Melanocyte Survival and Response to Treatment in Vitiligo Patients

by

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DECLARATION:

I, Dr Vanessa Lapiner, declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor part of it has been, is being, or is to be submitted for another degree in this or any other University.

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DEDICATION:

To my precious husband and best friend – your steadfast belief that I can achieve anything I set my heart on, is the driving force behind everything I do. I love you infinitely. You are truly my beshert.

To my incredible parents who I will spend my lifetime striving to emulate – your unconditional love and support for me have always provided a platform from which to leap without worry of consequence. I consider myself the luckiest girl in the world to be your daughter.

Finally, to the vitiligo patients who have taught me so much about the meaning of holistic medicine and who have made me understand that research is an integral part of practicing medicine. Your tales of heartbreak and social stigmatism will continue to fuel research into this distressing condition.

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ABSTRACT:

Vitiligo is a common depigmenting skin disorder with devastating psychological implications. Although evidence strongly suggests that melanocytes die in various forms of vitiligo, it is also evident, at least in certain cases, that melanocytes/melanoblasts survive in an undifferentiated stem cell form and it is from these that re-pigmentation is assumed to occur. Treatment of this distressing condition remains 'hit-or-miss' and patients' responses are unpredictable. This study aims to integrate prognostic variables such as the molecular profile of vitiliginous lesions and the patient's epidemiological profile, to develop a reliable and sensitive prognostic test enabling clinicians to plan an individualized, rational therapeutic approach for their vitiligo patients. The analysis of tyrosinase and TRP-2 mRNA expression in vitiliginous skin using quantitative RT-qPCR revealed the presence of melanoblasts/melanocytes in 60% of patients (n=21). When this survival was calculated as a percentage of the mRNA expression in the pigmented positive control specimen, it was found to range from 4% to 46% survival. Patients were subsequently treated with either potent topical corticosteroid ointment or 5% khellin cream in combination with daily sunlight exposure for 3 months and their clinical response was then assessed and correlated to the lesional melanocyte status. A good response (>50% repigmentation) was found in 3/5 patients demonstrating both tyrosinase and TRP-2 gene expression, in only 1/4 patients demonstrating TRP-2 gene expression alone and in 0/3 patients demonstrating only tyrosinase gene expression. The presence of both tyrosinase and TRP-2 mRNA expression is therefore a significant positive prognostic indicator. A significant correlation (R^2 =0.8919) was found between melanocyte survival and response to khellin therapy suggesting that therapeutic modalities stimulating melanocyte proliferation and migration should be employed in patients demonstrating a melanocyte/ melanoblast reservoir. Conversely, an absence of melanocyte/melanoblast markers is predictive of a poor response to treatment with 7/9 patients lacking lesional tyrosinase or TRP-2 mRNA expression demonstrating <25% repigmentation at the conclusion of the 3 month treatment period. Therapeutic options such as surgery or cosmetic camouflage techniques should be considered for these patients. The epidemiological profile was not found to be a significant prognostic factor in predicting treatment response.

1. CHAPTER 1:

1.1 A VIGNETTE

Mrs H. is an eighty-two year old woman who was first seen in 1995 at the Groote Schuur Hospital PUVA clinic. She presented with a short history of depigmented skin lesions predominantly on her trunk and limbs and also had a background history of rheumatoid arthritis. She had been a widow for fifteen years and it was noted at the initial consultation that she seemed depressed. A diagnosis of vitiligo was made and various treatments including potent corticosteroids and PUVA were initiated.

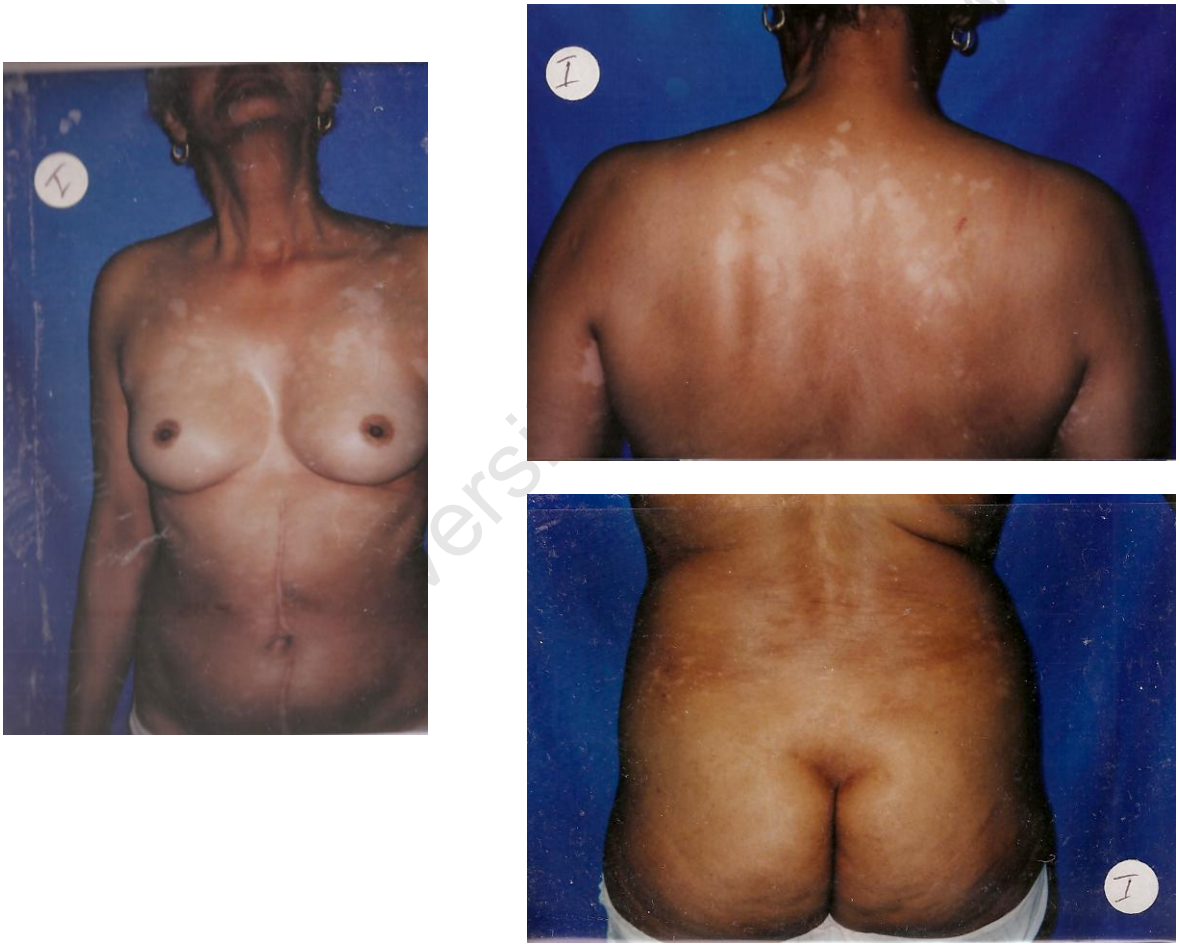


Fig 1. Photographs of Mrs H. at her initial consultation on 25/01/95 (consent for photographs obtained)

She demonstrated no response to therapy and her areas of depigmentation continued to expand, despite a minipulse of oral corticosteroids. It was noted that the patient was obsessed with "…is it (the pigment) coming or going?"

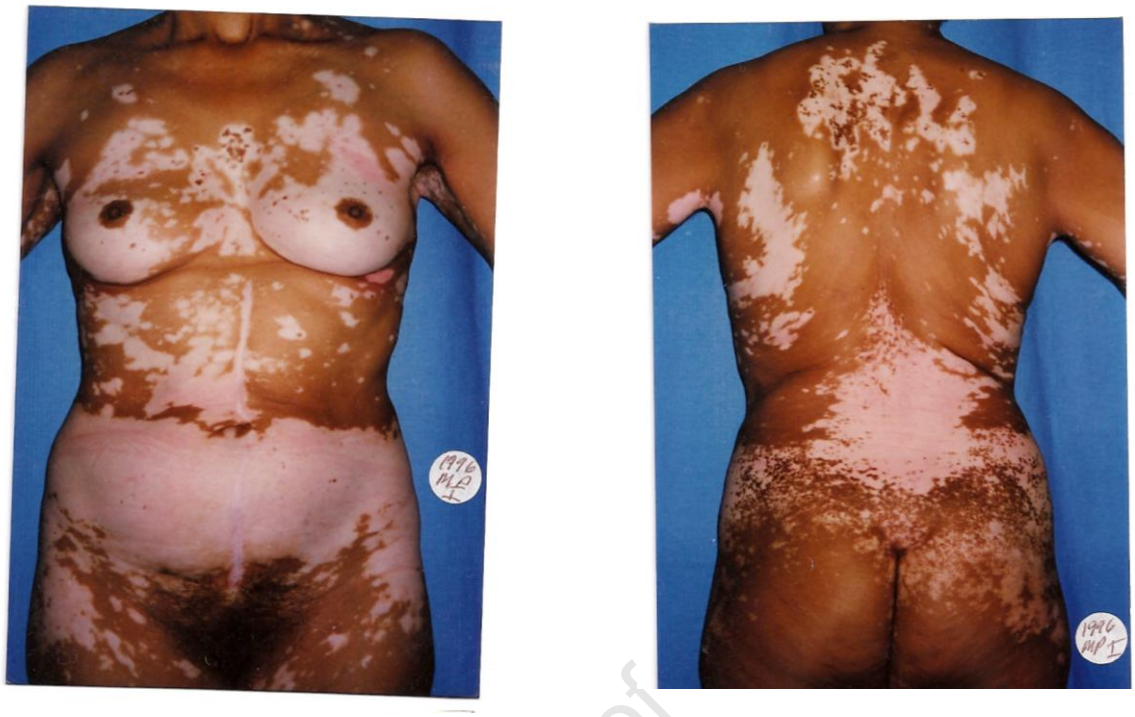


Fig 2. Photographs of Mrs H. taken on 19/01/96

One year after starting treatment, she confessed to suicidal ideation and she was urgently referred to psychiatry where Imipramine 175mg nocte and Alprazolam 0.25mg bd were commenced. Three months later, after sixty-five treatments of PUVA (an accumulative dose of 468.5) and a second minipulse of corticosteroids, a remarkable burst of repigmentation was seen. She met and fell in love with a new boyfriend and it was noted that '…at last I am happy with myself'.

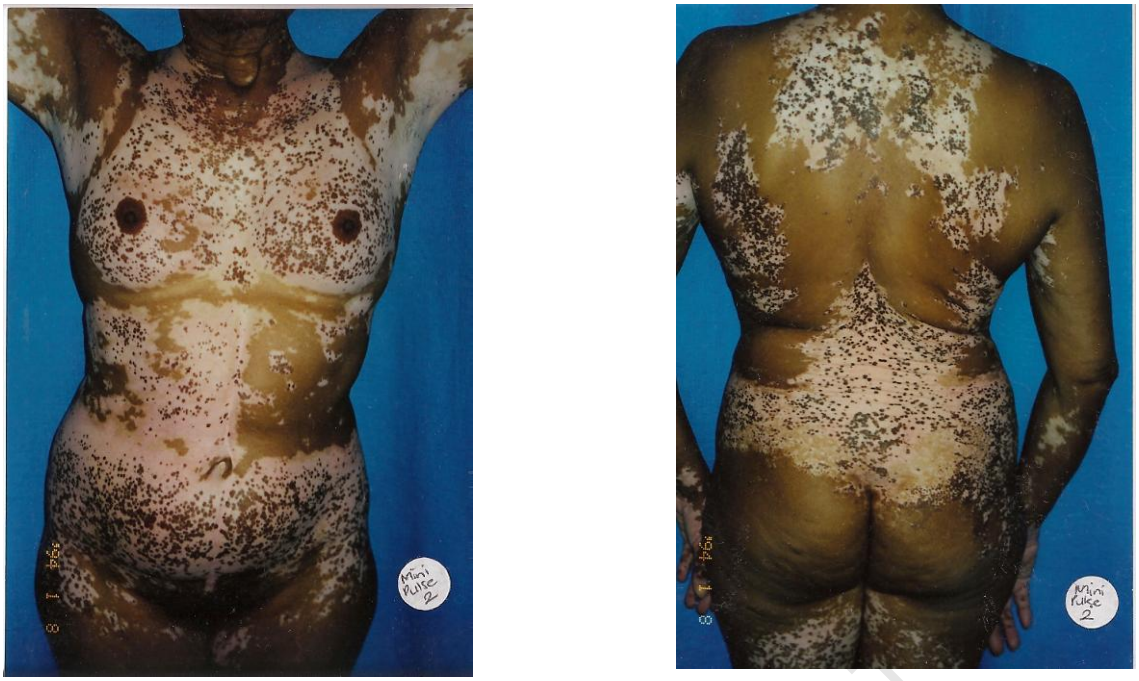


Fig 3. Photographs on Mrs H. taken on 04/04/96

In September 2000, following the sudden and devastating deaths of a close friend, her sister and her boyfriend, she experienced severe depression and once again, her pigment was lost.

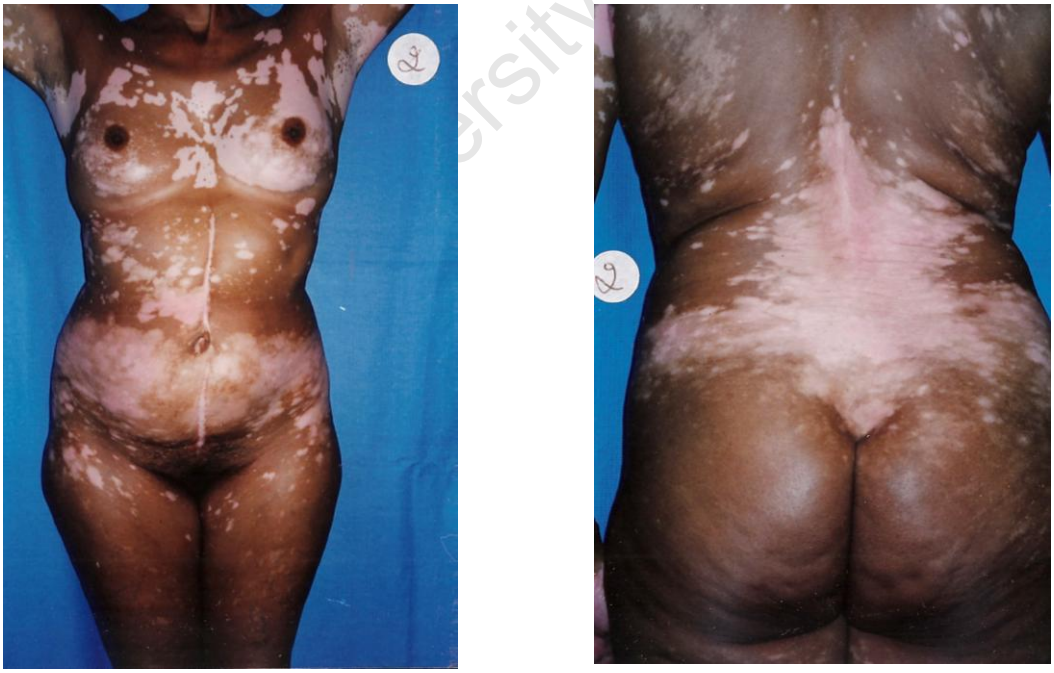


Fig 4. Photographs of Mrs H. taken on 15/02/01

Despite continuing with the PUVA treatments, she continued to depigment. This was exacerbated by a bout of pneumonia.

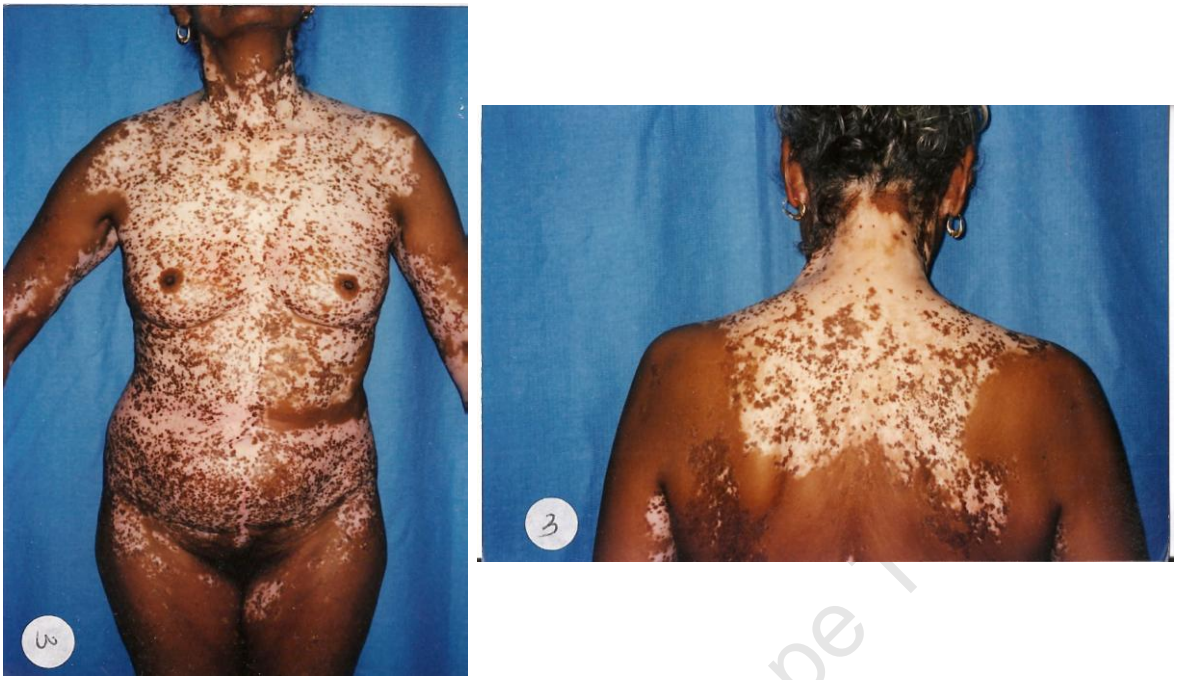


Fig 5. Photographs of Mrs H. taken on 27/09/01

In 2001, following review by the psychiatrists, her antidepressant medication was adjusted and it was decided to continue with her PUVA therapy despite her high accumulative dose of 2910 (visit no. 378). In April 2002, her pigment exploded.

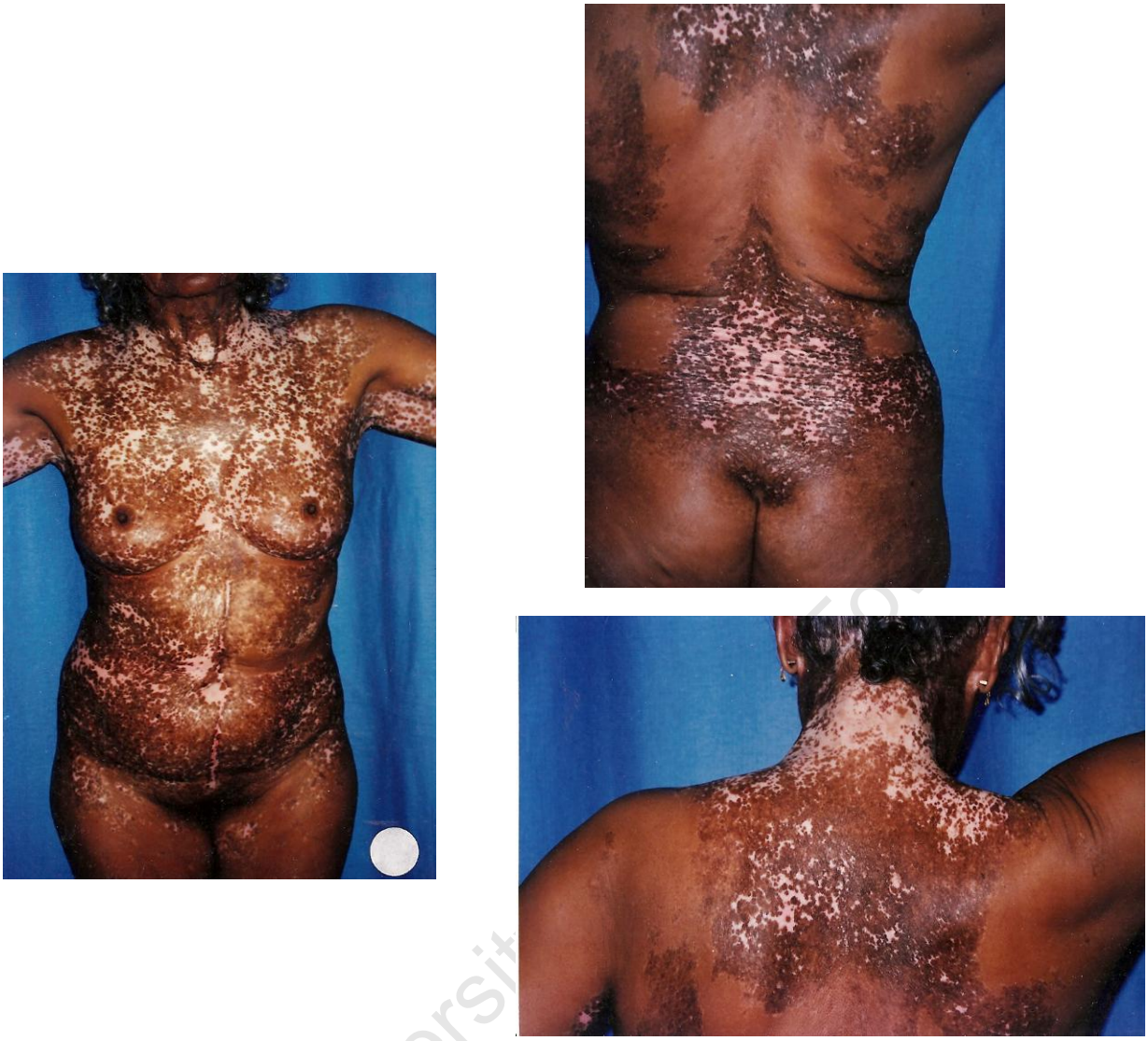


Fig 6. Photographs of Mrs H. taken on 04/04/02

Her clinical course continued to fluctuate with her mental state until in July 2004, it was decided to stop her therapy due to her increasing fragility and age.

