CHAPTER I

INTRODUCTION

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).

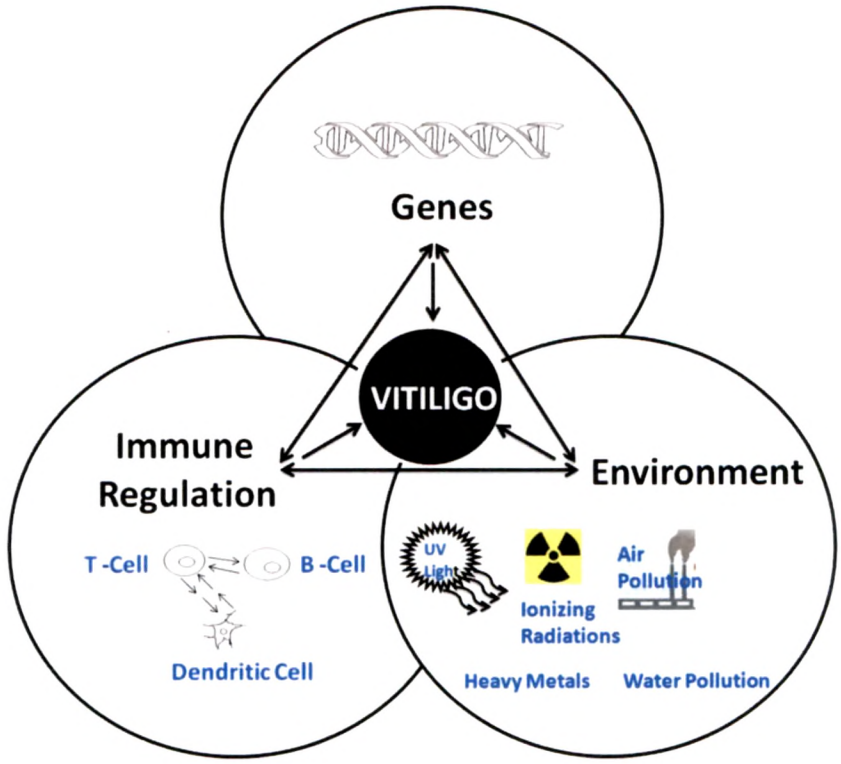


Figure 1. Interplay of genes, environment (ROS generation) and immune system in precipitation of Vitiligo.

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

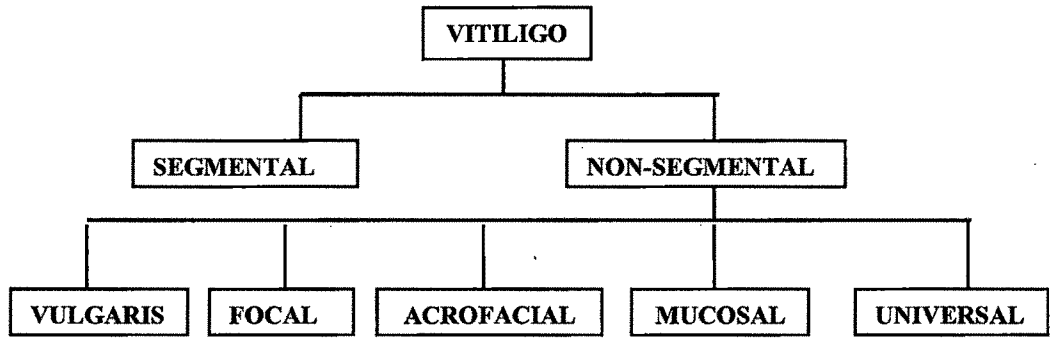
Vitiligo affects approximately 0.5 to 1% of the world population (Taieb *et al.,* 2007). The prevalence of the disease in United States has been estimated to be 1% (Lerner, 1971). In Denmark the prevalence is around 0.38% (Howitz *et al.,* 1977). The prevalence of vitiligo is estimated to be about 2% 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.

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 1. 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

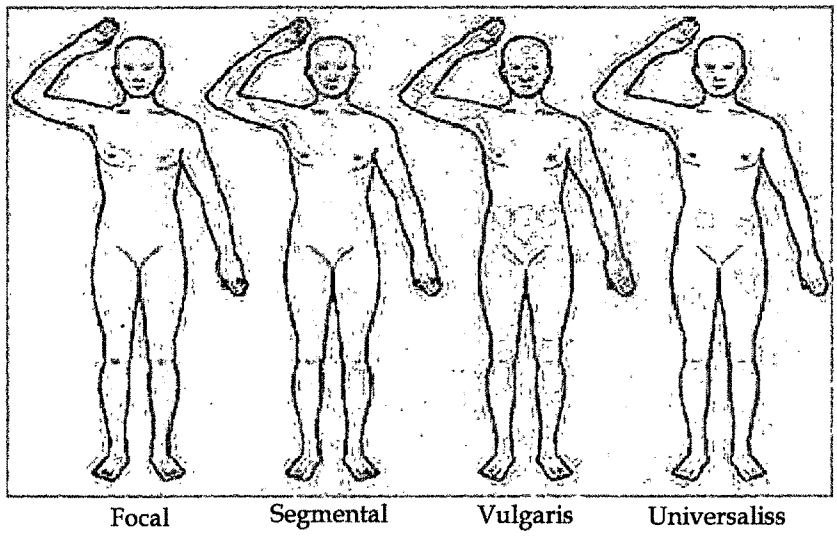


Figure 2. Clinical types of vitiligo

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).

