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The Pathogenesis of Vitiligo

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1. Introduction

The question, "What causes vitiligo?" remains ambiguous. The lay population generally accepts that it is the concept of the "autoimmune destruction of pigment-producing cells called melanocytes" – however, this assertion has not been fully substantiated. The exact pathogenesis is unknown, but research shows that it is complex, involving the interplay of multiple factors, many of which are not elucidated. In the last century, much research has been dedicated to vitiligo and several overarching theories of its pathogenesis have emerged.
In addition to genetics, the Neural Theory was first proposed by Lerner *et al* in the 1950s (Lerner, 1959), and since then, the Autoimmune Theory, Reactive Oxygen Species Model and the Melanocytorrhagy Hypothesis have been developed.

2. The role of genetics in the pathogenesis of vitiligo

Numerous studies have investigated the effect genetics engender on the onset and development of vitiligo. It is important to recognize the patterns of vitiligo and its physical distribution, as the genetic basis for each type of distribution can differ. *Trichrome vitiligo* refers to lesions that appear white, light brown, and dark brown concurrently, with each color representing a stage of disease progression. *Inflammatory vitiligo* lesions present with pruritus and have elevated, erythematous margins. Distribution of the disease follows two basic patterns: *focal vitiligo* involves one or several macules at a single site, whereas *generalized vitiligo* (GV) involves a widespread and largely symmetrical distribution of macules. When GV becomes extensive, or coalesces to which point the vast majority of the body is involved and very few pigmented areas remain, it is deemed *vitiligo universalis*. Both focal and generalized types are considered *non-segmental vitiligo*, whereas *segmental vitiligo* refers to disease that occurs and remains stable in one unilateral region, but at the same time can be associated with lesions elsewhere (Wolff & Johnson, 2009).

2.1 Family-based studies and patterns of vitiligo inheritance

Studies demonstrate that a family history for vitiligo exists in 6.25-38% of patients (Njoo *et al.*, 2001); however, the exact mode of inheritance remains unclear (Njoo *et al.*, 2001). A study by Majumder *et al* (1988) suggested that recessive alleles at multiple unlinked loci interact epistatically to cause the vitiligo phenotype. They employed their own Multiple Recessive Homozygosis Model (Li, 1987) with a data set of 274 families that had one affected individual to develop this hypothesis (Majumder *et al*, 1988). The model assumes that

vitiligo is a recessive trait, involving multiple, autosomal, and unlinked loci. After applying their population data to this model, they found no significant differences between the observed segregation probabilities and those calculated using the Multiple Recessive Homozygosis Model (1987). These results demonstrate that recessive alleles at multiple unlinked loci could be involved in the genetic pathogenesis of vitiligo.

Years later, researchers tested this hypothesis with another family-based study, gathering data on 194 affected families from the United States (Nath *et al.*, 1994). This study showed that approximately 20% of affected individuals, or "probands", had at least one first-degree relative also with vitiligo. After completing segregation analysis of their data, the researchers concluded that three epistatically interacting autosomal diallelic loci are involved, and individuals who maintain recessive homozygosity at these loci are affected by vitiligo (Nath *et al*, 1994).

A study examining 1,030 Korean vitiligo patients also demonstrated a clear pattern of familial aggregation. Of these patients, 120 had a family history of vitiligo and data from these patients was collected. They found clear father-to-son transmission in some families, effectively ruling out X-linked inheritance as a possible genetic etiology. If a threshold trait, in this case vitiligo, has a multifactorial mode of inheritance, its frequency in relatives of affected individuals approaches the square root of the trait's frequency in the general population. Using their data, they calculated that the threshold trait in first-degree relatives of vitiligo patients was similar to the square root of the trait's frequency in the general population. Thus, their findings suggest that the inheritance of vitiligo is polygenic (Kim *et al*, 1999).

2.2 Molecular genetics-based studies

Another group studied 102 families with more than one vitiligo-affected offspring (termed "multiplex" families) (Spritz *et al.*, 2004). Peripheral blood was collected, and genotyping was done on 660 people, and 300 were found to be affected with vitiligo. Following genome-wide linkage analysis of these individuals, and heterogeneity testing between families with autoimmune disorders and families with no history of autoimmune disorders, they concluded that, for generalized vitiligo, there are two phenotypic subcategories that involve different loci or alleles. For patients with vitiligo and other concomitant autoimmune diseases, associated loci include the auto-immune susceptibility (AIS)-1, AIS2 (on chromosome 7), and the systemic lupus erythematosus vitiligo-related gene (SLEV1, a locus on chromosome 17 that is detected in multiplex families with systemic lupus erythematosus). The other phenotypic category, involving patients with generalized vitiligo alone, is linked with the AIS3 locus (on chromosome 8) (Spritz *et al*, 2004).

In a study of 26 vitiligo patients from Jordan, researchers investigated NALP1 as a candidate gene for the pathogenesis of vitiligo (Alkhateeb *et al.*, 2010). NALP1 acts as a primary regulator of the innate immune system, primarily existing in Langerhans cells and T cells (Kummer 2007). Eight variants within the NALP1 genomic and promoter regions were genotyped and analyzed, of which two variants in the NALP1 promoter region (rs2670660 and rs1008588) were determined to have significant association with vitiligo and Caucasian patients. These results confirm findings by Jin *et al* in 2007 demonstrating the association between the single nucleotide polymorphism (SNP) rs2670660 and vitiligo in a Romanian population.

Another means of investigating the genetic basis of vitiligo predisposition is to carry out a genome-wide association study. Birlea *et al* (2011) used genotype data from 1,392 unrelated non-Hispanic white vitiligo patients and compared these to 2,629 non-Hispanic white

controls to determine genetic associations with GV. Of the thirty-three candidate loci tested, only three (FOXP3, TSLP, and XBP1) had a primary association with GV. Whereas the exact function of genes TSLP and XBP1 are unknown, FOXP3 is known to be erroneous in the X-linked recessive multiple autoimmune disease syndrome. Further meta-analysis suggested XBP1 is the most significant GV susceptibility locus. Lastly, they determined that the locus CTLA4 maintains a secondary association with GV, having its primary association with the autoimmune diseases epidemiologically related to vitiligo (Birlea *et al*, 2011).

Other studies have gone beyond identifying what genes are involved, to the mechanisms behind how the expression of those genes may be modified in order to create the vitiligo phenotype. Deoxyribunucleic acid (DNA) methylation is an epigenetic process that plays a role in gene transcription and genomic imprinting, among other mechanisms (Li 2002 and Reik *et al.*, 2001). The methylation itself is carried out by enzymes called DNA methyltransferases (DNMT1, -3a, -3b). Zhao *et al* (2010) examined peripheral blood mononuclear cells (PBMCs) from vitiligo patients and controls, and measured messenger ribonucleic acid (mRNA) levels of DNMTs, methyl-DNA binding domain proteins (MBDs) and interleukin-10 (IL-10). Since IL-10 has been associated with autoimmune reactivity, and demonstrated to be sensitive to alterations in methylation status, its levels were also examined (Balasa *et al.*, 1998, Dong *et al.*, 2007, Szalmas *et al.*, 2008). In vitiligo PBMCs, it was found that methylation was increased in comparison with controls, and, notably, the methylation-sensitive region in IL-10 was hypermethylated. At the same time, IL-10 expression was significantly reduced in the vitiligo PBMCs. These results suggest that in vitiligo, changes in DNA methylation activity can alter the expression of genes involved in autoimmunity, thereby providing a potential means for creating the vitiligo phenotype.

In a similar way, Yun *et al* (2010) assessed genetic interactions by looking into the transforming growth factor beta-receptor II (TGFBR2). This receptor has immunologic signaling that may cause autoimmune disease through a variety of mechanisms including inhibition of inflammatory pathways and lymphocyte activation (Basak *et al*, 2009). This was performed on a Korean sample that consisted of 415 controls and 233 non-segmental vitiligo (NSV) patients that were genotyped. Following age and gender adjustments and data analysis, three SNPs for the receptor gene were found to be significantly associated with the NSV group, suggesting a possible role for TGFBR2 signaling in the pathogenesis of vitiligo.