This case demonstrates the devastating psychological implications of vitiligo and the striking correlation between the patient's mental state and her response to therapy. It provokes a question as to whether the impact of the state of mind on a patient's therapeutic course has been underestimated. The negative impact of vitiligo on the patient's quality of life and mental well-being can be exacerbated by treatment failure. A prognostic test to identify those patients likely to be good candidates for therapy and those who are unlikely to respond would avoid the despondency implicit in a poor response to treatment. This study aims to develop such a test.

1.2 Melanocyte Biology

1.2.1 Embryology

The melanocyte is a neural crest-derived cell that migrates via the mesenchyme into the epidermis and hair follicles during embryogenesis (Li and Urmacher, 2007). The immediate unpigmented precursors of melanocytes are called melanoblasts. Other sites of melanocyte migration include the uveal tract of the eye, the leptomeninges and the inner ear (Tobin, 2006). As can be seen in Figure 7, melanocytes can be found in both the basal layer of the hair matrix where they actively produce melanin as well as in the outer root sheath where they remain amelanotic (Tobin and Bystryn, 1996).

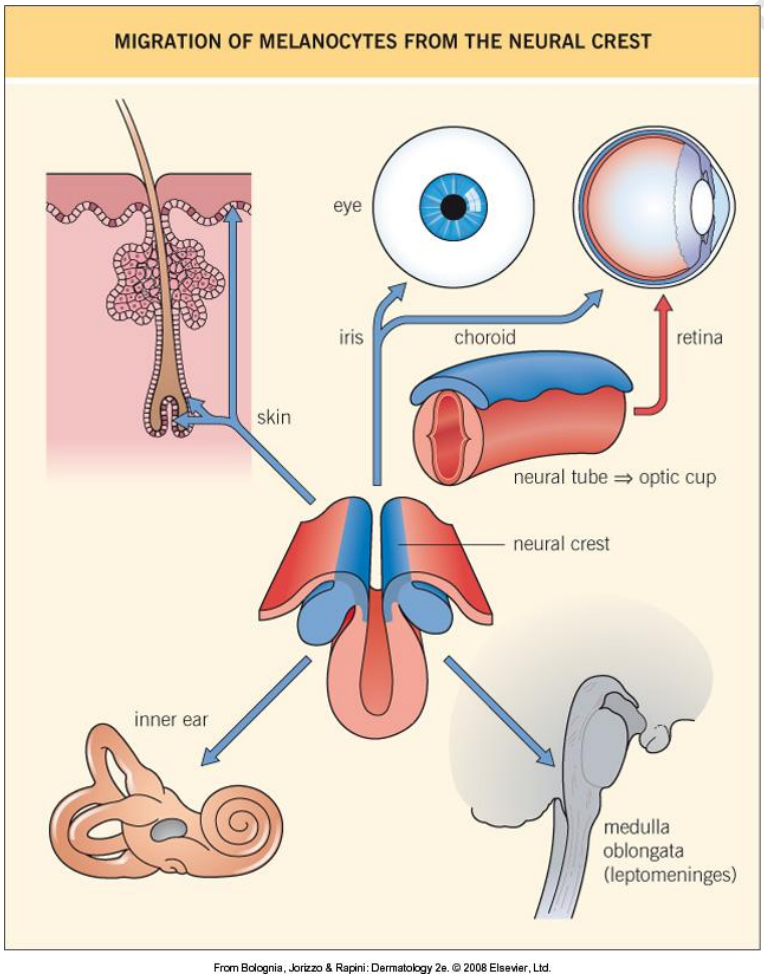


Fig 7. Melanocyte migration from the neural crest (Bologna and Orlow, 2008)

Melanocytes can be identified within the fetal epidermis by immunohistochemical staining as early as 50 days of gestation. By electron microscopy, melanin-containing melanosomes can be seen during the fourth month of gestation (Li and Urmacher, 2007). During embryogenesis, melanin-producing melanocytes can also be found diffusely throughout the dermis, appearing initially at ten weeks of gestation in the head and neck region (Holbrook et al., 1989). By birth, however, active dermal melanocytes remain only in the anatomic sites of the head and neck, the dorsal aspects of the distal extremities and the presacral area (Sagebiel and Odland, 1972). In the epidermis, melanocytes reside within the basal layer but their dendrites come into contact with keratinocytes as far away as the mid stratum spinosum (Figure 2). The epidermal melanin unit describes this association of a melanocyte with approximately 30-40 surrounding keratinocytes to which it transfers melanosomes.

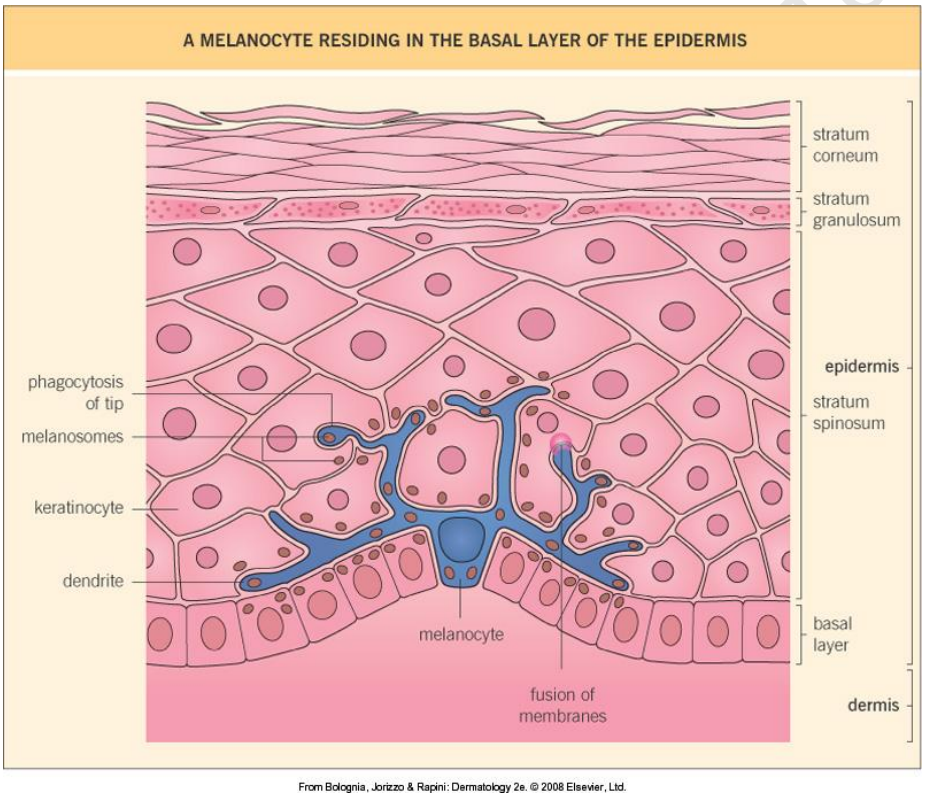


Fig 8. The epidermal melanin unit (Bolognia and Orlow, 2008)

1.2.2 Melanosome Formation

The melanosome is a unique organelle within the cytoplasm of the melanocyte which protects the melanocyte from melanin precursors (e.g. phenols, quinines) which can lead to lipid peroxidation. The melanosome contains both proteins regulating melanin biosynthesis as well as matrix proteins which form a scaffold upon which the melanin is deposited (Orlow, 1995). Several of the enzymes involved in melanogenesis undergo glycosylation and processing within the endoplasmic reticulum and Golgi apparatus and are then packaged into vesicles after which they combine with the matrix proteins (Thody, 1995). Melanosome formation can be divided into four stages from stage I where the melanosome is spherical and amelanotic to stage IV where the melanosome is oval with a heavy deposition of melanin. This process is under tight regulation and stimulated by environmental changes such as exposure to ultraviolet light. As melanin is deposited within the melanosome, it migrates via microtubules and by means of kinesin and dynein proteins into the dendrites (Hara et al., 2000). The melanosomes are then transferred to the neighbouring keratinocytes, either within the epidermis or within the anagen hair matrix (Seiberg et al., 2000). They form supranuclear caps over the keratinocyte nuclei in order to protect the nuclear DNA against solar ultraviolet radiation (UVR) (Jimbow et al., 1992). Normal skin colour is determined predominantly by melanocyte activity and not by their density. Melanocyte activity relates to the number of melanized melanosomes produced as well as the efficiency of their transfer to the neighbouring keratinocytes. Lightly pigmented skin has fewer and mainly stage II and III melanosomes whereas darkly pigmented skin contains a greater number of primarily stage IV melanosomes (Kadekaro et al., 2003).

1.2.3 Melanogenesis and Melanin

There are three types of melanin in mammals: brown, slightly soluble, intermediate molecular weight (MW) eumelanin, black, insoluble, high MW eumelanin and the reddish-yellow alkali-soluble, low MW phaeomelanin (Jimbow et al., 1976).

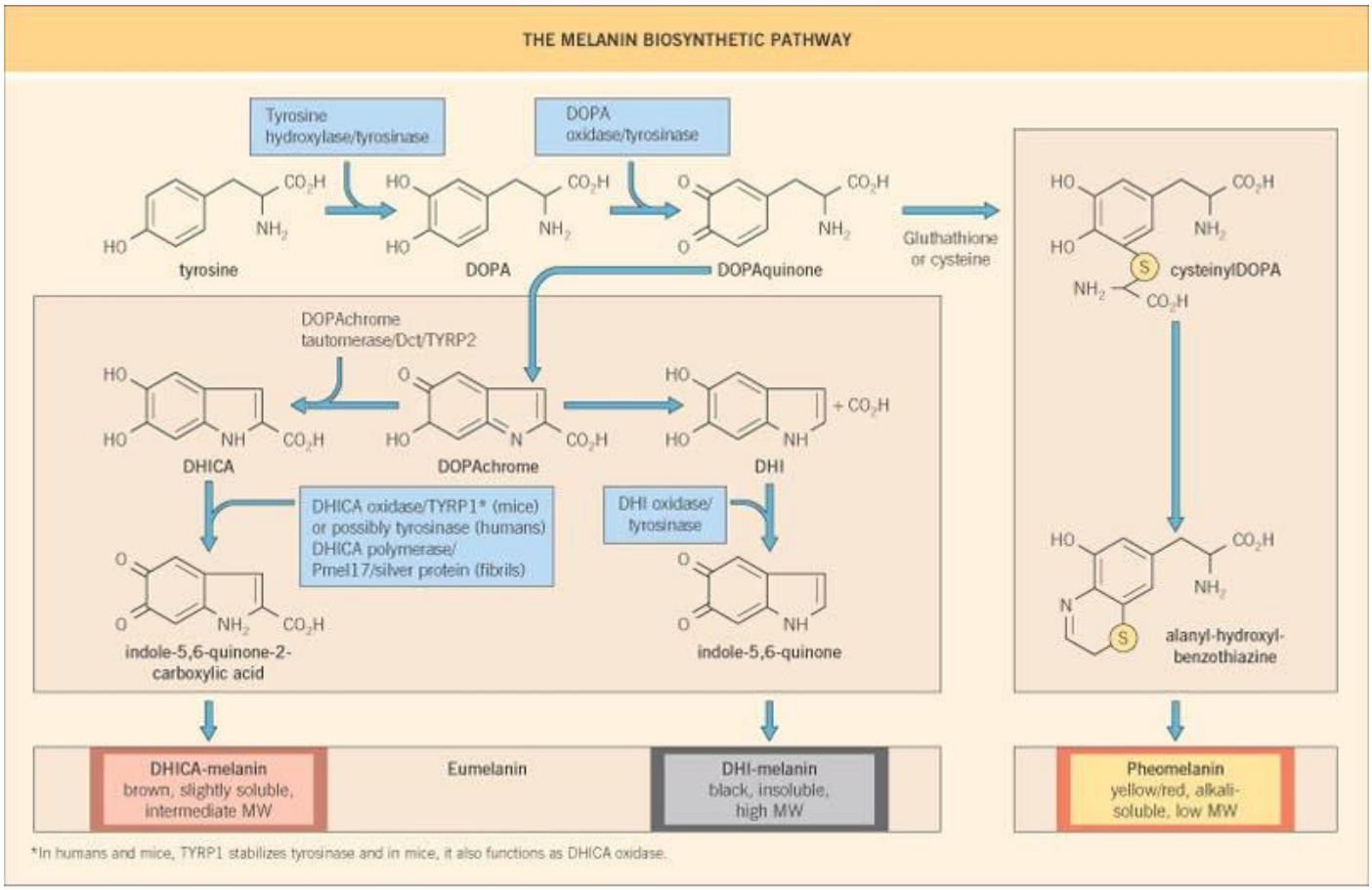
The synthesis of melanin can be divided into the following steps (Figure 3):

1.) The hydroxylation of L-tyrosine to L-dopa by the tyrosinase enzyme

2.) The oxidation of L-dopa to dopaquinone by tyrosinase – this is the precursor for both eu/pheomelanins and is the limiting step in melanogenesis

3.) The oxidation of DHI (dihydroxindole) to yield melanin pigment

Both eu/pheomelanogenesis require the oxidation of dopa to dopaquinone. Thereafter, the conversion of dopaquinone to leuko-dopachrome signals eumelanin production, while the addition of cysteine to dopaquinone to yield cysteinyldopa occurs in pheomelanin production (Kobayashi et al., 1995). In oculocutaneous albinism type 1A where there are mutations in both copies of the tyrosinase gene and thus complete loss of enzyme activity, the hair, skin and eyes contain no melanin pigment. In oculocutaneous albinism type 1B, however, where there is decreased enzyme activity, pheomelanin is produced (leading to the now obsolete term of 'yellow mutants') (Mange et al., 2000). As less tyrosinase activity is required for pheomelanogenesis compared to eumelanogenesis, the formation of pheomelanin can be thought of as a default pathway.



(Courtesy of Dr Vincent Hearing.)

Fig 9. The melanin biosynthetic pathway (Bolognia and Orlow, 2008)

Melanins are group of complex polymers which function to camouflage as well as to quench the oxidative free radicals generated via exposure to UVR. The type and amount of melanin production is a complex interplay of the various melanogenic enzymes' activities as well as the activity of proteins such as tyrosinase-related protein 1(TRP-1) which stabilize the activity of tyrosinase and the P protein. Several factors regulate the activity of these key melanogenic proteins including melanocyte stimulating hormone (MSH), basic fibroblast growth factor (bFGF), endothelin-1 and UV light (Luger et al., 1997; Gilchrest et al., 1996).

1.2.4 Melanogenic Proteins

The key regulatory and rate-limiting enzyme in the melanogenic pathway is tyrosinase which controls tyrosine hydroxylation, the initial biochemical reaction in this pathway. This is the rate-limiting step as the remainder of the pathway can proceed spontaneously at physiological pH (Hearing et al., 1991). Tyrosinase also catalyzes the additional steps of dopa oxidation as well as the oxidation of 5,6-dihydroxyindole to indole-5,6-quinone. Dopachrome tautomerase, also known as tyrosinase-related protein 2 (TRP-2) because of the similarity of its amino acid sequence to tyrosinase, converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Fang et al., 2001). Another melanogenic protein, tyrosinase-related protein 1 (TRP-1) is involved in the stabilization of tyrosinase (Del Marmol et al., 1993). A fourth transmembrane protein, P protein, is involved in the transport of small molecules across the melanosome membrane and regulates the processing and trafficking of tyrosinase, possibly via the control of pH or glutathione content within intracellular compartments (Abdel-Malek et al., 1993). Two of the melanogenic proteins pertinent to this study, namely tyrosinase and TRP-2, require further elaboration.

1.2.4.1 Tyrosinase

Tyrosinase is a copper requiring metalloenzyme with two putative copper-binding sites and is regarded as the rate-limiting enzyme for the melanin biosynthetic pathway described above. Thus, in patients with Menkes kinky hair disease which is characterized by a dysfunctional transmembrane copper-transporting ATPase, there is hypopigmentation of the hair. The human tyrosinase gene has been mapped to chromosome 11(q14-q21) and is comprised of five exons and four introns spanning a distance of over 65 kb (Giebel et al., 1991). This type I membrane glycoprotein is synthesized as a nascent 60-kDa polypeptide which then undergoes glycosylation in the endoplasmic reticulum (ER) to produce a 70-kDa polypeptide (Halaban et al., 2000). This glycosylation of tyrosinase is imperative for its translocation to premelanosomes (Imokawa et al., 1982). It is then targeted to the Golgi apparatus where it undergoes further modifications forming the 80-kDa mature wild-type (WT) isoform. The mature tyrosinase enzyme then proceeds to the melanosome where it is deposited on the melanosomal matrix (Setaluri, 2000).

Hydroquinone, used as a depigmenting agent in the treatment of melasma is a competitive inhibitor of tyrosinase activity (Bolognia and Pawelek, 1988). Another competitive inhibitor is L-phenylalanine and in phenylketonuria where there is a defect in the enzyme L-phenylalanine hydroxylase converting L-phenylalanine to L-tyrosine resulting in elevated levels of L-phenylalanine, a diffuse pigmentary dilution is evident (Bolognia and Pawelek, 1988).

1.2.4.2 Tyrosinase-Related Protein 2

Original descriptions of the melanogenic pathway reported tyrosinase as the only involved enzyme due to the fact that in vitro, L-dopa can spontaneously oxidize to form melanin. However, by the late 1970s, the role of TRP-1 was recognized and 1984 saw a breakthrough in the recognition of a new enzyme which catalyses the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid by means of a keto-enolic tautomerization. This enzyme was thus initially named dopachrome tautomerase and later, due to its sequence homology to tyrosinase, it was called tyrosinase-related protein 2. It has been mapped to chromosome 13q32 (Yokoyama et al., 1994). Steel et al., (1992) developed a probe derived from TRP-2 and were able to detect migratory melanoblasts following their emergence from the neural crest as early as 10 days post coitum in mice. This enzyme is therefore thought to be the earliest known melanocyte-specific marker capable of detecting even undifferentiated melanocytes and is therefore the most suitable marker of melanoblasts discovered to date.

1.3 Vitiligo

Vitiligo is a common depigmenting skin disorder that affects approximately 1% of the world's population and often has devastating psychological implications. The word 'vitiligo' has its origins from the Latin word 'vitulum' meaning 'small blemish' (Nordlund and Ortonne, 1998) and is characterised by well-circumscribed, irregularly shaped, white cutaneous macules (Ortonne, 2008). This leukoderma is due to the loss or decrease of functional pigment-producing melanocytes from otherwise healthy looking skin (Jimbow, 1998). This disorder is classified according to its extent and distribution into generalised and localised forms. The generalised form may be termed vitiligo vulgaris, where symmetrical, scattered macules involve the whole body or vitiligo universalis in which there is > 80% depigmentation. The localised form may be focal or may be termed segmental where macules are seen in a quasi-dermatomal distribution (Mason and Gawkrodger,

2005). There have been no reports on the prevalence of vitiligo in South Africa, but dermatologists report on high numbers of patients seeking therapy for this disfiguring disorder.

1.3.1 Aetiopathogenesis of Vitiligo

Although there are numerous theories regarding the pathogenesis of vitiligo, the cause remains unknown. The numerous clinical manifestations of the disorder suggest a multifactorial interplay of factors leading to melanocyte destruction.

Pigment cell research on the biology of melanocytes and melanogenesis during the 1960s and 1970s resulted in the development of three general theories regarding the aetiopathogenesis of vitiligo. These have subsequently been superceded by convergence theories.

1.3.1.1 The Autoimmune Theory

The autoimmune hypothesis proposes that melanocytes are destroyed due to alterations in humoral or cellular immunity.

The theory of dysfunctional **humoral** immunity is based on the association of vitiligo with various autoimmune syndromes and originated in Thomas Addison's report of the association of adrenal failure with vitiligo in 1855. The incidence of vitiligo is 10-15 times greater in patients with autoimmune disease, with 30% of patients being affected with at least one additional autoimmune disorder. Furthermore, these autoimmune diseases occurred at an increased frequency in the first-degree relatives of vitiligo patients. Similarly, in multiplex generalised vitiligo families, higher frequencies of psoriasis, rheumatoid arthritis and type I diabetes mellitus were noted in addition to autoimmune thyroid disease, Addisons's disease, systemic lupus erythematous and pernicious anaemia (Rezaei et al., 2007). It has also been reported that vitiligo patients have an increased prevalence of autoantibodies to endocrine organs without manifesting clinical disease (Auer-Grumbach and Stangl, 1993).

The most significant advance in promotion of this theory was the demonstration of specific antibodies to melanocyte cell-surface antigens present in the blood circulation of vitiligo patients. These antibodies were found initially in the sera of patients with vitiligo and mucocutaneous

candidiasis but were felt to be a non-specific marker of immune dysregulation (Nordlund et al., 1981). Naughton et al., (1983) published the first report to document convincingly the presence of antimelanocyte antibodies in vitiligo patients by means of immunoprecipitation. In further studies (Naughton et al., 1986), they found that the antibody levels were elevated more in actively progressing vitiligo as well as in patients with co-morbid autoimmune diseases. Further evidence of these antimelanocyte antibodies were provided by indirect immunofluorescence studies (Bystryn and Naughton, 1985), immunoblotting, enzyme-linked immunoabsorbent assays (ELISA) (Harning et al., 1991; Baharav et al., 1996) and by means of passive transfer experiments (Gilhar et al., 1995; Kemp et al., 1997; Kemp et al., 1998). In 1988, Norris et al., proved the functional significance of these autoantibodies by demonstrating that sera from vitiligo patients were cytotoxic for melanocytes both by complement lysis and by antibody-dependent cell-mediated cytotoxicity. The significance of these autoantibodies in vitiligo, however, is controversial. Some researchers have proposed that these antibodies are formed secondary to primary melanocyte destruction by other mechanisms (Halaban and Moellmann, 1993). The identification of antibodies to tyrosinase, an intracellular antigen, in 61% of patients with vitiligo suggests that the contents of a damaged melanocyte are released into the circulation, initiating an immune response (Song et al., 1994). Autoantibodies directed against TRP-1 and TRP-2 as well as against two transcription factors SOX9 and SOX10 and the melanin-concentrating hormone receptor-1 have also been identified (Kemp et al., 1998). Other authors propose that these antibodies are the markers of vitiligo rather than the cause of it. The role of humoral immunity in vitiligo is further supported by a study demonstrating the destruction of melanocytes from normal skin engrafted on to nude mice injected with vitiligo patient sera (Ongenae et al., 2003).