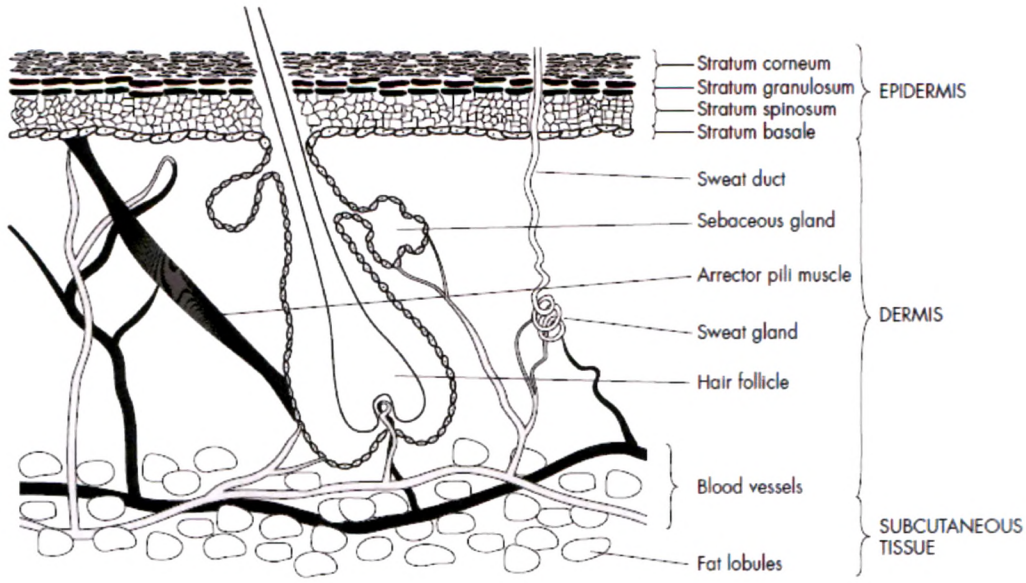


Figure 3. A diagrammatical cross-section of human skin

(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.

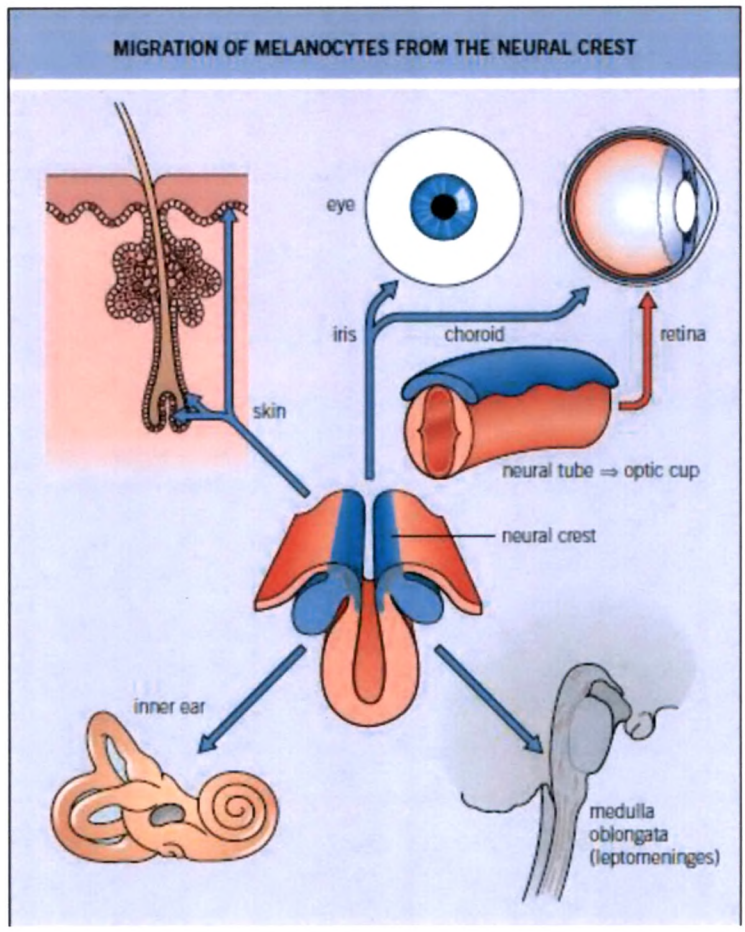


Figure 4. Migration of melanocytes from the neural crest: Melanocytes migrate to the uveal tract, the leptomeninges, and the cochlea, as well as the epidermis and hair follicle. The retina actually represents an outpouching of the neural tube (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

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 32 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit (Figure 5).

