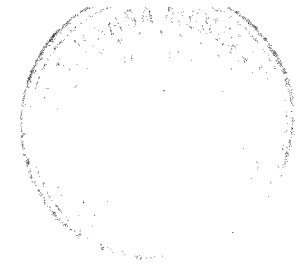


# CHAPTER I

# INTRODUCTION

Vitiligo is an acquired, idiopathic, hypomelanotic disease characterized by circumscribed depigmented macules (Ortonne and Bose 1993). The absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo (Le Poole et al 1993). The lesions may be progressive and may develop at any age, however in many cases the onset is reported in the second decade of the life coinciding with major hormonal changes. (Lerner 1959; Liu et al 2005). The disease appears most commonly on hands, feet, arms, face and lips although it can begin from any part of the body (Nordlund et al 1998). Vitiligo generally leads to psychological and social embarrassment particularly in brown and black people. The etiology of vitiligo is still unknown, but 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).

## I. GENERAL ASPECTS OF VITILIGO

**1.1. Historical References:** The earliest reports on patchy skin diseases that may be interpreted as today's vitiligo dates back to approximately 1500 BC.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 1-4% of the world population (Ortonne et al 1993). The prevalence of the disease in United States is 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). The Gujarat and Rajasthan states have the highest prevalence i.e. around 8.8% (Valia and Datta 1996).

**1.3. Psychological Effects:** Vitiligo carries social stigma in India (Handa and Kaur 1999), and affected persons and family particularly girls are socially ostracized for marital purpose (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).

**1.4. Types of Vitiligo:** Vitiligo is classified according to the distribution, pattern and extent of depigmentation. There are many reports on classification however, most investigators distinguished two subtypes of vitiligo; segmental vitiligo (SV) and non-segmental vitiligo (NSV) (Taieb 2000) as shown in Table 1.

**Table 1. Clinical subtypes of vitiligo**

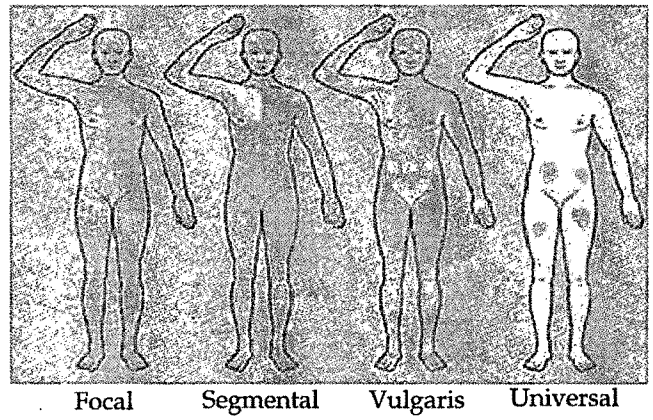
| Segmental vitiligo | Non segmental vitiligo |
|---|---|
| Begins often in the childhood | Later onset |
| Autoimmunity rare | Autoimmunity associated |
| Frequently facial | Trauma prone sites and koebnerisation |
| Stable results after autologus grafting | Unstable results after autologus grafting |
| Dermatomal, unilateral distribution | Non dermatomal, bilateral distribution |

According to another classification by Nordlund and Lerner (1982) based on the distribution and extension of lesions three types are identified i.e. localized, generalized and universal vitiligo (Table 2) (Nordlund and Lerner 1982). Localized vitiligo is further classified as shown in Table 2 into focal and segmental; generalized into acrofacial, vulgaris and mixed subtypes. Different clinical types of vitiligo are shown in Figure 1.

**Table 2. Clinical classification of vitiligo**

| Localized | | Generalized | | | Universal |
|---|---|---|---|---|---|
| Focal | Segmental | Acrofacial | Vulgaris | Mixed | |
| One or more patches in one area but not in segmental pattern | 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 | Involves more than 80 % of the body |

**Figure 1. Clinical types of vitiligo**



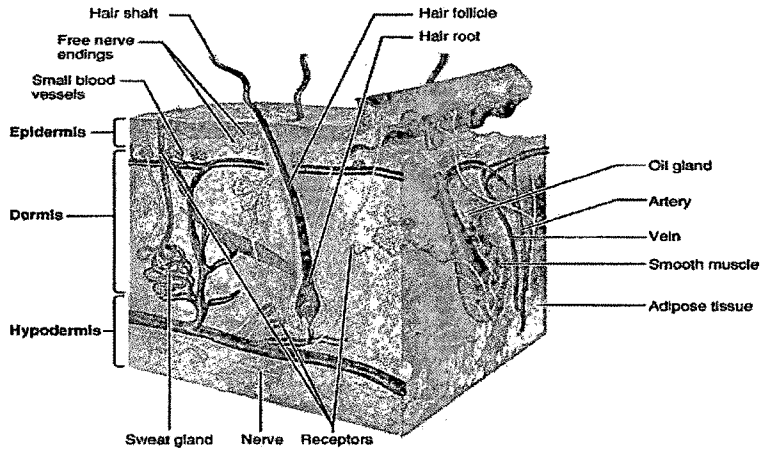
Focal Segmental Vulgaris Universal

**1.5. Genetics of Vitiligo:** The prevalence of familial cases of vitiligo varies from 6.25% - 38% in population worldwide (Ortonne and Bose 1993). About 20% of vitiligo patients have at least one first degree relative with vitiligo. The relative risk of vitiligo for the first-degree relatives of patients is increased by at least 7-10 fold (Nath et al 1994). The inheritance of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. A non Mendelian (complex) trait results from multiple genes acting in concert with each other and with environmental factors. Vitiligo seems to be a complex hereditary disease governed by a set of recessive alleles which may govern oxidative stress, melanin synthesis, autoimmunity etc. occurring at several unlinked autosomal loci that collectively confer the vitiligo phenotype (Majumder et al 1993; Nath et al 1994).

## 1.6. THE SKIN

**1.6.1. Structure of the Skin:** The skin is the largest body organ and functions as a metabolically active biological barrier separating internal homeostasis from the external environment. Depending on anatomic localization and environmental influences, the skin shows remarkable functional and structural diversity (Slominski and Wortsman 2000). 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 that secrete collagen, and a range of supporting structures, including blood vessels, inflammatory cells, nerves and ground substance (Figure 2). 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, the latter are bone marrow derived professional macrophages (Rees 2003)

**Figure 2. Structure of skin**




**1.6.2. Colour of the skin:** Skin color is primarily due to the presence of a pigment called melanin. Both light and dark complexioned people have this pigment. Under normal conditions it is not the number of melanocytes in the skin that determine the degree of pigmentation but their level of activity.

Although, there are regional variations in the density of epidermal melanocytes, their numbers are consistent even in different skin types and ethnic groups (Tsatmali et al 2002). Skin color is also affected by red cells in blood flowing close to the skin. To a lesser extent, the color is affected by the fat under the skin and carotene a reddish-orange pigment in the skin.

**1.6.3. Melanocytes:** The melanocytes are neural crest derived cells that migrate via mesenchyme into the epidermis and hair follicles during embryogenesis (Bolognia and Orlow, 2003). Apart form the skin, melanocytes are known to be present in other areas such as retinal pigment epithelium, uveal tract, inner ear and leptomeninges (Figure 3).

