a variety of intracellular and environmental signals. Characterization of the 5'-flanking genomic region from rat (Kuo et al., 1999), bovine (Meyrick *et al*., 1994), and human (Wan *et al*., 1994; Zhang *et al*., 1996; Yeh *et al*., 1998) indicates that the *SOD2* promoter is TATA and CAAT-less but contains GC-rich sequences immediately upstream from the transcription initiation site. Bioinformatics analysis and foot-printing assays reveal a number of putative binding sites for Sp1 and AP2 transcription factors in the proximal promoter of human *SOD2*. The two proteins have opposite effects on *SOD2* expression: while the Sp1 element positively promotes transcription, the AP2 proteins significantly repress the promoter activity (Zhu *et al*., 2001)

1.5.2.5.8.2.4 Stimuli upregulating SOD2 expression:

A wide variety of compounds induce transcription of *SOD2*. Cytokines such as interleukin (IL)-1 (Masuda *et al*., 1988; Visner *et al*., 1992; Dougall and Nick, 1991), IL4, IL6 (Dougall and Nick, 1991), TNFα (Visner *et al*., 1992; Wong and Goeddel, 1988), lipopolysaccharide (LPS) (Visner *et al*., 1990), and IFNγ (Harris *et al*., 1991) are potent activators of *SOD2* in different tissues and cell types. The cytokine inducible enhancer has been localized to the 236 bp sequence within intron 2 of murine (Jones *et al*., 1997), rat and human (Rogers *et al*., 2000) *SOD2* genes. The cytokine inducible enhancer regions contain binding sites for NF-kB, C/EBP, and NF-1 transcription factors. Protein kinase C stimulating agents such as TPA induce human *SOD2* expression via activation of a CREB-1/ATF-1 like factor, but not via NF-kB or AP1 (Kim *et al*., 1999). Interestingly, the microtubule-active anticancer drugs vinblastin, taxol and vincristine also induce *SOD2* expression via activation of protein kinase C (Das *et al*., 1998). Manganese ions, which at high concentrations are toxic to the cells, induce expression of *SOD2* in human breast cancer (Thongphasuk *et al*., 1999). Platelet-derived growth factor induces the expression of the *SOD2* gene in NIH3T3 cells, and its induction is associated with activation of Egr-1 transcription factor (Maehara *et al*., 2001).

1.5.2.5.8.2.5 Stimuli downregulating *SOD2*:

The expression of *SOD2* in many cancers is decreased due to methylation of particular sequences in the intronic region (Huang *et al*., 1997; Huang *et al*., 1999) and elevated levels of AP2 transcription factor, which interacts with the 5'-flanking sequences of *SOD2* gene (Zhu *et al*., 2001).

1.5.2.5.8.2.6 Translational regulation of SOD2:

SOD2 expression is regulated not only at the level of transcription, but also at the level of translation by a RNA-binding protein. The 41 bp region, located in the 3'-untranslated part of *SOD2* mRNA binds the specific protein that increases its translation efficiency (Chung *et al*., 1998). When this cis-element was positioned after the coding region of chloramphenicol acetyltransferase, it considerably increased the translation efficiency and enzymatic activity of the reporter gene (Knirsch *et al*., 2000). While the identity of RNA-binding protein has not been determined, but SOD2 binding protein undergoes phosphorylation by tyrosine kinase and dephosphorylation state is required for its binding activity (Knirsch *et al*., 2001).

The expression profile of SOD2 is somewhat similar to that of SOD1 and appears to be species-specific. In the sheep and guinea pig, kidney SOD2 activity and mRNA concentration increase in neonatal and adult animals compared to early and late gestation fetuses (Carbone *et al*., 1994; Vlessis *et al*., 1989). In humans the expression profiles of *SOD1* and *SOD2* almost coincide, increasing towards adulthood in lung and liver, but the activities do not always correlate with mRNA levels (Asikainen *et al*., 1998).

1.5.2.5.8.3 Superoxide dismutase 3 (SOD3)

1.5.2.5.8.3.1 Gene Structure:

The gene structure for human *SOD3* has been determined (Folz and Crapo, 1994). A partial genomic clone encoding the complete open reading frame for mouse SOD3 has been reported (Carlsson *et al*., 1995). Currently, *SOD3* cDNA clones for the human (Hjalmarsson *et al*., 1987), rat (Perry *et al*., 1993; Willems *et al*., 1993), mouse (Folz *et al*., 1997), and rabbit (Laukannen *et al*., 1995) have been isolated and sequenced. The *SOD3* gene shares 40–60% similarity with the *SOD1* gene at the exon level, but shows no similarity with *SOD2* (Figure 10). The mouse *SOD3* gene consists of two

exons separated by a 4 kb intron while in human three exons have been found. The promoter region of human and mouse *SOD3* apparently lacks classical TATA or CCAAT boxes (Folz and Crapo, 1994). In humans, several putative transcriptional response elements have been identified and include a metal regulatory element, an AP-1 site as well as two potential antioxidant response elements (Folz and Crapo, 1994). In contrast, the mouse proximal promoter, characterized by unusually GA-rich sequence, has multiple putative binding sites for Kruppel-like and Ets-family transcription factors. The functional importance of these sites is not clear.

1.5.2.5.8.3.2 Chromosomal localization and polymorphisms:

The *SOD3* gene has been localized to chromosome 4 (region 4p–q21) of the human (Hendrickson *et al*., 1990) and in the middle of chromosome 5, tightly linked to the QDPR locus in mouse (Folz *et al*., 1997; Suh *et al*., 1997). To date, only one mutation located in the center of the carboxyl-terminal cluster of positively charged amino acid residues, which defines the heparin binding domain, has been described for human *SOD3*. Substitution of arginine in position 213 to glycine causes an 8–15-fold increase in plasma SOD3 levels (Folz *et al*., 1994; Yamada *et al*., 1995; Sandstrom *et al*., 1994). The effect of this *SOD3* polymorphism, which has been found in 4% of Swedish (Marklund *et al*., 1997), 3% of Australian (Adachi and Wang, 1998) and 6% of Japanese (Yamada *et al*., 1995) subjects studied. Early studies suggest that this amino acid mutation impairs affinity for heparin and endothelial cell surface and may reduce susceptibility to trypsin-like proteases. Two additional polymorphisms have been identified in the human *SOD3* gene; a transition mutation of A to G at position 241 resulting in a Thr to Ala (T40A) substitution and a silent transition mutation of C to T at position 280 (Yamada *et al*., 1997). While the substitution of nucleotide A to G at position 241 creates a new BssHll restriction site, T to A at position 40 does not seem to affect heparin binding capacity or the specific activity of EC-SOD (Yamada *et al*., 1997]. *SOD3* null mutant mice show enhanced sensitivity to hyperoxia (Carlsson *et al*., 1995), worsened outcome from focal cerebral ischemia (Sheng *et al*., 1999), and have dramatic impairments in spatial learning (Levin *et al*., 1998).

1.5.2.5.8.3.3 Transcriptional regulation:

In contrast to intracellular *SOD1* and *SOD2*, the expression of *SOD3* appears restricted to only a few cell types in several tissues. High levels of *SOD3* expression

have been documented for alveolar type II cells (Folz *et al*., 1997),vascular smooth muscle cells (Stralin *et al*.,1995), lung macrophages (Loenders *et al*., 1998) and a few cultured fibroblast cell lines (Marklund, 1990). The features regulating such highly specific expression are not yet known, but analysis of the 5'- flanking region of human *SOD3* reveals several potential regulatory sequences such as a glucocorticoid response element, xenobiotic response element, and an antioxidant response element (Folz and Crapo, 1992). Bioinformatic analysis of murine *SOD3* proximal promoter reveals multiple putative binding sites for the ETs family of transcription factors. The importance of these proteins in regulating cell-specific expression has yet to be elucidated. The promoter region of *SOD3* lacks typical TATA or CAAT boxes but possesses purine-rich sequences.

1.5.2.5.8.3.4 Stimuli upregulating SOD3:

In human fibroblasts, the level of SOD3 was elevated by IFNγ and IL1β, while other cytokines such as IL2, IL3, IL4, IL6, and IL8 demonstrated no effect on its expression (Marklund, 1992). Similar results were reported for induction of SOD3 in rat sertoli cells, but IFNγ has no effect on *SOD3* expression (Mruk *et al*., 1998). TNFα and IFNγ together appear to have a role in induction of *SOD3* expression in rat alveolar type II pneumocytes through NF-kB activation (Brady *et al*., 1997). As SOD3 exerts an important protective role in the vascular wall, the vasoactive factors such as histamine, vasopressin, oxitocine, endothelin-1, serotonin, and heparin markedly increased enzyme level in the cultured arterial smooth muscle cells (Stralin and Marklund, 2001). Further, exercise training increases production of nitric oxide in mouse vessel endothelial cells, which in turn upregulates *SOD3* in adjacent smooth muscle cells (Fukai *et al*., 2001). Thus, increased concentration of SOD3 prevents the degradation of NO by oxygen radicals. Angiotensin II strongly induces SOD3 activity in mouse aortas (Fukai *et al*., 1999) and in cultured human smooth muscle cells (Stralin and Marklund, 2001) through transcriptional activation and stabilization of mRNA. Interestingly, the effect of angiotensin II on SOD3 expression is due to activation of p42/44 MAP kinase pathway, while nitric oxide exerts its effect through MAP kinase p38. There are contradictory data on regulation of *SOD3* expression by cyclic nucleotides. The exposure of rat glioma cells to cAMP increases SOD3 production while in mouse aortas it has no effect (Fukai *et al*., 2001; Nicolai *et al*., 1996). Interesting data on upregulation of *SOD3* mRNA level in Hep G2 cells

expressing nuclear receptor CAR have been published, but the physiological relevance of this regulation is not clear (Sugatani *et al*., 2001).

1.5.2.5.8.3.5 Stimuli downregulating SOD3:

The expression of *SOD3* is repressed by different types of growth factors. Transforming growth factor-β in human fibroblasts (Marklund, 1992) and platelet-derived growth factors and fibroblast growth factor in vascular smooth muscle cells (Stralin and Marklund, 2001) markedly downregulate expression and excretion of SOD3. These responses are slow and develop over several days.

The developmental expression of SOD3 has been documented only in rabbit lung at preterm, term, 8 days old, 1 month, and adult stages (Nozik-Grayck *et al*., 2000). While activity of SOD3 increases almost six times from preterm to adult, the SOD3 protein level remains constant during these times. In humans, plasma levels of SOD3 in children are considerably higher compared with adults and decreases toward adulthood about 2% per year reaching a plateau at age 20 (Adachi *et al*., 2000).

1.5.3 Autoimmune Hypothesis:

Generalized vitiligo is widely considered as an autoimmune disease, with involvement of humoral and cellular components of the innate and adaptive immune system. This hypothesis is supported by the following lines of epidemiological, clinical and investigational research: an association with other autoimmune disorders; chronic relapsing and remitting course so typical of autoimmune disorders; possible response to immunosuppressive therapies like topical and oral corticosteroids, and topical calcineurin inhibitors; 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 (Le Poole and Luiten, 2008; Ongenae *et al*., 2003). Autoimmune conditions associated with vitiligo include autoimmune polyendocrine syndrome types 1 and 2, pernicious anaemia, type 1 diabetes, Addison's disease, Graves' disease, alopecia areata, systemic lupus erythematosus, rheumatoid arthritis, psoriasis and myasthenia gravis. A survey of 2600 vitiligo patients showed increased frequencies of autoimmune thyroid disease, Addison's disease, systemic lupus

erythematosus and pernicious anaemia, with about 30% of patients having at least one of these disorders (Alkhateeb *et al*., 2003) In addition, family members who did not have vitiligo still had a tendency to the same autoimmune conditions, pointing to a genetic risk for a specific cluster of autoimmune diseases. Other studies report associations only with thyroid dysfunction and thyroid antibodies, regarding the other conditions as random concomitant events. Psoriasis or lichen planus occurring in vitiligo lesions has also been reported. Organ specific autoantibodies are reported with increased frequency in vitiligo patients, often in the absence of clinical symptoms. There is probably an increased risk of developing clinical or subclinical disease later (Ongenae *et al.,* 2003; Schallreuter *et al.,* 1994; Alkhateeb *et al.,* 2003; Jandus *et al*., 2008). Several immunogenetic factors predispose patients to autoimmune diseases, and some of these are associated with vitiligo, adding to the evidence that vitiligo may have an autoimmune basis. Various HLA class II alleles have been associated with vitiligo, in particular, HLADR4 (Foley *et al.,* 1983). The particular haplotype association varies according to ethnic origin. Genes involved in antigen presentation and processing have been associated with autoimmune diseases and in some cases with vitiligo (Casp *et al.,* 2003). Homozygous or heterozygous complement 2 and 4 deficiency is associated with autoimmunity, and this has been described in vitiligo (Venneker *et al.,* 1992). Certain *CTLA4* polymorphisms predispose to vitiligo in patients who already have other autoimmune conditions (Blomhoff *et al.,* 2005). Autoimmune polyendocrine syndrome type 1, which often includes vitiligo, is due to mutations in the autoimmune regulator gene, *AIRE* (Collins *et al.,* 2006). A missense mutation in the *PTPN22* gene, which encodes LYP (lymphoid protein tyrosine phosphatase), has been linked to several autoimmune diseases including vitiligo (Canton *et al.,* 2005). Loci on chromosomes 1, 7 and 8 have been linked with autoimmune diseases and termed *AIS* (autoimmune susceptibility loci) 1, 2 and 3, respectively. A locus designated *SLEV1* on chromosome 17p13 has also been linked to vitiligo (Spritz *et al.,* 2004). Animal models of vitiligo show prominent roles for anti-melanocyte antibodies. Some of these cross-react with mammalian TRP-1 (Austin *et al.,* 1995). Antibodies to melanocytes have been found in the circulation of patients with vitiligo (Farrokhi *et al.,* 2005). These antibodies correlate with disease activity and extent (Harning *et al.,* 1991). Targets of these antibodies include a variety of melanocyte and melanosomal antigens. Whatever their role in vitiligo, these antibodies have the capacity to injure pigment cells *in vivo* and *in vitro*. Hence study

of melanocyte antibodies and target antigens might refine the diagnostic and prognostic testing of vitiligo, reveal putative T-cell targets (Oyarbide-Valencia *et al.,* 2006). Circulating anti-parietal cell, thyroid and adrenal antibodies have been detected in vitiligo patients, as well as antinuclear antibodies and rheumatoid factor, again suggesting an autoimmune pathomechanism for the disease (Farrokhi *et al.,* 2005). Autoimmune diseases are the result of complex interactions between T and B cell subpopulations. A flow cytometric study of these cells in vitiligo did not show a pathological distribution of B cells, suggesting that T-cells might have a more dominant role.

1.5.3.1 Humoral immune response in vitiligo:

Antibodies against melanocyte antigens are detected in the sera of vitiligo patients mainly belonging to the IgG class. The principal antigen recognized by these antibodies is tyrosinase (Song *et al.,* 1994; Fishman *et al.,* 1993; Kemp *et al.,* 1997). The other melanocyte antigens recognized by autoantibodies are gp100/Pmel 17 (a melanosomal matrix glycoprotein), and tyrosinase related proteins 1 and 2 (TRP 1 and TRP 2) (Kemp *et al.,* 1998a; 1998b). These cell differentiation antigens are localized primarily to melanosomes (Hearing, 1999). The transcription factors SOX9 and SOX10 are identified as melanocyte autoantigens (Hedstrand *et al.,* 2001). Also autoantibodies against HLA Class I molecules are reported in vitiligo (Ongenae *et al.,* 2003). A summary of the autoantigens implicated in vitiligo is given in Table 5. A correlation is seen between the level of melanocyte antibodies and disease activity in vitiligo (Harning *et al.,* 1991). Also presence of these antibodies is also related to the extent of the skin area involved (Naughton *et al.,* 1986). *In vitro* studies showed that vitiligo antibodies are able to destroy melanocytes by complement mediated damage and antibody dependent cellular cytotoxicity (Gilhar *et al.,* 1995). Recently a surface receptor, melanin concentrating hormone receptor 1 (MCHR1) was detected as an autoantibody target in 16% vitiligo sera. Circulating organ specific autoantibodies particularly to thyroid, adrenal glands and gastric glands are commonly detected in the sera of vitiligo patients (Zauli *et al.,* 1986; Mandry *et al.,* 1996; Brostoff *et al.,* 1969; Betterle *et al.,* 1976).

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The exact role of antimelanocyte antibodies in the pathogenesis of vitiligo remains unresolved. Autoantibodies against pigment cells might result from a genetic predisposition to immune dysregulation at the T cell level (Kemp *et al.*, 2001).

Table 5. Antigens recognized by vitiligo autoantibodies.

Autoantigens	**Reference**
Tyrosinase	Song *et al.*,1994, Baharav *et al.*,1996; Xie *et al.*,1999; Kemp *et al.*,1997
TRP 1	Kemp *et al.*,1998b
TRP 2	Okamoto *et al.*,1998; Kemp *et al.*,1997
Pmel 17	Kemp *et al.*,1998a
Melan A/MART 1	Waterman *et a,.*,2002
MCHR 1	Waterman *et al.*,2002
SOX 9	Hedstrand *et al.*,2001
SOX 10	Hedstrand *et al,.*,2001

Alternatively cross-reacting antigens expressed either on other target cells or infecting microorganisms could elicit their production. Vitiligo antibodies could also result from an immune response to melanocyte antigens released following damage to pigment cells by other mechanisms, and these antibodies might then exacerbate the condition. The selective destruction of melanocytes might result from antibody reactivity directed to the antigens preferentially expressed on pigment cells (Kemp *et al.*, 1997) or from a antibody response against antigens expressed on a variety of cell types (Cui *et al.*, 1992) that might selectively destroy melanocytes because they are intrinsically more sensitive to immune mediated injury than other cells (Norris *et al.*, 1988).

1.5.3.2 Cell mediated immunity:

Histopathological investigations of the perilesional skin of vitiligo suggested the involvement of lymphocytes in the depigmentation process. 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; Gross *et al.,* 1987; Wijngaard *et al.,* 2000). By contrast, a decrease in the $CD4^+$ T cell population along with a reduced $CD4^+/CD8^+$ ratio has been observed (Grimes *et al.,* 1986; Halder *et al.,* 1986). A substantial number of infiltrating T cells express the cutaneous lymphocyte antigen (Al Badri *et al.,* 1993), CLA typical of skin homing T cells. Wijngaard *et al.,* (2000) reported the localization of CLA positive cytotoxic T cells in apposition to disappearing melanocytes in the perileisonal skin of vitiligo patients. High frequencies of Melan A/Mart 1 (a melanosomal antigen) specific $CD8^+$ T lymphocytes are identified in peripheral blood (Ogg *et al.,* 1998). Interestingly, MelanA/Mart1 specific $CD8^+$ T were identified in inflammatory lesions of melanocyte destruction following infusion of MelanA/Mart1 specific $CD8^+$ T cell clones in melanoma patients (Yee *et al.,* 2000). The above findings give direct evidence for T cell mediated melanocyte destruction in vitiligo. However, natural killer cells and lymphokine-activated cytotoxicity are shown to be normal in patients with progressive vitiligo (Durham-Pierre *et al.,* 1995).

Immunohistochemical studies of the perilesional area of generalized vitiligo mainly detects $CD4^+$ and $CD8^+$ T cells in the infiltrate which express the activated molecules such as interleukin 2 receptor (IL 2R and CD25), HLA DR and MHC II. They express cytokine interferon gamma, which enhances T cell trafficking to the skin by increasing ICAM-I expression (Abdel-Nazer *et al.,* 1994; Abdel-Nazer *et al.,* 1991; Al Badri *et al.,* 1993; Okada *et al.,* 1996; Horn *et al.,* 1997; Von Den Driesch *et al.,* 1992). In parallel, and in correlation with these local findings, activation of circulating T lymphocytes was observed. Increased expression of CD25 and or HLA DR (Mahmoud *et al.,* 1998; Abdel-Naser *et al.,* 1992) elevated CD45RO memory T cells (Mahmoud *et al.,* 2002) and decreased $CD45RA^+$ naïve subsets were demonstrated in non-segmental vitiligo (Abdel-Nazer *et al.,* 1992) although the latter observation was not confirmed by others (Mahmoud *et al.,* 1998). *In vitro* studies demonstrated an increased production of pro inflammatory cytokines IL6 and IL8 by monocytes of

patients with active vitiligo. These not only play an important role in effector cell migration and effector target attachment but also cause B cell activation (Yu *et al.*, 1997). An activation of T cell mediated immune system was confirmed in vitiligo by detecting significantly increased levels of soluble interleukin 2 receptors (SIL2R) especially in generalized, focal and non-dermatomal types of vitiligo (Honda *et al.*, 1997; Yeo *et al.*, 1999; Caixia *et al.*, 1999). The progressive loss of melanocytes from depigmenting vitiligo skin is accompanied by the cellular infiltrates containing both $CD4^+$ and $CD8^+$ T lymphocytes. Infiltrating cytotoxic T cells with high affinity T cell receptors may be escaped clonal deletion in the thymus, allowing such T cells to enter the circulation. Through the expression of CLA, these T cells home to the skin where they express type 1-cytokine and mediate melanocyte apoptosis via the granzyme/perforin pathway (Huang *et al.*, 2002). The possible cross talk between cellular and humoral immune mechanisms in vitiligo is given in Figure 11.

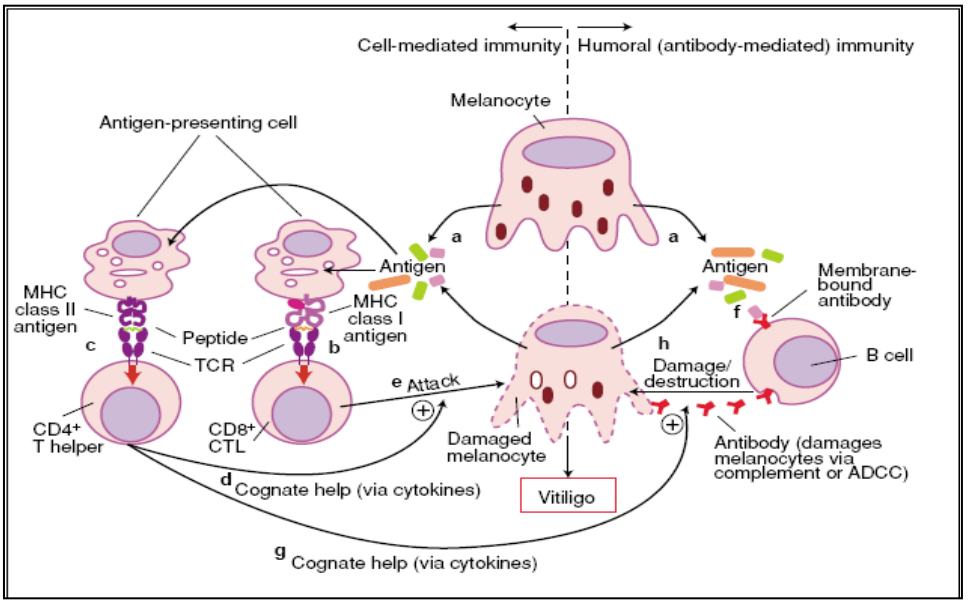


Figure 11. Possible cellular and humoral immune mechanisms in vitiligo.
(Kemp *et al.*, 2001)

1.5.3.3 Oxidative Stress and the Immune System in Vitiligo Pathogenesis

The potential role of oxygen free radicals in human autoimmune disease was reviewed by Ahsan *et al.* (2003). Agrawal *et al.* (2004) reported systemic oxidative stress in vitiligo patients due to an imbalance in enzymatic and non-enzymatic antioxidant systems. The study suggested different mechanisms of generation of

oxidative stress in different clinical types of vitiligo. Whereas low levels of catalase may contribute to the generation of oxidative stress in segmental vitiligo, generation of oxidative stress in non-segmental vitiligo appears attributable to lower levels of glutathione peroxidase (Shajil and Begum, 2006). Moreover, studies on neurochemical basis of vitiligo have documented significantly decreased systemic acetylcholine esterase (AChE) activity (Shajil *et al.*, 2006). This could be due to H_2O_2 mediated oxidation of AChE, which emphasizes the role of oxidative stress in precipitating vitiligo (Schallreuter *et al.*, 2004).

Natarajan *et al.,* 2010 have also shown the involvement of Nrf2-dependent Phase II detoxification pathway, considering its importance in the regulation of epidermal skin homeostasis. The role of enhanced Nrf2 effectors seems to be the prime protective function conferred by this pathway on skin homeostasis. Apart from the protective response, phase II genes could also be interestingly responsible for sustaining depigmented lesions in vitiligo patches. Thus, this study demonstrated the involvement of Nrf2 and phase II genes in homeostatic mechanisms of vitiligo skin and moreover, lesional skin from vitiligo patients recorded higher levels of oxidative stress compared to non-lesional pigmented skin (Natarajan *et al.,* 2010). Eskandani *et al.*, 2010 have reported increased DNA damage in leucocytes and lowered levels of tyrosinase activity in lesional skin of vitiligo patients compared to their non-lesional skin. Tyrosinase is an important sensitive enzyme in pigmentation process as a range of factors can influence its activity including oxidative agents such as H_2O_2. In presence of DOPA substrate and 3-methylbenzothiazolinone-2-hydrazone (MBTH), H_2O_2 can function as an inhibitor of tyrosinase or, the presence of H_2O_2 and DOPA substrate can generate a secondary complex that can bind and inhibit the enzyme. This study suggested a meaningful correlation between increased oxidative stress and decreased tyrosinase activity. In addition, Jalel *et al.*, 2009 for the first time showed an important role for oxidative stress in pathogenesis of vitiligo in experimental mice, suggesting that melanocyte damage in vitiligo might be linked to generalized oxidative stress.

Thus high ROS/RNS in melanocytes may cause the cell to undergo defective apoptosis and release of aberrated proteins from the cell. Failure of phagocytosis of these apoptotic cells causes expression of auto-antigens. These auto-antigens are

presented by MHC to T-cells leading to autoimmunity (Kühtreiber *et al.,* 2003). TNFα is known as a paracrine inhibitor of melanocyte growth and increased levels of TNFα cause maturation of dendritic cells and thus results in to development of autoimmunity (Clemens *et al.,* 2000). The intracellular levels of H_2O_2 and other ROS also increase in several cellular systems in response to external stimuli and cytokines such as TNFα and TGFβ1 (transforming growth factor β1) (Celia *et al.,* 2001). High ROS also increases the levels of cytokines, including IL2 which upregulates the expression of anti-apoptotic protein, Bcl2 thereby making T-cells resistant to apoptosis (Figure 3, pathway 2).These cytokines are potent inhibitors of melanogenesis in B16 melanoma cells and human melanocytes (Swope *et al.,* 1991; Martínez-Esparza *et al.,* 1997; Martínez-Esparza *et al.,* 1998). In addition, the ROS/RNS results in increased lipid peroxidation products that have been proposed for hair graying (Nordlund and Abdel-Malek, 1988) and several pathological conditions, like vitiligo (Passi *et al.,* 1998).

There is interplay between the oxidative stress and the immune system in vitiligo pathogenesis. Vitiligo pathogenesis is an extremely complex event involving both genetic susceptibility as well as environmental triggers. The two major theories of vitiligo pathogenesis include an autoimmune etiology for the disease and an oxidative stress mediated toxicity in the melanocyte. Although these two theories are often presented as mutually exclusive entities, it is likely that vitiligo pathogenesis may involve both oxidative stress and autoimmune events, for which there is variability within a patient.

ROS are produced as byproducts of melanogenesis in melanocytes, and controlled in the epidermis by several redundant antioxidant enzymes such as catalase and glutathione peroxidase, both of which are decreased in the epidermis of vitiligo patients (Schallreuter *et al.,* 1999). Oxidative stress plays a very important role in the immune system, as phagocytic cells generate reactive oxygen intermediates such as superoxide, hydrogen peroxide and nitric oxide, which are toxic to many pathogens, and at the same time they can be toxic to the host as well. Given the role of oxidative stress in both melanogenesis and in the immune system it can be hypothesized that biochemical defects in the melanin biosynthesis pathway, as well as possible defects in patient's antioxidant enzymes, are responsible for the generation of ROS in the

epidermis of vitiligo patients (Casp *et al.*, 2002). Build up of ROS along with possible immune system defects allow for the inappropriate autoimmune response against normal melanocytes.

In autoimmune disorders the immune system aberrantly targets host cells for destruction, often creating a chronic or relapsing inflammatory milieu. The effects of chronic inflammation can be devastating on th host, eventually causing damage and/or destruction of the target organ. In this inflammatory environment, ROS can accumulate with a toxic effect on surrounding cells. This can explain the pathogenesis of inflammatory vitiligo (Buckley, 1953). In this rare disorder a raised rim surrounds the depigmented lesion. The question that lies unanswered is what is causing this aberrant inflammatory response in autoimmunity and whether these ROS are a result of the chronic inflammation and autoimmunity, or part of the cause of the autoimmune response.

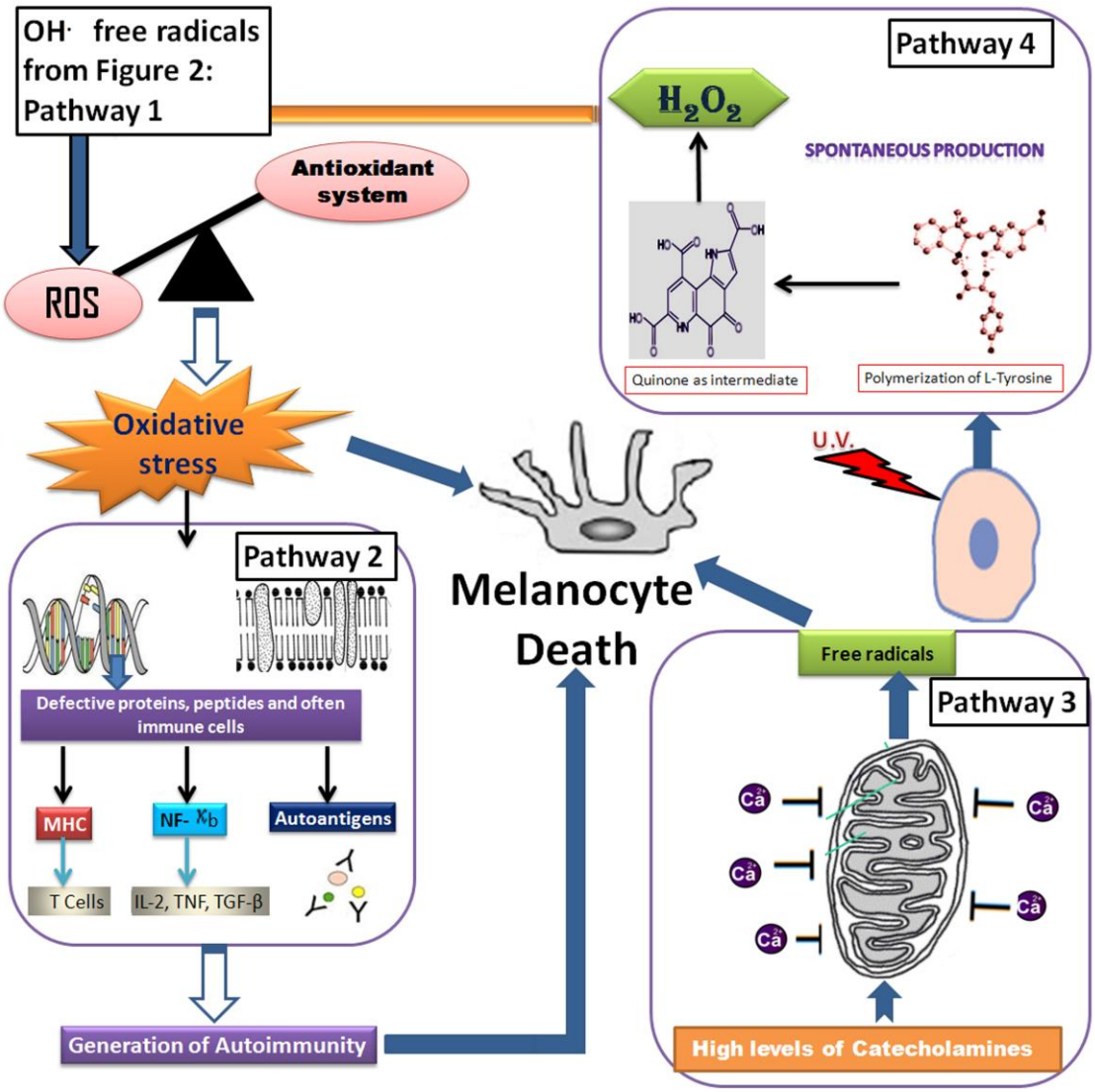


Figure 12.Different pathways for melanocyte destruction:

1. Generation of ROS by various metabolic processes. 2. Imbalance in ROS generation and antioxidant system leads to accumulation of free radicals resulting in oxidative stress. This accumulation causes DNA damage, synthesis of defective proteins and membrane disintegration which provokes immune system resulting in autoimmunity. 3. Increased catecholamines inhibits mitochondrial calcium uptake which results in free radicals generation 4. Exposure to UV radiation, leads in spontaneous production of quinones in melanocytes which in turn results in ROS generation.

1.5.3.4 Cytokines

Cytokines are a family of glycosylated or non- glycosylated polypeptides and proteins, secreted by cells in response to a stimulus which modulates the behavior of target cells (Dixon *et al.*, 1999). Cytokine studies of peripheral blood and skin in

patients with vitiligo have also yielded variable results, but with a trend to proinflammatory T-cell patterns. Earlier work showed increased IL6 and IL8 but decreased TNF*α* and IFN*γ* in serum, with elevated soluble IL2 receptor in blood and tissue (Yu *et al.,* 1997; Tu *et al.,* 1999). It was (Moretti *et al.,* 2002) found increased IL6, TNF*α* and minimal TGFβ in tissue, and increased IL6 but normal IL1*β*, IL8 and TNF*α* in blood (Tu *et al.,* 2003). Also, it is reported decreased soluble IL2 receptor in blood (Franczuk *et al.,* 2004). Grimes *et al.* (2004) noted increased tissue TNF*α*, IFN*γ* and IL10, and detected increased IL1, IL6, IL8 and TNF*α* (Zailaie, 2005) in blood, while Birol *et al.,* (2006) found elevated tissue TNFα but normal blood levels of this cytokine. It was noted that Imiquimod often causes vitiligo-like depigmentation when used to treat superficial basal cell carcinoma. Imiquimod binds Toll-like receptors 7 and 8 and evokes a Th-1 response with the production of IFN*α*, TNF*α* and IL12. Imiquimod also causes increased IL6, IL8 and IL10. Similar cytokines might be involved in vitiligo (Mashiah and Brenner, 2007). It is noted that topical tacrolimus (Taher *et al.,* 2009), used successfully to treat vitiligo, increases tissue IL10, which is an immunosuppressive Th-2 cytokine. This suggests that vitiligo might be a Th-1 type of autoimmune disease. Pichler *et al.,* 2009 found normal blood levels of TNF receptor with slightly elevated IL6, while Basak *et al.* (2009) reported significantly decreased serum levels of TGFβ in vitiligo, with potential inhibition of regulatory T-cell function.

1.5.3.4.1 Tumor necrosis factor *α* (TNF *α*)

TNFα is a multifunctional pleiotropic proinflammatory cytokine secreted predominantly by monocytes/macrophages. In addition to macrophages, TNF is also produced by a broad variety of other cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, neuronal tissue, fibroblasts, and keratinocytes. It was first isolated by Carswell *et al* (1975) in an attempt to identify tumor necrosis factors responsible for necrosis of the Meth A sarcoma. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. The cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self regulatory properties as well. For instance, TNFα induces neutrophil proliferation during inflammation, but it also induces

neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray, *et al.*, 1997). The cytokine is produced by several types of cells, but especially by macrophage. Tracey and Cerami, (1990) suggest two beneficial functions of TNFα which have lead to its continued expression. First, the low levels of the cytokine may aid in maintaining homeostasis by regulating the body's circadian rhythm. Furthermore, low levels of TNFα promote the remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine.

Additional beneficial functions of TNFα include its role in the immune response to bacterial, and certain fungal, viral and parasitic invasions as well as its role in the necrosis of specific tumors. Lastly it acts as a key mediator in the local inflammatory immune response. TNFα is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNFα secreted by the macrophage causes blood clotting which serves to contain the infection. Without TNFα, mice infected with gram negative bacteria experience septic shock (Janeway *et al.*, 1999).

The pathological activities of TNFα have attracted much attention. For instance, although TNFα causes necrosis of some types of tumors, it promotes the growth of other types of tumor cells. High levels of TNFα are correlated with increased risk of mortality (Rink & Kirchner, 1996). TNFα participates in both inflammatory disorders of inflammatory and non inflammatory origin (Strieter *et al.*, 1993).

1.5.3.4.1.1 Structure/binding sites

TNFα is a trimeric protein encoded within the major histocompatibility complex. Wang *et al.* (1985) and Shirai *et al.* (1985) independently cloned cDNA sequences corresponding to the human TNF gene. The deduced 233-amino acid protein has a long leader sequence of 76 residues. TNF is synthesized as a 26 kDa membrane bound protein (pro-TNF) that is cleaved by processing enzymes (Black *et al.*, 1997) to release a soluble 17 kDa TNF molecule. The soluble molecule can then bind to its main receptors TNFR1 and TNFR2 (Skoog *et al.*, 1999). TNFR1 is constitutively expressed in most tissues, and can be fully activated by both the membrane bound and

soluble trimeric forms of TNF, while TNFR2 is only found in cells of the immune system and respond to the membrane bound form of the TNF homotrimer.

TNFα shares only 36% amino acid sequence homology with TNFβ, also called lymphotoxin (LT) (Meager, 1991) but, the tertiary structures of the two proteins are remarkably similar and both bind to TNF receptors TNFR 55 and TNFR 75. These receptors are expressed on all somatic cells and both have similar biological activities. In addition to the transmembrane and soluble forms of TNFα which bind to the TNFR, TNFα can penetrate cell membranes and form ion channels across the membrane . Researchers speculate that the viral protein coat-like jelly role motif may facilitate membrane penetration (Kagan *et al.,* 1992).

1.5.3.4.1.2 Mechanism

Upon contact with their ligand, TNF receptors also form trimers, their tips fitting into the grooves formed between TNF monomers. This binding causes a conformational change to occur in the receptor, leading to the dissociation of the inhibitory protein SODD from the intracellular death domain. This dissociation enables the adaptor protein TRADD to bind to the death domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be initiated (Wajant *et al*., 2003).

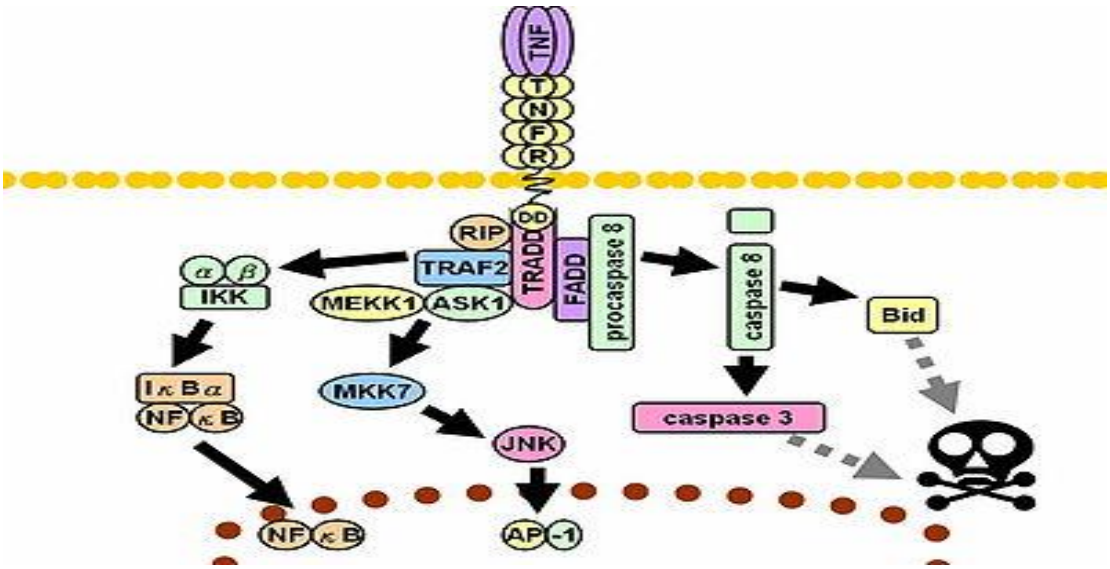


Figure 13.Mechanism of action of TNF-α.

Activation of NF-κB: TRADD recruits TRAF2 and RIP. TRAF2 in turn recruits the multicomponent protein kinase IKK, enabling the serine-threonine kinase RIP to

activate it. An inhibitory protein, IκBα that normally binds to NF-κB inhibits its translocation. IκBα is phosphorylated by IKK and subsequently degraded, releasing NF-κB. NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors.

Activation of the MAPK pathways: Of the three major MAPK cascades, TNF induces a strong activation of the stress-related JNK group, evokes moderate response of the p38-MAPK, and minimal activation of the classical ERKs. TRAF2 activates the JNK-inducing upstream kinases of MEKK1 and ASK1 (either directly or through GCKs and Trx, respectively), and these two kinases phosphorylate MKK7, which then activates JNK. JNK translocates to the nucleus and activates transcription factors such as c-Jun and ATF2. The JNK pathway is involved in cell differentiation, proliferation. Like all death-domain containing members of the TNFR superfamily, TNF-R1 is involved in death signaling (Gaur et. al., 2003). TRADD binds FADD, which then recruits the cysteine protease caspase-8. A high concentration of caspase-8 induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to cell apoptosis.

Kamata *et al.* (2005) found that TNFα induced ROS, whose accumulation could be suppressed by mitochondrial superoxide dismutase (SOD2), caused oxidation and inhibition of JNK-inactivating phosphatases by converting their catalytic cysteine to sulfenic acid. This resulted in sustained JNK activation, which is required for cytochrome c release and caspase-3 cleavage, as well as necrotic cell death. Treatment of cells or experimental animals with an antioxidant prevented H_2O_2 accumulation, JNK phosphatase oxidation, sustained JNK activity, and both forms of cell death. Antioxidant treatment also prevented TNFα mediated fulminant liver failure without affecting liver regeneration.

Other factors, such as cell type, concurrent stimulation of other cytokines, or the amount of ROScan shift the balance in favor of one pathway or another. Such complicated signaling ensures that whenever TNF is released, various cells with vastly diverse functions and conditions can all respond appropriately to inflammation.

1.5.3.4.1.3 Role of TNFα in vitiligo and other diseases

TNFα, a paracrine inhibitor of melanocytes, is especially important. Several single-nucleotide polymorphisms (SNP) have been identified in the human TNFα gene promoter. The polymorphisms at position -308 (TNF-308) and -238 (TNF-238) at the promoter region which involves substituting G for A and designing the AA genotype, leads to a higher rate of TNFα gene transcription than the wild-type GG genotype in invitro expression studies. It has also been linked to increased susceptibility to several chronic metabolic, degenerative, inflammatory and autoimmune diseases like rheumatoid arthritis, pernicious anemia, diabetes mellitius.

TNFα is also involved in the pathophysiology of a number of disorders including Crohn's disease, ankylosing spondylitis and psoriatic arthritis (Singh *et al.,* 2004). TNFα has also been suggested to be a key cytokine in controlling body weight (Argiles *et. al*., 1997). It also increases the expression of cell surface adhesion molecule of and can augment their procoagulant activity (Dosquet *et. al.,* 1992) Both TNFα and insulin can induce endothlein 1 production by vascular endothelial cell, which can lead endothelial dysfunction and vascular pathology observed in hyper insulinaemic state such as android obesity (Winkler *et. al.,* 1999).

TNFα is overexpressed in obesity and is a candidate mediator of obesity-induced insulin resistance. Complete lack of TNFα function through targeted mutations in *TNFα* gene or both of its receptors results in significant improvement of insulin sensitivity in dietary, chemical, or genetic models of rodent obesity. In addition to its antitumor and proinflammatory actions. (Haiyan *et. al.,* 2002).

TNFα can also modulates adipocyte biology and affects systemic glucose and lipid metabolism (Grunfeld *et. al.,* 1991). There is altered TNFα processing in adipocytes and increased expression transmembrane. Because this is an aberrant site of expression, it is feasible to postulate that the cellular machinery involved in the processing of this molecule operates differently because of multiple changes in obesity, therefore resulting in alterations in the ratio between transmembrane versus secreted forms of this molecule.

1.5.3.4.1.4 TNFα and clinical applications

TNFα seems to serve as a mediator in various pathologies. A few such examples include: septic shock, cancer, AIDS, transplantation rejection, multiple sclerosis, diabetes, rheumatoid arthritis, trauma, malaria, meningitis, ischemia-reperfusion injury, and adult respiratory distress syndrome.

As TNFα has a role in several diseases, TNFα therapies and anti- TNFα therapies can be attempted in controlling the disease conditions.

Research has focused upon inhibiting the effects of TNFα in diseases such as Rheumatoid Arthritis, Crohn's Disease, AIDS, bacterial septic shock (caused by certain gram negative bacteria), and bacterial toxic shock (caused by superantigens) as well as in prevention of alloreactivity and graft rejection. Anti-TNF monoclonal antibodies have been used to effectively reduce or inhibit TNFα activity (Beutler *et al.*, 1985b) in multiple types of inflammation. Strategies for preventing TNFα activity include neutralization of the cytokine via either anti-TNF antibodies, soluble receptors, or receptor fusion proteins; supression of TNFα synthesis via drugs such as cyclosporine A, glucocorticoides, or cytokine IL10; reduction of responsiveness to TNFα via repeated low dose stimulation; inhibition of secondary mediators such as IL1, IL6, or nitric oxide (Tracey *et al.*, 1993). However, the efficacy of preventing septic shock has been questioned as a result of recent research which suggests that, in the absence of TNFα, other cytokines will eventually initiate the inflammatory response. TNFα production may instead play a key kinetic role by amplifying release of cytokines ILα, ILβ and IL6 and thereby affecting the severity of a response to LPS (Amiot *et al.*, 1997). Additionally, eliminating the stimulatory affects of TNFα in diseases such as AIDS presents problems because inactivation of TNFα leaves the host at even greater risk for bacterial infections normally countered by TNFα activity.

1.5.3.4.1.5 Molecular Genetics of TNFα

Single-nucleotide polymorphisms in regulatory regions of cytokine genes have been associated with susceptibility to a number of complex disorders. TNF is a proinflammatory cytokine that provides a rapid form of host defense against infection but is fatal in excess. Because TNF is employed against a variety of pathogens, each

involving a different pattern of risks and benefits, it might be expected that this would favor diversity in the genetic elements that control TNF production.

TNFA is composed of four exons arranged over approximately 3 kb of DNA (Nedwin *et al.,* 1985). Regulation of *TNFA* production occurs at both transcriptional and post-transcriptional levels, with regulatory sequences within the 5' end of the gene, controlling the rate of transcription. SNP's within *TNFA* have the potential to cause

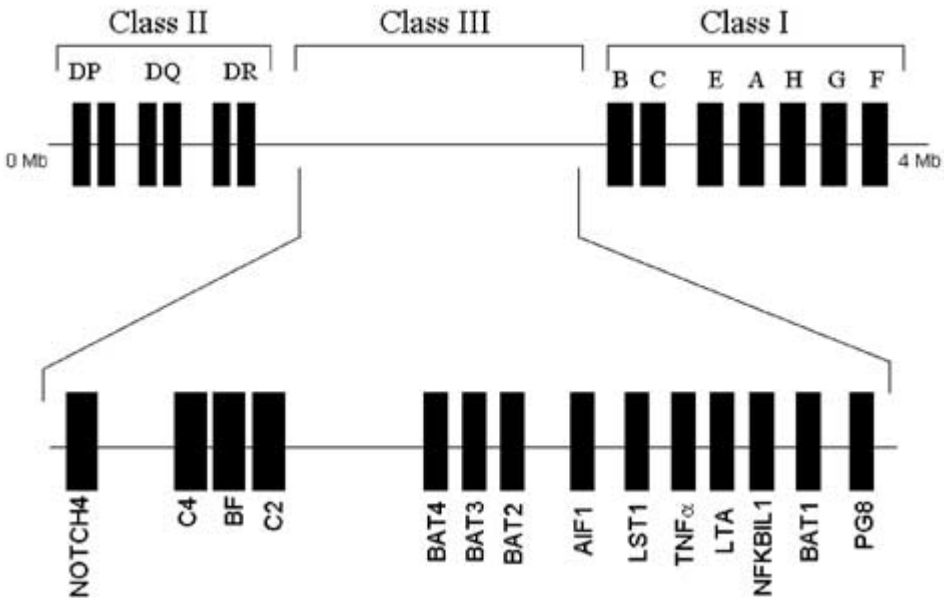


Figure 14. HLA region on chromosome 6q21: highlighting the different genes present within the HLA class III region that could be contributing to disease association.

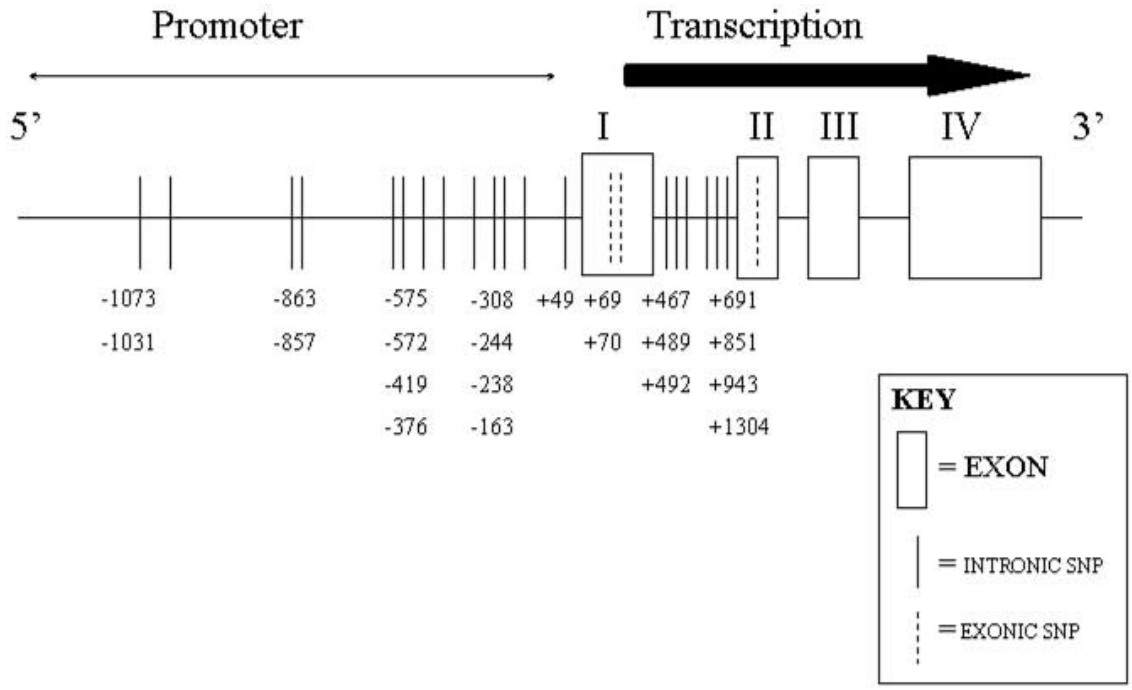


Figure 15. *TNFA* gene structure and known polymorphims.

structural changes within regulatory sites that could affect the activity or regulation of TNFα production. These factors combined could contribute to the autoimmune process making it an ideal candidate for the development of vitiligo.

Studies looking for association with disease have been performed only in small ethnically diverse data sets of different autoimmune diseases, including rheumatoid arthritis (Brinkman *et al.,* 1997), Crohn's disease (Louis *et al.,* 2000) and systemic lupus erythematosus (Fong *et al.,* 1996).

Attention within the HLA gene region has been mainly focussed on the class II genes, in particular DR and DQ. Little work has been performed on the Class III genes (Figure 14), which include components of the complement system, the heat shock proteins and the tumour necrosis factors including *TNFA.* Herrmann *et al.* (1998) used PCR-SSCP and sequencing to screen the entire coding region and 1,053 bp upstream of the transcription start site of the *TNFA* gene for polymorphisms. A number of SNPs have been detected in *TNFA* both within the promoter and the gene itself (Uglialoro *et al.,* 1998) (Figure 15). Five polymorphisms in *TNFA* were identified in the upstream region at positions -1031, -863, -857, -308, and -238 from the first transcribed nucleotide.

1.5.3.4.2 Tumor necrosis factor β (TNFβ)

TNFβ also known as Lymphotoxin (LTA) is an inducible, homotrimeric soluble protein secreted by activated T and B lymphocytes. It is also secreted by fibroblasts, astrocytes, myeloma cells, endothelial cells, epithelial cells and a number of transformed cell lines. The synthesis of TNFβ is stimulated by interferons and IL2 (Chen, 2005).

TNFβ is a potent mediator of inflammatory and immune responses. TNFβ also mediates antiviral responses. TNFβ is also involved in the formation of secondary lymphoid organs during development and plays a role in apoptosis. In TNFβ knockout mice, all Peyer's patches and lymph nodes will fail to develop indicating TNFβ's importance in immunological development (Eitan *et al.,* 2008). Genetic variations in this gene are associated with susceptibility to leprosy type 4, myocardial infarction, non-Hodgkin's lymphoma, and psoriatic arthritis. Alternatively spliced transcript variants have been observed for this gene.

TNFβ was first characterized as a biological factor in mitogen-stimulated lymphocytes having anticellular activity on neoplastic cell lines. Gray *et al.* (1984) isolated a chemically synthesized gene and natural complementary DNA coding for human lymphotoxin and engineered them for expression in *E. coli.* Cytotoxic and necrosis effects were observed in murine and human tumor cell lines *in vitro* and in murine sarcomas *in vivo*.

1.5.3.4.2.1 Gene structure

By analysis of deletions induced in lymphoblastoid cells by gamma-irradiation, Evans *et al.* (1989) showed that *TNFB* maps to the interval between C4 and HLA-B. Spies *et al.* (1989) showed that the *TNFA* and *TNFB* gene cluster is about 210 kb from HLA-B on 6p21.3. Jongeneel *et al.* (1991) described polymorphic microsatellites within a 12-kb region of the major histocompatibility complex that includes the *TNFB* locus. The human *TNFB* gene is located next to *HLA-C* and *HLA-B* loci in chromosome 6 (6p21.3) approximately 1.2 kb apart from the *TNFA* gene. The gene spans 2005 bp with 4 exons, which transcribes a *TNFB* mRNA with size of 1386 nucleotides. However, both the genes are regulated independently. The 5' region of the *TNFB* promoter contains a poly(dA-dT)-rich sequence that binds the nonhistone protein

HMG-1 which is involved in the regulation of the constitutive expression of the gene (Chen, 2005).

1.5.3.4.2.2 Protein characteristics

The human TNFβ is a glycoprotein protein and contains 205 amino acids. The soluble form of TNFβ is usually a homotrimer with a relative molecular mass of 60 to 70 kDa, whereas the membrane form of TNFβ is a heteromeric complex with lymphotoxin b (TNFc, LTb, TNFSF3). The biological function of TNFβ is mediated largely by TNFα receptor 1 and TNFα receptor 2. The human TNFβ shares 35% identity and 50% homology in amino acid sequence with the human TNFα. Murine and human TNFβ are highly homologous (74 %) (Chen, 2005).

1.5.3.5 Vitiligo and Apoptosis

The exact pathway of destruction of melanocytes is not yet known, however, apoptotic death has been suggested in vitiligo (Huang *et al.,* 2002). Cytokines such as ILI, IFNγ or TNFα that are released by lymphocytes, keratinocytes and melanocytes can initiate apoptosis (Huang *et al.,* 2002). Also an imbalance of cytokines in the epidermal microenvironment of lesional skin has been demonstrated which could impair the normal life and function of melanocytes. The observed increase of TNFα, a paracrine inhibitor of melanocytes could be related to its death (Moretti *et al.,* 2002). Birol *et al.* (2006) demonstrated that the level of cytokines IL1α and TNFα are significantly higher in lesional skin compared with the non-lesional skin in patients with vitiligo (Birol *et al.,* 2006). However, the exact mechanism of the effect of cytokines on pigmentation is not fully understood. It has been hypothesized that TNFα induces IL1α promoting B cell differentiation and immunoglobulin production. TNFα induces cell surface ICAM1 on melanocytes which is necessary for leucocyte-melanocyte attachment. ICAM1 can also induce B cell activation, increasing autoantibody production and may cause melanocyte damage in vitiligo. TNFα has the capacity to induce apoptosis in different cell types. Melanogenesis is also inhibited by TNFα through an inhibitory effect on tyrosinase and tyrosinase related protein (Birol *et al.,* 2006). Activated cytotoxic lymphocytes can also induce apoptosis through the perforin/ granzyme or Fas/Fas ligand pathway. The regulatory molecules of apoptosis seem to be well regulated in vitiligo and it was demonstrated that relative apoptotic

susceptibility of vitiligo melanocytes is comparable to that of normal control cells (Wijngaard *et al.,* 2000).

Nitric Oxide (NO) is a reactive endogenous molecule with multiple functions including inflammation and immunity. Studies have shown that nitric oxide could inhibit the *de novo* attachment of melanocytes to extra cellular matrix (ECM) suggesting that NO induced aberrant perturbation of melanocyte – ECM interaction could be a reason for melanocyte loss in vitiliginous lesions. Ivanova *et al.* (2005) showed that high concentrations of NO induce apoptosis mediated detachment of both normal melanocytes and vitiliginous melanocytes form fibronectin in a similar mechanism, suggesting that non-lesional vitiliginous melanocytes are not characterized by an increased proneness to NO induced apoptosis (Ivanova *et al.*, 2005).

1.5.4 Convergence Theory

Several hypotheses on the mechanism of pathogenesis of vitiligo have been combined and formulated a convergence theory to explain the etiopathogenesis of vitiligo (Le Poole *et al.,* 1993). This theory states that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can contribute to vitiligo pathogenesis in varying proportions.

According to the new hypothesis put forward by Dell'Anna and Picardo (2006) a compromised membrane could render the cell sensitive to external and internal agents differentially. According to this hypothesis, the melanocytes present biochemical defects, probably due to a genetic background, affecting the structure and functionality of the membranes. A compromised membrane could render the cell sensitive to external and internal agents differently (UV, cytokines, catechols, melanin intermediates, growth factor withdrawal) usually ineffective on cell activity and survival. The impaired arrangement of the lipids, involving fatty acids and cholesterol, may affect the transmembrane housing of proteins with enzymatic or receptor activities. The altered expression and release of transmembrane proteins could be the basis for the exposure of 'new antigens' triggering an immune response (Broquet *et al.,* 2003; Kroll *et al.,* 2005). The final result could depend on the

intensity or duration of the stimuli; a mild aggression leading to a reduction of ATP production impairs the adhesion function; a great stimulus acting as pro-apoptotic agent affects mitochondrial cell survival check points; finally, a strong stress directly causing the necrotic death with an inflammatory, or at least lymphocytic infiltrate (Dell'Anna and Picardo, 2006).

1.6 VITILIGO GENETICS

Vitiligo is characterized by multiple susceptibility loci, incomplete penetrance, and genetic heterogeneity (Spritz *et al*., 2008). The inheritance of vitiligo may involve genes associated with the biosynthesis of melanin, response to oxidative stress and regulation of autoimmunity. It is however not yet clear as to whether the abnormalities observed in neural pathways, oxidative stress and autoimmune events represent a cause or effect response of the disease. Nevertheless, it may involve both genetic and environmental factors. Recent studies suggest that genetic factors may play a major role in the pathogenesis of vitiligo. There is a positive family history in about 20% of cases and similar concordance in identical twins (Spritz, 2008). Shajil *et al* (2006) also reported that 21.93% of Gujarat vitiligo patients exhibited positive family history and 13.68% patients had at least one affected first-degree relative.

Vitiligo is a polygenic disease, and attempts have been made to identify genes involved in susceptibility include gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which involve immune regulation and immune targeting of melanocytes, suggesting that generalized vitiligo is a primary autoimmune disease however, the biological triggers of the autoimmune process remain unknown (Spritz *et al*., 2011).

Considerable progress has been made in the identification of candidate genes. Such studies have been carried out in different populations with differing susceptibility factors. Systematic studies in different populations would help identify candidate genes governing oxidative stress that contribute to the pathogenesis of vitiligo. As immune system also seems to play a major role in vitiligo, understanding the mechanism of immune responses involved and, identification of the potential antigen/s in a particular population would help in designing therapeutic regimens by

neutralizing the specific cell types or masking the specific antigen/s that are involved in the pathogenesis of vitiligo. The mammalian skin pigmentation is possibly controlled by more than a hundred genes; these genes as well as the genes regulating oxidative stress and immune responses qualify as potential candidate genes for vitiligo.

The role of genetic factors in vitiligo was also considered early because of the frequent clustering of cases among close relatives (Stuttgen, 1950; Teindel, 1950), and eventual genetic epidemiological studies by Das *et al.* (1985) supported multifactorial, polygenic inheritance, which currently is termed ‘‘complex disease’’. In 1960s and 1970s, ABO, haptoglobin, erythrocyte enzymes, and various serum proteins were tested as genetic markers for vitiligo, with negative results. In the 1970s, a plethora of analyses of HLA in vitiligo were reported, with equivocal and conflicting findings. Some of the earliest genetic studies of vitiligo were carried out in India, of ABO blood groups (Kareemullah et al., 1977), α1-antitrypsin, and haptoglobin (Mujahid et al., 1990). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC, ACE, CAT, CTLA-4, COMT, ESR, GCH1, MBL2, PTPN22,* and *VDR* (Spritz 2007, 2008). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA, NALP1, XBP1, FOXP1, IL-2RA* have been tested for genetic association with vitiligo (Spritz, 2010).

HLA molecules present peptides to T-cells, and it has been proposed that certain HLA haplotypes confer more efficient presentation of cognate autoantigen, thereby predisposing to autoimmunity; an example is HLADQB1*0301 (Gilhar *et al.,* 2007). *NALP1* is involved in the innate immune response to pathogens. Recent fine-mapping studies showed associations with chromosomes 7 and 9 (Jin *et al.,* 2009). Numerous other candidate genes and susceptibility loci bear ongoing scrutiny, including *CAT, GST, COMT, ACE,* mannose-binding lectin 2 and *XBP1* (Casp *et al.*,2002;Onay *et al.,* 2007; Liu *et al.,* 2009; Ren *et al.,* 2009). A recent genome-wide association study by Jin *et al.* (2010) using European white subjects and controls showed significant associations of generalized vitiligo with the following loci, which have been previously linked with autoimmune diseases: HLA class I and II molecules, *PTPN22,*

LPP, *IL2RA*, *UBASH3A* and *C1QTNF6*. Two additional immune-related loci identified were *RERE* and *GZMB*. The HLA class I association occurred in the regions between *HLA-A* and *HCG9*, consistent with previous reports of strong associations with the *HLA-A*02* allele, and the HLA class II gene association occurred in the region between *HLA-DRB1* and *HLADQA1*, in keeping with known associations to the *HLA-DRB1*04* allele. With the exception of *PTPN22*, the associations were similar whether patients had vitiligo alone or vitiligo as well as another autoimmune disease. An important association with a non-immune related gene, tyrosinase was identified. Tyrosinase is a melanocyte enzyme that catalyzes the rate limiting step in melanin biosynthesis and is a putative target autoantigen in vitiligo. Interestingly, certain *TYR* SNPs are associated with melanoma risk, and some of these are in linkage disequilibrium with vitiligo. Vitiligo *TYR* SNPs could be more antigenic than melanoma *TYR* SNPs, thereby conferring protection from melanoma through immune surveillance (Jin *et al*., 2010; Bishop *et al*., 2009).

1.7 SINGLE NUCLEOTIDE POLYMORPHISMS

Humans are 99.9% genetically identical (Venter *et al.,* 2001) and the most common type of genetic variability found in humans is in the form of Single Nucleotide Polymorphisms (SNPs). A SNP refers to a single base change in DNA. These SNPs occur when there are two or more possible nucleotides are seen at a specific mapped location in the genome, where in the least frequent allele has an abundance of 1% or more (Brookes *et al.,* 1999). An International Single Nucleotide Polymorphism Consortium (ISNPC) has currently identified over 6 million SNPs, approximately one at every 1-2 kilobase. SNPs may occur in non-coding regions as well as in coding regions. Some missense polymorphisms are more conservative than others e.g. a change in the codon CUU (leucine) to AUU (isoleucine) would have minimal structural impact, whereas modification of CAU (histidine) to CCU (proline) would be expected to have dramatic structural and/or functional influence on the protein.

SNPs act as potential useful markers for the gene mapping studies, particularly for identifying genes involved in complex diseases (Chakravarti *et al*., 2001). But the knowledge of frequency and distribution of these SNPs across ethnically diverse populations is essential in order to know their usefulness as markers for gene mapping

studies. Additionally, the density of SNPs needed for mapping complex diseases will likely vary across populations with distinct demographic histories (Tishkoff and Verrelli 2003).

The Common Disease/ Common Variant hypothesis states that common genetic disorders are affected by common disease susceptibility alleles at a few loci that are at high frequency across ethnically diverse populations e.g. the APOE ε 4 allele is associated with increased risk for Alzheimer's disease (Chakravarti *et al.,* 1999; Goldstein and Chikhi 2002). Thus, these alleles might arise prior to population differentiation. Alternatively, some complex diseases may be influenced by rare susceptibility alleles at many loci. If these disease predisposing alleles are geographically distributed due to mutation, drift, or regional specific selection pressure, then characterizing SNP diversity, haplotype structure and linkage disequilibrium across a broad range of ethnically diverse populations is of particular importance for identifying disease predisposing alleles (Tishkoff and Williams 2002).

1.7.1 SNP analysis

SNP analysis techniques fall into two distinct classes:

I. SNP Identification: Detection of novel polymorphisms

II. SNP Genotyping: Identifying specific allele in a known population.

1.7.1.1 SNP identification methods

The identification and characterization of large numbers of SNPs are necessary before their use as genetic tools. The following four methods are commonly used for SNP detection (Gray *et al*., 2000).

1.7.1.1.1 SSCP detection:

For single strand conformation polymorphism (SSCP) detection, the DNA fragment spanning the putative SNP is PCR amplified, denatured and run on denaturing polyacrylamide gel. During the gel run, the single-stranded fragments adopt secondary structures according to their sequences. Fragments bearing SNPs are identified by their aberrant migration pattern and are further confirmed by sequencing. Although SSCP is a widely used and relatively simple technique, it gives a variable success rate for SNP detection, typically ranging from 70 to 95%. It is labor intensive

and has relatively low throughput, although higher capacity methods are under development using capillary-rather than gel based detection (Orita *et al.,* 1989).

1.7.1.1.2 Heteroduplex analysis:

This relies on the detection of a heteroduplex formed during reannealing of the denatured strands of a PCR product derived from an individual heterozygous for the SNP. The heteroduplex can be detected as a band shift on a gel, or by differential retention on a HPLC column. HPLC has rapidly become a popular method for heteroduplex-based SNP detection due to simplicity, low cost and high rate of detection i.e. 95-100% (Lichten and Fox 1983).

1.7.1.1.3 Direct DNA sequencing:

The favored high-throughput method for SNP detection is direct DNA sequencing. SNPs may be detected *in silico* at the DNA sequence level. The wealth of redundant sequence data deposited in public databases in recent years, in particular expressed sequence tag (EST) sequences, allows SNPs to be detected by comparing multiple versions of the same sequence from different sources.

1.7.1.1.4 Variant detector arrays (VDA):

VDA technology is a relatively recent addition to the high throughput tools available for SNP detection. This technique allows the identification of SNPs by hybridization of a PCR product to oligonucleotides arrayed on a glass chip and measuring the difference in hybridization strength between matched and mismatched oligonucleotides. The VDA detection allows rapid scanning of large amounts of DNA sequences (Wang *et al.,* 1998).

1.7.1.1.5 High Resolution Melting (HRM):

High Resolution Melting (HRM) is a novel, homogeneous, close-tube, post-PCR method, enabling analysis of genetic variations (SNPs, mutations, methylations) in PCR amplicons. HRM characterizes nucleic acid samples based on their disassociation (melting) behavior. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified (Reed *et al*., 2004).

1.7.1.2 SNP GENOTYPING METHODS

SNP genotyping involves two components (Chen and Sullivan 2003) i.e. a method for discrimination between alternate alleles and a method for reporting the presence of the allele or alleles in the given DNA sample.

A typical genotyping protocol consists of the following steps.

1. Target fragment amplification by PCR.
2. Allelic discrimination reaction can be carried out by either of the following methods: primer extension, pyrosequencing, hybridization and sequence specific cleavage.
3. Allele specific product identification can be done by either of the following ways. Fluorescence resonance energy transfer (FRET), electrophoresis, microarray and mass spectroscopy.
4. Taqman assay for SNP genotyping: The TaqMan genotyping assay combines hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection. It allows screening, association, candidate region, candidate gene, and finemapping studies

1.7.2 Association studies:

SNP based studies can be performed mainly for two purposes:

a. Direct testing of a SNP with functional consequence for association with a disease trait.
b. Using a SNP as a marker for linkage disequilibrium.

1.8 CANDIDATE GENES ASSOCIATED WITH VITILIGO SUSCEPTIBILITY:

The complex genetics of vitiligo involves multiple susceptibility loci, genetic heterogeneity and incomplete penetrance with gene-gene and gene-environment interactions (Zhang, 2005). A few genes that are identified to contribute to vitiligo susceptibility are given in the Table 6.

Table 6. Genes that contribute to vitiligo susceptibility.

Gene	Method	SNP	Reference
ACE	*Candidate gene association*	rs1799752	Jin *et al.,* 2004a
AIRE	*Candidate gene association*	rs1800521	Nagamine *et al.,*1997
ASIP	*Candidate gene association*	rs6058017	Na *et al.*, 2003
CAT	*Candidate gene association*	rs769217 rs7943316	Casp *et al.* 2002, Gavalas *et al.* 2006, Park *et al.* 2006.
CD4	*Candidate gene association*	CD4 pentanucleotide repeat	Zamani *et al.,*2009 Kristiansen et al., 2004
CLEC11A	*Candidate gene association*	rs7246355	Lan *et al.*,2009
COMT	*Candidate gene association*	rs4680	Tursen *et al.,*2002
CTLA4	*Candidate gene association*	rs231775	Kemp *et al.,* 1999
C12orf10	*Candidate gene association*	rs7975232	Philips *et al.,*2010
DDR1	*Candidate gene association*	rs2267641	de Castro *et al.,*2010
EDN1	*Candidate gene association*	rs2071942–rs5370	Kim *et al.*,2007
ESR1	*Candidate gene association*	rs2234693 rs2234767	Jin *et al.,* 2004b Li *et al.*,2008
FAS	*Candidate gene association*		Li M *et al.*,2009

FOXD3		rs41285370	Alkhateeb et al., 2005
FOXP3	*Genome-wide linkage*	rs11798415	Hori *et al.*,2003
GSTM1	*Defective in IPEX*	rs2071487,	Uhm *et al.*,2007
GSTT1	*syndrome*	rs2234953	Liu *et al.*,2009
IL1RN	*Candidate gene association*	IL1RN VNTR	Pehlivan *et al.*,2009
IL10	*Candidate gene association*	rs689466	Abanmi *et al.*,2008
	Candidate gene association	rs1800872;	
	Candidate gene association	rs1800871	Lan *et al.*,2009
KITLG		rs11104947	Na *et al.*, 2003
MC1R	*Candidate gene association*	rs2228479	Onay *et al.*,2007
MBL2	*Candidate gene association*	rs6721961	Guan *et al.*,2008
NFE2L2	*Candidate gene association*	rs36901	Jin *et al.,* 2007
NALP1	*Candidate gene association*	rs6502867	Chen *et al.,* 2005
PDGFRA-KIT	*Candidate gene association*	rs689466	Canton *et al.,* 2005
PTPN22	*Candidate gene association*	rs2476601	Li K *et al.*,2009
PTGS2	*DNA sequencing*	rs7574865	Hu *et al.*,2010
STAT4	*Candidate gene association*	rs1135216	Casp *et al.*,2003
TAP1	*Candidate gene association*	rs2005061	Yun *et al.*,2010
TGFBR2	*Candidate gene association*	rs1800629	D'Alfonso *et l.,*1994
TNF	*Candidate gene association*	rs3806933	Pociot *et al.,* 1993
TSLP	*Candidate gene association*	rs1043784	
TXNDC5	*Candidate gene association*	rs1458836–	Birlea *et al.*,2011
UVRAG	*Candidate gene association*	rs7933235	Jeong *et al.*,2010a
VDR	*Candidate gene association*	rs7975232	Jeong *et al.*,2010b
XBP	*Candidate gene association*	rs2269577	Birlea *et al.*,2006
	Candidate gene association	rs2269577	Birlea *et al.*,2011

1.8.1 *AIRE*

Vitiligo is commonly associated with autoimmune polyglandular syndrome type I (APS I) (Ahonen *et al.*,1990) and mutation in *AIRE* gene causes this disease. *AIRE* gene is normally expressed in immune related organs such as thymus and lymph nodes. The function of AIRE protein is to act as a transcription factor (Nagamine *et al.,* 1997). Mutation analysis has identified two mutations in this gene in Swiss and Finnish APS I patients (Nagamine *et al.,* 1997).