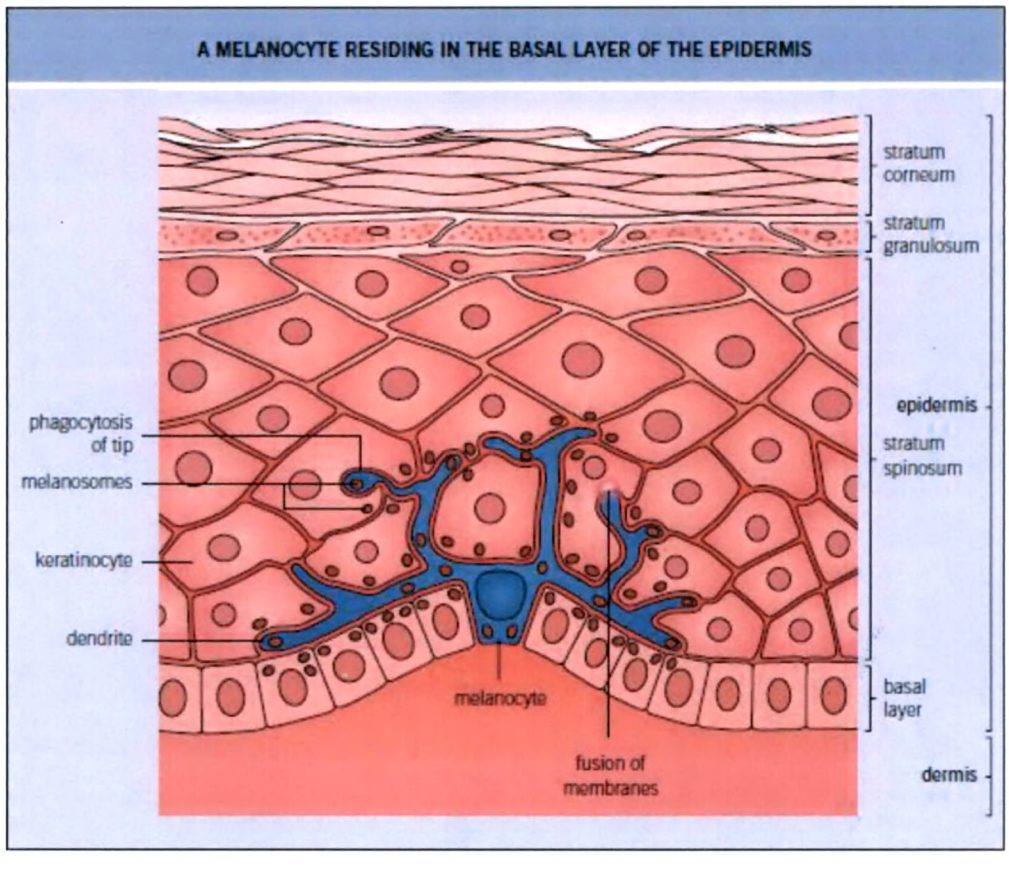


Figure 5. Epidermal Melanin unit showing a melanocyte residing in the basal layer of the epidermis: In normal skin, approximately every tenth cell in the basal layer is a melanocyte. Melanosomes are transferred from the dendrites of the melanocyte into neighboring keratinocytes of the epidermis, hair 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.

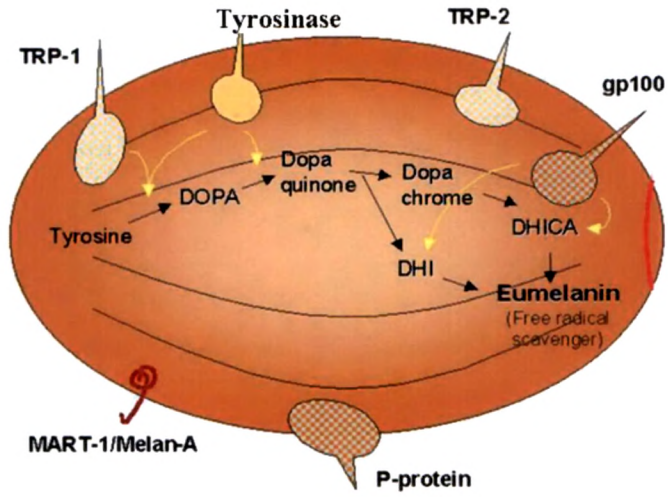


Figure 6. Structure of melanosome.

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.