**Figure 3. Embryonic origin of melanocyte populations**

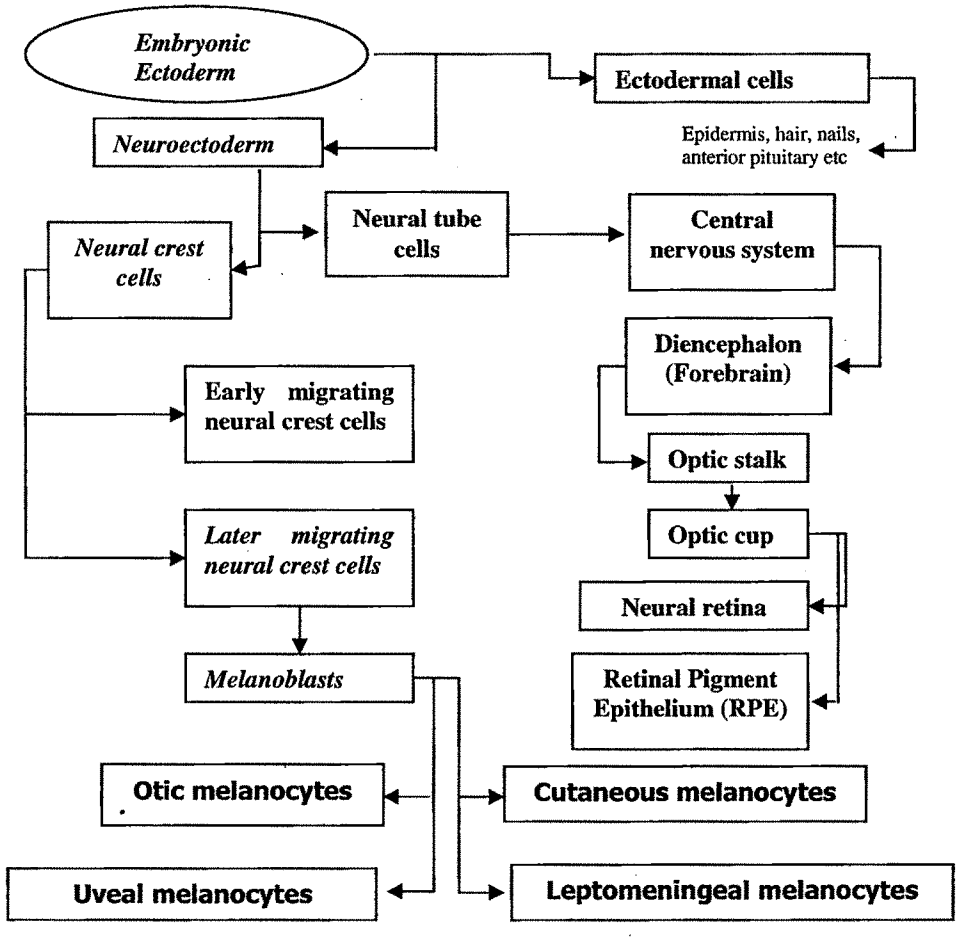
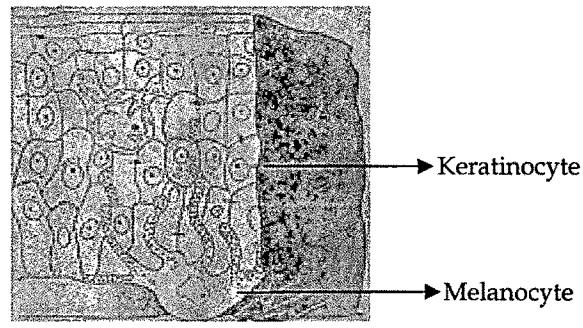



**Figure 3.** Embryonic origin of human melanocyte populations. Neural ectoderm differentiates to form the neural plate and neural fold. Upon closure of the neural fold, neural crest cells migrate through dorso-lateral pathways to form the peripheral nervous system, adrenal medulla, craniofacial structures, cardiac structures, and various melanocyte subpopulations. Melanocyte progenitor cell populations are shown in italics.

In the skin they reside in the basal layer of the epidermis and in the matrix of the hair follicle. They derive from the melanoblasts that originate from the neural crest from where they migrate during embryogenesis. Melanocytes are highly dendritic and these dendrites project into the malphigian layer of the epidermis where they transfer the melanosomes to keratinocytes (Jimbow et al 1999). Each epidermal melanocyte secretes melanosomes into approximately 36 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit (Figure 4).

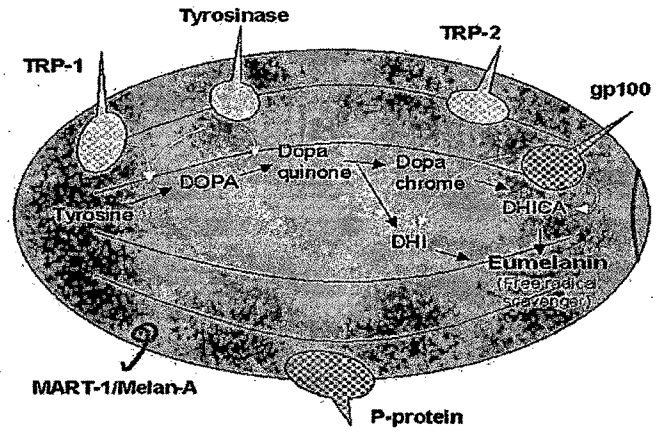
**Figure 4. Epidermal melanin unit**




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

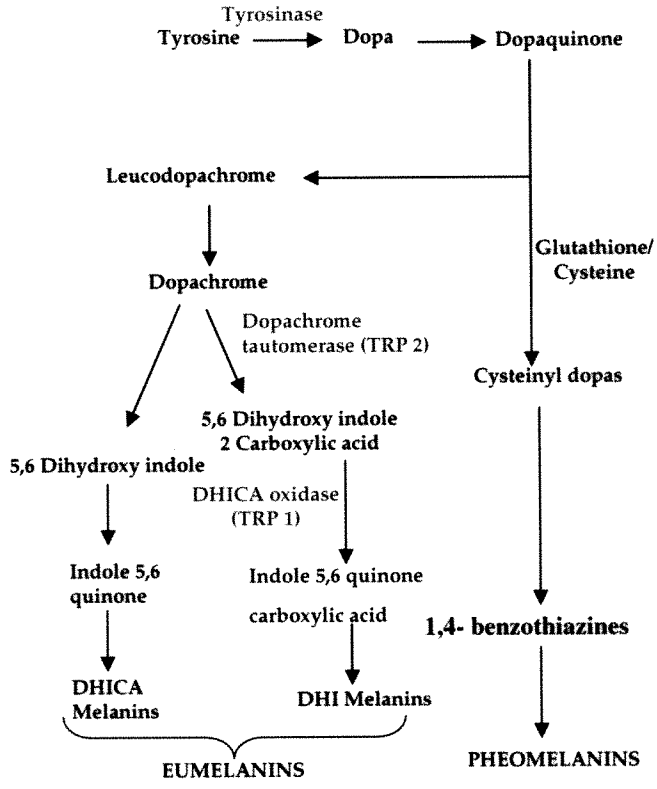
processing of Pmel 17, which in turn regulates the maturation of melanosomes (Hoashi et al 2005). The structure of melanosome is shown in Figure 5.

**Figure 5. Structure of melanosome**




Melanin synthesis, a multi step process takes place in melanosomes (Hearing 1999). Two major forms of melanin are produced in melanocytes i.e. brown black eumelanin and yellow red pheomelanin. The major function of melanin is attributed to be photo protection to the skin from the ionizing radiations (Hearing 2000). 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 dopaquinone stage. Cysteine/glutathione reacts with the dopaquinone to produce cysteinyldopas which may undergo further cyclisation to benzothiazines and higher condensates give rise to pheomelanins (Hearing 1999). The different steps of melanin production are shown in Figure 6.