The destruction of melanocytes results in the depigmentation observed in vitiligo. The ultraviolet radiation resistance-associated gene, or UVRAG, not only confers UV-damage resistance, but has also been demonstrated to play a role in autophagy – the process of cellular self-destruction that is potentially tied to autoimmune pathologies (Liang *et al.*, 2006). For these reasons, Jeong *et al* conducted a study to investigate a potential UVRAG association with NSV, or GV, in a Korean population. With 225 NSV patients and 439 controls, the researchers identified two SNPs of UVRAG that showed a significant genotype difference between the two groups, thereby suggesting a potential association between UVRAG and NSV (Jeong *et al.*, 2010).

Birlea *et al* (2010) did a genome-wide association study and located notable SNPs at 6q27. These SNPs were located near the insulin-dependent diabetes mellitus 8 locus (IDDM8), which is an association signal for type 1 diabetes and rheumatoid arthritis. In this study, 32 distantly related vitiligo patients from a Romanian founder population and 50 healthy controls from villages in its vicinity were genotyped. The region on 6q27 where the SNPs (specifically rs13208776) are located contains a single gene – SMOC2. This gene encodes a

protein whose exact function is unknown, but it is postulated to be involved in growth and development (Liu *et al.*, 2009) and cell matrix interactions (Maier *et al.*, 2008).

Kingo *et al* (2006) have demonstrated that messenger ribonucleic acid (mRNA) expression of melanocyte proliferating gene 1, or MYG1 (a gene involved in early developmental processes), is elevated in lesional skin of vitiligo patients. Nine SNPs are found within the MYG1 locus for susceptibility to vitiligo (Philips *et al.*, 2010). The MYG1 gene consists of seven exons, culminating as 7.5 kilo-base pair (kb) of DNA on chromosome 12. In total, 10 SNPs are apparent within the gene. The study examined 124 unrelated Caucasian vitiligo patients in Estonia. The -119 promoter SNP demonstrated an association with vitiligo. Two alleles exist at this SNP, a more common -119C allele and a minor -119G allele. They found a higher frequency of the -119G allele in vitiligo patients compared to controls and that this increase was most prevalent in patients with active vitiligo. The Kingo *et al* (2006) study found that MYG1 expression was the same in non-lesional skin of non-active vitiligo patients and in control skin. In active vitiligo patients, MYG1 expression was increased in both lesional and non-lesional skin, and within the lesional skin of non-active vitiligo patients. These results taken together thus suggests that the -119G allele of the MYG1 promoter sequence is a potential risk-allele for developing vitiligo and for the active state of the disease (Philips *et al.*, 2010).

2.3 Studies involving the human leukocyte antigen

A Chinese study genotyped 1,178 vitiligo patients and 1,743 controls for any association HLA-DRB1*07 had with vitiligo, and found that the HLA-DRB1*07 positive group showed a significantly higher frequency of early age of onset, positive family history, and vitiligo-associated autoimmune diseases than that of the negative group (Hu *et al*, 2010). Another study examined the influence of HLA susceptibility on familial versus non-familial vitiligo. One hundred and fourteen patients were studied, of which 84 had a family history and 30 did not. Familial or not, the vitiligo patients demonstrated no significant difference in the type, stability, and severity of the disease. Both groups showed an increase in HLA alleles A2, A11, A31, A33, B17, B35, B40 and B44. Familial vitiligo was specifically associated with increased HLA A2, A28, A31 and B44. The study also demonstrated that vitiligo with onset at younger than 20 years old was correlated with increased numbers of HLA A2, A11, B17, B35 and B44 (Misri *et al*, 2009). The latter study suggests that the genetic pathogenesis of familial versus non-familial vitiligo is different, albeit possibly overlapping.

Ying *et al* (2010) conducted a study genotyping 579,146 SNPs in 1,514 GV patients and compared the results with control genotypes. Significant associations included SNPs of genes encoding MHC class I (between HLA-A and HCG9) and class II (between HLA-DRB1 and HLA-DQA1) proteins. SNPs of significance were found in genes related to other autoimmune diseases (PTPN22, LPP, IL2RA, UBASH3A, C1QTNF6). The SNPs of genes RERE and GZMB (both involved in immunity in general) (Ying *et al.*, 2010), and the TYR locus (which encodes tyrosinase, an enzyme required for melanogenesis) (Spritz *et al.*, 2003) were also important. Overall, these candidate associations with NSV support the assertion that NSV susceptibility loci are shared with loci associated with other autoimmune diseases (Ying *et al.*, 2010).

In another genome-wide association study, susceptibility loci were found on chromosome 6 and in the MHC (Quan *et al*, 2010). Genotyping of 6,623 vitiligo patients and 10,740 controls was carried out, and analyzed for 34 SNPs which deemed promising from a

previous study. In the MHC region, two independent association signals were found (rs11966200 and rs9468925), the latter of which is potentially a novel HLA susceptibility allele. On chromosome 6, two significant SNPS were found at 6q27 in a block containing three separate genes. One of these genes, RNASET2, encodes a ribonuclease (RNAse). When this gene is overexpressed, it makes cells more vulnerable to oxidative stress (Thompson *et al.*, 2009), an important mechanism for melanocyte destruction. The two genes are FGFR10P, which encodes a fibroblast growth factor receptor and can play a role in cell cycle progression in some disorders (Acquaviva *et al.*, 2009), and the chemokine receptor 6 gene (CCR6) (Quan *et al*, 2010).

Another HLA-oriented study by de Castro *et al* (2010) examined the gene encoding the discoidin domain receptor 1 (DDR1). This gene encodes a tyrosine kinase receptor that affects cell differentiation, adhesion, and cytokine production (Yoshimura *et al.*, 2005). One of the three SNPs of DDR1 (rs2267641) was found to be significantly associated with vitiligo. No association with autoimmune disorders was observed in this study, which suggests that vitiligo susceptibility may or may not be aligned with autoimmune disease (de Castro *et al.*, 2010).

Thus, the genetics behind the pathogenesis of vitiligo appear multifactorial and causal associations are yet to be established.

3. The neural theory

3.1 Early development and important principles

Lerner's "Neural Theory" (1959) asserted that depigmentation in vitiligo results from increased discharge of a specific substance (*e.g.*, melatonin) at peripheral nerve endings in the skin; one that lightens pigment and discourages formation of new melanin. Lerner went on to report that many cases of segmental vitiligo followed a clear dermatomal pattern, and that vitiliginous lesions were found to exhibit hyperhidrosis at rest. His study of one hundred and twenty-eight vitiligo patients also found that 30% of patients reported significant emotional upset preceding onset of disease, and an additional 39% associated their onset with nervousness, accidents, illnesses, operations, or parturition. Overall, 69% patients associated vitiligo onset with stress (Lerner 1959).

To establish the role of stress and the onset of vitiligo, Manolache & Benea (2007) did a case control study with thirty-two vitiligo patients, forty-five alopecia areata patients, and controls suffering from skin disease clearly unrelated to stress (*e.g.*, infection). Data from vitiligo and alopecia areata patients were analyzed separately. Sixty-five percent of vitiligo patients noted stressful events at disease onset or exacerbation, compared to twenty-one percent of age and gender-matched controls. An odds ratio was calculated as 6.81 with a 95% confidence interval of 2.24-20.71. The majority of vitiligo patients reported their stressors were primarily associated with personal and financial issues. Overall, the study lends support to the notion that a stressful life event may contribute to the onset or exacerbation of vitiligo.

Koga & Tango (1988) described the clinical picture of vitiligo in 480 patients, and from their data they formulated a set of categories to better define the disease. *Type A vitiligo* is associated with autoimmune disease, halo-nevi, and the Koebner phenomenon. It can occur at any age, and it progresses continuously with periods of remission and exacerbation. On the other hand, *Type B vitiligo* has an early age of onset, and spreads rapidly for a short time and then ceases. More relevant to the neural hypothesis is *Type B*

vitiligo, which spreads over an affected dermatomal area (Koga & Tango, 1988). An additional classification germane to the neural theory is segmental vitiligo, in which lesions occur in the distribution, or "segment" of one or more nerves, albeit not necessarily affecting the entire segmental region (Lerner 1959).

3.2 Histopathological, microscopic and ultrastructural studies

Al'Abadie *et al* (1995) used electron microscopy to examine nerve fibers in the superficial dermis from vitiligo and control patients. Biopsies were taken from marginal (*i.e.*, peripheral) and central areas of vitiligo lesions as well as non-lesional skin. Vitiligo patients consistently demonstrated significantly thicker Schwann cell basement membranes surrounding nerve fibers in both lesional and non-lesional vitiligo skin, compared to controls. Finally, nerve ultrastructure was not dependent on the location (marginal or central) of the vitiliginous lesion. These findings suggest that, although in vitiligo the ultrastructural changes of superficial nerves are subtle, there is neural involvement in the pathogenesis of vitiligo (Al'Abadie *et al.*, 1995).