1.8.2 *CTLA4*

CTLA4 is considered as a candidate gene as it contributes to the development of T cell mediated autoimmune diseases and its expression or function is adversely affected by the mutations or polymorphic alleles. Studies suggest that vitiligo when not associated with an autoimmune disorder is not influenced by the *CTLA4* microsatellite polymorphism (Kemp *et al.,* 1999; Blomhoff *et al.,* 2005).

1.8.3 *CAT*

Catalase converts hydrogen peroxide to water and thereby prevents the cell damage from highly reactive oxygen derived radicals. The *CAT* gene is considered as a candidate gene because of the reduction in catalase activity and concomitant accumulation of H_2O_2 is observed in the epidermis of vitiligo patients (Schallreuter *et al.,* 1991). An association has been established between vitiligo and a SNP in exon 9 of *CAT* gene (Casp *et al.,* 2002; Gavalas *et al.,* 2004). It has been reported that C/T heterozygotes are more frequent among vitiligo patients than controls. The C allele is transmitted more frequently to patients than controls, which suggests that linked mutations in or near the *CAT* gene may contribute to a quantitative deficiency of catalase activity in vitiligo patients and the accumulation of H_2O_2.

1.8.4 *COMT*

In melanocytes, COMT prevents the formation of toxic o-quinones during melanin synthesis (Pavel *et al.,* 1983). It was found that epidermal homogenates from vitiligo patients expressed altered levels of COMT activity than homogenates from healthy controls (Le Poole *et al.,* 1994). A common biallelic polymorphism in the *COMT*

gene that determines high and low enzyme activity has been associated with neuropsychiatric disorders (Karayiorgou *et al.,* 1997). *COMT* polymorphism has not been detected in vitiligo patients compared to controls. However, *COMT*- LL (low activity homozygote) genotype was found to be significantly associated with acrofacial vitiligo (Tursen *et al.,* 2002).

1.8.5 *LMP* and *TAP*

Genes within the class II region of the major histocompatibility complex (MHC) are reported to be associated with several autoimmune diseases (Tanaka *et al.,* 1998; Pamer and Cresswell 1998). This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight protein polypeptide 2 and 7 (LMP 2 and 7) and transporter associated with antigen processing protein 1 (TAP 1). Casp *et al.,* (2003) showed genetic association of early onset of vitiligo with the *TAP1* gene. Moreover alleles from heterozygous parents were disequilibratedly transmitted to affected offspring for the *TAP1* gene, as well as for the closely linked *LMP2* and *LMP7* genes (Casp *et al.,* 2003).

1.8.6 *MC1R* and *ASIP*

Polymorphism studies in *MCIR* and *ASIP* revealed that G274A and A488G represented abundant forms of the SNPs of the *MCIR* in Korean population. The frequency of the A allele of G274A was higher in vitiligo patients; however this SNP was not statistically significant.The patients who carried both the SNPs of *MCIR* and *ASIP* were prone to vitiligo (Na *et al.,* 2003).

1.8.7 *ACE*

Neuropeptides such as substance P released from the sensory nerves in the presence of noxious stimuli may result in the destruction of melanocytes in the skin (Hann and Nordlund 2000). Angiotensin converting enzyme was capable of inactivating bradykinin, modulating cutaneous neurogenic inflammation and degrading substance P and other neuropeptides (Scholzen *et al.,* 2003). It was also reported that the *ACE* genotype distribution and allelic frequencies were significantly different between

vitiligo patients and controls suggesting a strong association of vitiligo and *ACE* gene polymorphism (Jin *et al.*, 2004a).

1.8.8 *ESR1*

It was reported that high estrogen levels in the serum was associated with increased skin pigmentation (Shahrad and Marks 1977). Successful treatment of vitiligo was shown with the steroid- thyroid hormone mixture containing estrogen (Nagai *et al.*, 2000; Ichimiya *et al.*, 1999). It was shown that *ESRI* (Estrogen receptor gene 1) intron 1 C/T polymorphism was associated with female or generalized vitiligo patients. *ESR1* gene may be a possible risk factor for the female or generalized type of vitiligo (Jin *et al.*, 2004b).

1.8.9 *PTPN22*

Lymphoid protein tyrosine phosphatase gene encodes lymphoid protein tyrosine phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill *et al.*, 2002). The missense polymorphism in the *PTPN22* gene at the nucleotide 1858 (1858 C> T) at codon 620 (620 Arg>Trp) was found to be associated with autoimmune diseases (Bottini *et al.*, 2004; Onengut-Gumuscu *et al.*, 2004; Ladner *et al.*, 2005; Velaga *et al.*, 2004; Kyogoku *et al.*, 2004; Orozco *et al.*, 2005; Begovich *et al.*, 2004). Studies on *PTPN22* gene showed that 1858T allele was significantly over represented in vitiligo patients compared to controls. This indicates that LYP missense polymorphism may have an influence on the development of generalized vitiligo, which further provides evidence for the autoimmunity as an etiological factor.

1.8.10 *KIT*

KIT encodes for a tyrosine kinase receptor named c-kit expressed on the surface of melanocytes, mast cells, germ cells and hematopoietic stem cells (Grabbe *et al.*, 1994). The c-kit ligand, SCF (stem cell factor) is involved in the proliferation and survival of melanoblasts and may be associated with the dysfunction and/or loss of melanocytes (Nishikawa *et al.*, 1991). The expression of c-kit and its down stream effector microphthalmia associated transcription factor (MITF) is reduced in vitiligo epidermis (Norris *et al.*, 1996; Kitamura *et al.*, 2004). It was also observed that

vitiligo had remained stable for many years after treatment with tyrosine kinase inhibitors (Passeron and Ortonne 2005). Moreover, several cases of vitiliginous depigmentation occurring after treatment with new tyrosine kinase inhibitors (STI-571 and SU 11428) are reported (Raanani *et al.*, 2002). BCL2 is a MITF dependent KIT transcriptional target in melanocytes (McGill *et al.*, 2002) and a decrease in *BCL 2* expression in melanocytes increases their susceptibility to apoptosis. Interestingly, SCF strongly protects melanocytes form TNF related apoptosis inducing ligand (TRAIL) (Larribere *et al.*, 2004). SCF/c – KIT thus brings new interesting potential clues regarding the physio-pathology of vitiligo.

1.8.11 *FOXD*3

Forkhead box D3 (*FOXD3*) is a transcription factor that suppresses melanoblast development from neural crest (Kos *et al.*, 2001). Therefore dysregulated expression might harm melanocytes. FOXD3 also regulates endodermal differentiation including thyroid, pancreas, adrenal gland and gut (Guo *et al.*, 2002). Also other FOX factors are involved in autoimmune syndromes (Jonsson and Peng 2005). Mutations in *FOXD3* leading to elevated FOXD3 transcription is recently reported in one AIS 1 linked family (Alkhateeb *et al.*, 2005).

1.8.12 *CD*4

The *CD4* gene plays an important role in the cell-mediated immune response and its association with type 1 diabetes mellitus has been previously reported. Zamani *et al.*, (2009) reported an association with a pentanucleotide variable number of tandem repeats polymorphism (VNTR) with vitiligo.

1.8.13 *CLEC*11*A*

C-type Lectin Domain Family 11, Member A (*CLEC11A)* is one of the relevant keratinocyte-growth related genes and its role has been implicated in the pathogenesis of vitiligo vulgaris (Lan *et al.*, 2009).

1.8.14 *MYG1* (C12orf10)

MYG1 (Melanocyte proliferating gene 1 or C12orf10) is a ubiquitous nucleo-mitochondrial protein, involved in early developmental processes as well as in stress conditions. MYG1 may participate in pathways proposed by autoimmune theory of vitiligo pathogenesis. Genetic variability in *MYG1* gene may be associated with its altered levels thereby increasing the risk for autoimmune diseases including vitiligo. Philips *et al.* (2010) showed that promoter polymorphism -119C/G polymorphism have a functional impact on the regulation of the *MYG1* gene. The promoter polymorphism (-119C/G) was related with suspectibility for actively progressing vitiligo in Estonian population.

1.8.15 *DDR1* (Discoidin domain receptor tyrosine kinase 1)

Receptor tyrosine kinases play a key role in the communication of cells with their microenvironment. These kinases are involved in the regulation of cell growth, differentiation and metabolism. The protein encoded by this gene belongs to a subfamily of tyrosine kinase receptors. Sakuma *et al.* (1996) cloned genomic DNA of the *DDR1* gene. The gene contains 15 exons spanning approximately 9 kb. The promoter region of the gene contains a consensus binding site for p53. Silva de Castro *et al.* (2010), reported association of *DDR1* gene with vitiligo. However, Kim *et al.* (2010) found no association of vitiligo with *DDR1*.

1.8.16 *EDN1* (Endothelin-1)

Endothelin-1, which is expressed by keratinocytes, has paracrine effects on melanocytes, influencing their homeostasis, proliferation and pigmentation. It is thought to play a role in the skin response to 311-nm, narrow-band ultraviolet irradiation. Interestingly, the haplotype frequencies of *EDN1* polymorphisms differed significantly between vitiligo patients and healthy controls. When analysed according to clinical type, the haplotype frequencies in the focal and segmental clinical types differed significantly from healthy controls (Kim *et al.*, 2007).

1.8.17 *FAS*

The FAS/FASLG system plays a key role in regulating apoptosis. Previous findings have shown that CD4-dependent destruction of melanocytes is partially inhibited by blocking FAS-FASLG interactions in autoimmune vitiligo. Functional polymorphisms of the FAS and FASLG genes can alter their transcriptional activities. Li *et al.* (2008) and Li *et al.* (2009) reported an association of vitiligo with the *FAS* gene.

1.8.18 *FBXO*11-*MSH*6

The involvement of FBXO11 (previously, VIT1) in vitiligo was suggested on the basis of differential expression analysis (Le Poole *et al.*, 2001). Putative mutations in the adjacent MSH6 gene were reported in a single patient with early-onset colorectal cancer, systemic lupus erythematosus, and vitiligo (Rahner *et al.*, 2008).

1.8.19 *FOXP*3

FOXP3, a member of the fork-winged helix family of transcription factors, plays an important role in the development and function of naturally occurring CD4 (186940)-positive/CD25 (IL2RA; 147730)-positive T regulatory cells (Tregs). Tregs are involved in active suppression of inappropriate immune responses. Due to similarities between the autoimmunity and inflammation produced by manipulation of CD25-positive/CD4-positive regulatory T (Tr, or Treg) cells and those induced by genetic defects in the *FOXP3* gene, Hori *et al.* (2003) investigated the contribution of Foxp3 to the development and/or function of Tr cells in mice. *FOXP3* is the defective gene in the X-linked recessive immunodysregulation, polyendocrinopathy, and enteropathy multiple autoimmune disease syndrome and vitiligo.

1.8.20 *GSTM* and *GSTT*1

The glutathione S-transferases (GSTs) are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik, 1985). Uhm *et al.* (2007) reported an association with a *GSTM1* INDEL polymorphism with vitiligo. Liu *et al.* (2009) reported an association with a *GSTT1* INDEL polymorphism

with vitiligo however, another study showed no association of the same marker with vitiligo (Uhm *et al.*, 2007).

1.8.21 *IL1RN* (IL1 receptor antagonist)

IL1RN is a protein that binds to IL1 receptors (IL1R1) and inhibits the binding of IL1-alpha and IL1-beta (IL1B). As a consequence, the biological activity of these two cytokines is neutralized in physiological and pathophysiological immune and inflammatory responses. IL1RN was the first-to-be described, naturally occurring cytokine or hormone-like molecule to function as a specific receptor antagonist (Arend, 1991). Pehlivan *et al.* (2009) reported an association with an *IL1RN* intronic VNTR polymorphism, however, this result was rendered not significant by appropriate multiple-testing correction.

1.8.22 *IL10* (Interleukin 10)

IL10 family of cytokines produced by activated macrophages that targets various leukocytes and mainly represses excessive inflammatory responses. This family of cytokines also plays a role in epithelial cell proliferation, anti-apoptotic responses, tissue remodeling and healing. As IL 10 acts as an anti- inflammatory cytokine and inhibits the production of several cytokines such as IFN- γ from Th1 cells, the low levels of this cytokine is been associated with autoimmune diseases. Abanmi *et al.* (2008) reported association of vitiligo with *IL10*.

1.8.23 *MBL2* (Mannose binding lectin 2)

Mannan binding lectin (MBL) helps in the clearance of apoptotic cells and it has a role in complement activation. MBL also plays an important role in innate immunity, hence structural and promoter polymorphisms in *MBL* gene may lead to autoimmune disorders such as vitiligo. Genetic variability in MBL2 gene is also reported to be associated with increased risk for several autoimmune diseases including vitiligo.

1.8.24 *PDGFRA-Kit* (Platelet-derived growth factor receptor alpha)

The *PDGFRA* gene is a proto-oncogene that maps to 4q12. It belongs to the human type III family of transmembrane receptors, with an intrinsic tyrosine kinase component. The PDGFRA protein has been shown to be important for several cellular and tissue processes, such as proliferation, apoptosis, chemotaxis, melanogenesis, hematopoiesis and gametogenesis (Mol *et al*., 2003). Several reports have documented PDGFRA in regulation of pigmentation. PDGF has been shown to be important for the differentiation and survival of melanocytes during embryonic development (Adamyeko *et al*., 2009). These reports lead us to speculate that the *PDGFRA* gene may be a candidate susceptibility gene of vitiligo mapped to the region of 4q12.

1.8.25 *TSLP* (Thymic stromal lymphopoietin)

TSLP induces naïve CD4+ T cells to produce Th2 cytokines. In addition, to low production of Th2 cytokines, strong Th1 response, which plays an important role in vitiligo development, has been induced by blockade of TSLP or TSLP receptor.Cheong *et al.* (2009) reported an association with the TSLP 50-flanking SNP rs3806933 (-847C4T).

1.8.26 *UVRAG* (UV radiation resistance-associated gene)

Teitz *et al.* (1990) identified a cDNA that partially complements the ultraviolet (UV) sensitivity of xeroderma pigmentosum complementation group C (278720) cells. Perelman *et al.* (1997) named this gene *UVRAG* and reported that the 4.0 kb *UVRAG* mRNA encodes a predicted 648-amino acid protein. Jeong *et al.* (2010b) reported association with a haplotype defined by SNPs rs7933235 and rs1458836 in vitiligo.

1.8.27 *VDR* (Vitamin D receptor)

VDR is an intracellular hormone receptor that specifically binds 1,25 (OH)2D3 and mediates its effects. In 31 GV cases and 33 controls, Birlea *et al.* (2006) reported an association with the VDR restricted fragment length polymorphism rs7975232.

1.8.28 *XBP1* (X box-binding protein 1)

XBP1, is a protein which in humans is encoded by the XBP1 gene.(Liou et al.,1990) The XBP1 gene is located on chromosome 22 .The XBP1 protein is a transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response (Yoshida *et al.*, 2006).Spritz *et al.* (2004) detected linkage of GV to microsatellites at 22q11–q11.22 and Liang *et al.* (2007) at 22q12 in Chinese families. Ren *et al.* (2009) tested XBP1 as a positional/biological candidate gene within the linkage interval.

1.9 LINKAGE AND ASSOCIATION STUDIES

Familial clustering and linkage disequilibrium studies showed that genetic factors predispose vitiligo although a clear transmission pattern and cosegregation of vitiligo with specific mutations have not been demonstrated.

1.9.1 *HLA* associations:

The frequent association of vitiligo with other autoimmune diseases has prompted the studies of HLA association with vitiligo predisposition. The *HLA* loci are strongly linked to other loci in the major histocompatibility region of chromosome 6p. Therefore, it may be that vitiligo associated *HLA* alleles are not disease causing but are genetic markers that are usually co inherited in the population (i.e. in strong linkage disequilibrium) with the actual disease allele at another locus within the major histocompatibility region (Zhang et al 2005). Linkage disequilibrium studies in different populations have consistently showed a significant association between the HLA system and vitiligo predisposition. There are several studies on the association between vitiligo and HLA complex. HLA subtypes vary with racial/ethnic background.

Association of *MHC* alleles with a disease gains importance because of the antigen-presenting function of the MHC. Recent genome wide association studies have implicated the role of MHC in vitiligo (Jin *et al*., 2010, Quan *et al*., 2010) where several SNPs in the *MHC* region were significantly associated with the disease. However, the authors imputed the Human Leukocyte Antigen (HLA) class-I alleles

based on the linkage disequilibrium (LD) of HLA alleles with the specific SNPs, but could not impute HLA class-II alleles due to limitations of HLA allele imputation in the CHB (Chinese Han from Beijing, China) samples (Quan *et al*., 2010). The association of multiple HLA class I and class II antigens have been suggested for vitiligo (de Vijlder et al., 2004, Zhang *et al*., 2004a, Zhang *et al*., 2004b) although no consensus could be reached, due to distinct ethnic groups (Orozco-Topete *et al*., 2005, Tastan *et al*., 2004, Zamani *et al*., 2001) small sample sizes and low resolution typing methods used to identify the HLA antigens. For example, HLA-DR4 is increased in blacks, HLA-B13 in Moroccan Jews, and HLA-B35 in Yemenite Jews. An association of HLA-B13 with anti-thyroid antibodies has been reported (Rezaei *et al*., 2007). However, using high resolution typing methods and large number of samples, Singh *et al*., 2012 suggested a consistent increase of A*33:01, B*44:03, and DRB1*07:01 in both initial and replication studies 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 population. Previous studies in Caucasians showed association of generalized vitiligo with both MHC class I (specifically, HLA-A*02:01) (Jin *et al*., 2010; Jin *et al*., 2012) and class II loci (Jin *et al*., 2010), whereas studies carried out in Chinese show no apparent association in the class I or II regions and instead favor association in the class III region (Quan *et al*., 2010). Together, these findings indicate that the principal MHC genetic associations with generalized vitiligo differ among different populations, and may in part mediate differing prevalence of this autoimmune disease in different groups around the world. The HLA association studies reported till now are listed in Table 7.

Table 7. HLA associations reported in vitiligo.

Positive association	Negative association	Reference
HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLA-DRB1*07:01	DRB1*03:01	Singh *et al*., 2012
HLA-A*02:01	-	Jin *et al*., 2012
DRB1*07:01	-	Ren *et al*., 2009

HLA-A2	-	Liu *et al*., 2007
DRB1*04-DQB1*0301	DRB1*15-DQB1*0602	Fain *et al.*, 2006
DQA1*0302,*0601, DQB1*0303, *0503	*0503 DQA1*0501	Yang *et al.*, 2005
A*2501, A*30, B*13, B*27, Cw*0602	A*66	Zhang *et al.*, 2004
DR4, DR53	DR3	de Vijlder *et al.*, 2004
DR3, DR4, DR7	-	Tastan *et al.*, 2004
DRB4*0101, DQB1*0303	-	Zamani *et al*., 2001
DRB1*0701, DQB1*0201, DPB1*1601	-	Buc *et al*., 1998
A2, A10, A30 + A31, B13, B15	A28, B46	Wang *et al.*, 2000
A2, Dw7	-	Buc *et al.*, 1996
B21, Cw6, DR53	A19, DR52	Al-Fouzan *et al.*, 1995
DR6	DQ2	Valsecchi *et al.*, 1995
Bw6, DR7	-	Venkataram *et al.*, 1995
DR6	Cw7	Venneker *et al.*, 1993
B46, A31, Cw4	-	Ando *et al.*, 1993
DR12, A2	-	Schallreuter *et al.*, 1993
A30, Cw6, DQ3	C4AQ0	Orecchia *et al.*, 1992
DR1	-	Poloy *et al.*, 1991
A30, Cw6, B27, DR7	DR1, DR3	Finco *et al.*, 1991
A2, A3	-	Dai *et al.*, 1990
DR4, DQ3	-	Dunston and Halder,1990
DR4	-	Foley *et al.*, 1983
BW35	-	Metzker *et al.*, 1980
A1, A2, A31	A10	Kachru *et al.*, 1978
Cw* 0602	-	Xia *et al.*, 2006

1.10 GENOME WIDE ASSOCIATION STUDIES (GWAS):

Genome wide linkage scans involve the typing of families using polymorphic markers that are positioned across the whole genome, followed by calculating the degree of linkage of the marker to a disease trait. Positional candidate genes can be identified by examining the regions around the peaks of linkage that are obtained by the study. Several genome wide linkage analyses of vitiligo have been performed and multiple linkages to vitiligo have been identified (Nath *et al.,* 2001; Fain *et al.,* 2003; Alkhateeb *et al.,* 2002; Spritz *et al.,* 2004). The susceptibility loci identified by genome wide linkage analyses are given in Table 8.

The most important recent vitiligo developments were two large-scale genomewide association studies of generalized vitiligo, one in Caucasians (Jin *et al*., 2010) and the other in Chinese (Quan *et al*., 2010), which together identified and confirmed at least 16 different loci that contribute to generalized vitiligo susceptibility. Jin *et al*., (2010 a,b) carried out a GWAS of GV in non-Hispanic white subjects, identifying and confirming different loci that contribute to GV risk, including *FOXP1, MYH15, CCR6, ICA1, TBC1D2, IKZF4, SH2B3* almost all of which have immunoregulatory functions (Jin *et al*., 2010a, b). Two of these loci and one additional signal in the MHC were also identified in a Chinese GWAS of GV (Quan *et al*., 2010).

All but one of these genes encode proteins involved in regulation of the immune system and/or have been genetically associated with susceptibility to other autoimmune diseases. The sole exception is *TYR*, encoding tyrosinase, the key enzyme of melanin biosynthesis and the principal vitiligo autoimmune antigen. In Caucasians, a common *TYR* missense variant, R402Q, confers both relative protection from generalized vitiligo and relative susceptibility to malignant melanoma, by modulating the presentation of the TYR peptide by HLA-A2*01, thereby modulating recognition of melanocytes by the immune system. These genes together account for a relatively small fraction of the genetic risk of generalized vitiligo, indicating that many additional vitiligo susceptibility genes undoubtedly remain to be discovered.

Recently, Jin *et al.,* 2012 reported a large GWAS (450 individuals with vitiligo and 3,182 controls), an independent replication study (1,440 cases and 11,316 controls) and a meta-analysis (3,187 cases and 6,723 controls) identifying 13 additional

vitiligo-associated loci. These include *OCA2-HERC2*, *MC1R*, a region near *TYR*, *IFIH1*, *CD80*, *CLNK*, *BACH2*, *SLA*, *CASP7*, *CD44*, *IKZF4*, *SH2B3* and *TOB2*. Most vitiligo susceptibility loci encode immunoregulatory proteins or melanocyte components that may mediate immune targeting and the relationships among vitiligo, melanoma and eye, skin and hair coloration.

Table 8. Susceptibility loci for vitiligo.

Susceptibility loci	Chromosomal Region	Reference
SLEV1	17p13	Spritz *et al.*, 2004, Nath *et al.*, 2001
AIS1	1p31.3-p32.2	Alkhateeb *et al.*, 2002
AIS2	7p	Spritz *et al.*, 2004
FOXP1	3p13	Jin *et al.*, 2010b
MYH15	3q13.13	Jin *et al.*, 2010b
CCR6	6q27	Jin *et al.*, 2010b
ICA1	7p21.3	Jin *et al*., 2010b
TBC1D2	9q22.33	Jin *et al*., 2010b
IKZF4	12q13.2	Jin *et al*., 2010b
SH2B3	12q24.12	Jin *et al*., 2010b
RNASET2	6q27	Quan *et al*., 2010
FGFR1OP	6q27	Quan *et al*., 2010
CCR6	6q27	Quan *et al*., 2010
IFIH1	2q24.2	Jin *et al*., 2012
CD80	3q13.33	Jin *et al*., 2012
CLNK	4p16.1	Jin *et al*., 2012
BACH2	6q15	Jin *et al*., 2012
SLA	8q24.22	Jin *et al*., 2012
CASP7	10q25.3	Jin *et al*., 2012
CD44	11p13	Jin *et al*., 2012
TYR	11q21	Jin *et al*., 2012
OCA2-HERC2	15q12-13.1	Jin *et al*., 2012
MC1R	16q24.3	Jin *et al*., 2012
TICAM1	19p13.3	Jin *et al*., 2012
TOB2	22q13.2	Jin *et al*., 2012

1.11 TREATMENT

Vitiligo is a difficult disease to treat. Although the treatment of vitiligo has improved during the last decade, it is still not satisfactory. Several treatment modalities are currently in use; however these methods usually induce incomplete pigmentation. Vitiligo treatment can be classified into two broad categories i.e. non-surgical therapies and surgical therapies (Nordlund *et al* 1993; Van Geel, 2001).

1.11.1 Non-surgical therapies

1.11.1.1 Psoralen photochemotherapy:

Psoralenes are furocoumarin tricyclic hydrocarbon compounds. Psoralen photochemotherapy consists of photosensitizing psoralen with ultraviolet A in the 320-400 nm range (PUVA). PUVA and UVB therapies are widely used in the treatment of many skin disorders including vitiligo. The rationale of PUVA is to induce remissions of skin diseases by repeated controlled phototoxic reactions (Matsumura and Ananthaswamy, 2004). These reactions occur only when psoralenes are photoactivated by UVA. In systemic treatment, 8 methoxypsoralen or 4,5,8 trimethoxypsoralen is administrated before radiation exposure. The UV dosage is gradually increased until minimal erythema of vitiligo lesions occurs. How PUVA therapy stimulates the inactive melanocytes is unknown (Kovacs, 1998). The mechanism underlying the therapeutic effects of the combination of psoralen plus UVA is generally assumed that UVA induced DNA psoralen photoadducts impair the cell replication (Honig *et al* 1994). Inhibition of cell proliferation is observed at psoralen concentration and UVA doses which do not affect the cell viability (Luftl *et al.*, 1998); on the other hand higher doses cause irreversible DNA damage, resulting in both apoptosis and necrosis (Johnson *et al.*, 1996). It has been confirmed that the repigmentation is derived from the melanocyte reservoir in the hair follicles (Cui *et al.*, 1991). It has been demonstrated that PUVA irradiation of normal melanocytes *in vitro* inhibits the DNA and protein synthesis and affects EGF receptor and vitiligo associated melanocyte antigen expression. It is difficult to explain the PUVA induced repigmentation of vitiligo on the basis of these different mechanisms. It has been proposed that PUVA could stimulate the production of melanocyte growth factor or may deplete antigens on vitiligo melanocytes, thus blocking the binding of specific

autoantibodies, (Kao and Yu 1992). PUVA is immunosuppressive and this action of PUVA on T lymphocytes could be the reason for its therapeutic effect on vitiligo (Akyol *et al.,* 2002). It was proposed that PUVA inhibits gene transcription, which ultimately results in the shut down of cytokine release. Neuner *et al* showed the effect of PUVA on the release of the pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNFα from human peripheral blood mononuclear cells resulting in a significant reduction in these cytokines, thus causing the anti-inflammatory activity of PUVA (Neuner *et al* 1994).

1.11.1.2 Water bath PUVA:

The most recent model in phototherapy is water bath PUVA, in which the patient lies in a bath tub containing psoralen water for 15 min so that the drug gets absorbed on the skin and then goes for light therapy. This kind of therapy is especially beneficial in children for whom oral medicines are not safe (Aragane *et al*., 2001). Another method of psoralen treatment, used rarely for pediatric patients with small, scattered vitiligo patches, involves the application of a very dilute solution of the drug directly to the affected skin area. This is then exposed to sunlight. Such topical treatment makes a person very liable to severe burn and blisters following too much sun exposure whereas water bath PUVA has the advantage of being done at home, and does not damage the entire skin surface.

1.11.1.3 Broadband UVB:

This phototherapy uses an emission spectrum of 290-320 nm (Koster and Wiskemann 1990).

1.11.1.4 Narrowband UVB:

In this phototherapy an emission spectrum of 310-315 nm is used (Westerhof and Korbotova 1997). Narrow band UVB therapy or TL-01 therapy is the latest in phototherapy for the treatment of vitiligo. In this therapy there is no need to take oral psoralen or apply psoralen. The therapy is very safe and can be safely administered even to children. Narrow band UVB is much safer than full spectrum UVB. If exposure to natural sunlight is equal to 100% UV radiation , using a narrow band UV light is roughly 1% UV radiation . The advantage of UVB therapy over PUVA regimen is reflected by shorter duration of treatment (Van Geel *et al.,* 2001).

1.11.1.5 Topical immunomodulators:

Topical immunomodulatory agents such as tacrolimus and pimecrolimus offer several advantages in the treatment of vitiligo. These agents are well tolerated in children and adults and they can be used for long duration without evidence of atrophy or telangiectasias, the common complications associated with long term steroid use (Grimes, 2005). Tacrolimus is a topical immunomodulatory agent that affects T cell and mast cell functions by binding to cytoplasmic immunophilins and by inactivating calcineurin. Tacrolimus inhibits the synthesis and release of pro inflammatory cytokines and vaso active mediators from basophils and mast cells (Tharp, 2002). Pimecrolimus, which has a mechanism of action similar to tacrolimus, also can induce repigmentation in vitiliginous lesions (Mayoral *et al* 2003). As tacrolimus, pimecrolimus induces maximal repigmentation on sun-exposed areas.

1.11.1.6 Calcipotriol:

It is a synthetic analogue of vitamin D3. Vitamin D3 binds to vitamin D receptors in the skin, affecting melanocyte and keratinocyte growth and differentiation. It also inhibits T cell activation (Dusso and Brown 1998). Melanocytes express 1, 25-dihydroxyvitamin D3 receptors, which may stimulate melanogenesis.

1.11.1.7 Pseudocatalase:

The discovery of low epidermal catalase levels in involved and uninvolved skin of patients with vitiligo suggested a major stress arising from increased epidermal H_2O_2 generation (Schallreuter *et al* 1991). However, catalase mRNA levels in melanocytes and keratinocytes from patients is normal compared to healthy controls (Maresca *et al*., 1997). One consequence of H_2O_2 accumulation is the oxidative degradation of the porphyrin active site of the catalase leading to its deactivation (Aronoff *et al.,* 1965). Pseudocatalase is a bis (Mn) bicarbonate complex for the removal of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.,* 1995). Pseudocatalase functions as a pro-drug requiring UV light for the full activation of the complex (Schallreuter *et al.,* 1999a). Successful removal of the high levels of epidermal H_2O_2 in vitiligo was shown with a topical application of pseudocatalase in several studies (Schallreuter *et al.,* 1995). It has been demonstrated that *in vitro* and *in vivo* use of pseudocatalase

leads to the recovery of $6BH_4$ whose recycling process is perturbed in vitiligo and thus leads to repigmentation (Schallreuter *et al.*, 2001).

1.11.1.8 Khellin and UVA:

Khellin is a furanochrome and combined with UVA, it is as effective as PUVA therapy in the treatment of vitiligo without having the phototoxicity associated with psoralens (Nordlund *et al*., 1993).

1.11.1.9 Fake tanning products:

Cover creams or self tanning products are special drug cosmetics that can be used to match most skin patches when medical treatment is not successful. Patients with vitiligo are required to protect their depigmented skin against excessive sun exposure by wearing protective clothing. Tattooing is rarely recommended. It works best for the lip area, particularly in people with dark skin. However, it is difficult to perfectly match the skin, and tends to look worse over time. Cosmetics can be used to improve the appearance of the white areas not covered by clothing. Sunscreens give coolness to the affected areas and also prevent the normal skin around the patches from becoming darker. Bleaching or depigmentation of the normal skin and autologous transplantation of skin are an option for those who are severely affected (Samantha *et al*., 2008).

1.11.2 Surgical therapies

Several treatment modalities such as PUVA, UVB and local corticosteroids are currently used in the treatment of vitiligo. However, these treatments usually induce incomplete repigmentation. Surgical methods intended to repigment vitiligo are an interesting therapeutic option if patients have stable disease (Ongenae *et al.,* 2001). All surgical techniques have the same basic principle: to transplant autologous melanocytes from a pigmented donor skin to regions without melanocytes (Ongeneae *et al.,* 2001). Basically there are two types of surgical techniques, tissue grafts and cellular grafts. Tissue grafts are full thickness punch grafts and, split thickness grafts and suction blister grafts. With tissue grafts, only a limited surface area can be treated but with good results in the majority of cases.

1.11.2.1 Full thickness punch grafts:

In this method punch grafts from normally pigmented skin are implanted in the affected area. Repigmentation is based on the 'pigment spread phenomenon' by grafted piece of normal skin. The grafts are implanted into perforations previously made at the recipient site using a biopsy punch under local anesthesia (Ongenae *et al*., 2001). The success rate of full thickness punch grafts is in between 68-82% (Malakar and Dhar 1999; Boersma *et al.*, 1995; Falabella *et al.*, 1988). Punch grafting is easy to perform and does not require special equipment or a laboratory set up. Difficult areas such as lips could be treated successfully; however it is not suitable for body folds (Malakar and Dhar 1999).

1.11.2.2 Split thickness grafts:

This technique has a high success rate of 78–91% (Olsson and Juhlin 1998; Kahn and Cohen 1998). After obtaining a split thickness skin graft using a dermatome it can be applied directly to the derma braded recipient area. Temporary small epithelial milia like cysts can be observed in the recipient area during the first months, especially on the face and neck. Scar or keloid formation at the donor site is reported in 12% of the patients treated with split thickness grafts. As donor tissue is limited more than one split skin grafting session can be necessary (Ozdemir *et al*., 2002).

1.11.2.3 Suction blister grafts:

Grafts are carefully removed with sharp scissors and forceps after harvesting the graft. This epidermal sheet is then grafted onto the denuded recipient site. The success rate is 73–88%. Pigment spread after epidermal blister grafting can be enhanced by pre operative radiation therapy of the donor site using PUVA. Temporary hyper pigmentation can be seen in the grafted sites in 2–65% (Ozdemir *et al*., 2002). The eyelids, lips and bony prominences can be treated using this method.

1.11.2.4 Cultured epidermal grafts:

A shave biopsy of normally pigmented skin is the source of epidermal cell culture. The cultured sheet is released by treatment with dispase and attached to petroleum gauze as support. Subsequently the gauze to which the epithelium adheres will be

applied onto the dermabraded recipient site and covered with occlusive dressing (Kumagai and Uchikoshi 1997). Success rate of this method is in between 33-54%.

1.11.2.5 Non-cultured keratinocytes and melanocytes:

Transplantation technique with a suspension of non cultured keratinocytes and melanocytes in the treatment of depigmented lesions is effective. Donor skin is obtained from the occipital area and immersed for 18 h in 0.25% trypsin solution. The following day the epidermis of the donor skin can be separated from the dermis *in vitro* using fine forceps. After several procedures a cellular suspension is obtained (Mysore and Salim, 2009). Liquid nitrogen is used to induce blisters in the recipient area. The cellular suspension from the donor site is injected into each blister at the recipient area after aspiration of the viscous blister fluid. The intact blister top is a natural dressing that holds the transplanted cells in place. It is important not to separate keratinocytes from melanocytes before grafting because factors furnished by keratinocytes sustain melanocyte growth (Ozdemir *et al*., 2002). The success rate of this therapy is more than 70% (Gauthier and Surleve-Bazeille 1992).

1.11.2.6 Cultured melanocytes:

Lerner *et al*., 1987 first described the use of cultured pure autologous human melanocytes. They cultured melanocytes of a shave biopsy from normally pigmented skin *in vitro* with the addition of several growth factors and chemical media. The success rates vary between 22-72%.

1.11.2.7 Depigmentation:

Depigmentation or the removal of remaining pigmentation is normally done in patients who have greater than 50% of their bodies affected and who have demonstrated recalcitrance to repigmentation. Depigmentation is permanent and irreversible. Monobenzylether of hydroquinone is used as a depigmenting agent (Nordlund *et al* 1993).

1.11.3 Herbal products

1.11.3.1 Anti-vitiligo® (True Herbals, Lahore, Pakistan):

Anti-vitiligo® (True Herbals, Lahore, Pakistan) is a traditional herbal formulation which was available internationally since November 2003. It is effective both in

disease of recent onset as well as long standing established cases. Formulation contains the following ingredients.

1.11.3.2 Psoralea corylifolia:

It is a rich source of naturally occurring psoralen. It sensitizes human skin to the tanning effect of UV and sun light. *P. corylifolia* has been traditionally used both orally as well as in the form of topical preparations. Oxidative stress is widely believed to be one of the likely causative factors in the initiation of white skin patches of vitiligo. Hence, the protective, anti-oxidative and anti stress properties of *P. corylifolia* may contribute to the improvement in the hypo-pigmented white skin patches of vitiligo.

1.11.3.3 Black cumin:

Seeds of Nigella sativa have also been having an immunomodulatory as well as anti cancer effect, which is due to augmentation of T cell and natural killer cell mediated immune responses.

1.11.3.4 Barberry root:

Barberry root or the root of *Berberis vulgaris* contains numerous chemicals and bioactive compounds of medical significance. It contains for example the alkaloids like berbamine, berberine, and oxyacanthine. Other compounds include tannins, chelidonic acid and resins. It is also quite rich in B-vitamin thiamine, lutein, vitamin C, beta-carotene, zeaxanthin, zinc, chromium, and cobalt. This herb has also been shown in scientific studies to possess antioxidant and cytoprotective properties.

1.11.3.5 Kalawalla® (American Life Style, New York, USA):

Kalawalla® (American Life Style, New York, USA) is a herbal product that works as a natural immunomodulator with proven immunomodulating effect. The product contains *Polypodium leucotomos* standardized extracts. *P. leucotomos* is a fern plant extract that has been used in Europe to treat vitiligo for over 10 years with encouraging results. The extract can help to regulate the immune system bringing it to its healthiest, strongest and balanced levels. Repigmentation results can be seen within the first month of taking the product. *P. leucotomos* standardized extract has

been known to increase the lymphocyte levels. It is also known to regulate the CD4/CD8 ratios to their normal values.

1.11.3.6 Piperine:

The synthetic derivatives of piperine can stimulate pigmentation in the skin especially when combined with UVR treatment. The studies have compared the effects of piperine and its analogues tetrahydropiperine (THP), cyclohexyl analogue of piperine (CHP) and reduced CHP (rCHP) when applied to the skin of mice, either alone or followed by UV treatment. CHP did not show significant results while piperine, THP and rCHP did induce pigmentation in the skin. When used alone, the compounds stimulated pigmentation to an even, light brown color within six weeks. However, by accompanying the use of piperine or THP with UV, the skin became significantly darker, and within only seven weeks as compared to other treatments which take a year.

1.12 REFERENCES

Abanmi A, Al Harthi F, Zouman A (2008). Association of Interleukin-10gene promoter polymorphisms in Saudi patients with vitiligo. *Dis Markers* 24: 51–7.

Abdel-Naser, M.B., Gollnick, H., and Orfanos, C.E (1991). Evidence of primary involvement of keratinocytes in vitiligo. *Arch Dermatol Res.* 283:47.

Abdel-Naser, M.B., Kruger-Krasagakes, S., Krasagakis, K., Gollnick, H., Abdel-Fattah, A. and Orfanos, C.E (1994). Further evidence for involvement of both cell mediated and humoral immunity in generalized vitiligo. *Pigment Cell Res.* 7: 1-8.

Adachi, T.; Wang, X. L (1998).Association of extracellular-superoxidedismutase phenotype with the endothelial constitutive nitric oxide synthase polymorphism. *FEBS Lett.* 433:166–168.

Adameyko I, Lallemend F, Aquino JB, Pereira JA, Topilko P, Muller T *et al* (2009). Schwann cell precursors from nerve innervation are a cellular origin of melanocytes in skin. *Cell* 139: 366–379.

Aggarwal, B. B., Eessalu, T. E., Hass, P. E (1985).Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. *Nature* 318: 665-667,

Agrawal, D., Shajil, E.M., Marfatia, Y.S., and Begum, R (2004). Study on the antioxidant status of vitiligo patients of different age groups in Baroda. *Pigment Cell Res.* 17:289-294.

Ahonen, P., Myllarniemi, S., Sipila, I. and Perheentupa, J (1990). Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med*. 322 :1829-1836.

Akyol, M., Celik, V.K., Ozcelik, S., Polat, M., Marufihah, M., and Atalay, A (2002). The effects of vitamin E on the skin lipid peroxidation and the clinical improvement in vitiligo patients treated with PUVA. *Eur J Dermatol*. 12: 24-26.

Al Badri, A.M., Foulis, A.K., Todd, P.M., Gariouch, 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. *J Pathol*. 169:203-206.

Al'Abadie MS, Senior HJ, Bleehen SS, et al. (1994). Neuropeptide and neuronal marker studies in vitiligo. *Br J Dermatol* 131:160–5.

Alkhateeb, A., Fain, P. R., Thody, A., Bennett, D. C. and Spritz, R. A (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their relatives. *Pigment Cell Res.* 16:208–214.

Alkhateeb, A., Fain, P.R, and Spritz, R.A (2005). Candidate functional promoter variant in the FOXD3 melanoblast developmental regulator gene in autosomal dominant vitiligo. *J Invest Dermatol*. 125: 388-391.

Amiot, F., Fitting, C., Tracey, K (1997). Lipopolysaccharide-Induced Cytokine Cascade and Lethality in LT-A/TNF-A Deficient Mice. *Molecular Medicine* 3 (12) : 864-875.

Aragane Y, Kawada A, Maeda A, Isogai R, Isogai N, Tezuka T (2001). Disseminated scleroderma of a Japanese patient successfully treated with bath PUVA photochemotherapy. *J Cutan Med Surg* 5:135–7.

Argilés JM, López-Soriano J, Busquets S, López-Soriano FJ (1997). Journey from cachexia to obesity by TNF. *FASEB J.* 11(10):743-51.

Arthur, J.R (2000). The glutathione peroxidases. *Cell Mol Life Sci.* 57:1825-1835.

Austin, L. M. and Boissy, R. E (1995). Mammalian tyrosinase-related protein-1 is recognized by autoantibodies from vitiliginous Smyth chickens. *Am. J. Pathol*. 146 :1529–1541.

Avraham, K. B.; Schickler, M.; Sapoznikov, D.; Yarom, R.; Groner, Y (1988). Down's syndrome: abnormal neuromuscular junction in tongue of transgenic mice with elevated levels of human. *Cell.* 54(6):823-9.

Badri, A.M., Todd, P.M., Garioch, J.J., Gudgeon, J.E., Stewart, D.G., and Goudie, R.B (1993). An immunohistological study of cutaneous lymphocytes in vitiligo, *J Pathol*. 170:149-155.

Baeyens, K., De Bondt, H., Raeymaekers, A., Fiers, W., De Ranter, C (1998a). MMDB Structure Summary.

Baeyens, K., De Bondt, H., Raeymaekers, A., Fiers, W., De Ranter, C (1998b) Protein Databank StructureExplorer.

Baharav, E., Merimski, O., Shoenfeld, Y., Zigelman, R., Gilbrund, B., and Yecheskel, G (1996). Tyrosinase as an autoantigen in patients with vitiligo. *Clin Exp Immunol*. 105:84-88.

Barisic-Drusko V, Rucevic I (2004) Trigger factors in childhood psoriasis and vitiligo. *Coll Antropol* 28:277–85.

Bar-Peled, O.; Yarom, R.; Rotshenker, S (1994). Cell damage by excess CuZnSOD and Down's syndrome. *Biomed. Pharmacother*. 48:231–240.

Basak, P. Y., Adiloglou, A. K., Ceyhan, A. M., Tas, T. and Akkaya, V. B (2009) The role of helper and regulatory T cells in the pathogenesis of vitiligo. *J. Am. Acad. Dermatol*. 60 :256–260.

Bast, A., Haenen, G.R., and Doelman, C.J (1991). Oxidants and antioxidants: state of the art. *Am J Med.* 91:2S-13S.

Beazley, W.D., Gaze, D., Panke, A., Panzig, E., and Schallreuter, K.U (1999). Serum selenium levels and blood glutathione peroxidase activities in vitiligo. *Br J Dermatol*. 141:301-303.

Beckman, J. S. and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol*. 271: C1424–C1437.

Begovich, A.B., Carlton, V.E., and Honigberg, L.A (2004). A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet.* 75:330-337.

Behl, P.N., and Bhatia, R.K (1972). 400 cases of vitiligo. A clinico-therapeutic analysis. *Ind J Dermatol.* 17:51-55.

Bell, G.I., Najarian, R.C., Mullenbach, G.T., and Hallewell. R.A (1986). cDNA sequence coding for human kidney catalase. *Nucleic Acids Res.* 14, 5561-5562.

Benedetto, M. T.; Anzai, Y.; Gordon, J. W(1991). Isolation and analysis of the mouse genomic sequence encoding Cu-Zn superoxide dismutase. *Gene* 99:191–195.

Betterle, C., Del Prete, G.F., Peserico, A., Bersani, G., Caracciolo, F., Trisotto, A and Poggi, F (1976). Autoantibodies in vitiligo. *Arch Dermatol*. 112 :1328.

Beutler, B., Greenwald, D., Hulmes, J., Chang, M., Pan, Y., Mathison, J., Ulevitch, R., Cerami, A (1985a.) Identity of Tumour Necrosis Factor and the Macrophage-Secreted Factor Cachectin. *Nature.* 316 (8): 552-554.

Beutler, B., Milsark, L., Cerami, A (1985b). Passive Immunization Against Cachectin/Tumor Necrosis Factor Protects Mice from Lethal Effects of Endotoxin. *Science* 229 : 867-871.

Bhawan, J. and Bhutani, L. K (1983) Keratinocyte damage in vitiligo. *J. Cutan. Pathol*. 10:207–212.

Bickers, D. R. and Athar, M (2006). Oxidative stress in the pathogenesis of skin disease. *J. Invest. Dermatol*. 126:2565–2575.

Bindoli, A., Rigobello, M.P., and Deeble, D.J (1992). Biochemical and toxicological properties of the oxidation products of catecholamines. *Free Rad Biol Med.* 13:391-405.

Bir LS, Aktan S (1991). Sympathetic skin response in psoriasis and vitiligo. *J Auton Nerv Syst* 77:68–71.

Birlea S, Birlea M, Cimponeriu D , Apostol P, Cosgarea R, Gavrila L, Tigan S, Costin G, Das P (2006). Autoimmune diseases and vitamin D receptor Apa-I polymorphism are associated with vitiligo in a small inbred Romanian community. *Acta Derm Venereol* 86:209–14.

Birlea S. A., Jin Y., Bennett D C., Herbstman D, Wallace M R,McCormack W T, Kemp EH , Gawkrodger D J, WeetmanA Y , Picardo M, Leone1 G, Taıeb A, Jouary T,Ezzedine K ,van Geel N, Lambert J, Overbeck A,Fain P R, Spritz R A (2011). Comprehensive Association Analysis of Candidate Genes for Generalized Vitiligo Supports XBP1,FOXP3, and TSLP. *J Invest Dermatol.* 1131: 371–381.

Birlea, S. A., Costin, G. -E. and Norris, D. A (2009). New insights on therapy with vitamin D analogs targeting the intracellular pathways that control repigmentation in human vitiligo. *Medicinal Res. Rev*. 29:514–546.

Birol, A., Kisa, U., Kurtipek, G. S., Kara, F., Kocak, M., Erkek, E. and Caglayan, O(2006). Increased tumor necrosis factor α (TNF-α) and interleukin 1α (IL1-α) levels in the lesional skin of patients with nonsegmental vitiligo. *Int. J. Dermatol*. 45:992–993.

Bishop DT, Demenais F, Iles MM, Harland M, Taylor JC, Corda E, Randerson-Moor J, Aitken JF, Avril MF, Azizi E, Bakker B, Bianchi-Scarrà G, Bressac-de Paillerets B, Calista D, Cannon-Albright LA, Chin-A-Woeng T, Debniak T, Galore-Haskel G, Ghiorzo P, Gut I, Hansson J, Hocevar M, Höiom V, Hopper JL, Ingvar C, Kanetsky PA, Kefford RF, Landi MT, Lang J, Lubiński J, Mackie R, Malvehy J, Mann GJ, Martin NG, Montgomery GW, van Nieuwpoort FA, Novakovic S, Olsson H, Puig S, Weiss M, van Workum W, Zelenika D, Brown KM, Goldstein AM, Gillanders EM, Boland A, Galan P, Elder DE, Gruis NA, Hayward NK, Lathrop GM, Barrett JH, Bishop JA (2009). Genome-wide association study identifies three loci associated with melanoma risk. *Nat. Genet*. 41: 920–925.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., Cerretti, D. P. **A** (1997). Metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385: 729-733 .

Blomhoff, A., Kemp, E. H., Gawkrodger, D. J., Weetman, A. P., Husebye, E. S., Akselsen, H. E., Lie, B. A. and Undlien, D. E. (2005). CTLA-4 polymorphisms are associated with vitiligo in patients with concomitant autoimmune diseases. *Pigment Cell Res*. 18 : 55–58.

Boisseau-Garsuad, A.M., Saint-Cyr, I., Quist, D., Arveiler, B. and Garsaud P. (2001). Familial aggregation of vitiligo in the French West Indies (Isle of Martinique). *Eur J Dermatol.* 11:554-556.

Boissy, R. E. and Spritz, R. A. (2009). Frontiers and controversies in the pathobiology of vitiligo: separating the wheat from the chaff. *Exp. Dermatol*. 18:583–585.

Boissy, R. E., Liu, Y. Y., Medrano, E. E. and Nordlund, J.J. (1991). Structural aberration of the rough endoplasmic reticulum and melanosome compartmentalization in long-term cultures of melanocytes from vitiligo patients. *J. Invest. Dermatol*. 97:395–404.

Bonifati, C., Ameglio, F(1999). Cytokines in psoriasis. *Int. J. Derm*. 38: 241-251.

Bottini, N., Musumeci, L. and Alonso, A. (2004). A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet*. 36: 337-338.

Boyman, O., Hefti, H. P., Conrad, C., Nickoloff, B. J., Suter, M., Nestle, F (2004). Spontaneous development of psoriasis in a new animal model shows an essential role for T cells and tumor necrosis factor-alpha. *J. Exp. Med*. 199: 731-736.

Brady, T. C.; Chang, L. Y.; Day, B. J.; Crapo, J. D(1997). Extracellular superoxide dismutase is upregulated with inducible nitric oxide synthase after NF-kappa B activation. *Am. J. Physiol*. 273: 1002–1006.

Brenner, D. A., O'Hara, M., Angel, P., Chojkier, M., Karin, M (1989). Prolonged activation of JUN and collagenase genes by tumour necrosis factor-alpha. *Nature* 337: 661-663.

Briganti, S. and Picardo, M (2003) Antioxidant activity, lipid peroxidation and skin diseases. What's new. *J. Eur.Acad. Dermatol. Venereol.* 17:663–669.

Brinkman BM, Huizinga TW, Kurban SS et al.(1997). Tumour necrosis factor alpha gene polymorphisms in rheumatoid arthritis: association with susceptibility to, or severity of, disease? *Br J Rheumatol* 36: 516–521.

Brookes, A.J (1999). The essence of SNPs. *Gene*. 234:177-86.

Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G., and Bachelet, M (2003). Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. *Biol Chem.* 278:21601-21606.

Brostoff, J. (1969). Autoantibodies in patients with vitiligo. *Lancet.* 2: 177-178.

Bruijn, L. I.; Houseweart, M. K.; Kato, S.; Anderson, K. L.; Anderson, S. D.; Ohama, E.; Reaume, A. G.; Scott, R. W.; Cleveland, D. W(1998). Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* 281:1851–1854.

Buckley, W.R., and Lobitz, and W.C. Jr.(1953). Vitiligo with a raised inflammatory border. *AMA Arch Derm Syphilol*. 67:16-20.

Caixia, T., Hongwen, F., and Xiran, L (1999). Levels of soluble interleukin-2 receptor in the sera and skin tissue fluids of patients with vitiligo. *J Dermatol Sci*. 21: 59-62.

Canton, I., Akhtar, S., Gavalas, N. G., Gawkrodger, D. J., Blomhoff, A., Watson, P. F., Weetman, A. P. and Kemp, E. H (2005). A single nucleotide polymorphism in the gene encoding lymphoid protein tyrosine phosphatase (PTPN22) confers susceptibility to generalized vitiligo. *Genes Immun*. 6:584–587.

Carbone, G. M.; St Clair, D. K.; Xu, Y. A.; Rose, J. C(1994). Expression of manganese superoxide dismutase in ovine kidney cortex during development. *Pediatr. Res*. 35:41–44.

Carlsson, L. M.; Jonsson, J.; Edlund, T.; Marklund, S. L(1995). Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc. Natl. Acad. Sci. USA* 92:6264–6268.

Carswell, E., Old, L., Kassel, R., Green, N., Fiore, N., Williamson, B (1975). An Endotoxin-Induced Serum Factor that Causes Necrosis of Tumors. *Proc. Natl. Acad. Sci. USA* 72 (9): 3666-3670.

Casp, C. B., She, J. X. and McCormack, W. T (2003). Genes of the LMP/TAP cluster are associated with the human autoimmune disease vitiligo. *Genes Immun*. 4: 492–499.

Casp, C. B., She, J. -X. and McCormack, W. T. (2002) . Genetic association of the Catalase gene (CAT) with vitiligo susceptibility. *Pigment Cell Res*. 15:62–66.

Chakravarti, A (1999). Population genetics-making sense out of sequence. *Nat Genet.* 21, 56-60.

Chakravarti, A (2001). To a future of genetic medicine. *Nature.* 15; 822-823.

Chance, B., Sies, H. and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev*. 59, 527-605.

Chaudiere, J., and Ferrari-Iliou, R (1999). Intracellular antioxidants: from chemical to biochemical mechanisms. *Food Chem Toxicol*. 37: 949-962.

Chavan, B., Beazley, W., Wood, J. M., Rokos, H., Ichinose, H. and Schallreuter, K. U (2009). H_2O_2 increases de novo synthesis of (6R)-L-erythro-5, 6,7,8-tetrahydrobiopterin via GTP cyclohydrolase I and its feedback regulatory protein in vitiligo. *J. Inherit. Metab. Dis.* 32: 86–94.

Chen F. LTA (Lymphotoxin-A). *Atlas Genet Cytogenet Oncol Haematol.* February 2005.

Chen JJ, Huang W, Gui JP et al. (2005). A novel linkage to generalized vitiligo on 4q13-q21 identified in a genome-wide linkage analysis of Chinese families. *Am J Hum Genet* 76:1057–65.

Chen, X., and Sullivan, P.F. (2003). Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. *Pharmacogenomics J.* 3 :77-96.

Cheong KA, Chae SC, Kim YS, Kwon HB, Chung HT, Lee AY (2009). Association of thymic stromal lymphopoietin gene 847C4T polymorphism in generalized vitiligo. *Exp Dermatol* 18:1073–5.

Cho, G.; Kang, S.; Seo, S. J.; Kim, Y.; Jung, G (1997). The transcriptional repression of the human Cu/Zn superoxide dismutase- (sod1) gene by the anticancer drug, mitomycin C(MMC). *Biochem. Mol. Biol. Int.* 42:949–956.

Cho, J. S.; Chang, M. S.; Rho, H. M (2001).Transcriptional activation of the human Cu/Zn superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through the xenobiotic-responsive element. *Mol. Genet. Genomics* 266:133–141.

Chung, D. J.; Wright, A. E.; Clerch, L. B. The 3' untranslated region of manganese superoxide dismutase RNA contains a translational enhancer element. Biochemistry 37:16298–16306; 1998.

Church, S. L.; Grant, J. W.; Meese, E. U.; Trent, J. M(1992). Sublocalization of the gene encoding manganese superoxide dismutase (MnSOD/SOD2) to 6q25 by fluorescence in situ hybridization. *Genomics* 14(3):823-5.

Clemens MJ, van Venrooij WJ, van de Putte (2000). Apoptosis and Autoimmunity. *Cell Death Differ* 7,131-133.

Clerch, L. B.; Massaro, D (1992). Rat lung antioxidant enzymes: differences in perinatal gene expression and regulation. *Am. J. Physiol*. 263:L466–L470.

Collins, S. M., Dominguez,M., Ilmarinen, T., Costigan, C. and Irvine, A. D (2006). Dermatological manifestations of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome. *Br. J. Dermatol*. 154, 1088–1093.

Covelli, V. and Jirillo, E (1988). Neuropeptides with immunoregulatory function: current status of investigations. *Functional Neurol*. 3 :253-261.

Crapo, J. D.; Oury, T.; Rabouille, C.; Slot, J. W.; Chang, L.-Y (1992). Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proc. Natl. Acad. Sci. USA* 89:10405–10409.

Creagan, R.; Tischfield, J.; Ricciuti, F.; Ruddle, F. H (1973). Chromosome assignments of genes in man using mouse-human somatic cell hybrids: mitochondrial superoxide dismutase (indophenol oxidase-B, tetrameric) to chromosome 6. *Humangenetik* 20: 203–209.

Cucchi M.L., Frattini, P., Santagostino, G., Preda, S., and Orecchia, G (2003). Catecholamines increase in the urine of non-segmental vitiligo especially during its active phase. *Pigment Cell Res*. 2 : 111-116.

Cucchi, M.L, Frattini, P. Santagostino, G and Orecchia, G (2000). Higher plasma catecholamines and metabolite level in the early phase of nonsegmetal vitiligo. *Pigment Cell Res*. 13 :28-32.

Cui, J., Harning, R., Henn, M. and Bystryn, J.C (1992). Identification of pigment cell antigens defined by vitiligo antibodies. *J Invest Dermatol*. 98 :162-165.

Cummings, M. P. and Nordlund, J. J (1995). Chemical leukoderma: fact or fancy. *Am. J. Contact Dermatitis* 6 :122–127.

D'Alfonso, S., and Richiardi, P.M (1994). A polymorphic variation in a putative regulation box of the TNFA promoter region. *Immunogenetics*. 39 :150-154.

Dammak, I., Boudaya, S., Ben Abdallah, F., Turki, H., Attia, H. and Hentati, B (2009). Antioxidant enzymes and lipid peroxidation at the tissue level in patients with stable and active vitiligo. *Int. J. Dermatol*. 48 :476–480.

Darr,D.,Fridovich, I (1994). Free radicals in cutaneous biology. *J. Invest. Dermatol*. 102: 671–675.

Das SK, Majumder PP, Majumdar TK , Haldar B (1985). Studies on vitiligo. II. Familial aggregation and genetics. *Genet Epidemiol* 2:255–62.

Das, K. C.; Guo, X. L.; White, C. W (1998). Protein kinase Cdelta dependent induction of manganese superoxide dismutase gene expression by microtubule-active anticancer drugs. *J. Biol. Chem.* 273:34639–34645.

Das, P. K., van den Wijngaard, R. M., Wankowicz-Kalinska, A. and Le Poole, I. C (2001) A symbiotic concept of autoimmunity and tumor immunity: lessons from vitiligo. *Trends Immunol.* 22:130–136.

de Haan, J. B.; Tymms, M. J.; Cristiano, F.; Kola, I (1994). Expression of copper/zinc superoxide dismutase and glutathione peroxidase in organs of developing mouse embryos, fetuses, and neonates. *Pediatr. Res.* 35:188–196.

De La Torre, R.; Casado, A.; Lopez-Fernandez, E.; Carrascosa, D.; Ramirez, V.; Saez, J (1996). Overexpression of copper-zinc superoxidedismutase in trisomy 21. *Experientia* 52:871–873.

de Vijlder HC, Westerhof W, Schreuder GM, de Lange P, Claas FH (2004). Difference in pathogenesis between vitiligo vulgaris and halo nevi associated with vitiligo is supported by an HLA association study. *Pigment Cell Res*.17: 270–4.

Dell'Anna, M. L. and Picardo, M (2006). A review and a new hypothesis for non-immunological pathogenetic mechanisms in vitiligo. *Pigment Cell Res*. 19 :406–411.

Dimmeler, S.; Hermann, C.; Galle, J.; Zeiher, A. M(1999). Upregulation of superoxide dismutase and nitric oxide synthase mediatesthe apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 19:656–664.

DiSilvestre, D.; Kleeberger, S. R.; Johns, J.; Levitt, R. C (1995). Structure and DNA sequence of the mouse MnSOD gene. *Mamm. Genome* 6:281–284.

Dixon MF and Philips Q (1993). Cytokines . In Aids to Pathology. 4th edition. Churchill Livingstone 9 – 14.

Dosquet C, Weill D, Wautier JL (1992). Molecular mechanism of blood monocyte adhesion to vascular endothelial cells. *Nouv Rev Fr Hematol*. 34 Suppl:S55-9.

Dougall, W. C.; Nick, H. S (1991). Manganese superoxide dismutase: a hepatic acute phase protein regulated by interleukin-6 and glucocorticoids. *Endocrinol* 129:2376–2384.

Durham-Pierre, D.G., Walters, C.S., and Halder, R.M (1995). Natural killer cell and lymphokine-activated killer cell activity against melanocytes in vitiligo. *J Am Acad Dermatol.* 33:26-30.

Dusso, A.S., and Brown, A.J (1998). Mechanism of vitamin D action and its regulation. *Am J Kidney Dis.* 32:S13-S24.

Dutta, A.K., and Mandal, S.B (1969). A clinical study of 650 cases of vitiligo and their classification. *Ind J Dermatol.* 14 :103-105.

Dynan, W. S (1986). Promoters for housekeeping genes. *Trends Genet.* 2:196–197.

El Maghraby GM, Barry BW, Williams AC (2008). Liposomes and skin : from drug delivery to model membranes. *Eur J Pharm Sci.* 34 : 203-22.

Eskandani M, Golchai J, Pirooznia N, Hasannia S (2010). Oxidative stress level and tyrosinase activity in vitiligo patients. *Indian J Dermatol* 55 : 15-19.

Estevez, A. G., Crow, J. P.,Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L.; Beckman, J. S (1999). Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. *Science* 286: 2498–2500.

Evans A. M., Petersen J. W., Sekhon, G. S., DeMars R (1989). Mapping of prolactin and tumor necrosis factor-beta genes on human chromosome 6p using lymphoblastoid cell deletion mutants. Somat. Cell Molec. *Genet*. 15: 203-213.

Eventoff W., Tanaka N., Rossmann M.G (1976). Crystalline bovine liver catalase. *Mol Biol.* 103; 799-801.

Ezzedine K., Diallo A., Léauté-Labrèze C., Mossalayi D., Gauthier Y., Bouchtnei S., Cario-André M., Seneschal J., Boralevi F., Jouary T., Taieb A (2011). Multivariate analysis of factors associated with early-onset segmental and nonsegmental vitiligo: a prospective observational study of 213 patients. *Br J Dermatol* 165:44-9.

Farin F. M., Hitosis Y., Hallagan S. E., Kushleika J., Woods, J. S., Janssen, P. S., Smith-Weller, T., Franklin G. M., Swanson P. D., Checkoway, H (2001). Genetic polymorphisms of superoxide dismutase in Parkinson's disease. *Mov. Disord* 16:705–707.

Farrokhi, S., Hojjat-Farsangi, M., Noohpisheh, M. K., Tahmasbi, R. and Rezaei, N (2005). Assessment of the immune system in 55 Iranian patients with vitiligo. *J. Eur. Acad. Dermatol. Venereol.* 19, 706–711.

Fishman, P., Azizi, E., Shoenfeld, Y., Sredni, B., Yesheskel, G., Ferrone, S., Zigelman, R., Chaitchik, S., Floro, S., and Djaldetti, M (1993). Vitiligo autoantibodies are effective against melanoma. *Cancer*. 72, 2365-2369.

Fitzpatrick, T. B., Miyomato, M and Ishikawa, K (1967). The evolution of concepts of melanin biology. *Arch. Dermatol*. 96, 305–323.

Foley, L. M., Lowe, N. J., Miseloff, E. and Tiwari, J. L (1983). Association of HLA-DR4 with vitiligo. *J. Am. Acad. Dermatol.* 38, 39–40.

Folz, R. J.; Crapo, J. D (1994). Extracellular superoxide dismutase Superoxide dismutase gene family 345 (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. *Genomics* 22:162–171.

Folz, R. J.; Guan, J.; Seldin, M. F.; Oury, T. D.; Enghild, J. J.; Crapo, J. D (1997). Mouse extracellular superoxide dismutase: primary structure, tissue-specific gene

expression, chromosomal localization and lung in situ hybridization. *Am. J. Respir. Cell Mol. Biol*. 17:393–403.

Folz, R. J.; Peno-Green, L.; Crapo, J. D (1994). Identification of a homozygous missense mutation (Arg to Gly) in the critical binding region of the human EC-SOD gene (SOD3) and its association with dramatic increased serum levels. *Hum. Mol. Genet*. 3:2251–2254.

Fong KY, Howe HS, Tin SK, Boey ML, Feng PH (1996). Polymorphism of the regulatory region of tumour necrosis factor alpha gene in patients with systemic lupus erythematosus. *Ann Acad Med* 25: 90–93.

Francke, U.; Taggart, R. T (1979). Assignment of the gene for cytoplasmic superoxide dismutase (Sod-1) to a region of chromosome 16 and of Hprt to a region of the X chromosome in the mouse. *Proc. Natl. Acad. Sci. USA* 76:5230–5233.

Franczuk, A., Szepietowski, J. C. and Noworolska, A (2004).Serum concentrations of interleukin-2 soluble receptor (IL-2 sR) in patients with vitiligo: relationship with type and extent of the disease. *Acta Dermatovenereol. Croat*. 12 :71–76.

Frank, L.; Groseclose, E. E (1984). Preparation for birth into an O2-rich environment: the antioxidant enzymes in the developing rabbit lung. *Pediatr. Res*. 18:240–244.

Frank, S.; Kampfer, H.; Podda, M.; Kaufmann, R.; Pfeilschifter, J (2000). Identification of copper/zinc superoxide dismutase as a nitric oxide-regulated gene in human (HaCaT) keratinocytes: implications for keratinocyte proliferation. *Biochem. J.* 346:719–728.

Fridovich, I (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem*. 64, 97-112.

Fukai, T.; Siegfried, M. R.; Ushio-Fukai, M.; Cheng, Y.; Kojda, G.; Harrison, D. G (2000). Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J. Clin. Invest.* 105:1631–1639.

Fukai, T.; Siegfried, M. R.; Ushio-Fukai, M.; Griendling, K. K.; Harrison, D. G (1999).Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension. *Circ. Res.* 85:23–28.

Gauthier, Y (1996). The importance of Koebner's phenomenon in the induction of vitiligo lesions. *Eur. J. Dermatol.* 5:704–708.

Gauthier, Y., and Surleve-Bazeille, J.E (1992). Autologous grafting with noncultured melanocytes: a simplified method for treatment of depigmented lesions. *J Am Acad Dermatol*. 26, 191-194.

Gavalas NG, Akhtar S, Gawkrodger DJ, Watsona PF, Weetmana AP, Kemp E H (2006). Analysis of allelic variants in the catalase gene in patients with the skin depigmenting disorder vitiligo. *Biochem Biophys Res Commun* 345:1586–91.

Gibbons, N. C., Wood, J. M., Rokos, H. and Schallreuter,K. U (2006). Computer simulation of native epidermal structures in the presence and absence of hydrogen peroxide (H_2O_2): potential and pitfalls. *J. Invest. Dermatol*. 126:2576–2582.

Gilhar A, Paus R, Kalish RS (2007). Lymphocytes, neuropeptides, and genes involved in alopecia areata. *J Clin Invest*. 117(8):2019-27.

Gilhar A, Zelickson B, Ulman Y, Etzioni A (1995). In vivo destruction of melanocytes by the IgG fraction of serum from patients with vitiligo. *J Invest Dermatol*. 105(5):683-6.

Gilhar, A., Paus, R., Kalish, R. S (2007). Lymphocytes, neuropeptides, and genes involved in alopecia areata. *J. Clin. Invest*. 117:2019–2027.

Glassman, S. J (2011) .Vitiligo, reactive oxygen species and T-cells. *Clinical Science* 120: 99–120.

Goldstein, D.B, Chikhi, L (2002). Human migrations and population structure: what we know and why it matters. *Annu Rev Genomics Hum Genet*. 3:129-152.

Gorman, J. D., Sack, K. E., Davis, J. C (2002). Treatment of ankylosing spondylitis by inhibition of tumor necrosis factor-alpha. *New Eng. J. Med*. 346: 1349-1356.

Grabbe, J., Welker, P., Dippel, E., Czarnetzki, B.M (1994). Stem cell factor, a novel cutaneous growth factor for mast cells and melanocytes. *Arch Dermatol Res*. 287:78-84.

Graham, A., Westerhof, W., Thody, A. J (1999). The expression of alpha-MSH by melanocytes is reduced in vitiligo. *Ann. N. Y. Acad. Sci.* 885 :470–473.

Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LP, et al.(1984). Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature.* 312(5996):721-4.

Gray, I.C., Campbell. D.A., Spurr, N.K (2000). Single nucleotide polymorphisms as tools in human genetics. *Hum Mol Genet.* 9 :2403-2408.

Grimes, P. E., Morris, R., Avaniss-Aghajani, E., Soriano, T., Meraz, M.,Metzger, A (2004). Topical tacrolimus therapy for vitiligo: therapeutic responses and skin messenger RNA expression of proinflammatory cytokines. *J. Am. Acad. Dermatol*. 51:52–61.

Grimes, P.E (2005). New insights and new therapies in vitiligo. *JAMA.* 293, 730-735.

Grisham, M.B (1992). Reactive Metabolites of Oxygen and Nitrogen in Biology and Medicine. *R.G. Landes Company, Austin*, pp: 104, ISBN: 187970224X.

Groner, Y.; Elroy-Stein, O.; Avraham, K. B.; Schickler, M.; Knobler, H.; Minc-Golomb, D.; Gross, A., Tapia, F.J., Mosca, W., Perez, R.M., Briceno, L., Henriquez, J.J., Convit, J (1987). Mononuclear cell subpopulations and infiltrating

lymphocytes in erythema dyschromicum and vitiligo. *Histol Histopathol.* 2 :277-283.

Guan CP, Zhou MN, Xu AE , Kang KF, Liu JF , Wei1 XD, Li YW, Zhao DK, Hong W S (2008). The susceptibility to vitiligo is associated with NF-E2-related factor2 (Nrf2) gene polymorphisms: a study on Chinese Han population.*Experimental Dermatol* 17 (12): 1059–1062.

Guan CP, Zhou MN, Xu AE, Kang KF, Liu JF, Wei XD, Li YW, Zhao DK, Hong WS (2008).The susceptibility to vitiligo is associated with NF-E2-related factor2 (Nrf2) gene polymorphisms: a study on Chinese Han population. *Exp Dermatol* 17:1059–62.

Guo, Y., Costa, R., Ramsey, H., Starnes, T., Vance, G., Robertson, K., Kelley, M., Reinbold, R., Scholer, H., and Hromas, R (2002). The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proc Natl Acad Sci U S A.* 99 :3663-3667.

Gurney, M. E.; Pu, H.; Chiu, A. Y.; Canto, M. C. D.; Polchow, C. Y.; Alexander, D. D.; Caliendo, J.; Hentati, A.; Kwon, Y. W.; Deng, H.-X.; Chen, W.; Zhai, P.; Sufit, R. L.; Siddique, T (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264:1772–1775.

Habig, W.H., Pabst, M.J., and Jakoby, W.B (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem.* 249 :7130-7139.

Hachiya, A., Kobayashi, A., Ohuchi, A., Takema, Y. and Imokawa, G (2001).The paracrine role of stem cell factor/c-kit signaling in the activation of human melanocytes in ultraviolet B-induced pigmentation. *J. Invest. Dermatol*. 116 :578–586.

Haiyan X., Uysal, K T., Becherer, JD., Arner P., Hotamisligil, G S (2002). Altered Tumor Necrosis Factor-α (TNF-α) Processing in Adipocytes and Increased Expression of Transmembrane TNF-α in Obesity. *Diabetes* 51: 1876-1883.

Halder, R.M., Walters, C.S., Johnson, B.A., Chakrabarty, S.G.,Kenney, J.A (1986). Aberrations in T lymphocytes and natural killer cells in vitiligo: a flow cytometric study. *J Am Acad Dermatol.* 14 :733-737.