Cellular immunity has also been strongly implicated in the pathogenesis of vitiligo (Palermo et al., 2001). T cells infiltrating perilesional epidermis are mostly $CD8^+$ T cells expressing the cutaneous lymphocyte-associated antigen (CLA) skin homing receptor. These cells are concentrated in the vicinity of disappearing melanocytes and express perforin and granzyme B suggesting that they play a role in melanocyte destruction. In addition, skin-homing, melanocyte-specific cytotoxic T lymphocytes (CTLs) have been found in the peripheral blood of vitiligo patients demonstrating specific cytotoxic responses against Melan-A/MART-1, tyrosinase and gp100. The presence of these CTLs has also been shown to be related to disease activity. These findings support the hypothesis of vitiligo being a $CD8^+$ T-cell mediated autoimmune disease (Mandelcorn-Monson et al., 2003).

Several immunogenetic factors predisposing patients to autoimmune disorders have been associated with vitiligo, adding credence to the theory that the disease may have an autoimmune component (Passeron and Ortonne, 2005). An association between major histocompatibility complex (MHC) class II human leucocyte antigen (HLA) alleles and vitiligo, particularly HLA-DR4 has been found. Other studies have revealed an association between vitiligo and HLA-DRB1*03, HLA-DRB1*04 and HLA-DRB1*07 alleles in Turkish patients (Tastan et al., 2004) and with HLA-DRB4*0101 and HLA-DRBa*0303 alleles in Dutch patients (Venneker et al., 1993). An association between several genes involved in antigen presentation including the *TAP-1* gene has also been found (Zhang et al., 2005).

Other factors such as an increased expression of intercellular adhesion molecule-1 (ICAM-1) involved in antigen presentation by perilesional melanocytes as well as the repigmentation of patients with immunosuppressive agents such as corticosteroids provide further support for the autoimmune hypothesis (Van den Wijngaard et al., 2000). An increased vulnerability to attack by complement due to an aberrant expression of membrane regulators of complement activation including decay acceleration factor and membrane cofactor protein in lesional melanocytes and keratinocytes has also been cited as contributing to the abnormal cellular immune responses against pigment cells (Van den Wijngaard et al., 2002).

1.3.1.2 The Neural Hypothesis

The neural hypothesis advocates that a neural dysfunction is responsible for the depigmentation seen in vitiligo and was initially based on anecdotal observations that stress may precipitate vitiligo. This hypothesis also rests on the feeble embryological fact that both melanocytes and neural cells are derived from the neural crest and that both cells types use the amino acid tyrosine for their major end products namely melanin and catechols respectively. The distribution of segmental vitiligo lesions in a quasi-dermatomal distribution has also been used as a validating fact (Nordlund and Ortonne, 1998). In addition, mild degenerative changes shown in a small proportion of axons and Schwann cells of nerves supplying vitiliginous lesions have been hypothesised to disturb the function of the melanocytes in these lesions (Al'Abadie et al., 1995). Hara et al., (1996) propose that these degenerative changes lead to an increase in neurotransmitters released from the nerve-endings at levels which are toxic to melanocytes. Segmental vitiligo has also been associated with a

dysfunction of the cholinergic sympathetic nerves and increased adrenergic tone in the affected skin (Wu et al., 2000). The discovery of the role of neuropeptides in melanocyte differentiation (dendricity) and in melanogenesis boosts this hypothesis. An increased immunoreactivity of neuropeptide Y as well as an altered balance of nerve growth factor receptors and calcitonin gene-related peptide has also been seen in vitiligo skin. In addition, vitiligo skin has been shown to have increased catechol-*o*–ethyltransferase and monoamino oxidase activities and an increased expression of β_2-adrenoreceptors which has been hypothesised to induce melanocyte dysfunction and injury by promoting the production of melanocytotoxic compounds and be decreasing the anti-oxidant capacities of melanocytes. Apart from this direct cytotoxic effect, these catecholamines could also cause indirect damage by activating α-receptors of skin arterioles leading to severe vasoconstriction. Severe or repeated hypoxic attacks lead to the production of toxic ROS resulting in depigmentation (Hsin-Su, 2002). Currently, however, the role of the nervous system in vitiligo, if any, is ill-defined.

1.3.1.3 The Autocytotoxic Hypothesis

The self-destruction hypothesis describes a toxin-mediated impairment of melanocytes suggesting that the leukoderma in vitiligo results from melanocyte destruction by toxic intermediates of melanin. It hypothesises that tyrosine analogues and intermediates including DOPA, DOPAchrome and 5,6-dihydroxyindole in melanin synthesis are toxic to melanocytes. Melanocytes have a protective mechanism leading to the successful elimination of toxic intermediates. Any disruption of this process leads to toxin accumulation, free radical generation and melanocyte destruction. This theory was first proposed due to the observation that hyperpigmented skin including periorificial skin seemed to be more susceptible to vitiligo than lighter skin (Lerner, 1971). The theory was further supported by the chemical leukoderma induced by hydroquinone derivatives like monobenzone which have been proposed to disrupt melanogenesis leading to the excessive production or leakage of toxic intermediates into the cytoplasm of the melanocyte and subsequent cytolysis (Cummings and Nordlund, 1995).

1.3.1.4 The Genetic Hypothesis

Both family and twin studies implicate genetic susceptibility to vitiligo. These studies indicate that the inheritance of vitiligo is characterised by genetic heterogeneity, multiple susceptibility loci and

incomplete penetrance rather than by simple Mendelian genetics. No clear association of vitiligo with any particular genetic locus has been found (Majumder et al., 1993). A number of candidate genes for vitiligo including *AIS1, AIS2, AIS3* and *PTPN22* however, have been proposed. In addition, several genome-wide linkage analyses have been performed by Spritz et al., (2007) and mutations in the *NALP1,* a gene on chromosome 17p has been found. This vitiligo susceptibility gene encodes *SLEV1,* a protein identified in families with vitiligo-related systemic lupus erythematosus and is a component of inflammasomes which regulate the activity of caspases.

1.3.1.5 The Oxidative Stress Theory

Schallreuter and Pittelkow, (1991) described a complex biochemical imbalance between keratinocytes and melanocytes resulting in an impaired anti-oxidant capacity as well as an increase in the levels or reactive oxygen species (ROS) in vitiligo patients. They demonstrated that keratinocytes cultured from vitiliginous skin had a low catalase activity resulting in an increase in hydrogen peroxide which in turn inhibited tyrosinase activity. Such high levels of epidermal hydrogen peroxide have been confirmed *in vivo* by non-invasive Fourier transform Raman spectroscopy of vitiligo skin. They also described an increased calcium uptake in these cultured keratinocytes leading to an inhibition of the activity of the thioredoxin reductase antioxidant (Schallreuter and Pittelkow, 1988). Other studies corroborating this theory showed a decrease in other anti-oxidants such as vitamin E, glutathione and total ubiquinone in vitiligo patients compared to healthy controls (Passi et al., 1998; Maresca et al., 1997). Red blood cells and peripheral lymphocytes as well as melanocytes in vitiligo patients have been shown to have an altered antioxidant pattern (decreased catalase and glutathione peroxidase activity, increased superoxide dismutase and xanthine oxidase activity) associated with increased lipid peroxidation (as demonstrated by a high level of malonylmaldehyde and decreased membrane polyunsaturated fatty acids) (Dell'Anna et al., 2001; Agrawal et al., 2004). The consequences of this elevated ROS production include oxidation of DNA bases and peroxidation of both intracellular and cytoplasmic membranes (Giovanelli et al., 2004). This in turn can result in cell membrane damage with loss or decreased activity of enzyme receptor sites (Kroll et al., 2005).

1.3.1.6 Reduced Melanocyte Survival

It has been proposed that the reduced melanocyte survival seen in vitiligo patients occurs as a result of a deficiency of factors imperative for their maintenance (Kitamura et al., 2004). The stem cell factor (SCF)/KIT/microphthalmia-associated transcription factor (MITF)/Bcl-2 pathway, in particular, has been implicated. Vitiligo keratinocytes have been shown to have a lower expression of keratinocyte-derived factors including SCF and MITF-M protein expression within the peri-lesional epidermis has been shown to be downregulated. This suggests that a dysfunction of the SCF/KIT/MITF survival pathway within melanocytes leads to apoptosis.

1.3.1.7 Transepidermal Melanocytorrhagy

A chronic detachment and transepidermal loss of melanocytes in patients with generalised vitiligo has recently been described in an *in vivo* study (Gauthier et al., 2003). This study theorises that mechanical stress leads to the detachment of melanocytes followed by a process of elimination via a passive transepidermal migration. This theory is supported by the observations of vitiligo occurring more frequently in sites predisposed to friction or excessive movement including the hands, knees and below the bra strap.

1.3.1.8 The Convergence Theory

The convergence theory (illustrated in Figure 10) reconciles the genetic, immune-mediated, auto-cytotoxic and neuronal hypotheses (Le Poole and Boissy, 1997; Passeron and Ortonne, 2005; Taieb, 2000). In 2005, a study by Le Poole et al., proposed a temporal sequence between the immune-mediated and toxic-mediated damage to melanocytes. They suggested that an immune response in the form of a specific cytotoxic T-cell reaction is mounted due to primitive intrinsic damage of the melanocytes resulting in the first evidence of depigmentation. *In vitro* evidence of this intrinsic 'fragility' of the melanocytes was provided by reports of a genetic polymorphism resulting in an elevated production of toxic intermediates such as 6- and 7-BH_4 (6- and 7- tetrahydrobiopterin) damaging the fragile melanocytes. These compounds lead to an inhibition of the antioxidant enzymes and of melanin synthesis as well as to an increased production of catecholamines with a subsequent process favouring oxidative stress. In addition, stressed melanocytes have been found to

activate dendritic cells with the resulting dendritic cell effector functions playing a role in the melanocyte destruction (Hsin-Su, 2002).

It is also imperative to note that other studies have focused on the role of keratinocytes and Langerhans cells in vitiligo. It has been noted that the keratinocytes in adjacent normal-appearing skin as well as perilesional skin are abnormal with cytoplasmic vacuolization and extracellular granular material can also be seen indicating keratinocyte damage (Moellmann et al., 1982). Degenerative changes and functional impairment have also been observed in Langerhans cells. This suggests that the entire keratinocyte-Langerhans cell-melanocyte unit is affected in vitiligo. An important study conducted by Bondanza et al., (2007) suggests that vitiligo may be a disorder primarily of keratinocytes resulting in melanocyte damage. They found that almost all involved keratinocytes in vitiligo patients had a decreased life span in vitro compared to uninvolved keratinocytes. They also found that they were unable to maintain melanocytes in culture in a physiological ratio and that there was an impaired senescence process in lesional keratinocytes with dysfunctional regulation.

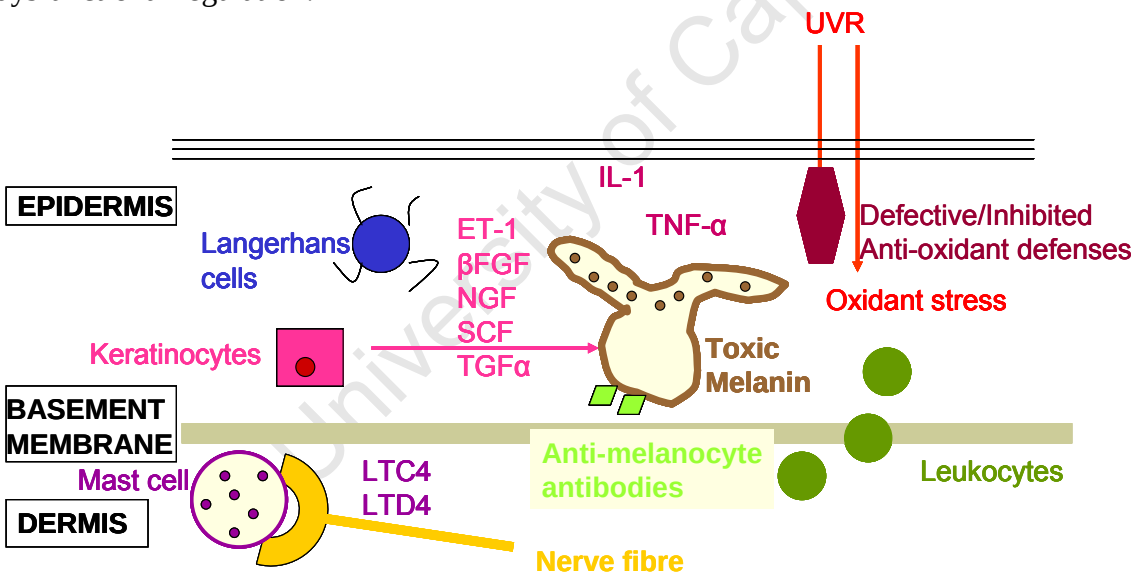


Fig 10. Summary of the convergence theory. Growth factors, ROS, antibodies, cytokines, T cells and inflammatory mediators combine to produce melanocyte destruction. Exogenous influences including ultraviolet light radiation (UVR), neural stimulation and cytotoxic lymphocytes and antibodies converge to influence melanocyte viability. (ET-1 = endothelin-1; βFGF = basic fibroblast growth factor; NGF = nerve growth factor; SCF = stem cell factor; TGF-α= transforming growth factor-α; IL-1 = interleukin-1; TNF-α= tumour necrosis factor-α; LTC4= leukotriene C4; LTD4=leukotriene D4)

1.3.2 Melanocyte Survival in Vitiligo and Repigmentation

The fundamental question of what exactly is happening to the melanocytes in vitiligo remains unresolved. Although evidence strongly suggests that melanocytes die in various forms of vitiligo, it is also evident, at least in certain cases, that melanocytes survive in an undifferentiated stem cell form and it is from these that re-pigmentation can occur (Nordlund and Ortonne, 1998; Gottschalk and Kidson, 2007). Husain et al., (1982) demonstrated the presence of the melanocyte-specific tyrosinase enzyme in vitiliginous skin using a sensitive fluorimetric method as well as by ^{14}C(U)-L-tyrosine incorporation into melanin, thus unequivocally proving survival of melanocytes in vitiliginous skin. A further study confirmed that this tyrosinase enzyme was responsible for the enzymatic hydroxylation of tyrosine to dopa in epidermal homogenates of lesional skin. An ultrastructural study conducted by Bartosik et al., (1998) observed the presence of stage III/IV melanosomes within basal keratinocytes even in vitiliginous lesions of long duration. In 2000, Tobin et al., obtained suction blister tissue from the lesional skin of 12 vitiligo patients and successfully established melanocyte cultures. In addition, light and electron microscopy were utilised to demonstrate the presence of pre-melanosomes in basal and supra-basal keratinocytes of vitiliginous skin as well as the presence of single melanocytes in lesional skin. Lastly, a study conducted by Gottschalk and Kidson in 2007 utilised the sensitive molecular technique of reverse transcription PCR (RT-PCR) using primers for tyrosinase and dopachrome tautomerase followed by Southern blotting to provide evidence of melanocyte survival in the lesional skin of 25% of vitiligo patients(n=12).

This issue of melanocyte survival is complicated by the presence of melanocyte stem cells in the 'niche' of the hair follicle. Stem cells are defined as cells that can generate mature cells through differentiation as well as maintaining themselves through self-renewal (Ohyama, 2007). It is known that melanoblasts from the neural crest destined for the hair follicle can be divided into two populations: differentiated melanocytes localised in the hair matrix region that are responsible for hair colour; and melanocyte stem cells (MSC) which can be found at the lower permanent portion of the hair follicle and which are responsible for the repopulation of pigment cells in the cycling hair (Osawa et al., 2005). Another subpopulation of melanocytes is located in the mid-portion of the hair follicle outer root sheath (the so-called 'bulge' region). Melanocytes in this region are poorly differentiated, unpigmented and are considered to be melanocyte stem cells. Starrico (1994) published the first study demonstrating a reservoir of DOPA-negative melanocytes in the outer root

sheath of hair follicles which could be activated by UV light. In PUVA-treated vitiligo patients, islands of perifollicular repigmentation appear which then extend and coalesce to normalise pigmentation. Thus, the melanocyte repopulation of lesional skin involves the activation and proliferation of these MSCs and their subsequent migration through the interfollicular epidermis (Norris et al., 1994).

1.4 Therapeutic Options for Vitiligo

The objective in the treatment of vitiligo patients is to reverse the depigmentation and to establish normal pigmentation in leukodermic macules. A systematic literature review of vitiligo treatment options reveals mostly uncontrolled pilot studies and case reports, with treatments being evaluated mainly in the short term with few comparative trials. The astonishingly small number of randomised control trials makes evaluation of treatment efficacy difficult and makes it impossible to name one single treatment modality as the most effective for a particular form of vitiligo. Current vitiligo therapies include topical and systemic corticosteroids, oral or topical psoralen plus UVA (PUVA) therapy, khellin plus UVA therapy (KUVA), narrow band (NB) UVB (TLO1) therapy (Hartmann et al., 2004), calcineurin inhibitors (Tjioe et al., 2006), topical calcipotriol, 308nm excimer laser, low energy (632.8nm) helium-neon laser (Lan et al., 2009), focused microphototherapy as well as surgical methods of repigmentation such as minigrafting or suction blisters (Jimbow, 1998). Additional controversial therapies include topical pseudocatalase with NB-UVB and systemic antioxidant therapy. Patients with generalised vitiligo with more than fifty to eighty percent body surface area involvement can opt to use depigmenting agents. A met-analysis of the literature concludes that class 3 gluococorticoids and UVB therapy are the most effective and safest therapies for localised and generalised vitiligo respectively (Njoo et al., 1999).

Repigmentation with medical treatment has been shown to occur in three manners:

1. **Perifollicular:** from the melanocyte reservoir in the outer root sheath of the hair follicle
2. **Marginal:** from the adjacent normal skin
3. **Diffuse:** from either spared epidermal melanocytes or from the reactivation of DOPA-negative melanocytes in the centre of the lesion (Falabella, 1997).

Treatment of vitiligo patients remains, on the whole, unsatisfactory. Spontaneous repigmentation occurs in no more than 15% to 25% of cases. Response is slow and the rate of response is low. The paucity of information regarding melanocyte behaviour, both in the development of vitiligo lesions and in the process of repigmentation following an appropriate stimulus, makes the refining of treatment options difficult.

1.4.1 Topical Corticosteroid Therapy

A light and electron microscopic study of the effects of topical corticosteroid treatment in vitiligo was conducted in 1976. Bleehen (1976) treated ten patients with either 0.1% betamethasone or 0.05% clobetasol priopionate creams and placebo preparations for three months. Biopsies taken from both steroid-treated and control areas were then studied histologically. A marked repopulation of functional melanocytes in the repigmenting vitiliginous skin was seen. These melanocytes were more dendritic and dopa-positive and contained numerous melanosomes of normal size, shape and melanization as compared to the control areas. He concluded that topical corticosteroids can induce repigmentation of lesional skin.

The mechanism of action of topical corticosteroids is hypothesised to be via their immunosuppressive and anti-inflammatory properties thus giving credence to the auto-immune hypothesis of vitiligo. Corticosteroids exert their action by binding to glucocorticoid receptors. This receptor then translocates to the nucleus and forms a dimer which binds to the glucocorticoid response element of the promoter region of steroid-responsive genes. The receptor also directly and indirectly inhibits transcription factors with central roles in the inflammatory response such as nuclear factor-κβ and activating protein-1. This leads to inhibition of pro-inflammatory cytokines, adhesion molecules, inflammatory enzymes and growth factors such as tumour necrosis factor-α, granulocyte-macrophage colony-stimulating factor and several interleukins as well as intercellular adhesion molecule-1 and E-selectin. The inflammatory response is thus dramatically reduced (Boumpas et al., 1993).

Their immunosuppressive effects occur due to a number of factors. Glucocorticosteroids induce a neutrophilia but lead to a reduction of these cells at inflammatory sites by means of inhibiting endothelial adhesion molecule expression and chemoattractants. A decreased IL-2 production and IL-2 gene transcription also leads to decreased T-cell activation leading to decreased proliferation

and function of helper, suppressor and cytotoxic T lymphocytes. Glucocorticoid use also leads to a decreased production and differentiation of the monocyte-macrophage cell lines as well as inhibiting the antigen presentation by dendritic cells to T lymphocytes (Nesbitt LT, 2008).

The 1974 trial of Kandil, using 0.1% betamethasone 17-valerate for four months was the first randomised control double blind trial investigating the efficacy of topical corticosteroids in vitiligo. He found that 6/23 patients experienced 90-100% repigmentation while 3/23 patients showed 25-90% repigmentation using the potent corticosteroid betamethasone. Side effects included hypertrichosis and acne. This limited efficacy of corticosteroids was confirmed by Clayton's study, (1977) using the highly potent clobetasol proprionate as therapy. This trial concluded that only 2/23 patients showed >75% repigmentation while 10/23 patients demonstrated 15-25% repigmentation. He noted that all patients developed skin atrophy following eight weeks of treatment. Another study conducted on Filipino vitiligo patients (n=25) had a remarkably different experience, finding that 88% of patients showed at least 90% repigmentation after six months of treatment with topical clobetasol proprionate cream (Geraldez and Gutierrez, 1987).

The best controlled study to date of topical corticosteroids is that of Westerhof et al., (1999). This study found that the potent topical steroid fluticasone used as monotherapy for nine months induced a mean repigmentation of only 9%, that UVA therapy alone led to a mean repigmentation of 8% while the two treatment modalities together induced a mean repigmentation of 31%. This trial also documented that no steroid atrophy occurred.

There have been numerous trials comparing a potent or highly potent topical steroid with other topical agents including topical pimecrolimus, topical tacrolimus, calcipotriol and PUVAsol. These studies found a comparable degree of repigmentation between clobetasol and topical pimecrolimus (Coskun et al., 2005) and tacrolimus (Lepe et al., 2003). They also found that the combination of topical betamethasone and calcipotriol had a higher efficacy than either agent alone (Kumaran et al., 2006) and noted that the use of clobetasol induced better repigmentation than PUVAsol alone (Khalid et al., 1995). Taken together, one can surmise that topical clobetasol used over two to six months repigments generalized symmetrical types of vitiligo to some degree. A level 1a evidence meta-analysis proved class III corticosteroids such as betamethasone to be the most effective therapy for localised vitiligo with a significant pooled odds ratio for topical class III corticosteroids versus placebo (Grimes, 2005). In the guidelines for vitiligo drawn up by Gawkrodger et al.,

(2008), however, it is noted that the evidence for clinically meaningful repigmentation with topical betamethasone used over a period of four months is weak. It is also important to note that as mid to super high-potency steroids are often required, side-effects such as atrophy can occur and have been observed in 14% of patients treated with class III corticosteroids (Frauman AG, 1996). Irreversible striae have been found to develop on the legs after as little as four months of continuous treatment. Thus topical glucocorticoids are recommended for use on limited lesions or isolated macules. The best response has been seen on thinner skinned areas such as the face while trunk and acral regions often prove resistant to this therapy. A study conducted by Kumari, (1984) (n=75) utilising clobetasol proprionate treatment found that more than 80% of patients with vitiligo of the face and more than 40% of patients with vitiligo on the trunk or limbs demonstrated 90-100% repigmentation over a three year period. He found that this repigmentation continued progressively even following the cessation of treatment.