Gokhale & Mehta (1983) further investigated the histopathology of skin from vitiligo patients. Their work examined the epidermis, dermal papillations, blood vessels, sweat glands, sweat ducts, hair follicles and sebaceous glands, dermal nerve and nerve endings, and the connective tissue of the dermis. Seventy-four patients were studied and researchers examined biopsies from depigmented areas and contralateral, pigmented areas from vitiligo patients and compared these with control biopsies from corresponding sites from unaffected individuals (Gokhale & Mehta, 1983). It was observed that more acute disease was associated with a high frequency of inflammatory changes and long-standing disease demonstrated significant degenerative changes in dermal nerves and sweat glands. The dermal nerves in 41% of patients were completely degenerated and 38% showed some degree of degeneration. Similar findings were present at the nerve endings. Gokhale & Mehta (1983) concluded that since melanocytes are of neural crest origin, the degeneration of dermal nerves and nerve endings could play a role in the development of vitiligo.

Another histological investigation searched for a relationship between vitiligo pathogenesis and Merkel cells, a type of neuroendocrine cell. These cells are localized to the epidermis and are more abundant in sun-exposed areas (Moll *et al.*, 1990, Lacour *et al.*, 1991). Moreover, these cells are continuous with nerve fibers. Bose investigated biopsies from five patients with stable vitiligo. Lesions and adjacent normal skin samples were compared to biopsies from unaffected control skin from normal subjects. All five patients had Type A vitiligo (*i.e.*, their lesions *did not* follow a strict dermatomal pattern). The monoclonal antibody TROMA 1 was used for indirect immunofluorescence study of the biopsies to detect the presence of Merkel cells. Bose (1994) found that in the adjacent normal skin biopsies of vitiligo patients, and in the normal skin biopsies of healthy controls, that TROMA 1 bound to an average of five Merkel cells on the basement membrane of hair follicles and skin. There was no binding of TROMA 1 to Merkel cells evident in lesional skin biopsies. Bose thereby observed the loss of Merkel cells in vitiliginous skin. Toxic metabolites resulting in melanocyte destruction could lead to the diminished number of Merkel cells, or an alternative mechanism may exist between melanocytes and Merkel cells that results in the loss of Merkel cells and eventually melanocyte loss.

3.3 The role of the sympathetic nervous system in depigmentation

The role of the sympathetic nervous system in tyrosinase activity and pigmentation was performed in an animal study. Laties and Lerner (1975) took twenty-eight brown-eyed, Dutch belted rabbits and resected the superior cervical ganglion on one side in 10, and interrupted the preganglionic nerve trunk in the remaining 18. Regardless of which sympathectomy procedure was used, the researchers considered signs of ptosis and miosis as indicators of successful surgical outcomes (*i.e.,* loss of sympathetic nervous system activity). They found that in all of the animals that survived longer than two months, the color of the eye ipsilateral to the surgery lightened compared to the other eye. The researchers also completed an assay for tyrosinase activity in the iris tissue and found that following both types of surgery, tyrosinase activity was diminished. This loss of enzyme activity could stop melanin production and result in depigmentation. This study suggests that there may be sympathetic nervous system dysfunction in vitiligo.

Wu *et* al (2000) sought to confirm whether the sympathetic nervous system was involved in the pathogenesis of vitiligo. They used laser Doppler flowmetry and iontophoresis to assess the level of microcirculation occurring in vitiligo lesions to in order to assess sympathetic nervous system activity. They examined ten patients with stable facial segmental-type vitiligo, and had two groups of controls. One control group contained ten stable non-segmental-type vitiligo patients, and the other control group had ten healthy, unaffected individuals. All patients were matched for age and gender, and "stable" was defined as no new lesions or changes in present lesions in at least 3 months. They found approximately three times the cutaneous blood flow on the lesional side compared to that of the contralateral normal skin in segmental vitiligo. No such differences were found in the non-segmental group, or the healthy controls. When the researchers administered sympathetic nervous system blockers (such as propranolol), the segmental type patients demonstrated a dramatic decrease in blood flow, whereas the other two groups did not. Notably, however, when the researchers measured baseline plasma levels of catecholamines (specifically adrenaline and noradrenaline), and adrenoceptor (alpha and beta) densities on blood cells, there were no significant differences across all three groups. Wu *et al.* contend that their results further support that the nervous system is indeed involved in the pathogenesis of vitiligo. In particular, they found that some level of sympathetic nerve dysfunction exists in segmental type vitiligo, and this possibly plays an important role in disease onset and progression.

3.4 Neuropeptide studies and neuronal marker investigations

Al'Abadie *et al* (1994) studied neuropeptides and neuronal markers in vitiligo patients. In 12 vitiligo patients and 7 unaffected control subjects, immunoreactivity for polyclonal general neuronal marker (PGP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY) was tested. Compared to normal controls, nerve fibers reactive to NPY were increased in the marginal areas of lesions in half of the patients studied. In lesional biopsies, 25% of patients also showed increased reactivity for NPY compared to control subjects. Overall, there was locally increased NPY reactivity around blood vessels and in the dermis of lesions (predominantly in marginal biopsies). NPY is associated with noradrenaline in human dermal nerves and is known to potentially exert a local autonomic effect. Furthermore, it is also a potential modulator of the sympathetic response. These results suggest that changes in neuropeptide reactivity in vitiligo patients could be a factor in the onset or progression of the disease.

Lazarova *et al* (2000) carried out a similar study several years later. This study employed indirect immunofluorescence techniques to identify the immunoreactivity of nerve fiber endings in the skin to neuropeptides and found similar findingss as Al'Abadie *et al* reported in 1994. In affected skin samples, NPY was most intensely reactive; however, Lazarova *et al* found that CGRP was also increased, although not as significantly. Both studies suggest that neuropeptides play a role in vitiligo pathogenesis. Lazarova *et al* postulated that a precipitating factor, for example, stress, causes a significant secretion of neuropeptides like NPY, which subsequently set off other reactions that trigger the onset of vitiligo.

Furthermore, Yehuda *et al* (2005) examined the relationship between the neuronal marker NPY and stress in vitiligo. The trial included thirty-four male veterans, eleven of whom were not exposed to any military trauma, eleven exposed (to military trauma), veterans without post-traumatic stress disorder (PTSD), and twelve veterans who were exposed with PTSD. Plasma NPY levels were determined. Upon regression analysis of collected data, high NPY levels were associated with symptom recovery and effective coping with trauma or stress. This finding suggests that NPY may have a protective role in stress exposure.

Rateb *et al* (2004) studied the role of nerve growth factor (NGF), a neuropeptide hormone, in a cohort of 20 vitiligo patients and 10 non-vitiliginous control subjects. All but two vitiligo patients had widespread disease. NGF is an amino acid peptide hormone that is required for sympathetic nervous system function (Lewin *et al.*, 1996). Rateb *et al* (2004) measured NGF levels in the lesions and non-affected skin in vitiligo patients and in the skin of control subjects. They found significantly increased levels of NGF in the lesional skin of affected patients when compared to non-lesional and control skin. At the same time, NGF levels were still higher in the non-lesional skin of vitiligo patients when compared to control skin samples. Thus, NGF may play a neurochemical role in the pathogenesis of vitiligo, and its presence could be an important factor in the maintenance or destruction of melanocytes (David, 2001 as cited in Rateb *et al.*, 2004).

Peters *et al* (2004) investigated the role of NGF in stress-induced neurogenic inflammation using a mouse model in which mice were subjected to sonic stress and then examined for subsequent hair growth termination. Skin tissue NGF levels and NGF receptors TrkA and p75 neurotrophin receptor (p75NTR) were measured using fluorescence immunohistochemistry. They found that stress upregulates NGF expression in hair follicles. Stress also increased expression of the low affinity p75NTR NGF-receptor and decreased that of the high affinity TrkA receptor. Using retrograde tracing, the researchers also found that NGF injections, which mimic stress, increased the proportion of Substance P neurons in the dorsal root ganglia. Since Substance P is involved in neurogenic inflammation, their overall findings suggest that stress-induced NGF expression can set off neurogenic inflammation.