Halliwell, B. and Gutteridge, J. M (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219 :1–14.

Halliwell, B., Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr*. 57: 715-725.

Handa, S., Kaur, I. (1999). Vitiligo: clinical findings in 1436 patients. *J Dermatol*. 10: 653-657.

Hann, S.K., Nordlund, J.J (2000). Clinical features of generalized vitiligo. In: Hann, S.K., and Nordlund, J.J. Eds. Vitiligo a monograph on the basic and clinical science. Blackwell *Science*. 35-48.

Hariharan, V., Klarquist, J., Reust, M. J., Koshoffer, A., McKee, M. D., Boissy, R. E. and Le Poole, I. C (2010). Monobenzyl ether of hydroquinone and 4-tertiary butyl phenol activate markedly different physiological responses in melanocytes: relevance to skin depigmentation. *J. Invest. Dermatol.* 130 :211–220.

Harman, D. (1993). Free radical involvement in aging. Pathophysiology and therapeutic implications. *Drugs Aging* 3: 60-80.

Harning, R., Cui, J. and Bystryn, J.C (1991). Relation between the incidence and level of pigment cell antibodies and disease activity in vitiligo. *J. Invest. Dermatol*. 97:1078–1080.

Harris, C. A.; Derbin, K. S.; Hunte-McDonough, B.; Krauss, M. R.; Chen, K. T.; Smith, D. M.; Epstein, L. B (1991). Manganese superoxide dismutase is induced by IFN-gamma in multiple cell types. Synergistic induction by IFN-gamma and tumor necrosis factor or IL-1. *J. Immunol*. 147:149–154.

Hass, M. A., Massaro, D (1987). Developmental regulation of rat lung Cu, Zn-superoxide dismutase. *Biochem. J.* 246:697–703.

Hass, M. A.; Massaro, D (1988). Regulation of the synthesis of superoxide dismutases in rat lungs during oxidant and hyperthermic stresses. *J. Biol. Chem*. 263:776–781.

Hasse, S., Gibbons, N. C., Rokos, H., Marles, L. K. and Schallreuter, K. U (2004). Perturbed 6-tetrahydrobiopterin recycling via decreased dihydropteridine reductase in vitiligo: more evidence for H_2O_2 stress. *J. Invest. Dermatol*. 122:307–313.

Hayashibe, H.; Asayama, K; Dobashi, K.; Kato, K (1990). Prenatal development of antioxidant enzymes in rat lung, kidney, and heart: marked increase in immunoreactive superoxide dismutases, glutathione peroxidase, and catalase in the kidney. *Pediatr. Res*. 27:472–475.

Hazneci, E., Karabulut, A. B., O¨ ztu¨ rk, C., Batcioglu, K., Dogan, G., Karaca, S. and Esrefoglu, M. (2005). A comparative study of superoxide dismutase, catalase, and glutathione peroxidase activities and nitrate levels in vitiligo patients. *Int. J. Dermatol*. 44 :636–640.

Hearing, V.J (2000). The melanosome: the perfect model for cellular responses to the environment. *Pigment Cell Res.* 8:23-34.

Hearing, V.J. (1999). The regulation of melanin production. In: Nordlund, J.J., Boissy, R.E., and Hearing, V.J. Eds. The Pigmentary system: Physiology and pathophysiology. *Oxford University Press, New York*. 423-428.

Hedstrand, H., Ekwall, O., Olsson, M.J., Landgren, E., Kemp, E.H., Weetman, A.P., and Rorsman, F (2001). The transcription factors SOX9 and SOX10 are melanocyte autoantigens related to vitiligo in autoimmune polyendocrine syndrome type 1. *J Biol Chem*. 276 :35390-35395.

Hendrickson, D. J.; Fisher, J. H.; Jones, C.; Ho, Y.-S.(1990). Regional localization of human extracellular superoxide dismutase gene to 4pter-q21. *Genomics* 8:736–738.

Herrmann, S.-M., Ricard, S., Nicaud, V., Mallet, C., Arveiler, D., Evans, A., Ruidavets, J.-B., Luc, G., Bara, L., Parra, H.-J., Poirier, O., Cambien, F (1998). Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. *Europ. J. Clin. Invest.* 28: 59-66.

Hill, R.J., Zozulya, S., Lu, Y.L., Ward. K., Gishizky, M. and Jallal, B (2002). The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T cell activation. *Exp Hematol*. 30: 237-244.

Hiroi, S.; Harada, H.; Nishi, H.; Satoh, M.; Nagai, R.; Kimura, A (1999). Polymorphisms in the SOD2 and HLA-DRB1 genes are ssociated with nonfamilial idiopathic dilated cardiomyopathy in Japanese. *Biochem. Biophys. Res. Commun*. 261:332–339.

Hjalmarsson, K.; Marklund, S. L.; Engstrom, A.; Edlund, T (1987). Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 84:6340–6344.

Ho, Y. S.; Gargano, M.; Cao, J.; Bronson, R. T.; Heimler, I.; Hutz, R. J (1998). Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *J. Biol. Chem*. 273:7765–7769.

Ho, Y.-S.; Howard, A. J.; Crapo, J. D (1991).Molecular structure of a functional gene for manganese-containing superoxide dismutase. *Am. J. Respir. Cell Mol. Biol*. 4:278–286.

Hoashi, T., Watabe, H., Muller, J., Yamaguchi, Y., Vieira, W.D., and Hearing, V.J (2005). MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. *J Biol Chem.* 280: 14006-14016.

Honda, Y., Okubo, Y. and Koga, M (1997). Relationship between levels of soluble interleukin-2 receptors and the types of activity of vitiligo. *J Dermatol*. 24:561-563.

Horn, T.D., Zahurak, M.K and Atkins, D (1997). Lichen Planus like histopathologic characteristics in the cutaneous graft vs host reaction independent of time course after allogenic bone marrow transplantation. *Arch Dermatol*. 133:961-965.

Howitz, J., Brodthagen, H., Schwarts, M., and Thomsen, K (1977). Prevalence of vitiligo. Epidemiological survey the Isle of Bornholm, Denmark. *Arch Dermatol*. 113: 47-52.

Hristakieva E, Lazarova R, Lazarov N, Stanimirović, A.; Shani, J (2000). Markers for vitiligo related neuropeptides in human skin nerve fibers. *Acta Med Croatica*. 54:53–7.

Hsu, J.-L.; Visner, G. A.; Burr, I. A.; Nick, H. S (1992).Rat copper/zinc superoxide dismutase gene: isolation, characterization, and species comparison. *Biochem. Biophys. Res. Commun*. 186:936– 943.

Hu K, Yang P, Jiang Z et al.(2010) STAT4 polymorphism in a Chinese Han population with Vogt-Koyanagi-Harada syndrome and Behc¸et's disease. *Hum Immunol* 71:723–6.

Huang, C.L., Nordlund, J.J., and Boissy, R (2002). Vitiligo: a manifestation of apoptosis? *Am J Clin Dermatol*. 3 :301-308.

Huang, Y.; He, T.; Domann, F. E.(1999). Decreased expression of manganese superoxide dismutase in transformed cells is associated with increased cytosine methylation of the SOD2 gene. *DNA Cell Biol*. 18:643–652.

Huang, Y.; Peng, J.; Oberley, L. W.; Domann, F. E (1997).Transcriptional inhibition of manganese superoxide dismutase (SOD2) gene expression by DNA methylation of the 5 CpG island. *Free Radic. Biol. Med*. 23:314–320.

Hutyrova´ B, Pantelidis P, Dra´bek J et al. (2002). Interleukin-1 gene cluster polymorphisms in sarcoidosis and idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med.* 165:148–51.

Ichimiya, M (1999). Immunohistochemical study of ACTH and alpha-MSH in vitiligo patients successfully treated with a sex steroid—thyroid hormone mixture. *J Dermatol*. 26:502-506.

Imlay, J. A. and Linn, S (1988) DNA damage and oxygen radical toxicity. *Science* 240 :1302–1309.

Imokawa, G., Yada, Y. and Kimura, M (1996). Signaling mechanisms of endothelin-induced mitogenesis and melanogenesis in human melanocytes. *Biochem. J.* 314 : 305–312.

Inoue, N.; Ramasamy, S.; Fukai, T.; Nerem, R. M.; Harrison, D. G (1996). Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *Circ. Res.* 79:32–37.

Isoherranen, K.; Peltola, V.; Laurikainen, L.; Punnonen, J.; aihia, J.; Ahotupa, M.; Punnonen, K (1997). Regulation of copper/zinc and manganese superoxide dismutase by UVB irradiation, oxidativestress and cytokines. *J. Photochem. Photobiol.* 40:288–293.

Ivanova, K., Block, I., Das, P. and Gerzer, R (2006). Role of cyclic GMP signaling in the melanocyte response to hypergravity. *Signal Transduct*. 6 :406–413.

Ivanova, K., van den Wijngaard, R., Gerzer, R., Lamers, W.H., and Das, P.K (2005). Non-lesional vitiliginous melanocytes are not characterized by an increased proneness to nitric oxide-induced apoptosis. *Exp Dermatol*. 14 : 445-53.

Iyengar, B (1989). Modulation of melanocytic activity by acetylcholine. *Acta Anat (Basel)* 2: 139-141.

Jackson, R. M.; Parish, G.; Ho, Y. S (1996).Effects of hypoxia on expression of superoxide dismutases in cultured ATII cells and lung fibroblasts. *Am. J. Physiol.* 271: L955–962.

Jain, D., Misra, R., Kumar, A. and Jaiswal, G (2008). Levels of malondialdehyde and antioxidants in the blood of patients with vitiligo of age group 11–20 years. *Indian J. Physiol. Pharmacol.* 52 :297–301.

Jalel,A., Yassine, M., Hamdaoui M. H (2009). Oxidative stress in experimental vitiligo C57BL/6 mice. *Indian J Dermatol* 54(3): 221–224.

Jandus, C., Bioley, G., Rivals, J. P., Dudler, J., Speiser, D. and Romero, P (2008). Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondyloarthritides. *Arthritis Rheum*. 58 : 2307–2317.

Janeway, c., Travers, P., Walport, M., Capra, J (1999). Immunobiology : The Immune System in Health and Disease. *New York, N.Y: Garland Publishers.*

Jeong KH, Shin MK, Uhm YK (2010) Association of TXNDC5 gene polymorphisms and susceptibility to nonsegmental vitiligo in the Korean population. *Br J Dermatol* 162:759–64.

Jeong TJ, Shin MK, Uhm YK et al. (2010) Association of UVRAG polymorphisms with susceptibility to non-segmental vitiligo in a Korean sample. *Exp Dermatol* 19: 323–5.

Jezek, P and Hlavata, L (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues and organism. *Int. J. Biochem. Cell Biol*. 37: 2478–2503.

Jimbow, K., Chen, H., Park, J. -S. and Thomas, P. D (2001). Increased sensitivity of melanocytes to oxidative stress and abnormal expression of tyrosinase-related protein in vitiligo. *Br. J. Dermatol.* 144 : 55–65.

Jimbow, K., Obata, H., Pathak, M. A. and Fitzpatrick, T.B (1974) Mechanism of depigmentation by hydroquinone. *J. Invest. Dermatol*. 62 :436–449.

Jimbow, K., Quevedo, W.C., Fitzpatrick, T.B., and Szabo, G (1999). Dermatology in general medicine, 4th edition. (New York. McGraw Hill Inc) 261.

Jimenez-Cervantes, C., Martinez-Esparza, M., Perez, C., Daum, N., Solano, F. and Garcia-Borron, J. C (2001). Inhibition of melanogenesis in response to oxidative stress: transient downregulation of melanocyte differentiation markers and possible involvement of microphthalmia transcription factor. *J. Cell Sci*. 114 : 2335–2344.

Jin Y, Birlea SA, Fain PR (2010). Common variants in FOXP1 are associated with generalized vitiligo. *Nat Genet.* 42:576–8.

Jin Y, Birlea SA, Fain PR, Gowan K, Riccardi SL, Holland PJ, Mailloux CM, Sufit AJ, Hutton SM, Amadi-Myers A, Bennett DC, Wallace MR, McCormack WT, Kemp EH, Gawkrodger DJ, Weetman AP, Picardo M, Leone G, Taieb A, Jouary T, Ezzedine K, van GN, Lambert J, Overbeck A, Spritz RA (2010). Variant of

TYR and autoimmunity susceptibility loci in generalized vitiligo. *N Engl J Med* 362:1686-1697.

Jin Y, Ferrara T, Gowan K, Holcomb C, Rastrou M, Erlich HA, Fain PR, Spritz RA (2012). Next-Generation DNA Re-Sequencing Identifies Common Variants of TYR and HLA-A that Modulate the Risk of Generalized Vitiligo via Antigen Presentation. *J Invest Dermatol* in press.

Jin, S.Y., Park, H.H., Li, G.Z., Lee, H.J., Hong, M.S and Hong, S.J (2004) Association of angiotensin converting enzyme gene I/D polymorphism of vitiligo in Korean population. *Pigment Cell Res*. 17: 84-86.

Jin, S.Y., Park, H.H., Li, G.Z., Lee, H.J., Hong, M.S., Park, H.J., and Lee, M.H. (2004). Association of estrogen receptor 1 intron 1 C/T polymorphism in Korean vitiligo patients. *J Dermatol Sci.* 35 :181-186.

Jin, Y., Birlea, S. A., Fain, P. R., Gowan, K., Riccardi, S. L., Holland, P. J., Mailloux, B. S., Sufit, A. J. D., Hutton, S. M., Amadi-Myers, A (2010). Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *N. Eng. J. Med.* 362:1686–1697.

Jin, Y., Riccardi, S. L., Gowan, K., Fain, P. R. and Spritz, R. A (2009). Fine-mapping of vitiligo susceptibility loci on chromosomes 7 and 9 and interactions with NLRP1 (NALP1). *J. Invest. Dermatol.* 130 :774–783.

Johnson, F., and Giulivi, C (2005). Superoxide dismutases and their impact upon human health. *Mol Aspects Med*. 26 :340-52.

Jones, E., Stuart, D., Walker, N (1989). Structure of Tumour Necrosis Factor. *Nature* 338 : 225-228.

Jones, N. C.; Rigby, P. W. J.; Ziff, E. B (1988). Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev* 2:267–281.

Jones, P. L.; Kucera, G.; Gordon, H.; Boss, J. M (1995). Cloning and characterization of the murine manganous superoxide dismutase- encoding gene. *Gene* 153:155–161.

Jones, P. L.; Ping, D.; Boss, J. M (1997). Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol. Cell. Biol.* 17:6970–6981.

Jongeneel, C. V., Briant, L., Udalova, I. A., Sevin, A., Nedospasov, S. A., Cambon-Thomsen, A (1991). Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes. *Proc. Nat. Acad. Sci*. 88: 9717-9721.

Jonsson, H., and Peng, S.L (2005). Forkhead transcription factors in immunology. *Cell Mol Life Sci.* 62 :397-409.

Kagan, B., Baldwin, R., Munoz, D., Wisnieski, J (1992). Formation of Ion-Permeable Channels by Tumor Necrosis Factor-Alpha. *Science.* 255: 1427-1430.

Kahn, A.M., and Cohen, M.J (1998). Repigmentation in vitiligo patients. Melanocyte transfer via ultra-thin grafts. *Dermatol Surg*. 24:365-367.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., Karin, M (2005).

Reactive oxygen species promote TNF-alpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120: 649-661.

Kanwar AJ, Dogra S (2005).Narrow-band UVB for the treatment of generalized vitiligo in children. Clinical dermatology. *Clin Exp Dermatol* 30:332–6.

Kao, C.H., and Yu, H.S (1992). Comparison of the effect of 8-methoxypsoralen (8-MOP) plus UVA (PUVA) on human melanocytes in vitiligo vulgaris and in vitro. *J Invest Dermatol* 98: 734-740.

Karayiorgou, M., Altemus, M., Galke, B.L., Goldman, D., Murphy, D.L., Ott, J. and Gogos, J.A (1997). Genotype determining low catechol-O-methyltransferase activity as a risk factor for obsessive-compulsive disorder. *Proc Natl Acad Sci U S A* 94 :4572-4575.

Kareemullah L, Taneja V, Begum S, Sarma PK, Baig HA (1977). Association of ABO blood groups and vitiligo. *J Med Genet* 14:211-213.

Kemp, E.H., Ajjan, R.A., Waterman, E.A., Gawkrodger, D.J., Cork, M.J., and Watson, P.F. (1999). Analysis of a microsatellite polymorphism of the cytotoxic T-lymphocyte antigen-4 gene in patients with vitiligo. *Br J Dermatol*. 140:73-78.

Khan, R., Satyam, A., Gupta, S., Sharma, V. K. and Sharma, A (2009). Circulatory levels of antioxidants and lipid peroxidation in Indian patients with generalized and localized vitiligo. *Arch. Dermatol. Res*. 301:731–737.

Kim HJ, Choi CP, Uhm YK (2007).The association between endothelin-1gene polymorphisms and susceptibility to vitiligo in a Korean population. *Exp Dermatol* 16:561–6.

Kim HJ, Uhm YK, Yun JY (2010). Association between polymorphisms of discoidin domain receptor tyrosine kinase 1 (DDR1) and non-segmentalvitiligo in the Korean population. *Eur J Dermatol* 20:231–2.

Kim, H. P.; Roe, J. H.; Chock, P. B.; Yim, M. B (1999). Transcriptional activation of the human manganese superoxide dismutase gene mediated by tetradecanoylphorbol acetate. *J. Biol. Chem.* 274: 37455–37460.

Kim, H. T.; Kim, Y. H.; Nam, J. W.; Lee, H. J.; Rho, H. M.;Jung, G (1994). Study of 5'-flanking region of human Cu/Zn superoxide dismutase. *Biochem. Biophys. Res. Commun* 201:1526–1533.

Kim, Y. H.; Park, K. H.; Rho, H. M (1996) Transcriptional activation of the Cu,Zn-superoxide dismutase gene through the AP2 site by ginsenoside Rb2 extracted from a medicinal plant, Panax ginseng. *J. Biol. Chem*. 271:24539–24543.

Kim, Y. H.; Yoo, H. Y.; Chang, M. S.; Jung, G.; Rho, H . M (1997). C/EBP alpha is a major activator for the transcription of rat Cu/Zn superoxide dismutase gene in liver cell. *FEBS Lett.* 401:267–270.

Kim, Y. H.; Yoo, H. Y.; Jung, G.; Kim, J. Y.; Rho, H. M (1993). Isolation and analysis of the rat genomic sequence encoding Cu/Zn superoxide dismutase. *Gene* 133:267–271.

Kitamura, R., Tsukamoto, K., Harada, K., Shimizu, A., Shimada, S., Kobayashi, T. and Imokawa, G (2004). Mechanisms underlying the dysfunction of melanocytes in vitiligo epidermis: role of SCF/KIT protein interactions and the downstream effector, MITF-M. *J. Pathol*. 202:463–475.

Klatt, E (1994). Inflammation. Chronic Inflammation. Version 4.0 (1994-1999, 2000).

Klaunig, J.E., and Kamendulis, L.M (2004). The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol*. 44:239-267.

Knirsch, L.; Clerch, L. B (2000). A region in the 3' UTR of MnSOD RNA enhances translation of a heterologous RNA. *Biochem. Biophys. Res. Commun*. 272:164–168.

Knirsch, L.; Clerch, L. B (2001). Tyrosine phosphorylation regulates manganese superoxide dismutase (MnSOD) RNA-binding protein activity and MnSOD protein expression. *Biochemistry* 40: 7890–7895.

Kobayashi, N., Nakagawa, A., Muramatsu, T., Yamashina, Y., Shirai, T., Hashimoto, M. W., Ishigaki, Y., Ohnishi, T. and Mori, T (1998). Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. *J. Invest. Dermatol*. 110:806–810.

Koca, R., Armutcu, F., Altinyazar, H. C. and Gurel, A (2004). Oxidant-antioxidant enzymes and lipid peroxidation in generalized vitiligo. *Clin. Exp. Dermatol.*29:406–409.

Kollias, N., Sayre, R. M., Zeise, L. and Chedekel, M. R (1991). Photoprotection by melanin. *J. Photochem. Photobiol. B*. 9:135–160.

Koranne, R.V., and Sachdeva, K.G (1988). Vitiligo. *Int J Dermatol.* 27:676-81.

Koranne, R.V., Sehgal, V.N., and Sachdeva, K.G (1986). Clinical profile of vitiligo in North India. *Ind J Deramtol Venereol Leprol* 52 :81-82.

Kos, R., Reedy, M.V., Johnson, R.L., Erickson, C.A (2001). The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. *Development.* 128:1467-79.

Koster, W., Wiskemann, A.Z (1990). Phototherapy with UV B in vitiligo. *Hautkr*.65:1022-1024.

Kovacs, S.O. (1998). Vitiligo. *J Am Acad Dermatol*. 38: 647-66.

Kristiansen OP, Karlsen AE, Larsen ZM (2004). Identification of a type 1diabetes-associated CD4 promoter haplotype with high constitutiveactivity. *Scand J Immunol* 59:582–91.

Kroll, J. (2005). Chaperones and longevity. *Biogerontology* 6, 357-361.

Kühtreiber, W. M., Hayashi, T., Dale, E. A., Faustman D L (2003). Central role of defective apoptosis in autoimmunity. *J Mol Endocrinol* 31, 373–399.

Kumagai, N., and Uchikoshi, T (1997). Treatment of extensive hypomelanosis with autologous cultured epithelium. *Ann Plast Surg*. 39:68-73.

Kuo, S.; Chesrown, S. E.; Mellott, J. K.; Rogers, R. J.; Hsu, J. L.;Nick, H. S (1999). In vivo architecture of the manganese superoxide dismutase promoter. *J. Biol. Chem* 274:3345–3354.

Ladner, M.B., Bottini. N., Valdes, A.M., and Noble, J.A (2005). Association of the single nucleotide polymorphism C1858T of the PTPN22 gene with type 1 diabetes. *Hum Immunol*. 66:60-64.

Lan CC, Ko YC, Tu HP (2009). Association study between keratinocyte derived growth factor gene polymorphisms and susceptibility to vitiligo vulgaris in a Taiwanese population: potential involvement of stem cell factor. *Br J Dermatol* 160:1180–7.

Larribere, L., Khaled, M., Tartare-Deckert, S., Busca, R., Luciano, F., Bille, K., and Bertolotto, C (2004). PI3K mediates protection against TRAIL-induced apoptosis in primary human melanocytes. *Cell Death Differ*. 11: 1084-1091.

Laukkanen, M. O.; Aittomaki, S. J.; Hiltunen, T. P.; Yla-Herttuala,S (1995). Genbank accession #Z67878.

Le Poole IC, Sarangarajan R, Zhao Y (2001). ‘‘VIT1’’ a novel gene associated with vitiligo. *Pigment Cell Res* 14:475–84.

Le Poole, C., Van den Wijngaard, R., Smit, N. P. M., Oosting, J.,Westerhof, W. and Pavel, S (1994). Catechol-O-methyl transferase in vitiligo. *Arch. Dermatol. Res*. 286: 81–86.

Le Poole, I. C. and Luiten, R. M. (2008).Autoimmune etiology of generalized vitiligo. *Curr. Dir. Autoimmun.* 10:227–243.

Le Poole, I. C., Mutis, T., van den Wijngaard, R. M., Westerhof, W., Ottenhoff, T., de Vries, R. R. and Das, P. K (1993). A novel, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. *J. Immunol*. 151: 7284–7292.

Le Poole, I.C. and Boissy, R. E. (1997). Vitiligo. *Semin. Cutan. Med. Surg*. 16: 3–14.

Lebovitz, R. M.; Zhang, H.; Vogel, H.; Cartwright, J. Jr. Dionne, L.; Lu, N.; Huang, S.; Matzuk, M. M (1996). Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. USA* 93:9782– 9787.

Leccia, M. T.; Yaar, M.; Allen, N.; Gleason, M.; Gilchrest, B. A (2001).Solar simulated irradiation modulates gene expression and activityof antioxidant enzymes in cultured human dermal fibroblasts.*Exp. Dermatol.* 10:272–279.

Leonardi, C. L., Powers, J. L., Matheson, R. T., Goffe, B. S., Zitnik, R., Wang, A., Gottlieb, A. B (2003). Etanercept as monotherapy in patients with psoriasis. *New Eng. J. Med*. 349: 2014-2022.

Lerner, A.B (1971). On the etiology of vitiligo and grey hair. *Am J Medicine*. 51, 141-147.

Lerner, A.B., Halaban, R., Klaus, S.N., Moellmann, G.E (1987) Transplantation of human melanocytes. *J Invest Dermatol*. 89, 219-224.

Levai, M. (1958). A study of certain contributory factors in the development of vitiligo in South Indian patients. *Arch of Dermatol*. 78, 364-371.

Levanon, D.; Lieman-Hurwitz, J.; Dafni, N.; Wigderson, M.; Sherman, L.; Bernstein, Y.; Laver-Rudich, Z.; Danciger, E.; Stein, O.; Groner, Y (1985). Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the Cu/Zn superoxide dismutase. *EMBO J*. 4:77–84.

Levin, E. D.; Brady, T. C.; Hochrein, E. C.; Oury, T. D.; Jonsson, L. M.; Marklund, S. L.; Crapo, J. D (1998). Molecular manipulations of extracellular superoxide dismutase: functional importance for learning. *Behav. Genet*. 28:381–390.

Li K, Li C, Gao L (2009). A functional single-nucleotide polymorphism in the catechol-O-methyltransferase gene alter vitiligo risk in a Chinese population. *Arch Dermatol Res* 301:681–7.

Li M, Gao Y, Li C (2009). Association of COX2 functional polymorphisms and the risk of vitiligo in Chinese populations. *J Dermatol Sci* 53: 176–81.

Li M, Sun D, Li C (2008). Functional polymorphisms of the FAS gene associated with risk of vitiligo in Chinese populations: a case-control analysis. *J Invest Dermatol* 128:2820–4.

Liang Y, Yang S, Zhou Y (2007). Evidence for two susceptibility loci on chromasomes 22q12 and 6p21-p22 in chinese generalized vitiligo families. *J Invest Dermatol* 127:2552–7.

Lichten, M.J., and Fox, M.S. (1983). Detection of non-homology-containing heteroduplex molecules. *Nucleic Acids Res.* 11, 3959-3971.

Linn, S (1998). DNA damage by iron and hydrogen peroxide in vitro and in vivo. *Drug Metab. Rev*. 30, 313–326.

Linthorst Homan, M. W., Spuls, P. I., de Korte, J., Bos, J. D., Sprangers, M. A. and van der Veen, M. A (2009). The burden of vitiligo: patient characteristics associated with quality of life. *J. Am. Acad. Dermatol*. 61, 411–420.

Liou HC, Boothby MR, Finn PW, Davidon R, Nabavi N, Zeleznik-Le NJ, Ting JP, Glimcher LH (1990). A new member of the leucine zipper class of proteins that binds to the HLA DR alpha promoter. *Science* 247 (4950): 1581–4.

Liu JB, Li M, Chen H (2007). Association of vitiligo with HLA-A2: a meta-analysis. *J Eur Acad Dermatol Venereol* 21:205–13.

Liu L, Li C, Gao J (2009). Genetic polymorphisms of glutathione S-transferase and risk of vitiligo in the Chinese population. *J InvestDermatol* 12:2646–52.

Liu PY, Bondesson L, Lontz W (1996). The occurrence of cutaneous nerve endings and neuropeptides in vitiligo vulgaris: a casecontrol study. *Arch Dermatol Res*; 288:670–5.

Liu, L., Li, C., Gao, J., Li, K., Gao, L. and Gao, T. (2009). Genetic polymorphisms of glutathione S-transferase and risk of vitiligo in the Chinese population. *J. Invest. Dermatol*. 129, 2646–2652.

Lloyd , D. R., Phillips D. H., Carmichael P L (1997). Generation of Putative Intrastrand Cross-Links and Strand Breaks in DNA by Transition Metal Ion-Mediated Oxygen Radical Attack. *Chem. Res. Toxicol* 10:393–400.

Loenders, B.; Mechelen, E. V.; Nicolai, S.; Buyssens, N.; Osselaer, N. V.; Jorens, P. G.; Willems, J.; Herman, A. G.; Slegers, H (1998). Localization of extracellular superoxide dismutase in rat lung: neutrophils and macrophages as carries of the enzyme. *Free Radic. Biol. Med.* 24:1097–1106.

Louis E, Peeters M, Franchimont D (2000). Tumour necrosis factor (TNF) gene polymorphism in Crohn's disease (CD): influence on disease behaviour? *Clin Exp Immunol* 119: 64–68.

Luftl, M., Rocken, M., Plewig, G., and Degitz, K. (1998). PUVA inhibits DNA replication, but not gene transcription at nonlethal dosages. *J Invest Dermatol*. 111, 399-405.

Maehara, K.; Oh-Hashi, K.; Isobe, K. I (2001). Early growth-responsive-1-dependent manganese superoxide dismutase gene transcription mediated by platelet-derived growth factor. *FASEB J.* 15:2025–2026.

Mahmoud, F., Abul, H., al-Saleh, Q, Haines, D., Burleson, J., and Morgan, G (1998). Peripheral T-cell activation in non-segmental vitiligo. *J Dermatol*. 25, 637-640.

Mahmoud, F., Abul, H., Haines, D., Al Saleh, C., Khajeji, M. and Whaley, K. (2002). Decreased total numbers of peripheral blood lymphocytes with elevated

percentages of CD4þCD45ROþ and CD4þCD25þ of T-helper cells in non-segmental vitiligo. *J Dermatol*. 29, 68-73.

Majumder, P.P (2001). Genetics and prevalence of vitiligo vulgaris in Vitiligo a monograph on the basic and clinical science. *(Blackwell Science)* 18.

Malakar, S., and Dhar, S. (1999). Treatment of stable and recalcitrant vitiligo by autologous miniature punch grafting: a prospective study of 1,000 patients. *Dermatology*. 198, 133-139.

Mandry, R.C., Ortiz, L.J., Lugo-Somolinos, A and Sanchez, J.L (1996). Organ-specific autoantibodies in vitiligo patients and their relatives. *Int J Dermatol*. 35, 18-21.

Manini, P., Napolitano, A.,Westerhof,W., Riley, P. A. and d'Ischia, M. (2009). A reactive ortho-quinone generated by tyrosinase-catalyzed oxidation of the skin depigmenting agent monobenzone: self-coupling and thiol-conjugation reactions and possible implications for melanocyte toxicity. *Chem. Res. Toxicol*. 22, 1398–1405.

Manolache L, Benea V (2007). Stress in patients with alopecia areata and vitiligo. *J Eur Acad Dermatol Venereol* ;21:921–8.

Marklund, S. L (1990) Expression of extracellular superoxide dismutase by human cell lines. *Biochem. J.* 266:213–219.

Marklund, S. L (1992) Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. *J. Biol. Chem*. 267:6696–6701.

Marklund, S. L.; Nilsson, P.; Israelsson, K.; Schampi, I.; Peltonen, M.; Asplund, K (1997). Two variants of extracellular-superoxide dismutase: relationship to cardiovascular risk factors in an unselected middle-aged population. *J. Intern. Med*. 242:5–14.

Marklund, S.L (1984). Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem J.* 222, 649-655.

Martínez-Esparza, M., Jiménez-Cervantes, C., Beermann, F., Aparicio, P., Lozano, J. A. and García-Borrón J. C (1997). Transforming growth factor-β1 inhibits basal melanogenesis in B16/F10 mouse melanoma cells by increasing the rate of degradation of tyrosinase and tyrosinase-related protein-1. *J. Biol. Chem*. 272, 3967-3972.

Martínez-Esparza, M., Jiménez-Cervantes, C., Solano, F., Lozano, J. A. and García-Borrón J. C. (1998). Mechanisms of melanogenesis inhibition by tumor necrosis factor-α in B16/F10 mouse melanoma cells. *Eur. J. Biochem*. 255, 139-146.

Marx, J. L. (1987). Oxygen free radicals linked to many diseases. *Science* 235, 529–531.

Mashiah, J and Brenner, S (2007) Possible mechanisms in the induction of vitiligo-like hypopigmentation by topical imiquimod. *Clin. Exp. Dermatol*. 33, 74–76.

Masuda, A.; Longo, D. L.; Kobayashi, Y.; Appela, E.; Oppenheim, J. J.; Matsushima, K (1988) Induction of mitochondrial manganese superoxide dismutase by interleukin 1. *FASEB J.* 2:3087 3091.

Matsumura, Y., and Ananthaswamy, H.N (2004). Toxic effects of ultraviolet radiation on the skin. *Toxicol Appl Pharmacol*. 195, 298-308.

Mayoral, F.A., Gonzalez, C., Shah, N.S., and Arciniegas, C (2003). Repigmentation of vitiligo with pimecrolimus cream: a case report. *Dermatology.* 207, 322-333.

McCord, J.M (1987). Oxygen-derived radicals: a link between reperfusion injury and inflammation. *Fed Proc*. 46, 2402-2406.

McCord, J.M., and Fridovich, I. (1969). Superoxide dismutase: An enzyme function of eythrocuprein (hemocuprin). *J Biol Chem.* 244, 6049-6055.

McCord, JM (1976). Iron and manganese containing superoxide dismutases: structure, distribution, and evolutionary relationships. *Adv Exp Med Biol.* 74, 540-550.

McElroy, M. C.; Postle, A. D.; Kelly, F. J (1992). Catalase, superoxide dismutase and glutathione peroxidase activities of lung and liver during human development. *Biochim. Biophys. Acta* 1117:153– 158.

McGill, G.G., Horstmann M., Widlund, H.R., Du, J., Motyckova, G., and Fisher, D.E. (2002). Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell.* 109, 707-718.

McGuire J (1970). Adrenergic control of melanocytes. *Arch Dermatol* 101:173–80.

McMillan, D.J, Davies, M.R. Good, M.F., and Sriprakash, K.S. (2004). Immune response to superoxide dismutase in group A streptococcal infection. *FEMS Immunol Med Microbiol*. 40, 249-256.

Meager, Anthony (1991). Cytokines. Englewood Cliffs, N.J. : Prentice Hall.

Mehta, N.R., Shah, K.C., Theodore, C., Vyas, V., and Patel, A. (1973). Epidemiological study of vitiligo in Surat area, South Gujarat. *Ind J of Med Res*. 61, 145-154.

Mellouli, F., Ksouri, H., Dhouib, N., Torjmen, L.,Abdelkefi, A., Ladeb, S., Ben Othman, T., Hmida, S., Ben Hassen, A. and B´ejaoui, M (2009) Possible transfer of vitiligo by allogeneic bone marrow transplantation: a case report. *Pediatr. Transplant*. 13, 1058–1061.

Meyrick, B.; Magnuson, M. A (1994) Identification and functional characterization of the bovine manganous superoxide dismutase promoter. *Am. J. Respir. Cell Mol. Biol*. 10:113–121.

Minc, E.; de Coppet, P.; Masson, P.; Thiery, L.; Dutertre, S.; Amor-Gueret, M.; Jaulin, C (1999) The human copper-zinc superoxide dismutase gene (SOD1) proximal promoter is regulated by Sp1, Egr-1, and WT1 via non-canonical binding sites. *J. Biol. Chem*. 274:503–509.

Mitrunen, K.; Sillanpaa, P.; Kataja, V.; Eskelinen, M.; Kosma, V. M.; Benhamou, S.; Uusitupa, M.; Hirvonen, A (2001). Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. *Carcinogenesis* 22:827–829.

Miyamoto, L. and Taylor, J. S. (2000). Chemical leukoderma. In Vitiligo: a Comprehensive Monograph on Basic and Clinical Science (Hann, S.-K. and Nordlund, J. J., eds), pp. 269–280, *Blackwell Science Ltd. Oxford*.

Moellmann, G., Klein-Angerer, S., Scollay, D. A.,Nordlund, J. J. and Lerner, A. B (1982). Extracellular granular material and degeneration of keratinocytes in the normally pigmented epidermis of patients with vitiligo. *J. Invest. Dermatol.* 79, 321–330.

Mol CD, Lim KB, Sridhar V, Zou H, Chien EY, Sang BC *et al* (2003). Structure of a c-kit product complex reveals the basis for kinase transactivation. *J Biol Chem* 278: 31461–31464.

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 Res*.15, 87–92.

Morrone, A., Picardo, M., de Luca, C., Terminali, O., Passi, S. and Ippolito, F (1992) Catecholamines and vitiligo. *Pigment Cell Res*. 5, 65–69.

Mottaz J H ,Zelickson A S(1967). Melanin Transfer: A Possible Phagocytic Process. *J. Invest. Dermatol* 49:605–610.

Mozzanica, N., Villa, M.L., Foppa, S., Vignati, G., Cattaneo, A., Diotti, R. and Finzi, A.F. (1992). Plasma alpha-melanocyte-stimulating hormone, beta-endorphin, met-enkephalin, and natural killer cell activity in vitiligo. *J Am Acad Dermatol*. 1, 693-700.

Mruk, D.; Cheng, C. H.; Cheng, Y. H.; Mo, M. Y.; Grima, J.; Silvestrini, B.; Lee, W. M.; Cheng, C. Y (1998) Rat testicular extracellular superoxide dismutase: its purification, cellular distribution, and regulation. *Biol. Reprod*. 59:298–308.

Mucos. 1998 August 4. Additional Information : Tumor Necrosis Factor. Accessed 2000 February 26.

Mujahid AM, Banu M, Waheed MA, Quadri GS, Habibullah CM (1990). Serum alpha 1-antitrypsin and haptoglobin phenotypes in vitiligo. *Arch Dermatol Res* 282:206-207.

Murray, J., Barbara, J., Dunkley, S., Lopez, A., Van Ostade, X., Condliffe, I., Haslett, C., Chilvers, E (1997). Regulation of Neutrophil Apoptosis by Tumor Necrosis

Factor-Alpha : Requirements for TNF-R55 and TNF-R75 for Induction of Apoptosis In Vitro. *Blood*. 90 (7) : 2772-2783.

Mysore V, Salim T (2009) Cellular grafts in management of leucoderma. *Indian J Dermatol*; 54(2):142–9.

Na, G.Y., Lee, K.H., Kim, M.K., Lee, S.J., Kim, D.W., and Kim, J.C (2003). Polymorphisms in Melanocortin-1 receptor (MCIR) and Agouti Signaling Protein (ASIP) genes in Korean vitiligo patients. *Pigment Cell Res*. *16*, 383-387.

Nadkarni, S., Mauri, C., Ehrenstein, M. R (2007). Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *J. Exp. Med*. 204: 33-39.

Nagai, K., Ichimiya, M., Yokoyama, K., Hamamoto, Y., and Muto, M. (2000). Successful treatment of non-segmental vitiligo: systemic therapy with sex hormone-thyroid powder mixture, *Horm Res*, 54, 316-317.

Nagamine, K., Peterson, P., and Scott, H.S. (1997). Positional cloning of the APECED gene. *Nat Genet*. 17, 393-398.

Namazi, M. R (2007) Neurogenic dysregulation, oxidative stress, autoimmunity, and melanocytorrhagy in vitiligo: can they be interconnected? *Pigment Cell Res*. 20, 360–363.

Namazi, M. R (2008). Proton pump inhibitors may trigger vitiligo by rendering melanocytes prone to apoptosis. *Br. J. Dermatol*. 158, 844–845.

Namazi, M. R (2009) Cytochrome-P450 enzymes and autoimmunity: expansion of the relationship and introduction of free radicals as the link. *J. Autoimmun*. *Dis*. 6, 4.

Namazi, M. R. (2005) Phenytoin as a novel anti-vitiligo weapon. *J. Autoimmun*. *Dis.* 2, 11–13.

Nappi, A. J. and E. Vass (1998). Hydroxyl radical formation via iron-mediated fenton chemistry is inhibited by methylated catechols. *Biochim. Biophys. Acta*. 1425(1):159-167.

Natarajan V T, Singh A, Kumar A A, Sharma P, Kar H K, Marrot L , Meunier J, Natarajan K, Rani R, Gokhale R.S (2010).Transcriptional Upregulation of Nrf2-Dependent Phase II Detoxification Genes in the Involved Epidermis of Vitiligo Vulgaris. *J Invest Dermatol* 130, 2781–2789.

Naughton, G.K., Reggiardo, M.D. and Bystryn, J.C (1986). Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. *J Am Acad Dermatol*. 15, 978-981.

Nedwin GE, Naylor SL, Sakaguchi AY (1985). Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. *Nucleic Acids Res* 13: 6361–6373.

Neuner, P., Charvat, B., Knobler, R., Kirnbauer, R., Schwarz, A., Luger, T.A., and Schwarz, T (1994). Cytokine release by peripheral blood mononuclear cells is affected by 8-methoxypsoralen plus UV-A. *Photochem Photobiol.* 59, 182-188.

Nicolai, S.; Willems, J.; Zwijsen, A.; Van Mechelen, E.; Slegers, H (1996) Cyclic AMP-induced differentiation increases the synthesis of extracellular superoxide dismutase in rat C6 glioma. *Free Radic. Biol. Med*. 21:481–486.

Nilakantan, V., Halligan, N.L., Nguyen, T.K., Hilton, G., Khanna, A.K., and Pieper GM (2005). Post-translational modification of manganese superoxide dismutase in acutely rejecting cardiac transplants: role of inducible nitric oxide synthase. *J Heart Lung Transplant*. 24, 1591-1599.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., Era, T., Sakakura, T., and Nishikawa, S. (1991). In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J*. 10, 2111-2118.

Njoo, M.D., and Westerhof, W (2001). Vitiligo: Pathogenesis and treatment. *Am J Clin Dermatol*. 2, 167-181.

Nordlund JJ, Ortonne JP (1998) The normal colour of human skin. In: JJ Nordlund, RE Boissy, VJ Hearing, JP Ortonne (eds). The Pigmentary System. *Oxford University Press*, pp. 475–487.

Nordlund, J. J, Abdel-Malek, Z (1988). Mechanisms for postinflammatory hyperpigmentation and hypopigmentation. *Prog. Clin. Biol. Res*. 256, 219-236

Nordlund, J.J., and Lerner, A.B. (1982). Vitiligo. Is it important? *Arch Dermatol*. 118, 5-8.

Norris, A., Todd, C., Graham, A., Quinn, A.G., and Thody, A.J (1996). The expression of the c-kit receptor by epidermal melanocytes may be reduced in vitiligo. *Br J Dermatol*. 134, 299-306.

Norris, D.A., Kissinger, R.M, Naughton, G.K., and Bystryn, J.C. (1988). Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocyes in vitro by complement-mediated damage and antibody-dependent cellular cytotoxicity. *J Invest Dermatol*. 90, 783-789.

Nozik-Grayck, E.; Dieterle, C. S.; Piantadosi, C. A.; Enghild,J. J.; Oury, T. D (2000). Secretion of extracellular superoxide dismutase in neonatal lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279:L977–L984.

Ogawa, F., Sander, C. S., Hansel, A., Oehrl, W., Kasperczyk, H., Elsner, P., Shimizu, K., Heinemann, S. H. and Thiele, J. J. (2006). The repair enzyme peptide methionine-S-sulfoxide reductase is expressed in human epidermis and upregulated by UVA radiation. *J. Invest. Dermatol.* 126, 947–949.

Ogg, G.S., Rod Dunbar, P., Romero, P., Chen, J.L., and Cerundolo, V. (1998). High frequency of skin homing melanocyte specific cytotoxic T lymphocytes in autoimmune vitiligo. *J Exp Med.* 188, 1203-1208.

Okada, T., Sakamoto, T., Ishibashi, T., and Inomata, H. (1996). Vitiligo in Vogt-Koyanagi-Harada disease: immunohistological analysis of inflammatory site. *Arch Clin Exp Ophthalmol*. 234, 359-363.

Okamoto, T., Irie, R.F., Fujii, S., Huang, S.K., Nizze, A.J., Morton, D.L., and Hoon, D.S.B (1998). Anti-tyrosinase-related protein-2 immune response in vitiligo and melanoma patients receiving active-specific immunotherapy. *J Invest Dermatol*. 111, 1034-1039.

Olsson, M.J., and Juhlin L (1998). Leucoderma treated by transplantation of a basal cell layer enriched suspension. *Br J Dermatol*. 138, 644-8.

Onay, H., Pehlivan, M., Alper, S., Ozkinay, F. and Pehlivan, S (2007). Might there be a link between mannose-binding lectin and vitiligo. *Eur. J. Dermatol*. 17, 146–148.

Onengut-Gumuscu, S., Ewens, K.G., Spielman, R.S. and Concannon, P (2004). A functional polymorphism (1858C/T) in the PTPN22 gene is linked and associated with type I diabetes in multiplex families. *Genes Immun*. 5, 678-680.

Ongenae, K., Van Geel, N. and Naeyaert, J. M (2003). Evidence for an autoimmune pathogenesis of vitiligo. *Pigment Cell Res*. 16, 90–100.

Orecchia, G., Frattini, P., Cucchi, M.L., and Santagostino, G (1994). Normal range plasma catecholamines in patients with generalized and acrofacial vitiligo: preliminary report. *Dermatol.* 189, 350-353.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A.* 86, 2766-2770.

Orlow, S.J (1995). Melanosomes are specialized members of the lysosomal lineage of organelles. *J Invest Dermatol*. 105, 3-7.

Orozco, G., Sanchez, E. and Gonzalez-Gay, M.A (2005). Association of a functional single-nucleotide polymorphism of PTPN22, encoding lymphoid protein phosphatase, with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Rheum*. 52, 219-224.

Orozco-Topete R, Córdova-López J, Yamamoto-Furusho JK, García-Benitez V, López-Martínez A, Granados J (2005) HLA-DRB1*04 is associated with the genetic susceptibility to develop vitiligo in Mexican patients with autoimmune thyroid disease. *J Am Acad Dermatol*. 52:182–3.

Ortonne, J.P., and Bose, S.K. (1993). Vitiligo: Where do we stand? *Pigment Cell Res*. 8, 61-72.

Oyarbide-Valencia, K., van den Boorn, J. G., Denman, C. J., Li, M., Carlson, J. M., Hernandez, C., Nishimura, M. I., Das, P. K., Luiten, R. M. and Le Poole, I. C (2006) Therapeutic implications of autoimmune vitiligo cells. *Autoimmun Rev.* 5, 486–492.

Ozdemir M, Cetinkale O, Wolf R, Kotogyan A, Mat C, Tuzun B (2002). Comparison of two surgical approaches for treating vitiligo: a preliminary study. *Int J Dermatol* 41(3):135–8.

Pamer, E., and Cresswell, P (1998). Mechanisms of MHC class I-restricted antigen processing. *Ann Rev Immunol*. 16, 323-358.

Papadopoulos B., Legg H (1998). Impact of life events on the onset of vitiligo in adults: preliminary evidence for a psychological dimension in aetiology. *Clinical and Experimental Dermatology 23(6)* : 243–248.

Parboosingh, J. S.; Rouleau, G. A.; Meninger, V.; McKenna- Yasek, D.; Brown, R. H. Jr.; Figlewicz, D. A (1995). Absence of mutations in the Mn superoxide dismutase or catalase genes in familial amyotrophic lateral sclerosis. *Neuromuscul. Disord*. 5:7–10.

Park HH, Ha E, Uhm YK (2006). Association study between catalase gene polymorphisms and the susceptibility to vitiligo in Korean population. *Exp Dermatol* 15:377–80.

Passeron, T., and Ortonne, J.P (2005). Physiopathology and genetics of vitiligo. *J Autoimmun*. 25, 63-68.

Passi, S., Grandinetti, M., Maggio, F., Stancato, A., De Luca, C (1998). Epidermal oxidative stress in vitiligo. *Pigment Cell Res.* 2, 81 - 85.

Pastore, A (1996). 3-D Images of MODULES in Extracellular Proteins. Accessed 2000 February 25.

Pavel, S., Muskiet, F.A.J., De Lay, L., The, T.H., and Van der Slik, W (1983). Identification of three indolic compounds on a pigmented melanoma cell culture supernatant by gas chromatography-mass spectrometry, *J Cancer Res Clin Oncol*. 105, 275-279.

Pehlivan S, Ozkinay F, Alper S (2009). Association between IL4 (-590)ACE (I)/(D) CCR5 (Delta32) CTLA4 (+49) and IL1-RN (VNTR in intron 2)gene polymorphisms and vitiligo. *Eur J Dermatol* 19:126–8.

Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., Kohr, W. J., Aggarwal, B. B., Goeddel, D. V (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 312: 724-729.

Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., Michael, K (1990). A Nonsecretable Cell Surface Mutant of Tumor Necrosis Factor (TNF) Kills by Cell-to Cell Contact. *Cell*. 63 : 251-258.

Perry, A. C. F.; Jones, R.; Hall, L (1993). Isolation and characterization of a rat cDNA clone encoding a secreted superoxide dismutase reveals the epididymis to be a major site of its expression. *Biochem. J.* 293:21–25.

Philips MA, Kingo K, Karelson M (2010). Promoter polymorphism119C/G in MYG1 (C12orf10) gene is related to vitiligo susceptibility and Arg4Gln affects mitochondrial entrance of Myg1. *BMC Med Genet* 11:56.

Picardi A, Pasquini P, Cattaruzza MS (2003) Stressful life events, social support, attachment security and alexithymia in vitiligo. A case-control study. *Psychother Psychosom*; 72:150–8.

Pichler, R., Sfetsos, K., Badics, B., Gutenbrunner, S., Berg, J. and Aubock, J (2009) Lymphocyte imbalance in vitiligo patients indicated by elevated CD4+/CD8+ T-cell ratio. *Wien. Med. Wochenschr*. 159, 337–341.

Pociot, F., Briant, L., Jongeneel, C.V., Molvig, J., Worsaae, H., Abbal, M., Thomsen, M., Nerup, J., and Cambon-Thomsen, A (1993). Association of tumor necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-alpha and TNF-beta by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. *Eur J Immunol*. 23, 224-231.

Quan C, Ren YQ, Xiang LH, Sun LD, Xu AE, Gao XH, Chen HD, Pu XM, Wu RN, Liang CZ, Li JB, Gao TW, Zhang JZ, Wang XL, Wang J, Yang RY, Liang L, Yu JB, Zuo XB, Zhang SQ, Zhang SM, Chen G, Zheng XD, Li P, Zhu J, Li YW, Wei XD, Hong WS, Ye Y, Zhang Y, Wu WS, Cheng H, Dong PL, Hu DY, Li Y, Li M, Zhang X, Tang HY, Tang XF, Xu SX, He SM, Lv YM, Shen M, Jiang HQ, Wang Y, Li K, Kang XJ, Liu YQ, Sun L, Liu ZF, Xie SQ, Zhu CY, Xu Q, Gao JP, Hu WL, Ni C, Pan TM, Li Y, Yao S, He CF, Liu YS, Yu ZY, Yin XY, Zhang FY, Xie, Z., Chen, D.L., Jiao, D. and Bystryn, J.C (1999). Vitiligo antibodies are not directed to tyrosinase. *Arch Dermatol*. 135, 417-422.

Raanani, P., and Ben-Bassat, I (2002). Immune-mediated complications during interferon therapy in hematological patients. *Acta Haematol*. 107,133-144.

Rahman I, Biswas SK, Jimenez LA, Torres M, Forman HJ.(2005). Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid Redox Signal* 7(1-2):42-59.

Rahman, I.; Clerch, L. B.; Massaro, D (1991). Rat lung antioxidant enzyme induction by ozone. *Am. J. Physiol*. 260:L412–418;

Rahner N, Ho¨efler G, Ho¨genauer C (2008) Compound heterozygosity for two MSH6 mutations in a patient with early onset colorectal cancer vitiligo and systemic lupus erythematosus. *Am J Med Genet* A 146A:1314–9.

Rameshwar, P., Gascon, P. and Ganea, D (1992). Immunoregulatory effects of neuropeptides: stimulation of interleukin2 production by substance *P. J Neuroimmunol.* 37, 65-74.

Ray, G., Husain, S.A (2002). Oxidants, antioxidants and carcinogenesis. *Indian J Exp Biol*. 11, 1213-1232.

Reaume, A. G.; Elliott, J. L.; Hoffman, E. K.; Kowall, N. W.; Ferrante, R. J.; Siwek, D. F.; Wilcox, H. M.; Flood, D. G.; Beal, M. F.; Brown, R. H. Jr.; Scott, R. W.;

Snider, W. D (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet*. 13:43–47.

Reedy, M.V., Parichy, D.M., Erickson, C.A., Mason, K.A., Frost-Mason, S.K (1998). Regulation of melanoblasts migration and differentiation. In: Nordlund, J.J., Boissey, R.E., Hearing, V.J., King, R.A., and Ortonne, J.P. Eds. The Pigmentary system. Physiology and pathophysiology. *New York: Oxford University Press*. 75-95.

Ren, Y., Yang, S., Xu, S., Gao, M.,Huang,W., Gao, T., Fang, Q., Quan, C., Zhang, C., Sun, L (2009). Genetic variation of promoter sequence modulates XBP1 expression and genetic risk for vitiligo. *PLoS Genet*. 5, e1000523.

Rezaei N, Gavalas NG, Weetman AP, Kemp EH (2007). Autoimmunity as an aetiological factor in vitiligo. *J Eur Acad Dermatol Venereol*. 21: 865-76.

Rice-Evans, C., and Burdon, R. (1993). Free radical-lipid interactions and their pathological consequences. *Prog Lipid Res*. 32, 71-110.

Rink, L., Kirchner, H (1996). Recent Progress in the Tumor Necrosis Factor-Alpha Field. *International Archives of Allergy and Immunology*. 111 (3): 199-209.

Rogers, R. J.; Chesrown, S. E.; Kuo, S.; Monnier, J. M.; Nick, H. S (2000) Cytokine-inducible enhancer with promoter activity in both the rat and human manganese-superoxide dismutase genes. *Biochem. J*. 347:233–242.

Rokos, H., Beazley, W. D. and Schallreuter, K. U (2002) Oxidative stress in vitiligo: photo-oxidation of pterins produces H2O2 and pterin-6-carboxylic acid. *Biochem. Biophys. Res. Commun.* 292, 805–811.

Rokos, H., Moore, J., Hasse, S., Gillbro, J. M., Wood, J. M. and Schallreuter, K. U (2004) In vivo fluorescence excitation spectroscopy and in vivo Fourier-transform Raman spectroscopy in human skin: evidence of H2O2 oxidation of epidermal albumin in patients with vitiligo. *J. Raman Spectrosc.* 35, 125–130.

Rokos, H., Wood, J. M., Hasse, S. and Schallreuter, K. U (2008) Identification of epidermal L-tryptophan and its oxidation products by in vivo FT-Raman spectroscopy further supports oxidative stress in patients with vitiligo. *J. Raman. Spectrosc*. 39, 1214–1218.

Rosen, D. R.; Siddique, T.; Patterson, D.; Figlewicz, D. A.; Sapp, P.; Hentati, A.; Donaldson, D.; Goto, J.; O'Regan, J. P.; Deng, H.-X.; Rahmani, Z.; Krizus, A.; McKenna-Yasek, D.; Cayabyab, A.; Gaston, S. M.; Berger, R.; Tanzi, R. E.; Halperin, J. J.; Herzfeldt, B.; Van den Bergh, R.; Hung, W.-Y.; Bird, T.; Deng, G.; Mulder, D. W.; Smyth, C.; Laing, N. G.; Soriano, E.; Pericak-Vance, M. A.; Haines, J.; Rouleau, G. A.; Gusella, J. S.; Horvitz, H. R.; Brown, R. H (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362:59–62.

Rosenblum, J. S.; Gilula, N. B.; Lerner, R. A (1996) On signal sequence polymorphisms and diseases of distribution. *Proc. Natl. Acad. Sci. USA* 93:4471–4473.

Ruuls, S. R., Sedgwick, J. D (1999) Unlinking tumor necrosis factor biology from the major histocompatibility complex: lessons from human genetics and animal models. *Am. J. Hum. Genet*. 65: 294-301.

Salem, M. M., Shalbaf, M., Gibbons, N. C. J., Chavan, B., Thornton, J. M. and Schallreuter, K. U (2009). Enhanced DNA binding capacity on up-regulated epidermal wild-type p53 in vitiligo by H_2O_2-mediated oxidation: a possible repair mechanism for DNA damage. *FASEB J.* 23, 3790–3807.

Samantha B, Torello L.(2008). Recent advances in the treatment of childhood vitiligo. *Expet Rev Dermatol* 3(6):691–9.

Sandstrom, J.; Nilsson, P.; Karlsson, K.; Marklund, S. L (1994). 10- Fold increase in human plasma extracellular superoxide dismutase content caused by a mutation in heparin-binding domain. *J. Biol. Chem*. 269:19163–19166.

Schallreuter KU, Wood JM, Pittelkow MR (1993). Increased in vitro expression of beta 2-adrenoceptors in differentiating lesional keratinocytes of vitiligo patients. *Arch Dermatol Res* ;285: 216–20.

Schallreuter, K. U. (1999). A review of recent advances on the regulation of pigmentation in the human epidermis. *Cell Mol. Biol.* 45, 943–949.

Schallreuter, K. U. and Rokos, H (2007). From the bench to the bedside: proton pump inhibitors can worsen vitiligo. *Br. J. Dermatol*. 156, 1371–1373.

Schallreuter, K. U., Bahadoran, P., Picardo, M., Slominski, A., Elassiuty, E. H., Kemp, C., Giachino, C., Liu, J. B., Luiten, R. M., Lambe, T (2008). Vitiligo pathogenesis: autoimmune disease, genetic defect, excessive reactive oxygen species, calcium imbalance, or what else? *Exp. Dermatol.* 17, 139–160.

Schallreuter, K. U., Chiuchiarelli, G., Cemeli, E., Elwary, S. M., Gillbro, J. M., Spencer, J. D., Rokos, H., Panske,A., Chavan, B., Wood, J. M. and Anderson, D (2006). Estrogens can contribute to hydrogen peroxide generation and quinone-mediated DNA damage in peripheral blood lymphocytes from patients with vitiligo. *J. Invest. Dermatol.* 126, 1036–1042.

Schallreuter, K. U., Elwary, S. M., Gibbons, N. C., Rokos, H. and Wood, J. M (2004). Activation/deactivation of acetylcholinesterase by H_2O_2: more evidence for oxidative stress in vitiligo. *Biochem. Biophys. Res. Commun*. 315, 502–508.

Schallreuter, K. U., Gibbons, N. C., Elwary, S.M., Parkin, S. M. and Wood, J. M (2007). Calcium-activated butyrylcholinesterase in human skin protects acetylcholinesterase against suicide inhibition by neurotoxic organophosphates. *Biochem. Biophys. Res. Commun*. 355, 1069–1074.

Schallreuter, K. U., Gibbons, N. C., Zothner, C., Abou Elloof, M. M. and Wood, J. M (2007). Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on

calmodulin: more evidence for oxidative stress in vitiligo. *Biochem. Biophys. Res. Commun*. 360, 70–75.

Schallreuter, K. U., Lemke, R., Brandt, O., Schwartz, R.,Westhofen, M., Montz, R. and Berger, J (1994). Vitiligo and other diseases: coexistence or true association. *Dermatology* 188, 269–275.

Schallreuter, K. U., Moore, J., Wood, J. M., Beazley, W.D., Gaze, D. C., Tobin, D. J., Marshall, H. S., Panske, A., Panzig, E. and Hibberts, N. A (1999) In vivo and in vitro evidence for hydrogen peroxide (H2O2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. *J. Invest. Dermatol. Symp. Proc.* 4, 91–96.

Schallreuter, K. U., Moore, J.,Wood, J. M., Beazley, W. D., Peters, E. M., Marles, L. K., Behrens-Williams, S. C., Dummer, R., Blau, N and Thony, B (2001). Epidermal H2O2 accumulation alters tetrahydrobiopterin (6BH4) recycling in vitiligo: identification of a general mechanism in regulation of all 6BH4-dependent processes? *J. Invest. Dermatol.* 116, 167–174.

Schallreuter, K. U., Wood, J. M. and Berger, J (1991). Low catalase levels in the epidermis of patients with vitiligo. *J.Invest. Dermatol.* 97, 1081–1085.

Schallreuter, K. U., Wood, J. M., Pittelkow, M. R., Swanson, N. N. and Steinkraus, V (1993). Increased in vitro expression of β2-adrenoceptors in differentiating lesional keratinocytes of vitiligo patients. *Arch. Dermatol. Res*. 285, 216–220.

Schallreuter, K. U., Wood, J. M., Ziegler, I., Lemke, K. R., Pittelkow, M. R., Lindsey, N. J. and G¨ utlich, M (1994). Defective tetrahydrobiopterin and catecholamine biosynthesis in the depigmentation disorder vitiligo. *Biochim. Biophys. Acta* 1226, 181–192.

Schallreuter, K. U., Zschiesche, M., Moore, J., Panske, A., Hibberts, N. A., Herrmann, F. H., Metelmann, H. R. and Sawatzki, J (1998). In vivo evidence for compromised phenylalanine metabolism in vitiligo. *Biochem. Biophys. Res. Commun*. 243, 395–399.

Schallreuter, K. U.,Wood, J. M., Pittelkow, M. R., Buttner, G., Swanson, N., Korner, C. and Ehrke, C (1996). Increased monoamine oxidase A activity in the epidermis of patients with vitiligo. *Arch. Dermatol. Res*. 288, 14–18.

Schallreuter, K.U. and Pittelkow, M.R. (1988). Defective calcium uptake in keratinocyte cell cultures from vitiliginous skin. *Arch Dermatol Res*. 3, 137-139.

Schisler, N. J.; Singh, S. M (1985) Tissue-specific developmental regulation of superoxide dismutase (SOD-1 and SOD-2) activities in genetic strains of mice. *Biochem. Genet.* 23:291–308.

Schmutz, S. M.; Cornwell, D.; Moker, J. S.; Troyer, D. L (1996). Physical mapping of SOD1 to bovine chromosome 1. *Cytogenet. Cell Genet.* 72:37–39.

Scholzen, T.E., Stander, S., Riemann, H., Brzoska, T., and Luger, T.A (2003). Modulation of cutaneous inflammation by angiotensin-converting enzyme. *J Immunol.* 170, 3866-3873.

Sehgal VN, Srivastava G (2007).Vitiligo: Compendium of clinico-epidemiological features. *Indian J Dermatol Venereol Leprol* 73:149-56.

Sehgal, V.N (1974). A clinical evaluation of 202 cases of vitiligo. *Cutis.* 14, 439-445.

Seo, S. J.; Kang, S. S.; Cho, G.; Rho, H. M.; Jung, G (1997). C/EBP alpha and C/EBPbeta play similar roles in the transcription of the human Cu/Zn SOD gene. *Gene* 203:11–15.

Seo, S. J.; Kim, H. T.; Cho, G.; Rho, H. M.; Jung, G (1996) Sp1 and C/EBP-related factor regulate the transcription of human Cu/ZnSOD gene. *Gene* 178:177–185.

Shahrad, P. and Marks, R (1977). A pharmacological effect of oestrogene on human epidermis. *Br J Dermatol*. 97, 383-386.

Shajil E.M. and Begum R (2006). Antioxidant status of segmental and non-segmental vitiligo. *Pigment Cell Res* 19 :179–180.

Shajil E.M., Agrawal, D.,Vagadia, K., Marfatia, Y.S., Begum, R (2006). Vitiligo: Clinical profiles in Vadodara, Gujarat. *Indian J Dermatol* 51:100-4.

Shaker, O. G. and El-Tahlawi, S. M (2008). Is there a relationship between homocysteine and vitiligo? A pilot study. *Br. J. Dermatol*. 159, 720–724.

Shalbaf, M., Gibbons, N. C., Wood, J. M., Maitland, D. J., Rokos, H., Elwary, S. M., Marles, L. K. and Schallreuter,K. U (2008). Presence of epidermal allantoin further supports oxidative stress in vitiligo. *Exp. Dermatol*. 17, 761–770.

Sheng, H.; Brady, T. C.; Pearlstein, R. D.; Crapo, J. D.; Warner, D. S (1999) Extracellular superoxide dismutase deficiency worsens outcome from focal cerebral ischemia in the mouse. *Neurosci. Lett.* 267:13–16.

Shigenaga, M. K., Hagen, T. M. and Ames, B. N (1994). Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. U. S. A.* 91, 10771–10778.

Shirai, T., Yamaguchi, H., Ito, H., Todd, C. W., Wallace, R. B (1985). Cloning and expression in Escherichia coli of the gene for human tumour necrosis factor. *Nature* 313: 803-806.

Silvan, M (2004). The psychological aspects in vitiligo. *Cutis*. 73, 163-167.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, Laddha NC, Dwivedi M, Begum R, Gokhale RS, Rani R (2012). HLA alleles and amino-acid signatures of the peptide-binding pockets of HLA molecules in vitiligo. *J Invest Dermatol* 132:124-134..

Singh J, Suruchi A (2004). Anti tnf-a strategy: Present status of this therapeutic paradigm. *Indian J Pharmacol* 36:10-4.

Skoog, T., van't Hooft, F. M., Kallin, B., Jovinge, S., Boquist, S., Nilsson, J., Eriksson, P., Hamsten, A (1999). A common functional polymorphism (C-A substitution at position -863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. *Hum. Molec. Genet*. 8: 1443-1449.

Slominski A, Wortsman J, Tuckey RC (2007). Differential expression of HPA axis homolog in the skin. *Mol Cell Endocrinol* 265-6:143–9.

Song, Y.H., Connor, E., Li, Y., Zorovich, B., Balducci, P., and Maclaren, N (1994). The role of tyrosinase in autoimmune vitiligo. *Lancet*. 344, 1049-1052.

Soubiran, P., Benzaken, S., Bellet, C., Lacour, J.P., and Ortonne, J.P (1985). Vitiligo: peripheral T cell subset imbalance as defined by monoclonal antibodies. *Br J Dermatol*. 113, 1985, 124-127.

Spencer, J. D., Chavan, B., Marles, L. K., Kauser, S., Rokos, H. and Schallreuter, K. U (2005) A novel mechanism in control of human pigmentation by β-melanocyte-stimulating hormone and 7-tetrahydrobiopterin. *J. Endocrinol*. 187, 293–302.

Spencer, J. D., Gibbons, N. C. J., Bohm, M. and Schallreuter, K. U (2008). The Ca2+-binding capacity of epidermal furin is disrupted by H2O2-medaited oxidation in vitiligo. Endocrinology 149, 1638–1645.

Spencer, J. D., Gibbons, N. C., Rokos, H., Peters, E. M., Wood, J. M. and Schallreuter, K. U (2007). Oxidative stress via hydrogen peroxide affects proopiomelanocortin peptides directly in the epidermis of patients with vitiligo. *J. Invest. Dermatol*. 127, 411–420.

Spies, T., Blanck, G., Bresnahan, M., Sands, J., Strominger, J. L (1989). A new cluster of genes within the human major histocompatibility complex. *Science* 243: 214-217.

Spriggs DR, Deutsch S, Kufe DW (1992). Genomic structure, induction, and production of TNF-alpha. *Immunol Ser* ; 56: 3–34.

Spritz RA (2007). The genetics of generalized vitiligo and associated autoimmune diseases. *Pigment Cell Res*; 20: 271-278.

Spritz RA (2008). The genetics of generalized vitiligo. *Curr Dir Autoimmunity* ; 10: 244-257.

Spritz RA (2010). The genetics of generalized vitiligo: autoimmune pathways and an inverse relationship with malignant melanoma. *Genome Medicine*; 2: 78.

Spritz RA (2011). Recent progress in the genetics of generalized vitiligo. *J Genet and Genom* ; 38: 271-278.

Spritz RA, Gowan K, Bennett DC (2004). Novel vitiligo susceptibility loci on chromosomes 7 (AIS2) and 8 (AIS3) confirmation of SLEV1 on chromosome 17 and their roles in an autoimmune diathesis. *Am J Hum.*

Spritz, R. A (2008). The genetics of generalized vitiligo. *Curr. Opin. Autoimmun*. 10, 244–257.

Spritz, R. A., Gowan, K., Bennett, D. C. and Fain, P. R (2004). Novel susceptibility loci on chromosomes 7 (AIS2) and 8 (AIS3), confirmation of SLEV1 on chromosome 17, and their roles in an autoimmune diathesis. *Am. J. Hum. Genet*. 74, 188–191.

Stites, D.P., Terr, A.I., and Parslow, T.G (1994). Clinical laboratory methods for detection of cellular immunity. In Basic and Clinical Immunology. *Appleton and Lange, Norwalk*, CT. 199.

Stohs SJ, Bagchi D (1995).Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med.* 18(2):321-36.

Stokes PE, Sikes CR (1988). The hypothalamic-pituitary-adrenocortical axis in major depression. *Endocrinol Metab Clin North Am* ; 17:1–19.

Stralin, P.; Marklund, S. L (2001). Vasoactive factors and growth factors alter vascular smooth muscle cell EC-SOD expression. *Am. J. Physiol. Heart Circ. Physiol*. 281:H1621–1629.

Strange, R. C.; Cotton, W.; Fryer, A. A.; Drew, R.; Bradwell, A. R.; Marshall, T.; Collins, M. F.; Bell, J.; Hume, R (1988). Studies on the expression of Cu, Zn superoxide dismutase in human tissues during development. *Biochim. Biophys. Acta* 964:260–265.

Strieter, R., Kunkel, S., Bone, R (1993). Role of Tumor Necrosis Factor-Alpha in Disease States and Inflammation. *Critical Care Medicine*. 21 ; S447-S463.

Stuttgen G (1950). Die Vitiligo in erbbiologischer Betrachtung. *Z Haut Geschlechtskr* 9:451–7.

Sugatani, J.; Kojima, H.; Ueda, A.; Kakizaki, S.; Yoshinari, K.; Gong, Q. H.; Owens, I. S.; Negishi, M.; Sueyoshi, T (2001). The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. *Hepatology* 33:1232–1238.

Suh, J.-G.; Takai, S.; Yamanishi, T.; Kikuchi, T.; Folz, R. J.; Tanaka, K.; Oh, Y.-S.; Wada, K (1997). Sequence analysis, tissue expression and chromosomal localization of a mouse secreted superoxide dismutase gene. *Mol. Cells* 7:204–207.

Swope, V., Abdel-Malek, Z., Kassem, L. and Norlund, J (1991).Interleukins 1α and 6 and tumor necrosis factor-α are paracrine inhibitors of human melanocyte proliferation and melanogenesis. *J. Invest. Dermatol.* 96: 180-185.

Taher, Z. A., Lauzon, G., Maguiness, S. and Dytoc, M. T (2009) Analysis of interleukin-10 levels in lesions of vitiligo following treatment with topical tacrolimus. *Br. J. Dermatol*. 161, 654–659.

Taieb A, Picardo M (2007).The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res* 20:27-35.

Taieb, A (2000). Intrinsic and extrinsic pathomechanisms in vitiligo. *Pigment Cell Res*. 13, 41–4788–91.

Tanaka, K (1998). Molecular biology of the proteasome. *Biochem Biophys Res Commun*. 247, 537-541.

Teindel H (1950). Familia¨ re Vitiligo. *Z Haut Geschlechtskr* 9:457–62.

Thannickal, V. J., Day, R. M., Klinz, S. G., Bastien, M. C., Larios, J. M. and Fanburg, B. L (2000). Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF-β1. *FASEB J*. 14, 1741–1748.

Tharp, M.D (2002). Calcineurin Inhibitors. *Dermatol Therapy*. 15, 325-332.

Thongphasuk, J.; Oberley, L. W.; Oberley, T. D (1999). Induction of superoxide dismutase and cytotoxicity by manganese in human breast cancer cells. *Arch. Biochem. Biophys*. 365:317–327.

Thony, B., Auerbach, G. and Blau, N (2000). Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J*. 347, 1–16.

Thorneby-Andresson, K., Sterner, O. and Hansson, C (2000). Tyrosinase-mediated formation of a reactive quinone from the depigmenting agents, 4-tertbutylphenol and 4-tert-butylcatechol. *Pigment Cell Res.* 13, 33–38.

Tishkoff, S.A., and Williams, S.M (2002). Genetic analysis of African populations: human evolution and complex disease. *Nat Rev Genet*. 3, 611-621.

Tobin, D. J., Swanson, N. N., Pittelkow, M. R., Peters, E. M. and Schallreuter, K. U (2000) Melanocytes are not absent in lesional skin of long duration vitiligo. *J. Pathol.* 191, 407–416.

Tolis G (1983). Stefanis C Depression: biological and neuroendocrine aspects. *Biomed Pharmacother* ; 37:316–22.

Tomblyn, M.; Kasarskis, E. J.; Xu, Y.; St. Clair, D. K (1998). Distribution of MnSOD polymorphisms in sporadic ALS patients. *J. Mol. Neurosci*. 10:65–66.

Tomkins, J.; Banner, S. J.; McDermott, C. J.; Shaw, P. J (2001). Mutation screening of manganese superoxide dismutase in amyotrophic lateral sclerosis. *Neuroreport* 12:2319–2322.