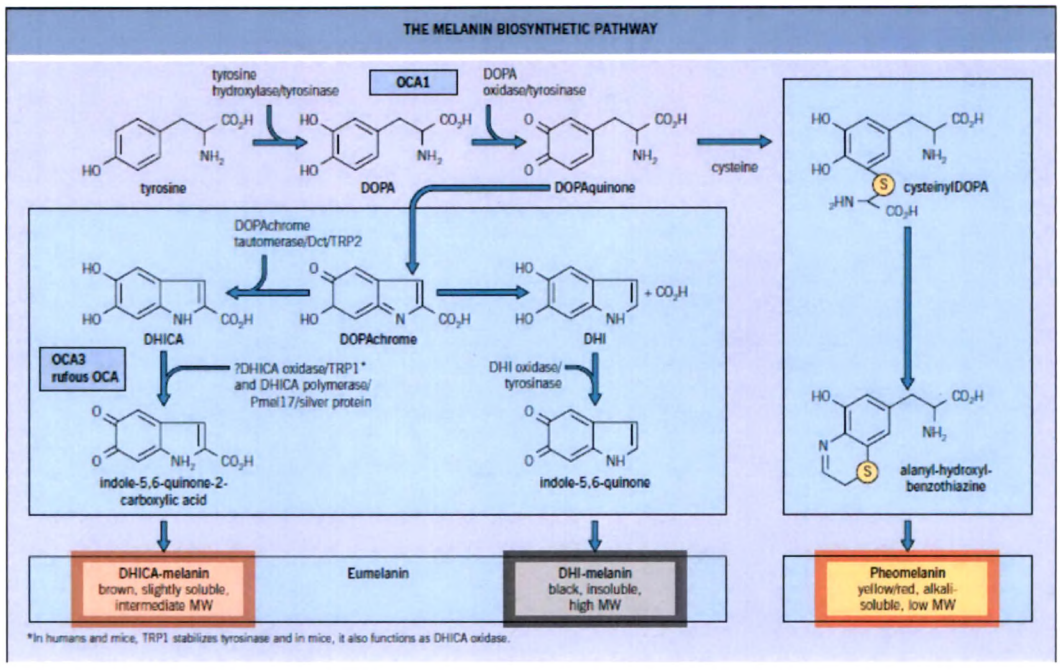


Figure 7. The melanin biosynthetic pathway: The pathway includes demonstration of the sites of dysfunction in OCA1 (tyrosinase), OCA3 (TRP1), and rufous OCA (TRP1). The two major forms of melanin in the skin and hair are brown–black eumelanin and yellow–red pheomelanin. DHI, 5,6-dihydroxyindole; DHICA, 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.