Another group of neuropeptides relevant to vitiligo includes catecholamines. Morrone *et al* (1992) measured catecholamine metabolite levels in the urine of vitiligo patients. The researchers argued that because many vitiligo patients associate their disease onset to a stressful event or injury, and that these situations often result in presynaptic release of catecholamines (namely dopamine, norepinephrine and epinephrine); therefore, catecholamines could play an important role in the pathogenesis of vitiligo. In particular, they measured the metabolites homovanillic acid (HVA), vanilmandelic acid (VMA), 3-methoxytyramine (MT), normetanephrine (NMN), metanephrine (MN), 3,4-dihydroxy mandelic acid (DOMAC), and 3,4-dihydroxy phenylacetic acid (DOPAC) in 24-hour urine samples. Their population included 150 patients and 50 healthy controls. Of the 150 patients, 15 had generalized vitiligo, 50 had segmental type, and 85 had acrofacial vitiligo. Three

groups were then created. Group 1 had 8 segmental and 18 acrofacial patients, all with early active phase (early onset) vitiligo or with disease progression (as indicated by number and size of lesions). The second group included patients who had no new lesions in the last 4-8 months (5 generalized, 10 segmental, and 19 acrofacial patients). The third and last group of patients had stable vitiligo lesions for 1-5 years. Twenty-four-hour urine collections from all groups showed that the first and second groups had HVA (a dopamine derivative) levels 4 to 10 times higher than controls, and VMA (an epinephrine and norepinephrine derivative) levels up to 3 times higher than controls. The long-term stable vitiligo patients showed no significant difference in any of the measured catecholamine metabolites when compared to controls. Overall, the results demonstrate that HVA and VMA urinary levels correspond to the onset and progressive active phases of vitiligo, regardless of the way the disease is distributed (segmental, generalized, or acrofacial). Morrone *et al* postulated from their results that the high urinary levels of HVA and VMA are markers of increased catecholamines in the circulation, and catecholamines are increased as a result of stress at the onset of disease. They also asserted that as patients grow accustomed to the lesions, their stress levels associated with the disease decreases, and consequently, so do levels of circulating catecholamines and urinary metabolites. This lends some support to the neural hypothesis, *i.e.,* that neurotransmitters may play a central role in the pathogenesis of vitiligo. They suggest that increased levels of catecholamines at autonomic nerve endings in the skin could be cytotoxic to melanocytes either directly or indirectly through their metabolites. Notable metabolites include melanotoxic phenols that can bind tyrosinase and interfere with melanogenesis. Morrone *et al* (1992) also suggested that stressful events could result in catecholamine discharge. These catecholamines could bind alpha-receptors in the skin and mucosa arterioles causing vasoconstriction, hypoxia, and overproduction of oxygen radicals that destroy melanocytes (Morrone *et al*, 1992).

The Neural Theory has been investigated internationally; however, substantial evidence supporting this has not yet been established. Mental stress can stimulate the secretion of catecholamines through stimulating the hypothalamic-pituitary-adrenal axis (Morrone *et al* 1992; Tolis & Stefanis, 1983; Stokes & Sikes, 1988). In addition, other neurogenic inflammatory mediators implicated in vitiligo pathogenesis, such as NPY (Ekblad *et al.,* 1984), NGF (Peters *et al.,* 2004), and NGF receptors (Tometten *et al.,* 2004) are also influenced by stress. These factors are postulated to result in melanocyte destruction via direct cytotoxic inflammatory or immune mechanisms. Therefore, pharmacologic agents and non-pharmacologic methods that alleviate mental stress and inhibit these neurogenic factors may be considered in the future as therapeutic targets for vitiligo.

4. The autoimmune hypothesis

As discussed previously, the neural hypothesis lends the most support for the pathogenesis of segmental-type vitiligo, whereas for non-segmental, or "generalized" vitiligo, the pathogenesis may be better explained by autoimmune mechanisms. One of the most apparent correlations between vitiligo and autoimmunity is the finding that patients with vitiligo often have autoimmune comorbidities. Another common finding in support of this hypothesis is that vitiligo often responds to immunosuppressive treatments (Lepe *et al.,* 2003). In this section, the pertinent research findings and arguments in support of the autoimmune theory of vitiligo pathogenesis will be discussed. The mechanisms of immunity are humoral (antibody-mediated), cell-mediated, or mediated by cytokines. Autoantibodies and their respective target cells are also relevant to the pathogenesis of vitiligo.

4.1 The role of autoantibodies, the humoral immune system and concomitant autoimmune disease

Kemp *et al* (2010) searched for autoantibodies against tyrosine hydroxylase (TH, an enzyme required for the production of catecholamine neurotransmitters) (Lewis *et al.*, 1993 as cited Kemp *et al.*, 2010). The researchers obtained sera from non-segmental vitiligo patients, 8 segmental patients, and 91 individuals with other autoimmune diseases (not including vitiligo), such as autoimmune thyroid disease, Addison's disease, and systemic lupus erythematosus (SLE). They also examined the sera of twenty-eight healthy controls with no history of autoimmune disease or vitiligo. Sera were tested for TH antibodies using a radioimmunoassay (RIA). They found that 23% of the patients with non-segmental vitiligo were positive for TH antibodies, whereas all control subjects and segmental-type patients were negative for TH antibodies. They also found a significant increase in TH positivity in patients with active disease over those with stable disease (defined here as no new or changing lesions in the previous 6 months). To confirm whether the TH antibodies were specific for TH, the researchers used absorption assays for several enzymes and found that the TH antibodies did not cross-react with phenylalanine hydroxylase (PAH) or tryptophan hydroxylase (TPH). Furthermore, in non-segmental patients, antibodies against MCHR1 (melanin-concentrating hormone receptor 1) and tyrosinase (Kemp *et al.*, 2010) were noted. These findings suggest autoimmunity plays a role in the development and activity level of non-segmental vitiligo.

Harning *et al* (1991) screened sera for antibodies against pigment cell-surface antigens and how their presence reflected vitiligo disease activity. Twenty-four vitiligo patients (10 with active and 14 with inactive disease), and nineteen healthy individuals who served as controls were included in this study. Active disease was defined as new or progressive disease within the 3 months prior to serum extraction. The researchers used a live-cell enzyme linked immunoabsorbant assay (ELISA) to detect relevant antibodies and their subtypes. They reported that the average level of pigment cell antibodies was notably greater in patients with active disease than in patients with inactive disease or in the controls, and there was no significant difference between inactive patients and controls. Results also indicated that immunoglobulin G (IgG)- and immunoglobulin M (IgM)-based pigment cell antibodies were found in 80% of active vitiligo patients. The control subjects and the inactive vitiligo patients demonstrated no IgG levels; however, 21% of inactive vitiligo patients and 16% of controls had notable levels of IgM. Immunoglobulin A (IgA) pigment cell antibodies were found in several individuals from the inactive and control groups – albeit in low levels (and the levels were not significantly different between these two groups). Harning *et al* demonstrate a relationship between pigment-cell antibody levels and vitiligo disease activity. This supports the idea that an autoimmune-mediated interaction with pigment cells exists in the pathogenesis of vitiligo.

As discussed earlier, vitiligo often occurs alongside other autoimmune disorders. Ingordo *et al* (2011) sought to decipher this relationship further by measuring circulating autoantibodies in a population of young southern Italian males. A total of 60 male vitiligo patients were included in the study. The average age was 19 years old, with an age range of 18-21. Circulating antibodies were found in 42.5% of these patients. Specifically, anti-thyroglobulin antibodies were detected in 27.5%, anti-thyroperoxidase in 22.5%, and anti-smooth muscle antibody in 17.3%. These antibodies are typically related to thyroid disease and other autoimmune diseases. They are of interest because vitiligo and thyroid disease are often associated with one another. In this study, only 5% of all patients presented with overt

thyroid disease. Their results, when taken along with patient histories and analyzed using Fisher's exact test and T-testing, revealed that circulating autoantibodies, in particular anti-thyroid antibodies, were correlated only with recent onset of vitiligo. Their results showed that, in vitiligo patients, autoantibodies are often present without overt autoimmune disease, and that their presence, albeit related to onset, is unrelated to the course or extent of the vitiligo itself. Circulating autoantibodies thus may have an early role in the mechanisms ultimately leading to melanocyte destruction.