Tracey, K., Cerami, A (1990). Metabolic Response to Cachectin/TNF. *Annals of the New York Academy of Sciences* : Vol. 587. Eds. Boland, B, Cullinan, J., Kimball, C. New York *: New York Academy of Sciences.* 325-330.

Tracey, K., Cerami, A (1994). Tumor Necrosis Factor : A Pleiotropic Cytokine and Therapeutic Target. *Annual Review of Medicine*. 45 : 491-503.

Tu, C. X., Fu, H. W. and Lin, X. R (1999) Levels of soluble interleukin-2 receptor in the sera and skin tissue fluids of patients with vitiligo. *J. Dermatol. Sci*. 21, 59–62

Tu, C. X., Gu, J. S. and Lin, X. R (2003). Increased interleukin-6 and granulocyte macrophage colony stimulating factor levels in the sera of patients with non-segmental vitiligo. *J. Dermatol. Sci.* 31, 73–78.

Tursen, U., Kaya, T.I., Erdal, M.E., Derici, E., Gunduz, O., and Ikizoilu. G (2002). Association between catechol-O-methyltransferase polymorphism and vitiligo. *Arch Dermatol Res.* 294, 143-146.

Ueda, A.; Hamadeh, H. K.; Webb, H. K.; Yamamoto, Y.; Sueyoshi, T.; Afshari, C. A.; Lehmann, J. M.; Negishi, M (2002). Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol. Pharmacol.* 61:1–6.

Uglialoro AM, Turbay D, Pesavento PA (1998). Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor-alpha gene promoter. *Tissue Antigens* ; 52: 359–367.

Uhm YK, Yoon SH, Kang IJ (2007). Association of glutathione S-transferase gene polymorphisms (GSTM1 and GSTT1) of vitiligo in Korean population. *Life Sci* 81:223–7.

Ursini, F., Maiorino, M., and Gregolin., C (1985). The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochim Biophys Acta.* 839, 62-70.

Valia, A.K, Dutta, P.K (1996). IADVL Text book and Atlas of Dermatology. Bombay: Bhalani Publishing House. 500 - 586.

Van Geel, N., Ongenae, K., and Naeyaert, J.M (2001). Surgical techniques for vitiligo: *A review. Dermatology*. 202, 162-166.

Van Landeghem, G. F.; Tabatabaie, P.; Beckman, G.; Beckman, L.; Andersen, P. M (1999). Manganese-containing superoxide dismutase signal sequence polymorphism associated with sporadic motor neuron disease. *Eur. J. Neurol*. 6:639–644.

Van Ostade, X., Vandenabeele, P., Everaerdt, B., Loetscher, H., Gentz, R., Brockhaus, M., Lesslauer, W., Tavernier, J., Brouckaert, P., Fiers, W (1993). Human TNF Mutants with Selective Activity on the P55 Receptor. *Nature.* 361 : 266-269.

Velaga, M.R, Wilson, V., and Jennings, C.E. (2004). The codon 620 tryptophan allele of the lymphoid tyrosine phosphatase (LYP) gene is a major determinant of Graves' disease. *J Clin Endocrinol Metab.* 89, 5862-5865.

Venneker, G. T.,Westerhof, W., de Vries, I. J., Drayer, N. M., Wolthers, B. G., de Waal, L. P., Bos, J. D. and Asghar, S. S (1992) Molecular heterogeneity of the fourth component of complement (C4) and its genes in vitiligo. *J. Invest. Dermatol*. 99, 853–858.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O (2001). The sequence of the human genome. *Science*. 291, 1304-1351.

Visner, G. A.; Chesrown, S. E.; Monnier, J.; Ryan, U. S.; Nick, H. S (1992). Regulation of manganese superoxide dismutase: IL-1 and TNF induction in pulmonary artery and microvascular endothelial cells. *Biochem. Biophys. Res. Commun*. 188:453–462.

Visner, G. A.; Dougall, W. C.; Wilson, J. M.; Burr, I. A.; Nick, H. S (1990). Regulation of manganese superoxide dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor. *J. Biol. Chem.* 265:2856–2864.

Vlessis, A. A.; Mela-Riker, L (1989). Perinatal development of heart, kidney, and liver mitochondrial antioxidant defense. *Pediatr. Res.* 26:220–226.

Von den Driesch, P., Fartasch, M., and Hornstein, O.P (1992). Chronic actinic dermatitis with vitiligo-like depigmentation. *Clin Exp Dermatol.* 17, 38-43.

Vuillaume, M (1987). Reduced oxygen species, mutation, induction and cancer initiation. *Mutat Res.* 186, 43-72.

Wajant,H., Pfizenmaier, K.,Scheurich P (2003). Tumor necrosis factor signaling. *Cell Death Different.* 10, 45–65.

Wan, X. S.; Devalaraja, M. N.; St. Clair, D. K (1994). Molecular structure and organization of the human manganese superoxide dismutase gene. *DNA Cell Biol.* 13:1127–1136.

Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdell, J. N., Yamamoto, R., Mark, D. F (1985). Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228: 149-154.

Waterman, E.A., Kemp, E.H., Gawkrodger, D.J., Watson. P.F. and Weetman, A.P (2002). Autoantibodies in vitiligo are not directed to the melanocyte differentiation antigen MelanA/MART1. *Clin Exp Immunol.* 129, 527-532.

Westerhof, W. and d'Ischia, M (2007) Vitiligo puzzle: the pieces fall in place. *Pigment Cell Res.* 20, 345–359.

Westerhof, W., and Nieuweboer-Krobotova, L (1997). Treatment of vitiligo with UV-B radiation vs topical psoralen plus UV-A. *Arch Dermatol*. 133, 1525-1528.

Wiedau-Pazos, M.; Goto, J. J.; Rabizadeh, S.; Gralla, E. B.; Roe, J. A.; Lee, M. K.; Valentine, J. S.; Bredesen, D. E (1996). Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science* 271:515–518.

Wijngaard, R.M., Wankowics-Kalinska, A., Le Poole, I.C., Tigges, B., Westerhof, W. and Das, P.K (2000). Local immune response in skin of generalized vitiligo patients: Destruction of melanocytes is associated with the prominent presence of CLA+T cells at the perilesional site. *Lab Invest.* 80, 1299-1309.

Willems, J.; Zwijsen, A.; Slegers, H.; Nicolai, S.; Bettadapura, J.; Raymackers, J.; Scarcez, T (1993). Purification and sequence of ratextracellular superoxide dismutase B secreted by C6 glioma.*J. Biol. Chem.* 268:24614–24621.

Wilson, A.G., Symons, J.A., McDowell, T.L., McDevitt, H.O., and Duff, G.W (1997). Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci USA.* 94, 3195-3199.

Winkler, G., Lakatos, P., Salamon, F., Nagy, Z., Speer, G., Kovács, M., Harmos, G., Dworak, O. and Cseh, K (1999). Elevated serum TNF-α level as a link between endothelial dysfunction and insulin resistance in normotensive obese patients. *Diabetic Medicine,* 16: 207–211.

Witz, G (1991). Active oxygen species as factors in multistage carcinogenesis. *Proc Soc Exp Biol Med.* 198, 675-682.

Wong, G. H. W.; Goeddel, D. V (1988). Induction of manganese superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science* 242:941–944.

Wood, J. M., Chavan, B., Hafeez, I. and Schallreuter, K.U (2004). Regulation of tyrosinase by tetrahydropteridines and H2O2. Biochem. Biophys. *Res. Commun.* 325, 1412–1417.

Wood, J. M., Decker, H., Hartmann, H., Chavan, B., Rokos, H., Spencer, J. D., Hasse, S., Thornton, M. J., Shalbaf, M., Paus, R. and Schallreuter, K. U (2009) Senile hair graying: H2O2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair. *FASEB J.* 23, 2065–2075.

Xu, Y.; Krishnan, A.; Wan, X. S.; Majima, H.; Yeh, C. C.; Ludewig, G.; Kasarskis, E. J.; St. Clair, D. K (1999). Mutations in the promoter reveal a cause for the reduced expression of the human manganese superoxide dismutase gene in cancer cells. Oncogene 18:93–102.

Yamada, H.; Yamada, Y.; Adachi, T.; Goto, H.; Ogasawara, N.; Futenma, A.; Kitano, M.; Hirano, K.; Kato, K (1995). Molecular analysis of extracellular-superoxide dismutase gene associated with high level in serum. *Jpn. J. Hum. Genet*. 40:177–184.

Yamada, H.; Yamada, Y.; Adachi, T.; Goto, H.; Ogasawara, N.; Futenma, A.; Kitano, M.; Miyai, H.; Fukatsu, A.; Hirano, K.; Kakumu, S (1997). Polymorphism of extracellular superoxide dismutase (EC-SOD) gene: relation to the mutation responsible for high EC-SOD level in serum. *Jpn. J. Hum. Genet.* 42:353–356.

Yamaoka, K.; Sato, E. F.; Utsumi, K (1994). Induction of two speciesof superoxide dismutase in some organs of rats by low dose X-irradiation. *Physiol. Chem. Phys. Med. NMR* 26:205–214.

Yang S, Zhou Y, Zhang XJ (2010). Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC. *Nat Genet* 42:614-618.

Yazici, A.C, Erdal, M.E., Kaya, T.I., Ikizoglu, G., Savasoglu, K., Camdeviren, H., and Tursen, U (2006). Lack of association with TNF-alpha-308 promoter polymorphism in patients with vitiligo. *Arch Dermatol Res.* 298, 46-49.

Yee, C., Thompson, J.A., Roche, P., Byrd, D.R., Lee, P.P., Piepkorn, M., Kenyon, K., Davis, M.M., Riddel, S.R., and Greenberg, P.D (2000). Melanocyte destruction after antigen specific immunotherapy of melanoma: Direct evidence of T cell mediated vitiligo. *J Exp Med.* 192, 1637-1644.

Yeh, C. C.; Wan, X. S.; St. Clair, D. K (1998). Transcriptional regulation of the 5' proximal promoter of the human manganese superoxide dismutase gene. DNA Cell Biol. 17:921–930.

Yeşilova, Y., Çalka, O., Akdeniz, N.,Berktaş, M (2012). Effect of Probiotics on the Treatment of Children with Atopic Dermatitis. *Ann Dermatol*. 2 : 189–193.

Yildirim, M., Baysal, V., Inaloz, H. S. and Can, M (2004). The role of oxidants and antioxidants in generalized vitiligo at tissue level. *J. Eur. Acad. Dermatol. Venereol.* 18, 683–686.

Yoo, H. Y.; Chang, M. S.; Rho, H. M (1999). Heavy metal-mediated activation of the rat Cu/Zn superoxide dismutase gene via ametal-responsive element. *Mol. Gen. Genet*. 262:310–313.

Yoo, H. Y.; Chang, M. S.; Rho, H. M (1999). Induction of the rat Cu/Zn superoxide dismutase gene through the peroxisome proliferator responsive element by arachidonic acid. *Gene* 234:87–91.

Yoo, H. Y.; Chang, M. S.; Rho, H. M (1999). The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. *J. Biol. Chem*. 274:23887–23892.

Yoo, H. Y.; Chang, M. S.; Rho, H. M (1999). Xenobiotic-responsive element for the transcriptional activation of the rat Cu/Zn superoxide dismutase gene. *Biochem. Biophys. Res. Commun.* 256: 133–137.

Yoshida H, Nadanaka S, Sato R, Mori K (2006).XBP1 is critical to protect cells from endoplasmic reticulum stress: evidence from Site-2 protease-deficient Chinese hamster ovary cells. *Cell Struct Funct* 31 (2): 117–25.

Yu, H. S., Chang, K. L., Yu, C. L.,Wu, M. T., Wu, C. S. and Wu, C. S (1997). Alterations in IL-6, IL-8, GM-CSF, TNF-α and IFN-γ release by peripheral mononuclear cells in patients with active vitiligo. *J. Invest. Dermatol.* 108, 527–529.

Yuan, H. T.; Bingle, C. D.; Kelly, F. J (1996). Differential patterns of antioxidant enzyme mRNA expression in guinea pig lung and liver during development. *Biochim. Biophys. Acta* 1305:163– 171.

Yucesoy, B., Vallyathan, V., Landsittel, D.P., Sharp, D.S., Weston, A., Burleson, G.R., Simeonova, P., McKinstry. M., Luster, M.I (2001). Association of tumor

necrosis factor-alpha and interleukin-1 gene polymorphisms with silicosis. *Toxicol Appl Pharmacol.* 172, 75-82.

Yun JY, Uhm YK, Kim HJ (2010). Transforming growth factor beta receptor II (TGFBR2) polymorphisms and the association with nonsegmental vitiligo in the Korean population. *Int J Immunogenet* 37:289–91.

Zailaie, M. Z. (2005). Decreased proinflammatory cytokine production by peripheral blood mononuclear cells from vitiligo patients following aspirin treatment. *Saudi Med. J.* 26, 799–805.

Zamani, M., Spaepen, M., Sghar, S.S., Huang, C., Westerhof, W., Nieuweboer-Krobotova, L., and Cassiman, J.J (2001). Linkage and association of HLA class II genes with vitiligo in a Dutch population. *Br J Dermatol.* 145, 90-94.

Zauli, D., Tosti, A., Biasco, G., Miseroccchi, F., Patrizi, A., Azzaroni, D., Andriani, G., Di Febo, G and Callegari, C (1986). Prevalence of autoimmune atrophic gastritis in vitiligo. *Digestion.* 34, 169-172.

Zhang XJ, Liu HS, Liang YH, Sun LD, Wang JY, Yang S (2004). Association of HLA class I alleles with vitiligo in Chinese Hans. *J Dermatol Sci.*; 35: 165–8.

Zhang XJ, Liu JB, Gui JP, Li M, Xiong QG, Wu HB (2004). Characteristics of genetic epidemiology and genetic models for vitiligo. *J Am Acad Dermatol* ; 51: 383–90.

Zhang, H. J.; Yan, T.; Oberley, T. D.; Oberley, L. W (1999). Comparison of effects of two polymorphic variants of manganese superoxide dismutase on human breast MCF-7 cancer cell phenotype. *Cancer Res.* 59:6276–6283.

Zhang, N (1996). Characterization of the 5' flanking region of the human MnSOD gene. Biochem. *Biophys. Res. Commun.* 220:171–180.

Zhang, X.J., Chen, J.J., and Liu, J.B (2005). The genetic concept of vitiligo. *J Dermatol. Sci.* 39, 137-146.

Zhou, Z., Li, C. Y., Wang, T., Zhang, B. and Gao, T.W (2009). Decreased methionine sulfoxide reductase A expression renders melanocytes more sensitive to oxidative stress: a possible cause for melanocyte loss in vitiligo. *Br. J. Dermatol.* 161, 504–509.

Zhu, C. H.; Huang, Y.; Oberley, L. W.; Domann, F. E (2001). A family of AP-2 proteins down-regulate manganese superoxide dismutase expression. *J. Biol. Chem.* 276:14407–14413.

Zhu, X., Ertel, W., Ayala, A., Morrison, M., Perrin, M., Chaudry, I (1993). Chloroquine Inhibits Macrophage Tumor Necrosis Factor-Alpha mRNA Transcription. *Immunology.* 80 (122): 122-126.

2.1 INTRODUCTION

Oxidative stress is a major form of assault on the skin. Skin exposure to ionizing and UV radiation and/or xenobiotics/drugs generates ROS in excessive quantity. It overwhelms tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS and increased lipid peroxidation are seen in multiple skin disorders such as atopic dermatitis, psoriasis, acne vulgaris, pemphigus vulgaris (PV), lichen planus, alopecia areata and vitiligo (Yesilova et al., 2012). Oxidative stress has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocyte destruction (Schallreuter et al 1999a; Maresca et al 1997) which is marked by accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter et al., 2001). Defective recycling of tetrahydrobiopterin in vitiligo epidermis is related with the intracellular production of H_2O_2 (Schallreuter et al 1994; Schallreuter et al., 1999b). In addition, a compromised antioxidant status with a significant reduction in catalase activity and increase in SOD activity has been demonstrated in both lesional and non-lesional epidermis (Schallreuter et al., 1991; Sravani et al., 2009) as well as in melanocytes (Maresca et al., 1997). Interestingly, antioxidant imbalance has also been observed in the peripheral blood mononuclear cells of active vitiligo patients. This was correlated with an increased intracellular production of reactive oxygen species due to mitochondrial impairment (Dell'Anna et al., 2001), supporting the concept of a possible systemic oxidative stress in vitiligo. Impaired redox status of the epidermal melanin unit acts as the primary defect leading to inappropriate immune response resulting in melanocyte destruction. Epidermal H_2O_2 generation in vitiliginous patients could be due to several factors and H_2O_2 overproduction leads to catalase inactivation in the epidermal melanocytes (Schallreuter et al. 1996, Maresca et al. 1997).

In normal cellular processes, SOD1 is a major antioxidant enzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen. Hydrogen peroxide in turn converted to oxygen and water by the action of catalase and glutathione peroxidase. As a result cell maintains its normal integrity and function. Sometimes equilibrium of antioxidants and oxidants gets imbalanced which leads to accumulation of free radicals resulting into the accumulation of oxidative stress. This accumulated oxidative stress causes DNA damage (Mohamed and Salem,

2009), lipid and protein peroxidation. Many proteins and peptides get altered or even show complete loss of functionality due to H_2O_2-mediated oxidation.

Vitiligo is an acquired hypomelanotic skin disorder, characterized by milky white patches of different size and shape. Though vitiligo is extensively addressed in the past six decades, its etiology is still being debated (Taieb 2000; Le Poole et al 1993; Ortonne and Bose 1993; Cucchi et al 2003; Ongenae et al 2003; Boisseau-Garsuad et al 2002). Vitiligo susceptibility is a complex genetic trait that may involve genes for melanin synthesis, response to oxidative stress and regulation to autoimmunity. In order to explore the genetic susceptibility, systematic study of each gene governing oxidative stress, melanin synthesis, and regulation of autoimmunity is essential. Several hypotheses were proposed to explain the pathogenesis of vitiligo and oxidative stress hypothesis considers a systemic involvement in the course of the disease (Picardo et al 1994; Yildirim et al 2003). Oxidative stress could act as the initial triggering event in melanocyte degeneration.

Superoxide dismutases are metalloenzymes found widely distributed in prokaryotic and eukaryotic cells (Fridovich 1995). Superoxide dismutases scavenge the superoxide radicals and reduce its toxicity by converting it to H_2O_2 (Schallreuter et al 1991). Three distinct isoforms of SOD have been identified in mammals. Two isoforms of SOD have Cu and Zn at their catalytic center and are localized to either intracellular cytoplasmic compartments (CuZn or SOD1) or to extracellular spaces (EC SOD or SOD3). SOD2 is localized to mitochondria and has Mn as the prosthetic group. SOD1 has a molecular mass of about 32 kDa and has been found in the cytoplasm, nuclear components and lysosomes of mammalian cells (Chang et al 1988; Keller et al 1991). SOD1 is a constitutively expressed gene, localized on chromosome 21(21q22) in humans. In addition, over 100 distinct SOD1 inherited mutations have been identified in the familial form of amyotrophic lateral sclerosis (ALS), a progressive degenerative disease of motor neurons. Despite the fact that SOD1 helps to eliminate toxic reactive species, its mutations in ALS have been described as gain-of-function. The mechanism by which mutant SOD1 induces the neurodegeneration observed in ALS is still unclear. Mutant SOD1 proteins become misfolded and consequently oligomerize into high molecular weight species that aggregate and end up in proteinaceous inclusions.

There are several reports which suggest that SOD1 activity is increased in vitiligo patients compared to controls (Hazneci et al 2005, Yildirim et al 2003, 2004; Dell'Anna et al 2001; Chakraborty et al 1996; Ines et al 2006). Increased activity of SOD1 results in overproduction of H_2O_2 which is toxic to the cell. Our earlier studies showed impairment in the systemic antioxidant system in Gujarat vitiligo patients (Agrawal et al. 2004, Shajil and Begum, 2006).

The objectives of this study were:

i.) To estimate lipid peroxidation (LPO) levels to assess oxidative stress in vitiligo patients and controls.

ii.) To estimate Superoxide dismutase 1 activity in vitiligo patients and controls.

iii.) To determine *SOD1* mRNA and protein levels in vitiligo patients and controls.

iv.) To investigate exon 2 C/T (Ile40Thr) single nucleotide polymorphism (SNP) of *SOD1* gene in vitiligo patients and controls.

v.) To identify novel mutations/SNPs in *SOD1* gene using High Resolution Melt Curve (HRM) analysis.

2.2 MATERIALS AND METHODS

2.2.1 Study Subjects:

The study group included 950 vitiligo patients comprised of 408 males and 542 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of 1650 ethnically, age and sex-matched unaffected individuals comprised of 742 males and 908 females were included as controls in this study (Table 1). None of the healthy individuals 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.

2.2.2 Blood collection:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). The blood was used for Genetic analysis (DNA and RNA preparation) and Biochemical analysis (estimation of LPO, SOD1 and quantification of SOD1 protein by western analysis) (Figure 1).

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 950)	(n = 1650)
Average age (mean age ± SD)	31.35 ± 14.28 yrs	29.42 ± 13.12yrs
Sex: Male	408 (42.95%)	742 (44.97%)
Female	542 (57.05%)	908 (55.03%)
Onset age (mean age ± SD)	22.32 ± 13.41 yrs	NA
Duration of disease (mean ± SD)	6.8 ± 5.2 yrs	NA
Type of vitiligo		
Generalized	684 (72.00)	NA
Localized	266 (28.00)	NA
Active vitiligo	705 (74.21)	NA
Stable vitiligo	245 (25.79)	NA
Family history	125 (13.16%)	NA

2.2.3 Estimation of LPO levels and SOD1 activity:

Sample preparation

Whole blood was centrifuged at 3000 rpm for 10 minutes. Plasma was separated and stored in deep freezer (-200C) until use. Erythrocyte sediment was washed thrice with PBS and hemolysate was prepared by adding distilled water corresponding to the amount of plasma separated.

Hemoglobin estimation: Hemoglobin estimation was done by cyanmethemoglobin method (Dacie and Lewis 1968).

Principle

Drabkin's reagent (ferricyanide) converts the hemoglobin to cyanmethemoglobin (CMG) and the absorbance of CMG is proportional to the hemoglobin concentration. The optical density was measured at 540 nm against distilled water.

Reagents

Drabkin's reagent
Cyanmethemoglobin standard (65mg/dl)

Protocol

Reagent	Blank	Control	Test
CMG standard	-	3 ml	-
Sample	-	-	20 µl
Drabkin's reagent	5 ml	-	5 ml

Mixed well and kept for 5 minutes. Read the absorbance of the test and CMG standard against distilled water at 540 nm separately.

Calculation

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \frac{251}{1000} \times 65$$

251 is the dilution factor

1000 is to convert mg/dl to gm/dl

Unit: gHb/ dl (gram hemoglobin/100 ml)

2.2.3.1 Lipid Peroxidation (LPO) levels:

Erythrocyte lipid peroxidation was estimated according to the procedure of Beuge and Aust (1978).

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS) which gives a characteristic pink color that can be measured colorimetrically at 532 nm.

Reagents:

PBS (pH 7.4)	0.1 M
TBA Reagent	TBA 100mg, EDTA 46 mg, 20% TCA 5ml, 2.5 N HCl 5ml and total volume was made up to 20 ml with distilled water
Tetra Methoxy Propene (TMP)	10mM, as standard

Sample preparation:

RBC pellet was prepared after centrifugation of the whole blood at 3000 rpm for 10 minutes. RBC pellet was washed thrice with PBS. 40µl of RBC pellet was added to 960µl of distilled water and shaked well.

Protocol

Reagents	Blank	Test
Sample	-	1.0ml
Distilled water	1.0ml	-
TBA reagent	1.0ml	1.0ml

The tubes were kept in boiling water bath for 20min, cooled under running tap water, centrifuged at 3000 rpm for 15 minutes and read the absorbance of the supernatant at 532nm.

Calculation: Calculation was done according to the slope calculated from the standard graph of TMP

$$\frac{\text{OD of the Test}}{\text{Slope}} \text{ X } \frac{100}{\text{gHb}}$$

Units – nmoles of MDA formed/ g Hb

2.2.3.2 Superoxide Dismutase 1 (SOD1) estimation:

The estimation of SOD1 activity in erythrocytes was carried out by the method of Marklund and Marklund (1974) with slight modification utilizing the inhibition of auto-oxidation of pyrogallol by SOD1 enzyme. Erythrocyte sediment was washed thrice with PBS. Hemolysate was prepared with hemoglobin concentration of about 1g Hb/dl. The final assay mixture contained 3 ml of Tris buffer (200 mM, pH 8.2) containing air equilibrated 0.2 mM pyrogallol (Merck, India), 1 mM EDTA and 2ul of 1:10 diluted erythrocyte lysate as an enzyme source.

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD1 present in hemolysate. Decrease in autooxidation shows indirect evidence of SOD1 activity.

Reagents:

1. Tris buffer 100mM (pH 8.2)
2. Pyrogallol (0.2 mM) dissolved in 0.5 N HCl

Protocol:

The 1g Hb/dl. of hemolysate was prepared and O.D. was adjusted to 0.08-0.12 by auto oxidation of pyrogallol at 420 nm and the assay system was followed as given below:

Reagents	Blank	Control	Test
Buffer	1.500 ml	1.500 ml	1.500 ml
DDW	1.350 ml	1.348 ml	1.348 ml
Pyrogallol	0.15 ml	0.15 ml	0.15 ml
Hemolysate (1g Hb/dl.)	-	2 ul	2 ul
Total volume	3 ml	3 ml	3 ml

In all tubes, the reaction was started by the addition of pyrogallol (0.2 mM) and the change in optical density was recorded for 3 mins at an interval of 5 sec at 420 nm. The initial 10 sec were considered as the induction period of the enzyme. The % inhibition of the test system was calculated from the standard graph generated from the auto-oxidation of pyrogallol. The SOD1 activity was expressed in units/ gHb/min. One unit of SOD1 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

2.2.4 Determination of *SOD1* and *GAPDH* mRNA expression:

2.2.4.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

2.2.4.2 Real-time PCR:

The expression of *SOD*1 and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out on the product formed (Figures 3C & D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

2.2.5 Western blot analysis of SOD1 and GAPDH:

The hemolysate containing 0.5 g% Hb was run on 15% SDS PAGE under reducing conditions (160 mM DTT) along with the low MW protein marker and electro blotted onto PVDF membrane. The membranes were cut into two pieces above cytochrome c (14 kDa) protein band i.e. the upper piece contained GAPDH (37 kDA) and the lower piece contained SOD1 (16 kDa). The membranes were blocked with 1% BSA in PBS for two hours, added to the lower membrane piece anti superoxide dismutase 1 (Cu/Zn) sheep polyclonal Ab (Calbiochem, Germany) diluted to 1: 5000 with PBS containing 0.1% Tween 20; added to the upper membrane piece anti-GAPDH Rabbit polyclonal Ab (Sigma, USA) diluted 1:10000 with PBS containing 0.1% Tween 20; and both the membrane pieces were incubated for two hours at room temperature. Washed three times with PBS containing 0.2% Tween 20; and added to the lower membrane piece 1:5000 diluted (PBS containing 0.1% Tween 20) secondary antibody (Anti-sheep IgG, Sigma, USA); added to the upper membrane piece 1:3000 diluted (PBS containing 0.1% Tween 20) secondary antibody (Anti-Rabbit IgG, Bangalore Genei, India); and incubated for 1 hour at room temperature. Washed both the membrane pieces thrice with PBS containing 0.15% Tween 20, and developed with

DAB (Diaminobenzidine tetrahydrochloride) in 5 ml PBS containing 50 µl of 50mM $NiCl_2$ and 5µl of 30% H_2O_2.

Reagents for SDS PAGE and Immunoblotting:

Acrylamide monomer solution (25 ml)	
Acrylamide	7.3 g
Bis acrylamide	200 mg
Double distilled water	25 ml

Running gel buffer, pH 8.8 (4X, 50 ml)	
Tris Base (1.5 M)	9.08 g

Stacking gel buffer, pH 6.8 (4X, 50ml)	
Tris Base (0.05 M)	3 g

Tank buffer, pH 8.3 (500 ml)	
Tris Base (0.025 M)	1.52 g
Glycine	7.20 g
SDS 0.1%	5 ml from 10% SDS stock solution

Treatment buffer (2 ml)	
Stacking gel buffer	500 µl
SDS 10%	800 µl
Glycerol	400 µl
2 Mercaptoethanol	200 µl
Bromophenol blue	4 mg
Double distilled water	200 µl

Water saturated butanol	
Butanol	50 ml
Double distilled water	5 ml

After shaking well used the top phase

Running gel, 8% (5ml)	
30% acrylamide solution	1.3 ml
Running gel buffer	1.3 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	3 µl
Double distilled water	2.3 ml

Stacking gel, 5% (4ml)	
30% acrylamide solution	670 µl
Double distilled water	2.7 ml
Stacking gel buffer	500 µl

10% SDS	40 µl
10% APS	40 µl
TEMED	4 µl

Transfer buffer (900 ml)	
Tris	2.72 g
Glycine	12.96 g
Double distilled water	720 ml
Methanol	180 ml

2.2.6 Genomic DNA preparation:

Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analysis.

2.2.7 Genotyping of *SOD1* C/T (Ile40Thr) polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Ile40Thr (C/T) polymorphism of *SOD1* gene (Figure 1A, B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzyme *Acu*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of Ile40Thr (C/T) polymorphism of *SOD1* gene (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with

100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose gel stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *SOD1* C/T (Ile40Thr) SNP genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1804450)				*Acu*I
*SOD*1 C/T F	CAGCCTGGGATTTGGACACAGA	57	360	(295 &
*SOD*1 C/T R	GTATGGGTCACCAGCACAGCA			65 bp)
*SOD*1 expression F	CGAGCAGAAGGAAAGTAATGGACCA	65	110	-
*SOD*1 expression R	AGCCTGCTGTATTATCTCCAAACT			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

2.2.8 High Resolution Melting (HRM) Analysis of *SOD1* gene:

Genomic DNA isolated from blood was used to amplify individual exons of *SOD*1 gene using the oligonucleotide primers (Table 3) using High Resolution Melting (HRM) technique on a Real time PCR machine. Real-time PCR was performed in 20 µl volume using LightCycler®480 HRM Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s,

primer dependent annealing for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a high resolution melting curve analysis was carried out on the product formed as per the manufacturer's instructions.

Table 3. Primers used for HRM analysis of *SOD1* gene.

***SOD*1 Gene**	**Forward Primer**	**Reverse Primer**
Exon 1	**5'AGTCATTCCCGGCCACTCGCGAC3'**	**5'CGGCCTCGCAAACAAGCCTCCGTC3'**
Exon 2	**5'GAGGACACAGGCCTAGAGCAG3'**	**5'CAGCACAGCACACCCACCT3'**
Exon 3	**5'GCTTATCCCAGAAGTCGTGATGC3'**	**5'GCAAAGGTGGGGGAAACACGG3'**
Exon 4	**5'GTGGCATCAGCCCTAATCCATCTG3'**	**5'CTGCAAGTACAGTTTATCTGGATC3'**
Exon 5 Fragment 1	**5'CATCTTTTGGGTATTGTTGGGAGG3'**	**5'GGATACATTTCTACAGCTAGCAGG3'**
Exon 5 Fragment 2	**5'CCTGCTAGCTGTAGAAATGTATCC3'**	**5'CTAAATCTGTTCCACTGAAGCTG3'**

2.2.9 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *SOD1* C/T (Ile40Thr) polymorphism in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *SOD*1 polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.05 were considered as statistically significant. LPO levels, SOD1 activity, relative expression of *SOD1* and SOD1 protein levels in patient and

control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

2.3 RESULTS

2.3.1 Estimation of lipid peroxidation (LPO) levels in vitiligo patients and controls:

Oxidative stress has been suggested to be a primary cause of vitiligo and high lipid peroxidation levels serve as marker for oxidative stress. Hence, LPO levels were estimated in 950 vitiligo patients and 1650 controls. Vitiligo patients showed a significant ($p<0.0001$) increase in LPO levels as compared to controls suggesting increased oxidative stress in vitiligo patients (Figure 1A). Moreover, vitiligo subgroups also showed significant difference in LPO levels. Generalized vitiligo patients had higher levels of LPO as compared to localized vitiligo ($p=0.001$) (Figure 1A). Interestingly, when LPO levels were analyzed based on progression of the disease, patients with active vitiligo showed significantly higher LPO levels as compared to stable vitiligo ($p<0.0001$) (Figure 1B).

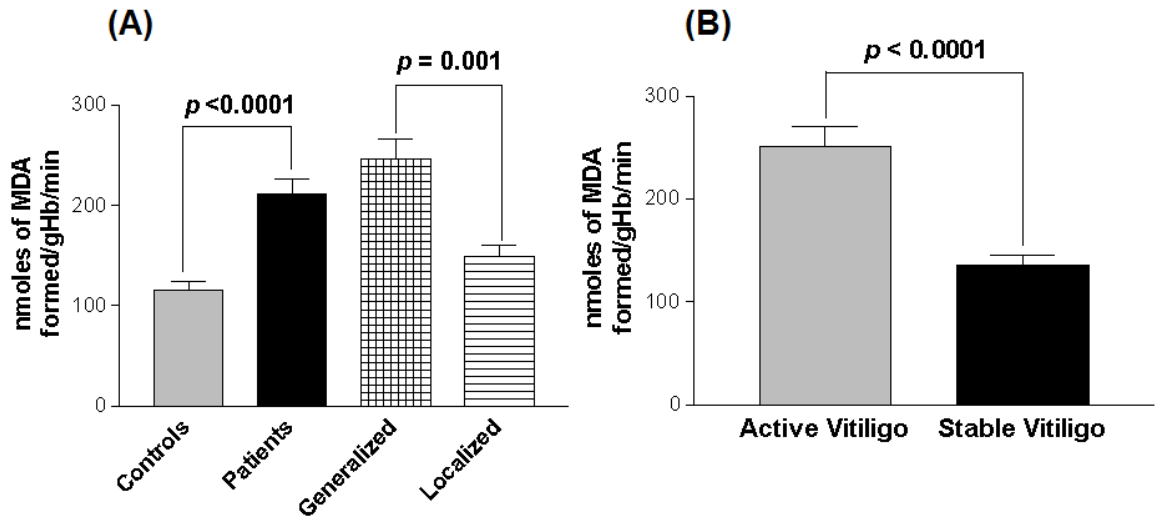


Figure 1. Lipid peroxidation (LPO) levels in controls and vitiligo patients:

(A) LPO levels in 950 vitiligo patients and 1650 controls. Vitiligo patients showed significantly increased LPO levels as compared to controls (Mean ± SEM: 211.6 ± 14.90 vs 115.6 ± 8.949; $p<0.0001$). Generalized vitiligo patients (n=684) showed

significantly higher LPO levels as compared to localized patients (n=266) (Mean ± SEM: 246.3 ± 20.58 vs 149.5 ± 11.16; p=0.001).

(B) LPO levels with respect to progression of the disease in 705 patients with active and 245 patients with stable vitiligo. Active vitiligo patients showed significantly higher LPO levels as compared to stable vitiligo patients (Mean ± SEM: 251.2 ± 19.55 vs 135.8 ± 9.874; p=0.026).

2.3.2 Estimation of Superoxide Dismutase 1 activity in vitiligo patients and controls:

The estimation of SOD1 activity in erythrocytes was carried out in 950 vitiligo patients and 1650 controls. The SOD1 activity was significantly (p<0.0001) higher in vitiligo patients as compared to controls (Figure 2A). Moreover, vitiligo subgroups also showed significant difference in SOD1 activity. Generalized vitiligo patients had significantly increased SOD1 activity as compared to localized vitiligo (p=0.0003) (Figure 2A). Interestingly, when SOD1 activity was analyzed based on progression of the disease, patients with active vitiligo showed significantly higher SOD1 activity as compared to stable vitiligo (p=0.0006) (Figure 2B).

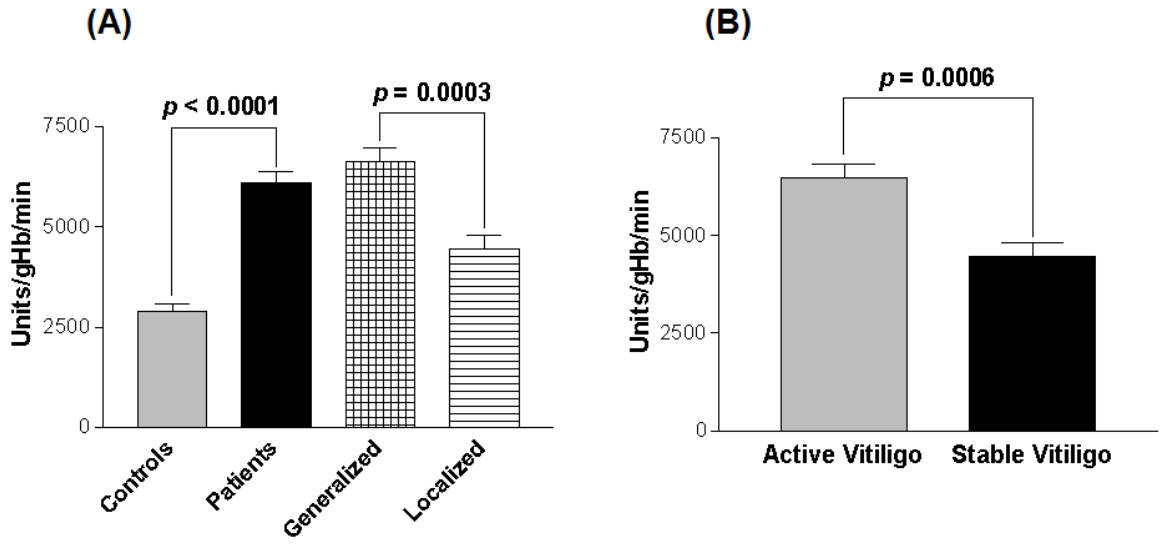


Figure 2. SOD1 activity in controls and vitiligo patients:

(A) SOD1 activity in 950 vitiligo patients and 1650 controls. Vitiligo patients showed significantly increased SOD1 activity as compared to controls (Mean ± SEM: 6088 ± 283.7 vs 2870 ± 193.5; p<0.0001). Generalized vitiligo patients (n=684) showed

significantly increased SOD1 activity as compared to localized patients (n=266) (Mean ± SEM: 6607 ± 365.2 vs 4452 ± 320.1; p=0.0003).

(B) SOD1 activity with respect to progression of the disease in 705 patients with active and 245 patients with stable vitiligo. Active vitiligo patients showed significantly increased SOD1 activity as compared to stable vitiligo patients (Mean ± SEM: 6471 ± 356.4 vs 4440 ± 378.2; p=0.0006).

2.3.3 Determination of SOD1 mRNA and protein levels in vitiligo patients and controls:

2.3.3.1 Relative gene expression of SOD1 in patients with vitiligo and controls:

*SOD1*gene expression studies showed no difference in expression of *SOD1* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.820) (Figure 3A). Moreover, there was no significant difference in expression of *SOD1* between generalized and localized vitiligo patients (p=0.250) (Figure 3A). In addition, we also checked the effect of *SOD1* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3B); however, no significant difference was observed between active and stable vitiligo patients (p=0.970). The specificity of the product formed for SOD1 and GAPDH was checked by melt curve analysis (Figure 3C & D).

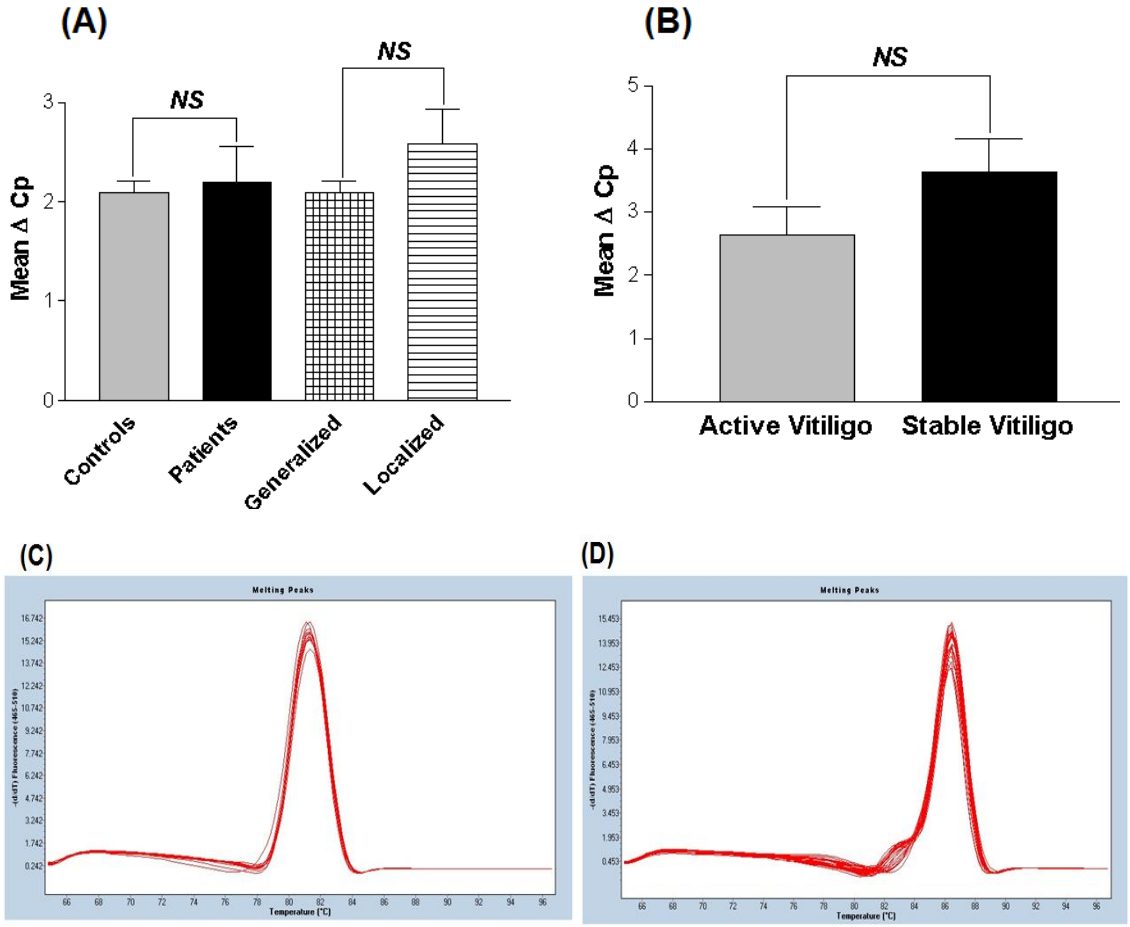


Figure 3. **Relative gene expression of *SOD1* in controls and vitiligo patients:**

(A) Expression of *SOD1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients did not show significant difference in mRNA levels of *SOD1* as compared to controls (Mean ΔCp ± SEM: 2.193 ± 0.3586 vs 2.090 ± 0.1173; p=0.820). No significant difference was observed for *SOD1* mRNA levels between generalized and localized vitiligo patients (Mean ΔCp ± SEM: 2.032 ± 0.1164 vs 2.581 ± 0.3518; p=0.250).

(B) Expression of *SOD1* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. No significant difference was observed for *SOD1* mRNA levels between active and stable vitiligo patients (Mean ΔCp ± SEM: 2.457 ± 0.4170 vs 2.479 ± 0.3443; p=0.970).

(C) & **(D)** Melt curve analysis of *SOD1* and *GAPDH* showing specific amplification.

2.3.3.2 SOD1 protein levels in patients with vitiligo and controls by western blot analysis:

SOD1 protein levels were monitored in human erythrocytes of 50 control subjects and 50 Gujarat vitiligo patients by western blot using GAPDH as an internal control (Figure 4 & 5). However, no significant difference was observed in SOD1 protein levels using densitometric analysis between patients and controls as suggested by mean IDV (integrated density value) (p=0.659; Figure 5A). Moreover, generalized and localized vitiligo groups did not show significant difference in SOD1 protein levels (p=0.214; Figure 5A). Also, no significant difference in SOD1 protein levels was observed between patients with active and stable vitiligo (p=0.256; Figure 5A).

Figure 4. **Western blot analysis of erythrocyte SOD1 and GAPDH protein levels in vitiligo patients and controls:** Representative western blot for SOD1 and GAPDH protein levels showing vitiligo patients as P1, P2, P3 and C1, C2 as healthy controls.

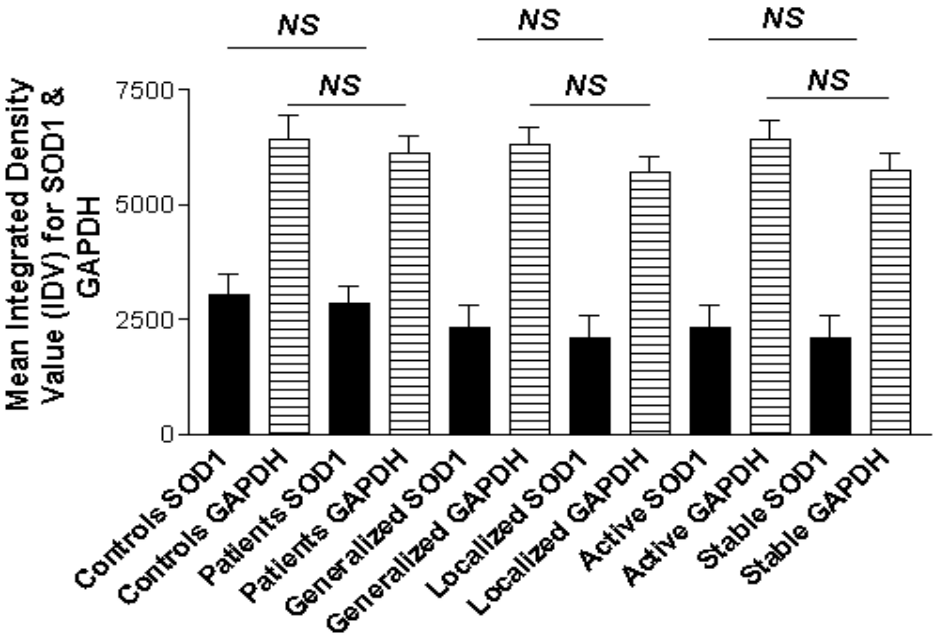


Figure 5. **Densitometric analysis of SOD1 and GAPDH protein levels in controls and vitiligo patients:**

GAPDH protein levels (as IDV) in 50 vitiligo patients and 50 controls. No significant difference was observed in SOD1 protein levels between vitiligo patients and controls (Mean ± SEM: 6130 ± 374.9 vs 6425 ± 530.0; p=0.659). No significant difference was observed in SOD1 protein levels between generalized (n=32) and localized (n=18) vitiligo patients (Mean ± SEM: 6307 ± 361.7 vs 5701 ± 322.1; p=0.214). No significant difference was observed in SOD1 protein levels between active (n=35) and stable (n=15) vitiligo patients (Mean ± SEM: 6404 ± 439.1 vs 5753 ± 364.5; p=0.256).

SOD1 protein levels (as IDV) in 50 vitiligo patients and 50 controls. No significant difference was observed in GAPDH protein levels between vitiligo patients and controls (Mean ± SEM: 2854 ± 382.7 vs 3026 ± 455.4; p=0.776). No significant difference was observed in GAPDH protein levels between generalized (n=32) and localized (n=18) vitiligo patients (Mean ± SEM: 2333 ± 475.9 vs 2110 ± 495.4; p=0.749). No significant difference was observed in GAPDH protein levels between active (n=35) and stable (n=15) vitiligo patients (Mean ± SEM: 2339 ± 459.1 vs 2082 ± 515.8; p=0.713) (NS=Non-significant).

2.3.4 Analysis of association between *SOD1* exon 2 C/T (Ile40Thr) polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD1* C/T (Ile40Thr) polymorphism yielded a 360 bp undigested product corresponding to C allele, 295 bp and 65 bp digested products corresponding to T allele. However, only one genotype 'CC' was identified by 2.5 % agarose gel electrophoresis (Figure 6).

The *SOD1* C/T (Ile40Thr) polymorphism was not found to be associated with vitiligo susceptibility (p=1.00) (Table 4). Interestingly, the polymorphism was found to be monogenic; being only CC alleles presented in control and both the patient groups (Table 4). Also, the distribution of genotype frequencies for *SOD1* C/T (Ile40Thr) polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups (p<0.0001) (Table 4).

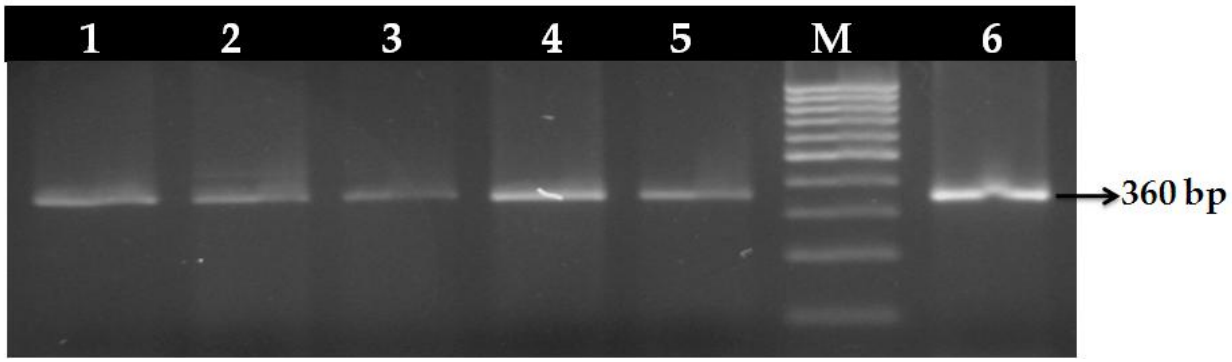


Figure 6. **PCR-RFLP analysis of *SOD1* C/T (Ile40Thr) polymorphism on 2.5 % agarose gel electrophoresis**: lanes: 1, 2, 3, 4, 5 & 6 show individuals with homozygous CC genotypes; lane M shows 100 bp DNA ladder. No individual with homozygous TT and heterozygous CT was found.

Table 4. **Association study for *SOD1* C/T (Ile40Thr) polymorphism in vitiligo patients and controls.**

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE
	Genotype	(n = 285)	(n = 300)		
	CC	285 (1.00)	300 (1.00)		<0.0001[a]
SOD1	CT	00 (0.00)	00 (0.00)	1.000	(P)
C/T	TT	00 (0.00)	00 (0.00)		
(rs1804450)	Allele				<0.0001[b]
	C	570 (1.00)	600 (1.00)	1.000	(C)
	T	00 (0.00)	00 (0.00)		

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

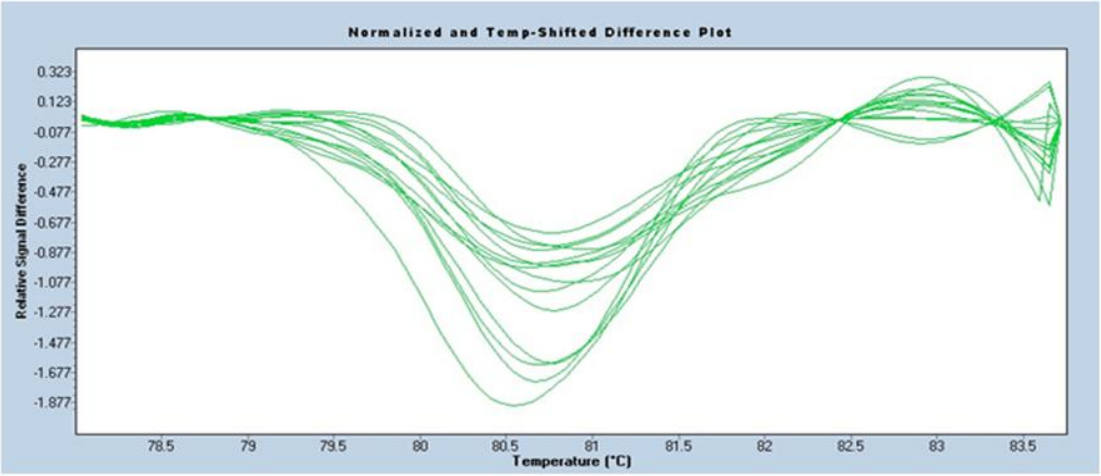
[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.05$.

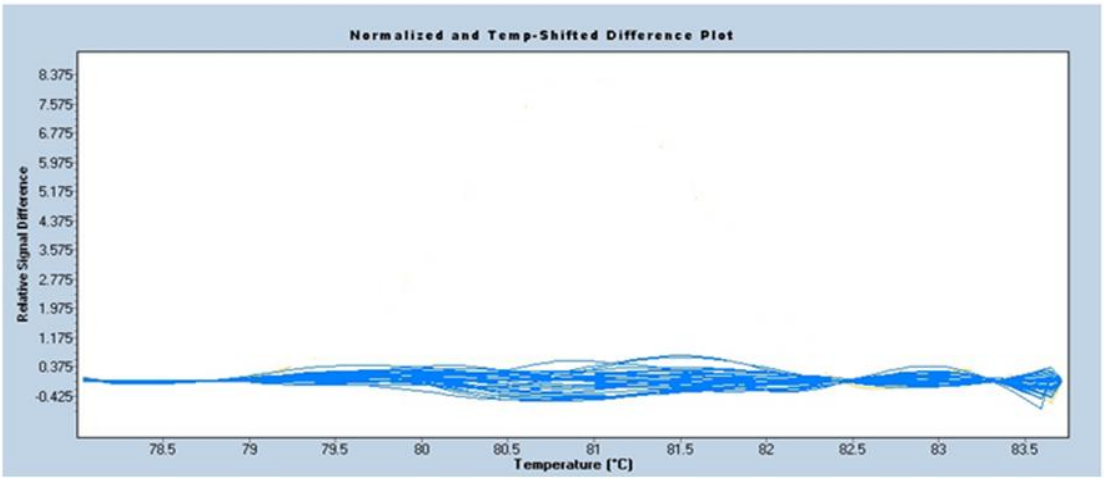
2.3.5 *SOD1* gene scanning for novel genetic variations using High Resolution Melt Curve (HRM) analysis:

Total 62 vitiligo patients and 30 controls were analyzed for any genetic variants in SOD1 gene. *SOD1* gene has five exons and four introns. Each exon of SOD1 amplified was subjected to HRM analysis using Real time PCR (Figure 7). All exons and their exon – intron boundries were amplified except exon 5 which was splitted into two fragments for amplification due to its large size. However, we could not find different groups generated by HRM analysis on difference plot and hence products from the single group were sequenced to confirm the results. Nevertheless, no mutations/SNPs detected based on BLAST search with Human SOD1 wild type sequence (Figure 8).

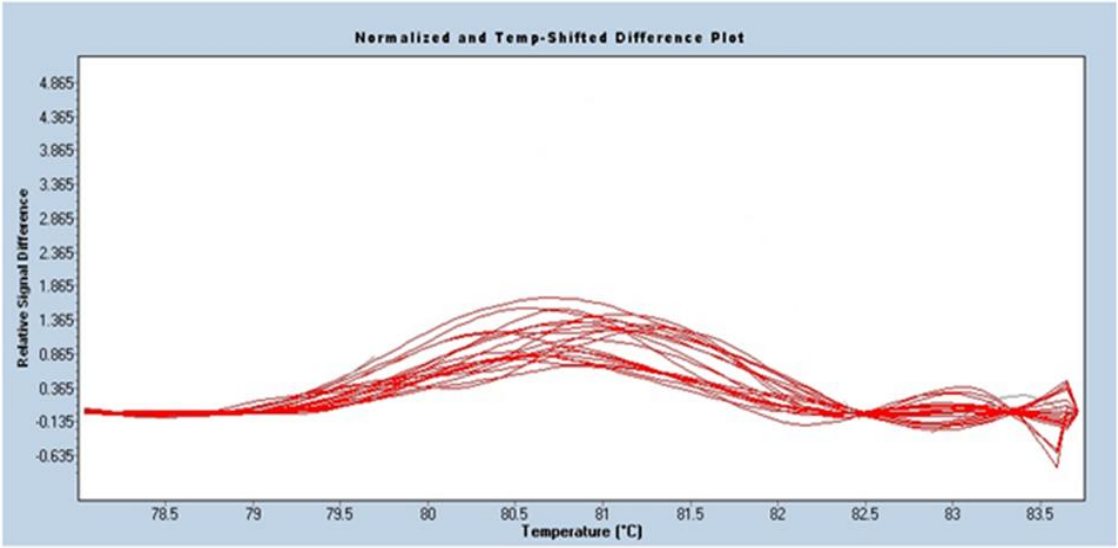
(A) Exon 1



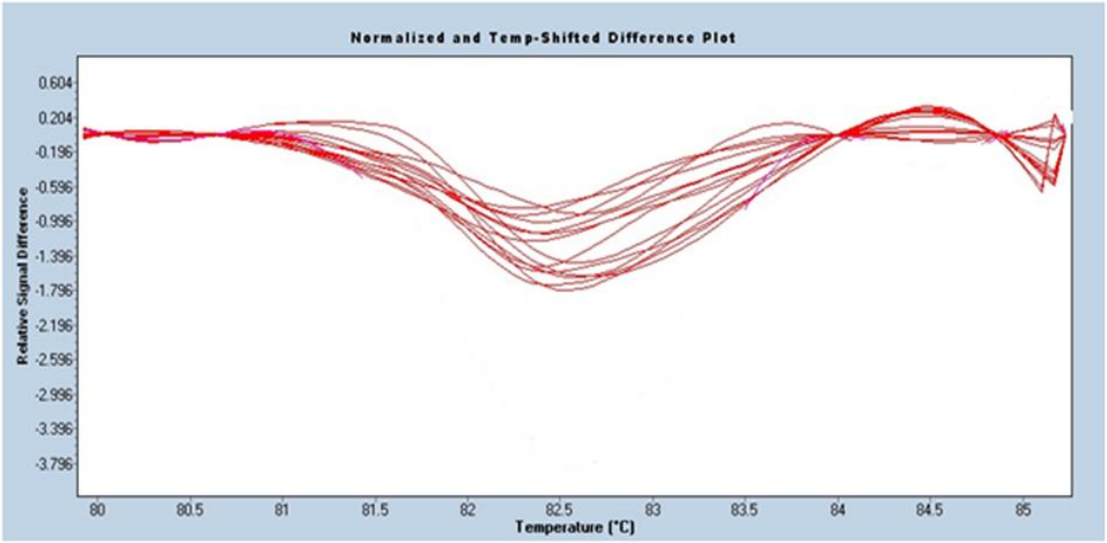
(B) Exon 2



(C) Exon 3



(D) Exon 4



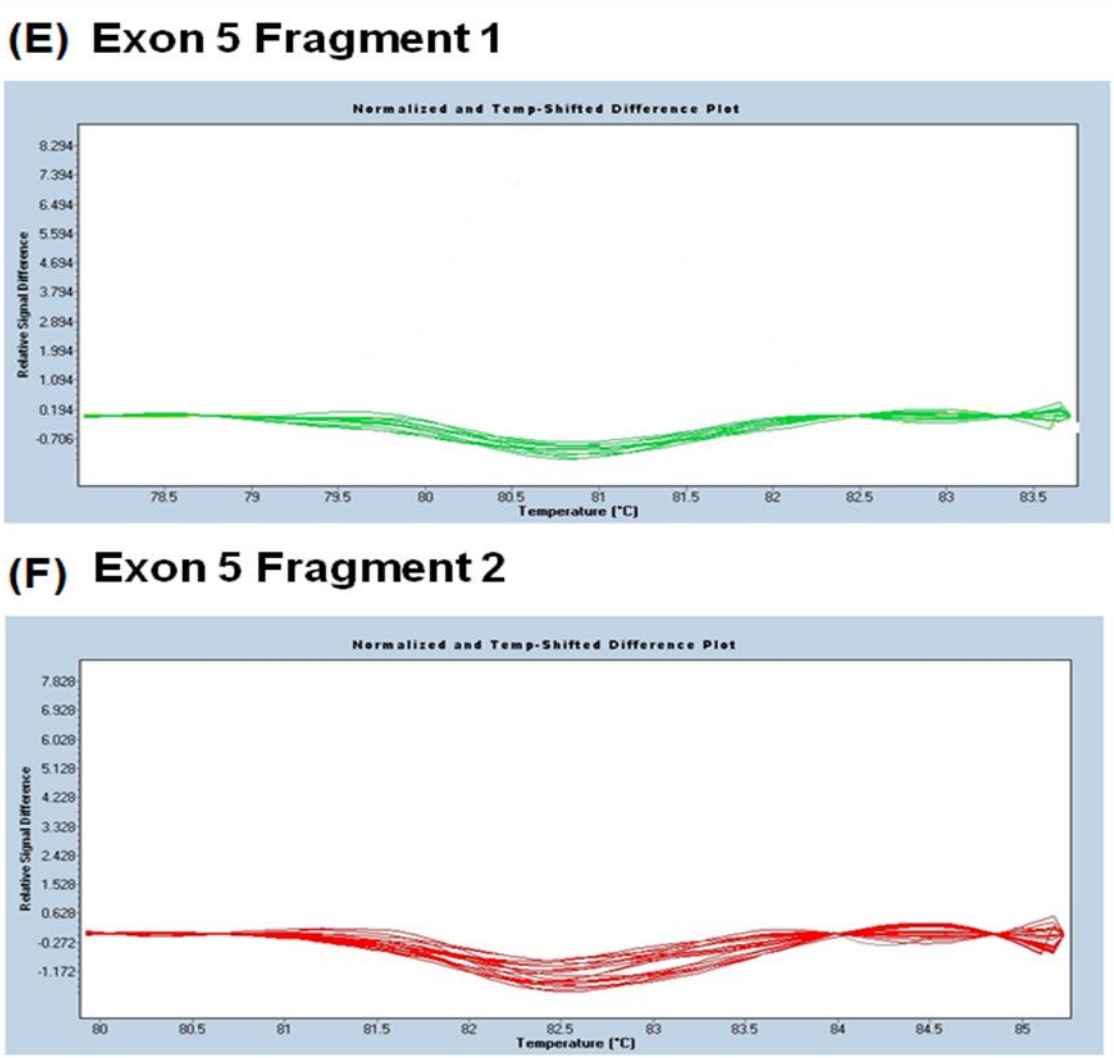


Figure 7. HRM analysis of *SOD1* gene in vitiligo patients and controls.

Figure 8. Representative BLAST analysis for *SOD1 sequence from* vitiligo patient showing 99% homology with the wild type sequence.

2.4 DISCUSSION

Oxidative stress acting as the triggering event in melanocyte degeneration is well established (Picardo et al 1994; Passi et al 1998). Different molecular events which lead to the accumulation of hydrogen peroxide are well documented (Schallreuter et al 1994; 1996; Rokos et al 2002; Gibson and Lilley 1997; Kaufman 1997; Dell'Anna et al 2001; Maresca et al 1997; Beazley et al 1999; Jimbow et al 2001; Schallreuter et al

1991). Hydrogen peroxide thus formed further inhibits epidermal catalase resulting in oxidative stress, which leads to the destruction of melanocytes (Schallreuter et al 1999). Low epidermal catalase levels in both lesional and non-lesional epidermis of vitiligo patients suggests that the entire epidermis may be involved in this disorder (Schallreuter et al 1999). Oxidative stress hypothesis also considers a systemic involvement during the course of the disease (Boisseu-Garsuad et al 2002; Picardo et al 1994; Yildirim et al 2003; Agrawal et al 2004). Thus oxidative stress could act as the initial triggering event in melanocyte degeneration. Local/systemic factors affect the homeostasis of the epidermal melanin unit in segmental vitiligo whereas an impaired redox status of the epidermal melanin unit acts as the primary defect further leading to inappropriate immune response in non-segmental vitiligo (Taieb 2000).

The present study showed significant increase in LPO levels in patients compared to controls suggesting high oxidative stress in vitiligo patients (Figure 1A). Our results are in line with earlier studies (Agrawal et al., 2004; Shajil and Begum, 2006, Shajil et al., 2007; Khan et al., 2009). Moreover, LPO levels were high in patients with generalized vitiligo as compared to localized, suggesting higher level of oxidative stress in generalized cases (Figure 1A). Analysis based on the progression of disease also showed increased oxidative stress in active cases compared to stable cases of vitiligo as LPO levels were significantly increased in active cases implicating the role of oxidative stress in progression of disease (Figure 1B).

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (McCord and Fridovich 1969). In the present study significant increase in erythrocyte SOD1 activity was observed in vitiligo patients (Figure 2A). Interestingly, generalized vitiligo patients in the present study exhibited increased SOD1 activity compared to localized group of patients suggesting the differential role of SOD1 activity in manifestation of vitiligo in different types (Figure 2A). Also an increase in SOD activity was reported by Chakraborty et al (1996), Yildirim et al (2003) and Agrawal et al (2004). However, Picardo et al (1994) reported that SOD activity in erythrocytes of vitiligo patients was not significantly different from the healthy age matched controls (Picardo et al 1994). Passi et al (1998) also showed that there is no significant change in the epidermal SOD levels in vitiligo patients compared to controls (Passi et al 1998). Nevertheless, significant decrease in serum SOD levels was reported by

Koca et al (2004) in generalized vitiligo (Koca et al 2004). Maresca et al (1997) have observed no difference in the SOD activity in cultured vitiliginous melanocytes compared to cultured melanocytes of normal subjects (Maresca et al 1997). The increased activity of SOD1 was consistently high in active cases of vitiligo as compared to stable cases suggesting that SOD1 has an important role in progression of disease (Figure 2B). Our results are in line with those of Jain et al. (2011) suggesting increased SOD1 activity in active cases of vitiligo in Indian population. Previously, Sravani et al. (2009) reported a significant increase in the levels of SOD in vitiliginous and non vitiliginous skin of patient group compared to the control group.

Further, the study was aimed to verify whether the increase in SOD1 activity in vitiligo patients is due to increased mRNA and/or protein levels, relative gene expression and western blot analysis of SOD1 was carried out. The relative gene expression of *SOD1* suggested no significant difference in *SOD1* mRNA levels in patients and controls (Figure 4A). This study was further extended for generalized, localized, active and stable cases of vitiligo which also could not find significant difference in *SOD1* mRNA levels between these groups (Figure 4A & B). Moreover, to confirm the *SOD1* expression results western blot analysis was performed and the results suggested no significant difference in SOD1 protein levels in patients as compared to controls (Figure 5 & 6A). Also the SOD1 protein levels did not differ in generalized, localized, active and stable groups of vitiligo patients (Figure 6 A). These two expression studies of SOD1 confirm that the increased activity of SOD1 in patients as well as different types of vitiligo was not due to increased mRNA and/or protein levels. There are no reports available which showed increased activity of SOD1 without a change in its protein content.

We speculated that the increased activity of SOD1 observed in patients might be due to genetic varaints present in *SOD1* gene exonic regions, as possibility of promoter variants was nullified due to non-significant difference in mRNA and protein levels of SOD1. Thus our results suggested that mutation/s in the exonic regions of *SOD1* gene may enhance the activity of this enzyme. One of the reported SNP in *SOD1* gene is exon 2 C/T (Ile40Thr) which substitutes Ile to Thr. We investigated this polymorphism to find its association with vitiligo; however, the SNP remained

uninformative as only single 'CC' wild type genotype was observed in both control and patient groups (Table 4). This result led us to screen the exonic regions of *SOD1* gene which may harbor novel mutations responsible for increased SOD1 activity. However, *SOD1* gene scanning through high resolution melting (HRM) curve analysis could not find any mutation in the five exonic regions of *SOD1* gene in patients. These results further suggest that increased SOD1 activity may be due to post translational modifications of SOD1.

From the current study, it is clear that increased activity of erythrocyte SOD could enhance the systemic production of H_2O_2 which affects the downstream antioxidant enzymes that neutralize H_2O_2 i.e. catalase and glutathione peroxidase as observed in vitiligo patients (Agrawal et al., 2004). Low levels of catalase or/and GPX in patients could result in excessive production of H_2O_2, which in turn leads to oxidative stress as evident by high LPO levels in vitiligo patients.

In conclusion, impairment of the systemic antioxidant system due to increased SOD1 activity results in oxidative stress in vitiligo patients indicating that melanocyte damage in vitiligo may be linked to generalized oxidative stress. Further, the study proposes that post translational modifications may play an important role in increasing the efficiency of SOD1.

2.5 REFERENCES

Agrawal D, Shajil EM, Marfatia YS and Begum R (2004). Study on the antioxidant status of vitiligo patients of different age groups in Baroda. *Pigment Cell Res.* 17, 289-294.

Beazley WD, Gaze D, Panke A, Panzig E and Schallreuter KU (1999). Serum selenium levels and blood glutathione peroxidase activities in vitiligo. *Br J Dermatol.* 141, 301-303.

Boisseau-Garsuad, P, Lejoly-Boissaeu, H., Robert, M., Quist, D., and Arveiler, B. (2002). Increase in the total antioxidant status and selenium levels in the black patients with active vitiligo. *Int J Dermatol*.10, 640-642.

Chakraborty, D.P., Roy, S., and Chakraborty, A.K (1996). Vitiligo, psoralen, and melanogenesis: some observations and understanding. *Pigment Cell Res*. 9, 107 - 116.

Chang, L.Y., Slot, J.W., Geuze, H.J., and Crapo, J.D. (1988). Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. *J Cell Biol*. 107, 2169–2179.

Cucchi M.L., Frattini, P., Santagostino, G., Preda, S., and Orecchia, G. (2003). Catecholamines increase in the urine of non segmental vitiligo especially during its active phase. *Pigment Cell Res*. 2, 111-116.

Dell'Anna ML, Maresca V, Briganti S, Camera E, Falchi M, and Picardo M (2001). Mitochondrial impairment in peripheral blood mononuclear cells during the active phase of vitiligo. *J Invest Dermatol*. 117, 908–913.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem.* 64, 97-112.

Gibson A., Lilley E (1997). Superoxide anions, free-radical scavengers, and nitrergic neurotransmission.Gen. Pharmacol. ;28:489–493.

Ines D, Sonia B, Riadh BM, Amel EG (2006). A comparative study of oxidant-antioxidant status in stable and active vitiligo patients. *Arch Dermatol Res*. 298:147–52.

Jain A, Mal J, Mehndiratta V, Chander R, Patra SK (2011). Study of oxidative stress in vitiligo. *Indian J Clin Biochem.* 26(1):78-81.

Jimbow, K., Chen, H., Park, J. -S. and Thomas, P. D (2001). Increased sensitivity of melanocytes to oxidative stress and abnormal expression of tyrosinase-related protein in vitiligo. *Br. J. Dermatol*. 144 : 55–65.

Kaufman S (1997): Tetrahydrobiopterin: Basic Biochemistry and Role in Human Disease. Baltimore: *John Hopkins University Press*. p 448.

Keller, G.A., Warner, T.G., Steimer, K.S., and Hallewell, R.A (1991). Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. *Proc. Natl. Acad. Sci. USA*. 88, 7381–7385.

Khan R, Satyam A, Gupta S, Sharma VK, Sharma A (2009). Circulatory levels of antioxidants and lipid peroxidation in Indian patients with generalized and localized vitiligo. *Arch Dermatol Res*. 301(10):731-7.

Koca, R., Armutcu, F., Altinyazar, H. C. and Gurel, A (2004). Oxidant-antioxidant enzymes and lipid peroxidation in generalized vitiligo. *Clin. Exp. Dermatol.* 29:406–409**.**

Le Poole, I.C., Das, P.K., Van Den Wijngaard, R.M., Bose, J.D., and Westerhof, W. (1993). Review of the etiopathomechanism of vitiligo: a convergence theory. *Exp Dermatol*. 2, 146-153.

Maresca, V., Roccella, M., and Roccella, F (1997). Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. *J Invest Dermatol* 109: 310–313

Mohamed L. Salem, C. Marcela Díaz-Montero, Amir A. AL-Khami, Sabry A. EL Naggar, Osama Naga, Alberto J. Montero, Ahmed Khafagy, and David J (2009). Cole Recovery from cyclophosphamide-induced lymphopenia results in expansion of immature dendritic cells which can mediate enhanced prime-boost vaccination anti-tumor responses in vivo when stimulated with the TLR3 agonist poly (I:C). *J Immunology*.(182)15: 2030-40

Ongenae, K., Van Geel, N., and Naeyaert, J.M. (2003). Evidence for an autoimmune pathogenesis of vitiligo. *Pigment Cell Res*. 2, 90-100.

Ortonne, J.P., and Bose, S.K. (1993). Vitiligo: Where do we stand? *Pigment Cell Res.* 8, 61-72.