**Figure 6. Melanin synthesis pathway**




## 1.7. ETIOPATHOGENESIS OF VITILIGO

Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated. The three main prevailing theories of pathogenesis of vitiligo are the neurochemical hypothesis, autoimmune hypothesis and oxidative stress hypothesis but none of them can explain the entire spectrum of this disorder.

### 1.7.1. 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

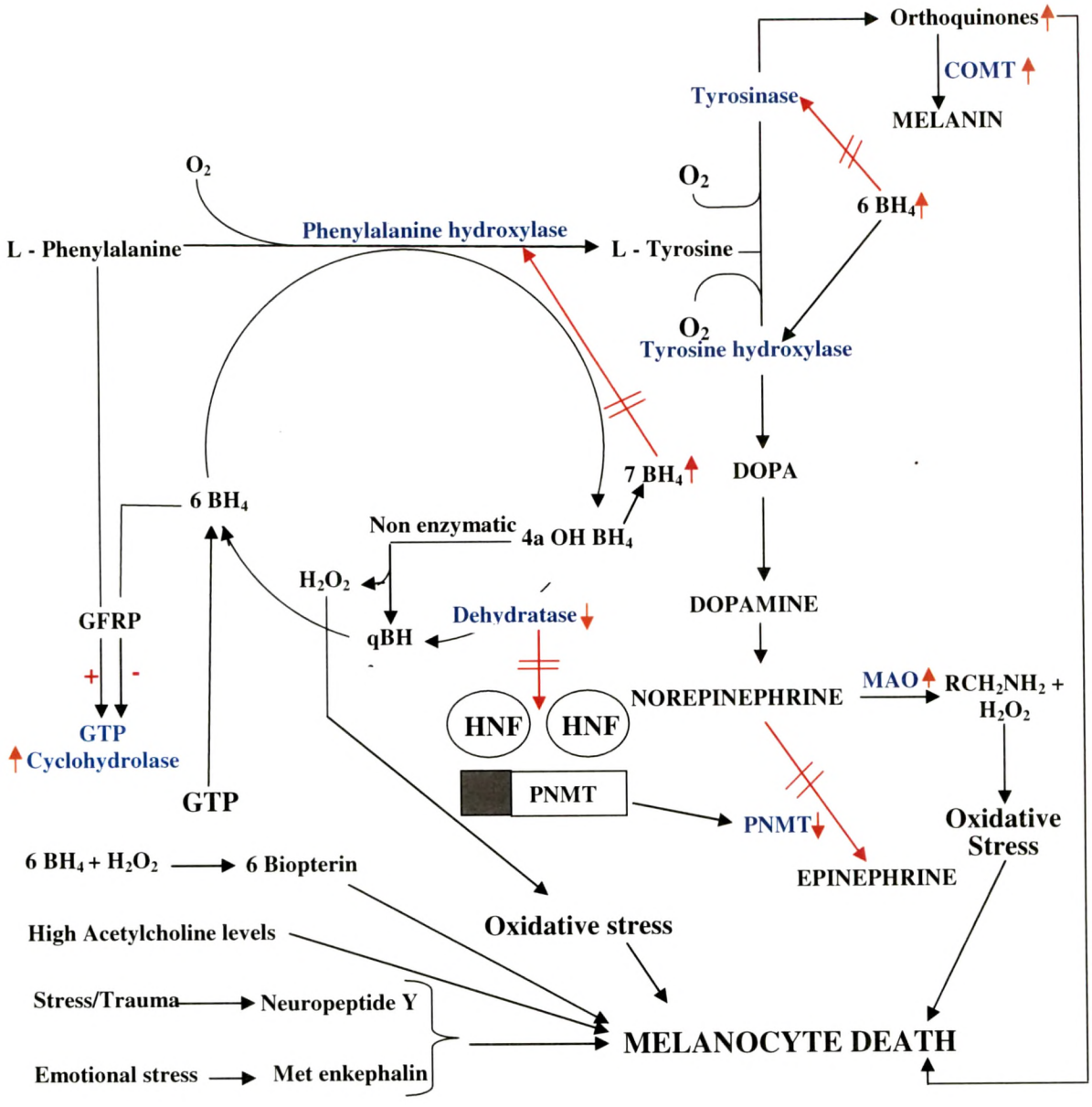
vitiliginous skin during depigmentation (Iyengar 1989). Different studies showed significantly higher levels of plasma and urinary catecholamines and their metabolites in vitiligo patients especially at the onset and in the active stage of the disease (Morrone et al 1992; Orecchia et al 1994; Cucchi et al 2000; Cucchi et al 2003). Increased catecholamine synthesis is observed with the disease activity suggesting a role for catecholamines in the process of depigmentation (Cucchi et al 2000). High levels of norepinephrine and its metabolites in vitiligo is related to decreased phenylethanolamine-N-methyl transferase (PNMT) activity and an increased activity of tyrosine hydroxylase (TH), which plays a key role in the catecholamine synthesis pathway, producing L-dopa form L-tyrosine (Schallreuter et al 1994). The rate limiting cofactor/electron donor for TH is (6R)-5,6,7,8-tetrahydribiopterin (6-BH4), which is increased due to a decreased 4a-hydroxy-6BH4 dehydratase (DH) activity (Schallreuter et al 2001) in vitiligo patients. There is a defective recycling of 6BH4 which leads to increased non-enzymatic production of 7BH4, 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). Moreover, the 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). In vitiligo, 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. 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 a role for the nervous system 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, which plays a role in the 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 and 6.5 - fold less epinephrine than control keratinocytes (Schallreuter et al 1996a). Norepinephrine is reported to be toxic to melanocytes.

A derangement of the enzymes involved in catabolism of adrenergic transmitters namely catechol O methyl transferase (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. Toxic products may be damaging melanocytes because of their low turn over rate (Le Poole et al 1994). The events that support the neurochemical pathogenesis of vitiligo are shown in Figure 7.

Figure 7. The events that support the neurochemical pathogenesis of vitiligo



### 1.7.2. Oxidative Stress Hypothesis

**1.7.2.1. 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 the oxidative stress, which has been implicated in the pathogenesis of several diseases.

**1.7.2.2. Reactive oxygen species (ROS):** Oxygen is vital for the aerobic life process. However about 5% or more of the inhaled $O_2$ is converted to reactive oxygen species (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 such as proteins, carbohydrates, lipids and DNA. In the sequential univalent process by which $O_2$ undergoes reduction, several reactive intermediates are formed such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and extremely reactive hydroxyl radical ($^{\cdot}OH$). These three are collectively known as ROS. The process can be represented as

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {}^{\cdot}OH \xrightarrow{e^-} H_2O$$

ROS can be produced by both endogenous and exognous sources. Potential endogenous sources include oxidative phosphorylation, cytochome P450 metabolism, peroxisomes and inflammatory cell activation.