1.4.2 Topical Khellin and Ultraviolet Light Therapy

Khellin (4,9 – dimethoxy – 7 - methyl – 5H - furo[3,2 - g] - [1] benzopyran – 5 – one) is a furanochrome produced from the seeds of the plant *Ammi visnaga* which is commonly found in the Eastern Mediterranean area and is known in Arabic as 'khella'. Its chemical structure resembles that of psoralen. Khellin was introduced as a vasodilator for the therapy of angina pectoris in the mid-1940's and 1950's and no long-term side effects were reported. The effectiveness of other treatments led to the discontinuation of the use of khellin in cardiac disease (Mandel et al., 1997).

The mechanism of khellin stimulation of melanogenesis is still largely unknown. In vitro studies have demonstrated that khellin stimulates mitosis as well as melanogenesis in human melanocytes and melanoma cells (Carlie et al., 2003). In combination, khellin and UVA have a synergistic mitotic and melanogenic effect. Khellin was consistently found to cause melanocyte proliferation and melanogenesis at concentrations between 0.01-0.5mM, with a peak stimulation at 0.01mM with 250 mJcm^2 UVA. Western blot, Northern blot and RT-PCR analysis showed that the increased melanogenic enzyme activity were not due to increases in gene expression or protein synthesis but rather that UVA treatment causes a significant increase in post-translational melanogenic enzyme glycosylation.

Clinical studies conducted using topical khellin have produced varied results. In 1992, Orecchia et al., evaluated the efficacy of topical khellin in combination with sunlight for vitiligo. Forty-one vitiligo patients entered the study. All patients were treated topically with 2% khellin dissolved in 90% acetone and 10% propylene glycol. As a control, 36/41 patients applied acetone-propylene glycol solution to lesions on the opposite side. In the remaining five patients, lesions on one half of the body were not treated and were shielded from sunlight. The therapy consisted of three treatments per week over a period of four months. No significant difference was noted between the khellin and placebo-treated sides.

In a subsequent, double-blind, vehicle controlled study using artificial UVA and a water/2-propanol/propylene glycol gel formulation, the same group found that repigmentation (>51%) occurred in 11/31 khellin-UVA-treated areas compared to 2/31 vehicle-UVA-treated areas (Orecchia et al., 1999). Repigmentation 26-50% occurred in 8/31 patients and 12/31 patients showed an 11-25% response. Only one patient failed to respond to khellin-WPG plus UVA. They concluded that the vehicle used in their previous study did not adequately facilitate the availability of khellin as there was an evident precipitation of khellin secondary to a rapid evaporation of the solvent following application to the skin. Their new, more appropriate pharmaceutical approach led to promising results. Furthermore, over the six months of therapy, short-term side-effects such as symptomatic erythema or phototoxic reactions were not reported.

Another study conducted by Procaccini et al., (1995) found khellin plus solar irradiation to be ineffective. They evaluated the efficacy of KUVA in the treatment of vitiligo using 3% and 5% khellin in two different vehicles: an oil/water cream and a liquid vehicle 1-methyl-2-pyrrolidinone (PYR). Repigmentation, estimated both clinically and photographically, was found to be similar on vitiligo patches treated with khellin and those treated only with the vehicle.

An important pilot study was carried out in 2003 by Valkova et al., on 33 patients. The efficacy of topical khellin (5% khellin in water/oil emulsion) plus UVA was compared to systemic PUVA therapy in terms of degree of repigmentation, side effects, duration of treatment and total UVA dose. The authors concluded that topical KUVA may effectively induce repigmentation of vitiligo-affected skin areas to a degree comparable to that achieved using systemic PUVA. Topical KUVA was followed by >50% repigmentation in 43.8% of patients. The authors re-iterated the many advantages of KUVA compared with the classical psoralens:

- Decreased photo-toxicity
- Decreased carcinogenesis
- No pigmentation of healthy skin
- No significant systemic side-effects
- Can safely be combined with solar irradiation without the risks of photo-toxic incidents
- Home-based therapy

This unresolved controversy regarding topical khellin efficacy and a need determined by the Groote Schuur Hospital Dermatology department for a safe, home-based therapy for vitiligo for many of their patients unable to utilise hospital based PUVA therapy, led in 1999 to a clinical trial. (Milne et al., Unpublished data). A double-blind, placebo-controlled trail was conducted to assess the efficacy of 5% khellin in aqueous cream combined with solar radiation. Sixty patients were involved in the study which ran over a 12 week period: 31 patients were given 5% khellin in aqueous cream and 29 patients were given aqueous cream only. Khellin was applied 30 mins prior to solar exposure for 15 mins daily, increasing to 30 mins over two weeks. Significant clinical improvement ($p<0.04$) using topical khellin was seen in the facial region but all other body regions showed no statistical difference when compared with placebo. The authors hypothesise that the face responds better than other areas as it has the highest concentration of melanocytes. No systemic side effects were reported and 7/60 patients complained of only temporary cutaneous side effects in the treated areas: four had burning (three khellin, one placebo) and three had itching (one khellin, two placebo).

In terms of KUVA's safety, khellin has been found to have lower photogenotoxicity in yeast compared to 5-methoxypsoralen (5-MOP) used in PUVA (Morliere et al., 1988). In comparison to results obtained with 5-MOP, khellin at a 100 times higher concentration, was found to be genetically inactive using doses of UVA for which 5-MOP was previously shown to exert significant genetic effects. Morliere et al. (1988) found that khellin's affinity for DNA is notably lower than that determined for psoralen derivatives: 5-MOP exhibits a 15 times higher binding constant and more binding sites as compared to khellin. In addition, khellin is unable to induce interstrand cross-links in DNA upon UVA irradiation. Trabalzini et al., (1990) also found an absence of photo-oxidation of DNA after treatment with khellin plus UVA suggesting that khellin does not have a photodynamic effect on DNA. Riccio et al., (1992) examined the photomutagenic

activity of khellin with the Salmonella mutagenicity test. When khellin was assayed with the Salmonella strain TA102, a 79% survival was demonstrated compared to only a 66% survival of the same strain with 8-MOP. The authors thus concluded that khellin has a weak photomutagenic potential and that khellin may be safer than psoralens for clinical use. The fact that khellin does not cause phototoxicity is thought to be due to the fact that khellin is neither a strong generator of reactive oxygen species nor a strong type 1 photodynamic sensitizer. Pan et al., (2001) investigated the mode of the khellin's photodynamic action on furanochromones. They found that khellin is a more stable photosensitizer as compared to 8-methoxypsoralen (8-MOP) also used in PUVA.

All the above-mentioned data support the notion of khellin's safety as a phototherapeutic agent and substantiate khellin's numerous advantages over the classical PUVA therapy.

1.5 Principles of Real-Time RT-PCR

Real-time RT-PCR is a quantitative technique for measuring the mRNA levels of a gene of interest relative to an internal control or housekeeping gene (Freeman et al., 1999). The fundamental principle of PCR is the selective amplification of a specific DNA product through the action of DNA dependent DNA polymerase over a number of cycles of denaturing, annealing and extension of a specific DNA primer in the presence of free nucleotides and a polymerase enzyme. In real-time RT-PCR, fluorescence is measured during each cycle, greatly increasing the dynamic range of the reaction since the fluorescence generated is proportional to the amount of PCR product. PCR products can be detected using either fluorescently labeled sequence-specific probes or fluorescent dyes. The threshold cycle (C_T) is the cycle at which there is a significant detectable increase in fluorescence i.e. the cycle at which the amplification plot crosses the threshold. This threshold cycle can then be used to determine the starting level of the target gene. Comparison of the C_T value of the gene of interest with that of the endogenous reference gene allows the gene expression level of the target gene to be normalized to the amount of input cDNA. The RNA encoding GAPDH is ubiquitously expressed and moderately abundant. It is frequently used as an endogenous control for quantitative RT-PCR because its expression is constant at different times and after experimental manipulation. Real-time RT-PCR has largely superceded methodologies such as semiquantitative and competitive RT-PCR which are reagent and labour intensive as well as being error prone due to the large number of sample manipulations involved. It is currently the most sensitive method for the detection of low-abundance mRNAs (Lekanne et al., 2002).

The qPCR method used in this study involves detection of the binding of the fluorescent SYBR Green dye to DNA. The SYBR Green dye exhibits fluorescence only when bound to double-stranded DNA during the elongation phase, however does not bind to denatured single-stranded DNA (Rajeevan et al., 2001). This property can be exploited to verify the specificity of the qPCR product through the generation of melting curves by means of plotting fluorescence as a function of temperature. This is performed by slowly increasing the temperature to 95ºC and measuring the fluorescence. A curve is produced as at the lower end of the temperature range there is only a slight decrease of fluorescence which then decreases much more quickly at higher temperatures where the melting temperature of the amplicon is reached and the DNA is denatured. The Lightcycler programme calculates the first derivatives of the curves, giving rise to curves with peaks at the respective melting temperatures. As the melting temperature of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product (Bustin, 2000). Curves with peaks at a lower melting temperature than that of the desired PCR product indicate the formation of primer-dimers. Non-specific products can be seen as diverse peaks with different melting temperatures.

1.6 AIMS:

1. To develop an efficient and practical protocol for the homogenisation of small skin biopsies and the extraction of high quality RNA for gene expression analyses

2. To develop a sensitive, reliable, quantitative measure of melanocyte and melanoblast survival in vitiliginous skin biopsies utilizing quantitative RT-PCR

3. To determine whether there is a correlation between the presence of melanocytes/ melanoblasts in lesional skin and the patient's response to either potent topical corticosteroid therapy or khellin therapy

4. To determine whether there is a correlation between the epidemiological profile of vitiligo patients and their treatment outcome

2. CHAPTER 2:

MATERIALS AND METHODS

2.1 Sources of human tissues

2.1.1 Cell lines and culture

The human melanotic (UCT Mel-1) melanoma cell line was obtained from melanomas excised at Groote Schuur Hospital, Cape Town and established by the Department of Clinical Science and Immunology, UCT (Hoal, 1981). The HERMES primary cultured melanocytes were a kind gift from Dr Dorothy Bennet, St Georges University of London. The keratinocyte cell line (HaCaTs) was generously provided by Dr Fusenig, Germany (Boukamp et al., 1988). Both melanoma and keratinocyte cell lines were grown in 100mm petri-dishes (Corning, Acton, USA) with Dulbeco's Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Highveld Biological, SA), 100IU/ml penicillin (Sigma, USA) and 100µg/ml streptomycin (Sigma, USA) and cultured at 37°C under 5% CO_2/95% air and 90% humidity. The medium was changed twice a week until cells were sufficiently confluent for RNA extraction. The melanoma cell lines were used to optimise the RNA extraction and develop the RT-qPCR assay and the keratinocyte cell line was used as a negative control.

2.1.2 Skin tissue samples

2.1.2.1 Skin samples for optimisation of protocols

Human skin tissue samples were obtained from Dr K. Adams, a consultant in the Department of Plastic Surgery, Groote Schuur Hospital, Cape Town. These were surplus skin samples from patients undergoing abdominoplasty procedures. These samples were transported on ice and 4mm^2 punch biopsies were taken using sterile instruments under sterile conditions. These biopsies were immediately placed into cryotubes and flash-frozen in liquid nitrogen. The entire protocol was optimised on these surgical samples prior to obtaining skin samples from vitiligo patients.

2.1.2.2 Skin samples from vitiligo patients

Vitiligo patients were recruited from the Dermatology outpatient department and PUVA clinics at Groote Schuur Hospital, Cape Town. Punch biopsies of 4mm^2 (mean tissue weight of 13.8 mg) were taken from the centre of a vitiligo lesion as well as from the surrounding pigmented skin. The depth of the punch biopsy was 7mm and included subcutaneous fat. Following cleaning of the skin with povidone iodine solution, the skin was infiltrated with 2% lidocaine and adrenaline solution. The punch biopsy was taken from the vitiliginous skin and then from the normal surrounding skin in order to prevent any contamination of the vitiligo sample with melanocytes from normal skin. Samples were removed using sterile forceps and scissors and immediately snap-frozen in a flask of liquid nitrogen. The biopsy samples were then transferred to the liquid nitrogen tank. Pressure and/or a 40% aluminium chloride solution were applied to the biopsy sites in order to gain haemostatic control. Persistent bleeding sites were sutured closed using 4/0 nylon suture material. The biopsy sites were then dressed using gauze dressings. Sutures were removed ten days later.

2.2 Homogenisation of human biopsy specimens

2.2.1 Polytron homogeniser

2.2.1.1 Preparation of the corex tubes

All corex tubes to be used during homogenisation were cleaned in chromic acid overnight, rinsed ten times with tap water and then three times with distilled water. They were then dried in an oven at 80°C for 30 mins. The tubes were then siliconised in order to prevent any tissue or RNA from sticking to the inside of the tube. In a fumehood, the tubes were filled with siliconising fluid (4% dichlorodimethyl silan in CCl_4) and allowed to soak for five to ten minutes. The siliconising fluid was then removed and the tubes baked in an oven at 180°C for two hours. They were allowed to cool and then rinsed three times with distilled water in order to remove any residual salin. Lastly, the tubes were soaked in 0.01% DEPC at 37°C for two hours or overnight, followed by autoclaving to inactivate the DEPC.

2.2.1.2 Cleaning of the Polytron homogeniser

Prior to use, the Polytron homogeniser (Kinematica GmbH., Littau, Switzerland) was washed in Deconex. This entailed submerging the Polytron head in a corex tube filled with Deconex and switching the machine on to ensure thorough cleansing. The homogeniser was then rinsed extensively with dH_2O followed by sterilisation with 70% ethanol. Lastly, the Polytron as well as the work surface, all reagent bottles and pipettes were wiped with RNAse AWAY (Molecular Bio-Products, Inc, San Diego, CA) in order to prevent RNA degradation. This procedure was repeated between each biopsy specimen.

2.2.1.3 Homogenisation of skin biopsy samples

The biopsy samples in their cryotubes were removed from liquid nitrogen and weighed prior to homogenisation. A sterile needle was used to place each biopsy sample into separate siliconized DEPC-treated corex tubes each filled with 1mL of TriPure extraction reagent (Boehringer Mannheim #1667 1665). These corex tubes were kept on ice. The cryotubes were re-weighed and the weight of the biopsy specimen alone was calculated. Each biopsy specimen was then homogenized three times in three second bursts. Between each three second homogenisation, the corex tubes containing the specimens were placed back on ice for five seconds in order to prevent any RNA degradation due to overheating of the samples. Some samples were more resistant to break-down, such as samples from sites such as the back with tough dermal collagen or samples with a thicker stratum corneum. These samples were subjected to further homogenisation with a maximum number of five bursts. It was noted that in samples homogenised more than five times RNA degradation occurred. The homogenised tissue samples in TriPure reagent were then pipetted into a labelled Eppendorf tube and kept on ice. If more than one patient's skin biopsy samples were homogenised at one time, all the vitiliginous skin specimens were biopsied first in order to prevent any contamination with melanocytes from the pigmented skin samples. Quality control experiments analysing the level of carry-over contamination during homogenisation consistently confirmed the efficacy of the cleaning procedure applied between the processing of each sample. The Eppendorf tubes were then incubated at room temperature for five minutes in order to ensure the complete dissociation of nucleoprotein complexes and RNA then extracted as described in 2.3.2.

2.2.2 Tissue breakdown by ball-beating with the Tekniva homogeniser

The cryotube containing the skin biopsy sample was removed from the liquid nitrogen tank and two sterile 8 mm stainless-steel balls (Bearing Man, Maitland, SA) as well as 1 mL of TriPure was added to the tube. This tube was then inserted into the Tekniva homogeniser (Tekniva, Cape Town, South Africa) which was then activated for a 49 second sequence. This comprised seven cycles of a five second 'on' phase and a two second 'rest' phase. The 'on' phase involved the transmission of high-speed (50 Hz), single plain harmonic motion to the stainless-steel ball and subsequent tissue breakdown. The purpose of the 'rest' phase was to avoid overheating of the biopsy samples. The homogenate was then transferred to a labelled eppendorf tube and kept on ice.

2.2.3 Enzymatic breakdown of skin biopsy samples

Skin biopsy specimens were placed in a McCartney bottle with 4 mL of 0.45% trypsin, 100IU/ml penicillin and 100µg/ml streptomycin as well as 500 µl of RNAse inhibitor (RNAsin). The samples were left at 4°C overnight. The following morning, the sample was transferred into a 100mm petri-dish and washed twice with PBS. Using sterile fine forceps, the epidermis was teased apart from the dermis and the dermis discarded. The epidermal fragment was placed into a labelled eppendorf tube containing 50 µl of a 0.02% trypsin and 0.05% EDTA solution as well as 15µl of RNAsin RNAse inhibitor (Promega, USA). The sample was placed on a shaker for four minutes at 37°C. Finally, 1 mL of TriPure was added.

2.3 Total RNA Isolation using Tripure reagent

All glassware was autoclaved before use, sterile disposable plasticware and pipettes/tips reserved for RNA work only was used and gloves were used at all times. This RNA extraction method utilised the TriPure isolation reagent, a monophasic solution of phenol and guanidine thiocyanate, in an enhancement of the single-step RNA isolation method developed by Chomczynski and Sacchi, (1987).

2.3.1 Total Cellular RNA Isolation

RNA was extracted from confluent 100mm petri-dishes of MEL-1 melanoma cell lines, HERMES primary melanocyte cultures and HaCaT keratinocyte cell lines. Medium was decanted from the petri-dishes and the cells were rinsed with 10 mL of PBS. This was followed by the addition of 1 mL trypsin/EDTA to disrupt the intercellular bonds and incubation at 37ºC for three minutes to facilitate cellular degradation as evidenced by rounding up of the cells. Another 1 mL of medium containing FCS was added to inactivate the trypsin followed by gentle trituration to create a single cell suspension. Cell suspensions were collected in 15ml tubes and centrifuged at 3000 rpm for five minutes at room temperature. The supernatants were discarded and the pellets resuspended in PBS, centrifuged again at 3000 rpm for five minutes and finally resuspended in 1ml cold TriPure RNA extraction reagent. The cell lysate was transferred to a 1.5ml Eppendorf tube and allowed to stand at room temperature for five minutes. The protocol continued as described below.

2.3.2 RNA isolation protocol for both skin tissue samples and cells

Following incubation of samples in TriPure reagent at room temperature, 200 µL of chloroform (Merck, Germany) was added to precipitate proteins and lipids. The tubes were vortexed for 20 seconds, allowed to stand at room temperature for a further 15 minutes, then followed by 15 minutes of being centrifuged at 12 000g at 4ºC in order to separate the solution into three phases. A volume of 400 µL of the upper clear aqueous phase was removed and transferred to clean 1.5 mL Eppendorf tubes. The RNA was then precipitated from this colourless aqueous phase by the addition of 500 µL of cold isopropanol (Merck, Germany) followed by overnight precipitation at -20ºC. The following morning, the samples were centrifuged at 12 000g at 4ºC for ten minutes and the supernatant discarded. Pellets were washed with 1 mL of 75% ethanol, vortexed and re-centrifuged at 12 000g at 4ºC for a further ten minutes. The ethanol was removed and the excess ethanol removed from the RNA pellet by means of vacuum-drying for five minutes followed by 15 minutes of air-drying and resuspension in 10 µL of distilled water. The RNA was quantified at A_{260} and purity determined with the $A_{260/280}$ ratio using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

2.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A mix containing 1 µg total RNA and 21 pmol/µL oligo$(dT)_{15}$ (Colter Group, UK) made up to a volume of 8.5 µL with distilled water was incubated at 70ºC for five minutes in a Hybaid PCR-Sprint thermal cycler (Hybaid, UK). The samples were then transferred to slushy ice for five minutes, followed by the addition of a reaction mixture containing 5mM of four deoxynucleotide triphosphates (dNTP) (ABgene, USA), 25mM $MgCl_2$ (ABgene, USA), 5 x Maloney-Murine leukaemia virus (MMLV) reverse transciptase buffer solution (Promega, USA), 200U/µL of reverse transcriptase (Maloney-Murine leukaemia virus; Promega, USA) and 40U/µL RNAsin RNAse Inhibitor (Promega, USA) to make up a total volume of 20 µL. Complementary DNA was then synthesised in the Hybaid PCR-Sprint thermal cycler at 42ºC for one hour and the cDNA stored at -20ºC. For all reactions, a negative control was included in order to verify a lack of amplicons. This was done by replacing the RNA template of the reverse transcriptase reaction with distilled water.

2.5 Quantitative Real-time Polymerase Chain Reaction (qPCR)

The polymerase chain reaction (20 µL total volume) was performed as follows: The 18 µL PCR-mix consisted of 10.4 µL of distilled water, 1.6 µL $MgCl_2$ (25 mM), 2 µL each of forward and reverse primers and 2 µL of SYBR Green (LightCycler DNA Master SYBR Green I kit, Roche, Mannheim, Germany). The primers (Whitehead Scientific, South Africa) used included:

- 5 µM GAPDH forward primer – 5’ GAAGGCTGGGGCTCATTT 3’
- 5µM GAPDH reverse primer – 5’ CAGGAGGCATTGCTGATGAT 3’
- 5 µM Tyrosinase forward primer – 5’ GTCTTTATGCAATGGAACGC 3’
- 5 µM Tyrosinase reverse primer – 5’ AGGCATTGTGCATGCTGCTT 3’
- 5µM TRP-2 forward primer – 5’ GAGGTGCGAGCCGACACAAG 3’
- 5 µM TRP-2 reverse primer – 5’ CGCTGCCAGGTAACAAATGC 3’

This PCR-mix was mixed and transferred to a LightCycler capillary to which 2 µL of the prepared cDNA was added followed by a short pulse of ten seconds. The capillaries were then transferred into the LightCycler Sample Carousel (LightCycler Faststart 2.0, Roche).