Passi S, Grandinetti M, Maggio F, Stancato A and De Luca C (1998). Epidermal oxidative stress in vitiligo. *Pigment Cell Res*. 2, 81 - 85.

Picardo, M., Passi, S., Morrone, A., and Grandinetti, M. (1994). Antioxidant status in the blood of patients with active vitiligo. *Pigment Cell Res*. 2, 110-115.

Rokos, H., Beazley, W. D. and Schallreuter, K. U. (2002) Oxidative stress in vitiligo: photo-oxidation of pterins produces H2O2 and pterin-6-carboxylic acid. Biochem. Biophys. Res. Commun. 292, 805–811

Schallreuter KU, Moore J, Wood JM and Beazley WD (2001). Epidermal H2O2 accumulation alters tetrahydrobiopterin (6BH4) recycling in vitiligo: identification of a general mechanism in regulation of all 6BH4-dependent processes. *J Invest Dermatol*. 1, 167-174.

Schallreuter KU, Moore J, Wood JM, Beazley WD and Gaze DC (1999b). In vivo and in vitro evidence for hydrogen peroxide accumulation in the epidermis of patients of vitiligo and its successful removal by UVB activated pseudocatalase. *J Invest Dermatol symposium proceedings*. 4, 91-96.

Schallreuter KU, Wood JM and Berger J (1991). Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol*. 6, 1081-1085.

Schallreuter KU, Wood JM, Pittelkow MR, Buttner G, Swanson N, Korner C and Ehrke C (1996a). Increased monoamine oxidase A activity in the epidermis of patients with vitiligo. *Arch Dermatol Res*. 288, 14-18.

Shajil EM and Begum R (2006). Antioxidant status in segmental and nonsegmental vitiligo. *Pigment Cell Res*. 19 (2), 179-180.

Sravani PV, Babu NK, Gopal KV, Rao GR, Rao AR, Moorthy B, Rao TR (2009). Determination of oxidative stress in vitiligo by measuring superoxide dismutase and catalase levels in vitiliginous and non-vitiliginous skin. *Indian J Dermatol Venereol Leprol*. 75(3):268-71.

Taieb, A. (2000). Intrinsic and extrinsic pathomechanisms in vitiligo. *Pigment Cell Res*. 8, 41-47.

Yildirim, M., Baysal, V., Inaloz, H. S. and Can, M (2004). The role of oxidants and antioxidants in generalized vitiligo at tissue level. *J. Eur. Acad. Dermatol. Venereol.* 18, 683–686.

Yildirim, M., Baysal, V., Inaloz, H.S., Kesici, D., Delibas, N. (2003). The role of oxidants and antioxidants in generalized vitiligo. J Dermatol. 2, 104-108.

3.1 INTRODUCTION

Aerobic organisms have several mechanisms to protect from harmful reactive oxygen species (ROS). The principle cellular anti-oxidants are the superoxide dismutase family (SOD, E.C. 1.15.1.1). These enzymes dismutate superoxide into hydrogen peroxide which is further detoxified by other cellular defences such as glutathione peroxidase and catalase. Superoxide and its products have been implicated in a wide range of diseases including cancer, inflammation, neurodegenerative diseases, diabetes and aging. The SOD family has three members, two of which are Cu-Zn type- the extracellular (EC) SOD3 and the cytoplasmic SOD1. The other member is the mitochondrial Mn type SOD2.

SOD2 is involved in controlling dioxygen toxicity in the mitochondria, an organelle of extreme oxidative load (Fridovich *et al*., 1995). Mitochondria are the primary source of endogenous ROS, since approximately 2% of NADH-derived electrons are transferred to molecular oxygen rather than to ubiquinone by Complex I of the respiratory chain, and this transfer results in the formation of the highly reactive superoxide anion (Turrens and Boveris, 1980). Superoxide is also a by-product of electron transfer by Complex III. Mitochondrial manganese superoxide dismutase is a critical antioxidant protein that reduces superoxide radical to hydrogen peroxide (Klug *et al.*, 1972). Within mitochondria, SOD2 provides a major defence system against oxidative damage by reactive oxygen species generated from mitochondrial electron transport chain during ATP synthesis process.

SOD2 protein is encoded by the nuclear genome and *SOD*2 gene is present on chromosome 6q25. The *SOD*2 gene consists of 5 exons interrupted by 4 introns with typical splice junctions (Wan *et al*., 1994). *SOD*2 mRNA translates in cytosol and translocates into the mitochondria via an N-terminal signal peptide, which plays a key role in targeting the enzyme to mitochondria (Church *et al*., 1990; Church *et al.*,1993). SOD2 docks in the mitochondrial matrix as a homotetramer of subunit mass 23 kDa. Amino acid sequences of SOD2 among species are highly conserved, and their homologies between human and rodent, and between rat and mouse are 93% and 96%, respectively (Ho *et al*., 1998; DiSilvestre *et al*., 1995). This conservation may be critical to maintain its enzymatic function.

SOD2 is regarded as one of the key enzymes involving the anti-oxidation in the clinical disorders. Several studies have suggested the importance of SOD2 in clinical disorders. The SOD2 has been known to be associated with cancers (Li *et al*., 2005), diabetic nephropathy (Nomiyama *et al*., 2003), dilated cardiomyopathy (Shimoda-Matsubayashi *et al*., 1996; Shimoda-Matsubayashi *et al*., 1997; Li *et al*., 1995; Huang *et al*., 2001), neurodegenerative diseases (Hinerfeld *et al*., 2004) and brain ischemia (Kim *et al*., 2002).

An alteration in the antioxidant system, with a significant reduction in catalase activity has been demonstrated in both lesional and non-lesional epidermis of vitiligo patients (Schallreuter *et al.,* 1991) as well as in melanocytes derived from patients (Maresca *et al.,* 1997). Antioxidant imbalance in peripheral blood mononuclear cells of active vitiligo patients is also observed. An increased intracellular production of ROS appeared to be due to mitochondrial impairment (Dell'Anna *et al.,* 2001). These findings support the concept of a possible systemic oxidative stress in vitiligo. Sravani *et al*., 2009 demonstrated significant increase in the levels of SOD and low catalase levels in vitiliginous and non vitiliginous skin of patient group compared to the control group suggesting high oxidative stress in vitiligo patients. Jain *et al*., 2011 reported significantly higher SOD activity in blood of both active and stable vitligo patients as compared to controls. Our previous reports have also shown an imbalance in the antioxidant enzyme system with increased activity of erythrocyte SOD in vitiligo patients from Gujarat suggesting high oxidative stress in patients (Agrawal *et al.,* 2004; Shajil and Begum, 2006).

Nevertheless, the exact genetic defects in antioxidant enzymes that lead to their altered levels/activity leading to oxidative stress mediated damage of melanocytes in vitiligo are still unknown. Also there are no reports on *SOD*2 polymorphisms in vititligo pathogenesis till date. Therefore, addressing the genetic polymorphisms in *SOD*2 gene is essential to understand its role in vitiligo pathogenesis.
We have addressed four *SOD*2 polymorphisms in the present study.

Val16Ala (T/C; rs4880): The SOD2 signal sequence is essential for correct transport activity of proteins by mitochondria (Nelson and Cox 2005). A polymorphism in the second exon of the *SOD*2 gene results in an alanine-to-valine change at amino acid position 16 (Val16Ala) (Shimoda-Matsubayashi *et al*., 1996). This polymorphism

may change the protein conformation and mitochondrial transport of SOD2 (Shimoda-Matsubayashi *et al*., 1996; Rosenblum *et al*., 1996). As a result, the alanine-containing protein shows normal transport and generates 30-40% more active enzyme than does the valine form of the enzyme (Sutton *et al*., 2003). Moreover, the Ala variant of the mitochondrial targetting signal (MTS) has been shown to improve the processing efficiency, resulting in increased protein tetramers of SOD2 in the mitochondria (Sutton *et al.,* 2003). Clinically different genetic variants were associated with different diseases, such as Ala/Ala with increased risk for cancers (Li *et al.*,2005; Mikhak *et al.*,2008) and Val/Val homozygosity with the development of diabetic nephropathy (Nomiyama *et al.*,2003; Liu *et al.*,2009). Fujimoto *et al*. (2008) found an association between the alanine variant of the signal peptide and increased mitochondrial SOD2 activity, which protects macrophages from the oxLDL-induced apoptosis and reduces the risk of acute coronary syndrome and cardiovascular diseases.

Thr58Ile (C/T; rs35289490): A polymorphic locus, amino acid codon +58 (T5777C, Ile or Thr allele) located in the exon 3, is related to the potency of SOD2. In Thr58Ile polymorphism, SOD2 with threonine has a weaker antioxidant activity than that with isoleucine (Ho *et al.*,1998). The polymorphism, Thr58Ile, affects the stability of protein tetrameric interface and reduces the biological activity of SOD2 (Borgstahl *et al*., 1996; Ho and Crapo, 1988). The +58Ile protein form showed a three-fold higher activity than the +58Thr form (Zhang *et al*., 1999). Besides, from the molecular viewpoint, the unique genetic expression of Ile/Ile homozygosity at codon +58 implies the conserved functional domain of SOD2 structure. The *SOD*2 gene with T at nucleotide position 5777 encodes a native form of SOD2, which has a stable tetrameric interface. However, *SOD*2 gene with a nucleotide sequence 5777C encodes a mutant form that has increased thermal instability and accelerated thermal inactivation (Borgstahi 1992; Borgstahl *et al.,* 1996).

Both the above polymorphisms with substituted amino acids altered the SOD2 enzyme activity (Martin *et al.*,2005; Zhang *et al.*,1999; Sutton *et al.*,2003) resulting in altered anti-oxidative metabolism. In addition, two non-synonymous SNPs [Leu84Phe (C/T; rs11575993) and Ile82Thr (T/C; rs1141718)] in *SOD*2 gene have also been

addressed in the present study. However, the functional significance of these two SNPs has not been reported.

In the present study, we have made an attempt to understand the role of SOD2 in vitiligo pathogenesis and the objectives of this study were:

i) To determine whether the following four polymorphisms of *SOD*2 are associated with vitiligo susceptibility and modulate SOD2 activity.
   a) Val16Ala (T/C; rs4880)
   b) Thr58Ile (C/T; rs35289490)
   c) Ile82Thr (T/C; rs1141718)
   d) Leu84Phe (C/T; rs11575993)

ii) To measure and compare *SOD*2 transcript levels and activity in patients with vitiligo and in unaffected controls.

iii) To correlate *SOD*2 polymorphisms, expression levels and its activity with progression of the disease.

3.2 MATERIALS AND METHODS

3.2.1 Study Subjects:

The study group included 520 vitiligo patients comprised of 228 males and 292 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of seven hundred and fifty ethnically, sex-matched unaffected individuals comprised of 338 males and 412 females were included as controls in this study (Table 1). None of the healthy individuals 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.

3.2.2 Blood collection and genomic DNA preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analysis.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 520)	(n = 750)
Average age (mean age ± SD)	32.45 ± 13.48 yr	28.23 ± 14.42 yr
Sex: Male	228 (43.85%)	338 (45.07%)
Female	292 (56.15%)	412 (54.93%)
Onset age (mean age ± SD)	21.25 ± 12.53 yr	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yr	NA
Type of vitiligo		
Generalized	364 (70.00)	NA
Localized	156 (30.00)	NA
Active vitiligo	385 (74.04)	NA
Stable vitiligo	135 (25.96)	NA
Family history	69 (13.27%)	NA

3.2.3 Genotyping of *SOD*2 Leu84Phe (C/T) and Thr58Ile (C/T) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Leu84Phe (C/T) and Thr58Ile (C/T) polymorphisms of *SOD*2 gene (Figure 1A, B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and

reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes *Ear*I and *Eco*RV (New England Biolabs, Beverly, MA) were used for digesting amplicons of Leu84Phe (C/T) and Thr58Ile(C/T) polymorphisms of *SOD*2 gene (Table 2) respectively. 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose or 20% polyacrylamide gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

3.2.4 Genotyping of *SOD*2 Val16Ala (T/C) and Ile82Thr (T/C) polymorphisms:

The genotyping of Val16Ala (T/C) and Ile82Thr (T/C) SNPs of *SOD*2 was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480 Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay IDs: C_8709053_10 and C_32382851_10 respectively; Life Technologies Corp., California, USA). Real-time PCR was performed in 10 µl volume using LightCycler® 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay.

Table 2. Primers and restriction enzymes used for *SOD*2 Leu84Phe (C/T; rs11575993) and Thr58Ile(C/T; rs35289490) SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs11575993)				
*SOD*2 C/T F	AGATCCCTGAGCCTAGCAG	54	280	*Ear*I (240 & 40 bp)
*SOD*2 C/T R	CAATCGATTCCTACTGTGCAC			
(rs35289490)				
*SOD*2 C/T F	CAGTCTGTATGTTGAGCCATAC	58	195	*Eco*RV (177 & 18 bp)
*SOD*2 C/T R	CAGTGCAGGCTGAAGAGAT			
*SOD*2 expression F	GTTGGCCAAGGGAGATGTTACAG	65	138	-
*SOD*2 expression R	CAACTCCCCTTTGGGTTCTCCAC			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

3.2.5 Estimation of Superoxide dismutase 2 activity:

The estimation of SOD2 activity in WBC lysate was carried out by the method of Marklund and Marklund (1974) with slight modifications utilizing the inhibition of auto-oxidation of pyrogallol by SOD2 enzyme. The final assay mixture contained 3 ml of Tris buffer (200 mM, pH 8.2) containing air equilibrated 0.2 mM pyrogallol (Merck, India), 1 mM EDTA and 2ul of 1:10 diluted WBC lysate as an enzyme source. 4mM of cyanide was used to inhibit SOD1 and SOD3 activity. The concentration of protein in the WBC lysate was estimated by Lowry's method (Lowry *et al*., 1951).

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD2 present in WBC lysate. Decrease in autooxidation shows indirect evidence of SOD2 activity.

Reagents:

1. Tris buffer 100mM (pH 8.2)

2. Pyrogallol (0.2 mM) dissolved in 0.5 N HCl

3. 4mM Cyanide

Protocol:

The 1:10 dilution of WBC lysate was prepared and O.D. was adjusted to 0.08-0.12 by auto oxidation of pyrogallol at 420 nm and the assay system was followed as given below:

Reagents	**Blank**	**Control**	**Test**
Buffer	1.500 ml	1.500 ml	1.500 ml
DDW	1.350 ml	1.348 ml	1.348 ml
Pyrogallol	0.15 ml	0.15 ml	0.15 ml
WBC lysate (1:10)	-	2 ul	2 ul
Total volume	3 ml	3 ml	3 ml

In all tubes, the reaction was started by the addition of pyrogallol (0.2 mM) and the change in optical density was recorded for 3 mins at an interval of 5 sec at 420 nm. The initial 10 sec were considered as the induction period of the enzyme. The % inhibition of the test system was calculated from the standard graph generated from the auto-oxidation of pyrogallol. The SOD2 specific activity was expressed in units/mg of WBC lyate protein.

Unit: One unit of SOD2 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation.

Specific activity: Units of enzyme/ mg of protein

Folin Lowry Method For Protein Estimation: The method involves both the Biuret reaction, where the peptide bonds of proteins react with Cu^{2+}under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent and the Folin-Ciocalteau reaction which is poorly understood but essentially involves the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids. The resultant strong blue color is therefore partly dependent on the tyrosine and tryptophan content of the protein sample.

Reagents:

BSA	1mg /ml
Solution A	10% Na_2CO_3, 2%NaOH, 0.1%NaK tartarate
Solution B	0.5%$CuS0_4.5H_20$
Solution C (freshly prepared): 10ml soln A+40 ml D/W+1 ml soln B	
FC Reagent: Folin Ciocalteau reagent (1:1 dilution)	

Briefly, 2.5 ml of solution C was added to 0.05 ml of WBC lysate, mixed well and incubated for 20 min. Then, 0.25 ml of FC Reagent was added, mixed well and incubated for 45 minutes in dark followed and absorbance was measured at 660nm.

3.2.6 Determination of *SOD*2 and *GAPDH* mRNA expression:

3.2.6.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

3.2.6.2 Real-time PCR:

The expression of *SOD*2 and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data

collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 3D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

3.2.7 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the four polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *SOD*2 polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.0125 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.

Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi et al., 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett et al., 2005).

SOD2 specific activity and relative expression of *SOD*2 gene in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al*., 2007).

3.3 RESULTS

3.3.1 Analysis of association between *SOD*2 C/T; Leu84Phe polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD*2 C/T Leu84Phe polymorphism yielded a 280 bp undigested product corresponding to T allele, and 240 bp and 40 bp digested products corresponding to C allele. The three genotypes identified by 2.5 % agarose gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for Leu84Phe polymorphism of *SOD*2 gene (Figure 1A).

The genotype and allele frequencies of the C/T Leu84Phe polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The Leu84Phe polymorphism of *SOD*2 gene was found to be in significant association with vitiligo patients ($p<0.0001$) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (T) of Leu84Phe polymorphism was more frequent in the vitiligo group compared to the control group (25.0% versus 11.0%, $p<0.0001$) (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism ($p=0.481$ and $p=0.066$ respectively) (Table 3).

Moreover, both generalized and localized vitiligo groups showed significant association of Leu84Phe polymorphism when the genotypes were compared with those of control group ($p<0.0001$) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group ($p<0.0001$) (Table 4). The distribution of *SOD2* Leu84Phe genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups ($p>0.05$).
Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Leu84Phe 'T' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (27.00% versus 11.00%, $p<0.0001$) (Table 5). Also, there was statistically significant difference in the distribution of the Leu84Phe 'T' allele between patients with stable vitiligo and control group (19.00% versus 11.00%, $p=0.001$) (Table 5). Interestingly, the Leu84Phe 'T' allele showed significant difference between patients with active vitiligo as compared to stable

vitiligo (27.00% versus 19.00%, p=0.009) (Table 5). This study has 88.76% statistical power for the effect size 0.8 to detect association of Leu84Phe polymorphism of *SOD*2 at p<0.05 in patients and control population.

3.3.2 Analysis of association between *SOD*2 C/T; Thr58Ile polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD*2 C/T (Thr58Ile) polymorphism yielded a 195 bp undigested product corresponding to 'C' allele, 177 bp and 18 bp digested products corresponding to 'T' allele. The three genotypes identified on 15 % polyacrylamide gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for C/T (Thr58Ile) polymorphism of *SOD*2 gene (Figure 1B).

The genotype and allele frequencies of the C/T (Thr58Ile) polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The C/T (Thr58Ile) polymorphism was found to be in significant association with vitiligo patients (p<0.0001) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (T) of Thr58Ile polymorphism was more frequent in the vitiligo group compared to the control group (41.0% versus 31.0%, p<0.0001) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.079 and p=0.068 respectively) (Table 3).

Moreover, both generalized and localized vitiligo groups showed significant association of Thr58Ile polymorphism when the genotypes were compared with those of control group (p<0.0001) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group (p<0.0001) (Table 4). The distribution of *SOD2* Thr58Ile genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Thr58Ile 'T' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (46.00% versus 31.00%, p<0.0001) (Table 5). However, there was no significant difference in the distribution of Thr58Ile 'T' allele between patients with stable vitiligo and control group (p=0.132) (Table 5).

Interestingly, the Thr58Ile 'T' allele showed significant difference between patients with active vitiligo as compared to stable vitiligo (46.00% versus 27.00%, p<0.0001) suggesting its role in progression of the disease (Table 5). This study has 88.76% statistical power for the effect size 0.8 to detect association of Thr58Ile polymorphism of *SOD2* at p<0.05 in patients and control population.

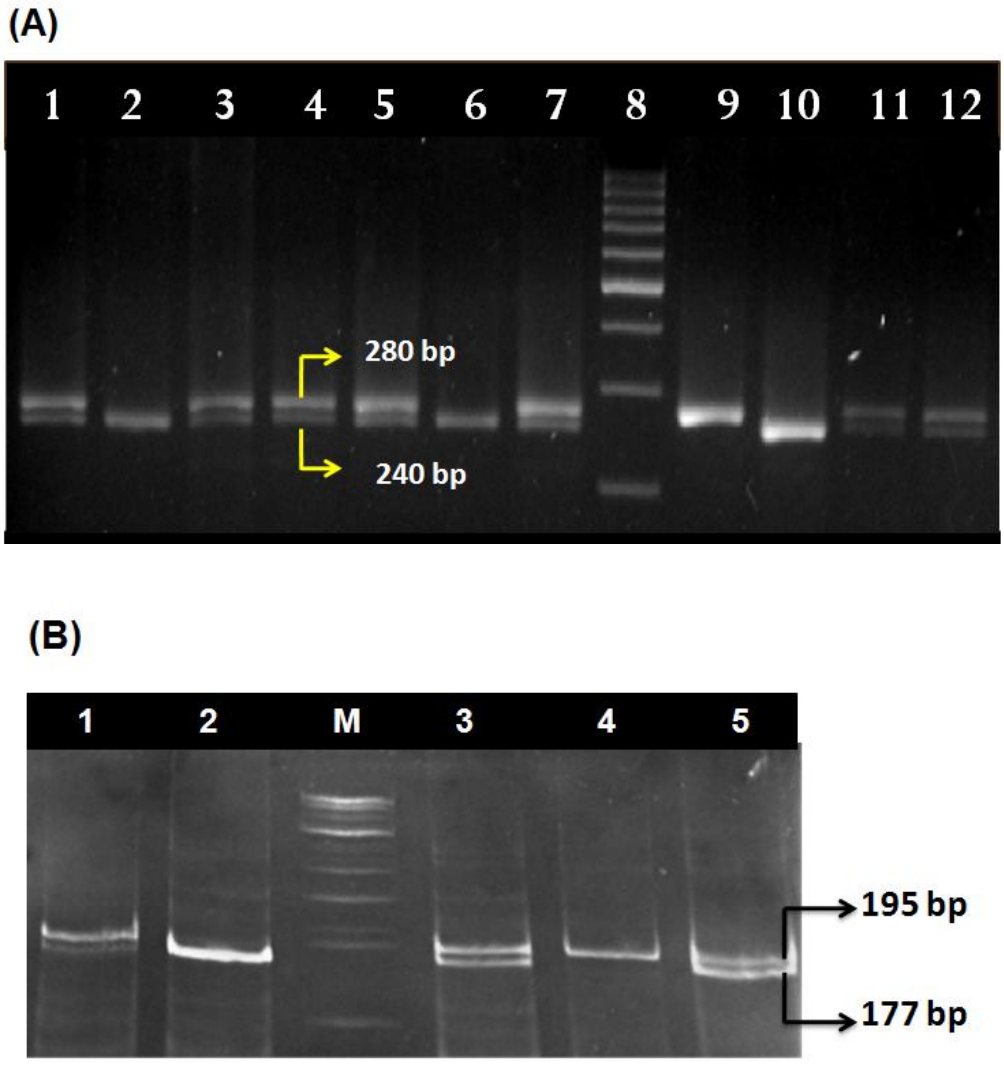


Figure 1. PCR-RFLP analysis of *SOD2* C/T (Leu84Phe) and C/T (Thr58Ile) polymorphisms:

(A) PCR-RFLP analysis of *SOD2* C/T (Leu84Phe) polymorphism on 2.5% agarose gel electrophoresis: lanes: 1, 3, 4, 5, 7, 11 & 12 show heterozygous (CT) genotypes; lane: 2, 6 & 10 show homozygous (CC) genotype; lane: 9 shows homozygous (TT) genotype; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *SOD2* C/T (Thr58Ile) polymorphism on 20% polyacrylamide gel electrophoresis: lanes: 1, 3, & 5 show heterozygous (CT) genotypes; lane: 2 shows homozygous (TT) genotype; lane: 4 shows homozygous (CC) genotype; lane M shows 100 bp DNA ladder.

3.3.3 Analysis of association between *SOD*2 T/C; Val16Ala polymorphism and susceptibility to vitiligo:

Real- time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'T' allele) and VIC (for 'C' allele) fluorophores for *SOD*2 T/C (Val16Ala) polymorphism which yielded the three genotypes (TT homozygous, TC heterozygous and CC homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2A).

The genotype and allele frequencies of the T/C (Val16Ala) polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The T/C (Val16Ala) polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.491; p=0.322) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.354 and p=0.973 respectively) (Table 3). Also, both generalized and localized vitiligo groups did not show significant association of T/C (Val16Ala) polymorphism when the genotypes were compared with those of control group (p=0.569; p=0.745 respectively) (Table 4). The distribution of *SOD*2 T/C (Val16Ala) genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Val16Ala 'C' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (51.00% versus 41.00%, p=0.006) (Table 5). However, there was no statistically significant difference in the distribution of the Val16Ala 'C' allele between both the groups of patients with active and stable vitiligo and control group (p=0.040; p=0.112) (Table 5). This study has 88.76% statistical power for the effect size 0.08 to detect the association of *SOD*2 T/C

(Val16Ala) polymorphism at $p<0.05$ in generalized vitiligo patients and control population.

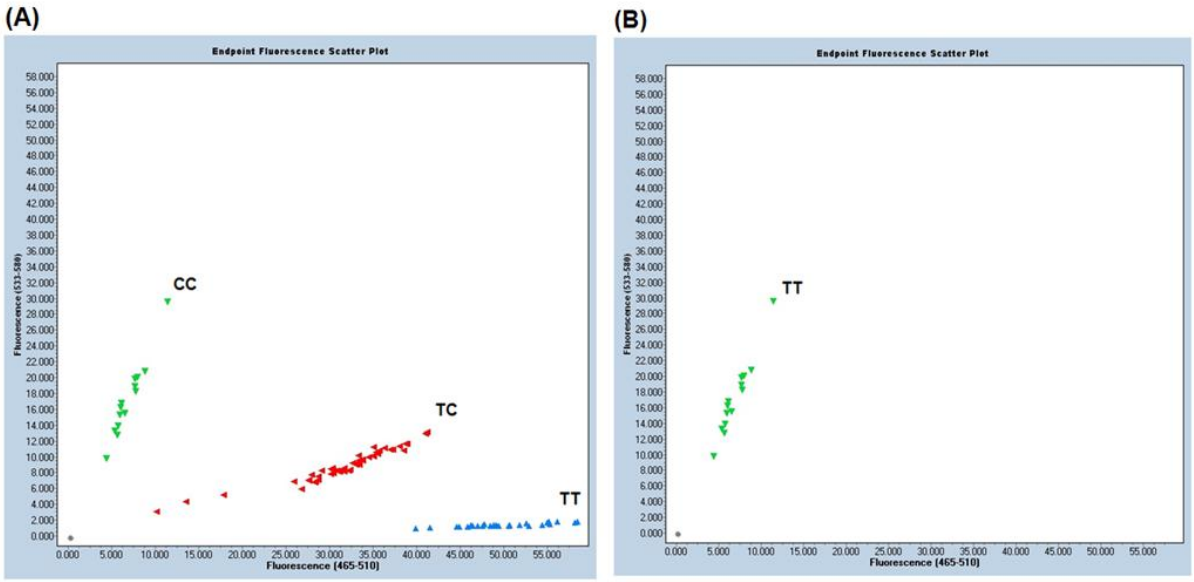


Figure 2. (A) TaqMan end point fluoroscence analysis for *SOD2* Val16Ala (T/C) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: TT, TC and CC, based on fluorescence with Channel 465-510 (FAM for 'T' allele) and Channel 536-580 (VIC for 'C' allele).

(B) TaqMan end point fluoroscence analysis for *SOD2* Ile82Thr (T/C) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. Only single genotype was identified as: TT based on fluorescence with Channel 536-580 (VIC for 'T' allele). There was no fluorescence with Channel 465-510 (FAM for 'C' allele). A no-template control (NTC) was used with each SNP genotyping assay (shown as grey spot).

3.3.4 Analysis of association between *SOD*2 T/C; Ile82Thr polymorphism and susceptibility to vitiligo:

Real time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'C' allele) and VIC (for 'T' allele) fluorophores for *SOD*2 T/C (Ile82Thr) polymorphism which yield the three genotypes

(TT homozygous, TC heterozygous and CC homozygous) however, in the present study only single genotype was identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2B).

The *SOD2* Ile82Thr (T/C) polymorphism was not found to be associated with vitiligo susceptibility (p=1.00) (Table 3). Interestingly, the polymorphism was found to be monogenic; being only TT alleles presented in control and both the patient groups and hence further analysis was not performed for this polymorphism (Table 4). Also, the distribution of genotype frequencies for *SOD2* Ile82Thr (T/C) polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups (p<0.0001).

Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the three polymorphisms [C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala)] investigated in the *SOD2* gene were in low LD association [**C/T: C/T** (D' =0.085, r^2=0.002); **C/T: T/C** (D'= 0.081, r^2=0.001); **C/T: T/C** (D'= 0.073, r^2=0.003)]. A haplotype evaluation of the three polymorphic sites was performed and the estimated frequencies of the haplotypes differed significantly between vitiligo patients and controls (global p-value=6.33e-032) (Table 6). Intersetingly, the frequency of susceptible haplotype 'TTC' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls (5.1% vs 1.1%; p=1.98e-008) and increased the risk of vitiligo by 4.6-fold [odds ratio (OR): 4.696; 95% confidence interval (CI): (2.601~8.477)] (Table 6). Also three other haplotypes with one or two susceptible alleles: 'TTT' and 'TCT' and 'TCC' were significantly increased in vitiligo patients as compared to controls (p=2.78e-006, p=1.13e-014; p=3.97e-015). However, the wild type haplotype 'CCT' was more frequently observed in control group as compared to the patient group (30.1% vs 21.5%; p=1.04e-005) (Table 6).

Table 3. Association studies for *SOD2* gene, C/T (Leu84Phe), C/T (Thr58Ile), T/C (Val16Ala) and T/C (Ile82Thr) polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 520)	(n = 750)			
	CC	297 (0.57)	599 (0.80)		0.481	
	CT	188 (0.36)	137 (0.18)	<0.0001	(P)	
rs11575993	TT	35 (0.07)	14 (0.02)			
(C/T;	Allele				0.066	0.3746
Leu84Phe)	C	782 (0.75)	1335(0.89)	<0.0001	(C)	(0.3023-
	T	258 (0.25)	165 (0.11)			0.4642)
	Genotype	(n = 520)	(n = 750)			
	CC	189 (0.36)	364 (0.49)		0.068	
	CT	232 (0.45)	302 (0.40)	<0.0001	(P)	
rs35289490	TT	99 (0.19)	84 (0.11)			
(C/T;	Allele				0.079	0.6473
Thr58Ile)	C	610 (0.59)	1030 (0.69)	<0.0001	(C)	(0.5490-
	T	430 (0.41)	470 (0.31)			0.7633)
	Genotype	(n = 520)	(n = 750)			
	TT	140 (0.27)	225 (0.30)		0.973	
rs4880	TC	260 (0.50)	360 (0.48)	0.491	(P)	
(T/C;	CC	120 (0.23)	165 (0.22)			
Val16Ala)	Allele				0.354	0.9200
	T	540 (0.52)	810 (0.54)	0.322	(C)	(0.7852-
	C	500 (0.48)	690 (0.46)			1.078)
	Genotype	(n=520)	(n = 750)			
	TT	520 (1.00)	750 (1.00)		<0.0001	
rs1141718	TC	0 (0.00)	0 (0.00)	1.000	(P)	
(T/C;	CC	0 (0.00)	0 (0.00)			
Ile82Thr)	Allele				<0.0001	
	T	1040(1.0)	1500 (1.00)	1.000	(C)	
	C	0 (0.00)	0 (0.00)			

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 4. Association studies for SOD2 gene, C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo (Freq.)	Localized Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 364)	(n = 156)	(n = 750)		0.875	
	CC	196(0.54)	101 (0.65)	599(0.80)	<0.0001[a]	(GV)	2.919[c]
	CT	143(0.39)	45 (0.29)	137(0.18)	<0.0001[b]		(2.317-
rs11575993	TT	25(0.07)	10 (0.06)	14 (0.02)		0.117	3.677)
(C/T;	Allele					(LV)	
Leu84Phe)	C	535(0.73)	247 (0.79)	1335(0.89)	<0.0001[c]		2.129[d]
	T	193(0.27)	65 (0.21)	165(0.11)	<0.0001[d]	0.066	(1.550-
						(C)	2.925)
	Genotype	(n = 364)	(n = 156)	(n = 750)		0.433	
	TT	105(0.29)	84 (0.54)	364(0.49)	<0.0001[a]	(GV)	0.4248[c]
	TC	174(0.48)	58 (0.37)	302(0.40)	<0.0001[b]		(0.3185-
rs35289490	CC	85 (0.23)	14 (0.09)	84 (0.11)		0.389	0.5665)
(C/T;	Allele					(LV)	
Thr58Ile)	T	384(0.53)	226 (0.72)	1030(0.69)	<0.0001[c]		0.5094[d]
	C	344(0.47)	86 (0.28)	470 (0.31)	<0.0001[d]	0.079	(0.4247-
						(C)	0.6110)
	Genotype	(n= 364)	(n = 156)	(n = 750)		0.978	
	TT	98 (0.27)	42 (0.27)	225(0.30)	0.569[a]	(GV)	0.9200[c]
rs4880	TC	182(0.50)	78 (0.50)	360 (0.48)	0.745[b]		(0.7705-
(T/C;	CC	84 (0.23)	36 (0.23)	165 (0.22)		0.985	1.099)
Val16Ala)	Allele					(LV)	
	T	378(0.52)	162 (0.52)	810 (0.54)	0.381[c]		0.9200 [d]
	C	350(0.48)	150 (0.48)	690 (0.46)	0.533[d]	0.354	(0.7206-
						(C)	1.175)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(GV) refers to Generalized Vitiligo, (LV) Localized Vitiligo and (C) refers to Controls,

[a] Generalized Vitiligo vs. Controls using chi-squared test with 3 × 2 contingency table,

[b]Localized Vitiligo vs. Controls using chi-squared test with 3 × 2 contingency table,

[c] Generalized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,

[d] Localized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 5. Association studies for *SOD2* gene, C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs11575993 (C/T; Leu84Phe)	Genotype	(n = 385)	(n = 135)	(n = 750)		0.831 (AV)	0.6334 [a] (0.4490-0.8934)
	CC	205 (0.57)	92 (0.68)	599 (0.80)	0.009[a]		
	CT	153 (0.40)	35 (0.26)	137 (0.18)	<0.0001[b]		
	TT	27 (0.07)	08 (0.06)	14 (0.02)	0.002[c]	0.074 (SV)	0.3362 [b] (0.2679-0.4219)
	Allele						
	C	563 (0.73)	219(0.81)	1335(0.89)	0.009 [a]		
	T	207 (0.27)	51 (0.19)	165 (0.11)	<0.0001[b]	0.066 (C)	0.5307[c] (0.3758-0.7495)
					0.001[c]		
rs35289490 (C/T; Thr58Ile)	Genotype	(n = 385)	(n = 135)	(n = 750)		0.439 (AV)	0.4185 [a] (0.3086-0.5676)
	TT	114 (0.30)	75 (0.56)	364 (0.49)	<0.0001[a]		
	TC	184 (0.48)	48 (0.36)	302 (0.40)	<0.0001[b]		
	CC	87 (0.22)	12 (0.08)	84 (0.11)	0.301[c]	0.291 (SV)	0.5251 [b] (0.4392-0.6280)
	Allele						
	T	412 (0.54)	198(0.73)	1030(0.69)	<0.0001 [a]		
	C	358 (0.46)	72 (0.27)	470 (0.31)	<0.0001[b]	0.079 (C)	1.255 [c] (0.9380-1.679)
					0.132[c]		
rs4880 (T/C; Val16Ala)	Genotype	(n= 385)	(n = 135)	(n = 750)		0.573 (AV)	0.6699 [a] (0.5058-0.8872)
	TT	91 (0.24)	49 (0.36)	225 (0.30)	0.012[a]		
	TC	198 (0.51)	62 (0.46)	360 (0.48)	0.072[b]		
	CC	96 (0.25)	24 (0.18)	165 (0.22)	0.283[c]	0.570 (SV)	0.8300 [b] (0.6974-0.9878)
	Allele						
	T	380 (0.49)	160(0.59)	810 (0.54)	0.006 [a]		
	C	390 (0.51)	110(0.41)	690 (0.46)	0.040[b]	0.354 (C)	1.239 [c] (0.9523-1.612)
					0.112[c]		

'n' represents number of Patients/ Controls

HWE refers to Hardy-Weinberg Equilibrium,

AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 6. Distribution of haplotypes frequencies for *SOD2* gene, C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala) polymorphisms among generalized vitiligo patients and controls.

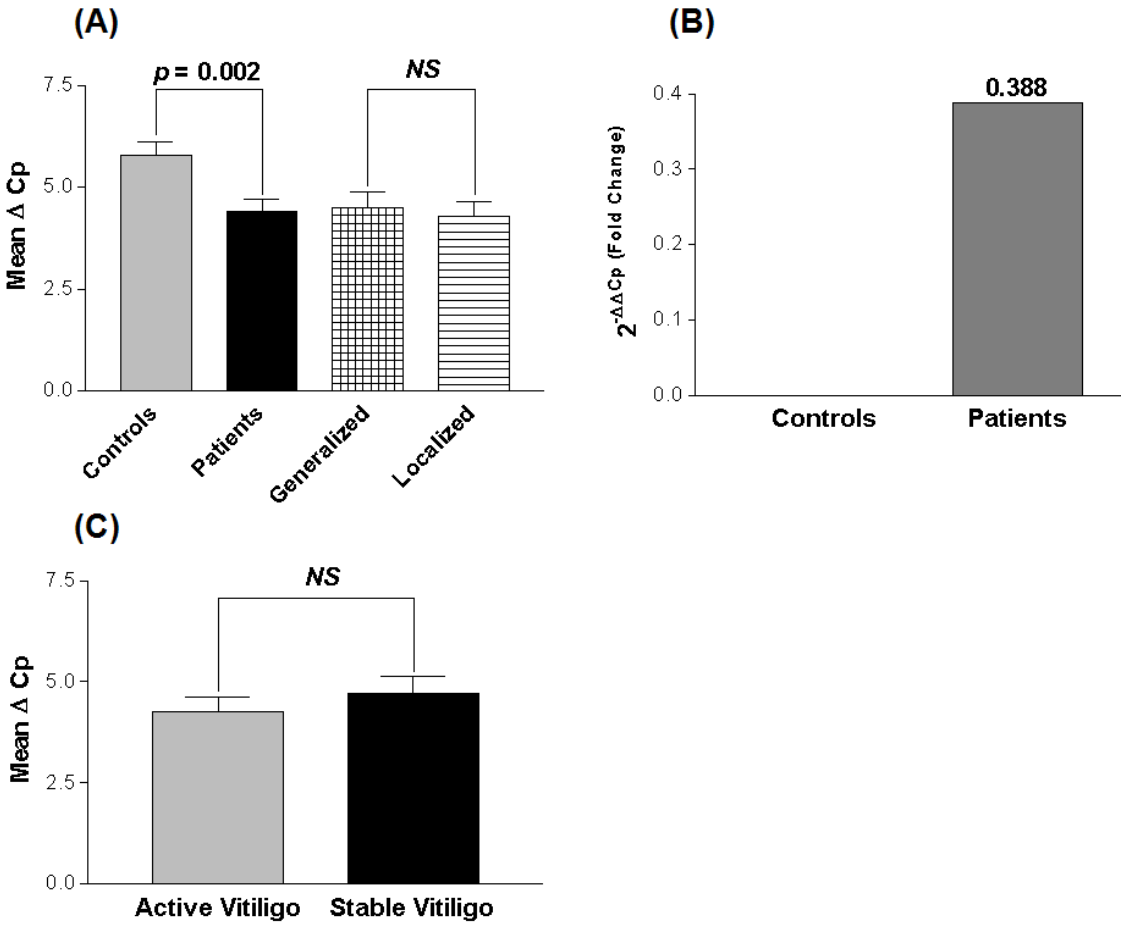
Haplotype (C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala)	Generalized Vitiligo Patients (Freq. %) (n=926)	Controls (Freq. %) (n=1358)	*p* for Association	p_{global}	Odds ratio (95% CI)
C C C	171.30(0.207)	390.25(0.287)	3.12e-005		0.647 [0.527~0.795]
C C T	177.61(0.215)	408.62(0.301)	1.04e-005		0.635 [0.519~0.778]
C T C	117.72(0.143)	198.39(0.146)	0.807022	6.33e-032	0.970 [0.758~1.241]
C T T	145.37(0.176)	286.73(0.211)	0.043846		0.797 [0.638~0.994]
T C C	68.81(0.083)	19.99(0.015)	3.97e-015		6.073 [3.662~10.072]
T C T	62.28(0.075)	16.14(0.012)	1.13e-014		6.772 [3.889~11.792]
T T C	42.17(0.051)	15.36(0.011)	1.98e-008		4.696 [2.601~8.477]
T T T	40.73(0.049)	20.51(0.015)	2.78e-006		3.377 [1.972~5.784]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

3.3.5 Relative gene expression of *SOD*2 in patients with vitiligo and controls:

Our *SOD*2 gene expression studies showed significant increase in expression of *SOD*2 transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.002) (Figure 3A). However, there was no significant difference in expression of *SOD*2 between generalized and localized vitiligo patients (p=0.726) (Figure 3A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.388 fold change in the expression of *SOD*2 transcript in patients as compared to controls (Figure 3B). In addition, we also checked the effect of *SOD*2 expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3C); however, no significant difference was observed between active and stable vitiligo patients (p=0.481).



(D)

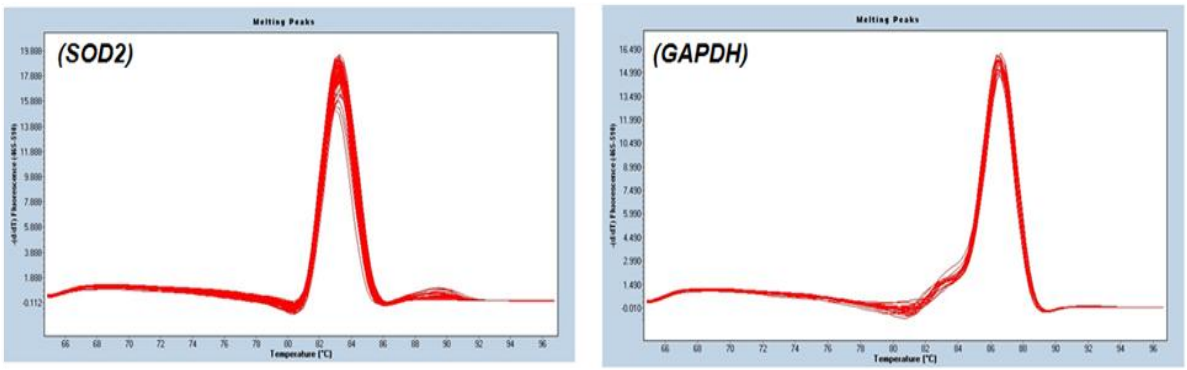


Figure 3. **Relative gene expression of *SOD*2 in controls and vitiligo patients:**

(A) Expression of *SOD*2 transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *SOD2* as compared to controls (Mean ΔCp ± SEM: 4.421 ± 0.2848 vs 5.788 ± 0.3236; p=0.002). Generalized vitiligo patients did not show difference in mRNA levels of *SOD*2 as compared to localized patients (Mean ΔCp ± SEM: 4.499 ± 0.4037 vs 4.291 ± 0.3606; p=0.726) [*NS* = non-significant].

(B) Expression fold change of *SOD*2 transcripts in 166 vitiligo patients against 175 controls showed 0.388 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *SOD*2 transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients did not show difference in mRNA levels of *SOD*2 as compared to stable vitiligo patients (Mean ΔCp ± SEM: 4.254 ± 0.3644 vs 4.700 ± 0.4365; p=0.481).

(D) Melt curve analysis of *SOD2* and *GAPDH* showing specific amplification.

3.3.6 Estimation of SOD2 activity in vitiligo patients and controls:

We have also analyzed the SOD2 specific activity in WBC-lysate from 520 vitiligo patients and 750 controls. SOD2 activity differed significantly between vitiligo patients and controls (p<0.0001) (Figure 4A). In particular, vitiligo patients showed 2.3 fold higher SOD2 activity as compared to controls. However, there was no significant difference in SOD2 activity between generalized and localized vitiligo patients (p=0.406) (Figure 4A).

Analysis based on the stage of progression of vitiligo revealed that active vitiligo patients had significant increase in SOD2 activity as compared to the patients with stable vitiligo (p=0.011) which suggests the involvement of SOD2 in disease progression (Figure 4B).

3.3.7 Correlation of *SOD*2 C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) genotypes with SOD2 activity in vitiligo patients:

Further, the SOD2 activity was correlated with the genotypes obtained for Leu84Phe, Thr58Ile and Val16Ala polymorphisms (Figure 4). Interestingly, SOD2 activity was significantly increased in patients with susceptible Thr58Ile TT genotypes and heterozygous CT genotypes as compared to controls (p=0.021; p=0.016) suggesting the involvement of Thr58Ile'T' allele in increased activity of SOD2.

No significant difference was observed in SOD2 activity in patients with CC genotypes as compared to controls (p=0.596) (Figure 4D).

However, SOD2 activity did not differ significantly between patients with Leu84Phe TT, CT and CC genotypes as compared to controls (p=0.854; p=0.712; p=0.817) (Figure 4C). Moreover, there was no significant difference in SOD2 activity in patients with Val16Ala TT, TC and CC genotypes as compared to controls (p=0.866; p=0.202; p=0.071) (Figure 4E) suggesting that Leu84Phe and Val16Ala polymorphisms are not involved in increased SOD2 activity in patients.

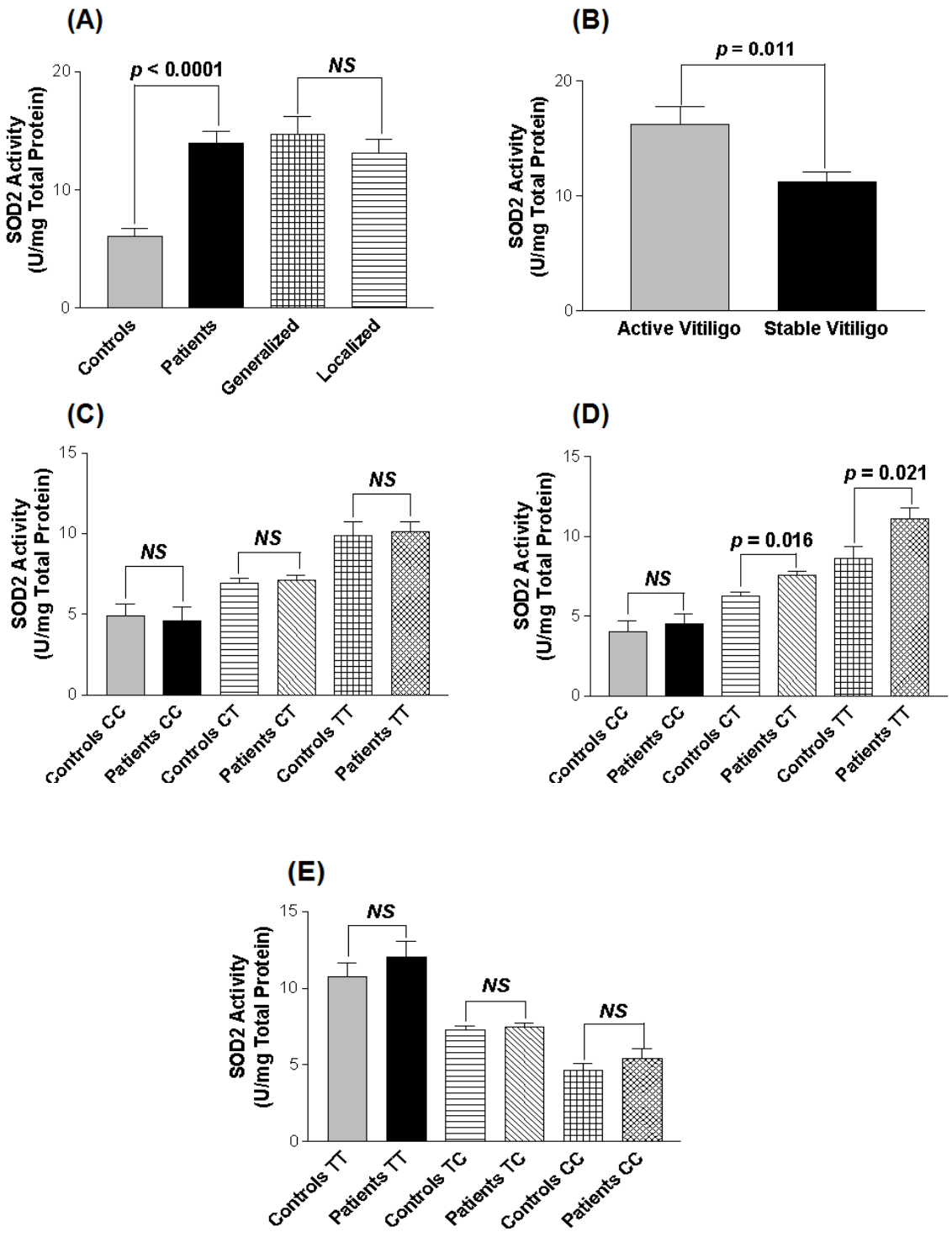


Figure 4. SOD2 specific activity in controls and vitiligo patients:

(A) SOD2 specific activity in 520 vitiligo patients and 750 controls. Vitiligo patients showed significantly increased SOD2 activity as compared to controls (Mean ± SEM: 13.98 ± 0.9810 vs 6.108 ± 0.6305; p<0.0001). Generalized vitiligo patients (n=364) did not show difference in SOD2 activity as compared to localized patients (n=156) (Mean ± SEM: 14.73 ± 1.488 vs 13.08 ± 1.211; p=0.406) [*NS* = non-significant].

(B) SOD2 specific activity with respect to activity of the disease in 385 patients with active vitiligo and 135 patients with stable vitiligo. Active vitiligo patients showed significant difference in SOD2 activity as compared to stable vitiligo patients (Mean ± SEM: 16.25 ± 1.601 vs 11.25 ± 0.8688; p=0.011).

(C) SOD2 specific activity with respect to *SOD*2 C/T (Leu84Phe) polymorphism in 520 vitiligo patients and 750 controls. No significant difference was observed in SOD2 activity with TT (Mean ± SEM: 10.12 ± 0.6632 vs 9.921 ± 0.8360; p=0.854), CT (Mean ± SEM: 7.106 ± 0.3319 vs 6.924 ± 0.3395; p=0.712) and CC (Mean ± SEM: 4.606 ± 0.8761 vs 4.883 ± 0.7670; p=0.817) genotypes between vitiligo patients and controls.

(D) SOD2 specific activity with respect to *SOD*2 C/T (Thr58Ile) polymorphism in 520 vitiligo patients and 750 controls. Vitiligo patients showed significant increase in SOD2 activity with TT (Mean ± SEM: 11.14 ± 0.6645 vs 8.601 ± 0.7934; p=0.021) and CT (Mean ± SEM: 7.566 ± 0.2746 vs 6.262 ± 0.2439; p=0.016) genotypes as compared to controls. There was no significant difference in the activity of SOD2 with CC genotypes (Mean ± SEM: 4.531 ± 0.6291 vs 4.009 ± 0.6917; p=0.596) as compared to controls.

(E) SOD2 specific activity with respect to *SOD*2 T/C (Val16Ala) polymorphism in 520 vitiligo patients and 750 controls. No significant difference was observed in SOD2 activity with CC (Mean ± SEM: 5.448 ± 0.5841 vs 4.683 ± 0.4531; p=0.817), TC (Mean ± SEM: 7.498 ± 0.2637 vs 7.290 ± 0.2752; p=0.595) and TT Mean ± SEM: 12.04 ± 0.9936 vs 10.74 ± 0.8725; p=0.361) genotypes between vitiligo patients and controls. [*NS* = non-significant]

3.4 DISCUSSION

ROS are produced because approximately 2–3% of the oxygen atoms taken up by the mitochondria are reduced insufficiently (Valko *et al*., 2004). ROS can oxidize and damage nucleic acids, proteins and lipids thereby altering their stability and function (Evans *et al*., 2004; Chen *et al*., 2008; Xie *et al*., 2008). Thus, protein modifications (such as protein carbonylation and nitration) and the formation of lipid peroxidation adducts (e.g. 4-hydroxynonenal) can be the results of ROS damage.

Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction as H_2O_2 accumulation is observed in the epidermis of active vitiligo

patients (Bindoli *et al.,* 1992; Schallreuter *et al.,* 1996). One of the important reasons for increased epidermal H_2O_2 levels is an imbalance in the antioxidant system. Previously, we have reported significant increase in erythrocyte lipid peroxidation levels in patients of vitiligo in all age groups as compared to healthy controls from Gujarat (Agrawal *et al.,* 2004; Shajil and Begum, 2006). The study showed that systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients (Agrawal *et al.,* 2004). Systemic oxidative stress is also reported to be elevated in patients (Hazneci *et al*, 2005). The mechanism underlying this etiology is not yet known but increased systemic oxidative stress is generated due to altered antioxidant system which affects the epidermal melanocytes leading to vitiligo manifestation.

Mitochondria are a major site of ATP production through oxidative phosphorylation, and they also participate in other cellular functions such as cell cycle regulation, stress response (Manoli *et al*., 2007), and different aspects of metabolism (Bowsher and Tobin, 2001; Chen *et al*., 2006; Eaton *et al*., 1996; Leverve, 2007; Manoli *et al*., 2007; Newsholme *et al*., 2007). Mitochondria are also important for apoptosis (Gulbins *et al*., 2003; Spierings *et al*., 2005) due to altered mitochondrial function (Green and Reed, 1998) and the presence of many factors that induce apoptosis upon release from mitochondria, including cytochrome c (Scheffler, 1999), apoptosis inducing factor (AIF), endonuclease G, Smac/Diablo, and Omi/Htr2 (Adams, 2003). Manganese superoxide dismutase (SOD2) is an antioxidant enzyme, located in the mitochondrial matrix that appears to have strategic advantage in providing an initial defense against oxidative damage by ROS. This enzyme catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Barra *et al.,* 1984). Given that mitochondria are the major sites for cellular metabolism and thus of ROS production, SOD2 is very important in protecting cells from ROS-induced oxidative damage.

A number of studies have identified the association of *SOD*2 genetic polymorphisms with various diseases e.g. type II diabetes, hypertension etc. (Nakanishi *et al*., 2008; Hirooka, 2008; Arsova *et al*., 2008; Mikhak *et al*., 2008). The presence of *SOD*2 single-nucleotide polymorphisms (SNPs) and the potential effects of these SNPs on human SOD2 have been briefly reviewed previously (St Clair and Kasarskis, 2003).

The present study investigated four non-synonymous SNPs: Leu84Phe (C/T; rs11575993), Thr58Ile(C/T; rs35289490), Val16Ala (T/C; rs4880) and Ile82Thr (T/C; rs1141718) in SOD2 gene, out of which two SNPs: Thr58Ile(C/T; rs35289490), Val16Ala (T/C; rs4880) are well documented in several diseases e.g. Parkinson's disease, diabetes mellitus and malignancies (Shimoda-Matsubayashi *et al.*,1996; Pociot *et al.*,1993; Ambrosone, *et al.*,1999).

The transport of SOD2 into mitochondria is mediated through the interaction of the mitochondrial targeting sequence with receptors on the mitochondrial membrane. The Val16Ala (T/C) polymorphism in the mitochondrial targeting sequence may influence the efficiency of SOD2 transport. Val16Ala is implicated in decreased efficiency of SOD2 transport into target mitochondria in Val allele carriers (Sutton *et al.,* 2003). The 16Ala polymorphism results in the formation of α-helix and the 16Val takes a β-sheet structure and Val allele was predicted to disrupt this structure (Shimoda-Matsubayashi *et al.,* 1996). The α-helix structure is important for the effective transport of precursor proteins into mitochondria (Lemire *et al.,* 1989). The amino acid substitution (Val/Ala) at position 16 of the mitochondrial targeting sequence may lead to misdirected trafficking, followed by the alteration of SOD2 activity in human mitochondria.

Several studies have suggested the association of the SOD2 Val16Ala 'T' allele with an increased risk of breast (Ambrosone *et al.,* 1999), prostate (Woodson *et al.,* 2003) and ovarian cancer (Olson *et al.,* 2004). In addition, the variant 'T'allele was associated with sporadic motor neuron disease (Van-Landeghem *et al.,* 1999), exudative agerelated macular degeneration (Kimura *et al.,* 2000), diabetic nephropathy (Nomiyama *et al.,* 2003) and ankylosing sypondylitis (Yen *et al.,* 2003), which are all related to oxidative stress and abnormal free radical defence mechanisms.

The present study suggests significant association of Val16Ala polymorphism with active vitiligo cases. In particular, the Val16Ala 'T' allele is prevalent in patient group as compared to controls indicating the important role played by 16Ala (T) allele in disease progression (Table 5). However, overall vitiligo patients did not show significant association with this polymorphism (Table 3). It is reported that carriers of 16Ala (T) allele have 30-40% more activity of SOD2 compared to 16Val (C) allele

carriers (Sutton *et al.*, 2003). The genotype-phenotype correlation study for this polymorphism revealed an increase in SOD2 activity with Val16Ala TT genotypes as compared to TC and CC genotypes which implicates the role of Val16Ala polymorphism in increased activity of SOD2; however, the activity was not significant between patients and controls harbouring these genotypes (Figure 4E).

Overall, vitiligo patients exhibited significantly higher (2.3 fold) SOD2 activity as compared to controls suggesting the high oxidative stress in patients (Figure 4A). Moreover, the SOD2 activity was also increased in patients with active vitiligo (1.5 fold) as compared to stable vitiligo, suggesting the important role of SOD2 in disease progression (Figure 4C). The SOD2 gene expression analysis showed significantly higher SOD2 transcripts levels (1 fold) as compared to controls (Figure 3A). However, there was no significant difference in SOD2 expression between active and stable patients suggesting that increased activity in active vitiligo patients may be mainly contributed by Val16Ala polymorphism (Figure 3C).

A wide variety of compounds induce transcription of *SOD*2. Cytokines such as interleukin (IL)-1 (Masuda *et al*., 1988; Visner *et al*., 1992; Dougall and Nick, 1991), IL-4, IL-6 (Dougall and Nick, 1991), TNF-α (Visner *et al*., 1992; Wong and Goeddel, 1988), lipopolysaccharide (LPS) (Visner *et al*., 1990), and IFN-γ (Harris *et al*., 1991) are potent activators of SOD2 in different tissues and cell types. The cytokine inducible enhancer regions in SOD2 contain binding sites for NF-kB, C/EBP, and NF-1 transcription factors. Our recent studies also suggest increased levels of cytokines such as TNF-α, TNF-β, IFN-γ (unpublished data) and IL-4 (Imran et al., 2012) in vitiligo patients which might be responsible for the induction of SOD2 as observed in the present study. The redox-sensitive transcriptional factor NF-κB acts as a regulator of genes by serving as an immediate responder to harmful cellular stimuli like high ROS. NF-κB is identified as the most crucial transcriptional factor regulating *SOD*2 induction (Eastgate *et al*., 1993).

The SOD2 Thr58Ile (C/T) polymorphism is related to the potency of SOD2. The Thr58Ile SNP disrupts the native tetrameric form of SOD2 and promotes a dimeric form in solution (Borgstach *et al*., 1996). In Thr58Ile polymorphism (C/T), SOD2 with threonine has a weaker antioxidant activity than that with isoleucine (Ho *et al.*,1998). The polymorphism, Thr58Ile, affects the stability of protein tetrameric

interface and reduces the biological activity of SOD2 (Borgstahl *et al*., 1996; Ho and Crapo, 1988). The 58Ile protein form showed three-fold higher activity than the 58Thr form (Zhang *et al*., 1999). However, Thr mutant form has increased thermal instability and accelerated thermal inactivation (Borgstahi 1992; Borgstahl *et al.*, 1996) which decreases the activity of SOD2. Intersetingly, we found significant association of Thr58Ile polymorphism with vitiligo being 'T' allele prevalent in vitiligo group (Table 3). This association was consistent with other subtypes of vitiligo i.e. generalized, localized, active and stable vitiligo patients (Table 4 & 5). This suggests the profound effect of 58Ile (T) allele in vitiligo susceptibility. The genotype-phenotype correlation of this SNP revealed significantly higher SOD2 activity in patients harbouring TT and CT genotypes as compared to those of controls (Figure 4D). Previous studies suggested the association of Ile58Thr polymorphism with neurodegenerative diseases involving a decrease in SOD2 levels (Checkoway *et al*., 1998). No association between the Ile58Thr polymorphism and Parkinson's disease, type 1 diabetes and bladder cancer was found (Grasbon-Frodl *et al*., 1999; Chistyakov *et al*., 2001; Paz-y-Min˜o *et al*. (2010). However this mutation is more likely to be involved in hereditary aging-related neurodegenerative disease (Borgstahl *et al*., 1996).

Furthermore, the present study found significant association of the non-synonymous SNP: Leu84Phe (C/T) with vitiligo. However, Ile82Thr (T/C) showed the presnce of only single allele i.e. 'T' in both control and patient groups (Table 3). There was no signifcant difference in SOD2 activity of patients harbouring the different genotypes of Leu84Phe polymorphism, suggesting that the polymorphism does not play an important role in vitiligo pathogenesis; however the SOD2 activity was increased with subjects harbouring TT and CT genotypes as compared to CC genotypes suggesting the effect of 'T' allele on increased activity of SOD2 (Figure 4C). However, the prevalence of Leu84Phe minor allele 'T' was significantly high in active patients of vitiligo as compared to stable patients suggesting the important role of 'T' allele in progression of the disease (Table 5). There are no reports available for these two non-synonymous SNPs in any disease pathogenesis. Intersetingly, the frequency of susceptible haplotype 'TTC' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls and increased the risk of vitiligo by 4.6-fold (Table 6). Also, three other haplotypes

with one or two susceptible alleles: 'TTT' and 'TCT' and 'TCC' were significantly increased in vitiligo patients as compared to controls suggesting the important role of susceptible alleles in vitiligo.

In conclusion, the present study signifies the important role of SOD2 in oxidative stress mediated pathogenesis and progression of vitiligo. In particular, the association of Thr58Ile had a profound effect on SOD2 activity in patients and the increased activity of SOD2 may in part be contributed by the polymorphism as well as increased SOD2 mRNA levels which in turn results in increased H_2O_2 production. As the downstream antioxidant system in vitiligo patients was found to be disturbed, the H_2O_2 might not be properly removed and may finally lead to oxidative damage to the melanocytes.

3.5 REFERENCES

Adams, J.M (2003). Ways of dying: multiple pathways to apoptosis. *Genes Dev*. 17:2481–2495

Agrawal, D., Shajil, E.M., Marfatia, Y.S., and Begum, R (2004). Study of the antioxidant statusof vitiligo patients of different age groups in Baroda. *Pigment Cell Res*. 17: 289-94.

Barrett, J.C., Fry, B., Maller, J., Dally, M.J. (2005). Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 21(2), 263-265.

Bindoli, A., Rigobello, M.P., and Deeble, D.J (1992). Biochemical and toxicological properties of the oxidation products of catecholamines. *Free Rad Biol Med*. 13:391-405.

Borgstahl GE, Parge HE, Hickey MJ, et al.,(1992). The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* 71:107- 18.

Borgstahl, G.E., Parge, H.E., Hickey, M.J., Johnson, M.J., Boissinot, M., Hallewell, R.A., Lepock, J.R., Cabelli, D.E., Tainer, J.A (1996). Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. *Biochemistry* 35 :4287–4297.

Bowsher, C.G., Tobin, A.K (2001). Compartmentation of metabolism within mitochondria and plastids. *J. Exp. Bot*. 53:513–527.

Chen, D., Yu, Z., Zhu, Z., Lopez, C.D (2006). The p53 pathway promotes efficient mitochondrial DNA base excision repair in colorectal cancer cells. *Cancer Res*. 66 :3485–3494.

Chistyakov DA, Savost'anov KV, Zotova EV, et al.,(2001).Polymorphisms in the SOD2 and EC-SOD and EC-SOD genes and their relationshipto diabetic neuropathy in type 1 diabetes mellitus. *BMC Med Genet* 2:4.

Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson PE, Meltzer PS, Trent JM (1993). Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. *Proc Natl Acad Sci U S A* 90:3113e7.

Church SL. (1990).Manganese superoxide dismutase: nucleotide and deduced amino acid sequence of a cDNA encoding a new human transcript *Biochim Biophys Acta* 1171:341.

Dell'Anna, M.L., Maresca, V., Briganti, S., Camera, E., Falchi, M., and Picardo, M (2001). Mitochondrial impairment in peripheral blood mononuclear cells during the active phase of vitiligo. *J Invest Dermatol*. 117:908–913.

Eaton, S., Bartlett, K., Pourfarzam, M (1996). Mammalian mitochondrial beta-oxidation. *Biochem. J.* 320 :345–357.

Evans, P.H (1993). Free radicals in brain metabolism and pathology. *Br. Med. Bull*. 49:577– 587.

Fridovich I. Superoxide radical and superoxide dismutases (1995). *Annu Rev Biochem* 64:97- 112.

Fujimoto H, Taguchi JI, Imai Y, Ayabe S, Hashimoto H, Kobayashi H (2005). Manganese superoxide dismutase polymorphism affects the oxidized low-density lipoprotein- induced apoptosis of macrophages and coronary artery disease. *Eur Heart J* 229:1267–74.

Grasbon-Frodl EM, Kösel S, Riess O, Müller U, Mehraein P, Graeber MB(1999). Analysis of mitochondrial targeting sequence and coding region polymorphisms of the manganese superoxide dismutase gene in German Parkinson disease patients. *Biochem Biophys Res Commun.* 255(3):749-52.

Green, D.R., Reed, J.C(1998) . Mitochondria and apoptosis. *Science* 281:1309–1312.

Gulbins, E., Dreschers, S., Bock, J (2003) . Role of mitochondria in apoptosis. *Exp. Physiol.* 88: 85–90.

Hazneci, E., Karabulut, A. B., O¨ ztu¨ rk, C., Batcioglu, K., Dogan, G., Karaca, S. and Esrefoglu, M (2005). A comparative study of superoxide dismutase, catalase, and glutathione peroxidase activities and nitrate levels in vitiligo patients. *Int. J. Dermatol* 44 :636–640.

Hinerfeld D, Traini MD, Weinberger RP, Cochran B, Doctrow SR, Harry J, et al. (2004). Endogenous mitochondrial oxidative stress: neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice. *J Neurochem* 88:657–67.

Ho YS, Crapo JD (1998). Isolation and characterization of complementary DNAs encoding human manganese-containing superoxide dismutase. *FEBS Lett* 229:256- 60.

Huang TT, Carlson EJ, Kozy HM, Mantha S, Goodman SI, Ursell PC, et al. (2001).Genetic modification of prenatal lethality and dilated cardiomyopathy in Mn superoxide dismutase mutant mice. *Free Radic Biol Med* 31:1101–10.

Jain A, Mal J, Mehndiratta V, Chander R, Patra SK (2011). Study of oxidative stress in vitiligo. *Indian J Clin Biochem*. 26(1):78-81.

Kim GW, Kondo T, Noshita N, Chan PH (2002). Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral ischemia/reperfusion in mice: implications for the production and role of superoxide radicals. *Stroke* 33:809–15.

Klug, D., Rabani, J., Fridovich, I (1972). A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. *J. Biol. Chem* 247.

Leverve, X.M (2007). Mitochondrial function and substrate availability. *Crit. Care Med* . 35:S454–S460.

Li H, Kantoff PW, Giovannucci E, Leitzmann MF, Gaiano JM, Stampfer MJ, et al.(2005). Manganese superoxide dismutase polymorphism, prediagnostic antioxidant status, and risk of clinical significant prostate cancer. *Cancer Res* 65:2498–504.

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, et al.(1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11:376–81.

Liu L, Zheng T, Wang N, Wang F, Li M, Jiang J, et al.,(2009). The manganese superoxide dismutase Val16Ala polymorphism is associated with decreased risk of diabetic nephropathy in Chinese patients with type 2 diabetes. *Mol Cell Biochem* 322:87–91.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). "Protein measurement with the Folin phenol reagent". *J. Biol. Chem*. 193: 265–75.

Manoli, I., Alesci, S., Blackman, M.R., Su, Y.A., Rennert, O.M., Chrousos, G.P (2007). Mitochondria as key components of the stress response. *Trends Endocrinol. Metab*. 18:190–198.

Maresca, V., Roccella, M., and Roccella, F (1997). Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. *J Invest Dermatol* 109: 310–313.

Martin RC, Lan Q, Hughes K. A, Martini BD, Lissowska J, et al.,(2005). No apparent association between genetic polymorphisms (-102 CNT) and (-9 TNC) in the human manganese superoxide dismutase gene and gastric cancer. *J Surg Res*124:92–7.

Mikhak B, Hunter DJ, Spiegelman D, Platz EA, Wu K, Erdman Jr JW, et al.,(2008). Manganese superoxide dismutase (SOD2) gene polymorphism, interactions with carotenoid levels and prostate cancer risk. *Carcinogenesis* 29:2335–40.

Nakanishi, S.; Yamane, K.; Ohishi, W.; Nakashima, R.; Yoneda, M.; Nojima, H.; Watanabe, H.; Kohno, N(2008). Manganese superoxide dismutase Ala16Val polymorphism is associated with the development of type 2 diabetes in Japanese-Americans. *Diabetes Res. Clin. Pract*. 81:381–385.

Nelson DL, Cox MM(2005). Lehninger principles of biochemistry. 4th ed. New York: Worth Publishers, New York.

Newsholme, P., Bender, K., Kiely, A., Brennan, L (2007). Amino acid metabolism, insulin secretion and diabetes. *Biochem. Soc. Trans.* 35:1180–1186 .

Nomiyama T, Tanaka Y, Piao L, Nagasaka K, Sakaim K, Ogihara T et al.(2003). The polymorphism of manganese superoxide dismutase is associated with diabetic nephropathy in Japanese type 2 diabetic patients. *J Hum Genet* 48:138–41.

Paz-y-Miño C, Muñoz MJ, López-Cortés A, Cabrera A, Palacios A, Castro B, Paz-y-Miño N, Sánchez ME (2010). Frequency of polymorphisms pro198leu in GPX-1 gene and ile58thr in SOD2 gene in the altitude Ecuadorian population with bladder cancer. *Oncol Res.* 18(8):395-400.

Rosenblum JS, Gilula NB, Lerner RA (1996). On signal sequence polymorphisms and diseases of distribution. *Proc Natl Acad Sci USA* 93:4471–3.

Schallreuter, K. U., Wood, J. M. and Berger, J. (1991). Low catalase levels in the epidermis of patients with vitiligo. *J.Invest. Dermatol*. 97:1081–1085.

Schallreuter, K. U.,Wood, J. M., Pittelkow, M. R., Buttner, G., Swanson, N., Korner, C. and Ehrke, C (1996). Increased monoamine oxidase A activity in the epidermis of patients with vitiligo. *Arch. Dermatol. Res*. 288:14–18.

Schallreuter, K.U., Wood, J.M., and Berger, J (1991). Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol*. 6 :1081-1085.

Scheffler, I.E (1999). Polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric polymorphisms in Parkinson's disease. *Neurotoxicol*. 19(4-5):635-43.

Shajil, E.M., and Begum. R (2006). Antioxidant status of segmental and non-segmental vitiligo. *Pigment Cell Res*. 19*:* 179-180.

Shi, Y.Y. and He, L. (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 15(2), 97-98.

Shimoda-Matsubayashi S, Hattori T, Matsumine H, Shinohara A, Yoritaka A, Mori H, et al. (1997). Mn SOD activity and protein in a patient with chromosome 6-linked autosomal recessive parkinsonism in comparison with Parkinson's disease and control. *Neurology* 49:1257–62.

Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, et al. (1996).Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. *Biochem Biophys Res Commun* 226:561- 5.

Spierings, D., McStay, G., Saleh, M., Bender, C., Chipuk, J., Maurer, U., Green, D.R (2005). Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. Science 310, 66–67.

Sravani PV, Babu NK, Gopal KV, Rao GR, Rao AR, Moorthy B, Rao TR(2009). Determination of oxidative stress in vitiligo by measuring superoxide dismutase and catalase levels in vitiliginous and non-vitiliginous skin. *Indian J Dermatol Venereol Leprol.* 75(3):268-71.

Sutton A, Imbert A, Igoudjil A, Descatoire V, Cazanave S, Pessayre D, et al.,(2005). The manganese superoxide dismutase Ala16Val dimorphism modulates both mitochondrial import and mRNA stability. *Pharmacogenet Genomics* 15:311–9.

Sutton A, Khoury H, Prip-Buus C, Cepanec C, Pessayre D, Degoul F (2003). The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. *Pharmacogenet.* 13:145–57.