**1.7.2.3. Sites of generation:** During mitochondrial oxidative metabolism, the majority of the $O_2$ consumed is reduced to water. However around 4-5% of the molecular oxygen is converted into ROS primarily superoxide anion which is formed by an initial one electron reduction of molecular oxygen (Klaunig and Kamendulis 2004). The source of mitochondrial ROS appears to involve a non-heme iron protein that transfers electron to oxygen. These occur at Complex I (NADH Coenzyme Q) and to a lesser extent, following the autooxidation of coenzyme Q from the Complex II (Succinate Coenzyme Q) and/or Complex III (Coenzyme $QH_2$-Cytochrome c reductases) sites. The precise contribution of each site to total mitochondrial ROS production is determined by local conditions including chemical or physical damage to mitochondria, oxygen availability and the presence of xenobiotics (Kehrer 2000). Superoxide can be dismutated by superoxide dismutase to yield $H_2O_2$ (Barber and Harris 1994). In the presence of partially reduced metal ions, in particular iron, $H_2O_2$ is subsequently converted through Fenton and Haber-Weiss reactions to a hydroxyl radical (Betteridge 2000). The hydroxyl radical is highly reactive and can interact with nucleic acid, lipids and proteins (Betteridge 2000).

$$Fe^{3+} + O^{2-} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \quad \text{(Fenton Reaction)}$$

$$O^{2-} + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \quad \text{(Haber-Weiss Reaction)}$$

In cytosol ROS may be formed by enzymes with peroxidase activity, such as cytochrome P450, which sequentially transfers two electrons from NADPH to bound molecular oxygen (Guengerich and Lieber 1985; Poulos and Raag 1992) or by dioxygenases, such as cyclooxygenases and lipoxygenases. In the course of electron transfer, some of the activated oxygen is released as superoxide and/or $H_2O_2$. For e.g. cytochrome P450 2E1 is involved in the oxygenation of substrates such as ethanol, and is capable of generating a prolonged burst of reactive oxygen species near the site of substrate oxidation (Ekstrom and Ingleman-Sundberg 1989).

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 due to the participation of peroxisomal oxidases, flavoproteins, D amino acid oxidase, L hydroxy acid oxidase and fatty acyl 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).

Reactive oxygen species can be produced by exogenous processes also. Environmental agents including nongenotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in 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). The endogenous and exogenous sources of ROS are summarized in Table 3.

**Table 3. Endogenous and exogenous sources of ROS**

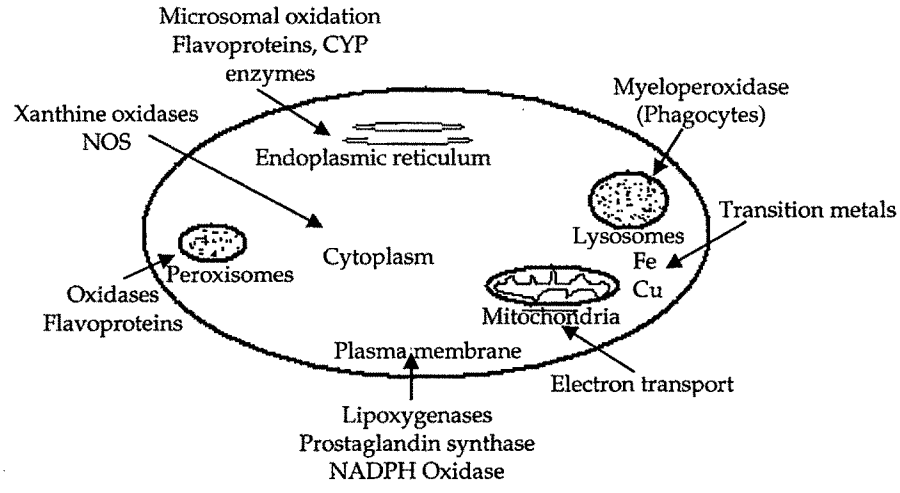
| Cellular oxidants | Source | Oxidative species |
|---|---|---|
| Endogenous | Mitochondria | $O_2^-$, $H_2O_2$, $^{\cdot}OH$ |
| | Cytochrome P450 | $O_2^-$, $H_2O_2$ |
| | Macrophage/Inflammatory cells | $O_2^-$, $\cdot NO$, $H_2O_2$, $OCl^-$ |
| | Peroxisomes | $H_2O_2$ |
| Exogenous | Redox cycling compounds | $O_2^-$ |
| | Metals (Fenton reaction) | $\cdot OH$ |
| | Radiation | $\cdot OH$ |

**1.7.2.4. Reactive nitrogen species (RNS):** The NO radical ($NO\cdot$) is produced in higher organisms by the oxidation of one of the terminal guanidonitrogen atoms of L-arginine. This process is catalyzed by the enzyme nitric oxide synthase. The target molecules of $NO\cdot$ are intracellular thiols, metal containing proteins and low molecular weight thiols like glutathione and cysteine etc (Irshad and Chaudhury 2002). Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation ($NO^+$), nitroxyl anion ($NO^-$) or peroxynitrite ($ONOO^-$). Peroxynitrite is a powerful oxidant that interacts with a wide range of targets to cause tyrosine nitration, thiol oxidation, lipid peroxidation, DNA strand break, guanosine nitration/oxidation and ultimately cell death (Irshad and Chaudhury 2002). The reaction of peroxinitrite with excess NO generates $NO_2$, which can combine with more NO to form $N_2O_3$ to cause nitrosative stress (Walker et al 2001).

To counteract the harmful effects of ROS and RNS, cellular antioxidant defense mechanism operates to detoxify or scavenge these reactive species. When overall generation of ROS and RNS exceeds than the total antioxidant activity in the body, the resulting condition is called oxidative stress. This oxidative stress may be mild or severe. The overproduction of ROS or breakdown of antioxidant system is the causative factor for the generation of

oxidative stress, which is linked to a number of clinical disease states including vitiligo. The endogenous sources of ROS and RNS are given in the Figure 8.

**Figure 8. Endogenous sources of ROS and RNS**




**1.7.2.5. 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.

**1.7.2.5.1. Superoxide dismutase (SOD) (EC 1.15.1.1)**

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. SOD I (CuZn SOD, cytoplasmic), SOD 2 (Mn SOD, mitochondrial), SOD 3 (CuZn 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).

**1.7.2.5.2. Catalase (CAT) (EC 1.11.1.6)**

Catalase is present in the peroxisomes of nearly all aerobic cells and serves to protect 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). The *CAT* gene is located on human chromosome 11 at 11p13. The *CAT* gene is 34 kb in length and contains 12 introns and 13 exons and encodes for a protein of 526 amino acids (Bell et al 1986; Quan et al 1986). The protein exists as a tetramer of four identical subunits (220 to 350 kDa) and each monomer contains a heme prosthetic group at the catalytic center. Catalase monomers also contain one

tightly bound NADP per subunit. This NADP may serve to protect the enzyme from oxidation by its $H_2O_2$ substrate (Eventoff et al 1976).

**1.7.2.5.3. Glutathione peroxidase (GPX) (EC 1.11.1.9)**

The *GPX* gene is located on human chromosome 3 at location 3p21.3. It is monomeric enzyme that contains one selenium atom at the active site as selenocysteine. The molecular weight of GPX is 18 kDa (Ursini et al 1985). Glutathione peroxidase catalyzes the reduction of various organic hydroperoxides, as well as hydrogen peroxide, with glutathione as hydrogen donor. Four distinct species of glutathione peroxidase have been identified in mammals to date, the classical cellular enzyme, the phospholipid hydroperoxide metabolizing enzyme, the gastrointestinal tract enzyme and the extracellular plasma enzyme. The cytosolic from of GPX (GPX 1) is the first and best characterized selenoprotein (Arthur 2000). This selenium dependent enzyme is ubiquitously expressed and detoxifies both hydrogen and lipid peroxides (Arthur 2000). 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

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

Glutathione peroxidase can directly reduce phospholipids and cholesterol hydroperoxides in cellular membranes and thus this enzyme plays a major role in protecting cells against the damaging effect of lipid peroxidation.

**1.7.2.5.4. Glutathione reductase (GR) (EC.1.8.1.7)**

Glutathione reductase (GR) is a ubiquitous enzyme associated with the hexose monophosphate shunt of glucose metabolism. It catalyses the 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

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

**1.7.2.5.5. Glutathione *S* transferase (GST) (EC.2.5.1.18)**

Glutathione *S* transferases (GSTs) are a super family of enzymes. In humans, there are at least 13 GST enzymes belonging to five families, namely α (GSTA), μ (GSTM), π (GSTP), σ (GSTS) and θ (GSTT) (Habig et al 1974).