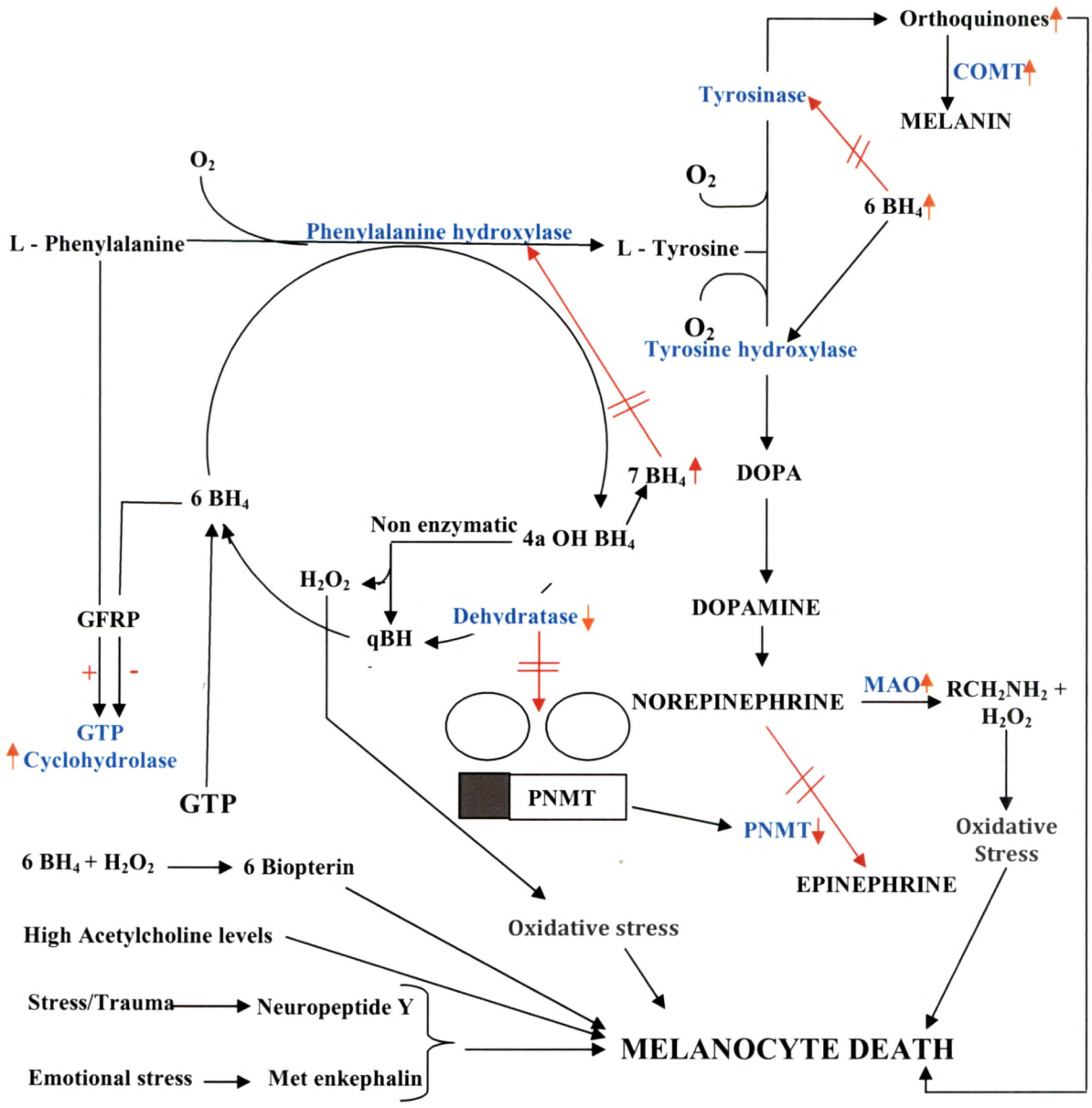


Figure 1. The events that support the neurochemical pathogenesis of vitiligo.

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).

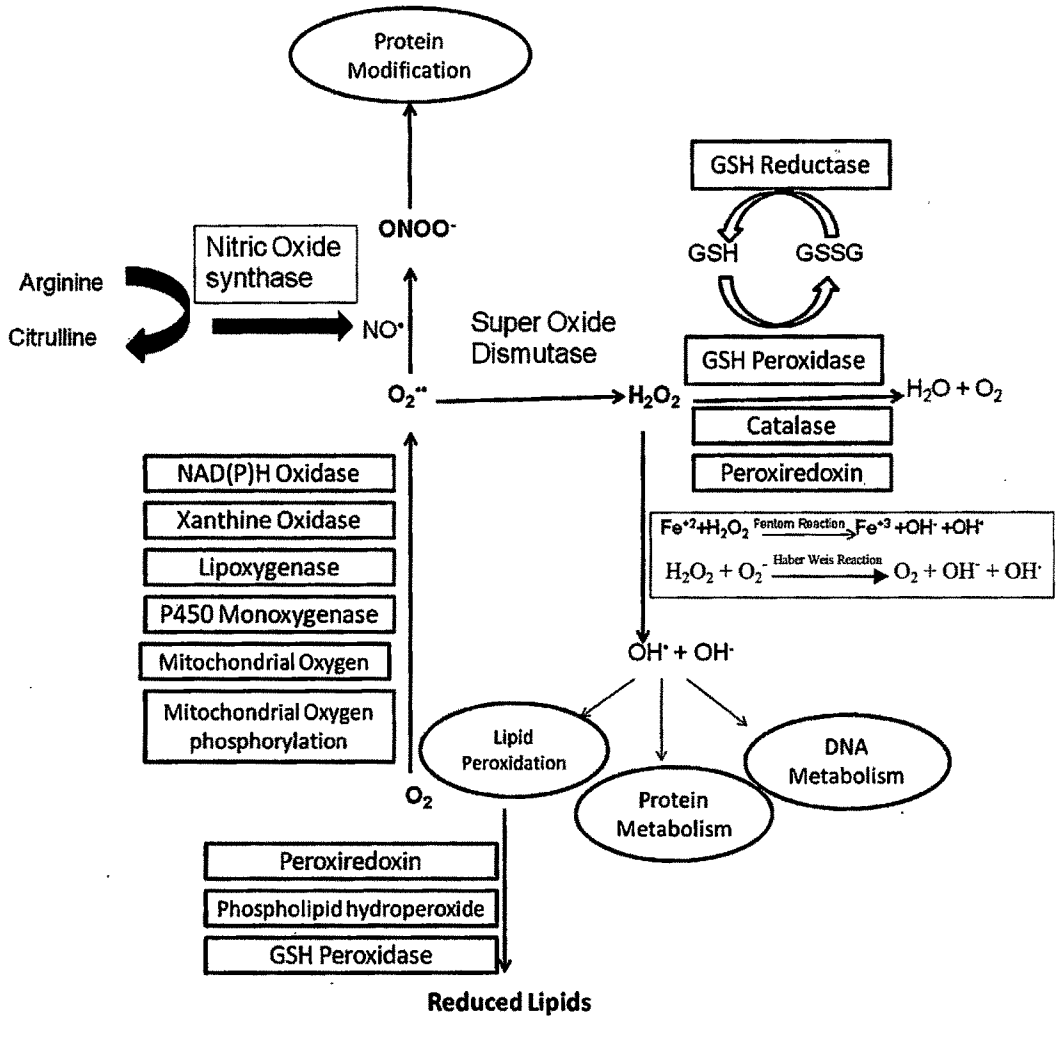


Figure 9. Intracellular generation of ROS.