Turrens JF & Boveris A (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *BiochemJ* 191 :421–427.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J., Telser, J (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell Biochem.* 266 :37–56.

Wan XS, Devalaraja MN, Clair DK (1994). Molecular structure and organization of the human manganese superoxide dismutase gene. *DNA Cell Biol* 13:1127- 36.

Zhang HJ, Yan T, Oberley TD, Oberley LW (1999).Comparison of effects of two polymorphic variants of manganese superoxide dismutase on human breast MCF-7 cancer cell phenotype. *Cancer Res* 59:6276–83.

4.1 INTRODUCTION:

Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction (Schallreuter *et al.,* 1999; Schallreuter *et al.,* 1994a) as H_2O_2 accumulation is observed in the epidermis of active vitiligo patients (Hasse *et al.,* 2004). An alteration in the antioxidant system, with a significant reduction in catalase activity has been demonstrated in both lesional and non-lesional epidermis of vitiligo patients (Schallreuter *et al.,* 1991) as well as in melanocytes derived from patients (Maresca *et al.,* 1997). Antioxidant imbalance in peripheral blood mononuclear cells of active vitiligo patients is also observed. An increased intracellular production of reactive oxygen species appeared to be due to mitochondrial impairment (Dell'Anna *et al.,* 2001). These findings support the concept of a possible systemic oxidative stress in vitiligo. Previously, systemic oxidative stress in vitiligo patients of Gujarat due to an imbalance in enzymatic and non-enzymatic antioxidant systems has been reported (Agrawal *et al.,* 2004; Shajil and Begum, 2006).

Reactive oxygen species are produced in the body by several different mechanisms, including cellular respiration, interactions between ionizing radiation and biological molecules, and phagocytosis (McCord, 1993). ROS produced at lower levels under normal conditions are important mediators of cell signaling events, including differentiation, cell cycle progression, growth arrest, apoptosis, and immunity (Oury et al., 1991). Under physiological conditions, a balance exists between the level of ROS produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruption of this balance, either through increased production of ROS or decreased levels of antioxidants, produces a condition referred to as oxidative stress and leads to variety of pathological conditions including cardiovascular diseases, neurological disorders, lung pathologies, accelerated aging and UV-induced skin inflammation (Bowler and Crapo 2002; Delanty and Dichter, 1998; Fukai et al 2002). Superoxide radicals produced from a one-electron reduction of oxygen can undergo either spontaneous or enzyme-catalyzed dismutation to hydrogen peroxide (H_2O_2) or can react with nitric oxide (NO) to form the toxic product peroxynitrite ($ONOO^-$). Either the combination of H_2O_2 with metal ions (iron) or the breakdown of $ONOO^-$ can produce the highly

toxic hydroxyl radical (•OH). These ROS can react with a variety of cellular macromolecules such as lipids, proteins and DNA leading to the disruption of cell membranes, inappropriate activation or inactivation of enzymes, and genetic mutations. Therefore, persistence of these diverse ROS in and around cells and tissues can have severe pathophysiological consequences including vitiligo.

The antioxidant enzyme superoxide dismutase (SOD) family comprises three distinct isoforms i.e. SOD1, SOD2 and SOD3 that are responsible for maintaining low levels of ROS by metabolizing superoxide anion into oxygen and hydrogen peroxide. The SOD3 is an extracellular (EC SOD) (Marklund et al., 1982) enzyme encoded on chromosome 4p16.3-q21. Human EC-SOD is a metalloprotein containing copper and zinc ions in its active site, which support enzymatic activities. EC-SOD contains four functional domains that include a signal peptide (amino acid residues 1-18), an amino-terminal domain (residues 19–95) with a glycosylation site, a central domain with active site that shows 50% homology to Cu/Zn SOD (residues 96–193) with binding sites for copper and zinc ions, and a heparin- binding domain at its carboxyl terminus (residues 194–222). EC-SOD exists as a tetramer composed of two interacting dimers, and the tetramer is held together by van der Waals and hydrogen bonding/salt bridge interactions. SOD3 is a secretory protein (Beckman *et al*., 1994). In mammals, 90-99% of the SOD3 is found in the interstitial spaces of tissues (Kroncke *et al.,* 1994; Squardrito *et al.,* 1995). However, the enzyme is also found in extracellular fluids and accounts for the majority of the SOD activity of plasma, lymph, and synovial fluid (Foalz *et al*., 1994; Sandsrom *et al.,* 1994). As a copper-containing enzyme, the activity of SOD3 is regulated by copper availability. Free intracellular copper is extremely limited (Rae *et al.,* 1999) and soluble carrier proteins or "copper chaperones" are used for copper trafficking to specific coper containing proteins.

The mature human SOD3 contains six cysteine residues (Hjalmarsson *et al.,* 1987) and occurs naturally in two different forms with evident disulfide bridges (Petersen *et al.,* 2003). One form is active (aSOD3) and has a free cysteine residue at position 195. The intrasubunit disulfide bridge formed by Cys107 and Cys189 is necessary for the SOD3 activity (Petersen *et al.,* 2003). The other form has a free cysteine residue at position 45 and is inactive (iSOD3). SOD3 is unusually resistant to high temperatures,

extreme pH, and high urea concentrations, although it can be inhibited by a variety of agents including azide and cyanide and inactivated by diethyldithiocarbamate and hydrogen peroxide.

SOD3 is also found in the extracellular matrix of mammalian tissues (Marklund, 1984; Oury et al., 1994). The C-terminal region is highly rich in basic amino acid residues (Hjalmarsson *et al.*, 1987). This polybasic region is involved in the binding to heparin/heparan sulfate (Adachi et al., 1992) and type I collagen (Petersen *et al.*, 2004) and is referred to as the extracellular matrix (ECM)-binding region (Petersen *et al.,* 2004). The ECM region is removed proteolytically just before secretion. This process is a two step event involving an initial cleavage of the protein by furin or another member of the proprotein convertase family of procesing proteinases followed by the action of an unknown Arg/Lys-specific carboxypeptidase (Bowler *et al.*, 2002; Enghild *et al.*, 1999; Olsen *et al*., 2004). A fundamental and distinguishing property of SOD3 is its affinity for certain glycosaminoglycans such as heparin and heparan sulfate. The last 20 amino acids of the 222-amino acids long SOD3 C-type subunit contains 6 arginines, 3 lysines and 1 histidine. The cluster of 6 positively charged amino acids at positions 210-215 forms the essential part of the heparin binding domain (Sandstrom *et al*, 1992). It binds to the negatively charged molecules: heparin and heparan sulphate (which is of electrostatic nature) (Karlsson *et al*, 1988) and coverts superoxide anion to H_2O_2. Superoxide anion can easily react with nitric oxide (NO) to form the toxic product peroxynitrite ($ONOO^-$) and SOD3 is important in preserving NO function by maintaining low superoxide concentrations in blood vessels (Fattman *et al.,* 2003). The heparin binding domain has been proposed to act as a nuclear localization signal in certain cell types (Ookawara *et al.,* 2003), suggesting that SOD3 may also provide antioxidant protection to DNA and nuclear proteins.

In *SOD*3 gene two missense mutations i.e. Arg213Gly (C/G; rs8192291) and Ala40Thr (G/A; rs2536512), have been reported (Yamada *et al.*, 1997; Yamada *et al.*, 1995). Additional variants of the human *SOD*3 gene include three missense mutations: Phe131Cys, Val160Leu, and Arg202Leu. A silent mutation, Leu53Leu (CTG to TTG), has also been found in samples of Japanese and Mediterranean populations (Qin et al., 2008; Campo et al., 2005; Tamai et al., 2006).

The Arg213Gly (C/G) SNP present in exon3 of *SOD*3 gene at codon 213 inhibits ionic interactions between heparin and SOD3 which in turn results in 10 fold increase in SOD3 concentration in plasma (Sandstrom *et al.*, 1994). The Arg213Gly SNP located at the heparin-binding domain has been identified in a small proportion of the healthy population (Folz *et al*., 1994) and has been associated with very high plasma SOD3 levels due to altered affinity of SOD3 for heparin at the endothelial cell surface (Sandstrom *et al*., 1994; Adachi *et al.*, 1996; Karlsson *et al*., 1994).
The Ala40Thr polymorphism is located in the amino terminal domain, where it is thought to work for the tetramerization of the enzyme. The findings of Tamai *et al.*, (2006) suggested the possibility that the Thr allele of the Thr40Ala polymorphism is associated with impaired function of SOD3.

However, the exact genetic defects in antioxidant enzymes that lead to their altered levels/activity leading to oxidative stress mediated damage of melanocytes in vitiligo are still unknown. In the present study, we have made an attempt to understand the role of SOD3 in vitiligo pathogenesis. Hence, the objectives of this study were:

i.) To determine whether the Arg213Gly (C/G; rs8192291) and Ala40Thr (G/A; rs2536512) polymorphisms of *SOD3* are associated with vitiligo susceptibility and modulate *SOD3* activity.

ii.) To measure and compare *SOD3* transcript levels and activity in patients with vitiligo and in unaffected controls.

iii.) To correlate *SOD3* polymorphisms, expression levels and its activity with progression of the disease.

4.2 MATERIALS AND METHODS

4.2.1 Study Subjects:

The study group included 482 vitiligo patients comprised of 208 males and 274 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of five hundred and sixty four ethnically, and sex-matched unaffected individuals comprised of 249 males and 315 females were included as controls in this study (Table 1). None of the healthy individuals 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.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n =482)	(n =564)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42yrs
Sex: Male	208 (43.15%)	249 (44.15%)
Female	274 (56.85%)	315 (55.85%)
Onset age (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yrs	NA
Type of vitiligo		
Generalized	328 (68.05)	NA
Localized	154 (31.95)	NA
Active vitiligo	352 (73.03)	NA
Stable vitiligo	130 (26.97)	NA
Family history	63 (13.07%)	NA

4.2.2 Blood collection and genomic DNA Preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analysis.

4.2.3 Genotyping of *SOD*3 Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms of *SOD*3 gene (Figure 1A, B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes *Mwo*I and *Pau*I (New England Biolabs, Beverly, MA) were used for digesting amplicons of Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms of *SOD*3 gene (Table 2) respectively. 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 20% polyacrylamide or 2.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *SOD*3 Arg213Gly (C/G; rs1799895) and Ala40Thr (G/A; rs2536512) SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1799895)				*Mwo*I
*SOD*3 C/G F	CGCCAGGCGCGGGAACACTCAG	64	63	(35 & 28
*SOD*3 C/G R	GGCGGACTTGCACTCGCTCTCG			bp)
(rs2536512)				*Pau*I
*SOD*3 G/A F	GGTTCTGCGATAATGGGGTCCCT	58	396	(274 &
*SOD*3 G/A R	GGCGAAGAAGGCGTCGAGCTT			121 bp)
*SOD*3 expression F	GCTGGAAAGGTGCCCGACTC	65	162	-
*SOD*3 expression R	CAGATCTCCGTGACCTTGGCG			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

4.2.4 Estimation of Superoxide dismutase 3 activity:

The estimation of SOD3 activity in plasma was carried out by the method of Marklund and Marklund (1974) with slight modifications utilizing the inhibition of auto-oxidation of pyrogallol by SOD3 enzyme. The final assay mixture contained 3 ml of tris buffer (200 mM, pH 8.2) containing air equilibrated 0.2 mM pyrogallol [E merck, India], 1 mM EDTA and 2ul of 1:10 diluted plasma as an enzyme source. The concentration of protein in the plasma was estimated by Lowry's method (Lowry et al., 1954) (as described in Chapter III).

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD3 present in plasma sample. Decrease in autooxidation shows indirect evidence of SOD3 activity.

Reagents:

1. Tris buffer 100mM (pH 8.2)
2. Pyrogallol(0.2 mM) dissolved in 0.5 N HCl

Protocol:

The 1:10 dilution of plasma was prepared and O.D. was adjusted to 0.08-0.12 by auto oxidation of pyrogallol at 320 nm and the assay system was followed as given below:

Reagents	**Blank**	**Control**	**Test**
Buffer	1.500 ml	1.500 ml	1.500 ml
DDW	1.350 ml	1.348 ml	1.348 ml
Pyrogallol	0.15 ml	0.15 ml	0.15 ml
Plasma (1:10)	-	2 ul	2 ul
Total volume	3 ml	3 ml	3 ml

In all tubes, the reaction was started by the addition of pyrogallol (0.2 mM) and the change in optical density was recorded for 3 min at interval of 5 sec at 320 nm. The initial 10 sec were considered as the induction period of the enzyme. The % inhibition of the test system was calculated from the standard graph. The SOD3 activity was expressed in units/mg of plasma protein. One unit of SOD3 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

Unit: One unit of SOD3 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation.

Specific activity: Units of enzyme/ mg of protein

4.2.5 Determination of *SOD*3 and *GAPDH* mRNA expression:

4.2.5.1.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis/ ethidium bromide staining and purity by monitoring 260/280 absorbance ratio of >1.95. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

4.2.5.1.2 Real-time PCR:

The expression of *SOD*3 and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 2D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

4.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *SOD*3 polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.025 were considered as statistically significant due to

Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.

SOD3 specific activity and relative expression of *SOD*3 gene in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al*., 2007).

4.3 RESULTS:

4.3.1 Analysis of association between *SOD*3 C/G; Arg213Gly polymorphism and susceptibility to vitiligo

PCR-RFLP for *SOD*3 C/G Arg213Gly polymorphism yielded a 63 bp undigested product corresponding to G allele and 35 bp and 28 bp digested products corresponding to C allele. The three genotypes identified by 20% polyacrylamide gel electrophoresis were: CC homozygous, CG heterozygous and GG homozygous for Arg213Gly polymorphism of *SOD*3 gene (Figure 1A).

The genotype and allele frequencies of the C/G Arg213Gly polymorphism in 455 vitiligo patients and 548 controls are summarized in Table 3. The Arg213Gly polymorphism of *SOD*3 gene was found to be in significant association with vitiligo patients (p=0.001) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of Arg213Gly polymorphism was more frequent in the vitiligo group compared to the control group (16.0% versus 8.0%, p=0.001; OR 1.363, 95% CI 1.134- 1.639) consistent with a susceptibility effect (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.881 and p=0.081 respectively) (Table 3).

Moreover, generalized vitiligo group showed significant association of Arg213Gly polymorphism when the genotypes were compared with those of control group (p=0.003); however localized vitiligo group did not show significant association of this polymorphism (p=0.047) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the

alleles were compared with those of control group (p=0.004); however, localized vitiligo group showed marginal significance difference in allele frequencies (p=0.021) (Table 4). The distribution of *SOD3* Arg213Gly genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Arg213Gly 'G' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (41.00% versus 32.00%, p=0.0002) (Table 5). However, there was no statistically significant difference in the distribution of the Arg213Gly 'G' allele between patients with stable vitiligo and control group (34.00% versus 32.00%, p=0.487) (Table 5). Also, the Arg213Gly 'G' allele did not show significant difference between patients with active vitiligo as compared to stable vitiligo (41.00% versus 34.00%, p=0.119) (Table 5). This study has 81.46% statistical power for the effect size 0.8 to detect association of Arg213Gly polymorphism of *SOD*3 at p<0.05 in patients and control population.

4.3.2 Analysis of association between *SOD*3 G/A; Ala40Thr polymorphism and susceptibility to vitiligo

PCR-RFLP for *SOD*3 G/A (Ala40Thr) polymorphism yielded a 396 bp undigested product corresponding to G allele, 274 bp and 121 bp digested products corresponding to A allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: GG homozygous, GA heterozygous and AA homozygous for G/A (Ala40Thr) polymorphism of *SOD*3 gene (Figure 1B).

The genotype and allele frequencies of the G/A (Ala40Thr) polymorphism in 448 vitiligo patients and 550 controls are summarized in Table 3. The G/A (Ala40Thr) polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.802; p=0.873) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.070 and p=0.315 respectively) (Table 3). Also, both generalized and localized vitiligo groups did not show significant association of G/A (Ala40Thr) polymorphism when the genotypes were compared with those of control group (p=0.615; p=0.746 respectively) (Table 4). The

distribution of *SOD*3 G/A (Ala40Thr) genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

There was no statistically significant difference in the distribution of the Ala40Thr 'A' allele between both the groups of patients with active and stable vitiligo and control group (p=0.816; p=0.304) (Table 5). Also, the Ala40Thr 'A' allele did not show significant difference between patients with active vitiligo as compared to stable vitiligo (p=0.241) (Table 5).

This study has 81.30% statistical power for the effect size 0.08 to detect the association of *SOD*3 G/A (Ala40Thr) polymorphism at p<0.05 in generalized vitiligo patients and control population.

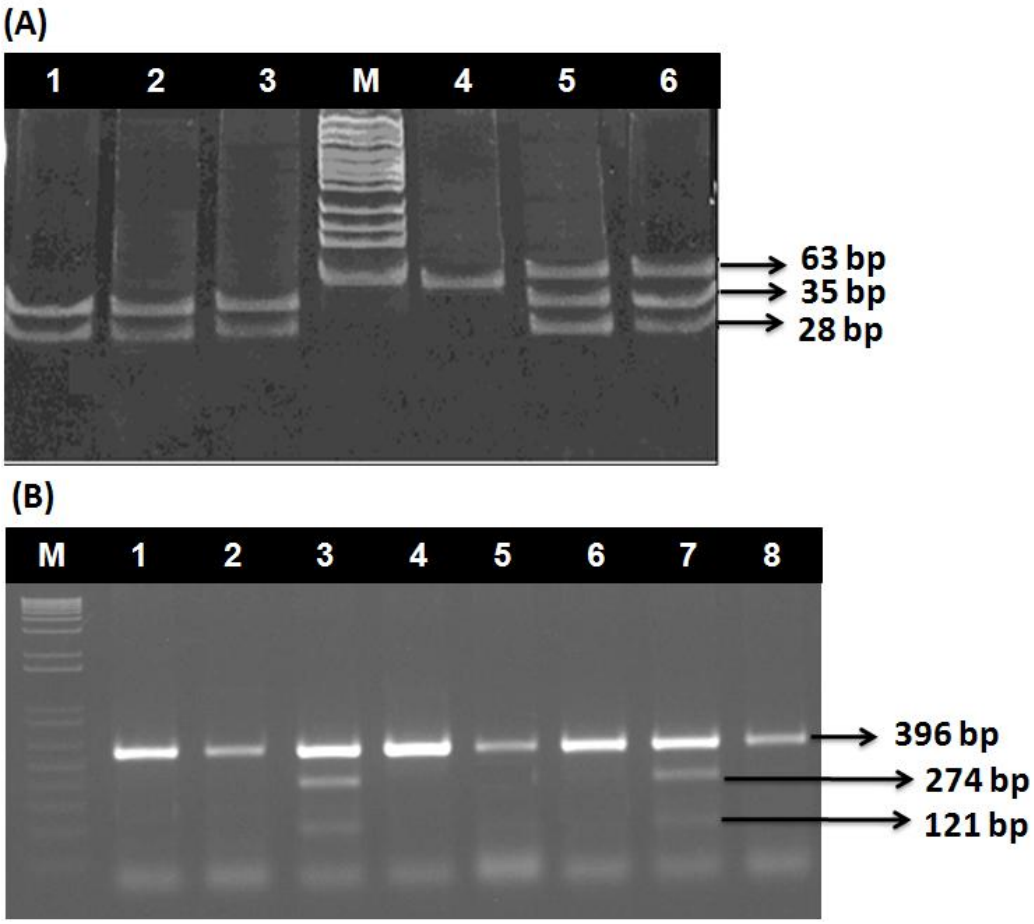


Figure 1. PCR-RFLP analysis of *SOD*3 C/G (Arg213Gly) and G/A (Ala40Thr) polymorphism

(A) PCR-RFLP analysis of *SOD*3 C/G (Arg213Gly) polymorphism on 20% polyacrylamide gel electrophoresis: lanes: 1, 2 & 3 show homozygous (CC) genotypes; lane: 5 shows homozygous (GG) genotype; lanes: 6 & 7 show

heterozygous (CG) genotypes. lane: 4 shows a 50 bp ladder. (B) PCR-RFLP analysis of *SOD*3 G/A (Ala40Thr) polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 4, 5, 6 & 8 show homozygous (AA) genotypes; lanes: 3 & 7 show heterozygous (GA) genotypes; lane M shows 50 bp DNA ladder.

Table 3. Association studies for *SOD*3 gene C/G (Arg213Gly) and G/A (Ala40Thr) polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs1799895 (C/G; Arg213Gly)	Genotype	(n = 455)	(n = 548)			
	CC	170 (0.37)	245 (0.45)		0.881	
	CG	215 (0.47)	256 (0.47)	0.001[a]	(P)	
	GG	70 (0.16)	47 (0.08)			
	Allele				0.081	1.363
	C	555 (0.61)	746 (0.68)	0.001[b]	(C)	(1.134-
	G	355 (0.39)	350 (0.32)			1.639)
rs2536512 (G/A; Ala40Thr)	Genotype	(n = 448)	(n = 550)			
	GG	268 (0.60)	328 (0.60)		0.070	
	GA	148 (0.33)	188 (0.34)	0.802[a]	(P)	
	AA	32 (0.07)	34 (0.06)			
	Allele				0.315	0.9786
	G	684 (0.76)	844 (0.77)	0.873[b]	(C)	(0.7948-
	A	212 (0.24)	256 (0.23)			1.205)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 4. Association studies of *SOD*3 gene for C/G (Arg213Gly) and G/A (Ala40Thr) polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs8192291 (C/G; Arg213Gly)	Genotype	(n = 310)	(n = 145)	(n = 548)			
	CC	119 (0.38)	51 (0.35)	245 (0.45)	0.003[a]	0.45 (GV)	0.7378 (0.6008-0.9061) (GV)
	CG	141 (0.45)	74 (0.51)	256 (0.47)			
	GG	50 (0.17)	20 (0.14)	47 (0.08)	0.047[b]		
	Allele					0.402 (LV)	0.7243 (0.5542-0.9467) (LV)
	C	379 (0.61)	176(0.61)	746 (0.68)	0.004[c]		
	G	241 (0.39)	114(0.39)	350 (0.32)			
					0.021[d]	0.081 (C)	
rs2536512 (G/A; Ala40Thr)	Genotype	(n = 315)	(n = 133)	(n = 550)			
	GG	184 (0.58)	84 (0.63)	328 (0.60)	0.615[a]	0.086 (GV)	0.9216 (0.7332-1.158) (GV)
	GA	106 (0.34)	42 (0.32)	188 (0.34)			
	AA	25 (0.08)	07 (0.05)	34 (0.06)	0.746[b]		
	Allele					0.564 (LV)	1.137 (0.8207-1.576) (LV)
	G	474 (0.75)	210(0.79)	844 (0.77)	0.482[c]		
	A	156 (0.25)	56 (0.21)	256 (0.23)			
					0.465[d]	0.315 (C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(GV) refers to Generalized Vitiligo, (LV) Localized Vitiligo and (C) refers to Controls,

[a] Generalized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,

[b]Localized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,

[c] Generalized Vitiligo vs. Controls using chi-squared test with 2×2 contingency table,

[d] Localized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies of *SOD*3 gene for C/G (Arg213Gly) and G/A (Ala40Thr) polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs8192291 (C/G; Arg213Gly)	Genotype	(n = 339)	(n = 116)	(n = 548)			0.7713 [a]
	CC	122 (0.36)	48 (0.41)	245(0.45)	0.194[a]	0.616	(0.5650-
	CG	159 (0.47)	56 (0.48)	256(0.47)	0.0002[b]	(AV)	1.053)
	GG	58 (0.17)	12 (0.11)	47 (0.08)	0.731[c]		
	Allele					0.461	0.6875 [b]
	C	403 (0.59)	152(0.66)	746(0.68)	0.119 [a]	(SV)	(0.5634-
	G	275 (0.41)	80 (0.34)	350(0.32)	0.0002[b]		0.8390)
					0.487[c]	0.081	
						(C)	0.8914 [c]
							(0.6610-
							1.202)
rs2536512 (G/A; Ala40Thr)	Genotype	(n = 335)	(n = 113)	(n = 550)			1.232 [a]
	GG	206 (0.61)	62 (0.55)	328(0.60)	0.462[a]	0.073	(0.8712-
	GA	106 (0.32)	42 (0.37)	188(0.34)	0.714[b]	(AV)	1.741)
	AA	23 (0.07)	09 (0.08)	34 (0.06)	0.588[c]		
	Allele					0.617	1.034 [b]
	G	518 (0.77)	166(0.73)	844(0.77)	0.241 [a]	(SV)	(0.8224-
	A	152 (0.33)	60 (0.27)	256(0.23)	0.816[b]		1.299)
					0.304[c]	0.315	
						(C)	0.8392 [c]
							(0.6053-
							1.164)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,

[a]Active Vitiligo vs. Stable Vitiligo,

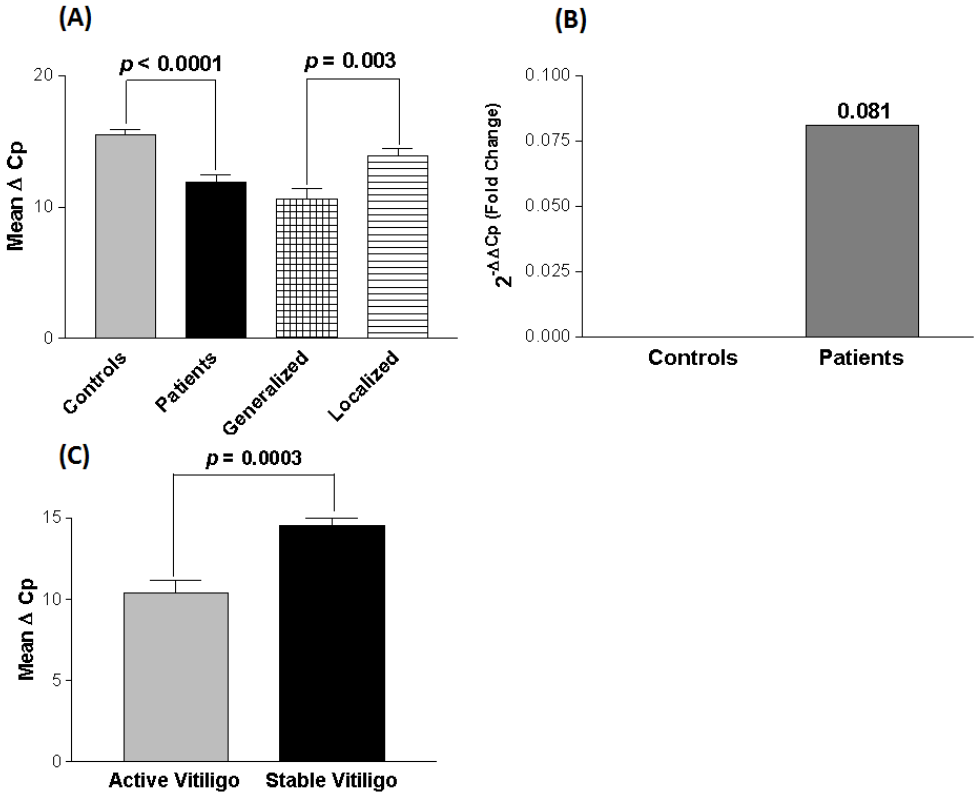
[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

4.3.3 Relative gene expression of *SOD*3 in patients with vitiligo and controls:

Our *SOD*3 gene expression studies showed significant increase in expression of *SOD*3 transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($p<0.0001$) (Figure 2A). Moreover, generalized vitiligo patients showed significant higher expression of *SOD*3 transcripts as compared to localized vitiligo patients ($p=0.003$) (Figure 2A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.081 fold change in the expression of *SOD*3 transcript in patients as compared to controls (Figure 2B). In addition, we also checked the effect of *SOD*3 expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 2C). Interestingly, active vitiligo patients showed significant increase in expression of *SOD*3 transcripts as compared to the patients with stable vitiligo ($p=0.0003$) suggesting the involvement of *SOD*3 in disease progression.



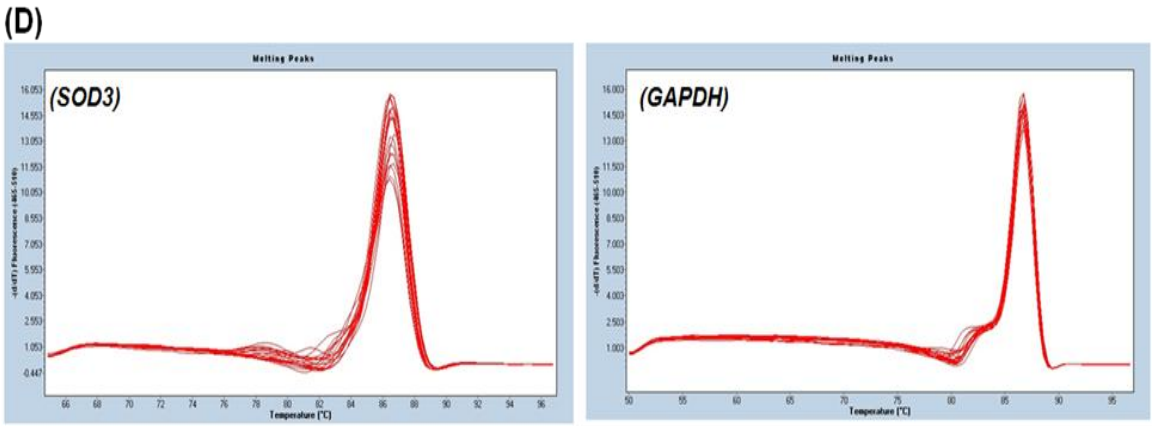


Figure 2. Relative gene expression of *SOD*3 in controls and vitiligo patients:

(A) Expression of *SOD*3 transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *SOD*3 as compared to controls (Mean ΔCp ± SEM: 11.93 ± 0.5535 vs 15.55 ± 0.3914; $p<0.0001$). Generalized vitiligo patients showed significant increase in mRNA levels of *SOD*3 as compared to localized patients (Mean ΔCp ± SEM: 10.64 ± 0.7918 vs 13.92 ± 0.5248; $p=0.003$).

(B) Expression fold change of *SOD*3 transcripts in 166 vitiligo patients against 175 controls showed 0.081 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *SOD*3 transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significant increase in mRNA levels of *SOD*3 as compared to stable vitiligo patients (Mean ΔCp ± SEM: 10.42 ± 0.7306 vs 14.50 ± 0.4859; $p=0.0003$).

(D) Melt curve analysis of *SOD3* and *GAPDH* showing specific amplification.

4.3.4 Estimation of SOD3 activity in vitiligo patients and controls:

We have also analyzed the SOD3 specific activity in plasma samples of 482 vitiligo patients and 564 controls. SOD3 activity differed significantly between vitiligo patients and controls ($p<0.0001$) (Figure 3A). In particular, vitiligo patients showed 2 fold higher SOD3 activity as compared to controls. Also, patients with generalized

vitiligo showed significantly higher activity of SOD3 as compared to localized vitiligo (p=0.025) (Figure 3A).

Analysis based on the stage of progression of vitiligo revealed that active vitiligo patients had significant increase in SOD3 activity as compared to the patients with stable vitiligo (p=0.026) which suggests the involvement of SOD3 in disease progression (Figure 3B).

4.3.5 Correlation of *SOD*3 C/G (Arg213Gly) and G/A (Ala40Thr) genotypes with SOD3 activity in vitiligo patients:

Further, the SOD3 activity was correlated with the genotypes obtained for Arg213Gly and Ala40Thr polymorphisms (Figure 3). Interestingly, SOD3 activity was significantly increased in patients with susceptible Arg213Gly GG genotypes and heterozygous CG genotypes as compared to controls (p=0.022; p=0.041) suggesting the involvement of Arg213Gly 'G' allele in increased activity of SOD3. However, no significant difference was observed in SOD3 activity in patients with CC genotypes as compared to controls (p=0.651) (Figure 3C). Moreover, there was no significant difference in SOD3 activity in patients with Ala40Thr GG, GA and AA genotypes as compared to controls (p=0.866; p=0.202; p=0.071) (Figure 3D) suggesting that Ala40Thr G/A polymorphism is not involved in increased SOD3 activity in patients.

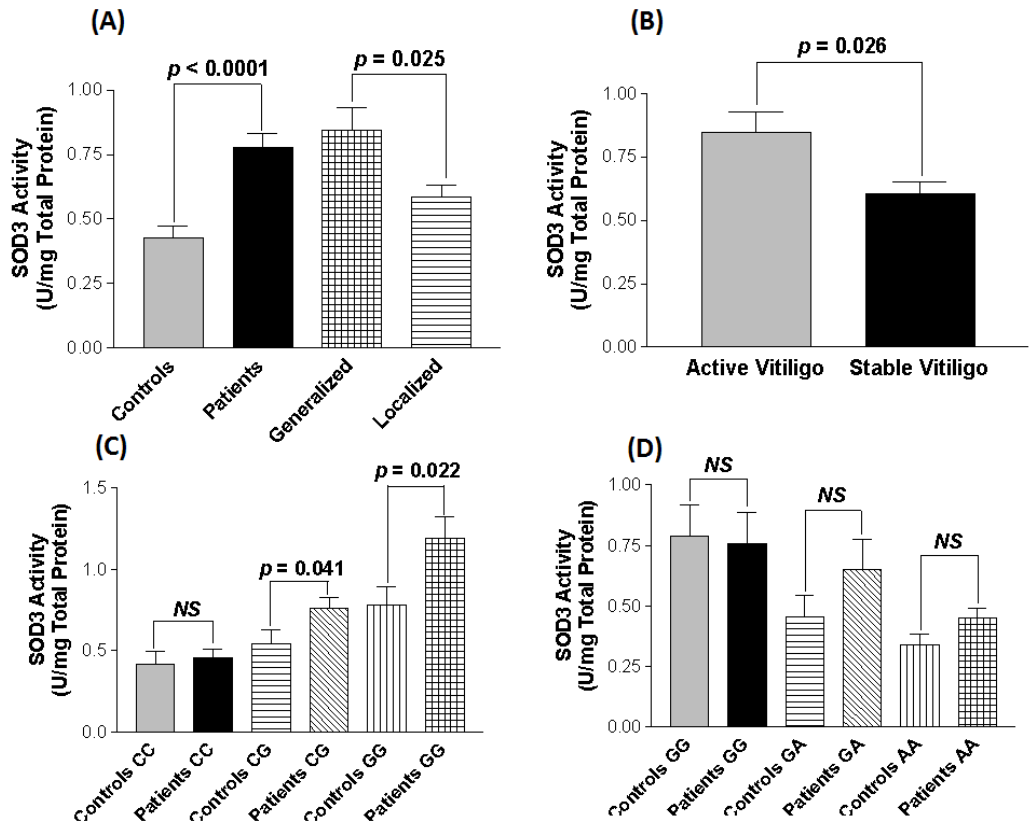


Figure 3. **SOD3 activity in controls and vitiligo patients:**

(A) SOD3 specific activity in 482 vitiligo patients and 564 controls. Vitiligo patients showed significant increase in SOD3 activity as compared to controls (Mean ± SEM: 0.7785 ± 0.05568 vs 0.4255 ± 0.04589; p<0.0001). Generalized vitiligo patients (n=328) showed significant increase in SOD3 activity as compared to localized patients (n=154) (Mean ± SEM: 0.8448 ± 0.08551 vs 0.5881 ± 0.04563; p=0.025).

(B) SOD3 specific activity with respect to progression of the disease in 352 patients with active vitiligo and 130 patients with stable vitiligo. Active vitiligo patients showed significant increase in SOD3 activity as compared to stable vitiligo patients (Mean ± SEM: 0.8490 ± 0.08076 vs 0.6040 ± 0.04752; p=0.026).

(C) SOD3 specific activity with respect to *SOD3* C/G (Arg213Gly) polymorphism in 455 vitiligo patients and 548 controls. Vitiligo patients showed significant increase in SOD3 activity with GG (Mean ± SEM: 1.194 ± 0.1316 vs 0.7793 ± 0.1126; p=0.022) and CG (Mean ± SEM: 0.7644 ± 0.06393 vs 0.5451 ± 0.08456; p=0.041) genotypes as compared to controls. There was no significant difference in the activity of SOD3 with CC genotypes (Mean ± SEM: 0.4592 ± 0.05117 vs 0.4179 ± 0.07670; p=0.651) as compared to controls.

(D) SOD3 specific activity with respect to *SOD3* G/A (Ala40Thr) polymorphism in 448 vitiligo patients and 550 controls. Vitiligo patients did not show significant difference in SOD3 activity with GG (Mean ± SEM: 0.7588 ± 0.1256 vs 0.7896 ± 0.1276; p=0.866), GA (Mean ± SEM: 0.6506 ± 0.1241 vs 0.4556 ± 0.08755; p=0.202) and AA genotypes (Mean ± SEM: 0.4525 ± 0.03683 vs 0.3391 ± 0.04543; p=0.071) as compared to controls. [*NS* = non-significant]

4.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may involve genes important for melanin biosynthesis, response to oxidative stress and regulation for autoimmunity as well as environmental factors. Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction as H_2O_2 accumulation is observed in the epidermis of active vitiligo patients (Bindoli *et al.,* 1992; Schallreuter *et al.,* 1996). One of the important reasons for increased epidermal H_2O_2 levels is an imbalance in the antioxidant system. Previously, we have reported significant increase in erythrocyte lipid peroxidation levels in patients of vitiligo in all age groups as compared to healthy controls from Gujarat (Agrawal *et al.,* 2004; Shajil and Begum, 2006). The study showed that systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients (Agrawal *et al.,* 2004). Systemic oxidative stress is also reported to be elevated in patients (Hazneci *et a.l,* 2005). The mechanism underlying this etiology is not yet known but increased systemic oxidative stress is generated due to altered antioxidant system which affects the epidermal melanocytes leading to vitiligo manifestation.

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (Schallreuter *et al.,* 1991). SOD3 is found predominantly in the extracellular matrix of mammalian tissues (Marklund, 1984; Oury et al., 1994). The C-terminal region is highly rich in basic amino acid residues (Hjalmarsson *et al*., 1987). This polybasic region or the extracellular matrix (ECM)-binding region (Petersen *et al.,* 2004) is involved in its binding to heparin/heparan sulfate (Adachi et al., 1992) and type I collagen (Petersen *et al.,* 2004). The heparin binding domain has also been proposed to act as a nuclear localization signal in certain cell types (Ookawara *et al.,* 2003), suggesting that SOD3 may provide antioxidant protection to DNA and nuclear

proteins. The C/G SNP present in *SOD3* gene which substitutes Arg to Gly at codon 213 inhibits ionic interaction between heparin and SOD3 which in turn results in 10 fold increase in SOD3 concentration in plasma (Sandstrom *et al*., 1994). The Arg213Gly SNP located at the heparin-binding domain has been identified in a small proportion of the healthy population (Folz *et al*., 1994) and has been associated with very high plasma SOD3 levels due to altered affinity of SOD3 for heparin at the endothelial cell surface (Sandstrom *et al*., 1994; Adachi *et al.*, 1996; Karlsson *et al*., 1994). The present study reports that C/G Arg213Gly polymorphism of SOD3 is significantly associated with vitiligo susceptibility. In particular, the Arg213Gly 'G' allele is prevalent in patient group as compared to controls. Moreover, patients with active vitiligo had higher frequency of Arg213Gly 'G' allele as compared to controls suggesting that 'G' allele may be involved in progression of the disease. Interstingly, vitligo patients showed approximately two fold higher activity of SOD3 as compared to controls. Also, generalized vitiligo patients showed increased SOD3 activity as compared to patients with localized vitiligo. The genotype-phenotype correlation further confirms the association of Agr213Gly 'G' allele with increased activity of SOD3 which can modulate the susceptibility of an individual towards vitliligo. Furthermore, patients with active vitiligo showed increased SOD3 activity as compared to stable vitiligo suggesting SOD3 involvement in the progression of the disease. The increased SOD3 activity may lead to increased H_2O_2 production which in turn result in substrate inhibition of the down stream antioxidant enzyme e.g. catalase ultimately leading to H_2O_2 mediated cell damage. Previously, Shajil and Begum (2006) reported low activity of catalse and glutathione peroxidase in vitiligo patients from Gujarat, supporting these results. We have also monitored *SOD*3 expression in vitligo patients and controls to know whether the increased activity of SOD3 is due to an increase in *SOD*3 transcript levels. The expression results suggested that vitligo patients had increased *SOD*3 transcript levels as compared to controls. Also the expression of *SOD*3 was higher in generalized vitligo patients as compared to patients with localized vitilgio. Moreover, active vitligo patients exhibited increased *SOD*3 transcript levels as compared to stable vitligo patients suggesting the possiblity that increased activity of SOD3 in patients may be due to increased expression of *SOD*3. Thus increased levels of SOD3 in vitiligo patients could enhance the systemic production of H_2O_2 which in turn results in increased oxidative stress in patients.

The second polymorphism G/A Ala40Thr addressed in this study is located in the amino terminal domain, which may affect the tetramerization of SOD3. The findings of Tamai et al. (2006) suggested the possibility that the Thr allele (Thr40Ala) is associated with impaired function of SOD3. However, the present study could not achieve significant association of this polymorphism with vitiligo susceptibility. Moreover, there was no significant difference in allele distribution of this polymorphism between different types of vitiligo i.e. active vs stable and generalized vs localized. In addtion, the genotype-phenotype correlation for this polymorphism remained uninformative as there was no significant difference in the SOD3 activity with different genotypes of Ala40Thr polymorphism in patients suggesting that this polymorphism may not be involved in increased activity of SOD3 as observed in vitiligo patients.

Interestingly, the present study suggested an increase in *SOD*3 transcripts in vitiligo patients as compared to controls. This increased *SOD*3 expression may be responsible for the increased SOD3 activity especially with respect to Arg213Gly polymorphism. Expression of *SOD*3 can be induced by various factors. *In vitro* studies in skin fibroblasts showed that heparin and heparan sulfate could induce *SOD*3 mRNA as well as protein expression. Moreover, in vascular smooth muscle cells (VSMC) and lung alveolar type 2 cells, inflammatory cytokines such as TNF-α and IFN-γ showed induction of *SOD*3 mRNA and protein expression. While certain stimuli can also induce *SOD*3 expression e.g. a variety of growth factors have been shown to repress *SOD*3 mRNA expression, such as TGF-β in human fibroblasts (Marklund, 1992) and PDGF and FGF in vascular smooth muscle cells (Stralin & Marklund, 2001). Expression of the human *SOD*3 is regulated by transcription factors such as Sp1 and Sp3 in the lung (Zelko *et al*, 2008). A putative NF-κB motif in the human *SOD*3 promoter region has been proposed as the functional transcriptional binding site contributing to induction and coregulation of inducible nitric oxide synthase and SOD3 (Folz and Crapo, 1994; Fattman et al., 2003, Brady et al., 1997).

In conclusion, the present study signifies the imorptant role of SOD3 in pathogenesis and progression of vitiligo. In particular, Arg213Gly polymorphism of *SOD*3 and increased levels of *SOD*3 transcripts may in combination are responsible for

increased activity of SOD3 in extracelluar fluids resulting in increased H_2O_2 production. As the downstream antioxidant system in vitiligo patients was found to be disturbed, the H_2O_2 is not properly removed and may finally lead to oxidative damage to the melanocytes.

4.5 REFERENCES

Agrawal, D., Shajil, E.M., Marfatia, Y.S., and Begum, R. (2004). Study of the antioxidant status of vitiligo patients of different age groups in Baroda. Pigment Cell Res. *17*, 289-94.

Beuge, J.A., and Aust, S.D. (1978). Microsomal lipid peroxidation. Methods in Enzymol. *52*, 302-310.

Bhatia, P.S., Mohan, L., Pandey, O.N., Singh, K.K., Arora, S.K., and Mukhija, R.D. (1992). Genetic nature of vitiligo. J Derm Sci. *4*, 181-184.

Bowler, R. P.; Crapo, J. D. Oxidative stress in airways: is there a role for extracellular superoxide dismutase? *Am. J. Respir. Crit. Care Med*. **166:**S38–S43; 2002.

Brady, T. C.; Chang, L. Y.; Day, B. J.; Crapo, J. D. Extracellular superoxide dismutase Am. J. Physiol. 273:L1002–1006; 1997.

Casp, C.B., She, J.X., and McCormack, W.T. (2002) Genetic association of the catalase gene (CAT) with vitiligo susceptibility, Pigment Cell Res. *15*, 62-66.

Das, S.K., Majumder, P.P., Chakraborty, R., Majumdar, T.K., Haldar, B. (1985). Studies on vitiligo. Epidemiological profile in Calcutta, India. Genet. Epidemiol. *1*, 71-78.

Delanty, N.; Dichter, M. A. Oxidative injury in the nervous system. *Acta Neurol. Scand*. **98:**145–153; 1998.

Dell'Anna, M.L., Maresca, V., Briganti, S., Camera, E., Falchi, M., and Picardo, M. (2001). Mitochondrial impairment in peripheral blood mononuclear cells during the active phase of vitiligo. J Invest Dermatol. *117*, 908–913.

Dell'Anna, M.L., Urbanelli, S., Mastrofrancesco, A., Camera, E., Iacovelli, P., Leone, G., Manini, P., D'iscia, M., and Picardo, M. (2003). Alterations of mitochondria in peripheral blood mononuclear cells of vitiligo patients. Pigment Cell Res. *16*, 553-559.

Fain, P.R., Gowan, K., and LaBerge, G.S. (2003). A genome wide screen for generalized vitiligo: confirmation of AIS1 on chromosome 1p31 and evidence for additional susceptibility loci. Am J Hum Genet. *72*, 1560-1564.

Fattman C.L., Schaefer L.M., Oury T.D. Extracellular superoxide dismutase in biology and medicine, Free Radic. Biol.Med. 35 (2003) 236–256.

Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* 2007; **39**: 175-191.

Folz, R. J.; Crapo, J. D. Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. Genomics 22:162–171; 1994.

Fukai, T.; Folz, R. J.; Landmesser, U.; Harrison, D. G. Extracellular superoxide dismutase and cardiovascular disease. *Cardiovasc.Res*. **55:**239–249; 2002.

Gavalas, N.G., Akhtar, S., Gawkrodger, D.J., Watson, P.F., Weetman, A.P., and Kemp, E.H. (2006). Analysis of allelic variants in the catalase gene in patients with the skin depigmenting disorder vitiligo. Biochem. Biophys. Res. Commun. *345*, 1586-1591.

Goth, L., Rass, P., and Pay, A. (2004). Catalase enzyme mutations and their association with diseases. Mol Diagn. *8*, 141-149.

Handa S and Kaur I. Vitiligo: clinical findings in 1436 patients. *J Dermatol* 1999; **10**: 653-657.

Handa, S., and Kaur, I. (1999). Vitiligo: clinical findings in 1436 patients. J Dermatol. *10*, 653-657.

Hasse, S., Gibbons, N.C.J., Rokos, H., Marles, L.K., and Schallreuter, K.U. (2004). Perturbed 6-tetrahydrobiopterin recycling via decreased dihydropteridine reductase in Vitiligo: more evidence for H_2O_2 stress. J Invest Dermatol. *122,* 307-313.

Hazneci, E., Karabulut, A.B., Ozturk, C., Batcioglu, K., Dogan, G., Karaca, S., and Esrefoglu, M. (2005). A comparative study of superoxide dismutase, catalase and glutathione peroxidase activities and nitrate levels in vitiligo patients. Int J Dermatol. *44*, 636-640.

Ines, D., Sonia, B., Riadh, B.M., Amel, G., Slaheddine, M, Hamida, T., Hamadi, A., Basma, H. (2006) A comparative study of oxidant-antioxidant status in stable and active vitiligo patients. Arch. Dermatol. Res. *298*, 147-152.

is upregulated with inducible nitric oxide synthase after NF-kappa B activation.

Kemp, E.H. (2004). Autoantibodies as diagnostic and predictive markers of vitiligo. Autoimmunity *37,* 287-290.

Kim, S.M., Chung, H.S., and Hann. S.K. (1998). The genetics of vitiligo in Korean patients. Int J Dermatol. *37*, 908-910.

Koca, R., Armutcu, H., Altinyazar, H.C., and Gurel, A. (2004). Oxidant antioxidant enzymes and lipid peroxidation in generalized vitiligo. Clin and Exp Dermatol. *29*, 406-409.

Le Poole, I.C., Das, P.K., Van Den Wijngaard, R.M., Bose, J.D., and Westerhof, W. (1993). Review of the etiopathomechanism of vitiligo: a convergence theory. Exp Dermatol. *2*, 146-153.

Le Poole, I.C., Das, P.K., Van Den Wijngaard, R.M., Bose, J.D., Westerhof, W. (1993). Review of the etiopathomechanism of vitiligo: a convergence theory. Exp Dermatol. *2*, 146-153.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). "Protein measurement with the Folin phenol reagent". J. Biol. Chem. 193: 265–75.

Maresca, V., Roccella, M., and Roccella, F. (1997). Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. J Invest Dermatol. *109*, 310–313.

Marklund, S. L. (1992). Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. *J. Biol. Chem.*, *267*(10), 6696–6701.

McCord, J. M. Human disease, free radicals, and the oxidant/ antioxidant balance. *Clin. Biochem*. **26:**351–357; 1993.

Nath, S.K., Majumder, P.P., and Nordlund, J.J. (1994). Genetic epidemiology of vitiligo: multilocus recessivity cross-validated. Am J of Hum Genet. *55*, 981-990.

Nordlund, J.J. (1997). The epidemiology and genetics of vitiligo. Clin Dermatol. *15*, 875-878.

Ortonne, J.P., and Bose, S.K. (1993). Vitiligo: Where do we stand? Pigment Cell Res. *8*, 61-72.

Ortonne, J.P., and Bose, S.K. (1993). Vitiligo: Where do we stand? Pigment Cell Res. *8*, 61-72.

Oury, T. D.; Day, B. J.; Crapo, J. D. Extracellular superoxide dismutase: a regulator of nitric oxide bioavailability. *Lab. Invest*. **75:**617–636; 1996.

Park, H.H., Ha, E., Uhm, Y.K., Jin, S.Y., Kim, Y.J., Chung, J.H., and Lee, M.H. (2006). Association study between catalase gene polymorphisms and the susceptibility to vitiligo in Korean population. Exp. Dermatol. *15*, 3 77-380.

Passi, S., Grandinetti, M., Maggio, F., Stancato, A., and De Luca, C. (1998). Epidermal oxidative stress in vitiligo. Pigment Cell Res. *2*, 81 - 85.

Picardo, M., Passi, S., Morrone, A., and Grandinetti, M. (1994). Antioxidant status in the blood of patients with active vitiligo. Pigment Cell Res. *2*, 110-115.

Picardo, M., Passi, S., Morrone, A., and Grandinetti, M. (1994). Antioxidant status in the blood of patients with active vitiligo. Pigment Cell Res. *2*, 110-115.

Schallreuter, K.U., Moore, J., Wood, J.M., Beazley, W.D., and Gaze, D,C. (1999). In *vivo* and *in vitro* evidence for hydrogen peroxide accumulation in the epidermis of patients of vitiligo and its successful removal by UVB activated pseudocatalase, J of Invest Dermatol symposium proceedings. *4*, 91-96.

Schallreuter, K.U., Wood, J.M., and Berger, J. (1991). Low catalase levels in the epidermis of patients with vitiligo. J Invest Dermatol. 6, 1081-1085.

Schallreuter, K.U., Wood, J.M., Ziegler, I., Lemke, K.R., Pittelkow, M.R., Lindsey, N.J., and Gutlich, M. (1994a). Defective tetrahydrobiopterin and catecholamine

biosynthesis in the depigmentation disorder vitiligo. Biochim Biophys Acta. *2*, 181-192.

Shajil, E.M., Agrawal, D., Vagadia, K., Marfatia, Y.S., and Begum, R. (2006) Vitiligo: Clinical profiles in Vadodara, Gujarat. Ind. J. Dermatol. *51*, 100-104.

Shajil, E.M., and Begum. R. (2006). Antioxidant status of segmental and non-segmental vitiligo. Pigment Cell Res. *19*, 179-180.

Shajil, E.M., Chatterjee, S., Agrawal, D., Bagchi, T., and Begum, R. (2006). Vitiligo: pathomechanisms and genetic polymorphism of susceptible genes. Ind. J. Exp. Biol. *44*, 526-539.

Shajil, E.M., Marfatia, Y.S., and Begum, R. (2006). Acetylcholine esterase levels in different clinical types of vitiligo in Baroda, Gujarat. Ind. J. Dermatol. *51*, 289-291.

Stralin, P., & Marklund, S. L. (2001). Vasoactive factors and growth factors alter vascular smooth muscle cell EC-SOD expression. *Am. J. Physiol. Heart Circ. Physiol.*, *281*(4), 1621–1629.

Taieb A, Picardo M, VETF Members. The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res* 2007; **20**: 27-35.

Taieb, A. (2000). Intrinsic and extrinsic pathomechanisms in vitiligo. Pigment Cell Res. *8*, 41-47.

Tamai M, Furuta H, Kawashima H, Doi A, Hamanishi T, Shimomura H, Sakagashira S, Nishi M, SasakiH, Sanke T, Nanjo K (2006) Extracellular superoxide dismutase gene polymorphism is associated with insulin resistance and the susceptibility to type 2 diabetes. Diabetes Res Clin Pract 71:140–145

Valia AK, Dutta PK. IADVL. Text book and Atlas of Dermatology. *Bombay: Bhalani Publishing House* 1996; 500-586.

Valia, A.K., and Dutta, P.K. (1996). IADVL Text book and Atlas of Dermatology. Bombay: Bhalani Publishing House. pp. 500 – 586.

Yamada H., Y. Yamada, T. Adachi, H. Goto, N. Ogasawara, A. Futenma, et al. Molecular analysis of extracellular-superoxide dismutase gene associated with high level in serum, Jpn. J. Hum. Genet. 40 (1995) 177–184.

Yamada H., Y. Yamada, T. Adachi, H. Goto, N. Ogasawara, A. Futenma, et al. Polymorphism of extracellular superoxide dismutase (EC-SOD) gene: relation to the mutation responsible for high EC-SOD level in serum, Jpn. J. Hum. Genet. 42 (1997) 353–356.

Yildirim, M., Baysal, V., Inaloz, H.S., Kesici, D., Delibas, N. (2003). The role of oxidants and antioxidants in generalized vitiligo. J Dermatol. *2*, 104-108.

5.1 INTRODUCTION

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from the epidermis. The mechanism of melanocyte loss from the epidermis to cause vitiligo is not yet clearly understood (Taieb *et al*., 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are oxidative stress, autoimmune and neurochemical hypotheses (Ortonne and Bose, 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp *et al*., 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp *et al.,* 2001). Also the immune suppressive effect of a number of repigmenting therapies (steroids) indirectly supports the autoimmune mediated process of depigmentation (Ongenae *et al*., 2003). However, the exact mechanism involved in vitiligo pathogenesis remains obscure.

The aim of this study was to find whether oxidative stress or autoimmunity plays a major role for the initiation of vitiligo pathogenesis and its progression. Lipid peroxidation levels, the index of oxidative stress was assessed to evaluate the oxidative stress hypothesis and the levels of antimelanocyte antibodies were measured to evaluate the autoimmune hypothesis in patients with the onset of vitiligo (<3 months) and compared with the patients suffering from the disease for long duration of (>3 months). For the progression of the disease, patients with active vitiligo and stable vitiligo were compared for LPO levels and antimelanocyte antibody levels.

5.2 MATERIALS AND METHODS

5.2.1 Study subjects:

The study group included 300 vitiligo patients and comprised of 138 males and 162 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 400 ethnically sex-matched unaffected individuals (188 males and

212 females) were included in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo or 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.

Table 1. **Demographic characteristics of vitiligo patients and unaffected controls.**

	Vitiligo Patients	**Controls**
	(n = 300)	(n = 400)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	138 (46.0%)	188 (47.00%)
Female	162 (54.0%)	212 (53.00%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Patients with		
<3 months onset	124 (41.33%)	NA
>3 months onset	176 (58.67%)	NA
Active vitiligo	215 (71.67%)	NA
Stable vitiligo	85 (28.33%)	NA
Family history	41 (13.66%)	NA

5.2.2 Estimation of lipid peroxidation (LPO) levels:

Erythrocyte LPO levels were estimated according to Beuge and Aust (1978) method as described in Chapter 2.

5.2.3 Estimation of antimelanocyte antibody levels by enzyme linked immunosorbent assay (ELISA):

In the present study, plasma from vitiligo patients was examined for the reactivity with the human melanoma cell line (SK Mel 28) to find the levels of antimelanocyte antibodies in vitiligo patients compared to controls. We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA) and plasma was extracted. Human melanoma cell line SK Mel 28 was obtained from NCCS, Pune and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mg/L gentamycin at 37°C under 5% CO2 in a humidified atmosphere (Hann and Kim, 1995).

Cells were harvested by scraping and lysed by adding lysis buffer (HEPES 20 mM, EGTA 1 mM, PMSF 1 mM, MgCl2 1.5 mM, NaCl 150 mM, CuSO4 1mM, Trition -X 100 1%, Glycerol 1%). Protein estimation in the lysate was done by Lowry's method (Lowry *et al*., 1951). Fifty µl of the cell lysate containing 1 µg protein was loaded in each well of the microtiter plate and kept at 4^oC overnight. Excess antigen was discarded, washed 3 times with PBS, blocked with 1% BSA in PBS and incubated for 1 hour. Excess blocking reagent was discarded and washed 3 times with PBS. Fifty µl of plasma (1:10,000 diluted in PBS) was added to the micro titer plates and incubated for 2 hours at room temperature. Excess plasma was discarded and washed 3 times with PBS containing 0.2% Tween 20. Added 50 µl of 1: 2000 diluted secondary antibody (Rabbit anti human IgG HRP conjugate, Bangalore Genei, India) and incubated for 1 hour. Excess antibody was discarded and washed 3 times with PBS containing 0.2% Tween 20. Fifty µl substrate (TMB-H_2O_2) was added and incubated for 5 minutes for the color development. The reaction was stopped by adding 200 µl of 1N H_2SO_4 as the color developed and OD was read at 405 nm.

5.2.4 Statistical analysis:

LPO and antimelanocyte antibody levels were plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.05 were considered as statistically significant.

5.3 RESULTS

Antimelanocyte antibody and LPO levels were measured in 300 vitiligo patients and 400 controls. Vitiligo patients showed significant increase in LPO levels and antimelanocyte antibody levels as compared to controls (p=0.009; p<0.0001 respectively) (Figure 1A & B).

Further, we compared lipid peroxidation levels to evaluate the oxidative stress hypothesis and antimelanocyte antibody levels to evaluate the autoimmune hypothesis in patients at the onset of vitiligo (< 3 months) and with those suffering from vitiligo for a long duration (>3 months). Significant low (p=0.0008) levels of antimelanocyte antibodies were observed in <3 months patients compared to >3 months patients (Figure 2B). On the contrary, significant high (p=0.009) LPO levels were observed in vitiligo patients at the onset (>3 months) compared to vitiligo patients with long duration of the disease (Figure 2A).

Moreover, analysis of antimelanocyte antibody levels and LPO levels were monitored based on progression of the disease i.e. active vitiligo and stable vitiligo. Interestingly, patients with active vitiligo showed significant increase in antimelanocyte antibody (p<0.0001) and LPO levels (p=0.002) as compared to stable vitiligo patients (Figure 2C & D).

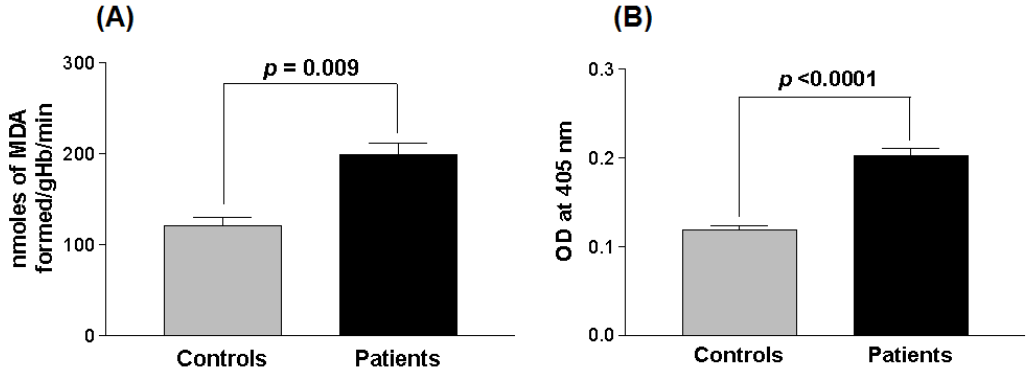


Figure 1. Lipid peroxidation (LPO) and antimelanocyte antibody levels in controls and vitiligo patients:

(A) LPO levels in 300 vitiligo patients and 400 controls. Vitiligo patients showed significantly increased LPO levels as compared to controls (Mean ± SEM: 199.5 ± 12.84 vs 120.7 ± 9.199; p<0.0001).

(B) Antimelanocyte antibody levels in 300 vitiligo patients and 400 controls. Vitiligo patients showed significantly increased antimelanocyte antibody levels as compared to controls (Mean ± SEM: 0.2029 ± 0.0081 vs 0.1186 ± 0.0042; p<0.0001).

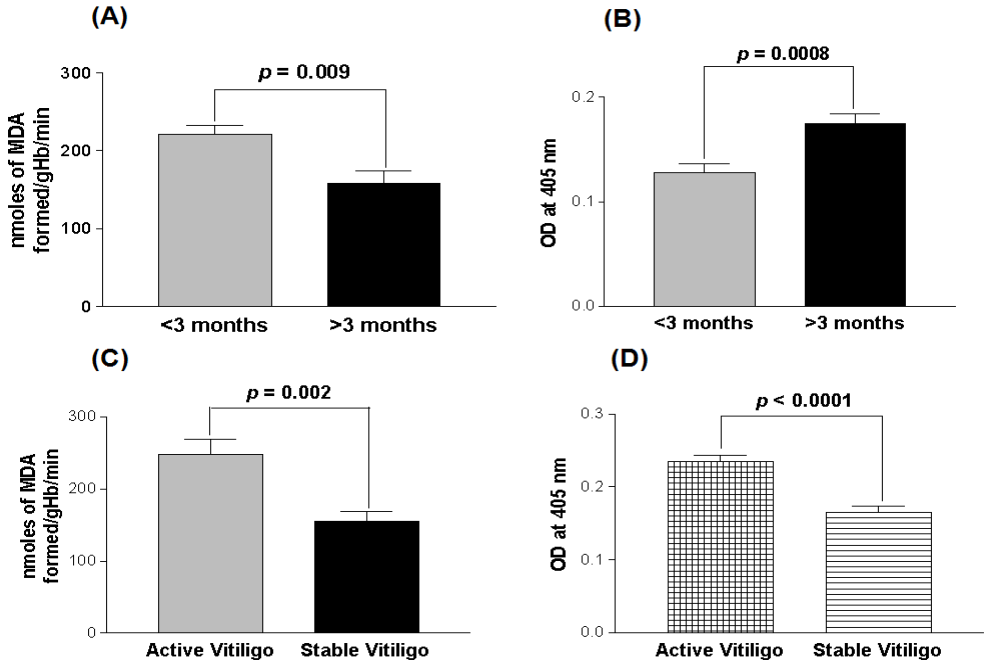


Figure 2. Lipid peroxidation (LPO) and antimelanocyte antibody levels with respect to onset and progression of disease:

(A) LPO levels in vitiligo patients with <3 months onset (n=124) and >3 months (n=176) of disease. Patients with <3 months onset showed significantly higher levels of LPO as compared to >3 months onset (Mean ± SEM: 221.1 ± 11.65 vs 158.5 ± 15.51; p=0.009).

(B) Antimelanocyte antibody levels in vitiligo patients with <3 months onset (n=124) and >3 months (n=176) of disease. Patients with <3 months onset showed significantly lower levels of antimelanocyte antibody as compared to >3 months of disease (Mean ± SEM: 0.1280 ± 0.0084 vs 0.1743 ± 0.0097; p=0.0008).

(C) LPO levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of LPO as compared to stable vitiligo (Mean ± SEM: 247.7 ± 20.94 vs 155.1 ± 13.84; p=0.002).

(D) Antimelanocyte antibody levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of antimelanocyte

antibody as compared to stable vitiligo (Mean ± SEM: 0.2350 ± 0.0086 vs 0.1653 ± 0.0079; $p<0.0001$).

5.4 DISCUSSION

There are several reports on the antimelanocyte antibody levels in vitiligo patients compared to controls. However, no study has been performed to evaluate both oxidative stress and autoimmune hypotheses simultaneously at the onset of vitiligo to find out which factor/s play a major role in triggering vitiligo. In addition, it is also not clear which hypothesis is prevailing in understanding the progression of the disease.

Naughton *et al.,* (1983) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation (Naughton *et al.,* 1983a). In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton et al 1983b). However, Grimes *et al.,* (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Farrokhi *et al.,* (2005) also showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients.

The present study reports that patients with long duration (>3 months) exhibited significantly lower (p=0.009) LPO levels compared to patients with early onset (<3 months) of the disease (Figure 2A). However, patients at the onset (<3 months) showed significantly lower (p=0.0008) levels of antimelanocyte antibodies compared to patients with long duration (>3 months) of vitiligo (Figure 2B). These results suggest that oxidative stress rather than autoantibodies plays a major role in the initiation of vitiligo pathogenesis. Moreover, our results on neurochemical hypothesis also showed that significant decrease in acetylcholine esterase levels in vitiligo patients compared to controls (Shajil *et al.,* 2006). This could be due to H_2O_2 mediated oxidation of AChE, thus emphasizing the role of oxidative stress in precipitating vitiligo (Schallreuter *et al.,* 2004). Interestingly, analysis of antimelanocyte antibody levels and LPO levels were monitored based on progression of the disease revealed that both LPO and antimelanocyte antibodies were higher in active cases of vitiligo compared to stable vitiligo (Figure 2C & D). This signifies the equal contribution of both oxidative stress and autoimmunity in disease progression.

There are several ways by which high oxidative stress, besides having a direct melanocytotoxicity, can induce an autoimmune attack against melanocytes. The structures of melanocytic macromolecules such as Melan-A and tyrosinase, may markedly change by acute or chronic oxidative stress and can act as antigens (neo-antigens). Neo-antigens with sufficient homology or identity to host antigenic proteins induce auto-reactivity. This phenomenon is referred to as 'molecular mimicry' (Kannan, 2006). Aldehydic products, mainly the 4-hydroxy-2-alkenals, form adducts with proteins and make them highly immunogenic (Kurien *et al*., 2006). Hydroxyl radicals are also very highly reactive and could attack a wide range of targets. The presence of lesions in vitiligo patients can be explained by this mechanism (Vahedi Darmian *et al*., 2004). Over time, chronic oxidative stress could generate several adducted molecules that could act as 'neo-antigens'. This is consistent with the slow maturation of auto-antibodies during the evolution of the disease. More neo-antigens are involved in amplifying the autoaggressive lymphocytes by a process referred to as 'antigen spreading'. This is an autoimmune reaction initially directed against a single autoantigen that spreads to other autoantigens, causing the T helper cells to recognize them (Kannan, 2006).

During chronic oxidative stress, neo-antigens potentially cause tissue damage and release a plethora of sequestered auto-antigens. This process is referred to as the 'bystander effect'. Such an outburst of auto-antigens from the target tissue would potentially amplify the effect of the neo-antigens, leading to the breakdown of self-tolerance (Kannan, 2006).

In conclusion, 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.

5.5 REFERENCES:

Beuge, J.A., and Aust, S.D. (1978). Microsomal lipid peroxidation. Methods in Enzymol. *52*, 302-310.

Farrokhi, S., Hojjat-Farsangi, M., Noohpisheh, M.K., Tahmasbi, R., and Rezaei, N. (2005). Assessment of the immune system in 55 Iranian patients with vitiligo. J Eur Acad Dermatol Venereol. *19*, 706-711.

Kemp, E.H., Waterman E.A., and Weetman A.P (2001). Autoimmune aspects of vitiligo. Autoimmunity. *34*, 65-77.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with Folin phenol reagent. J Biol Chem. *193*, 265-275.

Naughton, G.K., Eisinger, M., and Bystryn, J.C. (1983a). Antibodies to normal human melanocytes in vitiligo. J Exp Med. *158*, 246-251.

Naughton, G.K., Eisinger, M., and Bystryn, J.C. (1983b). Detection of antibodies to melanocytes in vitiligo by specific immunoprecipitation. J Invest Dermatol. *81*, 541-542.

Ongenae, K., Van Geel, N., and Naeyaert, J.M. (2003). Evidence for an autoimmune pathogenesis of vitiligo. Pigment Cell Res. *2*, 90-100.

Ortonne, J.P., and Bose, S.K. (1993). Vitiligo: Where do we stand? Pigment Cell Res. *8*, 61-72.

Schallreuter, K.U., Elwary, S.M., Gibbons, C.J., Rokos, H., and Wood, J.M. (2004). Activation/deactivation of acetylcholine esterase by H_2O_2: More evidence for oxidative stress in vitiligo. Biochem Biophys Res Commun. *315*, 502-508.

Shajil, E.M., Marfatia, Y.S., and Begum, R. (2006). Acetylcholine esterase levels in different clinical types of vitiligo in Baroda, Gujarat. Ind. J. Dermatol. *51*, 289-291.

Taieb, A. (2000). Intrinsic and extrinsic pathomechanisms in vitiligo. Pigment Cell Res. *8*, 41-47.

Kannan, S. (2006). Free radical theory of autoimmunity. Theor Biol Med Model. *3*, 22.

Vahedi Darmian, F., Joubeh, S., Doroudchi, M., Abdollahi, B., and Ghaderi, A. (2004). Detection of rheumatoid factors in sera and biopsy lesions of vitiligo patients. Iran J Immunol. *1*, 48–55.

Kurien, B.T., Hensley, K., Bachmann, M., and Scofield, R.H. (2006). Oxidatively modified autoantigens in autoimmune diseases. Free Radic Biol Med *41*, 549–556

6.1 INTRODUCTION:

Vitiligo is an acquired, non-contagious disease in which progressive, patchy, multifocal loss of pigmentation of skin, overlying hair, and often mucous membranes results from loss of melanocytes from the involved areas (Taieb and Picardo, 2009). It affects 0.2-1% of the world population (Spritz, 2008). In India, the incidence of vitiligo is found to be 0.5% (Das *et al.,* 1985). It is associated with increased risk of several other autoimmune diseases such as: autoimmune thyroid disease (Graves' disease and autoimmune hypothyroidism), rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al*., 2003; Laberge *et al*., 2005). The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.*, 2006a; Kemp *et al*., 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997).

Vitiligo is a polygenic disease; however, recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which almost universally involve immune regulation and immune targeting of melanocytes, that have led to the general consensus that generalized vitiligo is a primary autoimmune disease, though the biological triggers of the autoimmune process remain unknown (Spritz, 2011). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC*, *ACE*, *CAT*, *CTLA-4*, *COMT*, *ESR*, *GCH1*, *MBL2*, *PTPN22*, and *VDR* (Das *et al.,* 1985; Spritz, 2007). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA*, *PTPN22, NALP1, XBP1, FOXP1, IL2RA have* been tested for genetic association with vitiligo (Spritz, 2010).
Cytokines are important mediators of immunity and their response due to imbalance or deficiency in the cytokine network may largely determine autoimmune disease susceptibility and severity. Tumor necrosis factor (TNF)α is a multifunctional, proinflammatory cytokine which plays an important role in several autoimmune diseases like rheumatoid arthritis, pernicious anemia, diabetes mellitus etc.

TNFα plays an important role in apoptosis through activation of the receptor-mediated apoptosis pathway in numerous cell types (Gupta and Gollapudi, 2006). It is produced by many different cell types, including activated T cells, fibroblasts, adipocytes, smooth muscle cells and keratinocytes. In the epidermal melanin unit of epidermis, a melanocyte is in close interaction with ~32 keratinocytes. The keratinocytes synthesize cytokines, such as TNFα, interleukin (IL) 1α, IL-6, and transforming growth factorβ (TGFβ), which are paracrine inhibitors of human melanocyte proliferation and melanogenesis. TNFα also affects the apoptotic pathway of melanocytes and its level may play an important role in vitiligo pathogenesis. Moreover, TNF-α can inhibit melanocyte stem cell differentiation (Alghamdi *et al.*, 2012; Huang *et al.*, 2002).

Additionally, IFN-γ and TNF-*α* induce the expression of intercellular adhesion molecule-1 (*ICAM1*) on the cell-surface of melanocytes (Yohn *et al.*, 1990). Increased expression of this adhesion molecule on the melanocytes enhances T cell- melanocyte attachment in the skin and may play a role in the destruction of melanocytes in vitiligo (Al Badri, 1993).