The PCR reaction was carried out under the following conditions for the first cohort of patients: one cycle of denaturation at 95ºC for 30 seconds followed by 45 cycles of denaturation (5 seconds

at 95º), annealing (3 seconds at 58ºC) and extension (45 seconds at 72ºC) at which point the amount of PCR template was quantified. This was followed by a melting curve where the temperature was increased from 65ºC to 95ºC at 0.1ºC/second to determine PCR specificity and finally a cooling cycle of 30 seconds at 40ºC. The PCR cycling conditions for the second cohort of patients included an additional step following extension where the target gene was quantified at the higher temperatures of 84ºC for tyrosinase and 86ºC for TRP-2 for 5 seconds at the end of each cycle. Each sample was run in duplicate and the RT-qPCR protocol repeated to ensure both technical and biological reproducibility. The crossing threshold values were analysed to measure the target gene expression by normalizing the gene's mean C_T value to the mean C_T value of GAPDH and utilizing the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The PCR products, to which 4 µL of fluorescent SYBR Gold (Invitrogen, Eugene, Oregon, USA) was added, were then electrophoresed through a 1.2% agarose gel for two hours at 80V as an additional confirmation of product specificity. A negative control of distilled water was included in each RT-qPCR run in order to exclude false-positives which may have arisen from contamination of RT-qPCR reagents with cDNA or amplicons. During the optimization of the RT-qPCR protocol, a positive control using UCT-Mel-1 RNA as RT-PCR template was included.

2.6 Patient treatment and follow-up

Twenty-one vitiligo patients signed informed consent forms to participate in this study (Appendix). This sample included a random subset of patients with vitiligo attending either the Groote Schuur Hospital (GSH) Dermatology outpatient clinics or the PUVA clinic between August 2007 and February 2008. Groote Schuur Hospital is a tertiary hospital with referrals from primary care physicians, peripheral district hospitals, private dermatologists as well as from other specialities within GSH. Patients recruited for this study therefore came from diverse backgrounds. Both male and female patients, regardless of normal skin colour as well as the type and extent of the vitiligo were included. Exclusion criteria included patients who had received any form of topical or systemic treatment within the prior six months, patients with leukoderma secondary to other causes, pregnant women, as well as patients under the age of 21. The nature of the study was carefully explained and confidentiality and anonymity were assured. The study was approved by the Research Ethics Committee, UCT Medical School, University of Cape Town, South Africa (Ethics number 265/2004). All 21 patients completed the study.

At the first interview, a full history was elicited from each patient with regard to epidemiological factors, possible precipitating factors and facts about the duration and progression of the disorder. Thereafter a clinical examination was conducted documenting the sites of the vitiliginous lesions and their extent. An assessment as to the total body surface area involved was determined. Baseline photographs of all vitiliginous lesions were taken using a Sony digital camera (Sony Digital Mavica MVC-FD51).

Therapeutic modalities were discussed with patients and a treatment was chosen based on previous attempted therapies and patient preference. Financial constraints were also considered as the expense of khellin precluded its use in several patients. Khellin powder was mixed as a 5% concentration in aqueous cream by the hospital pharmacy in 100g containers while the clobetasol propionate (Dovate, Aspen Pharmacare: Pharma)) and betamethasone valerate (Lenovate, Aspen Pharmacare: Pharma) ointments were dispensed in 25g and 15g tubes respectively. Patients using topical corticosteroids were instructed to apply clobetasol propionate ointment to the sun-exposed areas of the fore-arms and hands and betamethasone valerate ointment to the face and neck twice daily. These sun-exposed sites were targeted for treatment as lesions in these visible sites resulted in a greater degree of distress and self-consciousness. Vitiligo patients in the khellin arm of the study were to apply topical 5% khellin in an aqueous cream vehicle once daily to the same sites, 30 minutes prior to sun exposure. Patients were instructed to apply preparations every day including cloudy or rainy days. Patients receiving both therapies were to receive daily sun exposure at noon for 15 minutes or until a slight erythema was noted. These treatments were initiated one week following the punch biopsy procedures.

The efficacy of the two treatment modalities was analysed at a three month follow-up visit by the principal investigator as well as by the experienced sister conducting the PUVA clinic. These analyses involved evaluation of the extent of repigmentation as well as the assessment of adverse effects related to treatment including atrophy, telangiectasia or any burning sensation. The treatment outcome was visually assessed by means of comparison to the patients' baseline photographs. In addition, this clinical assessment was supplemented by photographs taken under the same conditions at the three month follow-up visit. This allowed for verification at a later date. Repigmentation was evaluated as follows: poor response (0-25% repigmentation), average response (25-50% repigmentation), good response (50-75% repigmentation) and excellent response (75-100% repigmentation). This represented the conclusion of the three month trial period.

3 CHAPTER 3

RESULTS

3.1 Optimisation of protocols

3.1.1 Optimisation of the RNA extraction protocol

3.1.1.1 Techniques for mechanical disruption of the skin biopsy samples

In order to develop a protocol for determination of melanocyte/precursor cell survival, the biopsy samples first needed to be homogenized for RNA extraction. This is a particular challenge for skin samples which are very tough due to their high levels of collagen and which contain high levels of RNAses. For these optimization studies, skin samples from patients undergoing breast reduction surgery were supplied by the Department of Plastic Surgery, Groote Schuur Hospital. As detailed in Materials and Methods, 4mm^2 punch biopsies were taken from the samples which were then snap-frozen in liquid nitrogen. RNA extraction proved to be difficult as the small 4mm^2 punch biopsy samples were resistant to shearing forces. This necessitated prolonged homogenization times which in turn led to increased heat production and thus RNA degradation as shown by degraded RNA visible on agarose gels (not shown).

Therefore, three alternative methods of RNA extraction were attempted:

1. Homogenization using a Polytron homogenizer (Kinematica GmbH., Littau, Switzerland) with the specimen being kept on ice between short bursts of homogenization (as detailed in Chapter 2.2.1)
2. Tissue homogenization using a Tekniva homogenizer (Tekniva, Cape Town, South Africa) which provides high speed (50 Hz), single plain harmonic motion to a sterile 8mm stainless-steel ball (Bearing Man, Maitland, SA) located in a cryovial containing the skin biopsy (as detailed in Chapter 2.2.2).
3. Enzymatic splitting of the skin tissue by means of trypsinisation followed by RNA extraction from the epidermis (as detailed in Chapter 2.2.3).

Each extraction method was carried out a number of times on three different samples each time. The average weight of the skin biopsies was 13.8mg. The quantity and quality of RNA from the

three extraction techniques were determined using the Nanodrop spectrophotometer and an average quantity of RNA per biopsy was calculated (Table 1).

In order for RNA to be used for quantitative RT-PCR measurements, an excellent RNA purity is important and an OD260/280 of 2.0 is optimal. RNA with an OD260/280 < 1.8 is inadequate for qPCR and a minimum yield of 1µg of RNA is necessary for RT-PCR. The RNA yield and purity (as an OD260/280) is shown in Table 1.

	Average RNA yield per biopsy	**RNA 260/280 ratio**
Polytron homogenizer	**3.3 µg**	**1.9**
Bead beating	**3 µg**	**1.66**
Skin splitting	**0.5 µg**	**1.91**

Table 1. For each method of tissue disruption, the quantity of RNA (µg) and quality of RNA (as an OD260/280 ratio) is shown. The RNA was quantified using the Nanodrop spectrophotometer.

It can be seen that the Polytron homogenizer yielded the most RNA per biopsy with an excellent RNA purity. The method of tissue homogenization using the Tekniva homogeniser produced a poor RNA purity while enzymatic skin splitting resulted in an insufficient RNA yield per biopsy.

Therefore, for all further studies, the polytron homogenizer was used for the mechanical disruption of the skin biopsy samples.

3.1.1.2 Cleaning protocol of the Polytron homogenizer

Since the same Polytron homogenizer had to be used for all samples, it was essential to ensure that there was no carry-over of RNA between samples. In particular, any carry-over from a pigmented skin sample to a lesional skin sample could have led to a false positive gene expression result. In order to avoid this eventuality, all lesional skin samples were homogenized prior to the pigmented samples on any given day. In addition, the cleaning protocol between individual samples was

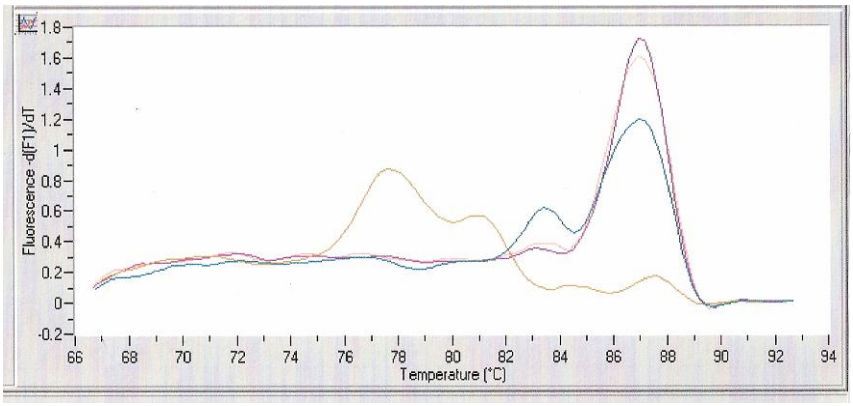
stringent. The protocol involved rinsing the homogenizer extensively in sterile distilled water followed by one rinse with 70% ethanol. Finally, the homogenizer was rinsed with RNAse AWAY.

In order to demonstrate that no carry-over contamination had occurred, the cleaning protocol was carried out following the homogenization of three skin samples. The polytron tip was then rinsed with a control sample of 1ml extraction reagent alone which subsequently underwent the entire qPCR protocol using primers for the housekeeping gene GAPDH, tyrosinase as well as TRP-2.

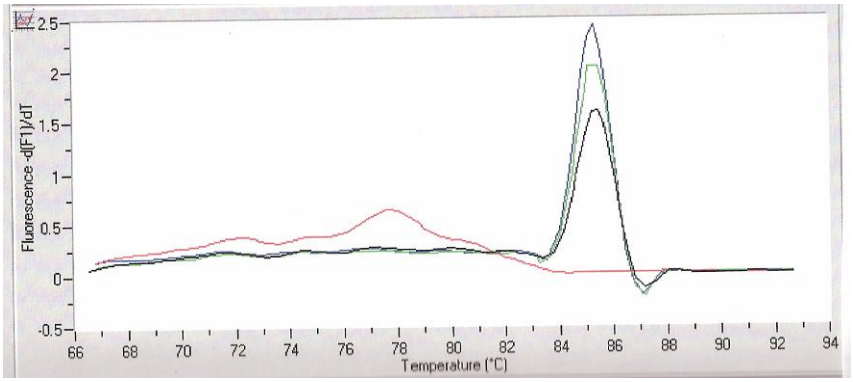
As can be seen in Fig11a, the products of the three skin samples (purple, pink and blue curves) melt at the specific temperature of 87-88ºC for GAPDH. The product from the extraction reagent control sample (yellow curve) demonstrates only primer-dimer formation. A similar picture can be seen in Fig11b in which the products of the three skin samples using the primers for tyrosinase (blue, green and black curves) melt at the specific temperature of 86ºC. The lack of a curve at 86ºC for the extraction reagent control sample (red curve) indicates that there was no RNA contamination in this sample. This effectiveness of the cleaning protocol was reiterated in the experiment for TRP-2 as can be seen in Fig11c. The products of the three skin samples (green, pink and grey curves) demonstrate the expected melting temperature of 89ºC for TRP-2. The extraction reagent control sample (blue curve), however, does not demonstrate a peak at this melting temperature indicating that no contamination has occurred.

In this way, the experiment using the control extraction reagent yielded consistent negative results thereby verifying the efficacy and reliability of the cleaning protocol.

a.



b.



c.

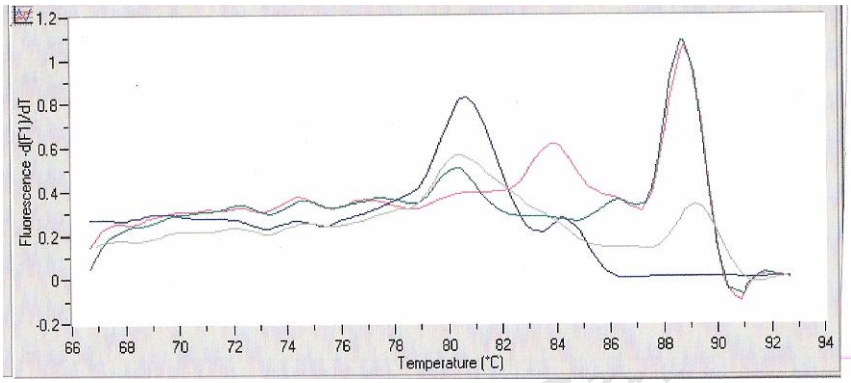


Fig 11. Melting curves of the quantitative PCR products using primers for the housekeeping gene GAPDH (**a.)**, tyrosinase (**b.)** and TRP-2 (**c.**).

3.1.2 Optimisation of the RT-qPCR methodology for the detection of TRP-2 mRNA

A skin biopsy is made up of many different cell types of which melanocytes represent only a small number. A specific and highly sensitive RT-qPCR protocol for the detection of melanocytes therefore needed to be developed. The established RT-qPCR cycling conditions for the detection of GAPDH and tyrosinase mRNA consistently produced specific products of 119bp (Fig 12, lanes 2 and 3) and 238bp (lanes 4 and 5) respectively on agarose gel electrophoresis. The initial RT-qPCR cycling conditions for the detection of TRP-2 mRNA however, produced only primer-dimers and a low level of product of the incorrect size as evidenced by non-specific product formation on agarose gel electrophoresis (Fig 12, lanes 6 and 7).

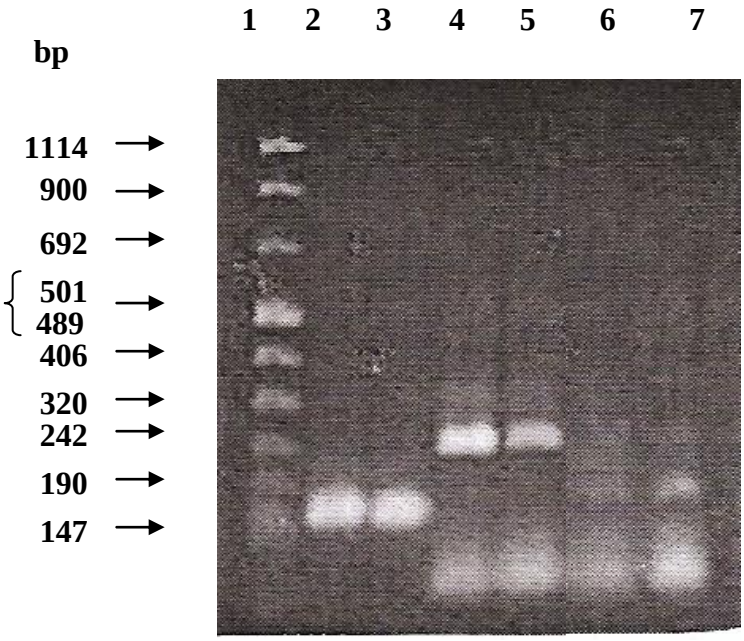


Fig. 12. Agarose gel electrophoresis demonstrating GAPDH, tyrosinase and TRP-2 mRNA expression

LANE 1: 1kb marker
LANE 2: GAPDH mRNA expression in skin sample 1
LANE 3: GAPDH mRNA expression in skin sample 2
LANE 4: Tyrosinase mRNA expression in skin sample 1
LANE 5: Tyrosinase mRNA expression in skin sample 2
LANE 6: TRP-2 mRNA expression in skin sample 1
LANE 7: TRP-2 mRNA expression in skin sample 2

This chapter details the optimization of the RT-qPCR cycling conditions for TRP-2 mRNA which was critical in the development of a highly accurate molecular assay for the detection of melanocyte survival in human skin biopsies.

The quantitative PCR to detect TRP-2 was initially carried out under the following conditions (protocol A): an initial denaturation step at 95ºC for 30s followed by 45 cycles of denaturation at 95ºC for 5 seconds, annealing at 60ºC for 3 seconds and elongation at 72ºC for 5 seconds at which

stage the fluorescence was quantified. This was followed by a melting curve generated using temperatures from 65-95ºC in 1ºC increments in order to verify the specificity of amplification.

These cycling conditions were carried out using a melanoma cell line, primary melanocyte cultures, a pigmented skin sample, a lesional skin sample and a keratinocyte cell line. The RT-qPCR products were analysed by gel electrophoresis (Fig 13).

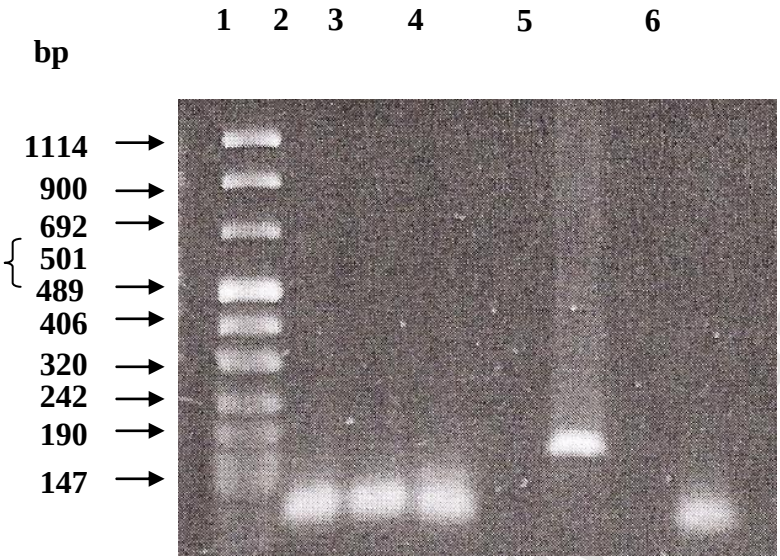


Fig 13. Agarose gel electrophoresis demonstrating TRP-2 gene expression in a pigmented skin sample, lesional skin sample, HERMES primary cultured melanocytes, MEL-1 melanoma cell line and HaCaT keratinocyte cell line.

LANE 1: 1kb marker
LANE 2: Pigmented skin sample
LANE 3: Lesional skin sample
LANE 4: HERMES primary melanocyte culture
LANE 5: MEL-1 melanoma cell line
LANE 6: HaCaT keratinocyte cell line

It can be seen that there are no bands that correlated with the expected product size of 476bps. The pigmented (lane 2) and lesional (lane 3) skin samples as well as the primary melanocytes (lane 4) and keratinocytes (lane 6) demonstrated primer-dimer formation. The MEL-1 melanoma cell line (lane 5) produced a single band but with an incorrect product size. In order to optimize the detection of TRP-2 gene expression, the RT-qPCR cycling conditions were modified to allow for a longer elongation time of twenty seconds (Protocol B).

Increasing the elongation time resulted in a product size of 476bp for melanoma (lane 2) and immortalized melanocytes (lane 3). However, non-specific products were also seen. No product was seen in the keratinocyte lane (lane 4).

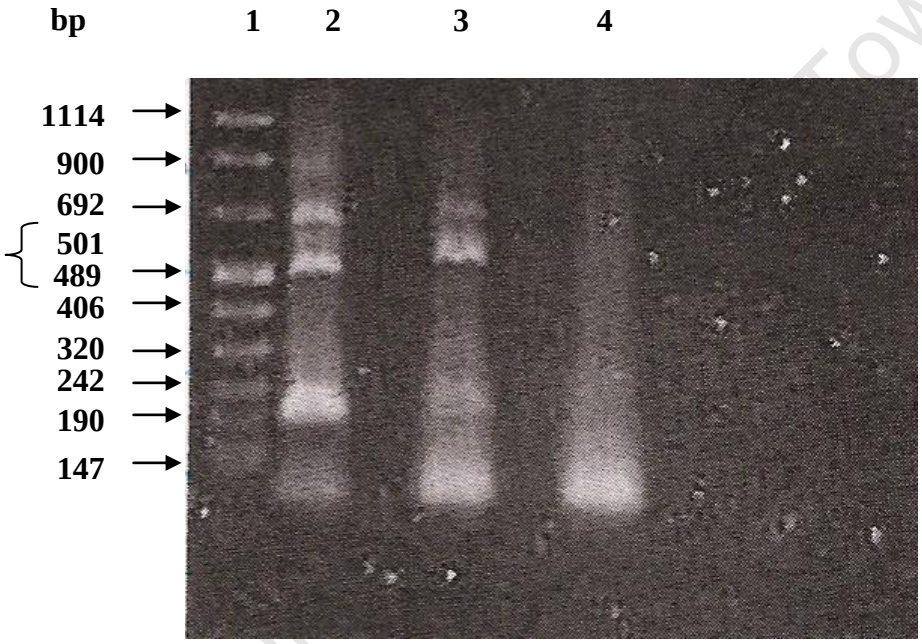


Fig 14. Agarose gel electrophoresis analyzing TRP-2 gene expression in SYBR gold stained qPCR products.

LANE 1: 1kb marker
LANE 2: MEL-1 melanoma cell line
LANE 3: HERMES primary melanocyte culture
LANE 4: HaCaTS keratinocyte cell line

To further optimize, the annealing temperature was decreased to 58ºC and the elongation time was increased to 45 seconds (Protocol C, Fig 15).

Both the MEL-1 melanoma sample (lane 2) and the HERMES primary melanocyte sample (lane 3) produced a clear single band of approximately 476 bps, confirming the specificity of the transcripts. Faint bands at a smaller bp size of approximately 242-320bps indicate a small amount of primer-dimer formation. The HaCaT keratinocyte sample (lane 4) shows only non-specific product or primer-dimer formation with a size of approximately 190 bps.

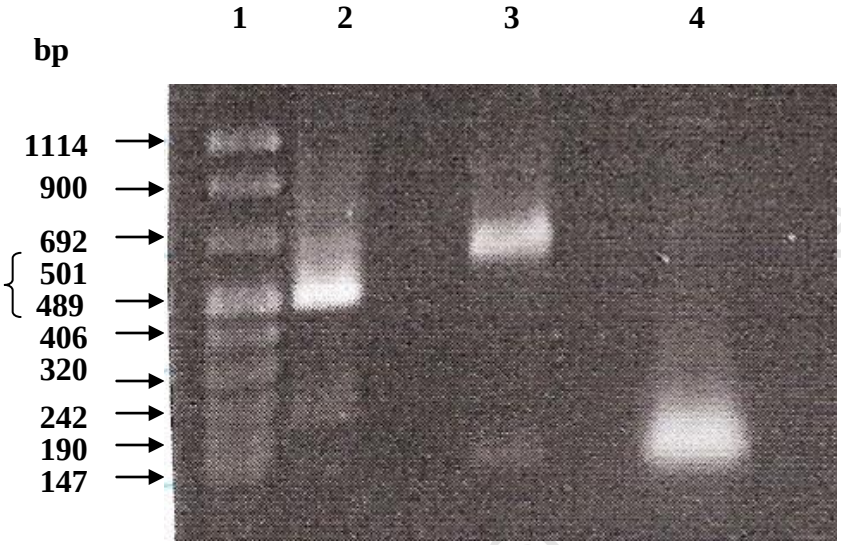


Fig 15. Agarose gel electrophoresis of the RT-qPCR products obtained using the modified Protocol C cycling conditions.