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 2. 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 3. 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 :

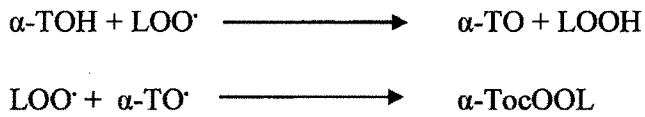
Glutathione (γ-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the living system. Glutathione often 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·) 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·) 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).



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).

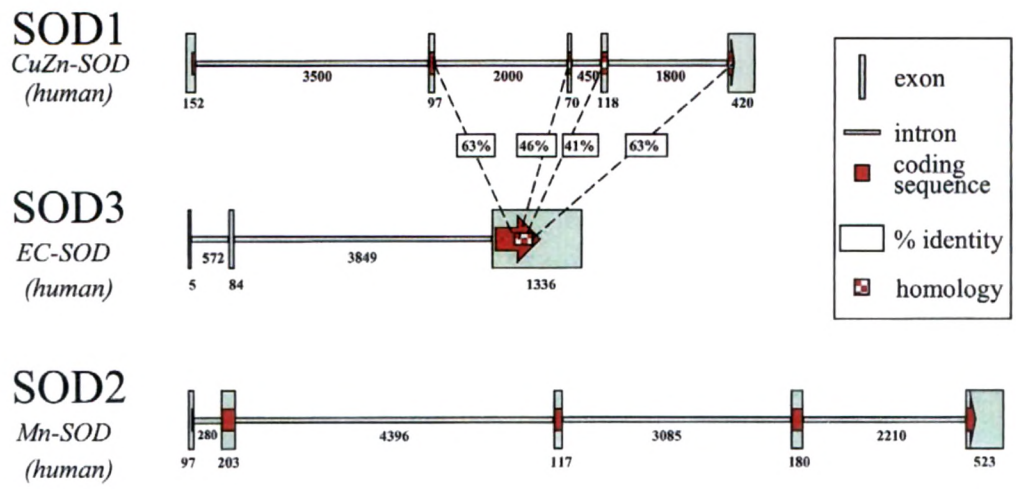


Figure 10. Genomic organization of the three known members of the human *SOD* family. *SOD3* was placed in the middle in order to demonstrate areas of amino acid sequence homology between *SOD1* and *SOD3*. *SOD2* has no significant amino acid sequence homology with either *SOD1* or *SOD3*. The size 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 4. 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.

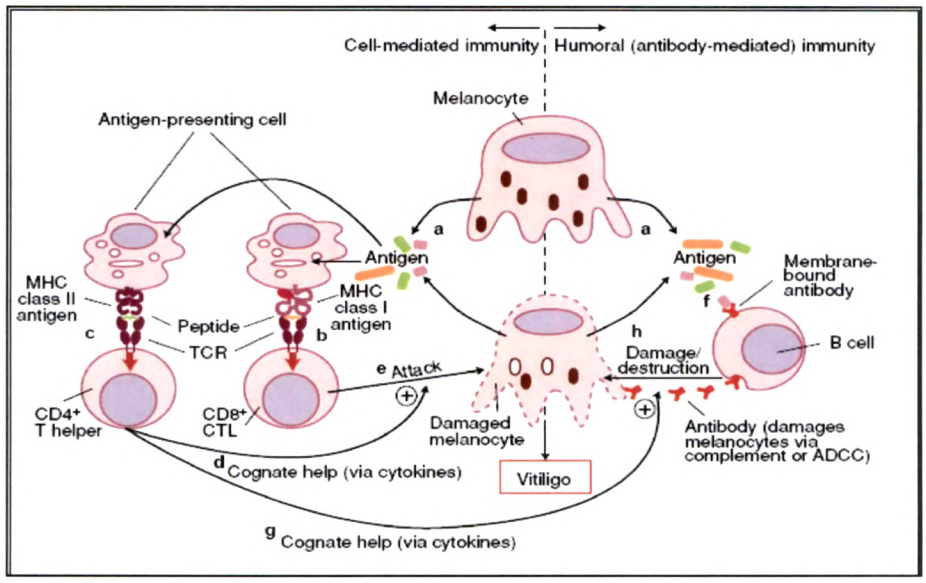

Figure 11. Possible cellular and humoral immune mechanisms in vitiligo.
(Kemp *et al.*, 2001)

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.