Similarly, Uncu *et al* (2011) examined the incidence of thyroid disorders in children with vitiligo using thyroid-specific tests. Fifty children with vitiligo (with an average age of 9.5 years, 26 males and 24 females) and fifty control children (25 males and 25 females, with an average age of 8.6 years and no history of autoimmune disorders) were examined for serum levels of free triiodothyronine, free thyroxine, (T3 and T4 respectively), TSH and antibodies to thyroperoxidase and thyroglobulin. All major subtypes of vitiligo were included in the patient population; however, generalized vitiligo was the most common. None of the subjects had overt thyroid disease; however 8% of the vitiligo group tested positive for autoimmune thyroiditis. No healthy controls were diagnosed with autoimmune thyroiditis. In addition, the researchers concluded that having concomitant autoimmune thyroiditis was more likely if the patient was female and if the duration of vitiligo was longer. This study further supports the association of vitiligo with autoimmune thyroid dysfunction, and that vitiligo may be caused by an autoimmune pathomechanism.

The humoral immune system likely plays roles in the autoimmune pathogenesis of vitiligo. The potential targets of these antibodies have been studied. A specific cell-surface target worthy of discussion is the melanin concentrating hormone receptor 1 (MCHR1). Using IgG from the sera of vitiligo patients and phage-display technology with a melanocyte complementary deoxyribonucleic acid (cDNA) phage-display library, Kemp *et al* (2002) first identified MCHR1 as a novel target for vitiligo autoantibodies in 2002. In total, 55 patients with vitiligo were enrolled, 41 with no autoimmune disorders, and 14 with one or more. Using radio-binding assays, immunoreactivity against MCHR1 was confirmed in sera from all of the patients. Antibodies to MCHR1 were found in 16.4% of the patients with vitiligo, whereas control sera exhibited no reactivity. Although this suggests that MCHR1 antibodies have a high disease-associated specificity for vitiligo, how they arise is unknown, and no obvious correlations between the presence of the antibodies and age of onset, gender, duration, subtype, or existence of concomitant autoimmune disease was determined. Normally, melanin concentrating hormone (MCH) binds MCHR1 (a G-protein-coupled receptor) to mobilize intracellular calcium (Chambers *et al.*, 1999) and acts as an antagonist of α-melanocyte-stimulating hormone (α-MSH). Studies by Hoogdijn *et al* (2001) suggest that MCH partially inhibits the induction of melanogenesis by α-MSH in human melanocytes (Hoogdijn *et al.*, as cited in Kemp *et al.*, 2002). Thus, signaling pathways involving MCHR1 could be involved in melanocyte regulation and melanin production (Kemp *et al.*, 2002).

Although MCHR1 was successfully identified as a target, how the antibodies interacted at this target was not elucidated. Gottumukkala *et al* (2006), sought to determine whether MCHR1 autoantibodies activate or block the MCHR1 response to MCH by studying nine vitiligo patients with MCHR-binding autoantibodies, nine vitiligo patients without these autoantibodies, ten patients with SLE (due to their tendency to exhibit notable autoantibody reactivity), and twenty healthy individuals ascontrols. IgG samples were taken from all participants, and fluorometry was used to detect intracellular calcium levels which would

reflect MCHR1 activity. No control or SLE patient samples blocked MCHR1 receptor activity; however 56% of the IgG samples of vitiligo patients inhibited the function of MCHR1. No MCHR-activating autoantibodies were detected in any participant. The researchers found no correlation between the presence of MCHR-autoantibodies and vitiligo subtype, activity, age of onset, duration, or presence of concomitant autoimmune disease. This demonstrates that MCHR1-binding autoantibodies can block the function of MCHR1, and MCHR1 is a relevant B-cell auto-antigen in vitiligo (Gottumukkala *et al.*, 2006).

4.2 The role of cell-mediated immunity in vitiligo

Le Poole *et al* (1996) sought to elucidate what specific types of immune mechanisms were taking place in vitiligo. The perilesional skin of patients suffering from inflammatory vitiligo was evaluated. Inflammatory vitiligo is a relatively rare subtype of vitiligo in which perilesional skin is red, itchy, and irritated, and inflammation progresses outwards into unaffected skin. Consequently, the investigators hypothesized that the inflammatory process may play a role in the elimination of melanocytes. Thus, using antibodies, they examined the inflammatory infiltrates of the perilesional skin and determined their composition. Specifically, they used antibodies for melanocytes, T-cells (CD2, CD3, CD4, and CD8), Langerhans cells, and macrophages (CD36 and CD68). Each of these components was assessed immunohistologically by single and double immunostaining of the perilesional biopsies. Three inflammatory vitiligo patients were biopsied and results were compared to healthy control skin. The researchers found that melanocyte densities were 2.5 times greater in control skin than in the pigmented non-lesional skin of vitiligo patients. In perilesional skin, 66% of the patients demonstrated a marked decrease in melanocyte density when compared to non-lesional skin. CD3 staining of T cells was significantly greater in perilesional skin when compared to non-lesional or lesional skin. Also in perilesional skin, T cell infiltrates were substantially increased in the epidermal compartment and mostly concentrated to where melanocyte destruction occurs (at the epidermal basal layer). The epidermis-infiltrating T cells found in perilesional skin demonstrated an increased CD8:CD4 ratio, and increased interleukin-2 receptor (IL-2) expression. Interestingly, patients with generalized vitiligo have also been found to have an increased CD8:CD4 T cell ratio.

This finding suggests that the destruction of melanocytes could be cytotoxic CD8 T-cell mediated. All of the vitiligo patients also exhibited perilesional HLA-DR expression (MHC class II receptor), particularly along basal and suprabasal keratinocytes, which could be attributed to local T cell reactivity. Finally, Le Poole *et al* found that CD68+OKM5-type macrophages were more abundant in lesional and non-lesional skin when compared to controls, whereas the CD36 subset of macrophages were more abundant in control skin. From an autoimmune perspective, these results suggest that a melanocyte-specific immune reaction, most notably involving T cells, may play a role in the evolution of vitiligo.

4.3 The role of cytokines in vitligo

The immune system involves a complex interplay of many factors beyond lymphocytes and antibodies; this includes cytokines, which may also play a role in the development of vitiligo. Tacrolimus (FK-506 or Fujimycin) is an immunomodulatory drug thought to inhibit T cell activation and consequently diminish the production and secretion of pro-inflammatory cytokines (Schreiber & Crabtree, 1992 as cited in Grimes *et al.*, 2004). Grimes *et al.* (2004) performed a twenty-four week study that tested the effectiveness of 0.1%

tacrolimus ointment on nineteen patients with generalized vitiligo. The pre- and post-treatment cytokine expression in lesional and non-lesional skin compared to the expression in skin of normal controls was also assessed. A topical preparation of tacrolimus was applied twice daily for the 24-week study duration. Punch biopsies were performed at baseline from depigmented, non-sun-exposed lesional skin and adjacent non-lesional skin, and similar biopsies were taken from non-sun-exposed control skin. Following the 24-week treatment period, repeat biopsies were performed. A total of nineteen patients completed the study. Some level of repigmentation occurred in 89% of patients, most of which occurred in the face and neck regions. Overall, 68% of patients achieved between 76% and 100% repigmentation. In terms of cytokine expression, at baseline, both the involved and uninvolved skin of vitiligo patients demonstrated significantly increased expression of IL-10, IFN-γ, and TNF-α compared to expression in control skin. Post-treatment, the only significant difference was that expression of TNF-α was decreased compared to baseline in both lesional and non-lesional skin of the vitiligo patients.

These findings suggest that a cytokine imbalance is likely to be involved in the pathogenesis of vitiligo, and that the apparent suppression of TNF-α by tacrolimus may facilitate repigmentation. It is important to consider that repigmentation with tacrolimus was most notable in sun-exposed areas (*i.e.*, the face and neck). Therefore, it could be suggested that the suppression of cytokines, namely TNF-α, facilitates UV-stimulation of melanogenesis and ultimately, the repopulation of melanocytes in vitiliginous skin (Grimes *et al.*, 2003).