Glutathione transferases play 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

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

**1.7.2.5.6. Glucose 6 phosphate dehydrogenase (G6PDH) (1.1.1.49)**

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. It is required to make NADPH in red blood cells but not in other 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 GST is given below

Glucose 6-phosphate + $NADP^+$ ⟶ Glucono-1, 5-lactone 6-phosphate + NADPH + $H^+$

**1.7.2.5.7. Reduced glutathione (GSH)**

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. It is the most abundant non protein thiol compound in mammalian cell. 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 the cell and outside, 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.

Glutathione is an essential cofactor for antioxidant enzymes, namely the GSH peroxidases (both Se-dependent and non-Se-dependent forms exist) and the more recently described phospholipid hydroperoxide GSH peroxidases. The GSH peroxidases serve to detoxify peroxides (hydrogen peroxide, other peroxides) in the water-phase, by reacting them with GSH; the latter enzymes use GSH to detoxify peroxides generated in the cell membranes and other lipophilic cell phases. Enzymes collectively known as GSH transhydrogenases use GSH as a cofactor to reconvert dehydroascorbate to ascorbate, ribonucleotides to deoxyribonucleotides, and for a variety of S-S to S-H inter-conversions.

**1.7.2.5.8. 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 step 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)

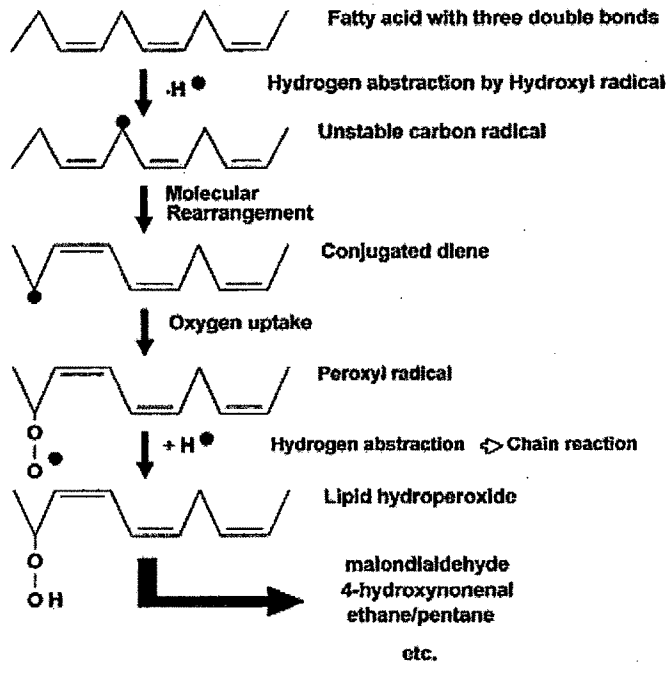
$$\alpha\,TOH + LOO\cdot \longrightarrow \alpha\,TO + LOOH$$

$$LOO\cdot + \alpha\,TO\cdot \longrightarrow \alpha\,TocOOL$$

### 1.7.2.5.9. Lipid peroxidation

Oxygen radicals catalyze the oxidative modification of lipids (Gardner 1989). This peroxidation reaction is given in the Figure 9. The presence of double bond adjacent to a methylene group makes the methylene C-H bonds of polyunsaturated fatty acids (PUFA) weaker and therefore the hydrogen becomes more prone to abstraction. Lipid peroxidation is initiated by ·OH, alkoxy radicals (RO·) and peroxy radicals (ROO·) (Turrens and Boveris 1980). This can lead to a self-perpetuating process since peroxy radicals are both reaction initiators as well as the products of lipid peroxidation. Lipid peroxy radicals react with other lipids, proteins and nucleic acids; propagating thereby the transfer of electrons and bringing about the oxidation of substrates. All membranes, which are structurally made up of large amounts of PUFA, are highly susceptible to oxidative attack, which results in the changes in membrane fluidity, permeability, and cellular metabolic functions. The mechanism of lipid peroxidation is given in the Figure 9.

**Figure 9. Lipid peroxidation mechanism**



Oxidative stress has been suggested to be the initial pathogenic event in the melanocyte destruction (Schallreuter et al 1999a; Maresca et al 1997), with $H_2O_2$ accumulation in the epidermis of the patients with vitiligo (Schallreuter et al 1999a; Schallreuter et al 2001). Defective recycling of tetrahydrobiopterin in vitiligo epidermis is associated with the intracellular production of $H_2O_2$ (Figure 7) (Schallreuter et al 1994; Schallreuter et al 1999b). In addition, an alteration in the antioxidant pattern, with a significant reduction in the catalase activity has been demonstrated in both lesional and non-lesional epidermis of patients (Schallreuter et al 1991) as well as in the melanocytes (Maresca et al 1997). The antioxidant imbalance in the peripheral blood mononuclear cells of active vitiligo patients also has been observed. It was correlated to an increased intracellular production of reactive oxygen species and appeared to be due to mitochondrial impairment (Dell'Anna et al 2001). These findings support the concept of a possible systemic oxidative stress in vitiligo.

The inhibition of thioredoxin reductase, a free radical scavenger located in the membrane of melanocytes also contributes to oxidative stress generation in the epidermis of vitiligo epidermis (Schallreuter and Pittelkow 1988). Higher extracellular calcium levels cause increased superoxide radicals that lead to inhibition of tyrosinase (Schallreuter et al 1996b). Several sources for the unusual epidermal $H_2O_2$ production /accumulation have been documented (Passi et al 1998; Schallreuter et al 1991; 1996a; 1999a; Rokos et al 2002). Moreover, millimolar levels of $H_2O_2$ are reported to result in the inactivation of catalase (Aronoff 1965; Schallreuter et al 1991; Dell'Anna et al 2001) and reduced glutathione peroxidase activity (Beazley et al 1999; Agrawal et al 2004). 4 α carbinolamine dehydratase is also found to be inhibited by higher concentration of $H_2O_2$ which disrupts the recycling of the essential cofactor (6R)- L – erythro- 5,6,7,8 tetrahydrobiopterin ($6BH_4$) for the aromatic amino acid hydroxylases as well as the nitric oxide synthases (Hasse et al 2004). Table 4 summarizes the sources of $H_2O_2$ documented to date in vitiligo. Giovannelli et al (2004) showed a significantly higher level of oxidative DNA damage in

mononuclear leukocytes in active vitiligo patients compared to controls. (Giovannelli et al 2004).

**Table 4. Sources for epidermal/systemic $H_2O_2$ generation/accumulation in vitiligo**

| Source and Reference | $H_2O_2$ generation/ Accumulation | Increase/ Decrease |
|---|---|---|
| Monoamine Oxidase A (Schallreuter et al 1996a) | Epidermal | Increase |
| Superoxide dismutase (Agrawal et al 2004, Hazneci et al 2005) | Blood | Increase |
| Glucose 6 phosphate dehydrogenase (Agrawal et al 2004) | Blood | Decrease |
| NADPH Oxidase (Schallreuter et al 1999b) | Epidermal | Increase |
| Photoxidation of pterins (Rokos et al 2002) | Epidermal | Increase |
| Nitric oxide synthases (Gibson and Liley 1997) | Epidermal | Increase |
| Short circuit in 6BH4 recycling (Schallreuter et al 1994 d; Kaufman et al 1997) | Epidermal | Increase |
| Catalase (Dell'Anna et al 2001; 2003; Schallreuter et al 1991; Maresca et al 1997) | Blood and Epidermal | Decrease |
| Glutathione peroxidase/Reduced glutathione (Beazley et al 1999; Dell'Anna et al 2001; Agrawal et al 2004; Yildirim et al 2003) | Blood | Decrease |
| Tyrosinase related protein 1 (Jimbow et al 2001) | Epidermal | Decrease |
| Xanthine oxidase (Koca et al 2004) | Blood | Increase |

**1.7.2.6. Enzymatic and non-enzymatic antioxidant levels in vitiligo:** The major enzymatic and non-enzymatic antioxidant levels reported in vitiligo till now are summarized in the tables given below.