LANE 1: 1kb marker
LANE 2: MEL-1 melanoma cell line
LANE 3: HERMES primary melanocyte culture
LANE 4: HaCaT keratinocyte cell line

Having established product specificity, the next step was to analyse these samples by RT-qPCR. In Figure 16 it can be seen that both the MEL-1 melanoma cell line (blue curve) and the HERMES primary melanocytes (green curve) demonstrate a single peak at the specific T_m of 89-90ºC

verifying that the cycling conditions were optimal. The HaCaT keratinocyte cell line – a negative control – predictably exhibited a different melting profile with a lower T_m of 85ºC.

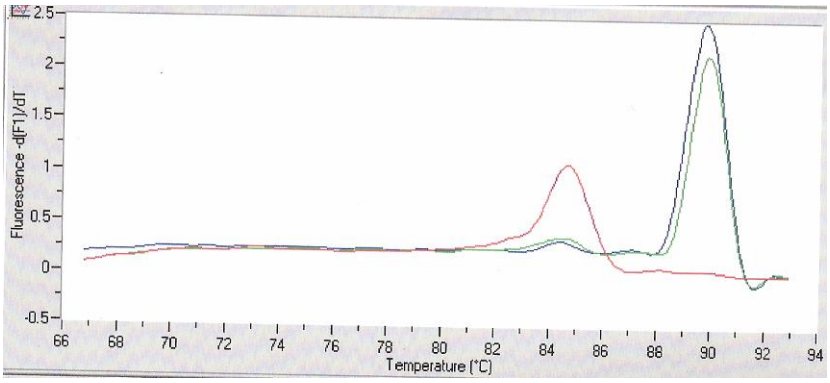


Fig 16. Melting curve analysis of the TRP-2 transcript in a MEL-1 melanoma cell line (blue curve), HERMES primary melanocyte culture (green line) and HaCaT keratinocyte cell line (pink curve) using the modified qPCR cycling conditions (Protocol C).

The next step was to utilise Protocol C to analyse GAPDH, tyrosinase and TRP-2 gene expression in skin samples obtained from the Plastic Surgery Department from excess skin removed during abdominoplasty procedures.

The qPCR products obtained from these samples were analysed by gel electrophoresis (Figure 17). Analysis of the gel indicates specific product formation for all 3 primers with product sizes of 119bps (lanes 2-4), 240bps (lanes 5-7) and 476bps (lanes 8-10) for GAPDH, tyrosinase and TRP-2 respectively. These results were consistently shown in 3 separate skin samples. While a single band only is seen with the GAPDH products, some primer-dimer formation can be seen in the tyrosinase and in the TRP-2 products. In addition, non-specific products can also be seen in the TRP-2 lanes.

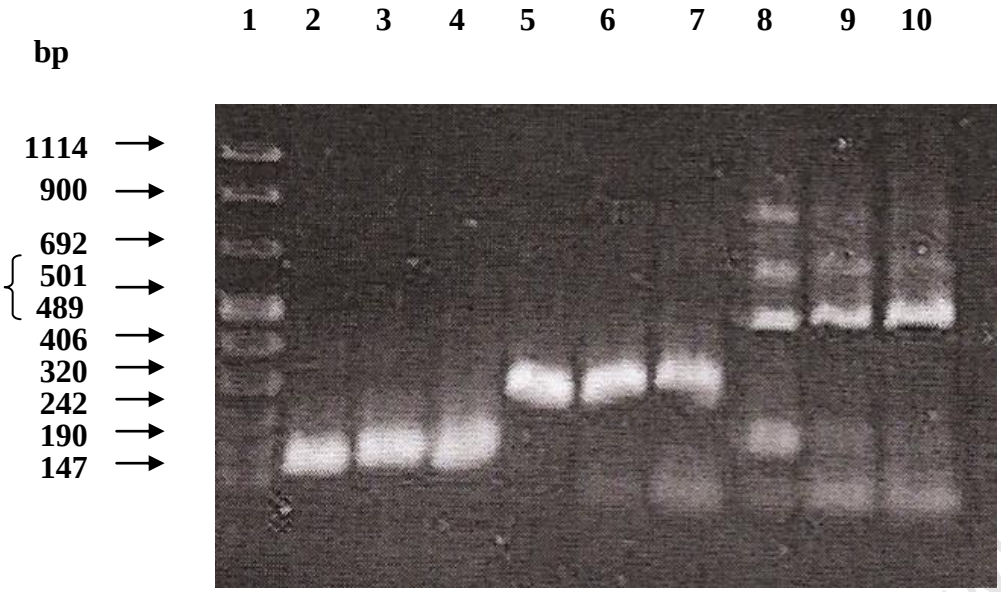


Fig 17. Agarose gel electrophoresis of GAPDH, tyrosinase and TRP-2 transcripts obtained during qPCR.

LANE 1: 1kb marker
LANE 2: Skin sample 1: GAPDH
LANE 3: Skin sample 2: GAPDH
LANE 4: Skin sample 3: GAPDH
LANE 5: Skin sample 1: Tyrosinase
LANE 6: Skin sample 2: Tyrosinase
LANE 7: Skin sample 3: Tyrosinase
LANE 8: Skin sample 1: TRP-2
LANE 9: Skin sample 2: TRP-2
LANE 10: Skin sample 3: TRP-2

It was thus established that Protocol C was sufficiently sensitive to detect tyrosinase and TRP-2 mRNA expression even in skin samples containing numerous cell types apart from melanocytes.

3.2 Analysis of tyrosinase, TRP-2 and GAPDH in lesional and non-lesional skin samples by RT-qPCR

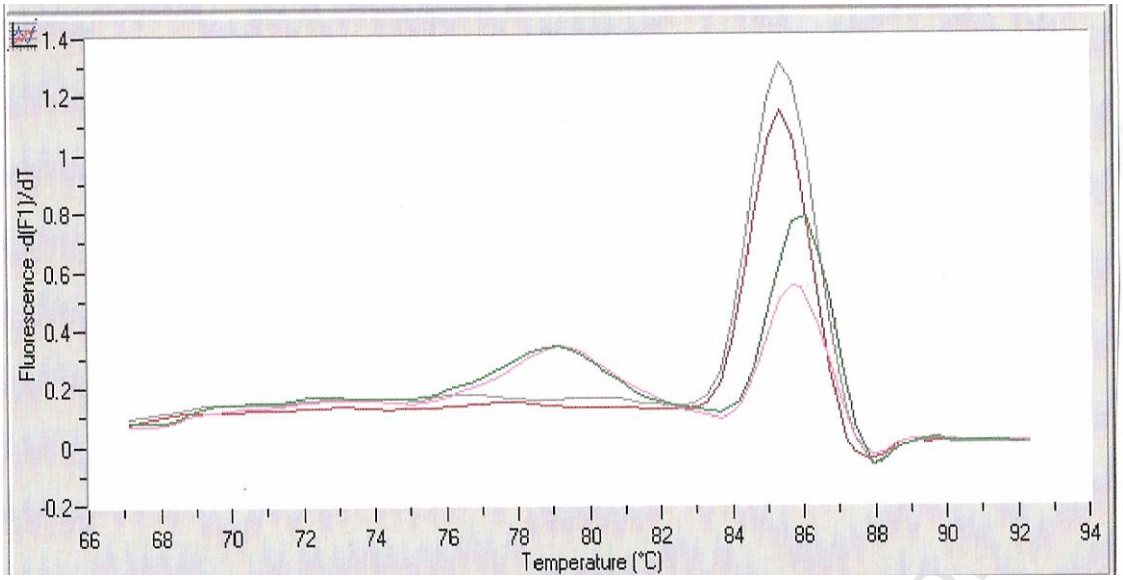
The aim of this specific experiment was to utilise Protocol C to analyse vitiliginous and non-lesional skin samples from vitiligo patients for the presence of melanocyte survival and to assess whether there was any correlation between this survival and the patient's response to treatment. Tyrosinase was chosen as a marker of differentiated melanocytes as it is an enzyme involved in melanogenesis. TRP-2 was chosen as a marker of melanocytes as well as of melanoblasts as this enzyme is expressed by undifferentiated melanoblasts at day 10 post-coitum in mice.

Vitiliginous and non-lesional skin samples were obtained from ten vitiligo patients prior to the instigation of treatment. These skin samples were then analysed using the optimized RNA extraction and RT-qPCR Protocol C cycling conditions as described previously.

Typical melting curve results for tyrosinase and TRP-2 are shown in Figures 18 and 19. It was established that the GAPDH gene product has a melting temperature of 88ºC, the tyrosinase gene product a melting temperature of 86ºC (as can be seen in Figure 18) and the TRP-2 gene product a melting temperature of 89º-90ºC (as can be seen in Figure 19).

Quantitative PCR was conducted in duplicate for each vitiliginous and non-lesional skin sample. Both the pigmented and lesional skin samples demonstrated positive results for GAPDH with the amplicons melting at the above-mentioned melting temperature. The pigmented skin samples also demonstrated positive results for tyrosinase and TRP-2 without exception and thus could be used as positive controls. With regard to the lesional skin samples, the presence of a peak at the correct melting temperature of the gene of interest was interpreted as a positive result (Figure 18a and Figure 19a). The absence of a peak at this melting temperature was interpreted as a negative result (Figure 18b and Figure 19b).

a.



b.

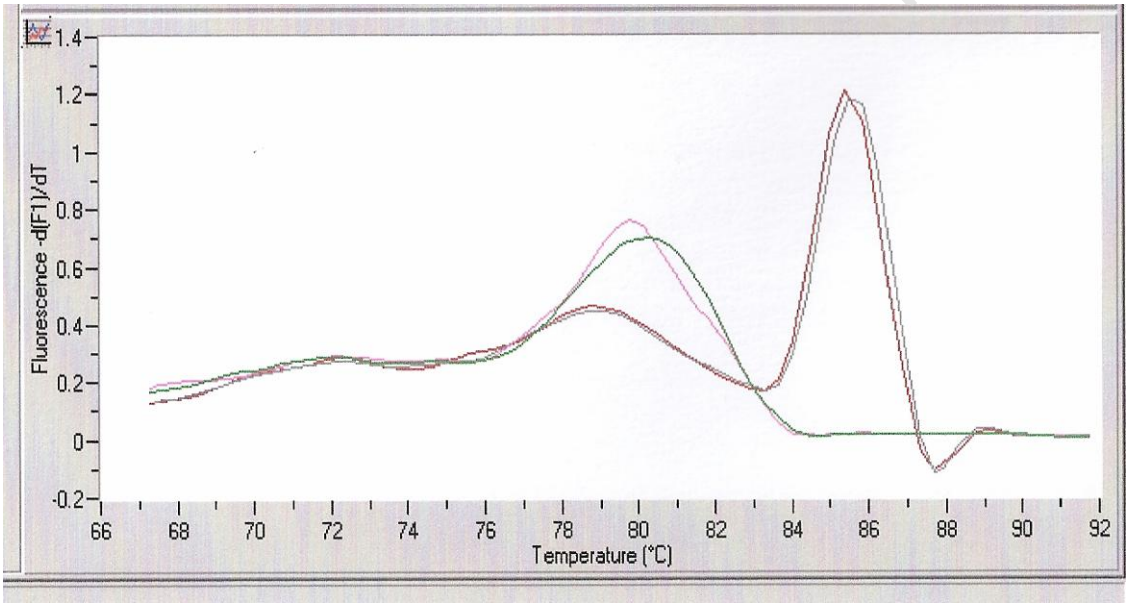
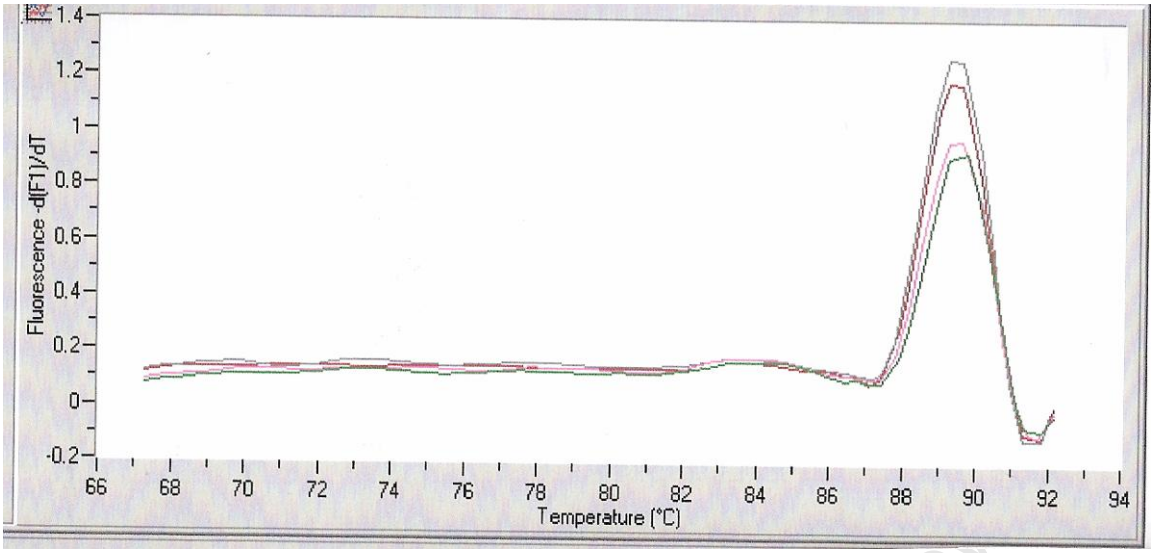


Fig 18. Melting curves of the tyrosinase gene product for pigmented (red and grey curves) and lesional (green and pink curves) skin samples. The pigmented skin samples demonstrate the specific T_m of 86ºC. **a.** Lesional skin samples are positive for tyrosinase as demonstrated by the melting peaks at the specific melting temperature of 86ºC of the tyrosinase gene product. **b.** Lesional skin samples are negative for tyrosinase as demonstrated by the lack of any peak at the 86ºC melting temperature.

a.



b.

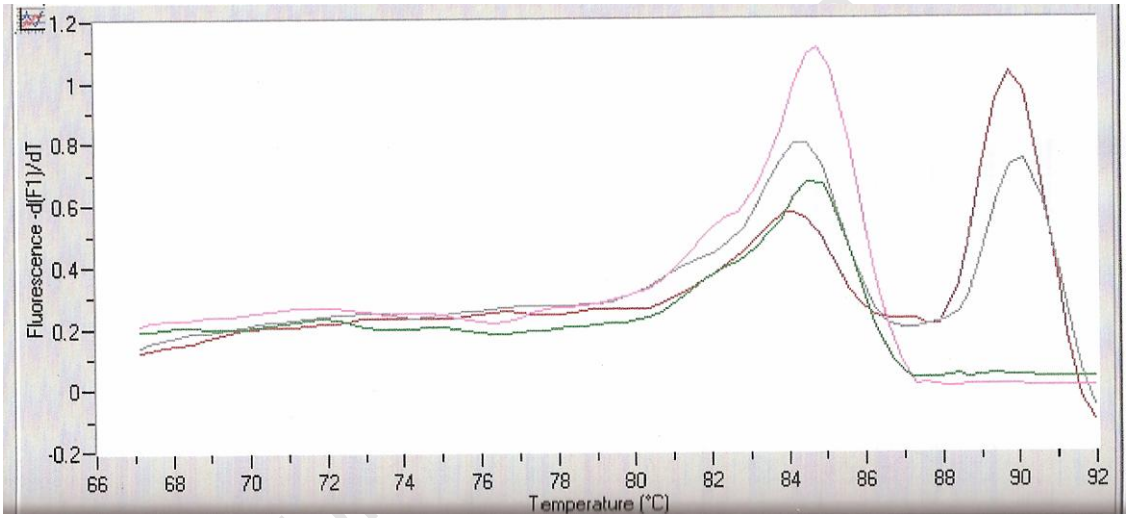


Fig 19. Melting curves of the TRP-2 gene product for pigmented (red and grey curves) and lesional (green and pink curves) skin samples. The pigmented skin samples demonstrate the specific T_m of 89º-90ºC. **a.** Lesional skin samples are positive for TRP-2 as demonstrated by the melting peaks at the specific melting temperature of 89º-90ºC of the TRP-2 gene product. **b.** Lesional skin samples are negative for TRP-2 as demonstrated by the lack of any peak at the 89º-90ºC melting temperature.

These interpretations could now be used to analyse skin samples collected from the first cohort of patients.

3.3 Profile of the first patient cohort

Ten patients with generalized vitiligo were recruited from the Groote Schuur Hospital outpatient department. These patients had received various treatments in the past, including PUVA, corticosteroids or khellin with variable responses ranging from no response (subjective rating of '0') to complete repigmentation (subjective rating of '10'). The patients were predominantly female (70%) with a mean age of 47 years (with a range of 23 to 70 years). The mean duration of the vitiligo was 16 years (ranging from one year to 45 years) with 4/10 patients (40%) classifying their disease activity as stable. For the purposes of this study, vitiligo is classified as 'stable' when the following three parameters are fulfilled:

- There have been no new areas of depigmentation or enlargement of pre-existing lesions during the previous six months
- There has been an absence of the Koebner phenomenon during the previous six months. The Koebner phenomenon refers to the development of new depigmented vitiliginous lesions in lines of trauma.
- The patients have reported episodes of spontaneous repigmentation within leukodermic lesions within the previous six months

The majority of the patients had Fitzpatrick skin type IV. A family history of vitiligo was found in 2/10 (20%) of patients. The number of patients with concomitant auto-immune disorders was higher than the current statistics of 13% (James WD et al., 2006) with 5/10 patients (50%) having been diagnosed and treated for conditions including rheumatoid arthritis, hypothyroidism, diabetes, asthma and allergic rhinitis.

Pt	Age	Sex	Disease duration (years)	Classification	Activity	Fitz-patrick skin type	FH	Concomitant auto-immune disorders	Previous treatments	Subjective response to prior treatment (0-nil 10-100%)
1	54	M	45	Generalised	Stable	IV	Nil	Nil	2001: PUVA	6
2	41	F	18	Generalised	Stable	IV	Nil	Rheumatoid arthritis	1997: PUVA	0
3	70	F	1	Generalised	Active	V	Nil	Nil	2006: PUVA	0
4	51	F	20	Generalised	Active	IV	Nil	Hypo-thyroidism	1999: PUVA 1999: Khellin	0 7
5	56	F	17	Generalised	Stable	VI	Daughter	Nil	1993: Cortico-steroids 1993: PUVA 1998: Cortico-steroids	10 1 1
6	23	M	5	Generalised	Active	VI	Nil	Nil	2002: Cortico-steroid 2003: PUVA 2006: PUVA	2 3 5
7	43	M	25	Generalised	Active	IV	Nil	Diabetes	2004: PUVA	1
8	23	F	12	Generalised	Active	II	Nil	Nil	1998: Cortico-steroids	1
9	42	F	14	Generalised	Stable	IV	Brother	Allergic rhinitis	2001: PUVA	7
10	66	F	5	Generalised	Active	III	Nil	Diabetes Asthma	2006: Cortico-steroid	0

Table 2. Epidemiological profile of the first cohort of vitiligo patients

3.4 Melanocyte survival and response to treatment in the first cohort of patients

The next aim was to utilise the optimised RT-qPCR protocol for assessment of melanocyte survival in vitiligo skin biopsies and to determine whether there was any correlation between this melanocyte survival and response to treatment in vitiligo patients.

Ten patients with generalized vitiligo were recruited from the adult outpatient and PUVA clinics at Groote Schuur Hospital in Cape Town, South Africa. Pregnant female and lactating mothers, patients with serious concomitant diseases, patients with a history of keloidal tendency or koebnerisation and patients less than 18 years of age were excluded from the study. The study protocol was discussed with the patients and signed consent was obtained (See Addendum).

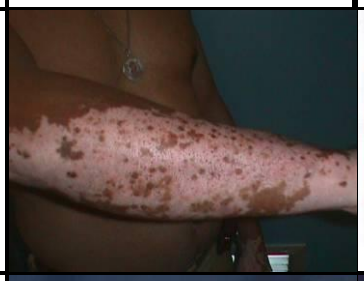
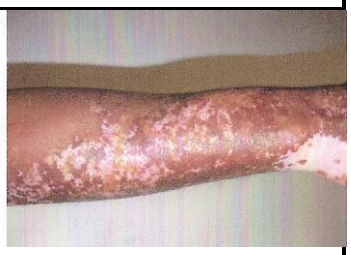
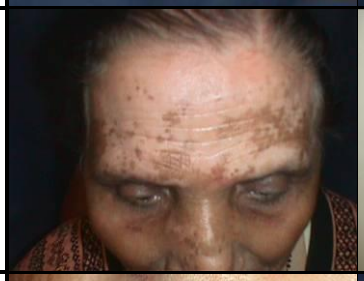
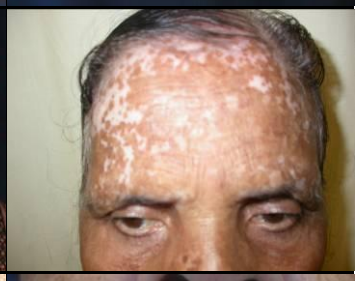
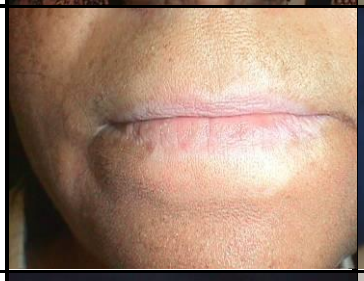
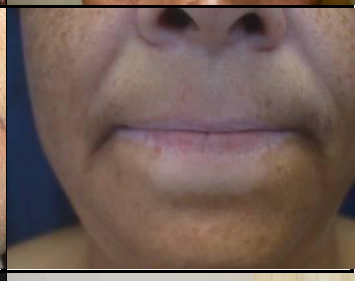
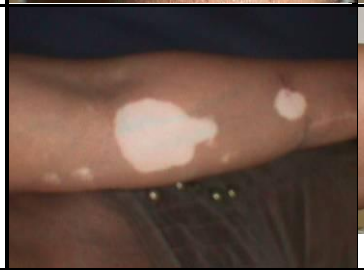
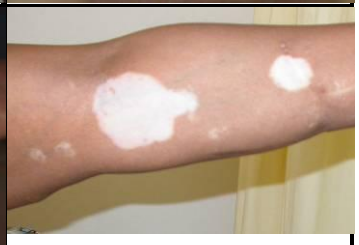
In consultation with the patient, the two biopsy sites were chosen. In order to prevent an unsightly cosmetic result, the face and hands were excluded as biopsy sites. This was an unavoidable limitation whereby the vitiligo lesions analysed for melanocyte survival were not always the sites treated. However, it was hoped to assess whether analysis of any single vitiligo lesion could be used to prognosticate on the leukoderma on the whole. A $4mm^2$ punch biopsy was taken from the centre of a vitiligo lesion as well as from an adjacent area of pigmented skin. (as detailed in Chapter 2.1.2.2) These pigmented and lesional samples were then analysed by the optimized RT-qPCR protocol for tyrosinase and TRP-2 mRNA expression developed as described in Results Section 3.1.2. Both technical and biologic repeat experiments were conducted. Melting curves were examined to confirm specificity of product. A sample was considered positive for tyrosinase if the melting temperature peak was at 86ºC and positive for TRP-2 if the melting temperature peak was 89-90ºC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive internal control.