Considering that TNF-α and IFN-γ are both T helper cell-1 (Th1) cytokines, Taher *et al* (2006) suggest that vitiligo is mediated by the Th1 response. They also argue that tacrolimus could promote repigmentation by potentially suppressing the Th1 response via upregulating the immunosuppressive Th2 cytokine, IL-10 (Taher *et al.*, 2006). The researchers measured Th2-related cytokine IL-10 levels before and after treating twenty vitiligo patients with tacrolimus. Following three months of treatment, of the seventeen patients who completed the study, all experienced a significant decrease in lesion size, and all noted follicular repigmentation. On average, patients experienced a 41% decrease in the size of their lesions after the course of the treatment. In addition, IL-10 levels were significantly increased in lesional skin post-treatment, compared to normal control skin and lesional untreated skin. These results further supported tacrolimus as an effective vitiligo treatment. Bassiouny and Shaker (2011) further investigated the putative role of cytokines in vitiligo by studying interleukin 17 (IL-17). IL-17 is a cytokine that interacts with many cell types: keratinocytes, macrophages, and fibroblasts, amongst others. Furthermore, IL-17 works to activate the production of other cytokines, including IL-1 and IL-6, and can potentiate other local inflammatory mediators like TNF-α (Kolls & Linders, 2004, as cited in Bassiouny & Shaker, 2011). Using a similar ELISA technique as Harning *et al.*, the Bassiouny research team took a population of thirty patients with vitiligo and twenty healthy controls and examined their sera and tissue for the cytokine IL-17. They found increased levels of IL-17 in both the lesional skin and sera of the vitiligo patients, compared to that of the controls. In addition, they found a statistically significant positive correlation between disease duration and the level of IL-17 in both the sera and tissue samples. Although the exact function of IL-17 overexpression is unclear, this study affords further support for cytokine-involvement in the development of vitiligo (Bassiouny & Shaker, 2011).

The autoimmune hypothesis for vitiligo is has provided the basis for a vast number of experimental designs and studies. The immune system is complex, involving cell-mediated and humoral mechanisms – both of which appear to play roles in the manifestation of

vitiligo. Identifying pathways involved in the immune reactions in vitiligo will help in understanding the cause of vitiligo and pave the way for developing specific immune targets to combat the disease.

5. The reactive oxygen species model

The theory that oxidative stress is a cause for vitiligo suggests that patients with vitiligo have an imbalanced redox (reduction-oxidation) state of the skin, resulting in the excess production of reactive oxygen species (ROS, *e.g.*, H_2O_2). These disturbances and ROS accumulation can have toxic effects on all components of the cell (*e.g.*, proteins, lipids), and could potentially result in the destruction of melanocytes creating the depigmented macules observed in vitiligo (Khan *et al.*, 2009).

5.1 Establishing the redox status of vitiligo patients

An early study relevant to this theory examined the anti-oxidant defense enzymes catalase (CAT), glutathione reductase (GR), and thioredoxin reductase (TR) in lesional and non-lesional skin using suction blisters from vitiligo patients (Schallreuter *et al.*, 1991). They found that TR levels were similar between patients and healthy controls; however, CAT levels were significantly decreased in both lesional and non-lesional skin of patients compared to healthy controls. Lastly, GR levels were also notably higher in patient skin compared to controls, with a significantly higher amount in the non-lesional, or pigmented skin of the patients compared to levels in the lesional skin. Since GR can facilitate some level of oxygen metabolism, the authors suggest that GR is upregulated as an attempt to compensate for the lack of catalase. Catalase is involved in oxygen metabolism, and these results suggest that catalase levels are decreased throughout the epidermis (spanning both affected and unaffected skin) in vitiligo patients. Thus, it is probable that oxygen metabolism is defective in vitiligo (Schallreuter *et al.*, 1991).

Ines *et al* (2006) examined the serum of thirty-six vitiligo patients (eighteen with stable and eighteen with active disease), and forty healthy controls for markers of redox status including malondialdehyde (MDA), selenium, vitamins E and A, and the erythrocyte activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and CAT. SOD scavenges superoxide radicals and reduces their toxicity (converts O_2^- to O_2 and H_2O_2) and catalase converts hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O) (Ines *et al.*, 2006). MDA is a product of lipid peroxidation and is an indicator of oxidative stress (Latha & Babu, 2001, Yildirim *et al.*, 2004). Selenium is required for GPx activity and vitamins E and A are important in antioxidant activity. Ines *et al* found that SOD and MDA activity as well as serum selenium were increased in both stable and active disease, however, all were maximally increased in the active disease state. Erythrocyte CAT activity and serum vitamin A and E levels were not significantly different from controls. The researchers suggest that enhanced SOD activity results in the accumulation of H_2O_2. Furthermore, GPx is a downstream enzyme that detoxifies H_2O_2, and GPx levels were found to be decreased in vitiligo patients, which could compound H_2O_2 accumulation (Ines *et al.*, 2006).

Ines *et al* (2009) then sought to determine if disease activity was associated with oxidative stress at the tissue level. Tissue levels of MDA, CAT, SOD, and GPx from 10 stable and 10 active vitiligo patients were compared to levels found in twenty healthy volunteers. Overall, SOD, GPx, and MDA levels were all increased in both active and stable disease with consistently higher increases in the active group. Conversely, CAT activity was significantly

decreased in both active and stable disease with a more notable decrease in the active group. This suggests that increased SOD activity in vitiligo patient tissue could be an adaptation to increased oxidative stress; however, the increased SOD ultimately results in H_2O_2 accumulation that can not be broken down by CAT because it is present in subnormal levels (Ines *et al.*, 2009).

Ines *et al* had some conflicting results between the 2006 and 2009 studies. The latter found decreased CAT levels and increased GPx levels in tissue, whereas previously, CAT was unchanged and GPx was decreased in patient serum. To substantiate the more recent Ines *et al* findings, Yildirim *et al* (2004) also found increased SOD, MDA, and GPx when examining the tissue of vitiligo patients. In addition, Khan *et al* (2009) found increased MDA, but significantly lower levels of SOD, GPx, and non-enzymatic antioxidant agents vitamins C and E in vitiligo patient serum. From these results, GPx is arguably increased in tissue, but its activity is decreased in the serum of vitiligo patients. The low SOD activity found by Khan *et al* is controversial, considering that the other studies discussed all found increased SOD activity. To reinforce this assertion, Sravani *et al* (2009) found statistically significant increases in SOD and CAT levels in both lesional and non-lesional skin of vitiligo patients compared to levels measured from skin from healthy controls. Furthermore, they found that CAT was decreased in both vitiligo skin typescompared to controls (Sravani *et al.*, 2009). Thus, the results from measuring similar markers vary somewhat from study to study. Khan *et al* suggested that these discrepancies could be due to differences between serum and tissue levels, duration, and activity of disease, as well as differences in laboratory techniques (Khan *et al.*, 2009). Nonetheless, when compared to controls, markers of oxidative stress in vitiligo patients are found at aberrant levels indicating that the balance between ROS and the anti-oxidant defense system is disrupted.

5.2 Characterizing the redox disruption in vitiligo

Dell'Anna *et al* (2001) suggested that the source of this disruption lies at the level of mitochondria. The research team retrieved and examined red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs) from forty non-segmental vitiligo patients and forty age- and sex-matched controls. They assessed the PBMCs for ROS generation using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay and flow cytometry analysis. They found significantly higher ROS generation in cells from active vitiligo subjects. Dell'Anna *et al* suggested that this ROS hyperproduction could be caused by opening of mitochondrial permeability transition pores (PTPs). They found that when they added a PTP inhibitor cyclosporin A, (CsA) to the cells, the DCFH-DA staining significantly decreased in the active vitiligo patients, reinforcing the notion that excess ROS production resulted from PTP opening at the level of mitochondria (Dell'Anna *et al.*, 2001).

To further characterize the redox imbalance in vitiligo and changes of mitochondria, blood samples were taken from fifty vitiligo patients (thirty-five with active and fifteen with stable vitiligo) and thirty healthy controls (Dell'Anna *et al.*, 2003). They measured the oxidative stress markers CAT, reduced glutathione (GSH), and SOD, and similar to previous research, found decreased CAT and GSH and increased SOD levels. Consequently, the SOD/CAT ratio was significantly higher in active disease and unchanged in stable and normal patients. ROS generation was significantly higher in active disease only. ROS levels correlated with the SOD/CAT ratio in controls and stable patients; however, the ratio was found to be inversely related to ROS activity in active patients. Thus, the researchers contend that excess

ROS production is an established phenomenon in vitiligo and when the disease is stable, this excess is balanced out by the body's cellular antioxidant system. Conversely, when the disease is active, and an oxidative stimulus is present, cells increase their ROS production and the redox balance is lost (Dell'Anna *et al.*, 2003).