**Table 5. SOD levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Hazneci et al 2005 | Erythrocyte | Increase |
| Yildirim et al 2004 | Tissue | Increase |
| Koca et al 2004 | Serum | Decrease |
| Yildirim et al 2003 | Erythrocyte | Increase |
| Dell'Anna et al 2001 | Erythrocyte | No change |
| Dell'Anna et al 2001 | PBMC | Increase |
| Passi et al 1998 | Epidermis | No change |
| Maresca et al 1997 | Cultured melanocytes of normal skin of vitiligo and controls | No change |
| Chakraborty et al 1996 | Serum | Increase |
| Picardo et al 1994 | Erythrocyte | No change |
| Ines et al 2006 | Erythrocyte | Increase |

**Table 6. Catalase levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Hazneci et al 2005 | Erythrocyte | No change |
| Dell'Anna et al 2001 | Erythrocyte | No change |
| Dell'Anna et al 2001 | PBMC | Decrease |
| Passi et al 1998 | Epidermis | Decrease |
| Masreca et al 1997 | Cultured melanocytes of normal skin of vitiligo and controls | Decrease |
| Schallreuter et al 1991 | Epidermis (Suction blister roofs) | Decrease |
| Ines et al 2006 | Erythrocyte | No change |

**Table 7. Glutathione peroxidase levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Hazneci et al 2005 | Erythrocyte | No change |
| Yildirim et al 2004 | Tissue | Increase |
| Yildirim et al 2003 | Erythrocyte | Decrease |
| Passi et al 1998 | Epidermis | No change |
| Picardo et al 1994 | Blood | No change |
| Beazley et al 1999 | Blood | Decrease |
| Ines et al 2006 | Erythrocyte | Decrease |

**Table 8. GSH levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Dell'Anna et al 2001 | Erythrocyte | No change |
| Dell'Anna et al 2001 | PBMC | Decrease |
| Passi et al 1998 | Epidermis | Decrease |
| Picardo et al 1994 | Blood | No change |

**Table 9. LPO levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Ines et al 2006 | Plasma | Increase |
| Koca et al 2004 | Serum | Increase |
| Yildirim et al | Tissue | Increase |
| Picardo et al 1994 | Blood | No change |
| Yildirim et al 2003 | Serum | Increase |
| Passi et al 1998 | Epidermis | Increase |

**Table 10. Vitamin E levels in vitiligo**

| Reference | Blood/ Epidermis | Increase/ Decrease |
|---|---|---|
| Picardo et al 1994 | Blood | No change |
| Dell'Anna et al 2001 | Blood | No change |
| Ines et al 2006 | Plasma | No change |

### 1.7.3. Autoimmune Hypothesis

The basis of autoimmune hypothesis is developed from the studies that demonstrated an association between vitiligo and other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata. In addition, circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients. A recent study performed on 2624 vitiligo probands from North America and UK confirmed the significant increase of frequencies of six autoimmune disorders in vitiligo probands and their first degree relatives: vitiligo itself, autoimmune thyroid disease (particularly hypothyroidism), pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease (Alkhateeb et al 2003).

These associations indicate that vitiligo shares common genetic etiologic link with these other autoimmune diseases (Passeron and Ortonne 2005).

**1.7.3.1. 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 11. A correlation is seen between the level of melanocyte antibodies and disease activity in vitiligo (Harning et al 1991). Also the 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). According to Kemp et al (1998a), the low frequencies of autoantibodies to tyrosinase and Pmel 17 reflect their minor role in autoimmune response in vitiligo (Kemp et al 1998a). Furthermore, MelanA/Mart 1 a melanosomal autoantigen is not a target in the autoantibody response of vitiligo patients (Waterman et al 2002). Recently a surface receptor, melanin concentrating hormone receptor 1 (MCHR 1) was detected as an autoantibody target in 16% vitiligo sera.

**Table 11: 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 al 2002 |
| MCHR 1 | Waterman et al 2002 |
| SOX 9 | Hedstrand et al 2001 |
| SOX 10 | Hedstrand et al 2001 |

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).

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). 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).

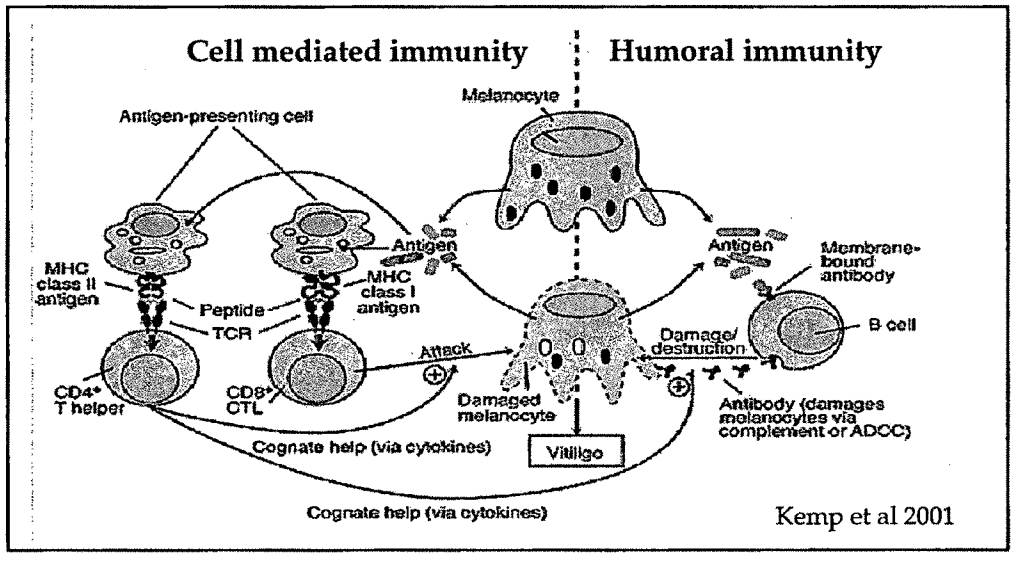
**1.7.3.2. Cell mediated immunity:** Histopathological investigations of the perilesional skin of vitiligo suggested the involvement of lymphocytes in the depigmentation process. Immunohistochemical studies have confirmed the presence of infiltrating T cells (Le Poole et al 1996). T cell infiltrates 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). Autoimmune diseases are often associated with an expansion of peripheral CD4+ T cells (Stites et al 1994). In vitiligo an increase in the number of CD4+ cells and an elevated CD4+/CD8+ ratio is reported in patients with stable vitiligo and their first-degree relatives (Venneker et al 1993; Soubiran et al 1985; D'Amelio et al 1990). By contrast, a decrease in the CD4+ T cell population along with a reduced CD4+/CD8+ ratio is also 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) has 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, Melan-A/Mart 1 specific CD8+ T were identified in inflammatory lesions of melanocyte destruction following infusion of Melan-A/Mart 1 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 of 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; Hợrn et al 1997; Von Den Driesch et al 1992). In parallel, and supposedly 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 IL-6 and IL-8 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 (SIL-2R) 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 the Figure 10.