TNFA gene locus is located within the Class III region of the human major histocompatibility complex (MHC) on chromosome 6 (6p21.31) spanning about 3 kb and contains 4 exons. Regulation of TNFα production occurs at both the transcriptional and post-transcriptional levels, with regulatory sequences within the 5' end of the gene controlling the rate of transcription (Spriggs *et al.*, 1992). Several single-nucleotide polymorphisms (SNPs) have been identified in the human *TNFA* gene promoter region having the potential to cause structural changes within regulatory sites that could affect the function or regulation of TNFα production. The location of its gene within major histocompatibility complex and biological activities has raised the possibility that polymorphisms within this locus may contribute to the pathogenesis of wide range of autoimmune and infectious diseases.

The promoter polymorphisms at positions: -238, -308, -857, and -1031 may lead to a higher rate of *TNFA* gene transcription whereas -863 leads to decrease the transcription. These polymorphisms combined could contribute to the autoimmune process making it an ideal candidate for the development of vitiligo.

In the present study, we have made an attempt to understand the role of TNFα in vitiligo pathogenesis. Hence, the objectives of this study were:

i.) To determine whether the promoter polymorphisms of *TNFA* [-238 (G/A; rs361525), -308 (G/A; rs1800629), -857 (C/T; rs1799724), -863 (C/A; rs1800630) and -1031 (T/C; rs1799964)] are associated with vitiligo susceptibility and modulate *TNF*-α transcript and protein levels.

ii.) To measure and compare *TNFA* and *ICAM1* transcript and serum TNFα levels in patients with vitiligo and in unaffected controls.

iii.) To correlate *TNFA* polymorphisms/levels with onset and progression of the disease.

6.2 MATERIALS AND METHODS

6.2.1 Study Subjects:

The study group included 977 vitiligo patients [733 generalized (including acrofacial vitiligo and vitiligo universalis) and 244 localized vitiligo cases] comprised of 451 males and 526 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 990 ethnically and sex-matched unaffected individuals (447 males and 543 females) were included in the study (Table 1). 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.

6.2.2 Blood collection and DNA extraction:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA

was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analyses.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls of Gujarat.

	Vitiligo Patients	**Controls**
	(n = 977)	(n = 990)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42 yrs
Sex: male	451 (46.16%)	447 (45.15%)
female	526 (53.84%)	543 (54.85%)
Age of onset (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yrs	NA
Type of vitiligo		
Generalized	733 (75.03%)	NA
Localized	244 (24.97%)	NA
Active vitiligo	682 (69.81%)	NA
Stable vitiligo	295 (30.19%)	NA

6.2.3 Genotyping of *TNFA* promoter polymorphisms:

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype all five promoter polymorphisms of *TNFA* gene (Figure 1). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for

10 minutes followed by 30 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) used were: *Bam*HI, *Nco*I, *Tai*I and *Bbs*I for digesting amplicons of -238 G/A, -308 G/A, -857 C/T, -863 C/A and -1031 T/C of *TNFA* gene (Table 2). 5 μL of the amplified products were digested with 5 U of the corresponding restriction enzyme in a total reaction volume of 25 μL as per the manufacturer's instruction. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 3.5% agarose gels or 15% polyacrylamide gels stained with ethidium bromide and visualized under UV transilluminator.

More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

6.2.4 Determination of *TNFA, ICAM1* and *GAPDH* mRNA expression:

6.2.4.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis/ ethidium bromide staining and O.D. 260/280 absorbance ratio >1.95. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

6.2.4.2 Real-time PCR:

The expression of *TNFA, ICAM1* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2.

Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (94°C for 15 s, 63°C for 30 s, 72°C for 30 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out on the product formed (Figure 7). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

Table 2. Primers for *TNFA* promoter SNPs genotyping and gene expression analysis.

Gene/SNP* Primer	Sequence (5' to 3')	Annealing Temp. (°C)	Product size (bp)	Restriction Enzyme (Digested Products)
(rs361525)				*Bam*HI
TNFA -238G/A F	CTGTCCCAGGCTTGTCCTGCTAC	66	376	(352bp &
TNFA -238G/A R	CTCACACTCCCCATCCTCCC**G**G**A**TC			24bp)
(rs1800629)				*Nco*I
TNFA -308G/A F	GAGGCAATAGGTTTTGAGGG**C**CAT	57	360	(339bp &
TNFA -308G/A R	TCTGCTGTCCTTGCTGAGGGA			21bp)
(rs1799724)				*Tai*I
TNFA -857C/T F	GCATCTGCACCCTCGATGAAG	58	325	(306bp &
TNFA -857C/T R	CCTCTACATGGCCCTGTCTAC			19bp)
(rs1800630)				*Tai*I
TNFA -863C/A F	GCTCAAAGGGAGCAAGAGCTG	65	323	(302bp &
TNFA -863C/A R	CTACATGGCCCTGTCTTCGTTACG			21 bp)
(rs1799964)				*Bbs*I
TNFA -1031T/C F	GCTCAAAGGGAGCAAGAGCTG	66	481	(313bp &
TNFA-1031T/C R	GCTGGTTTCAGTCTTGGCTTCC			168bp)
TNFA gene expression F	GCCCCCAGAGGGAAGAGTTCCCCA	65	124	-
TNFA gene expression R	GCTTGAGGGTTTGCTACAACATGGGC			
ICAM1 gene expression F	TCTGTTCCCAGGACCTGGCAATG	65	282	-
ICAM1 gene expression R	GGAGTCCAGTACACGGTGAGGAAG			
GAPDH gene expression F	CATCACCATCTTCCAGGAGCGAG	65	122	-
GAPDH gene expression R	CCTGCAAATGAGCCCCAGCCT			

*The nucleotide change was from the ancestral (major) to the derived (minor) allele.

Bold letters within the primer sequences represent a forced mismatch.

6.2.5 Estimation of serum TNFα levels by enzyme-linked immunosorbent assay:

Serum levels of TNF*α* in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Immunotech Human TNF*α* ELISA kit (Immunotech SAS, Marseille Cedex 9, France) as per the manufacturer's protocol.

6.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all five polymorphisms of *TNFA* in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *TNFA* promoter polymorphisms for patients and control subjects were compared using the chi-square test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.01 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi and He, 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r^2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al.*, 2005). Relative gene expression of *TNFA* and serum TNF*α* levels in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.*, 2007).

6.3 RESULTS

6.3.1 Association of *TNFA* promoter polymorphisms with generalized vitiligo:

The genotype and allele frequencies of the investigated *TNFA* promoter polymorphisms in 733 generalized vitiligo patients and 990 controls are summarized in Table 3. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups (p>0·05).

The five promoter polymorphisms of *TNFA* were found to be in significant association with generalized vitiligo patients (p<0.0001) when genotypes were compared using chi-square test-3x2 contingency table with Bonferroni's correction for multiple testing (Table 3). Also, there was significant difference in allele frequencies of these polymorphisms between generalized patients and controls when compared with 2x2 contingency table (p<0.0001) (Table 3). Interestingly, -238A and -308A alleles were found to increase the risk of generalized vitiligo by 6.35 and 4.326 fold respectively [odds ratio (OR): 6.35; 95% confidence interval (CI): (5.320-7.590); odds ratio (OR): 4.326; 95% confidence interval (CI): (3.623-5.165)] (Table 3). However, -857T, -863A and -1031C alleles were found to increase the risk of generalized vitiligo by 2.181, 2.231 and 1.960 fold respectively [odds ratio (OR): 2.181; 95% confidence interval (CI): (1.885-2.524); odds ratio (OR): 2.231; 95% confidence interval (CI): (1.894-2.629); odds ratio (OR): 1.960; 95% confidence interval (CI): (1.675-2.294)] (Table 3). This study has 95.0% statistical power for the effect size 0.08 to detect association of *TNFA* promoter polymorphisms at p<0.05 in generalized vitiligo patients and control population.

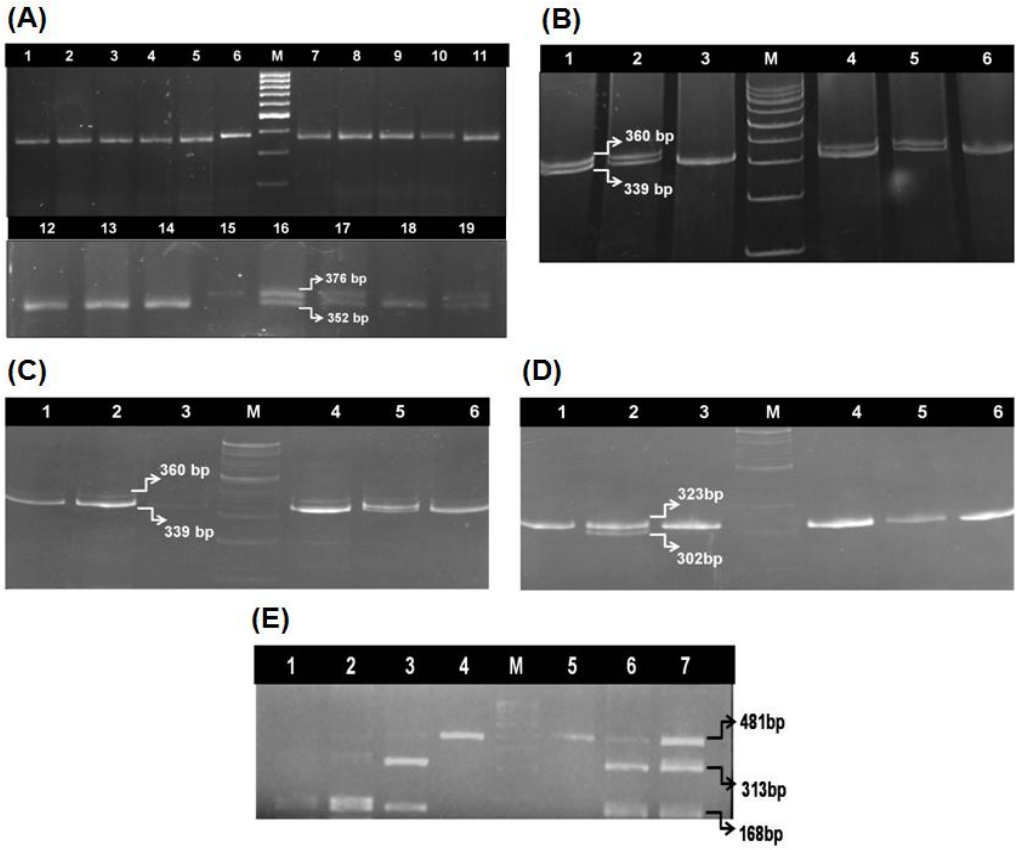


Figure 1. (A) PCR-RFLP analysis of *TNFA* -238 G/A polymorphism on 3.5 % agarose gel: lanes: 1, 2, 3, 4, 5, 11, 12, 13, 14 & 18 show homozygous (GG) genotypes; lanes: 16, 17 & 19 show heterozygous (GA) genotypes; lanes: 6, 7, 8, 9 10 & 15 show homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *TNFA* -308 G/A polymorphism on 10% polyacrylamide gel: lanes: 1, 2, 4 & 5 show heterozygous (GA) genotypes; lanes: 3 & 6 show homozygous (GG) genotypes; lane: 11 shows homozygous (AA) genotype; lane M shows 100 bp DNA ladder.

(C) PCR-RFLP analysis of *TNFA* -857 C/T polymorphism on 10% polyacrylamide gel: lanes: 1, 2, 4, 5 & 6 show heterozygous (CT) genotypes; lane: 3 shows homozygous (GG) genotype; lane M shows 100 bp DNA ladder.

(D) PCR-RFLP analysis of *TNFA* -863 C/A polymorphism on 10% polyacrylamide gel: lanes: 1, 3, 4, 5 & 6 show homozygous (CC) genotypes; lane: 2 shows heterozygous (CA) genotype; lane M shows 100 bp DNA ladder.

(E) PCR-RFLP analysis of *TNFA* -1031 T/C polymorphism on 2.0 % agarose gel: lanes: 4 & 5 show homozygous (TT) genotypes; lanes: 6 & 7 show heterozygous (TC)

genotypes; lanes: 1, 2 & 3 show homozygous (CC) genotype; lane M shows 100 bp DNA ladder.

Table 3. Association study for *TNFA* promoter polymorphisms in patients with generalized vitiligo from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 729)	(n = 990)		0.192	
	GG	250 (0.34)	798 (0.81)	<0.0001	(P)	
rs361525	GA	339 (0.47)	178 (0.18)			
(-238;	AA	140 (0.19)	14 (0.01)		0.263	
G/A)	Allele				(C)	6.35
	G	839 (0.58)	1774(0.90)	<0.0001		(5.320-
	A	619 (0.42)	206 (0.10)			7.590)
	Genotype	(n = 728)	(n = 981)		0.093	
	GG	317 (0.44)	780 (0.80)		(P)	
rs1800629	GA	311 (0.43)	184 (0.19)	<0.0001		
(-308;	AA	100 (0.13)	17 (0.01)		0.114	
G/A)	Allele				(C)	4.326
	G	945 (0.65)	1744 (0.89)	<0.0001		(3.623-
	A	511 (0.35)	218 (0.11)			5.165)
	Genotype	(n = 728)	(n = 984)		0.563	
	CC	249 (0.34)	563 (0.57)		(P)	
rs1799724	CT	347 (0.48)	352 (0.36)	<0.0001		
(-857;	TT	132 (0.18)	69 (0.07)		0.173	
C/T)	Allele				(C)	2.181
	C	845 (0.58)	1478 (0.75)	<0.0001		(1.885-
	T	611 (0.42)	490 (0.25)			2.524)
	Genotype	(n = 728)	(n = 984)		0.084	
	CC	365 (0.50)	698 (0.71)		(P)	
rs1800630	CA	287 (0.40)	253 (0.26)	<0.0001		
(-863;	AA	76 (0.10)	33 (0.03)		0.094	
C/A)	Allele				(C)	
	C	1017(0.70)	1649 (0.84)	<0.0001		2.231
	A	439 (0.30)	319 (0.16)			(1.894-
						2.629)
	Genotype	(n = 733)	(n = 989)		0.063	
	TT	354 (0.48)	653 (0.66)		(P)	
rs1799964	TC	295 (0.40)	295 (0.30)	<0.0001		
(-1031;	CC	84 (0.12)	41 (0.04)		0.296	
T/C)	Allele				(C)	
	T	1003(0.68)	1601(0.81)	<0.0001		1.960

C	463 (0.32)	377 (0.19)	(1.675-2.294)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

Statistical significance was considered at p value ≤ 0.01 due to Bonferroni's correction for multiple testing.

6.3.2 Association of *TNFA* promoter polymorphisms with localized vitiligo:

The genotype and allele frequencies of the investigated *TNFA* promoter polymorphisms in 244 localized vitiligo patients and 990 controls are summarized in Table 4. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups (p>0·05) except for -238G/A and -863C/A in patients (p=0.0001 and p=0.014 respectively).

The five promoter polymorphisms of *TNFA* were found to be in significant association with localized vitiligo patients (p<0.0001) when genotypes were compared using chi-square test-3x2 contingency table with Bonferroni's correction for multiple testing (Table 4). Also, there was significant difference in allele frequencies of these polymorphisms between localized patients and controls when compared with 2x2 contingency table (p<0.0001) (Table 4). Although, all five promoter polymorphisms of *TNFA* were found to be associated with localized vitiligo patients none of the susceptible alleles of these polymorphisms were found to be a risk for localized vitiligo as suggested by the odds ratio (Table 4). This study has 88.0% statistical power for the effect size 0.08 to detect association of *TNFA* promoter polymorphisms at p<0.05 in localized vitiligo patients and control population.

Table 4. Association study for *TNFA* promoter polymorphisms in patients with localized vitiligo from Gujarat.

SNP	Genotype or allele	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 241)	(n = 990)		0.0001	
	GG	75 (0.31)	798(0.81)		(P)	
rs361525	GA	91 (0.38)	178(0.18)	<0.0001		
(-238;	AA	75 (0.31)	14 (0.01)		0.263	
G/A)	Allele				(C)	0.116
	G	241 (0.50)	1774(0.90)	<0.0001		(0.092-
	A	241 (0.50)	206(0.10)			0.146)
	Genotype	(n = 241)	(n = 981)		0.278	
	GG	79 (0.33)	780 (0.80)		(P)	
rs1800629	GA	125 (0.52)	184 (0.19)	<0.0001		
(-308;	AA	37 (0.15)	17 (0.01)		0.114	
G/A)	Allele				(C)	0.178
	G	283 (0.59)	1744 (0.89)	<0.0001		(0.141-
	A	199 (0.41)	218 (0.11)			0.224)
	Genotype	(n = 241)	(n = 984)		0.864	
rs1799724	CC	86 (0.36)	563 (0.57)		(P)	
(-857;	CT	117 (0.49)	352 (0.36)	<0.0001		
C/T)	TT	38 (0.15)	69 (0.07)		0.173	
	Allele				(C)	0.496
	C	289 (0.60)	1478 (0.75)	<0.0001		(0.403-
	T	193 (0.40)	490 (0.25)			0.612)
	Genotype	(n = 242)	(n = 984)		0.014	
rs1800630	CC	137 (0.57)	698 (0.71)		(P)	
(-863;	CA	80 (0.33)	253 (0.26)	<0.0001		
C/A)	AA	25 (0.10)	33 (0.03)		0.094	
	Allele				(C)	
	C	354 (0.73)	1649 (0.84)	<0.0001		0.527
	A	130 (0.27)	319 (0.16)			(0.417-
						0.666)
	Genotype	(n = 244)	(n = 989)		0.607	
rs1799964	TT	112 (0.46)	653 (0.66)		(P)	
(-1031;	TC	104 (0.43)	295 (0.30)	<0.0001		
T/C)	CC	28 (0.11)	41 (0.04)		0.296	
	Allele				(C)	0.483
	T	328 (0.67)	1601(0.81)	<0.0001		(0.388-
	C	160 (0.33)	377 (0.19)			0.601)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,
Statistical significance was considered at p value ≤ 0.01 due to Bonferroni's correction for multiple testing.

6.3.3 Linkage disequilibrium (LD) and haplotype analyses of *TNFA* promoter polymorphisms:

The LD analysis revealed that the five promoter polymorphisms investigated in the *TNFA* gene were in low to moderate LD association in both generalized as well as localized vitiligo patients (Figures 2A & B). In particular, -238G/A and -308G/A polymorphisms were in moderate LD association with D'= 0.485 and 0.484 in generalized and localized vitiligo patients respectively (Tables 5 & 6).
A haplotype evaluation of the five polymorphic sites was performed and the estimated frequencies of the haplotypes were differed significantly between generalized vitiligo patients and controls (global p<0.0001). Also, localized vitiligo patients exhibited significantly different frequencies of haplotypes as compared to controls (global p<0.0001) (Tables 7 & 8).

The susceptible haplotypes: AACAT, AACCT, AATCC, AATCT and AGCCT were more frequently observed in generalized vitiligo patients as compared to controls and were found to increase the risk of generalized vitiligo as suggested by odds ratio (Table 7). However, the non-susceptible haplotypes: GGCAT, GGCCC, GGCCT, GGTCC, GGTCT were more frequently observed in controls as compared to generalized vitiligo patients (Table 7).

Furthermore, susceptible haplotypes: AACAT, AACCT, AATCC, AATCT, AGCCC and AGCCT were more frequently observed in localized vitiligo patients as compared to controls and were found to increase the risk of localized vitiligo as suggested by odds ratio (Table 8); however, the non-susceptible haplotypes: GGCAT, GGCCC, GGCCT, GGTAT and GGTCC were more frequently observed in controls as compared to localized vitiligo patients (Table 8).

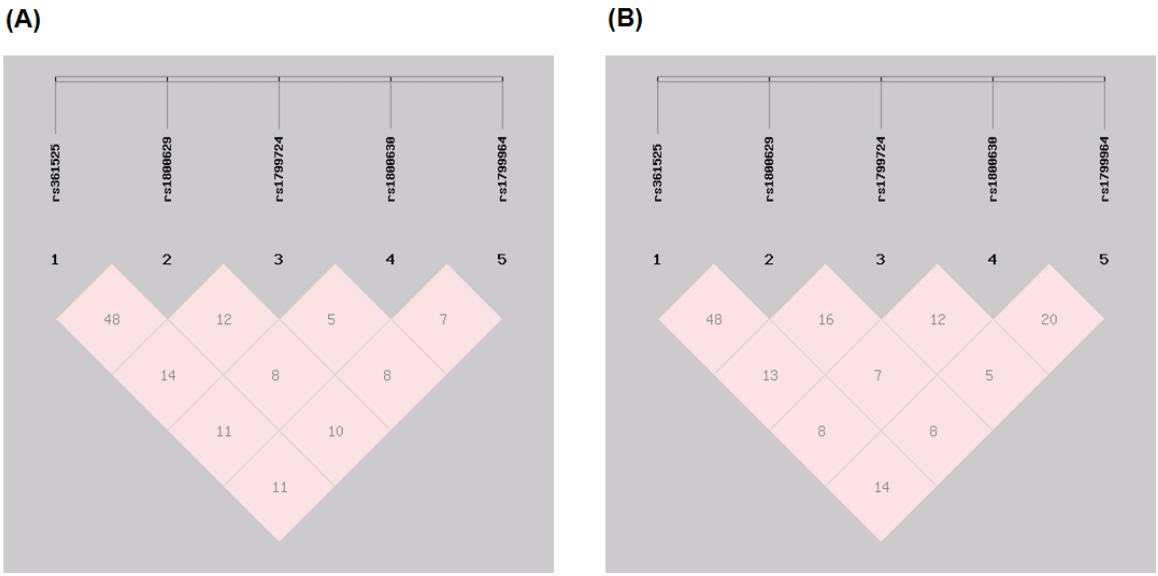


Figure 2. (A) Linkage disequilibrium (D') among *TNFA* promoter SNPs in generalized vitiligo patients and controls from Gujarat population.

(B) Linkage disequilibrium (D') among *TNFA* promoter SNPs in localized vitiligo patients and controls from Gujarat population.

Table 5. Pairwise linkage disequilibrium (D') values between *TNFA* SNPs with >3% minor allele frequencies within generalized vitiligo patients and controls from Gujarat population.

	rs1800629 (-308 G/A)	**rs1799724 (-857 C/T)**	**rs1800630 (-863 C/A)**	**rs1799964 -1031 T/C**
rs361525 (-238 G/A)	**0.485**	0.138	0.106	0.106
rs1800629 (-308 G/A)	-	0.123	0.080	0.100
rs1799724 (-857 C/T)	-	-	0.052	0.075
rs1800630 (-863 C/A)	-	-	-	0.074

*Bold value represents moderate LD.

Table 6. Pairwise linkage disequilibrium (D') values between *TNFA* SNPs with >3% minor allele frequencies within localized vitiligo patients and controls from Gujarat population.

	rs1800629 (-308 G/A)	**rs1799724 (-857 C/T)**	**rs1800630 (-863 C/A)**	**rs1799964 -1031 T/C**
rs361525 (-238 G/A)	**0.484**	0.131	0.083	0.135
rs1800629 (-308 G/A)	-	0.159	0.073	0.076
rs1799724 (-857 C/T)	-	-	0.123	0.052
rs1800630 (-863 C/A)	-	-	-	0.200

*Bold value represents moderate LD.

Table 7. Distribution of haplotypes frequencies for *TNFA* promoter polymorphisms among generalized vitiligo patients and controls.

Haplotype (-238G/A, -308 G/A, -857 C/T, -863 C/A and -1031 T/C)	**Generalized Vitiligo Patients (Freq. %) (n=1454)**	**Controls (Freq. %) (n=1936)**	***p* for Association**	**$p_{(global)}$**	**Odds ratio (95% CI)**
A A C A T	49.92 (0.034)	13.88 (0.007)	2.33e-012	<0.0001	6.547 [3.593~11.929]
A A C C T	94.79 (0.065)	21.40 (0.011)	5.67e-025		8.420 [5.232~13.551]
A A T C C	60.38 (0.042)	2.04 (0.001)	1.48e-023		54.818 [13.554~221.703]
A A T C T	71.17 (0.049)	13.93 (0.007)	4.31e-020		9.511 [5.326~16.982]
A G C C T	60.72 (0.042)	68.55 (0.035)	0.010890		1.580 [1.108~2.251]
G A C C T	31.38 (0.022)	83.64 (0.043)	0.036942		0.643 [0.423~0.977]
G G C A T	83.30 (0.057)	181.62 (0.094)	0.075190		0.782 [0.596~1.026]
G G C C C	72.25 (0.050)	206.78 (0.107)	0.000113		0.579 [0.438~0.766]
G G C C T	218.00 (0.150)	778.39 (0.402)	7.54e-033		0.347 [0.291~0.414]
G G T A T	65.08 (0.045)	51.56 (0.027)	9.25e-006		2.284 [1.571~3.320]
G G T C C	56.39 (0.039)	81.71 (0.042)	0.271883		1.215 [0.858~1.722]
G G T C T	144.86 (0.100)	254.60 (0.132)	0.934445		0.991 [0.795~1.235]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

Table 8. Distribution of haplotypes frequencies for *TNFA* promoter polymorphisms among localized vitiligo patients and controls.

Haplotype (-238G/A,-308 G/A, -857 C/T, -863 C/A and -1031 T/C)	Localized Vitiligo Patients (Freq. %) (n=482)	Controls (Freq. %) (n=1936)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
A A C A T	22.81 (0.047)	13.88 (0.007)	9.51e-012	<0.0001	7.520 [3.824~14.787]
A A C C T	40.56 (0.084)	21.40 (0.011)	9.52e-022		9.047 [5.296~15.453]
A A T C C	37.87 (0.079)	2.04 (0.001)	6.66e-016		88.928 [21.64~365.32]
A A T C T	33.24 (0.069)	13.93 (0.007)	2.89e-020		11.220 [5.944~21.180]
A G C C C	20.19 (0.042)	21.79 (0.011)	7.93e-007		4.193 [2.267~7.758]
A G C C T	17.72 (0.037)	68.55 (0.035)	0.648433		1.132 [0.664~1.930]
G A C C T	5.60 (0.012)	83.64 (0.043)	0.002174		0.282 [0.119~0.668]
G G C A C	20.16 (0.042)	20.87 (0.011)	4.39e-007		4.373 [2.348~8.144]
G G C A T	21.90 (0.045)	181.62(0.094)	0.002400		0.499 [0.316~0.788]
G G C C C	19.17 (0.040)	206.78(0.107)	3.43e-005		0.375 [0.232~0.606]
G G C C T	74.89 (0.155)	778.39(0.402)	3.35e-021		0.291 [0.223~0.380]
G G T A T	15.29 (0.032)	51.56 (0.027)	0.369915		1.304 [0.729~2.330]
G G T C C	17.54 (0.036)	81.71(0.042)	0.795328		0.933 [0.550~1.580]
G G T C T	51.54 (0.107)	254.60(0.132)	0.375415		0.865 [0.628~1.192]

CI represents Confidence Interval,
(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

6.3.4 Age of onset of vitiligo and *TNFA* promoter haplotypes in patients with vitiligo:

When age of onset of the disease was correlated with the *TNFA* promoter haplotypes, patients with AACAT, AACCT, AATCC and AATCT haplotypes showed early onset of the disease as compared to GGCAT, GGCCT, GGTCC and GGTCT (p=0.001, p=0.0004, p<0.0001 and p=0.005 respectively) (Figure 3A). Patients with haplotype AATCC had an early onset of the disease as compared to GATCC haplotype (p=0.04). Moreover, patients with haplotype AATCT showed early onset of the disease as compared to AGTCT and GATCT haplotypes (p=0.001 and p=0.025 respectively) (Figure 3A). Also, patients with AGCCC haplotype had an early onset of the disease as compared to GGCCC haplotype (p=0.045); however, there was no significant difference in age of onset of the disease for haplotype AGCCT as compared to GACCT and GGCCT haplotypes (p=0.147 and p=0.481 respectively) (Figure 3A). Patients with haplotypes GGTCT and GGCCC showed no significant difference in age of onset of the disease as compared to GGTAT and GGCAC haplotypes (p=0.248 and p=0.582 respectively) (Figure 3A). Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients (p<0.0001) (Figure 3B).

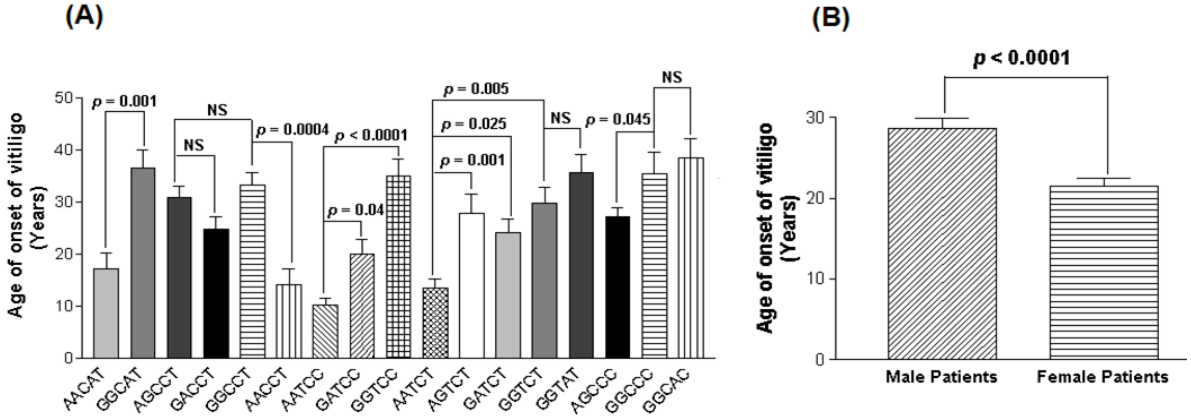


Figure 3. Age of onset of the disease in vitiligo patients.

(A) Comparison of age of onset of the disease (years) with respect to *TNFA* promoter haplotypes in 977 vitiligo patients.

(B) Comparison of age of onset of the disease (years) with respect to gender differences in 451 male patients and 526 female patients with vitiligo.

6.3.5 Relative gene expression of *TNFA* in patients with vitiligo and controls:

Comparison of the findings showed significantly increased expression of *TNFA* transcripts in 157 vitiligo patients than in 174 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.0005) (Figure 4A). Moreover, generalized vitiligo patients showed significant higher expression of *TNFA* transcripts as compared to localized vitiligo patients (p=0.0295) (Figure 4A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.445 fold change in the expression of *TNFA* transcript in patients as compared to controls (Figure 4E).

Further, the expression levels of *TNFA* were analyzed with respect to haplotypes generated from the five investigated promoter polymorphisms of *TNFA* (Figure 4B). Interestingly, *TNFA* expression was significantly increased for the haplotypes: GATCT, GATCC, AATCC and AACCT in vitiligo patients as compared to controls (p=0.013, p=0.006, p=0.023 and p=0.004 respectively); however, no significant difference was observed in *TNFA* expression for the haplotypes: GGTCC, GGCAC, GGCCT and GGTCT (p=0.517, p=0.258, p=0.790 and p=0.456 respectively).

In addition, we analyzed the *TNFA* expression based on the progression of the disease i.e. active vitiligo and stable vitiligo (Figure 4C). Active vitiligo patients showed significantly increased expression of *TNF*-α transcripts as compared to the patients with stable vitiligo (p<0.0001). To check the susceptibility of the disease based on the gender differences *TNFA* expression was analyzed for male and female vitiligo patients. Female patients with vitiligo showed significantly higher *TNFA* expression as compared to male patients (p=0.0073) (Figure 4D).

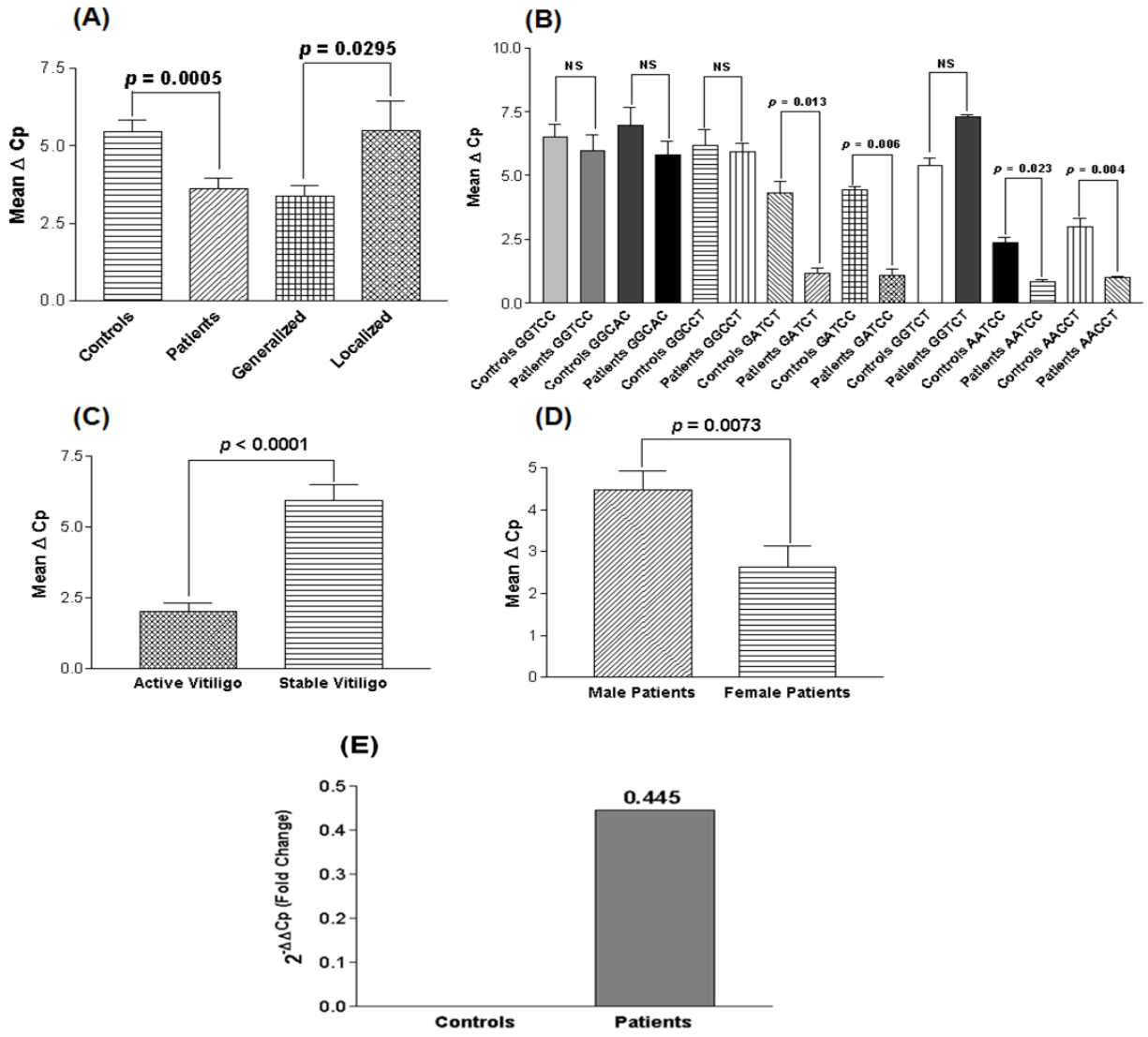


Figure 4. **Relative gene expression of *TNFA* in controls and vitiligo patients.**

(A) Expression of *TNFA* transcripts in 174 controls, 157 vitiligo patients, 115 generalized vitiligo patients and 42 localized vitiligo patients, as suggested by Mean ΔCp.

(B) Expression of *TNFA* transcripts with respect to *TNF*-α promoter haplotypes in 157 vitiligo patients and 174 controls, as suggested by Mean ΔCp.

(C) Expression of *TNFA* transcripts with respect to activity of the disease in 108 patients with active vitiligo and 49 patients with stable vitiligo, as suggested by Mean ΔCp.

(D) Expression of *TNFA* transcripts with respect to gender differences in 68 male patients and 89 female patients with vitiligo, as suggested by Mean ΔCp.

(E) Expression fold change of *TNFA* transcripts in 157 vitiligo patients against 174 controls showed 0.445 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

6.3.6 Functional correlation of *TNFA* promoter polymorphisms with its levels in the serum:

To find any functional correlation of the investigated *TNFA* promoter polymorphisms with its level in the serum, TNFα levels were measured in 214 vitiligo patients and 236 unaffected controls. Vitiligo patients showed significant increased serum TNFα (sTNFα) levels as compared to controls (p=0.0003) (Figure 5A). Moreover, when the patient subgroups were analyzed with respect to sTNFα levels, patients with generalized vitiligo had significantly higher sTNFα levels as compared to localized vitiligo (p=0.014) (Figure 5A).

In vitiligo patients, the *TNFA* haplotypes: GATCC, AGTCT, AGCCT and AACCT were found to increase sTNFα levels (p=0.031, p=0.003, p=0.009 and p=0.007 respectively) with susceptible alleles (-238A, -308A, -857T and -1031C) as compared to controls (Figure 5B). However, no significant difference was observed in sTNFα levels for the haplotypes: GGCCT, GGTAT, GGCCC, and GGTCC (p=0.217, p=0.150, p=0.153 and p=0.868 respectively) (Figure 5B).

Furthermore, when haplotypes were analyzed for sTNFα levels in the patients based on the disease activity, patients with active vitiligo showed significantly higher sTNFα levels as compared to stable vitiligo (p<0.0001) (Figure 5C). Additionally, when the male and female patients were analyzed with respect to sTNFα levels, female patients had significantly higher levels of sTNFα as compared to male patients (p=0.0066) (Figure 5D).

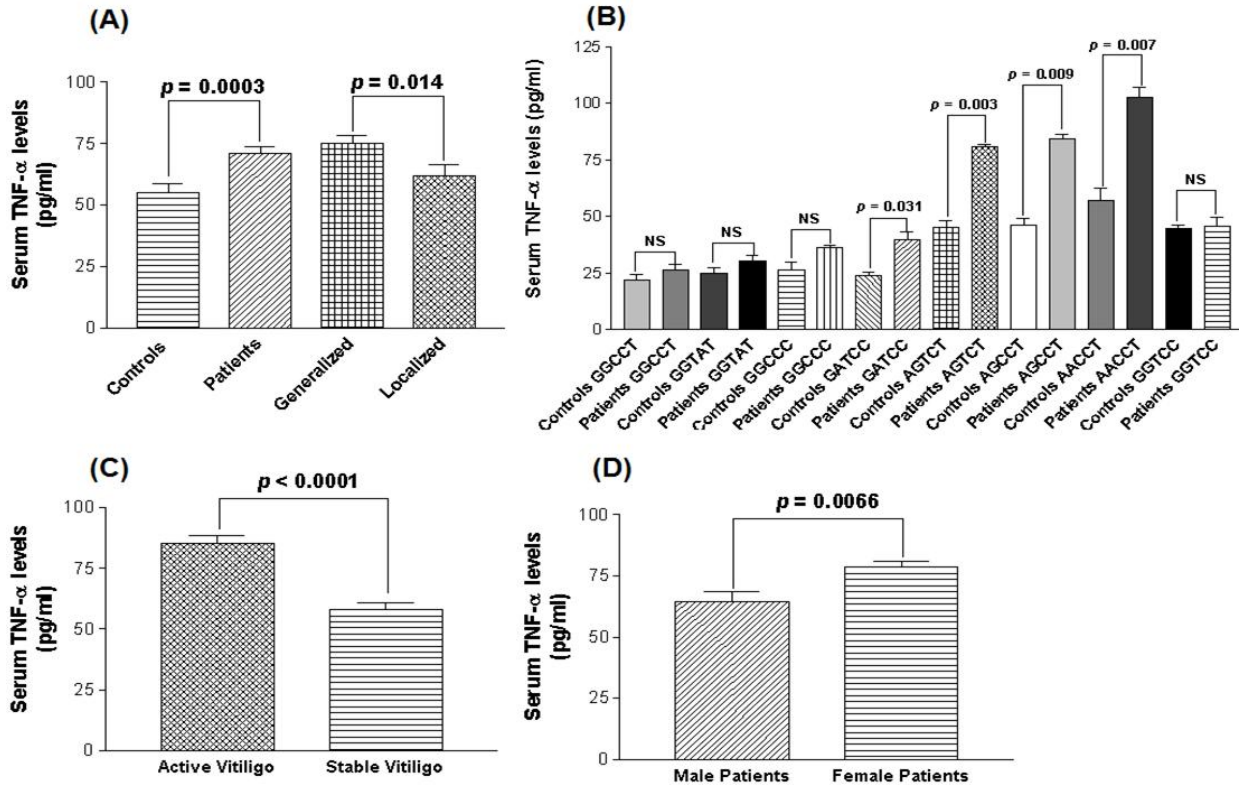


Figure 5. **Serum TNFα levels in controls and vitiligo patients:**

(A) Comparison of sTNFα levels (pg/ml) in 236 controls, 214 vitiligo patients, 158 generalized vitiligo patients and 56 localized vitiligo patients, as determined by ELISA.

(B). Comparison of sTNFα levels (pg/ml) with respect to *TNFA* promoter haplotypes in 214 vitiligo patients and 236 controls, as determined by ELISA.

(C) Comparison of sTNFα levels (pg/ml) with respect to activity of the disease in 150 patients with active vitiligo and 64 patients with stable vitiligo, as determined by ELISA.

(D) Comparison of sTNFα levels (pg/ml) with respect to gender differences in 97 male patients and 117 female patients with vitiligo, as determined by ELISA.

6.3.7 Relative gene expression of *ICAM1* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *ICAM1* transcripts in 166 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.008) (Figure 6A). Generalized vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to localized patients (p=0.002) (Figure 6A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.369 fold change in the expression of *ICAM1* transcript in patients as compared to controls (Figure 6B).

In addition, the effect of *ICAM1* expression on progression of the disease i.e. active and stable cases (Figure 6C) revealed that active vitiligo patients had significantly increased expression of *ICAM1* transcripts as compared to patients with stable vitiligo (p=0.008) suggesting the involvement *ICAM1* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *ICAM1* expression as compared to male patients (p=0.006) (Figure 6D). When *ICAM1* expression was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *ICAM1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.0002, p<0.0001 and p<0.0001 respectively) suggesting the importance of *ICAM1* in early onset of the disease (Figure 6E).

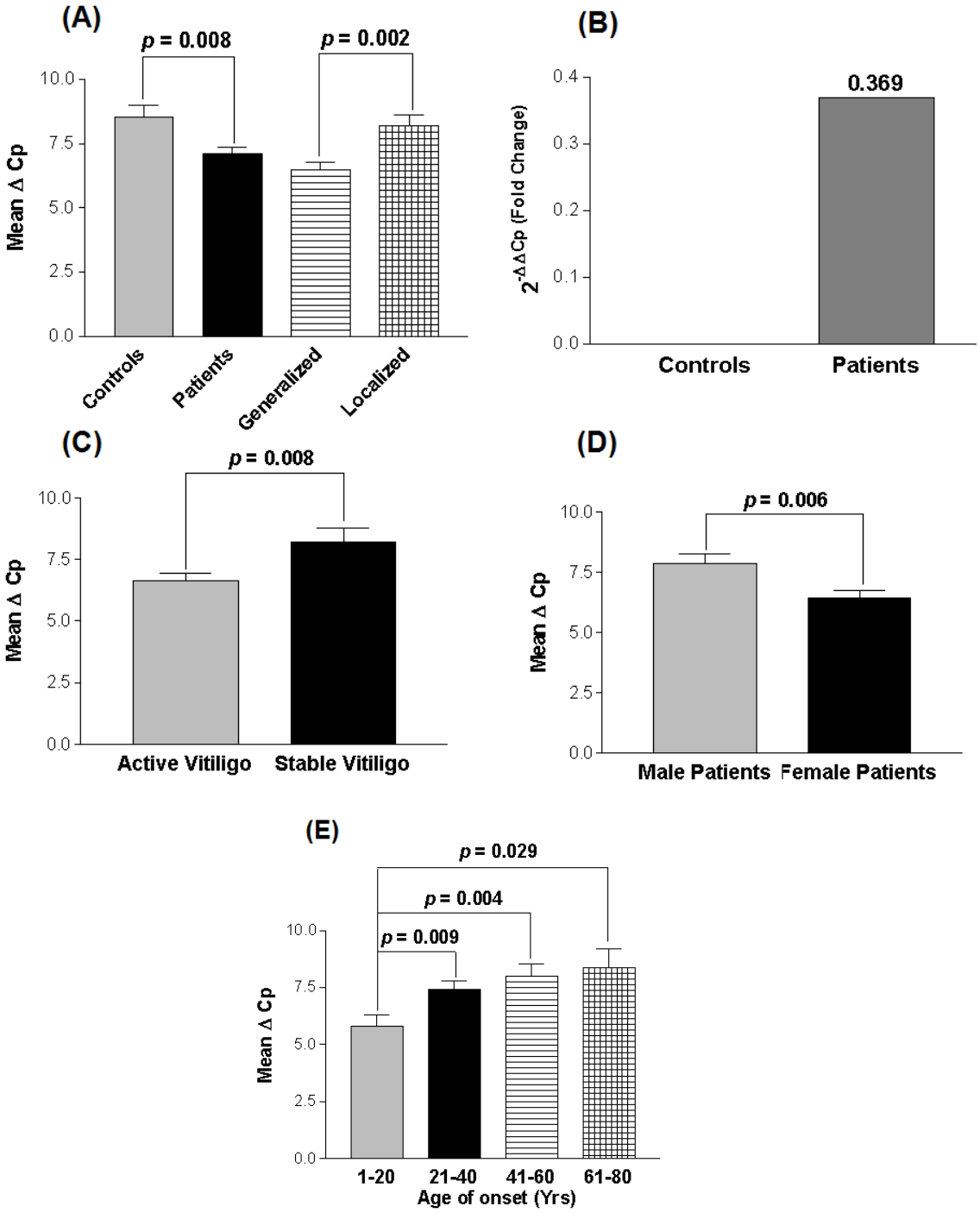


Figure 6. **Relative gene expression of *ICAM1* in controls and vitiligo patients:**

(A) Expression of *ICAM1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to controls (p=0.008). Generalized vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to localized patients (p=0.002).

(B) Expression fold change of *ICAM1* transcripts in 166 vitiligo patients against 175 controls showed 0.369 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *ICAM1* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to stable vitiligo patients (p=0.008).

(D) Expression of *ICAM1* transcripts with respect to gender differences in 87 male patients and 79 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significantly increased mRNA levels of *ICAM1* as compared to male vitiligo patients (p=0.006).

(E) Expression of *ICAM1* transcripts with respect to different age groups in 166 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased expression of *ICAM1* mRNA as compared to the age groups 21-40 yrs (p=0.009), 41-60 (p=0.004) and 61-80 yrs (p=0.029).

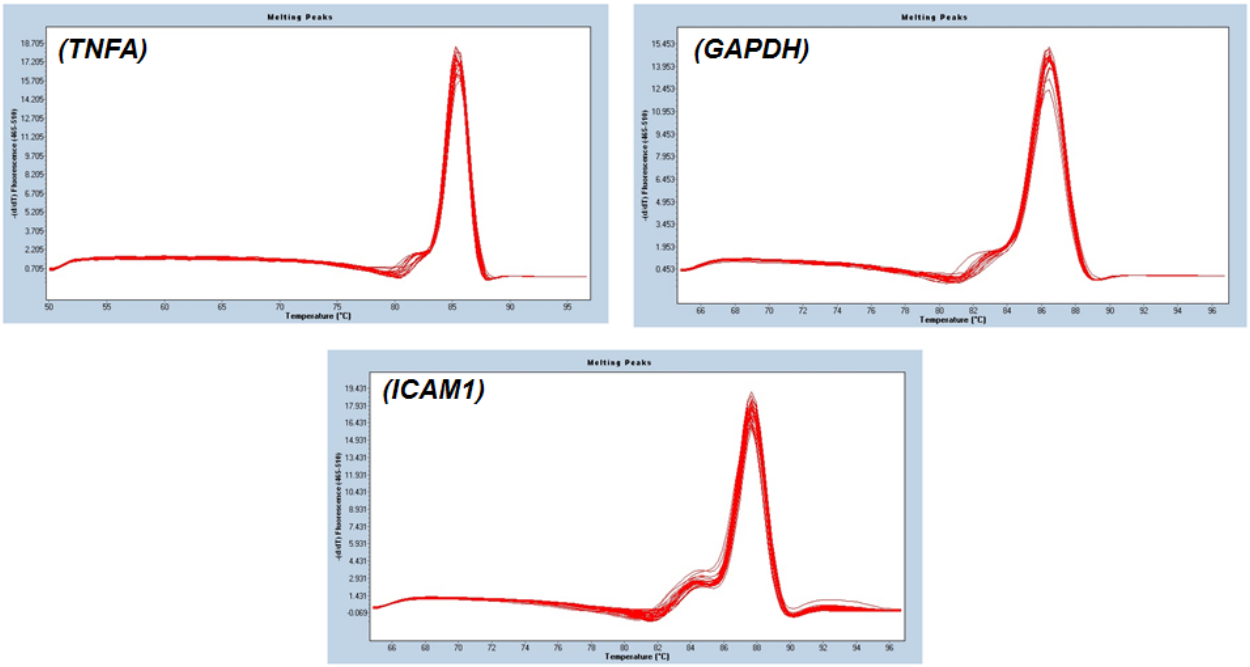


Figure 7. Melt curve analysis of *TNFA*, *ICAM1* and *GAPDH* showing specific amplification.

6.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997; Kim *et al.,* 1998). Our previous study suggests that 22% of Gujarat vitiligo patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.,* 2006b). Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo. Destruction of melanocytes due to an autoimmune response in vitiligo can be either through cellular and/or humoral immune response (Shajil *et al*., 2006a; Kemp *et al*., 2001). We have also shown that 66% of vitiligo patients possessed anti-melanocyte antibodies in their circulation as compared to control population (Shajil, 2007).

Association of Major Histocompatibility Complex (MHC) alleles with the disease gains importance because of the antigen-presenting function of the MHC. Recent genome wide association studies have implicated the role of MHC in vitiligo (Jin *et al.,* 2010, Quan *et al.,* 2010) where several SNPs in the *MHC* region were significantly associated with the disease. *TNFA* is located in *MHC* region and has strong linkage disequilibrium with *HLA* alleles. 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). Moreover, the study 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). Moreover, our recent study confirms genetic association of generalized vitiligo with SNPs in the MHC class II region in the Indian subcontinent. We have also 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.

The genotype-phenotype correlation of *CTLA4* and *IL4* gene polymorphisms also supported the autoimmune pathogenesis of vitiligo in Gujarat population (Dwivedi *et*

al., 2011; Imran *et al.*, 2012) whereas our earlier studies on *MBL2*, *ACE, PTPN22* polymorphisms did not show significant association (Dwivedi *et al*., 2009; Laddha *et al*., 2008).

Cytokines are important mediators of immunity and there is now convincing evidence that cytokines also have an important role in the pathogenesis of autoimmunity (Feldmann *et al.*, 1998). The cytokines mRNA and protein levels depend on both genetic and environmental factors. Analysis of cytokine gene polymorphisms would be able to detect genetic abnormality of cytokine regulation and hence establishment of genotype-phenotype correlation may be important in unraveling the disease pathogenesis. The promoter polymorphisms of *TNFA* are reported to be involved in modulating expression of *TNFA* gene which may be responsible for melanocyte death. TNFα, is an important multifunctional cytokine secreted by macrophages, T-lymphocytes, fibroblasts and keratinocytes with wide-ranging biological effects of protection from infection, surveillance against tumors and stimulation of inflammatory responses. In the epidermis, the epidermal melanin unit consists of the close interaction of a melanocyte and an associated pool of keratinocytes. Close relationship between these two cell types is important for melanocyte survival and differentiation mainly as keratinocyte-derived cytokines act on melanocytes via specific receptors (Moretti *et al*., 2002). Keratinocytes synthesize cytokines, such as TNFα, IL-1α, IL-6, and transforming growth factorβ (TGFβ), which are paracrine inhibitors of human melanocyte proliferation and melanogenesis (Moretti *et al*., 2002). However, primary role of TNFα is in the regulation of immune cells and its overproduction has been implicated in a variety of human diseases including autoimmune disorders and cancer (Locksley *et al*., 2001) *In vitro*, direct analysis of skin T cells from margins of vitiliginous skin show that polarized type-1 T cells (CD4+ and particularly CD8+), which predominantly secrete interferon (IFN)γ and TNFα are associated with the destruction of melanocytes during active vitiligo (Wajkowicz-Kalijska *et al*., 2003). In vitiligo affected skin, a significantly higher expression of TNFα (Moretti *et al*., 2002; Grimes *et al*., 2004), IL-6 (Moretti *et al*., 2002), IFNγ 32 (Grimes *et al*., 2004) was detected compared with healthy controls and perilesional, non-lesional skin (Moretti *et al*., 2002) indicating that cytokine imbalance plays an important role in the depigmentation process of vitiligo.

It has been reported that cytokines such as IFNγ and TNFα can initiate apoptosis and thus lead to melanocyte death in the context of autoimmunity and (Huang *et al.*, 2002). In addition, IFNγ and TNFα induce the expression of intercellular adhesion molecule-1 (ICAM-1) on the cell-surface of melanocytes (Yohn *et al.*, 1990). The increased expression of ICAM-1 on the melanocytes enhances T cell/melanocyte attachment in the skin and thereby may result in destruction of melanocytes in vitiligo (Al Badri *et al.*, 1993; Morelli *et al.*, 1993). TNFα also has the capacity to inhibit melanogenesis through an inhibitory effect on tyrosinase and tyrosinase related proteins (Martinez-Esparza *et al.*, 1998).

Thus, it becomes pertinent to study all *TNFA* promoter polymorphisms in adequate number of vitiligo patients and controls to elucidate the role of these polymorphisms in vitiligo susceptibility and to analyze the possible genotype - phenotype correlation. Here, we report that *TNF-α* -238, -308, -857, -863 and -1031 promoter polymorphisms are significantly associated with Gujarat vitiligo patients. Our results clearly suggest the important role of TNFα in pathogenesis of vitiligo. Vitiligo patients showed significant increase in *TNFA* transcripts and protein levels as compared to controls suggesting that melanocyte death in patients could be triggered due to the increased TNFα levels.

For the first time we report that generalized vitiligo has significantly higher *TNFA* transcript and protein levels as compared to localized vitiligo patients which indicate involvement of autoimmunity in precipitation of generalized vitiligo. Our results also indicate that active vitiligo patients have significantly higher *TNFA* transcript and protein levels as compared to the patients with stable vitiligo which signifies the role of TNFα in disease progression. Our results also suggest that there are significantly higher transcript and protein levels of TNFα in female patients as compared to male patients. Moreover, female patients have an early onset as compared to male patients suggestive of the fact that females have increased susceptibility towards vitiligo as compared to males, implicating sex bias in the development of autoimmunity (Whitacre, 2001; Afshan *et al.*, 2012).

TNFα and IFN-γ stimulates the expression of intercellular adhesion molecule 1 (ICAM1), which is important for activating T cells and recruiting leukocytes (Hedley et al., 1998; Ahn et al., 1994). ICAM1 protein levels are upregulated in vitiligo skin

and in melanocytes from perilesional vitiligo skin (al Badri et al., 1993). The present study also showed increased expression of ICAM1 in vitiligo patients suggesting that increased TNFα 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 in vitiligo (Al Badri, 1993). 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.

Interestingly, *TNF*-α -308 G/A and -238 G/A polymorphisms were found to influence serum TNFα levels in patients with sarcoidosis of Asian Indian population (Sharma *et al*., 2008) and our results are in line with this study. Furthermore, a genotype-phenotype study carried out on SLE patients showed increased *TNFA* transcript levels with -238 AA and GA genotypes as compared to GG genotypes 42 (Suárez *et al*., 2005). In particular, in the present study when combined effect of various genotypes was analyzed in the form of haplotypes, AATCC haplotype was found to be the highest risk combination observed for the disease. Intrestingly it has all susceptible alleles except -863A which is reported to decrease the levels of TNF-α. The -863 C/A polymorphism was associated with serum TNFα levels, carriers of the rare 'A' allele having a significantly lower TNFα levels in Swedish population (Skoog *et al.*, 1999). The -863A allele was associated with 31% lower transcriptional activity in chloramphenicol acetyltransferase (*CAT*) reporter gene studies in human hepatoblastoma (HepG2) cells (Skoog *et al.*, 1999). Moreover, the haplotype analysis revealed the degree of susceptibility to the disease as predicted by the odds ratio with generalized vitiligo: AATCC >AATCT >AACCT >AACAT >AGCCT and AATCC >AATCT >AACCT >AACAT >AGCCC for localized vitiligo. Also, the age of onset analysis of the disease suggested the haplotypes involved in the early age of onset in patients with vitiligo are those involved in high degree of susceptibility of the disease: AATCC >AATCT >AACCT >AACAT >AGCCC >AGCCT.

LD analysis suggests that *TNFA* -238 G/A & -308 G/A polymorphisms in moderate LD association as compared to the other investigated polymorphisms and are strongly associated with the disease risk in patients as suggested by the odds ratio. Moreover, the haplotype analysis showed the presence of haplotypes involving the susceptible alleles of *TNFA* -238 and -308 polymorphisms, having increased levels of TNFα in patients as compared to controls.

Kroeger *et al.* (1997) first showed that -308A allelic form gave a two-fold greater level of transcription than the -308G form in PMA-stimulated Jurkat and U937 cells suggesting that the -308 G/A polymorphism may play a role in the altered *TNFA* gene expression. The study of *TNFA* -308 G/A polymorphism in Iranian population have revealed significant association of -308A allele with vitiligo patients (Namian *et al.*, 2009) and these results are in line with our study however, a previous study of Turkish population suggested that *TNFA* -308 G/A polymorphism has no significant influence on vitiligo susceptibility (Yazici *et al.*, 2006). These contradictory reports may be because of the differences in ethnicity of the studied populations. However, both the studies involved less sample size and hence the association results needed further confirmation. Furthermore, there are no reports available on the effect of these *TNFA* promoter polymorphisms on its expression in vitiligo patients and the present study revealed the significant role of these promoter polymorphisms on the levels of TNFα which might be playing a central role in vitligo pathogenesis.

It has been known that the ROS microenvironment decides the fate of a cell for TNFα mediated apoptosis (Kim *et al.*, 2010). Our earlier reports with other studies suggest that the high oxidative environment prevails in vitiligo patients for the melanocyte destruction (Agrawal *et al.*, 2004; Schallreuter *et al.*, 1991). The destruction of melanocytes might be due to the increased secretion of TNF-α which further increases ROS and thus may lead to an early/defective apoptosis of the melanocytes via TNFα mediated pathway. The possiblity of the TNFα secretion is very high since the keratinocytes (a source of TNFα) surround these melanocytes forming a melanin epidermal unit and thus affect its proliferation and melanogenesis process.

Disturbances in TNFα metabolism have been well documented and found to be associated with several other autoimmune and infectious diseases such as rheumatoid arthritis (Elliot *et al*. 1994), systemic lupus erythematosus (Jacob *et al*., 1990), crohn's

disease (Van Dullemenn *et al*. 1995), cerebral malaria (Mc Guire *et al*., 1994) and lesihmaniasis (Carbera *et al.*, 1995). Previously North Indian and Caucasian studies revealed strong association of -308 G/A polymorphism with T1DM (Kumar *et al*., 2007; Noble *et al*., 2006) A study with psoriatic arthritis patients in Caucasian population for the five promoter polymorphisms suggested significant association of -238 G/A polymorphism with patients being -238 (A) variant, a significant risk factor for the disease (Rahman *et al*., 2006). The *TNFA* -308 G/A polymorphism was significantly associated with susceptibility to asthma in patients of South Iran and with susceptibility to inflammatory bowel disease in European population (Kamali-Sarvestani *et al.*, 2007; Ferguson *et al.,* 2008). A metaanalysis study suggested that *TNFA* -238G/A and -308G/A polymorphisms might be used as biomarkers for psoriasis risk prediction (Li *et al.*, 2007). Furthermore, a study involving 22 SNPs in Caucasian patients with Graves' disease (GD) showed significant association of *TNFA* -238G/A and -308G/A polymorphisms (Simmonds *et al.*, 2004).

Simon and Burgor-Vargas (2008) described a patient with ankylosing spondylitis (AS) and vitiligo who was treated with infliximab (a chimeric monoclonal anti-TNF antibody), which resulted in gradual fading of vitiligo lesions suggesting that *TNFA* was involved in the pathogenesis of vitiligo. Alghamdi et al. (2012) also showed the effect of anti TNFα agents: infliximab, etanercept, and adalimumab in generalized vitiligo patients. The patients did not develop any new depigmented patches during treatment or at the six-month follow-up. These reports signify the involvement of TNFα in vitiligo pathogenesis.

In conclusion, our findings suggest that the increased TNFα levels in vitiligo patients could result, at least in part, from variations at the genetic level. For the first time, we show that the promoter polymorphisms of the *TNF*α gene influence the expression both at transcriptional as well as translational levels in vitiligo which in turn results into increased *ICAM1* expression. The study also emphasizes the influence of TNFα on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding role of TNFα in precipitation of vitiligo and the development of effective anti-TNFα agents may prove to be useful as preventive/ameliorative therapies.

6.5 REFERENCES

Afshan G, Afzal N, and Qureshi S (2012). CD4+CD25(hi) regulatory T cells in healthy males and females mediate gender difference in the prevalence of autoimmune diseases. *Clin Lab.* 58*:* 567-571.

Agrawal D, Shajil EM, Marfatia YS and Begum R (2004). Study on the antioxidant status of vitiligo patients of different age groups in Baroda. *Pigment Cell Res.* 17: 289-294.

Ahn SK, Choi EH, Lee SH, Won JH, Hann SK, Park YK (1994). Immunohistochemical studies from vitiligo: comparison between active and inactive lesions. Yonsei Med J;35:404–10.

Al Badri AM (1993). Abnormal expression of MHC class ll and ICAM-l by melanocytes in vitiligo. *J Pathol.* 169: 203-206.

Alghamdi KM, Khurrum H, Taieb A, and Ezzedine K (2012). Treatment of generalized vitiligo with anti-TNF-α Agents. *J Drugs Dermatol*. 11: 534-539.

Alkhateeb A, Fain PR, Thody A, Bennett DC and Spritz RA (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their relatives. *Pigment Cell Res.* 16: 208-214.

Barrett JC, Fry B, Maller J and Dally MJ (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 21: 263-265.

Carbera M, Shaw MA, Sharples C, Williams H, Castes M et al. (1995). Polymorphism in TNF genes associated with mucocutaneous leishmaniasis. *J.Exp.Med.* 182: 1259-1264.

Das SK, Majumder PP, Chakraborty R, Majumdar TK, Haldar B (1985). Studies on vitiligo: Epidemiological profile in Calcutta, India. *Genet. Epidemiol.* 2: 71-78.

Dwivedi M, Gupta K, Gulla KC, Laddha NC, Hajela K (2009). Lack of genetic association of promoter and structural variants of Mannan-binding lectin (MBL2) gene with susceptibility to generalized vitiligo. *Brit J Dermatol.* 161: 63-69.

Dwivedi M, Laddha NC, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) in isolated vitiligo: a genotype-phenotype correlation. *Pigment cell Melanoma Res.* 24: 737-740.

Dwivedi M, Laddha NC, Shajil EM, Shah BJ and Begum R (2008). The ACE gene I/D polymorphism is not associated with generalized Vitiligo Susceptibility in Gujarat population. *Pigment cell Melanoma Res.* 21: 407-408.

Elliot MJ, Mani RN and Feldmann M (1994). Repeated therapy with monoclonal antibody to tumour necrosis factor-α in patients with rheumatoid arthiritis. *Lancet.* 344: 1125-1127.

Faul F, Erdfelder E, Lang AG and Buchner A (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods.* 39: 175-191.

Feldmann M, Brennan FM and Maini R (1998). Cytokines in autoimmune disorders. *Int Rev Immunol.* 17: 217-228.

Ferguson LR, Huebner C, Petermann I, Gearry RB, Barclay ML et al. (2008). Single nucleotide polymorphism in the tumor necrosis factor-alpha gene affects inflammatory bowel diseases risk. *World J Gastroenterol.* 14: 4652-4661.

Grimes PE, Morris R, Avaniss-Aghajani E, Soriano T, Meraz M et al. (2004). Topical tacrolimus therapy for vitiligo: therapeutic responses and skin messenger RNA expression of proinflammatory cytokines. *J Am Acad Dermatol.* 51: 52-61.

Gupta S and Gollapudi S (2006). Molecular mechanisms of TNF-a-induced apoptosis in 136 naive and memory T cell subsets. *Autoimmun Rev.* 5: 264-268.

Hedley SJ, Metcalfe R, Gawkrodger DJ, Weetman AP, Mac Neil S (1998). Vitiligo melanocytes in long-term culture show normal constitutive and cytokine-induced expression of intercellular adhesion molecule-1 and major histocompatibility complex class I and class II molecules. Br J Dermatol;139:965–73.

Huang CL, Nordlund JJ and Boissy R (2002). Vitiligo. A manifestation of apoptosis. *Am J Clin Dermatol.* 3: 301-308.

Imran M, Laddha NC, Dwivedi M, Mansuri MS, Singh J et al. (2012). Interleukin-4 genetic variants correlate with its transcript and protein levels in vitiligo patients. *Brit J Dermatol.* 167: 314-323.

Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA et al. (1990). Heritable major histocomptibility complex class 2 associated difference in production of TNF-α relevance to genetic predisposition to systemic lupus erythematosus. *PNAS.* 87: 1233-1237.

Jin, Y., Birlea, S.A., Fain, P.R., Gowan, K., Riccardi, S.L., Holland, P.J., Mailloux, C.M., Sufit, A.J., Hutton, S.M., Amadi-Myers, A., et al. (2010). Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. N Engl J Med. 362,1686–1697.

Kamali-Sarvestani E, Ghayomi MA and Nekoee A (2007). Association of TNF-alpha -308 G/A and IL-4 -589 C/T gene promoter polymorphisms with asthma susceptibility in the south of Iran. *J Investig Allergol Clin Immunol.* 17: 361-366.

Kemp EH, Waterman EA and Weetman AP (2001). Immunological pathomechanisms in vitiligo. *Expert. Rev. Mol. Med.* 23: 1-22.

Kim JJ, Lee SB, Park JK and Yoo YD (2010). TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L). *Cell Death Differ.* 17: 1420-1434.

Kim SM, Chung HS and Hann SK (1998). The genetics of vitiligo in Korean patients. *Int J Dermatol.* 37: 908-910.

Kroeger KM, Carville KS, and Abraham LJ (1997). The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. *Mol Immunol.* 34: 391-399.

Kumar R, Goswami R, Agarwal S, Israni N, Singh SK et al. (2007). Association and interaction of the TNF-alpha gene with other pro- and anti-inflammatory cytokine genes and HLA genes in patients with type 1 diabetes from North India. *Tissue Antigens*. 69: 557-567.

Laberge G, Mailloux CM, Gowan K, Holland P, Bennett DC et al. (2005). Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. *Pigment Cell Res.* 18: 300-305.

Laddha NC, Dwivedi M, Shajil EM, Prajapati H, Marfatia YS et al. (2008). Association of PTPN22 1858C/T polymorphism with vitiligo susceptibility in Gujarat population. *J Dermatol Sci.* 49: 260-262.

Li C, Wang G, Gao Y, Liu L and Gao T (2007). TNF-alpha gene promoter -238G>A and -308G>A polymorphisms alter risk of psoriasis vulgaris: a meta-analysis. *J Invest Dermatol.* 127: 1886-1892.

Locksley RM, Killeen N and Lenardo MJ (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell.* 104: 487-501.

Martinez-Esparza M, Jimenez-Cervantes C, Solano F, Lozano JA and Garcia-Borron JC (1998). Mechanisms of melanogenesis inhibition by tumor necrosis factor-α in B16–F10 mouse melanoma cells. *Eur J Biochem.* 255: 139-146.

Mc Guire W, Hill AV, Allsopp CE, Greenwood BM and Kwiatkowski D (1994). Variation in TNF-α promoter region associated with susceptibility to central malaria. *Nature.* 371: 508-510.

Morelli JG and Norris DA (1993). Influence of inflammatory mediators and cytokines on human melanocyte function. *J Invest Dermatol.* 100: 191-195.

Moretti S, Spallanzani A, Amato L, Hautmann G, Gallerani I et al. (2002). New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell Res.* 15: 87-92.

Namian AM, Shahbaz S, Salmanpoor R, Namazi MR, Dehghani F et al. (2009). Association of interferon-gamma and tumor necrosis factor alpha polymorphisms with susceptibility to vitiligo in Iranian patients. *Arch Dermatol Res.* 301: 21-25.

Noble JA, Valdes AM, Lane JA, Green AE and Erlich HA (2006). Linkage disequilibrium with predisposing DR3 haplotypes accounts for apparent effects of tumor necrosis factor and lymphotoxin-alpha polymorphisms on type 1 diabetes susceptibility. *Hum. Immunol.* 67: 999-1004.

Nordlund JJ (1997) The epidemiology and genetics of vitiligo. *Clin Dermatol.* 15: 875-878.

Panchanathan R and Choubey D (2012). Murine BAFF expression is up-regulated by estrogen and interferons: Implications for sex bias in the development of autoimmunity. *Mol Immunol.* 53: 15-23.

Quan, C., Ren, Y.Q, Xiang, L.H., Sun, L.D., Xu, A.E., Gao, X.H., Chen, H.D., Pu, X.M., Wu, R.N., Liang, C.Z., et al. (2010). Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC. Nat Genet. 42, 614–618.

Rahman P, Siannis F, Butt C, Farewell V, Peddle L et al. (2006). TNFalpha polymorphisms and risk of psoriatic arthritis. *Ann Rheum Dis.* 65: 919-923.

Rosenberg SA (1997). Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol. Today*. 18: 175-182.

Schallreuter KU, Wood JM and Berger J (1991). Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol.* 97: 1081-1085.

Shajil EM (2007). Biochemical basis and genetic association studies of selected single nucleotide polymorphisms in catalase and glutathione peroxidase genes in vitiligo. *Ph.D. thesis*. The M. S. University of Baroda, Vadodara, Gujarat, India.

Shajil EM and Begum R (2006) Antioxidant status of segmental and non segmental vitiligo. *Pigment Cell Res.* 19: 179-180.

Shajil EM, Agrawal D, Vagadia K, Marfatia YS and Begum R (2006b). Vitiligo: Clinical profiles in Vadodara, Gujarat. *Ind. J. Dermatol.* 51: 100-104.

Shajil EM, Chatterjee S, Agrawal D, Bagchi T and Begum R (2006a). Vitiligo: Pathomechanisms and genetic polymorphism of susceptible genes. *Ind. J. Exp. Biol.* 44: 526-539.

Sharma S, Ghosh B and Sharma SK (2008). Association of TNF polymorphisms with sarcoidosis, its prognosis and tumour necrosis factor (TNF)-alpha levels in Asian Indians. *Clin Exp Immunol.* 151: 251-259.

Shi YY and He L (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res.* 15: 97-98.

Simmonds MJ, Heward JM, Howson JM, Foxall H, Nithiyananthan R et al. (2004). A systematic approach to the assessment of known TNF-alpha polymorphisms in Graves' disease. *Genes Immun.* 5: 267-273.

Simon JA and Burgos-Vargas R (2008). Vitiligo improvement in a patient with ankylosing spondylitis treated with infliximab. *Dermatology.* 216: 234-235.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK et al. (2012) HLA. alleles and amino acid signatures of the peptide binding pockets of HLA molecules in Vitiligo. *J Invest. Dermatol.* 132: 124-134.

Skoog T, van't Hooft FM, Kallin B, Jovinge S, Boquist S et al. (1999). A common functional polymorphism (C-->A substitution at position -863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. *Hum Mol Genet.* 8: 1443-1449.

Spriggs DR, Deutsch S and Kufe DW (1992). Genomic structure, induction, and production of TNF-alpha. *Immunol Ser.* 56: 3-34.

Spritz RA (2007). The genetics of generalized vitiligo and associated autoimmune diseases. *Pigment Cell Res.* 20: 271-278.

Spritz RA (2008). The genetics of generalized vitiligo. *Curr. Dir. Autoimmun.* 10: 244-257.

Spritz RA (2010). The genetics of generalized vitiligo: autoimmune pathways and an inverse relationship with malignant melanoma. *Genome Medicine.* 2: 78.

Spritz RA (2011). Recent progress in the genetics of generalized vitiligo. *J Genet and Genom.* 38: 271-278.

Suárez A, López P, Mozo L and Gutiérrez C (2005). Differential effect of IL10 and TNF{alpha} genotypes on determining susceptibility to discoid and systemic lupus erythematosus. *Ann Rheum Dis.* 64: 1605-1610.