Therapeutic options were discussed with each patient. It was decided to treat only the areas of the body receiving sun exposure, namely the face, neck, fore-arms and hands. Potent topical corticosteroid treatment was initiated in 9/10 patients due to its affordability. Patients were instructed to apply betamethasone ointment to the face and neck region and clobetasol priopionate ointment to the forearms and hands twice daily. In 1 patient, a 5% khellin solution was to be applied once daily prior to sun exposure. All patients were to receive a daily dose of sun exposure at noon either for 15 minutes or until a mild erythema was noted. All ten patients completed the 3 month trial.

In all patients, clinical photographs were taken and an assessment of body surface area involvement of the face, forearms and hands was made by both the principal investigator and an experienced sister conducting the PUVA clinic. This was performed both prior to the initiation of treatment as well as at the end of the 3 months. At the conclusion of the treatment period, the response to treatment in each patient was assessed as being either a poor response (<25% repigmentation), average response (25-50% repigmentation), good response (50-75% repigmentation) or excellent response (>75% repigmentation) as has been detailed in Chapter 2.6.

Table 3 records the results obtained for the detection of tyrosinase and TRP-2 in lesional skin of vitiligo patients as well as the patient's response to 3 months of either corticosteroid or khellin

therapy. It can be seen that melanocyte survival was found in 6/10 (60%) of vitiligo patients. A poor response to treatment (<25% repigmentation of the sun exposed areas after 3 months of treatment) was seen in 7/10 (70%) of patients while 1 patient (10%) had an average response to treatment (25-50% repigmentation) and 2/10 patients (20%) had a good response to treatment (50-75% repigmentation). No patient achieved an excellent (>75% repigmentation) response to treatment. No correlation between melanocyte survival and response to treatment was found in this cohort of patients.

Pt	Biopsy Site	Tyrosinase	TRP-2	Treatment	% Repig	Before	After
1	Calf	+ve	+ve	Cortico-Steroids	50-75%		
2	Fore-Arm	-ve	-ve	Cortico-Steroids	<25%		
3	Thigh	-ve	-ve	Cortico-steroids	50-75%		
4	Abd	+ve	-ve	Cortico-Steroids	<25%		
5	Fore-Arm	-ve	-ve	Cortico-Steroids	<25%		

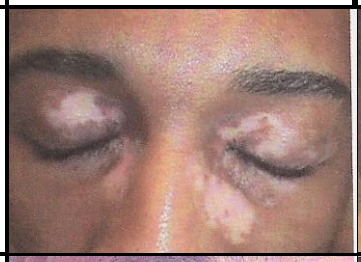
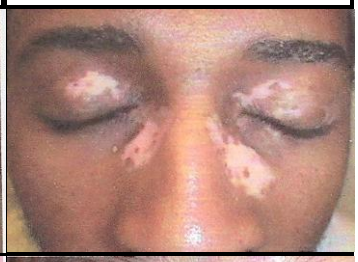
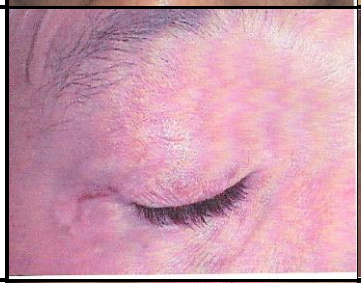
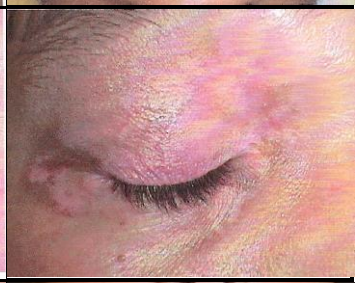
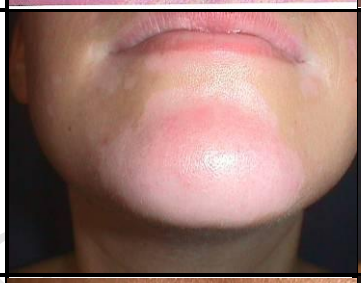
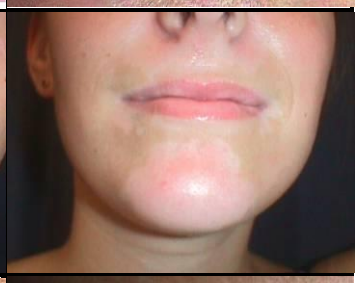
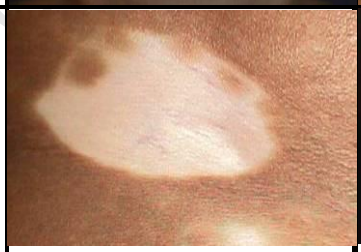
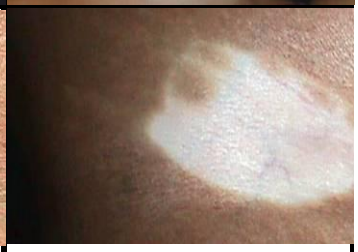
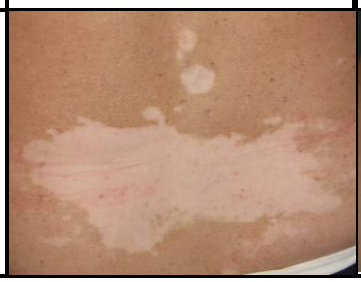
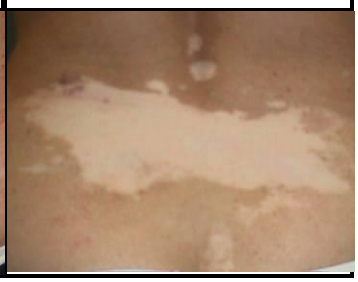
Pt	Biopsy Site	Tyrosinase	TRP-2	Treatment	% Repig	Before	After
6	Abd	+ve	-ve	Cortico-Steroids	25-50%		
7	Calf	-ve	+ve	Cortico-Steroids	<25%		
8	Back	-ve	+ve	Khellin	<25%		
9	Thigh	-ve	+ve	Cortico-Steroids	<25%		
10	Back	-ve	-ve	Cortico-Steroids	<25%		

Table 3. Presence of melanocytes/melanoblasts in lesional skin and response to treatment in the first cohort of vitiligo patients.

When analyzing tyrosinase and TRP-2 expression in the vitiliginous skin biopsies, it was found that only the presence or absence of transcript in these skin samples could be evaluated due to a technical problem encountered. RT-qPCR using SYBR Green works on the principle of

measurement of fluorescence yielded by the dye binding to dsDNA during extension. Analysis of the melting curves (Figure 18a) and of agarose gel electrophoresis (Figure 17) indicated that at the extension temperature of 72ºC, high levels of primer-dimers were present and there was still significant non-specific product formation with TRP-2. This would increase the fluorescence, thereby giving incorrect crossing threshold values and thus incorrect quantitation. Since this was a critical aspect in the development of an accurate prognostic assay for vitiligo patients, it was crucial to achieve finer optimization. Therefore, prior to conducting a study with a new cohort of patients, it was decided to optimize the RT-qPCR cycling conditions further in order to eliminate these confounding contaminants.

3.5 Elimination of primer-dimers

The crossing threshold (C_T) in quantitative PCR is defined as the point at which the fluorescent signal is first detected as statistically significant above background. In this way, the greater the amount of template present at the beginning of each reaction, the fewer the number of cycles it takes to reach the crossing threshold (Freeman et al., 1999). This crossing threshold value can then be used to analyse the relative change in gene expression of an experimental sample to that of a control sample by means of the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

The presence of primer-dimers or any non-specific product, however, contributes to the fluorescent signal resulting in a lower C_T value and consequently invalidating any relative quantification. The aim was thus to eliminate the fluorescence produced by primer-dimers during RT-qPCR. .

As previously described, the melting curve analyses revealed that the primer-dimers melted at a lower temperature compared to the desired amplicon due to their smaller size. The T_m of the tyrosinase transcripts was 86ºC compared to the approximate T_m of 80ºC of the primer-dimers (as seen in Fig 18). In the case of TRP-2, the melting temperatures of the transcripts vs primer-dimers were 89ºC and 84ºC respectively (as seen in Fig 19). The strategy was therefore to utilize the LightCycler's ability to measure fluorescence at a higher temperature than the melting point of the primer-dimers in order to ensure that the fluorescence of only the desired amplicon was measured. The initial cycling conditions of Protocol C was comprised of 45 cycles of denaturation at 95ºC for 5 s, annealing at 58ºC for 3 s and elongation at 72ºC for 40 s after which fluorescence was measured (Figure 20).

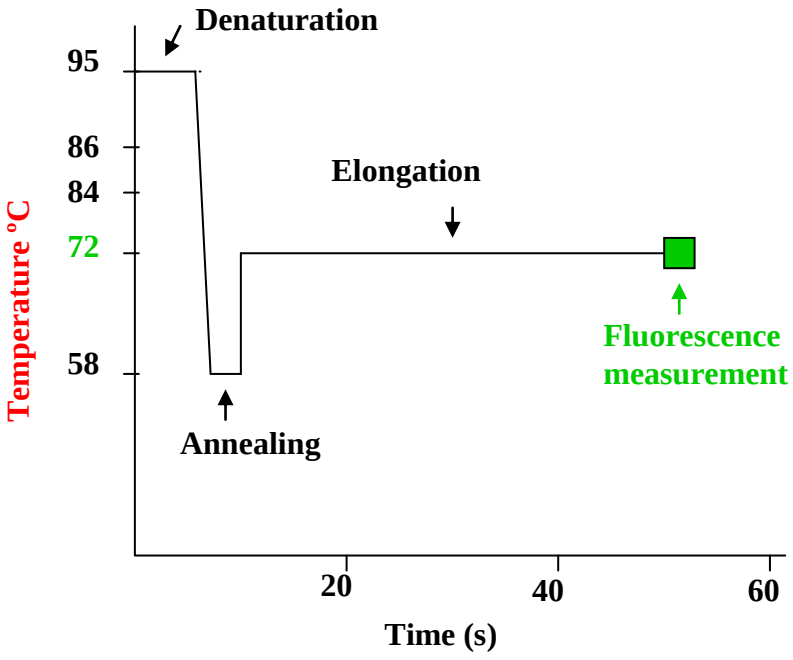


Fig 20. The initial qPCR cycling conditions of Protocol C for amplification of tyrosinase and TRP-2 cDNA.

In order to take advantage of the lower melting temperature of the primer-dimers and the Lightcycler's ability to measure fluorescence at any temperature, an additional step was added to the qPCR cycling conditions of Protocol C in order to prevent primer-dimer interference. This modified protocol – Protocol D – is depicted in Figure 21.

Following the initial qPCR cycle, the temperature was raised to 84ºC (tyrosinase) or to 86ºC (TRP-2) for 5 s after which the fluorescence was measured (Figure 21). At these temperatures there is denaturation of the primer-dimers but not of the specific qPCR product. As SYBR Green binds only to double-stranded DNA, the measured fluorescence was thus generated only by the desired amplicon.

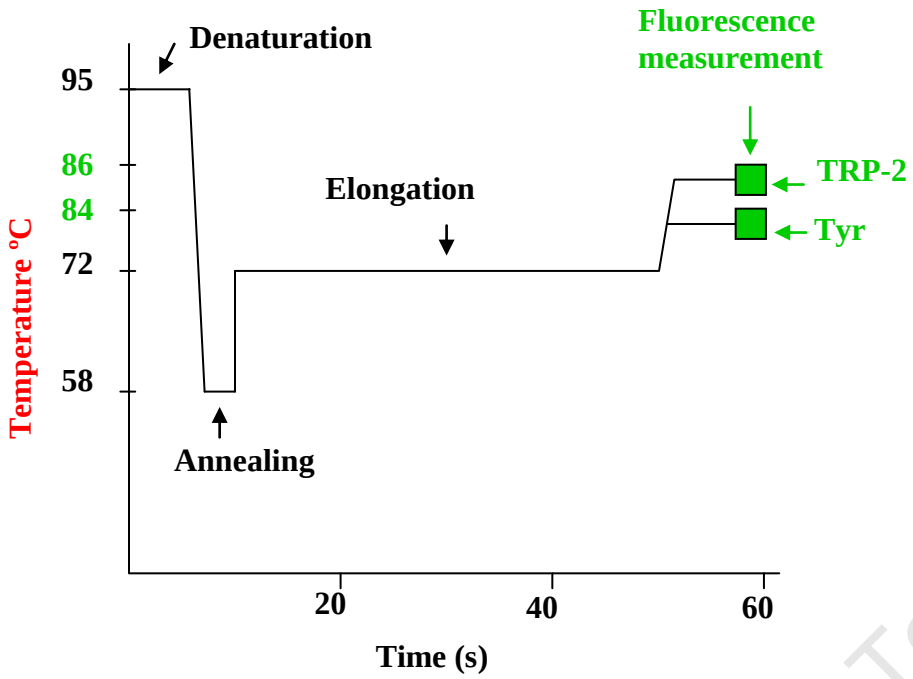


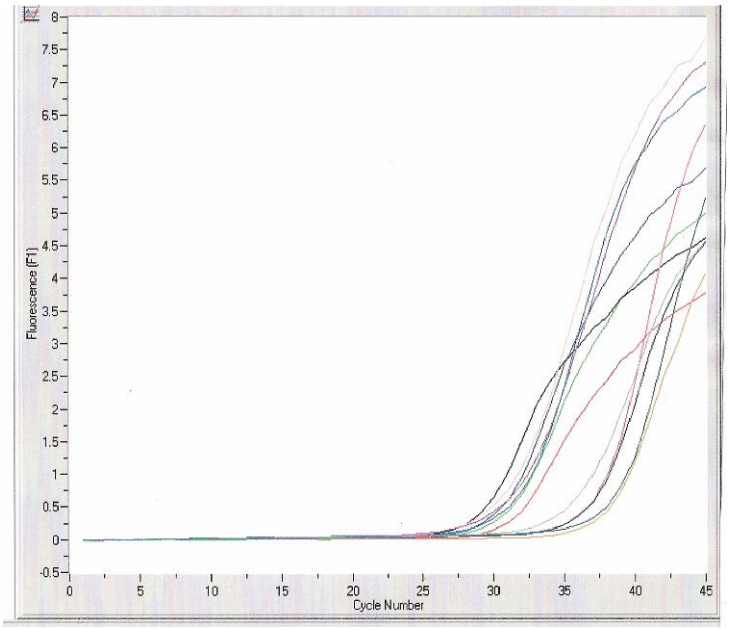
Fig 21. The modified qPCR cycling conditions used to eliminate the fluorescence elicited by primer-dimers (Protocol D).

In order to demonstrate that the modified cycling conditions of Protocol D successfully eliminate primer-dimer contribution to the total fluorescence, the amplification data from Protocol C and D were compared.

The same skin samples were analysed using the initial (Protocol C) and modified (Protocol D) cycling conditions (Fig 22). Figure 22a depicts the amplification data using Protocol C. The fluorescence can be seen to increase to almost 8 after 45 cycles with an average crossing threshold value of 36.2.

In contrast, the amplification data using Protocol D indicates a much lower relative fluorescence (as can be seen in Figure 22b). The fluorescence was measured as approximately 4.5 after 45 cycles with an average crossing threshold value of 29. This decrease in fluorescence and subsequent decrease of approximately 20% in the crossing threshold values proves that the elimination of primer-dimers is accomplished by means of this strategy. For the purposes of this study, all further RT-qPCR experiments were conducted using the optimized Protocol D cycling conditions.

a.



b.

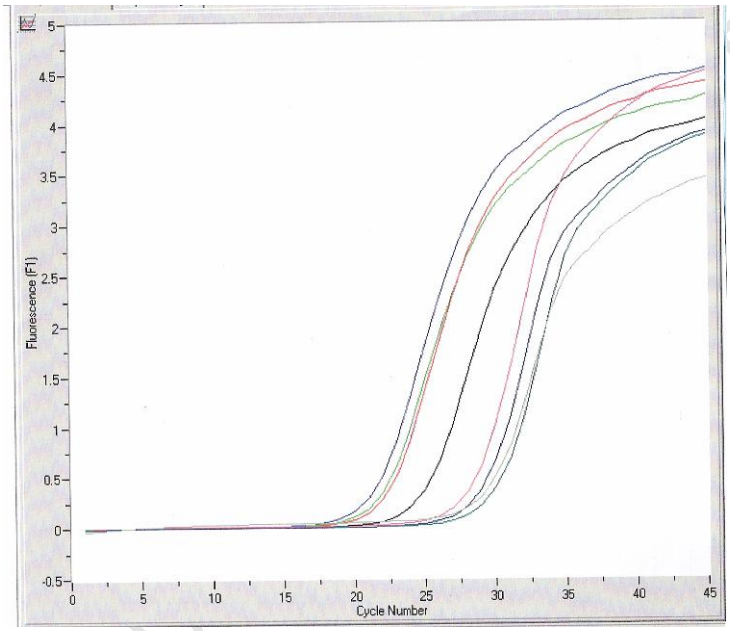


Fig 22. a. Representative experiment showing total fluorescence measured at the end of each qPCR cycle using the initial qPCR protocol (Protocol C). **b.** Representative experiment showing total fluorescence measured at the end of each qPCR cycle using the modified qPCR protocol (Protocol D).

The resolution of the above technical problem of primer-dimers allows quantification of melanocyte/melanoblast survival in vitiliginous skin. An additional patient cohort was therefore recruited in order to repeat the experimental model using the modified qPCR protocol.

3.6 Profile of the second patient cohort

Eleven patients consented to participate in the study and all eleven completed the three months of treatment. The ages of the patients ranged from 25 to 69 years (mean age of 45 years) and the duration of the disease varied from 2 to 49 years (mean duration of 12 years). Ten patients had generalized vitiligo vulgaris while 1 patient had vitiligo universalis. In 7/11 patients (64%), the disease was progressive, while in 4/11 patients (36%), vitiligo was stable. Stable vitiligo was defined as showing neither spread of existing lesions nor development of new lesions during the previous 6 months. Most patients were classified as having Fitzpatrick skin type III or IV. The majority of the patients – 9/11 (82%) – were male while 2/11 patients (18%) were female. A family history of vitiligo was found in 3/11 patients (27%), while 4/11 patients (36%) had additional concomitant auto-immune disorders including diabetes, asthma, psoriasis and rheumatoid arthritis. The questionnaire completed by the patients prior to the initiation of treatment included a history of previous therapies as well as their subjective response to that therapy rated on a scale from 0 (no response) to 10 (100% repigmentation.) Prior therapies included khellin, corticosteroids, PUVA and elocon and the subjective assessment of response to treatment ranged from 0 to 10.

Pt	Age	Sex	Disease duration (years)	Classification	Activity	Fitz-patrick skin type	FH	Concomitant auto-immune disorders	Previous treatments	Subjective response to prior treatment (0-nil 10-100%)
1	49	M	7	Generalised	Active	III	Nil	Diabetes	2006: Khellin	5
2	46	M	10	Generalised	Active	III	Nil	Nil	1997: Elocon	7
3	55	F	49	Vitiligo universalis	Stable	IV	Nil	Nil	PUVA	Unsure
4	43	M	5	Generalised	Stable	III	Nil	Asthma	PUVA	6
5	33	M	6	Generalised	Stable	VI	GM	Nil	Khellin	10
6	25	M	2	Generalised	Active	V	Nil	Psoriasis	2006: Cortico-steroid	4
7	70	F	3	Generalised	Active	IV	Nil	Rheumatoid Arthritis	2004: Cortico-steroid 2005: Khellin	5 8
8	64	M	6	Generalised	Active	IV	Uncle Cousin	Nil	2005: Khellin	4
9	45	M	28	Generalised	Active	IV	GF	Nil	2006: PUVA 2007: Khellin	7 0
10	45	M	2	Generalised	Active	III	Nil	Nil	2006: Khellin	0
11	25	M	10	Generalised	Active	IV	Nil	Nil	2003: Cortico-steroids	3

Table 4. Epidemiological profile of second cohort of vitiligo patients.