**Figure 10. Possible cellular and humoral immune mechanism in vitiligo**



### 1.8. Oxidative Stress and the Immune System in Vitiligo Pathogenesis

There is an interplay between the regulation of 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.

Reactive oxygen species 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 1999a). 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 antioxidant enzymes, are responsible for the generation of reactive oxygen species 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 the 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.

**1.9. 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 IL I, IFN gamma or tumor necrosis factor α (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 this hypothesis (Moretti et al 2002). Birol et al (2006) has demonstrated that the level of cytokines IL 1 α 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 IL 1α promoting B cell differentiation and immunoglobulin production. TNFα induce cell surface ICAM I on melanocytes which is necessary for leucocyte- melanocyte attachment. ICAM I 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. However a study using TUNEL assay in marginal skin of patients with active vitiligo did not demonstrate the presence of apoptosis (Boisseau-Garsuad et al

2001). Indirect evidence for the role of apoptosis comes from the expression of granzyme and perforin in the inflammatory infiltrate neighboring the melanocytic remnants in perilesional skin (Wijngaard et al 2000). 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).

**1.10. 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 (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

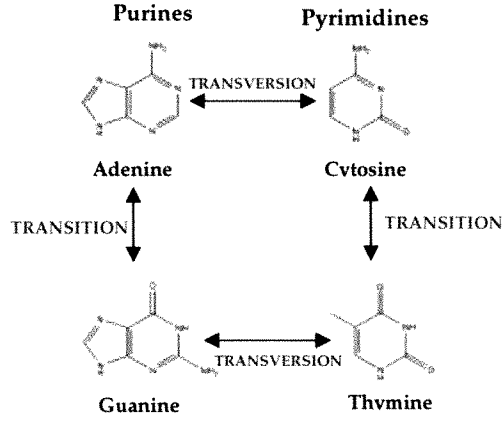
external and internal agents differently (UV, cytokines, catechols, melanin intermediates, growth factor withdrawal) usually ineffective on cell activity and survival. The impaired arrangement of the lipids, involving fatty acids and cholesterol, may affect the transmembrane housing of proteins with enzymatic or receptor activities. The altered expression and release of transmembrane proteins could be 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).

### 1.11. 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 kilobases (http://www.ncbi.nlm.nih.gov/SNP/snp summary.cgi). SNPs may occur in non-coding regions as well as in coding regions. There are two types of SNPs i.e. transitional and transversional. Nucleotide changes between purine and pyrimidine bases are transversions, while same class changes (purine to purine or pyrimidine to pyrimidine) are called transitions (Figure 11). Modification of a nucleotide within the coding region of a gene may be synonymous, resulting in no amino acid change or non-synonymous resulting in the production of a

different residue (missense) or premature termination (nonsense) of the polypeptide chain. The precise impact of a missense SNP can be equally variable, depending upon the physico-chemical properties of the residue and the functional and/or structural importance of the residue in the resulting protein. 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.

**Figure 11. Types of SNPs**




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).

**1.11.1. SNP analysis:** SNP analysis techniques fall into two distinct classes

- SNP Identification: Detection of novel polymorphisms
- SNP Genotyping: Identifying specific allele in a known population

**1.11.1.1. SNP IDENTIFICATION:** 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).

**1.11.1.1.1. 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).

**1.11.1.1.2. 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).

**1.11.1.1.3. 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.

**1.11.1.1.4. 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).

**1.11.1.2. 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.

### 1.11.2. SNP BASED ASSOCIATION STUDIES:

SNP based studies can be performed in two ways:

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

**1.12. 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 et al 2005). A few genes that are reported to contribute to vitiligo susceptibility are given in the Table 12.

**Table 12. Genes that contribute to vitiligo susceptibility**

| Gene | Reference |
|---|---|
| *AIRE* | Nagamine et al 1997 |
| *CTLA4* | Kemp et al 1999 |
| *CAT* | Casp et al 2002, Gavalas et al 2006, Park et al 2006. |
| *COMT* | Tursen et al 2002 |
| *LMP* and *TAP* | Casp et al 2003 |
| *MC1R* and *ASIP* | Na et al 2003 |
| *ACE* | Jin et al 2004a |
| *ESR1* | Jin et al 2004b |
| *PTPN 22* | Canton et al 2005 |
| *KIT* | Kitamura et al 2004 |
| *FOXD3* | Alkhateeb et al 2005 |

**1.12.1.** ***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; The Finnish–German APECED 1997)

**1.12.2.** ***CTLA 4:*** *CTLA 4* is considered as a candidate gene as it contributes to the development of T cell mediated autoimmune disease and its expression or function is adversely affected by the mutations or polymorphic alleles. Studies suggest that vitiligo at least when not associated with an autoimmune disorder is not influenced by the *CTLA 4* microsatellite polymorphism (Kemp et al 1999; Blomhoff et al 2005).

**1.12.3.** ***Catalase***: Catalase converts hydrogen peroxide to water and thereby prevents the cell damage from highly reactive oxygen derived radicals. The catalase gene is considered as a candidate gene because of the reduction of 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 catalase 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 catalase gene may contribute to a quantitative deficiency of catalase activity in vitiligo patients and the accumulation of $H_2O_2$. Other allelic variants in the catalase gene are given in Table 13.

**Table 13. Allelic variants in the catalase gene in vitiligo**

| Allele variants | Significant/ Non significant Association | Reference |
|---|---|---|
| Silent substitution<br>Codon 389 in exon 9<br>GAC (Asp) → GAT (Asp) | Significant<br><br>Non significant | Casp et al 2002,<br>Gavalas et al 2006<br>Park et al 2006 |
| Missense mutation<br>Codon 354 in exon 9<br>CGC (Arg) → CAC (His) | Non significant | Gavalas et al 2006 |
| Silent substitution<br>Codon 419 in exon 10<br>CTG (Leu) → TTG (Leu) | Non significant | Gavalas et al 2006 |
| Splice site mutation<br>Intron 7 position 5<br>G → T transition | Non significant | Gavalas et al 2006 |
| Frame shift mutation<br>Exon 2 position 79<br>G insertion | Non significant | Gavalas et al 2006 |
| 5′ Non-coding region<br>Position -18 A → T<br>Position -20 C → A<br>Position -21 C → T | Non significant | Gavalas et al 2006 |
| 5′ Non-coding region<br>Position -262 C → T | Non significant | Gavalas et al 2006 |

**1.12.4.** ***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 higher 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).

**1.12.5.** ***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 antigen 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 a genetic association of early onset of vitiligo with the TAP 1 gene. Moreover alleles from heterozygous parents were disequillibratedly transmitted to affected offspring for the TAP 1 gene, as well as for the closely linked LMP 2 and LMP 7 genes (Casp et al 2003).

**1.12.6.** ***MC1R*** and ***ASIP:*** Polymorphism studies in *MCIR* and *ASIP* revealed that Val92Met (G274A) and Arg163Gln (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 SNP studies on *ASIP* showed that g.8818A>G was higher in vitiligo but it was also not statistically significant. The patients who carried both the SNPs of *MCIR* and *ASIP* were prone to vitiligo (Na et al 2003).

**1.12.7. Angiotensin Converting Enzyme Gene (*ACE*):** According to the neural hypothesis of vitiligo pathogenesis, 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).