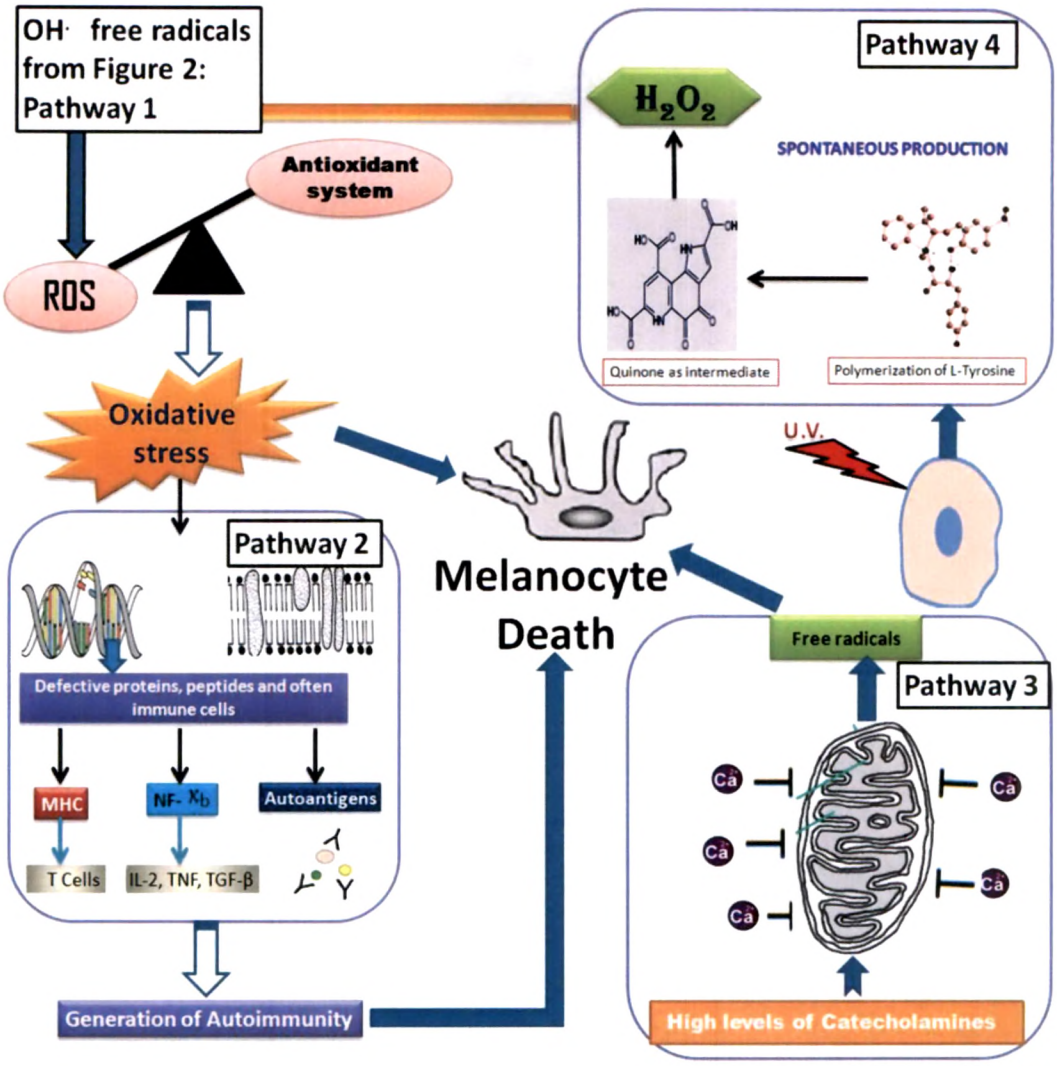

Figure 12.Different pathways for melanocyte destruction:

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 TNFα and IFNγ 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, TNFα and minimal TGFβ in tissue, and increased IL6 but normal IL1β, IL8 and TNFα 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 TNFα, IFNγ and IL10, and detected increased IL1, IL6, IL8 and TNFα (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 IFNα, TNFα 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).

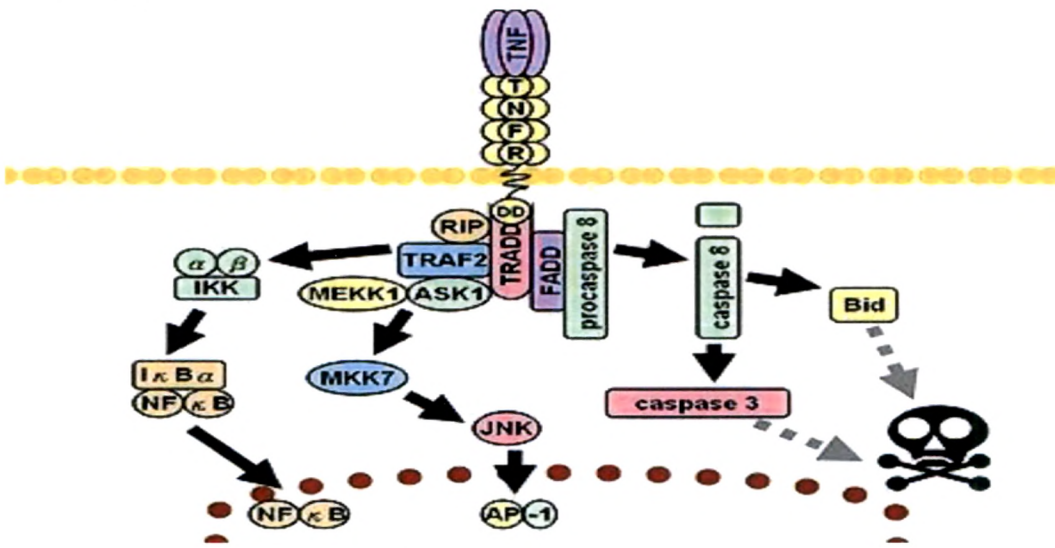

Figure 13.Mechanism of action of TNFα.