In the same study, due to the function of mitochondria as the main intracellular source of ROS, mitochondrial function was also evaluated (Dell'Anna *et al.*, 2003). The researchers found a significant decrease in membrane potential across mitochondria in both active and stable vitiligo patients compared to controls. They also assessed the electron transport chain, or ETC, with a series of tests involving inhibitors of each complex. They found that PBMCs from vitiligo patients are susceptible to rotenone, an inhibitor of complex I. They also evaluated the Krebs cycle efficiency in mitochondria, and found the mitochondrial isoform of malate dehydrogenase activity was notably increased in vitiligo patients. These findings further support the assertion that mitochondrial dysfunction is involved in the pathogenesis of vitiligo (Dell'Anna *et al.*, 2003).

At the time, the cause of ETC impairment and mitochondrial dysfunction was yet to be elucidated. Thus, Dell'Anna *et al* investigated further using punch biopsies from five vitiligo patients and five healthy controls, and focused this study on characterizing lipid membranes. Confocal microscopy and fluorescence-activated cell sorting (FACS) revealed that epidermal primary vitiligo melanocytes had significant membrane peroxidation and that the pattern of fluorescence retrieved was specifically suggestive of the involvement of mitochondrial membranes (Dell'Anna *et al.*, 2007). To investigate the mitochondrial membrane changes more thoroughly, the content and transmembrane portion of cardiolipin, or CL was assessed. CL is a phospholipid that has four fatty acyl chains that is associated with mitochondria and with proteins that conduct oxidative phosphorylation (Haines & Dencher, 2002). There was a reduced percentage of CL and a modified distribution of CL in the melanocyte mitochondria of vitiligo patients, particularly in comparison with particulary in comparison with controls controls. Furthermore, to assess the ETC, they performed a semiquantitative analysis of complex 1 (CxI) activity and found CxI was decreased in melanocytes from vitiligo subjects when compared to the controls. These results provide a plausible mechanism for vitiligo pathogenesis in which there is a primitive defective arrangement of membrane lipids (namely altered CL distribution) that results in impaired ETC activity. Hyperproduction of ROS ensues, and the redox imbalance ultimately causes melanocytes destruction (Dell'Anna *et al.*, 2007).

5.3 The role of tetrahydrobiopterin recycling and other indicators of oxidative stress in vitiligo

The accumulation of hydrogen peroxide (H_2O_2) in the skin of vitiligo patients is an important finding, with many implications. One particular cellular pathway affected by H_2O_2 involves tetrahydrobiopterin. Tyrosinase is a hallmark enzyme in the synthesis of melanin (Prota, 1992). L-tyrosine is formed from L-phenylalanine by the enzyme phenylalanine hydroxylase (PAH). The essential cofactor for this process is 5,6,7,8-tetrahydrobiopterin or 6BH₄. Defective recycling of 6BH₄ yields excess levels of 7BH₄, which is an inhibitor of PAH. This uncoupling of PAH and presence of 7BH₄ was found in suction blister material from the skin of vitiligo patients (Schallreuter *et al.*, 1994, 1998). Kowlessur *et al* (1996) also found that 7BH₄ production can lead to the formation of H_2O_2. To investigate this defective recycling of 6BH₄ evident in vitiligo, Haase *et al* (2004) studied the enzyme dihydropteridine reductase, or DHPR, which is responsible for the final steps in normal 6BH₄ recycling. They examined whole blood samples from twenty-seven untreated vitiligo patients and eight unaffected controls. The researchers

also determined the effect of H_2O_2 concentration on DHPR activity. They found that concentrations of H_2O_2 greater than 30 μM decreased DHPR activity, whereas concentrations less than 30μM activated or increased DHPR activity. From this relationship it can be suggested that the accumulation of H_2O_2 through its concentration-dependent association with DHPR, results in defective $6BH_4$ recycling. They confirmed the concentration-dependent association between H_2O_2 and DHPR using Fourier transform-Raman spectroscopy. Interestingly, when patients were treated with topical pseudocatalase (low-dose narrow-band ultraviolet B-activated pseudocatalase PC-KUS – a treatment to remove epidermal H_2O_2), their whole blood DHPR activities normalized. This finding suggests that the removal of epidermal H_2O_2 affects systemic H_2O_2 balance. Overall, this illustrates the role ROS, namely H_2O_2, plays in the pathogenesis of vitiligo.

Schallreuter *et al* considered the effect of H_2O_2 on acetylcholinesterase (AchE). This enzyme was of interest because AchE levels were found to be lower in patients with vitiligo when compared with healthy controls, suggesting cholinergic involvement in the disease (Iyengar, 1989 as cited in Schallreuter *et al.*, 2004). Skin biopsies from sun-unexposed areas from four healthy controls and four vitiligo patients. Similar to the findings of Iyengar, Schallreuter *et al* found that depigmented vitiligo skin showed marked decreases in AchE levels compared to controls while repigmenting patients treated with PC-KUS demonstrated an increase in AchE throughout the epidermis. Untreated depigmented skin showed very little catalase activity, and PC-KUS-treated skin showed significantly higher catalase expression throughout the epidermis compared to controls. Thus, H_2O_2 levels were also found to have a concentration-dependent influence on AchE, *i.e.,* low H_2O_2 concentrations (approximately 10^{-6}M or mol/L) activate AchE whereas high concentrations (10^{-3}M or mol/L) deactivate AchE (Schallreuter *et al.*, 2004). Butyrylcholinesterase (BchE) is an enzyme that mediates the hydrolysis of acetylcholine. The hydrolysis reaction is one of the rate-limiting steps in cholinergic signal transduction (Rakonczay & Brimijoin, 1988 as cited in Schallreuter *et al.*, 2006). Using immunofluorescence, Schallreuter *et al* (2006) demonstrated that BchE is present in the keratinocytes and melanocytes of the human epidermis; however the BchE protein is much lower in skin from vitiligo patients. Upon removal of epidermal H_2O_2 using PC-KUS, vitiligo patient skin demonstrated a higher level of BchE expression than controls. When AchE and BchE activities were tested at the same time on the same samples, BchE activity levels were greater than the AchE levels. The overall decreased activities of BchE and AchE were apparent in both lesional and non-lesional skin of vitiligo patients demonstrating that the effects of H_2O_2 occur throughout the entire epidermal compartment.

Considering the previous research on H_2O_2 accumulation in vitiligo, Shalbaf *et al* (2008) investigated xanthine oxidase (XO) as a source of H_2O_2 because it produces H_2O_2 in its reaction pathway. XO is found in many tissues and catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and then xanthine to uric acid, which is accompanied by H_2O_2 production (Mathews *et al.*, 2000 as cited in Shalbaf *et al.*, 2008). XO also oxidizes uric acid to allantoin, a substance that acts a marker of oxidative stress (Benzie *et al.*, 1999). Using skin biopsies from vitiligo patients, the presence of XO was confirmed in melanocytes and keratinocytes, and regulation of XO by H_2O_2 was also confirmed; high concentrations of H_2O_2 inhibit the activity of XO, whereas low concentrations activate it, making the relationship concentration-dependent. Epidermal cell extracts from suction blister tissue showed that allantoin was present in patients with acute vitiligo; however, it was entirely absent in healthy controls. Thus, XO may be a contributor of H_2O_2 ROS in vitiligo.

The vast majority of studies germane to the ROS model recruited patients with generalized vitiligo, and ROS-mediated damage may be applied to the pathogenesis of non-segmental, generalized vitiligo until further research is done in other types of vitiligo.

6. The melanocytorrhagy hypothesis

Compared to the other hypotheses discussed, the melanocytorrhagy hypothesis is a relatively new approach to explaining the pathogenesis of vitiligo. First proposed by Gauthier *et al* in 2003, this theory describes the pathogenesis of non-segmental vitiligo (NSV) as from the result of "melanocytorrhagy", or a chronic detachment and loss of melanocytes resulting from altered melanocytes responses to trauma and other stressors. The theory also attempts to tie together concepts from the theories previously discussed to create a single, integrated explanation of vitiligo pathogenesis, and suggests that stressors could include catecholamines, ROS, or autoimmune elements (Gauthier *et al.*, 2003).

Early studies by Le Poole *et al* demonstrated that melanocytes loss occurs in vitiligo lesions (Le Poole *et al.*, 1993). Gauthier *et al* countered that although melanocyte loss is well-established, direct demonstration of the physical destruction of melanocytes is not.