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

**1.12.9. Lymphoid protein tyrosine phosphatase (*PTPN* 22):** PTPN 22 gene encodes lymphoid protein tyrosine phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill et al 2002). The missense R620W polymorphism in the PTPN22 gene at the nucleotide 1858 (1858 C> T) in 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 R620W polymorphism may have an influence on the development of generalized vitiligo, which further provides evidence for the autoimmunity as an etiological factor.

**1.12.10. *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 observed that a marked progression of vitiligo that 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). BCL 2 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 physiopahology of vitiligo.

**1.12.11. 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 2001). Mutations in *FOXD3* leading to elevated FOXD3 transcription is recently reported in one AIS 1 linked family (Alkhateeb et al 2005).

**1.12.12. TNFα:** TNFα expression may depend on polymorphisms in the *TNFa* promoter region or a linkage association with the HLA genotype (D'Alfonso et al 1994; Pociot et al 1993). In this respect, the -308 allele is associated with the HLA A1, B8, DR3, DR4 and the DQ2 haplotypes; the DR2 is associated with low TNFα responses; and the DR3 and DR4 genotypes are associated with high TNFα production (Wilson et al 1997; Yucesoy et al 2001). The increased HLA associations in vitiligo patients were: HLA-A2, A31, B13, B27, B56, B60, CW6, DR4, DR5, DR7, DR53 and DQ3 (Zhang et al 2005). Therefore, the increased production of TNFα could contribute to the increased incidence of vitiligo observed in individuals with DR4 haplotype. However, a study in the Turkish population shows that –308 *TNFa* promoter polymorphism has no significant influence on vitiligo susceptibility and clinical manifestations (Yazici et al 2006).

## 1.13. 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.

**1.13.1. 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. The HLA association studies reported till now are listed in the Table 14.

**Table 14: HLA associations reported in vitiligo.**

| Positive association | Negative association | Reference |
|---|---|---|
| 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 |

### 1.13.2. Genome wide linkage analyses

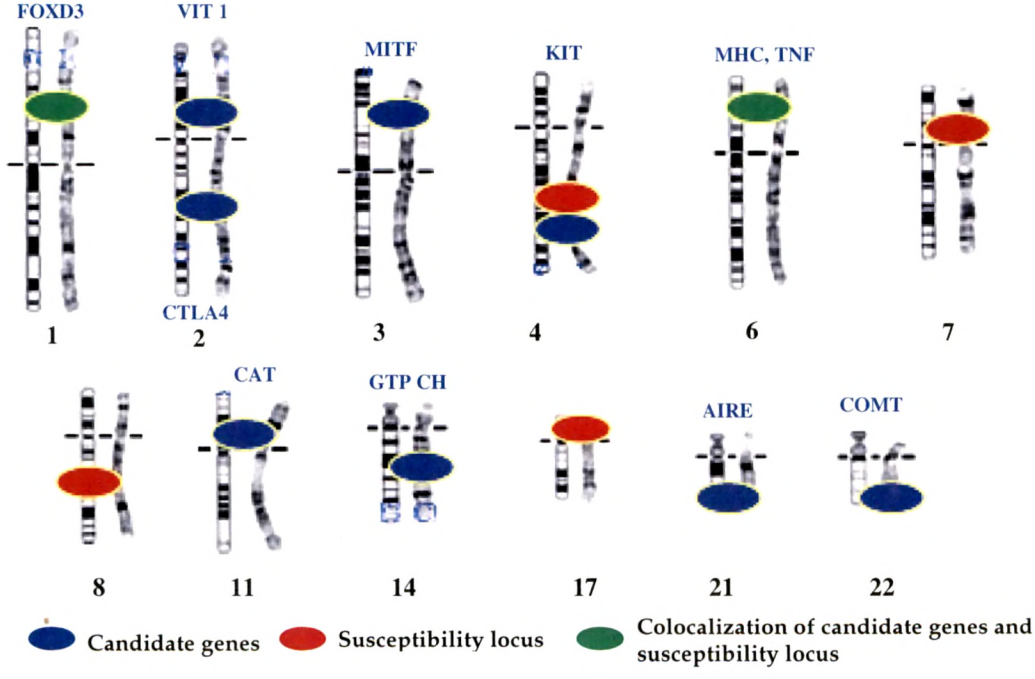
Genome wide linkage scans involve the typing of families using polymorphic markers that are positioned across the whole genome, followed by calculating the degree of linkage of the marker to a disease trait. Positional candidate genes can be identified by examining the regions around the peaks of linkage that are obtained by the study. Several genome wide linkage analyses of vitiligo have been performed in recent years 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 is given in the Table 15. The representation of reported susceptibility loci and candidate genes for vitiligo is also shown in Figure 12.

**Table 15: 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 |
| AIS3 | 8q | Spritz et al 2004 |
| | 6p21.3-21.4 | Arcos Burgos et al 2002 |
| | 4q13-q21 | Chen et al 2005 |

**Figure 12. Representation of reported susceptibility loci and candidate genes for vitiligo**



## 1.14. 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).

### 1.14.1. Non-surgical therapies

**1.14.1.1. 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 still 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). One study has 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 explanation for the therapeutic effect of PUVA on vitiligo (Akyol et al 2002). It was proposed that PUVA inhibits gene transcription, which ultimately results in the shut down of cytokine release. One study showed the effect of PUVA on the release of the pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF α 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).

**1.14.1.2. Broadband UVB:** This phototherapy uses an emission spectrum of 290-320 nm (Koster and Wiskemann 1990).

**1.14.1.3. Narrowband UVB:** In this phototherapy an emission spectrum of 310-315 nm is used (Westerhof and Korbotova 1997). The advantage of UVB therapy over PUVA regimen is reflected by shorter duration of treatment (Van Geel et al 2001).

**1.14.1.4. 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 with tacrolimus, pimecrolimus induces maximal repigmentation on sun-exposed areas.

**1.14.1.5. Calcipotriol:** It is a synthetic analogue of vitamin $D_3$. Vitamin $D_3$ 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 are thought to express 1 α dihydroxyvitamin $D_3$ receptors, which may have a role in stimulating melanogenesis.

**1.14.1.6. 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 the expression of catalase mRNA in melanocytes and keratinocytes from this patient group 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 the $6BH_4$ recycling process which is perturbed in vitiligo and thus leads to repigmentation (Schallreuter et al 2001).

**1.14.1.7. Khellin and UVA:** Khellin is a furanochrome and combined with UVA, it is reported to be as effective as PUVA therapy in the treatment of

vitiligo without having the phototoxicity associated with psoralens (Nordlund et al 1993).

### 1.14.2. 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.

**1.14.2.1. 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).

**1.14.2.2. Split thickness grafts:** After obtaining a split thickness skin graft using a dermatome it can be applied directly onto the dermabraded recipient area. The success rate of this method is in between 78-91% (Olsson and Juhlin 1998; Kahn and Cohen 1998).

**1.14.2.3. Suction blister grafts:** After harvesting the blister manually or using the specially designed equipment, the grafts are carefully removed. These epidermal sheets are grafted onto the denuded recipient site. The eyelids, lips and bony prominences can be treated using this method. The success rate of this method is in between 73-88%.

**1.14.2.4. 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%.

**1.14.2.5. Non-cultured keratinocytes and melanocytes:** Epidermis of donor skin was separated and after several procedures cellular suspension is obtained. Liquid nitrogen was used to induce blisters in the recipient area. The cellular suspension was injected into each blister at the recipient area after aspiration of the blister fluid. The success rate of this therapy is more than 70% (Gauthier and Surleve-Bazeille 1992).

**1.14.2.6. Cultured melanocytes:** Cultured pure autologous human melanocytes are used in this method (Lerner et al 1987). The success rates vary between 22-72%.

**1.14.3. 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).

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