Taieb A and Picardo M (2009). Vitiligo. *N. Engl. J. Med*. 360: 160-169.

van Dullemenn HM, van Daventer SJ, Hommes DW, Bijl HA, Jansen J et al. (1995). Treatment of Crohn's disease with anti TNF factor chimeric monoclonal antibody. *Gasenterology.* 109: 129-135.

Wajkowicz-Kalijska A, van den Wijngaard RM, Tigges BJ, Westerhof W, Ogg GS et al. (2003). Immunopolarization of CD4+ and CD8+ T cells to Type-1-like is associated with melanocyte loss in human vitiligo. *Lab Invest.* 83: 683-695.

Whitacre CC (2001). Sex differences in autoimmune disease. *Nat. Immunol.* 2: 777-780.

Yazici AC, Erdal ME, Kaya TI, Ikizoglu G, Savasoglu K et al. (2006). Lack of association with TNF-a-308 promoter polymorphism in patients with vitiligo. *Arch Dermatol Res.* 298: 46-49.

Yohn JJ, Critelli M, Lyons MB and Norris DA (1990). Modulation of melanocyte intercellular adhesion molecule-1 by immune cytokines. *J Invest Dermatol.* 90: 233-237.

7.1 INTRODUCTION

Cytokines are important mediators of immunity and there is now convincing evidence that cytokines also have an important role in the pathogenesis of autoimmunity (Feldmann *et al*, 1998). Analysis of cytokine gene polymorphisms would be able to detect genetic abnormality of cytokine regulation and hence establishment of genotype-phenotype correlation may be important in unraveling the disease pathogenesis.

Tumour necrosis factor (TNF)-β also known as lymphotoxin α (LTα), is a close homologue of TNFα. TNFβ has close structural homology and about 30% amino acid sequence identity to TNFα. Both of these cytokines are recognized by the same widely distributed cellular TNF receptors (Smith, 1994) and as a consequence, many of their numerous effects are similar. TNFβ is a Th1 cytokine, primarily produced by activated T and B lymphocytes. TNFβ is a potent mediator of inflammatory and immune responses and it is also involved in the regulation of various biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, coagulation, and neurotransmission.

The *TNFB* gene is of 3 kb and contains four exons. It encodes a primary transcript of 2038 nucleotides yielding a mRNA of 1.4 kb. The genes for the cytokines TNFA and TNFB are tandemly arranged in the class III region of the 3.6 Mb major histocompatibility complex (MHC) between the *HLA*-B and *HLA*-D on chromosome 6p21.3-21.1. *TNFB* is present approximately 1.2 kb apart from the *TNFA* gene. However, both genes are regulated independently. The 5' region of the *TNFB* promoter contains a poly(dA-dT)-rich sequence that binds the nonhistone protein HMG-1 which is involved in its constitutive expression. The synthesis of TNFβ is stimulated by interferons and IL-2. TNFβ is a protein of 171 amino acids with N-glycosylation at position 62. TNFβ is secreted as a soluble polypeptide, but can form heterotrimers with lymphotoxin-beta, which effectively anchors the TNFβ to the cell surface. The soluble TNFβ can interact with TNF receptors 1 (TNFR1) and 2 (TNFR2), similar to TNFα (Funahashi *et al*., 1991). TNFβ induces cell apoptosis upon binding to TNF receptor type 1, but it induces inflammatory responses by activating NF-kB nuclear protein upon binding to TNF receptor type 2 (Lucas *et al*

1997). TNFβ is involved in a large variety of inflammatory, immune-stimulatory, and antiviral responses (Vassalli, 1992).

Polymorphisms of the proinflammatory and immune-regulatory cytokines, *TNFA* and *TNFB* genes have been shown to affect their production and hence can be associated with several autoimmune diseases (Vassalli, 1992; Beutler and Bazzoni, 1998). It is possible that individuals who naturally produce higher levels of these cytokines might exhibit different susceptibility, or severity towards autoimmune diseases.

Two well-characterized variants of *TNFB* are in tight linkage disequilibrium; +252A/G polymorphism in the first intron (IVS1+90 A/G) and (Thr26Asn) causing the substitution of a threonine residue with an asparagine residue at codon 60 in exon 3 (Messer *et al*., 1991) which was found to influence *in vitro TNFB* expression (Messer *et al*., 1991; Whichelow *et al*., 1996). The +252A/G polymorphism also reported to influence TNFβ plasma levels. This single nucleotide polymorphism (C252 A/G) affects a phorbol ester-response element and distinguishes the two alleles that have been designated as *TNFB1* (5.5 kb) and *TNFB2* (10.5 kb) alleles (Messer *et al*., 1991). *TNFA2* and TNFB2 alleles are much more powerful transcription activators than *TNFA1* and *TNFB1* alleles, respectively (Messer *et al*. 1991). Other *in vitro* experiments have shown that the Thr26Asn polymorphism is associated with a twofold increase in the induction of several cell-adhesion molecules including VCAM1 and ICAM1 in the smooth muscle cells of human coronary arteries (Ozaki *et al*., 2002).

In the present study, we have made an attempt to understand the role of TNFβ in vitiligo pathogenesis. Hence, the objectives of this study were:

i.) To determine whether the promoter polymorphisms of *TNFB* [intron 1 +252G/A (rs909253) and exon 3 A/C (rs1041981; Thr26Asn)] are associated with vitiligo susceptibility and modulate *TNFB* transcript levels.

ii.) To measure and compare *TNFB* transcript levels in patients with vitiligo and in unaffected controls.

iii.) To correlate *TNFB* polymorphisms and its transcript levels with the onset and progression of disease.

7.2 MATERIALS AND METHODS

7.2.1 Study Subjects:

The study group included 524 vitiligo patients [360 generalized (including acrofacial vitiligo and vitiligo universalis) and 164 localized vitiligo cases] comprised of 224 males and 300 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 592 ethnically sex-matched unaffected individuals (267 males and 325 females) were included in the study (Table 1). 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 the patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 524)	(n = 592)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	224 (42.75%)	267 (45.10%)
Female	300 (57.25%)	325 (54.90%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Generalized	360 (68.70%)	NA
Localized	164 (31.30%)	NA
Active vitiligo	393 (75.00%)	NA
Stable vitiligo	131 (25.00%)	NA
Family history	68	NA

7.2.2 Genomic DNA preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analyses.

7.2.3 Genotyping of *TNFB* +252G/A polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype +252G/A polymorphisms of *TNFB* gene (Figure 1). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzyme *Nco*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of +252G/A of *TNFB* gene (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.0% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *TNFB* +252G/A SNP genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs909253)				
TNFB +252G/A F	GGTGGTGTCATGGGGAGAACC	62	417	*Nco*I (284 & 137 bp)
TNFB +252G/A R	GGGCCTTGGTGGGTTTGGTT			
TNFB expression F	GGGCCTTGGTTCTCCCCATG	65	232	-
TNFB expressionR	CTGGGGTCTCCAATGAGGTGA			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expressionR	CAAATGAGCCCCAGCCTTCT			

7.2.4 Genotyping of *TNFB* Thr26Asn A/C polymorphism:

The genotyping of Thr26Asn A/C SNP of *TNFB* was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay ID: C_7514870_20; Life Technologies Corp., California, USA). Real-time PCR was performed in 10 µl volume using LightCycler® 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay.

7.2.5 Determination of *TNFB* and *GAPDH* mRNA expression:

7.2.5.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis/ ethidium bromide staining and RNA purity by confirming 260/280 absorbance ratio >1.95. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

7.2.5.2 Real-time PCR:

The expression of *TNFB* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (94°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 5). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

7.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the polymorphisms in patients and controls by comparing the observed and expected

frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *TNFB* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.025 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.
Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi et al., 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett et al., 2005).

Age of onset analysis and relative expression of *TNFB* in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al*., 2007).

7.3 RESULTS

7.3.1 Analysis of association between *TNFB* +252G/A polymorphism and susceptibility to vitiligo:

PCR-RFLP for *TNFB* +252G/A polymorphism yielded a 417 bp undigested product corresponding to A allele and 284 bp and 133 bp digested products corresponding to G allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: GG homozygous, GA heterozygous and AA homozygous for +252G/A polymorphism of *TNFB* gene (Figure 1).

The genotype and allele frequencies of the +252G/A polymorphism in 524 vitiligo patients and 592 controls are summarized in Table 3. The *TNFB* +252G/A polymorphism was found to be in significant association with vitiligo patients (p=0.0024) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of +252G/A was more frequent in the vitiligo

group compared to the control group (27.0% versus 21.0%, p=0.0005; OR 1.424, 95% CI 1.171-1.732) consistent with a susceptibility effect (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.389 and p=0.109 respectively) (Table 3). Moreover, generalized vitiligo group showed significant association of +252G/A polymorphism when the genotypes were compared with those of control group (p<0.0001); however localized vitiligo group did not show significant association of the polymorphism (p=0.826) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the alleles were compared with those of control group (p<0.0001); however, localized vitiligo group did not show significant difference in allele frequencies (p=0.759) (Table 4). The distribution of *TNFB* +252G/A genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05). In addition, gender based analysis of *TNFB* +252G/A polymorphism suggested significant association of minor +252G allele with female vitligo patients as compared to male patients (30.0% versus 23.0%, p=0.021); however, genotype frequencies could not achieve significance due to Bonferroni's correction for multiple testing (p=0.039) (Table 5). This study has 84.96% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at p<0.05 in generalized vitiligo patients and control population.

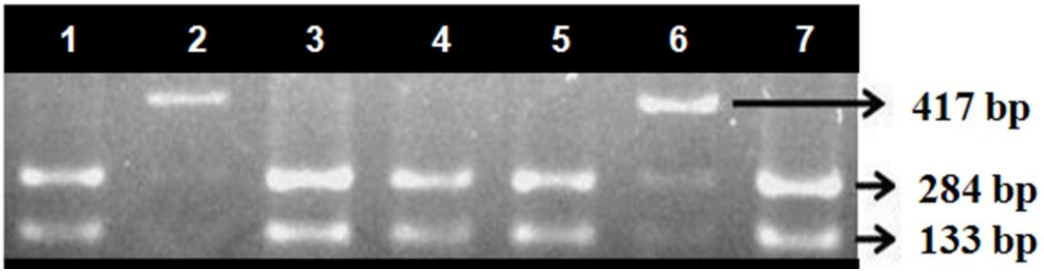


Figure 1. PCR-RFLP analysis of *TNFB* +252G/A polymorphism on 2.0 % agarose gel electrophoresis: lanes: 1, 3, 4, 5 & 7 show homozygous (GG) genotypes; lane: 2 shows homozygous (AA) genotype; lane: 6 shows heterozygous (GA) genotype.

Table 3. Association studies for *TNFB* gene +252G/A and exon 3 A/C polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 524)	(n = 592)		0.109	
	GG	46 (0.09)	29 (0.05)		(P)	
rs909253	GA	193 (0.37)	188(0.32)	0.0024[a]		
(+252G/A)	AA	285 0.54)	375(0.63)		0.389	
	Allele				(C)	1.424
	G	285 (0.27)	246(0.21)	0.0005[b]		(1.171-
	A	763 (0.73)	938(0.79)			1.732)
	Genotype	(n = 524)	(n = 592)			
	AA	46 (0.09)	29 (0.05)		0.109	
	AC	193 (0.37)	188(0.32)	0.0024[a]	(P)	
rs1041981	CC	285 (0.54)	375(0.63)			
(Exon 3	Allele				0.389	1.424
A/C;	A	285 (0.27)	246(0.21)	0.0005[b]	(C)	(1.171-
Thr26Asn)	C	763 (0.73)	938(0.79)			1.732)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 4. Association studies for *TNFB* gene +252G/A and exon 3 A/C polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs909253 (+252G/A)	Genotype	(n = 360)	(n = 164)	(n = 592)			
	GG	39 (0.11)	10 (0.06)	29 (0.05)		0.083 (GV)	1.623 (1.312-2.008) (GV)
	GA	137 (0.38)	51 (0.31)	188(0.32)	<0.0001[a]		
	AA	184 (0.51)	103 (0.63)	375(0.63)	0.826[b]		
	Allele					0.286 (LV)	1.053 (0.7819-1.419) (LV)
	G	215 (0.30)	71 (0.22)	246(0.21)	<0.0001[c]		
	A	505 (0.70)	257 (0.78)	938(0.79)	0.759[d]		
						0.389 (C)	
rs1041981 (Exon 3 A/C; Thr26Asn)	Genotype	(n = 360)	(n = 164)	(n = 592)			
	AA	39 (0.11)	10 (0.06)	29 (0.05)		0.083 (GV)	1.623 (1.312-2.008) (GV)
	AC	137 (0.38)	51 (0.31)	188(0.32)	<0.0001[a]		
	CC	184 (0.51)	103 (0.63)	375(0.63)	0.826[b]		
	Allele					0.286 (LV)	1.053 (0.7819-1.419) (LV)
	A	215 (0.30)	71 (0.22)	246(0.21)	<0.0001[c]		
	C	505 (0.70)	257 (0.78)	938(0.79)	0.759[d]		
						0.389 (C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(GV) refers to Generalized Vitiligo,

(LV) Localized Vitiligo and (C) refers to Controls,

[a] Generalized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,

[b]Localized Vitiligo vs. Controls using chi-squared test with 3 × 2 contingency table,

[c] Generalized Vitiligo vs. Controls using chi-squared test with 2×2 contingency table,

[d] Localized Vitiligo vs. Controls using chi-squared test with 2×2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies for *TNFB* gene +252G/A and exon 3 A/C polymorphisms in male and female patients with vitiligo.

SNP	Genotype or allele	Male Patients (Freq.)	Female Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs909253 (+252G/A)	Genotype	(n = 224)	(n= 300)			
	GG	12 (0.05)	34 0.11)		0.909 (M)	
	GA	81 (0.37)	112(0.38)	0.039[a]		
	AA	131(0.58)	154(0.51)			
	Allele				0.054 (F)	0.7143 (0.5399-0.9449)
	G	105(0.23)	180(0.30)	0.021[b]		
	A	343(0.77)	420(0.70)			
rs1041981 (Exon 3 A/C; Thr26Asn)	Genotype	(n = 224)	(n = 300)			
	AA	12 (0.05)	34 (0.11)		0.909 (M)	
	AC	81 (0.37)	112(0.38)	0.039[a]		0.7143 (0.5399-0.9449)
	CC	131(0.58)	154(0.51)			
	Allele				0.054 (F)	
	A	105(0.23)	180(0.30)	0.021[b]		
	C	343(0.77)	420(0.70)			

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(M) refers to Male Patients and (F) refers to Female Patients,

[a] Male Patients vs. Female Patients using chi-squared test with 3×2 contingency table,

[b]Male Patients vs. Female Patients using chi-squared test with 2×2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

7.3.2 Analysis of association between *TNFB* A/C*;* Thr26Asn polymorphism and susceptibility to vitiligo:

Real time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'A' allele) and VIC (for 'C' allele) fluorophores for *TNFB* A/C polymorphism which yield the three genotypes (AA homozygous, AC heterozygous and CC homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2).

The genotype and allele frequencies of the *TNFB* A/C polymorphism in 524 vitiligo patients and 592 controls are summarized in Table 3. The *TNFB* A/C polymorphism

was found to be in significant association with vitiligo patients (p=0.0024) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (A) of *TNFB* A/C was more frequent in the vitiligo group compared to the control group (27.0% versus 21.0%, p=0.0005; OR 1.424, 95% CI 1.171-1.732) consistent with a susceptibility effect (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.389 and p=0.109 respectively) (Table 3). Moreover, generalized vitiligo group showed significant association of *TNFB* A/C polymorphism when the genotypes were compared with those of control group (p<0.0001); however localized vitiligo group did not show significant association of the polymorphism (p=0.826) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the alleles were compared with those of control group (p<0.0001); however, localized vitiligo group did not show significant difference in allele frequencies (p=0.759) (Table 4). The distribution of *TNFB* A/C genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05). In addition, gender based analysis of *TNFB* A/C polymorphism suggested significant association of minor (A) allele with female vitligo patients as compared to male patients (30.0% versus 23.0%, p=0.021); however, genotype frequencies could not achieve significance due to Bonferroni's correction for multiple testing (p=0.039) (Table 5). This study has 84.96% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at p<0.05 in generalized vitiligo patients and control population.

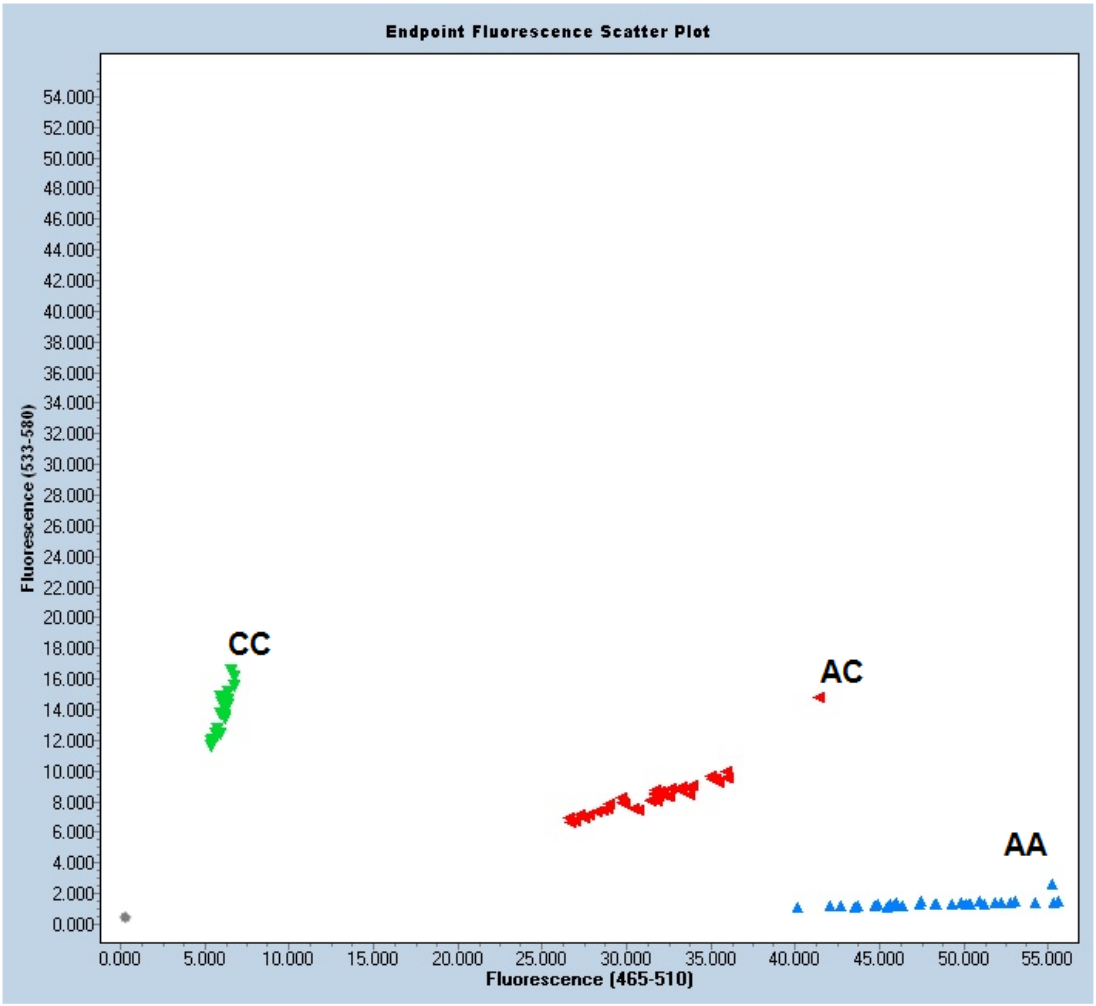


Figure 2. TaqMan end point fluoroscence analysis for *TNFB* A/C; (Thr26Asn) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: AA, AC and CC, based on fluorescence with Channel 465-510 (FAM for 'A' allele) and Channel 536-580 (VIC for 'C' allele). A no-template control (NTC) was used with each SNP genotyping assay (shown as grey spot).

7.3.3 Effect of *TNFB* +252G/A genotypes on age of onset of vitiligo and its progression:

When age of onset of the disease was correlated with the *TNFB* +252G/A genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to AA and GA genotypes (p=0.0001 and p=0.03 respectively) (Figure 2A). Moreover, patients with genotype GA showed early onset of the disease as compared to AA genotypes (p=0.009) (Figure 2A) suggesting the effect of the susceptible allele G on the early onset of disease. Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients (p<0.0001) (Figure 2B).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the +252G allele occurred prevalently in the group of patients with active vitiligo compared to the control group (30.00% versus 21.00%, p<0.0001) (Table 6). However, there was no statistically significant difference in the distribution of the +252G allele between patients with stable vitiligo and control group (20.00% versus 21.00%, p=0.8002) (Table 3). Interestingly, the +252G allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (30.00% versus 20.00%, p=0.002) suggesting the important role of +252G allele in progression of the disease (Table 6).

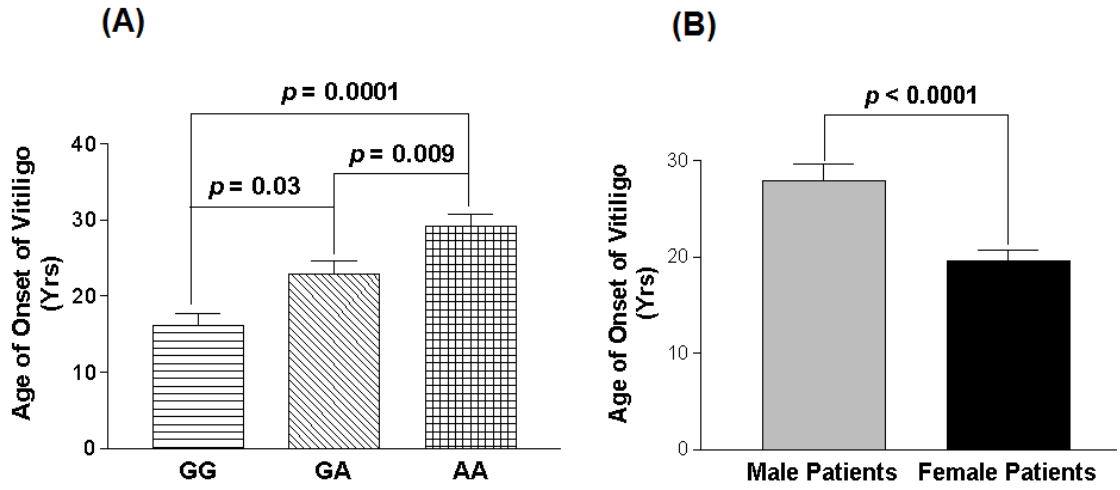


Figure 3. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *TNFB* +252G/A in 524 vitiligo patients. Vitiligo patients with +252GG genotype showed early age of onset of disease as compared to AA (Mean age of onset ± SEM: 16.15 ± 1.504 vs 29.23 ± 1.497; p=0.0001) and GA genotypes (Mean age of onset ± SEM: 16.15 ± 1.504 vs 22.83 ± 1.798; p=0.027). Patients with +252GA genotype showed early age of onset of disease as compared to AA genotype (Mean age of onset ± SEM: 22.83 ± 1.798 vs 29.23 ± 1.497; p=0.009).

(B) Comparison of age of onset of the disease (Years) with respect to gender differences in 224 male patients and 300 female patients with vitiligo. Female patients showed an early onset of disease as compared to male patients (Mean age of onset ± SEM: 19.63 ± 1.077 vs 27.94 ± 1.785; p<0.0001).

Table 6. Association studies for *TNFB* gene +252G/A and exon 3 A/C polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs909253 (+252G/A)	Genotype	(n = 393)	(n = 131)	(n = 592)			1.691[a]
	GG	40 (0.10)	6 (0.05)	29 (0.05)	0.012[a]	0.163	(1.204-
	GA	152 (0.39)	40 (0.30)	188(0.32)	< 0.0001[b]	(AV)	2.376)
	AA	201 (0.51)	85 (0.65)	375(0.63)	0.945[c]		
	Allele					0.645	1.597 [b]
	G	232 (0.30)	52 (0.20)	246(0.21)	0.002 [a]	(SV)	(1.297-
	A	554 (0.70)	210(0.80)	938(0.79)	<0.0001[b]		1.966)
					0.8002[c]	0.389	
						(C)	0.9442 [c]
							(0.6757-
							1.319)
rs1041981 (Exon 3 A/C; Thr26Asn)	Genotype	(n = 393)	(n = 131)	(n = 592)			1.691[a]
	AA	40 (0.10)	6 (0.05)	29 (0.05)	0.012[a]	0.163	(1.204-
	AC	152 (0.39)	40 (0.30)	188(0.32)	< 0.0001[b]	(AV)	2.376)
	CC	201 (0.51)	85 (0.65)	375(0.63)	0.945[c]		
	Allele					0.645	1.597 [b]
	A	232 (0.30)	52 (0.20)	246(0.21)	0.002 [a]	(SV)	(1.297-
	C	554 (0.70)	210(0.80)	938(0.79)	<0.0001[b]		1.966)
					0.8002[c]	0.389	
						(C)	0.9442 [c]
							(0.6757-
							1.319)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

7.3.4 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms (+252G/A and exon 3 A/C) investigated in the *TNFB* gene were in strong LD association (G/A: A/C; D' =1.00, r^2=1.00). A haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes did not differ between vitiligo patients and controls (global *p*-value =5.24e-033) (Table 7). However, the susceptible GA haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.6-fold [*p* = 1.03e-005; odds ratio (OR): 1.635; 95% confidence interval (CI): (1.519~1.778)] (Table 7).

Table 7. **Distribution of haplotype frequencies for *TNFB* gene, +252 T/C and Exon 3 C/A polymorphisms among generalized vitiligo patients and controls.**

Haplotype (+252 G/A and Exon 3 A/C)	Generalized Vitiligo Patients (Freq. %) (n=426)	Controls (Freq. %) (n=592)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
AC	274.30(0.69)	340.25(0.77)	2.13e-005	5.24e-033	0.647 [0.527~0.795]
GA	177.61(0.31)	108.62(0.23)	1.03e-005		1.635 [1.519~1.778]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

7.3.5 Relative gene expression of *TNFB* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *TNFB* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.0005) (Figure 3A). Moreover, generalized vitiligo patients showed significant higher expression of *TNFB* as compared to localized vitiligo patients (p=0.018) (Figure 3A).

The 2-ΔΔCp analysis showed approximately 0.492 fold change in the expression of *TNFB* in patients as compared to controls (Figure 3B).

7.3.6 Correlation of *TNFB* transcripts with the *TNFB* +252G/A genotypes:

Further, the expression levels of *TNFB* were analyzed with respect to +252G/A genotypes (Figure 3C). Interestingly, *TNFB* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.015). Also, patients with genotypes GA showed increased *TNFB* transcripts as compared to controls (p=0.039); however, no significant difference was observed in *TNFB* expression in patients as compared to controls with AA genotypes (p=0.168) (Figure 3C).

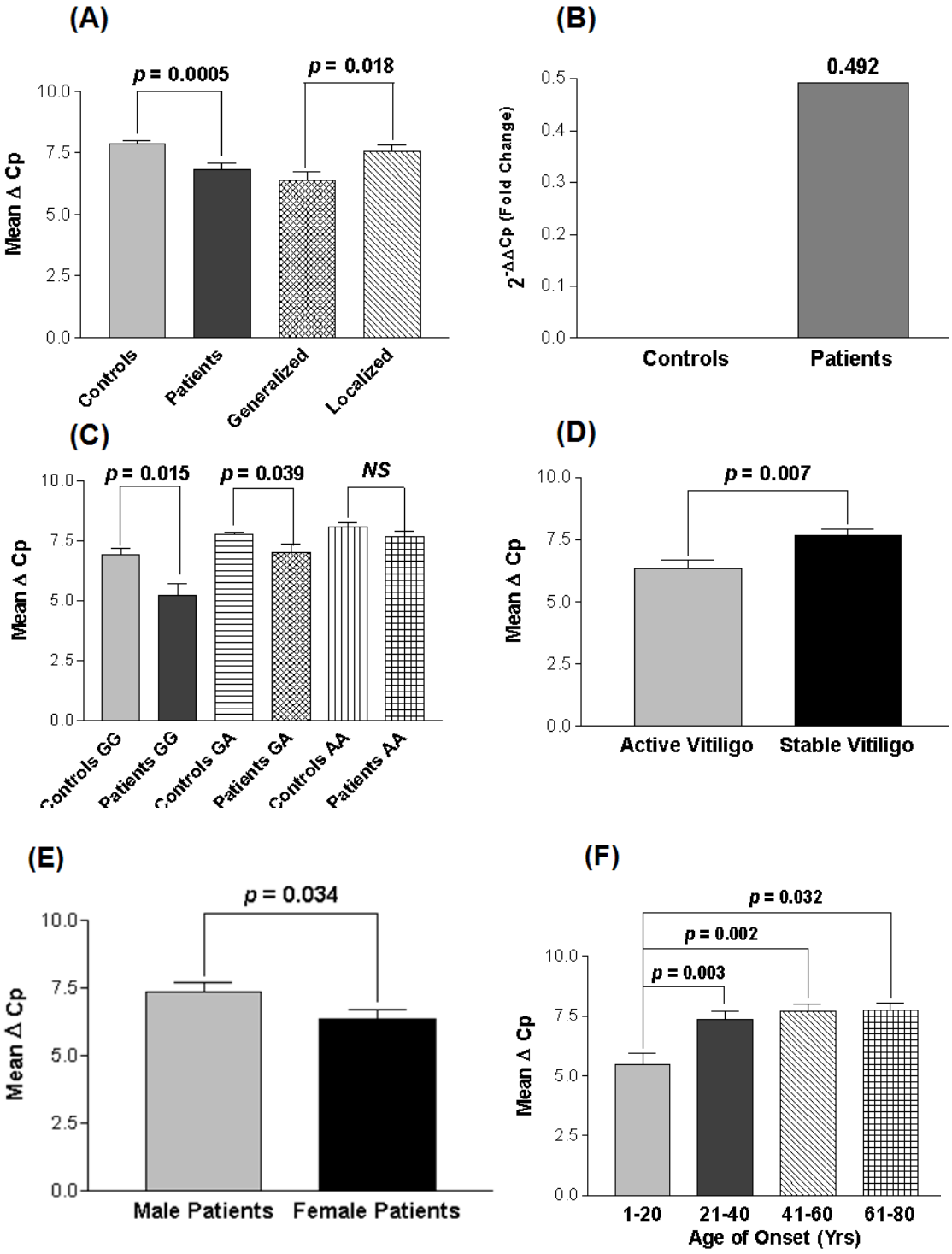
7.3.7 Correlation of *TNFB* transcripts with the *TNFB* A/C genotypes:

Further, the expression levels of *TNFB* were also analyzed with respect to **A/C** genotypes (Figure 3G). Interestingly, *TNFB* expression was significantly increased in patients with susceptible AA genotypes as compared to controls (p=0.015). Also, patients with genotypes AC showed increased *TNFB* transcripts as compared to controls (p=0.039); however, no significant difference was observed in *TNFB* expression in patients as compared to controls with CC genotypes (p=0.168) (Figure 3G).

7.3.8 Effect of *TNFB* expression on disease progression:

In addition, we also checked the effect of *TNFB* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3D). Interestingly, active vitiligo patients showed significant increase in expression of *TNFB* transcripts as compared to the patients with stable vitiligo (p=0.007) suggesting the involvement of *TNFB* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *TNFB* expression as compared to male patients (p=0.034) (Figure 3E).

When *TNFB* expression was monitored in different patient groups of age of onset, patients with the age group 1-20 yrs showed significant increase in expression of *TNFB* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.003, p=0.002 and p=0.032 respectively) suggesting the importance of *TNFB* in early onset of the disease (Figure 3F).



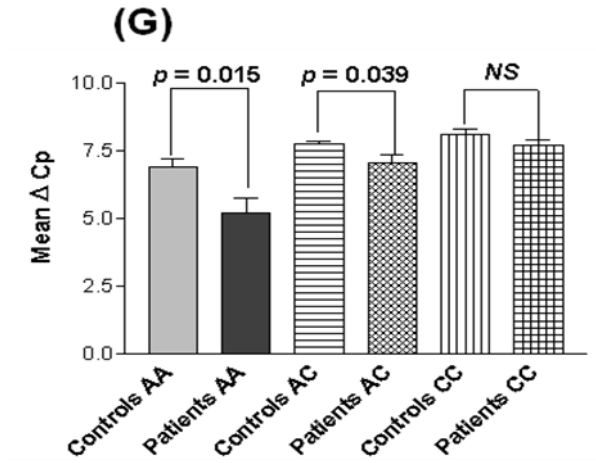


Figure 4. Relative gene expression of *TNFB* in controls and vitiligo patients:

(A) Expression of *TNFB* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* as compared to controls (Mean ΔCp ± SEM: 6.830 ± 0.2435 vs 7.853 ± 0.1244; p=0.0005). Generalized vitiligo patients showed significant increase in mRNA levels of *TNFB* as compared to localized patients (Mean ΔCp ± SEM: 6.372 ± 0.3498 vs 7.549 ± 0.2516; p=0.018).

(B) Expression fold change of *TNFB* transcripts in 166 vitiligo patients against 175 controls showed 0.447 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *TNFB* transcripts with respect to *TNFB* +252G/A in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* with GG (Mean ΔCp ± SEM: 5.217 ± 0.5087 vs 6.918 ± 0.2808; p=0.015) and GA (Mean ΔCp ± SEM: 7.028 ± 0.3376 vs 7.761 ± 0.0952; p=0.039) genotypes as compared to controls. There was no significant difference in the expression of *TNFB* with AA genotypes (Mean ΔCp ± SEM: 7.678 ± 0.2379 vs 8.102 ± 0.1747; p=0.168) as compared to controls. [*NS* = non-significant]

(D) Expression of *TNFB* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significant increase in mRNA levels of *TNFB* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 6.314 ± 0.338 vs 7.676 ± 0.2629; p=0.007).

(E) Expression of *TNFB* transcripts with respect to gender differences in 87 male patients and 79 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significant increase in mRNA levels of *TNFB* as compared to male vitiligo patients (Mean ΔCp ± SEM: 6.343 ± 0.3494 vs 7.374 ± 0.3165; p=0.034).

(F) Expression of *TNFB* transcripts with respect to different age groups in 166 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significant increase in expression of *TNFB* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.364 ± 0.3224; p=0.003), 41-60 (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.689 ± 0.3293; p=0.002) and 61-80 yrs (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.743 ± 0.2944; p=0.032).

(G) Expression of *TNFB* transcripts with respect to *TNFB* A/C in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* with AA (Mean ΔCp ± SEM: 5.217 ± 0.5087 vs 6.918 ± 0.2808; p=0.015) and AC (Mean ΔCp ± SEM: 7.028 ± 0.3376 vs 7.761 ± 0.0952; p=0.039) genotypes as compared to controls. There was no significant difference in the expression of *TNFB* with CC genotypes (Mean ΔCp ± SEM: 7.678 ± 0.2379 vs 8.102 ± 0.1747; p=0.168) as compared to controls. [*NS* = non-significant]

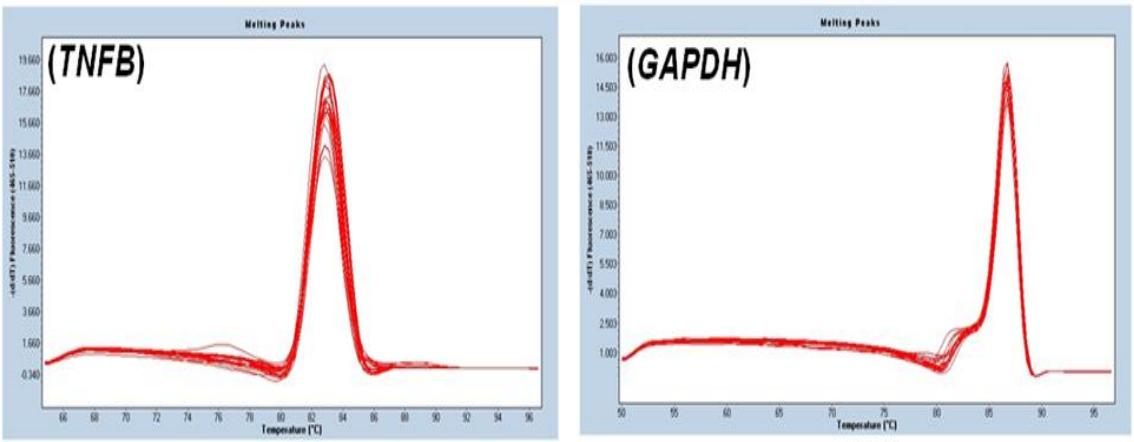


Figure 5. Melt curve analysis of *TNFB* and *GAPDH* showing specific amplification.

7.4 DISCUSSION

Cytokines are important mediators of immunity and their response due to imbalance or deficiency in the cytokine network may largely determine autoimmune disease susceptibility and severity. TNF plays a central role in the so-called ‘‘cytokine storm’’ characteristic of several autoimmune diseases. Since many aspects of the cellular and humoral immune responses are under genetic control and account for individual differences in immune response patterns, there is increasing interest in genetic polymorphisms that affect inflammatory cytokines, which might explain the well-known diversity among clinical findings and outcomes in critically ill patients affected by the same disease.

TNFA and *TNFB* polymorphisms are reported to be associated with the inflammatory and immunomodulatory responses and are involved in the modulation of gene expression, and thus affect the precipitation and progression of the diseases (Kaluza *et al*., 2000; Wilson *et al*., 1997; Gonza´ lez *et al*., 2003; Messer *et al*., 1991; Ozaki *et al*., 2002).

The polymorphism (A→G) of the *TNFB* gene is present in intron 1 at position +252 (Messer *et al* 1991). Although the *TNFB* +252 G/A polymorphic site is located in an intron, it is linked to an amino acid substitution at position 26 of the TNFβ protein, the substitution being conserved as asparagine in the *TNFB* +252G and as threonine in the *TNFB* +252A allele (Messer *et al*., 1991). The *TNFB* +252G allele has been shown to be associated with increased expression and the *TNFB* +252A allele with decreased production of the TNFβ protein (Messer *et al*., 1991).

The *TNFA* -308A and the *TNFB* +252G alleles are constituents of one of the extended ancestral haplotypes (AH8.1) located in the chromosomal region 6p21.3-21.1 (MHC). This haplotype is known to be associated with serious disorders of the immune system (Dawkins *et al*., 1983; Candore *et al*., 2002; Candore *et al*., 2003). The *TNFB* +252G allele was also associated with several autoimmune diseases such as type 1 diabetes mellitus (Dawkins *et al*., 1983; Aly *et al*., 2006) and systemic lupus erythematosus (Smerdel-Ramoya *et al*., 2005; McHugh *et al*., 2006). The present study for the first time reports significant association of *TNFB*+252G/A polymorphism with vitiligo susceptibility. In particular, we found that +252G allele was prevalent in generalized

vitiligo patients as compared to localized vitiligo patients and healthy controls. We also found that +252G allele was prevalent in active cases of vitiligo as compared to patients with stable vitiligo and controls suggesting the involvement of the allele in progression of the disease. Moreover, +252G allele was significantly associated with female patients as compared to male patients suggestive of the fact that females are more prone to autoimmune diseases. The age of onset analysis further suggested that patients with GG genotype had an early onset of the disease as compared to patients with GA and AA genotypes indicating the important role of +252G allele in disease susceptibility.

The whole *TNFB* gene is in strong linkage disequilibrium, therefore the +252G allele naturally coexists with the 804A allele of exon 3 A/C polymorphism (Thr26Asn) (Clarke *et al*., 2006). Ozaki *et al*. investigated the functionality of the +252G/A and 804A/C SNPs in the *TNFB* gene (Ozaki *et al*., 2002). The 804A/C polymorphism causes an amino-acid change from threonine (T) to asparagine (N) at codon 26. They found that the variant protein 26N is associated with a two fold increase in the induction of cell adhesion molecules in vascular smooth muscle cells (Ozaki *et al*., 2002). We found that *TNFB* exon 3 A/C polymorphism (Thr26Asn) was significantly associated with vitiligo susceptibility in Gujarat population. In particular, allele 'A' was prevalent in generalized vitiligo patients as compared to localized vitiligo patients and healthy controls. We also found that 'A' allele was prevalent in active cases of vitiligo as compared to patients with stable vitiligo and controls suggesting the involvement of the 'A' allele in progression of the disease. Moreover, the 'A' allele was significantly associated with female patients as compared to male patients suggestive of the fact that females are more prone to autoimmune diseases. The age of onset analysis further suggested that patients with AA genotype had an early onset of the disease as compared to patients with AC and CC genotypes indicating the important role of 'A' allele in disease susceptibility.

It has been reported that cytokines such as IFN-γ, TNFα and TNFβ can initiate apoptosis and thus lead to melanocyte death in the context of autoimmunity (Huang *et al*., 2002). TNFα/β also has the capacity to inhibit melanogenesis through an inhibitory effect on tyrosinase and tyrosinase related protein (Martinez-Esparza *et al*., 1998). In addition, IFN-γ and TNFα/β induce the expression of intercellular adhesion

molecule-1 (ICAM-1) on the cell-surface of melanocytes (Yohn *et al*., 1990). The increased expression of ICAM-1 on the melanocytes enhances T cell/melanocyte attachment in the skin and thereby resulting in destruction of melanocytes in vitiligo (Al Badri, 1993; Morelli and Norris, 1993).

Our study also confirmed that the *TNFB* mRNA levels have increased significantly in vitiligo patients as compared to controls. Our recent study also suggested an increase in *ICAM1* mRNA levels in vitiligo patients compared to controls. Furthermore, in patients with early age of onset had increased *ICAM1* expression as compared with patients with late onset of disease. These results are in concordance with the previous reports (Reimann *et al*., 2012; Ahn *et al*., 1994; al Badri *et al*., 1993; Yagi *et al*., 1997). Therefore, ICAM1 is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

The *TNFB* +252 G/A and Thr26Asn A/C SNPs gene could affect susceptibility/ resistance to vitiligo through its influence on the production of TNFα and TNFβ. As *TNFA* and *TNFB* genes are tandemly arranged within the HLA complex, and it has been shown that the *TNFB* gene polymorphisms influence the level of production of the TNFα protein. Studies indicate that variation at both *TNFA* -308 G/A and *TNFB* +252 G/A can affect TNFα production levels (Abraham and Kroeger, 1999; Ozaki *et al*., 2002).

Pociot *et al*, (1993) showed that secretion of TNFα was shown to be the highest in *TNFB* +252GG homozygotes, the lowest in the *TNFB* +252AA homozygotes, and intermediate in the *TNFB* +252GA heterozygous individuals. *In vitro*, direct analysis of skin T cells from margins of vitiliginous skin showed that polarized type-1 T cells ($CD4^+$ and particularly $CD8^+$), which predominantly secrete IFNγ and TNFα are associated with the destruction of melanocytes during active vitiligo (Wajkowicz-Kalijska *et al*., 2003). In vitiligo affected skin, a significantly higher expression of TNFα (Grimes *et al*, 2004; Moretti *et al*, 2002), IL6 (Moretti *et al*, 2002), IFNγ (Grimes *et al*, 2004) was detected compared with healthy controls and perilesional, non-lesional skin (Moretti *et al*, 2002) indicating that cytokine imbalance plays an important role in the depigmentation process of vitiligo. Since elevated levels of TNFα have been associated with vitiligo, this could explain the association of *TNFB*

+252G allele with susceptibility to vitiligo in the present study. Homozygosity for the *TNFB* +252A allele will be protective because of its association with lower TNFα/β levels. Previously, Hasegawa *et al*., also suggested the correlation of *TNFB* +252GA polymorphism with elevated levels of TNFα in pulmonary fibrosis in scleroderma (Hasegawa *et al*., 1997). *TNFB* +252GG homozygosity has also been shown to be associated with susceptibility to systemic lupus erythematosus in some populations from Germany, Korea, and China (Bettinotti *et al*., 1993; Kim *et al*., 1996; Lee *et al*., 1996; Zhang *et al*., 1997).

The variant of *TNFB* gene present in exon 3 leads to the substitution Asp to Thr at the amino acid position 26 (Kobayashi *et al*., 1986) and correlated with an altered level of TNFβ production (Messer *et al*., 1991). The exon 3 Asp to Thr is in strong linkage disequilibrium with +252G/A polymorphism of intron 1 and combination of these allelic forms may lead to the different levels of TNF production in response to various physiological and pathological stimuli (Steinman, 1995) and in turn might result in predisposition to the development of vitiligo and involved in the different clinical aspects of the disease. Evidence implicating the polymorphism in the *TNFB* gene (T26N) with increased susceptibility to T2DM has emerged from Japanese and Danish studies (Yamada *et al*., 2004; Hamid *et al*., 2005). In the present study we also found that +252G/A and exon 3 A/C SNPs were in strong LD association. The susceptible GA haplotype was more frequently observed in vitiligo patients as compared to controls (31% vs 23%) and increased the risk of vitiligo by 1.6-fold.

There are no reports available for gene expression analysis of *TNFB* in vitiligo patients hence the present study also focused on the *TNFB* expression in vitiligo patients. Our results clearly suggest the important role of *TNFB* in pathogenesis of vitiligo. Vitiligo patients showed significant increase in *TNFB* transcripts levels as compared to controls suggesting that melanocyte death in patients could be triggered due to the increased *TNFB* levels. For the first time we report that generalized vitiligo has significantly higher *TNFB* transcript levels as compared to localized vitiligo patients which indicate involvement of autoimmunity in precipitation of generalized vitiligo. Our results also indicate that active vitiligo patients have significantly higher *TNFB* transcript levels as compared to the patients with stable vitiligo which signifies the role of *TNFB* in disease progression. Our results also suggest that there are

significantly higher transcript levels of *TNFB* in female patients as compared to male patients. Moreover, female patients have an early onset as compared to male patients suggestive of the fact that females have increased susceptibility towards vitiligo as compared to males, implicating sex bias in the development of autoimmunity (Whitacre, 2001; Panchanathan and Choubey, 2012; Afshan *et al*., 2012). Furthermore, the genotype-phenotype analysis for +252G/A polymorphism indicated that patients with GG and GA genotypes had higher expression of *TNFB* transcripts suggesting the crucial role of +252G allele in pathogenesis of vitiligo. Also, the genotype-phenotype analysis of *TNFB* exon 3 A/C showed that patients harboring AA and AC genotypes had higher expression of *TNFB* suggesting that allele 'A' plays an important role in increased expression of *TNFB.*

In addition, such gene polymorphisms might provide useful biomarkers for the screening of patients at risk, as well in the identification of those most likely to benefit from specific therapeutic choices (Marshall and Reinhart, 2009).

In conclusion, our findings suggest that the increased *TNFB* levels in vitiligo patients could result, at least in part, from variations at the genetic level which in turn leads to increased ICAM1. For the first time, we show that the +252G/A polymorphism of the *TNFB* gene influences the *TNFB* expression levels in vitiligo. The study also emphasizes the influence of *TNFB* on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding the role of *TNFB* in precipitation of vitiligo and the development of effective anti-TNF agents may prove to be useful as preventive/ameliorative therapies.

7.5 REFERENCES

A.G. Porter (1990). Human tumor necrosis factor-alpha and -beta: differences in their structure, expression and biological properties. *FEMS Microbiol. Immunol.* 2 (4) 193–199.

Abraham LJ, Kroeger KM (1999).Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. *J Leukoc Biol* 66:562.

Afshan, G., Afzal, N., and Qureshi, S. (2012). CD4+CD25(hi) regulatory T cells in healthy males and females mediate gender difference in the prevalence of autoimmune diseases. *Clin Lab.* 58:567-571.

Aggarwal BB, Eessalu TE, Hass PE (1985). Characterization of receptors for human tumor necrosis factor and their regulation by c-interferon. *Nature* 318:665–7.

Ahn SK, Choi EH, Lee SH, Won JH, Hann SK, Park YK. (1994).Immunohistochemical studies from vitiligo—comparison between active and inactive lesions. *Yonsei Med J* 35:404–10.

Al Badri AM, Foulis AK, Todd PM, Gariouch JJ, Gudgeon JE, Stewart DG, *et al*. (1993).Abnormal expression of MHC class II and ICAM-1 by melanocytes in vitiligo. *J Pathol* 169:203–6.

Al Badri, A.M. (1993). Abnormal expression of MHC class ll and ICAM-l by melanocytes in vitiligo. *J Pathol*. 169 : 203-206.

Aly TA, Ide A, Jahromi MM, Barker JM, Fernando MS, Babu SR, *et al*. (2006). Extreme genetic risk for type 1A diabetes. *Proc Natl Acad Sci USA* 103:14074–9.

Barrett, J.C., Fry, B., Maller, J. and Dally, M.J (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 21 : 263-265.

Belch, J.J., Shaw, J.W., Kirk, G., McLaren, M., Robb, R., Maple, C., Morse, P (1997). The white blood cell adhesion molecule E-selectin predicts restenosis in patients with intermittent claudication undergoing percutaneous transluminal angioplasty. *Circulation* 95 (8) : 2027–2031.

Bettinotti MP, Hartung K, Deicher H, Messer G, Keller E, Weiss EH, *et al* (1993).Polymorphism of the tumor necrosis factor beta gene in systemic lupus erythematosus: TNFBMHC haplotypes. *Immunogenetics* 37:449.

Beutler B, Bazzoni F (1998). TNF, apoptosis and autoimmunity: a common thread? Blood Cells *Mol Dis* 24:216, 1998.

Candore G, Lio D, Colonna Romano G, Caruso C (2002). Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: effect of multiple gene interactions. *Autoimmun Rev* 1:29–35.

Candore G, Modica MA, Lio D, Colonna-Romano G, Listi F, Grimaldi MP, *et al*.(2003). Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: a genetically determined defect of C4 influences immunological parameters of healthy carriers of the haplotype. *Biomed Pharmacother* 57:274–7.

Clarke, R., Xu, P., Bennett, D., Lewington, S., Zondervan, K., Parish, S., Palmer, A., Clark, S., Cardon, L., Peto, R., Lathrop, M., Collins, R (2006). Lymphotoxin-alpha gene and risk of myocardial infarction in 6,928 cases and 2,712 controls in the ISIS case-control study. *PLoS Genet.* 2 (7): e107.

D'Alfonso S, Colombo G, Della Bella S, Scorza R, Momigliano-Richiardi P (1996). Association between polymorphisms in the TNF region and systemic lupus erythematosus in the Italian population. *Tissue Antigens* 47:551

Dawkins RL, Christiansen FT, Kay PH, Garlepp M, McCluskey J, Hollingsworth PN, *et al*. (1983).Disease associations with complotypes, supratypes and haplotypes. *Immunol Rev* 70:1–22.

Dawkins, R.L., Spies, T., Strominger, J.L., Hansen, J.A (1989). Some disease-associated ancestral haplotype carry a polymorphism of TNF. *Hum. Immunol*. 26 :91–94.

Faul, F., Erdfelder, E., Lang, A.G. and Buchner, A (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods*. 39 :175-191.

Feldmann, M., Brennan, F.M. and Maini, R (1998). Cytokines in autoimmune disorders. *Int Rev Immunol.* 17: 217-228.

Gonza´ lez S, Rodrigo L, Martı´nez-Borra J, Lo´ pez-Va´ zquez A, Fuentes D, Nin˜ o P, *et al*.(2003). TNF-a -308A promoter polymorphism is associated with enhanced TNF-a production and inflammatory activity in Crohn's patients with fistulizing disease. *Am J Gastroenterol* 98:1101e6.

Grimes, P.E., Morris, R., Avaniss-Aghajani, E., Soriano, T., Meraz, M. and Metzger, A(2004). Topical tacrolimus therapy for vitiligo: therapeutic responses and skin messenger RNA expression of proinflammatory cytokines. *J Am Acad Dermatol*. 51:52-61.

Hamid YH, Urhammer SA, Glqmer C, *et al*.(2005). The common T60N polymorphism of the lymphotoxin-alpha gene is associated with type 2 diabetes and other phenotypes of the metabolic syndrome. *Diabetologia* 48:445- 51.

Hasegawa M, Fujimoto M, Kikuchi K, Takehara K(1983). Elevated serum tumor necrosis factor-a levels in patients with systemic sclerosis: association with pulmonary fibrosis. *J Rheumatol* 24:663.

Huang, C.L., Nordlund, J.J. and Boissy, R (2002). Vitiligo. A manifestation of apoptosis. *Am J Clin Dermatol.* 3 :301-308.

Hwang, S.J., Ballantyne, C.M., Sharrett, A.R., Smith, L.C., Davis, C.E., Gotto, Jr.A.M., Boerwinkle, E (1997). Circulating adhesion molecules VCAM-1, ICAM-1, and Eselectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 96 (12) :4219–4225.

I. Funahashi, M. Kawatsu, T. kajikawa, K. Takeo, T. Asahi, T. Kakutani, *et al* (1991). Usefulness of glycosylated and recombinant human lymphotoxin for growth inhibition of human and murine solid tumors and experimental metastasis in mice. *J. Immunother*. 10 :28–38.

Kaluza W, Reuss E, Grossmann S, Hug R, Schoopf RE, Galle PR, *et al* (2000). Different transcriptional activity and in vitro TNF-alpha production in psoriasis patients carrying the TNFalpha 238A promoter polymorphism. *J Invest Dermatol* 114:1180e3.

Kim TG, Kim HY, Lee SH, Cho CS, Park SH, Choi HB, *et al*(1996). Systemic lupus erythematosus with nephritis is strongly associated with the TNFB*2 homozygote in the Korean population. *Hum Immunol* 46:10.

Kobayashi, Y., Miyomotu, D., Asada, M., Obinata, M., Osawa, T (1986). Cloning and expression of human lymphotoxin mRNA derived from a human T cell hydrydoma. *J. Biochem*. 100 :727–730.

Lanas A, Garcı´a-Gonza´ lez MA, Santolaria S, Crusius JBA, Serrano MT, Benito R, *et al*.(2001). TNF and LTA gene polymorphisms reveal different risk in gastric and duodenal ulcer patients. *Genes Immun* 2:415e21.

Lee SH, Park SH, Min JK, Kim SI, Yoo WH, Hong YS, *et al* (1997). Decreased tumour necrosis factor-beta production in TNFB*2 homozygote: an important predisposing factor of lupus nephritis in Koreans. *Lupus* 6:603.

Lucas R, Lou J, Morel DR, Ricou B, Suter PM, Grau GE, *et al* (1997).TNF receptors in the microvascular pathology of acute respiratory distress syndrome and cerebral malaria. *J Leukocyte Biol* 61:551–8.

M.C. Warle, A. Farhan, H.J. Metselaar, W.C. Hop, C. Perrey, P.E. zondervan, *et al* (2003).Are cytokine gene polymorphisms related to in vitro cytokine production profile? *Liver Transplant*. 9 (2):170–181.

Marshall JCE, Reinhart K (2009).Biomarkers of sepsis. *Crit Care Med* 37:2290–8.

Martinez-Esparza, M., Jimenez-Cervantes, C., Solano, F., Lozano, J.A. and Garcia-Borron, J.C (1998). Mechanisms of melanogenesis inhibition by tumor necrosis factor-α in B16–F10 mouse melanoma cells. *Eur J Biochem*. 255:139-146.

McDevitt H, Munson S, Ettinger R, Wu A (2004).Multiple roles for tumor necrosis factor-alpha and lymphotoxin alpha/beta in immunity and autoimmunity. *Arthritis Res* 4(Suppl 3):S141–52.

McHugh NJ, Owen P, Cox B, Dunphy J,Welsh K (2006).MHCclass II, tumour necrosis factor alpha, and lymphotoxin alpha gene haplotype associations with serological subsets of systemic lupus erythematosus. *Ann Rheum Dis* 65:488–94.

Messer G, Spengler U, Jung MC, Honold G, Blo¨mer K, Pape GR, Riethmu¨ ller G, Weiss EH (1991).Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-b gene correlates with a variant amino acid in position 26 and a reduced level of TNF-b production. *J Exp Med* 173:209.

Morelli, J.G., Norris, D.A. (1993). Influence of inflammatory mediators and cytokines on human melanocyte function. *J Invest Dermatol*. 100: 191-195.

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 Res*. 15:87-92.

Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, *et al*(2002). Functional SNPs in the lymphotoxinalpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 32:650.

Panchanathan, R. and Choubey, D. (2012). Murine BAFF expression is up-regulated by estrogen and interferons: Implications for sex bias in the development of autoimmunity. *Mol Immunol. 53*:15-23.

Pociot F, Briant L, Jongeneel CV, Mo¨lvig J, Worsaae H, Abbal M, Thomsen M, Nerup J, Cambon-Thomsen A(1993).Association of tumor necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-a and TNF-b by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. *Eur J Immunol* 23:224.

Reimann E, Kingo K, Karelson M, Reemann P, Loite U, Sulakatko H, Keermann M, Raud K, Abram K, Vasar E, Silm H, Koks S(2012). The mRNA expression profile of cytokines connected to the regulation of melanocyte functioning in vitiligo skin biopsy samples and peripheral blood mononuclear cells. *Human Immunology* 73: 393-398.

Shi, Y.Y. and He, L (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res*. 15 :97-98.

Smerdel-Ramoya A, Finholt C, Lilleby V, Gilboe IM, Harbo HF, Maslinski S, *et al*.(2005). Systemic lupus erythematosus and the extended major histocompatibility complex—evidence for several predisposing loci. *Rheumatology* (Oxford) 44:1368–73.

Smith C A(1994). The TNF receptor super family of cellular and viral proteins-activation, co stimulation and death, *Cell* 76: 959–962.

Steinman, L (1995). Escape from 'horror autotoxicus': pathogenesis and treatment of autoimmune disease. *Cell* 80 :7–10.

Vassalli P (1992). The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411.

Villar J. (2002). Genetics and the pathogenesis of adult respiratory distress syndrome. *CurrOp Crit Care* 8:1–5.

Wajkowicz-Kalijska, A., van den Wijngaard, R.M., Tigges, B.J., Westerhof, W., Ogg, G.S., Cerundolo, V., Storkus, W.J. and Das, P.K(2003). Immunopolarization of CD4+ and CD8+ T cells to Type-1-like is associated with melanocyte loss in human vitiligo. *Lab Invest 83*:683-695.

Whichelow CE, Hitman GA, Raafat I, Bottazzo GF, Sachs JA (1996). The effect of TNF*B gene polymorphism on TNF-alpha and –beta secretion level in patients with insulin dependent diabetes mellitus and healthy controls. *Eur J Immunogenet* 23:425–35.

Whitacre, C.C (2001). Sex differences in autoimmune disease. *Nat. Immunol*. 2 :777-780.

Wilson A, Symons J, McDowell TL, McDevitt HO, Duff GW (1997).Effects of a polymorphism in the human tumor necrosis factor-a promoter on transcriptional activation. *Proc Natl Acad Sci U S A* 94:3195e9.

Yagi H, Tokura Y, Furukawa F, Takigawa M. Vitiligo with raised inflammatory borders (1997). Involvement of T cell immunity and keratinocytes expressing MHC class II and ICAM-1 molecules. *Eur J Dermatol Clin* 7:19–22.

Yamada A, Ichihara S, Murase Y, *et al* (2004). Lack of association of polymorphisms of the lymphotoxin alpha gene with myocardial infarction in Japanese. *J Mol Med* 82:477- 83.

Yohn, J.J., Critelli, M., Lyons, M.B. and Norris, D.A (1990). Modulation of melanocyte intercellular adhesion molecule-1 by immune cytokines. *J Invest Dermatol*. 90 :233-237.

Z. Qin, T. Blankenstein, Tumor growth inhibition mediated by lymphotoxin(1995). Evidence of B lymphocyte involvement in the anti-tumor response.*Cancer Res.* 55 : 4747–4751.

Zhang J, Ai R, Chow F (1997). The polymorphisms of HLA-DR and TNF B loci in northern Chinese Han nationality and susceptibility to systemic lupus erythematosus. *Chin Med Sci J* 12:107.

Zuniga J, Vargas-Alarcon G, Hernandez-Pacheco G, Portal- Celhay C, Yamamoto-Furusho JK, Granados J (2001). Tumor necrosis factor-alpha promoter polymorphisms in Mexican patients with systemic lupus erythematosus (SLE). *Genes Immun* 2:363.

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 IL-2. IL-2 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 IL-1, IFN-γ and TNF-α 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 TNF-α cause maturation of dendritic cells and thus results in development of autoimmunity. The increased TNF-α 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.

TNF*α* 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.

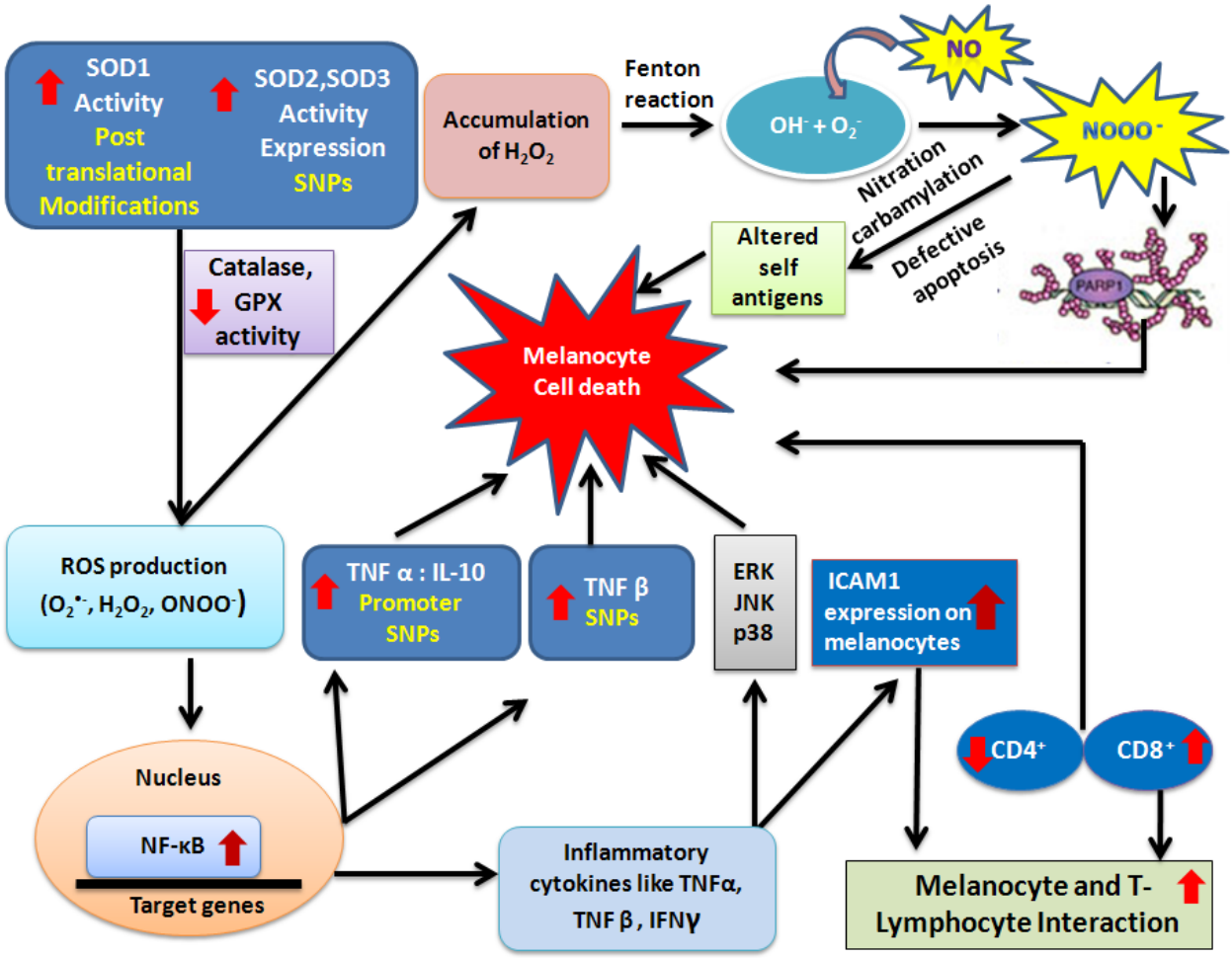


Figure 1. Possible molecular and cellular events responsible for melanocyte destruction in vitiligo:

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.

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):
    _____

15. Associated diseases:

_____

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16. Family history
    A. Ist degree relatives: father/mother/sister/brother/daughter/son
    B. 2nd degree relatives: paternal grandmother/paternal grandfather/ maternal grandmother/maternal grandfather/maternal or paternal uncles or aunts
    C. 3rd 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:

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

4. Treatment:
   _____
   _____
   _____

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

Signature

Date:

List of Publications

Imran M*, **Laddha NC***, Dwivedi M, Mansuri MS, Singh J, Rani R, Gokhale RS, Sharma VK, Marfatia YS and Begum R (2012). Interleukin-4 genetic variants correlate with Its transcript and protein levels in vitiligo patients. ***Brit J Dermatol.*** 167: 314-323. (*Equal contribution)

Dwivedi M, **Laddha NC**, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) in isolated vitiligo: a genotype-phenotype correlation. ***Pigment Cell Melanoma Res.*** 24: 737-740.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, **Laddha NC**, Dwivedi M, Begum R, The Indian Genome Variation Consortium, Gokhale RS and Rani R (2012). HLA alleles and amino acid signatures of the peptide binding pockets of HLA molecules in Vitiligo. ***J Invest. Dermatol.*** 132: 124-134.

Dwivedi M, Gupta K, Gulla KC, **Laddha NC**, Hajela K and Begum R (2009). Lack of genetic association of promoter and structural variants of mannan-binding lectin (*MBL*-2) gene with susceptibility to generalized vitiligo. ***Brit J Dermatol.*** 161: 63-69.

Dwivedi M*, **Laddha NC***, Shajil EM, Shah BJ and Begum R (2008). The *ACE* gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population. ***Pigment Cell Melanoma Res.*** 21: 407-408. (*Equal contribution)

Laddha NC, Dwivedi M, Shajil EM, Prajapati H, Marfatia YS and Begum R (2008). Association of *PTPN*22 1858C/T polymorphism with vitiligo susceptibility in Gujarat population. ***J Dermatol Sci.*** 49: 260-262.

Shajil EM, **Laddha NC**, Chatterjee S, Gani AR , Malek RA , Shah BJ 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 Res.*** 20: 405-407.

Manuscripts under Communication

Laddha NC, Dwivedi M, Mansuri MS, Ansarullah, Ramachandran AV, Dalai S and Begum R. Vitiligo: interplay between oxidative stress and immune system.

Laddha NC, Dwivedi M and Begum R. Increased Tumor Necrosis Factor (*TNF*)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo.

Dwivedi M, **Laddha NC**, Mansuri MS, Shah K, Begum R. Correlation of increased *MYG*1 expression and its promoter polymorphism with disease progression and higher susceptibility in vitiligo patients.

Birlea SA, Ahmad FJ, Uddin RM, Ahmad S, Begum R, **Laddha NC**, Dwivedi M, Mansuri MS, Jin Y, Gowan K, Riccardi SL, Ben S, Fain PR and Spritz RA. Association of Generalized Vitiligo with HLA Class II Loci in Patients from the Indian Subcontinent.

Oral and Poster Presentations

Laddha NC, Dwivedi M, M. Imran, Shah K, Shah BJ and Begum R. "Role of TNF-α promoter polymorphisms in vitiligo susceptibility: a genotype-phenotype correlation." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Dwivedi M, **Laddha NC**, M. Imran, Shah K, Shah BJ and Begum R. "Role of interferon-γ polymorphisms in vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Mansuri MS, **Laddha NC**, Dwivedi M, Imran M, Agarwal N, Vasan K, Marfatia YS and Begum R. "Association of glutathione peroxidase 1 structural polymorphisms in relation to its activity and oxidative stress with vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Agarwal N, **Laddha NC**, Dwivedi M, Imran M, Mansuri MS , Patel HH , Shah AM , Marfatia YS and Begum R "Genetic polymorphisms of Neuropeptide-Y and Interleukin-1 beta may confer susceptibility to vitiligo." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Rani R, Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, **Laddha NC**, Dwivedi M, Begum R, Indian Genome Variation Consortium, Gokhale RS. "Functional implications of MHC associations in vitiligo." **37th Annual Meeting of The American Society for Histocompatibility and Immunogenetics (ASHI),** Sheraton New Orleans, October 17-21, 2011.

Laddha NC, Dwivedi M, Imran M, Gani AR, Patel KJ, Sinh MK, Nateshan N, Parmar SS and Begum R. "Genetic polymorphisms in promoter region of TNF-α gene may confer susceptibility to vitiligo in Gujarat population." 4th

Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

Dwivedi M, **Laddha NC**, Imran M, Ichhaporia V and Begum R. "Association of Cytotoxic T Lypmhocyte Associated Antigen-4 (*CTLA*-4) 3' UTR CT60A/G and exon1 +49A/G single nucleotide polymorphisms with vitiligo susceptibility." 4th Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

Imran M, **Laddha NC**, Dwivedi M, Parmar CB, Khan F, Raimalani VM, Panchal VN and Begum R. "Genetic polymorphisms -590 C/T and intron 3 indel in interleukin 4 gene are in association with vitiligo in Gujarat population." HGM, 2011. 4th Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

Dwivedi M, **Laddha NC**, Imran M, Nateshan N, Parmar SS, and Begum R. "Genetic association of functional and structural variants in *MYG*1 gene with vitiligo susceptibility." **International Symposium on "Molecular medicine" (MOLMED-2011)**, at CHARUSAT, Changa, Gujarat, India held on 9th -11th January, 2011.

Ichhaporia V, Dwivedi M, **Laddha NC**, Imran M, and Begum R. "To Study the genotypic association of the CTLA4 CT60A/G single nucleotide polymorphism with vitiligo susceptibility." Oral presentation at **Science Excellence-2011** (SCIXL-2011) the State level Paper Presentation Competition held on 8th January, 2011 at Gujarat University, Ahmedabad.

Laddha NC, Dwivedi M, Gani AR, Patel KJ and Begum R. "Role of Tumor Necrosis Factor-α (TNF-α) and Mannan Binding Lectin (MBL) genes polymorphisms in Vitiligo susceptibility." Oral presentation at **National Conference on**

CME in Immunology, The M. S. University of Baroda, November 27-28, 2009. **(Awarded the First Prize)**

Imran M, **Laddha NC**, Shah AM, Panchal VN, Sinh MK, Parmar CB, Raimalani VM and Begum R. "Association of Interleukin-4 Intron 3 INDEL polymorphism with Vitiligo susceptibility in Gujarat population." **Oral presentation at National Conference on CME** in Immunology, The M. S. University of Baroda, November 27-28, 2009. **(Awarded the First Prize)**

Laddha NC, Dwivedi M, Gani AR, Patel KJ, Begum R. "Role of Tumor Necrosis Factor-α (TNF-α) and Mannan-binding lectin (MBL) genes polymorphisms in vitiligo susceptibility.") Poster presentation at International symposium on **"Advances in Molecular Medicine and Clinical Implications"**, January 24 and 25, 2009 at Reliance Institute of Life sciences. ***(Awarded the First Prize)***

Laddha NC, Dwivedi M, Gani AR, Patel KJ, Oza TG, Jain B, Begum R. "Association of TNF-α-308 and -238 promoter polymorphisms with vitiligo susceptibility in Gujarat population" Poster presentation at **13th Human Genome meeting "Genomics and the future of medicine"** at HICC, Hyderabad, India held on 27th-30th September, 2008.

Dwivedi M, Gupta K, Gulla KC, **Laddha NC**, Hajela K and Begum R. "Genetic association of promoter and structural gene variants of Mannan-Binding Lectin (MBL2) gene with susceptibility to vitiligo" Poster presentation at **13th Human Genome meeting "Genomics and the future of medicine"** at HICC, Hyderabad, India held on 27th-30th September, 2008.

Gani AR, Dwivedi M, **Laddha NC**, Shajil EM and Begum R. "*PTPN22* Single Nucleotide Polymorphism and *ACE* I/D Polymorphism in Gujarat vitiligo patients." Oral presentation at Science Excellence-2008 (SCIXL-2008) the State level Paper Presentation Competition held on 5th January 2008 at Gujarat University, Ahmedabad.. **(Awarded the First Prize)**

Shajil EM, Dwivedi M, **Laddha NC**, Thakker M and Begum R. "Evaluation of oxidative stress, autoimmune and neurochemical hypotheses of vitiligo in patients of Gujarat." Poster presentation at **National Syposium on "Apoptosis and Cancer"** held on 28-29th December, 2007 at Department of Biochemistry, The M. S. University of Baroda.