A study supporting the concept of melanocyte loss in vitiligo was done by Tobin *et al* in 2000. Twenty-seven patients with non-segmental vitiligo and ten healthy controls were enrolled. Seven patients received pseudocatalase treatment prior to the study, whereas twenty had received no previous treatment. The researchers acquired epidermal melanocytes and keratinocyte cultures from both lesional and non-lesional skin from vitiligo patients and normal controls. Light and transmission electron microscopy, as well as immunohistochemistry were used to evaluate the cultures for morphology. In the untreated vitiligo patient samples, they found vacuolation and degeneration of basal keratinocytes, melanocytes, and Langerhans cells. Also observed was an increased number of Langerhans cells in the basal layer of the epidermis near dysfunctional melanocytes, and dilated endoplasmic reticulum, intracellular granular debris, and fatty degeneration. Tobin *et al* attributed these changes to the oxidative stress caused by H_2O_2.

More importantly, there were signs suggesting melanocytes were never entirely absent. Evidence of rare clear cells in the epidermis of lesional skin from vitiligo patients with disease duration of up to twenty-five years was observed. These cells were small and amelanotic, although a portion of the clear cells did contain irregular melanosomes. These clear cells were deemed melanocytic as they contained tyrosinase (due to positive dopa reactivity). Although these cells were in significantly low numbers, these findings suggest that melanocytes are not entirely eliminated from vitiliginous skin and that they persist in some form, even in long-standing disease (Tobin *et al.*, 2000).

Gauthier *et al* (2003) also purports that defective cell adhesion plays a role in the pathogenesis of vitiligo as the production extracellular matrix components may be altered by keratinocytes. Basal membrane structure dysfunction is observed in vitiligo, in particular, the presence of focal gaps in the basement membrane and redundant production of basement membrane. These alterations could weaken the basal anchoring of melanocytes, making them vulnerable to detachment. Trauma could exacerbate this vulnerability, leading to the chronic melanocyte loss that has been described as melanocytorrhagy.

Le Poole *et al* argued that the protein tenascin may be involved in diminishing melanocyte adhesion in the pathogenesis of vitiligo. Skin biopsies of lesional and control skin were examined. In normal culture conditions, melanocytes most easily adhere to the extracellular matrix (ECM) protein fibronectin. There was an observed relationship between tenascin

concentration and melanocyte adhesiveness to fibronectin: an abundance of tenascin inhibited melanocyte to fibronectin adhesion. In general, the vitiligo patients were found to express higher levels of tenascin compared to controls. Whether the increased tenascin expression is a cause or consequence of vitiligo is unclear; however, from these results it is arguable that modified cellular adhesion is evident in vitiligo and could contribute to its pathogenesis (Le Poole *et al.*, 1997).

A pivotal study regarding the melanocytorrhagy hypothesis investigated how trauma could elicit vitiligo lesions, a process also called the Koebner phenomenon. Light and reproducible friction for four minutes on the forearms of eighteen patients with extensive vitiligo and on five healthy controls was performed. Biopsies were retrieved from the test region from all sets of patients at 1, 4, 24, and 48 hours after the friction was imposed. Each biopsy was evaluated using standard light microscopy, transmission electron microscopy, histochemistry, and immunohistochemistry. Control skin showed no changes; however, at 4 and 24 hours post-friction in vitiliginous skin, some melanocytes were detached and apparent in suprabasal regions, *i.e.,* the stratum spinosum, granular layer, and the stratum corneum. The researchers thought that vitiligo arising from the Koebner phenomenon is likely caused by the "transepidermal migration" observed in their study. They also concluded that this mechanism of melanocyte loss could provide an explanation for the chronic melanocyte loss evident in vitiligo, but may be instigated by another stressor other than friction or trauma (Gauthier *et al.*, 2003).

The melanocytorrhagy hypothesis was tested by recreating the initiating events leading up to melanocytorrhagy (Cario-André *et al.*, 2007). Epidermis was reconstructed on dead de-epidermized dermis (DDD) using control cells and cells from non-lesional NSV patients to form "new" epidermis. Since the "new" epidermis was weakly attached to the DDD, it was assumed that physical friction would not be required to initiate melanocytorrhagy. The reconstructs were subject to a variety of stressors, for example, epinephrine, norepinephrine, and H_2O_2. Reconstructs made with non-lesional vitiligo melanocytes had fewer basal melanocytes when compared to reconstructs made with normal melanocytes, suggesting that non-lesional NSV skin is affected by the disease process.

The reconstructs from vitiligo patients and found that 65% of the sera samples tested were able to induce melanocyte detachment. Epinephrine was also found to cause melanocyte detachment of normal and non-lesional vitiligo melanocytes; however norepinephrine had no effect on detachment at any concentration. Furthermore, H2O2 caused normal melanocyte…" (add space between H2O2 and caused) detachment, with variable effects on non-lesional vitiligo melanocytes. An intrinsic melanocyte defect limits melanocyte adhesion in reconstructed epidermis, and transepidermal migration melanocytes can occur in response to certain stressors (Cario-André *et al.*, 2007). Although an early *in vitro* study, these results provide some support for the melanocytorrhagy model.

Gauthier *et al* also indirectly support this theory in their early research. Their findings show that chronic melanocyte loss and defective adhesion could be the result of the dendritic function of melanocytes. Melanocyte dendrites are thought to be required for melanosome transfer as these processes connect melanocytes to numerous keratinocytes. Dendrites may play a role in melanocyte adhesion and anchoring within the basal layer of the epidermis, and dendrite retraction is commonly understood as the first step before melanocyte detachment and death. Morphologically, established vitiligo melanocytes demonstrate large perikaryon and "stubby" dendrites (Jimbow *et al.*, 2000). Exposing cultures of vitiligo or control melanocytes to catecholamines could result in dendrite retraction and loss over a

twenty-four hour period. Consequently, the research group concluded that oxyradicals or catecholamines could cause dendrite loss, thereby compounding the transepidermal melanocyte loss caused by an isomorphic or the Koebner phenomenon, resulting in the depigmented macules observed in vitiligo.

The melanocytorrhagy theory for the pathogenesis of vitiligo takes a new stance on how melanocyte loss occurs, and attempts to unify ideas from several pathogenesis theories to do so. Being a novel proposal, however, further research is needed to substantiate its postulations.

7. Conclusion

The pathogenesis of vitiligo has yet to be elucidated; however, years of research have provided us with a framework. A genetic predisposition to developing the disease is involved. The Neural Hypothesis suggests that the nervous system is involved, likely through the release of neurogenic factors in response to a stress event, and that these factors affect the survival of melanocytes. Cytotoxic and immune mechanisms are proposed to underlie the destruction of melanocytes through neuropeptides. The Autoimmune Theory argues that the loss of melanocytes observed in vitiligo is the result of an autoimmune reaction. The Reactive Oxygen Species Model suggests that faulty oxygen metabolism results in the excess production of reactive oxygen species, which causes melanocyte destruction. In addition, the Melanocytorrhagy Theory states that melanocyte loss occurs from defective cell adhesion coupled with friction or other types of stress. These mechanisms underlying vitiligo pathogenesis likely overlap and may vary depending on the type of vitiligo. Genetic factors likely precede neurogenic factors which, influenced by mental stress, may act via the aforementioned cytotoxic and immune mechanisms to cause destruction of melanocytes and resulting skin depigmentation. Future research would elucidate if these theories occur in a sequential fashion. Strategies targeting these pathways would potentially advance our therapeutic armament against vitiligo.

8. References

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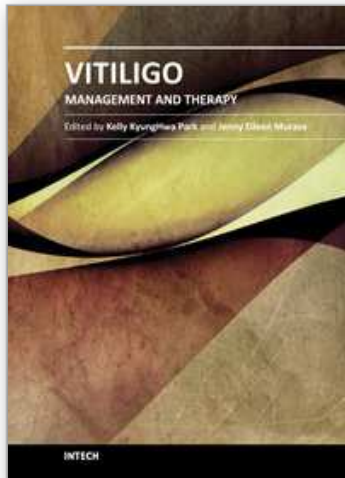
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Vitiligo - Management and Therapy

Edited by Dr. Kelly KyungHwa Park

Vitiligo: Management and Therapy is a practical guide to vitiligo that reflects current research related to the fundamentals of vitiligo and its management. Vitiligo experts and researchers from all over the world have contributed to this text, accounting for its comprehensive nature and diverse array of topics. The recent advances in medicine and technology have led to a better understanding of the disease and have broadened available treatment options. The essentials are captured in this book and are complemented by useful clinical photographs and reference tables. This concise tool will serve as an invaluable resource for clinicians in daily practice.

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