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

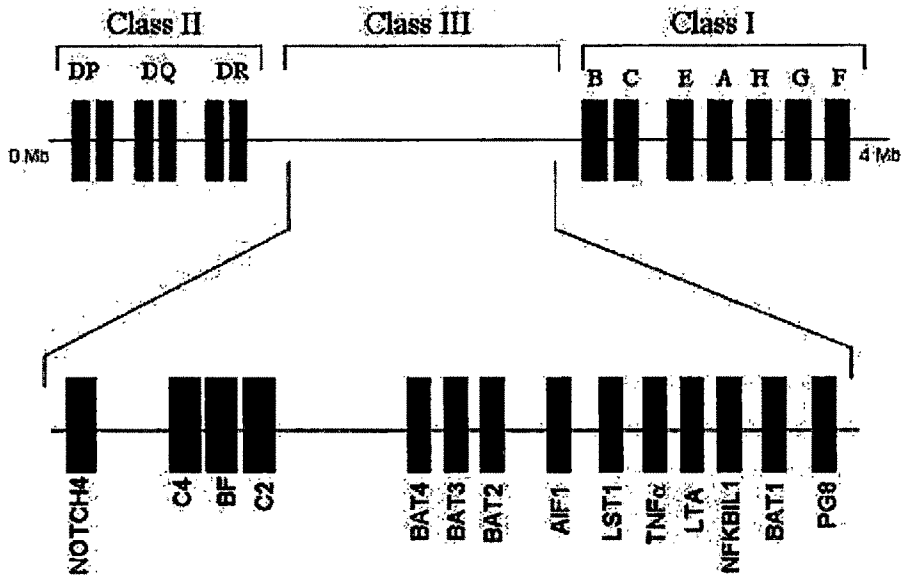

Figure 14. HLA region on chromosome 6q21: highlighting the different genes present within the HLA class III region that could be contributing to disease association.

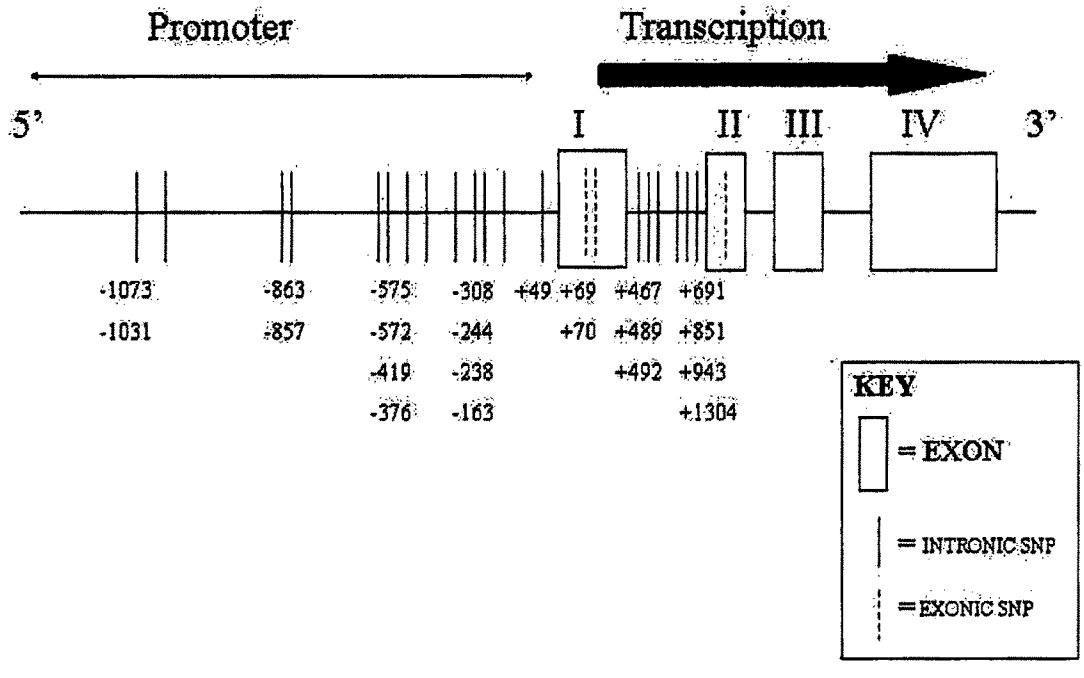

Figure 15. *TNFA* gene structure and known polymorphims.

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 5. 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 *MCIR* 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 *FOXD3*

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 *CD4*

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 *CLEC11A*

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 *FBXO11-MSH6*

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 *FOXP3*

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 *GSTT1*

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 6. 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 7. 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.

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