3.7 Melanocyte survival and response to treatment in the second patient cohort

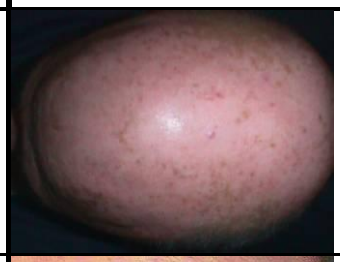
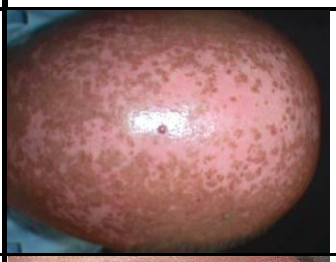
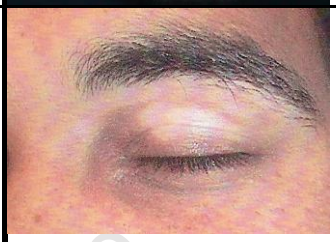
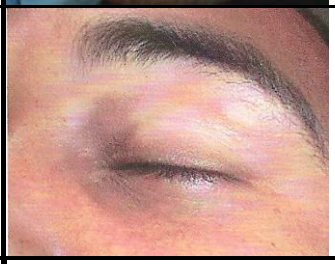
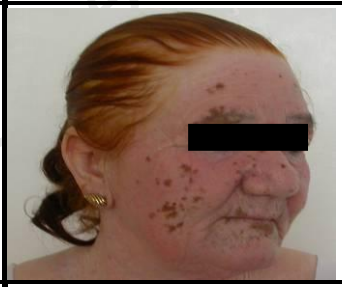
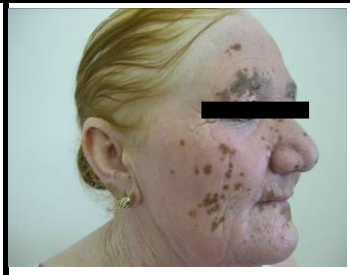
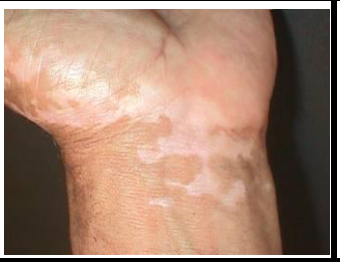
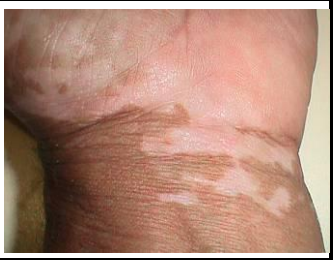
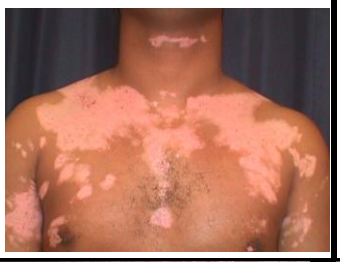
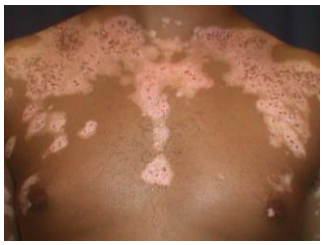
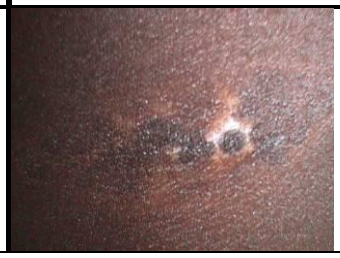
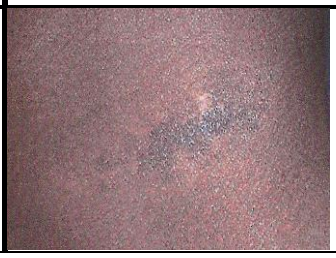
The next aim was to investigate whether quantification of melanocyte survival could be used as a prognostic assay for vitiligo patients prior to the initiation of therapy. The ability to predict a vitiligo patient's response to therapy would enable the clinician to develop an appropriate management plan. The clinician would also be able to advise the patient as to whether the time-consuming and expensive treatment options available would be judicious.

Eleven patients from the Groote Schuur outpatient and PUVA clinics consented to participate in this study. The same exclusion criteria described in Results Section 3.4 were applied. The optimized RT-qPCR protocol which effectively eliminated primer-dimers and non-specific products (Protocol D) was then used to analyse lesional and pigmented skin samples obtained by means of 4mm^2 punch biopsies. Lesional skin samples which demonstrated a peak at the correct T_m of 86ºC for tyrosinase gene expression and 89-90ºC for TRP-2 gene expression were assessed as exhibiting melanocyte/melanoblast survival. In these samples, the percentage melanocyte survival in vitiliginous skin was calculated in relation to expression in non-lesional skin using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) by calculating the relative changes in tyrosinase and TRP-2 expression.

The eleven patients were randomized into two groups: one group receiving potent corticosteroid ointment as described in Results Section 3.4 (group I) and the other group receiving a topical 5% khellin solution (group II). Both groups were instructed to have daily sunlight exposure at noon for 15 mins or until a mild erythema developed. The response to treatment in both groups was evaluated by serial photographic record. Repigmentation of the treated areas was recorded as poor (<25%), average (25-50%), good (50-75%) or excellent (>75%) depending on the extent of repigmentation.

Table 5 illustrates the relative changes in tyrosinase and TRP-2 gene expression of lesional skin samples determined from the RT-qPCR experiments as well as the patients' responses to either potent corticosteroid or khellin treatment. It can be seen that in 6/10 vitiliginous samples, the presence of melanocytes was demonstrated. In 4 patients both tyrosinase and TRP-2 gene expression was found, while 1 patient demonstrated tyrosinase gene expression alone and another patient demonstrated only TRP-2 gene expression. When melanocyte survival in lesional skin was

calculated as a percentage of the expression in pigmented skin, it was found to range from 4% to 46% survival.

Pt	Biopsy Site	Tyrosinase	TRP-2	Treatment	% Repig	Before	After
1	Lower Limb	23%	11%	Khellin	50-75%		
2	Axilla	-ve	-ve	Khellin	<25%		
3	Fore-Arm	8%	10%	Cortico-steroids	<25%		
4	Thigh	-ve	-ve	Khellin	<25%		
5	Flank	-ve	-ve	Corticosteroids	50-75%		
6	Lower Limb	-ve	30%	Khellin	>75%		

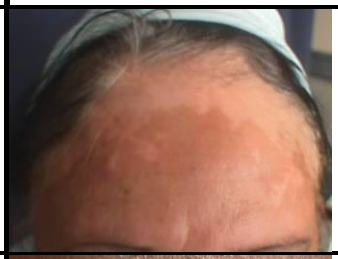
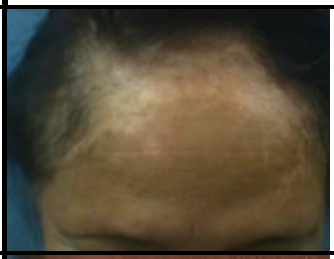
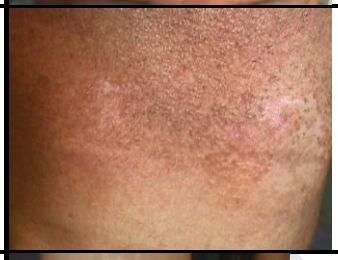
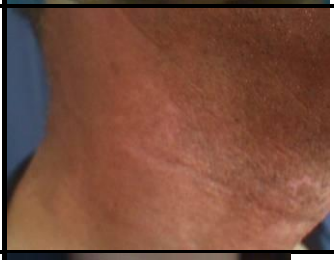
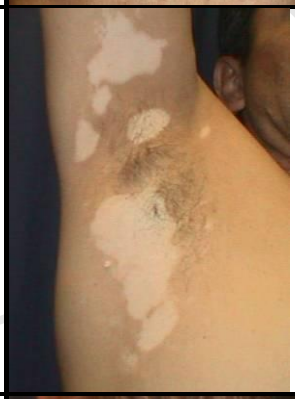
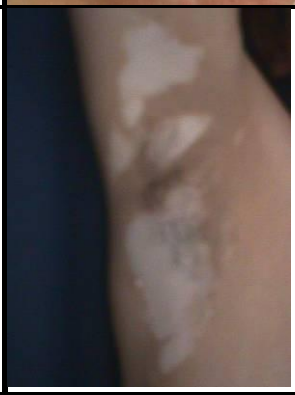
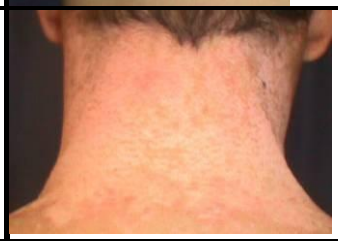
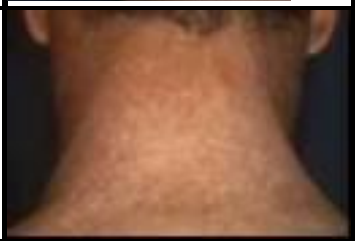
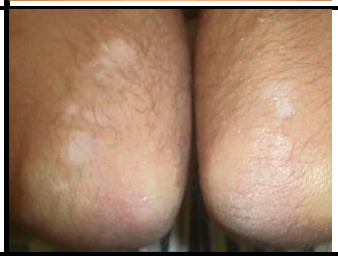
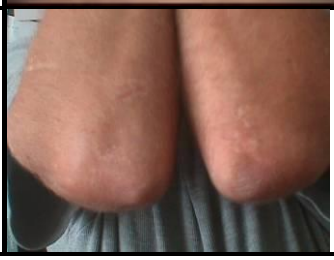
Pt	Biopsy Site	Tyrosinase	TRP-2	Treatment	% Repig	Before	After
7	Fore-Arm	-ve	-ve	Cortico-steroids	<25%		
8	Fore-Arm	-ve	-ve	Cortico-steroids	<25%		
9	Lower Limb	46%	-ve	Cortico-Steroids	<25%		
10	Lower Limb	14%	10%	Cortico-steroids	50-75%		
11	Knee	15%	4%	Khellin	25-50%		

Table 5. Percentage of melanocyte/melanoblast survival in lesional skin and response to treatment in the second cohort of vitiligo patients.

In order to determine whether a correlation between melanocyte survival and percentage repigmentation exists, a scatter graph was plotted (Fig 23). The Y axis depicts the percentage tyrosinase and TRP-2 gene expression found in lesional skin while the X axis demonstrates the percentage repigmentation following three months of topical corticosteroid or khellin therapy. The percentage gene expression shown here represents an average of the tyrosinase and TRP-2 gene expression found in lesional skin samples as calculated using the $2^{-\Delta\Delta CT}$ method. For example, in patient 3, RT-qPCR demonstrated a lesional tyrosinase and TRP-2 gene expression of 8% and 10% respectively relative to the gene expression found in the pigmented control samples. The percentage gene expression value plotted on to the scatter graph for patient 3 was therefore the value of 9%. The need to use an average of tyrosinase + TRP-2 gene expression was unavoidable as a disparity existed between the RT-qPCR values in vitiliginous skin for each gene separately. This is especially evident in patient 6 (Table 5) who demonstrated no lesional TRP-2 expression but who had a relative tyrosinase value of 30% compared to the pigmented skin sample. Similarly patient 9 exhibited a TRP-2 relative gene expression of 46% but no tyrosinase expression. Since both these genes represent markers of melanocyte survival, an average of the two values was felt to best represent the extent of this survival. The objective was to ascertain the value of the average vitiliginous skin gene expression value above which a patient was likely to demonstrate >50% repigmentation. For example, the scatter graph shown in Fig 25a depicting the correlation between % melanocyte survival and % repigmentation following treatment with khellin demonstrated a correlation coefficient $R^2 = 0.8919$. From this correlation curve, it could be deduced that an average tyrosinase and TRP-2 gene expression value of 10% indicates an adequate melanocyte reservoir whereby >50% repigmentation following treatment with the appropriate therapeutic agent could be expected. Although the correlation curve in Fig 23 shows that no correlation exists ($R^2 = 0.1257$), there does appear to be a trend between the presence of melanocytes and response to treatment. It can be seen that an absence of melanocytes appears to predict a poor response to therapy.

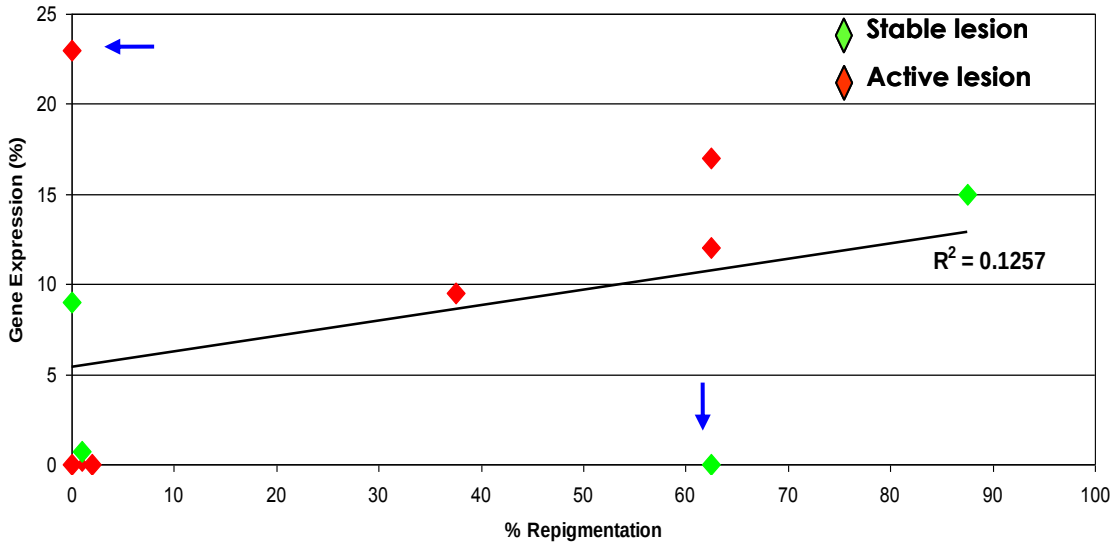


Fig 23. Scatter graph demonstrating the correlation between the % tyrosinase and TRP-2 gene expression in stable(◊) and active (◊) vitiliginous lesions and % repigmentation in vitiligo patients following either potent corticosteroid or khellin therapy ($R^2 = 0.1257$).

From the scatter graph depicted in Figure 23, it can be seen that there are 2 conspicuous outlying values influencing the correlation curve (see blue arrows). There are explanations for both of these cases.

In the first case (patient 5), the biopsy sample was taken from the flank area whereas the treated sites showing excellent (>75%) repigmentation were those only which were sun-exposed (face, neck and fore-arms). It is important to keep in mind that the biopsy of visible sites such as the face or hands is undesirable in all patients. It was therefore of value to establish whether the biopsy from any site could be used to prognosticate the state of melanocyte survival in general. It can be hypothesized however, that the fate of the melanocytes varies from lesion to lesion in vitiligo patients, particularly when taking the age of the lesion into account.

With regards to the second case (patient 9), it can be seen from Table 4 that while this patient had showed poor response to treatment with khellin, he had demonstrated a good response to PUVA therapy in 2006. This therapy was stopped due to financial constraints. It is a common and inexplicable finding that vitiligo patients will respond to one treatment and not to another. These

results emphasise this heterogeneity of patient responses to different treatment options. Evidence of melanocyte survival should therefore prompt the physician to persevere with alternative treatment modalities should one therapy fail.

In Figure 24, the scatter graph depicted in Figure 23 was re-plotted excluding the two outlying values. As can be seen, there is a significant correlation ($R^2 = 0.77$) between melanocyte survival and response to treatment. Thus this small-scale study suggests that analysis of vitiligo lesions prior to initiating treatment might have value in predicting patients' responses to therapy. Also of note is that the activity of the vitiligo lesion (active vs stable) does not seem to impact the correlation curve.

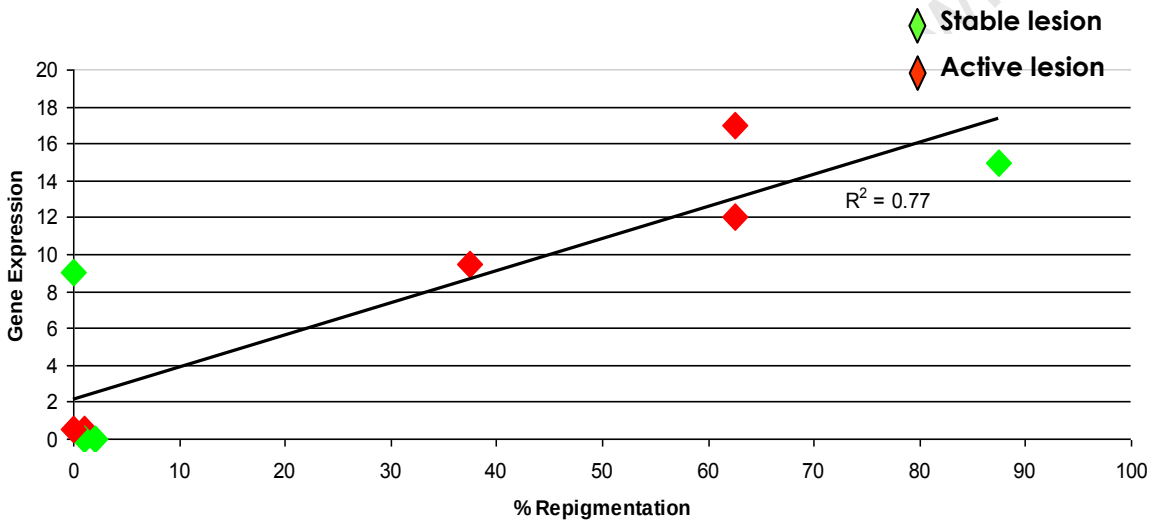


Fig. 24. Scatter graph demonstrating a significant correlation ($R^2 = 0.77$) between the % tyrosinase and TRP-2 gene expression in stable (◊) and active (◊) vitiliginous lesions and % repigmentation following treatment.

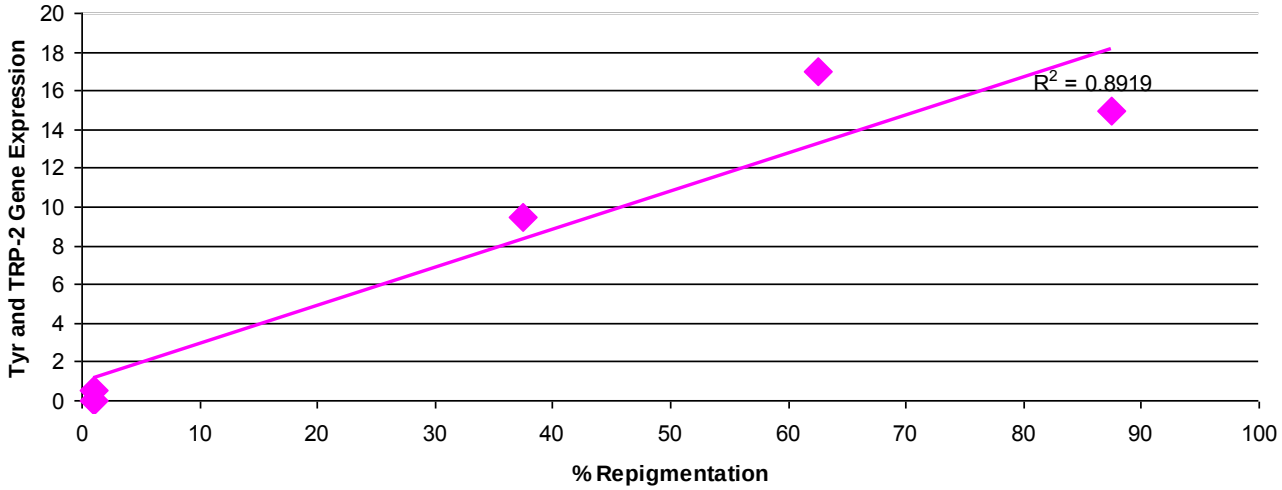
The next aim was to assess whether the different treatment modalities of khellin and corticosteroids had any impact on the degree of repigmentation.

In 2/5 (40%) of patients treated with khellin and 2/6 (33%) of patients treated with corticosteroids, more than 50% repigmentation was seen. It appears that patients in both the corticosteroid and khellin arms of the study demonstrated comparable degrees of repigmentation.

Independent scatter graphs were then plotted in order to determine whether the modality of treatment affected the correlation between melanocyte survival and % repigmentation. It can be

seen in Figure 25a that a strong correlation ($R^2 = 0.8919$) exists in patients receiving khellin therapy. Conversely, in Figure 25b, no correlation can be found in patients applying corticosteroid ointments ($R^2 = 0.169$). It is important to take into account, however, that the 2 outlying values discussed above were both in the corticosteroid arm of the study, thereby affecting this correlation curve.

a.



b.

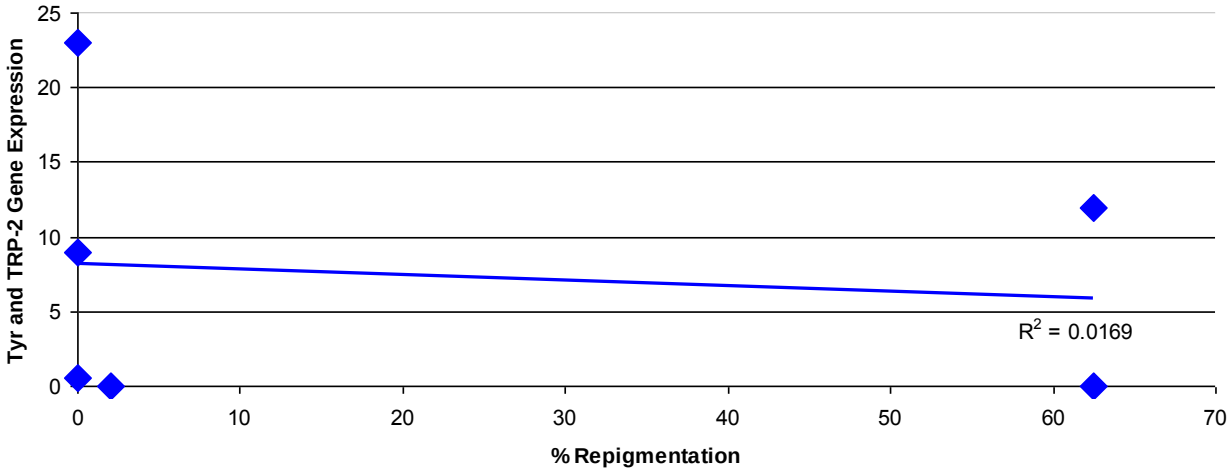


Fig 25. a. Scatter graph depicting the correlation between % melanocyte survival and % repigmentation following treatment with khellin ($R^2 = 0.8919$). **b.** Scatter graph depicting the lack of correlation between % melanocyte survival and % repigmentation following corticosteroid therapy ($R^2 = 0.0169$).

4 CHAPTER 4

DISCUSSION

Vitiligo is a depigmentation disorder of cosmetic importance with bearing on the psychological well-being and self-esteem of its sufferers. The treatment of this condition remains frustrating for innumerable reasons. The most salient of these is a lack of insight into the aetiopathogenesis of this condition. This is compounded by a lack of understanding of the mechanisms of actions of the available treatment options. Together, these critical gaps in our knowledge result in empirical therapies which might or might not target the underlying pathology. The ground-breaking discovery of the melanocyte stem cell 'niche' in the hair follicle explains the typical pattern of perifollicular repigmentation seen in vitiligo patients following PUVA therapy. But it still doesn't explain the dynamics of repigmentation or the question of why certain patients respond to treatment and others do not. In an attempt to marry science and clinical experience, the experimental approach of this study was to analyse the status of melanocytes in vitiliginous skin prior to the initiation of treatment utilizing the molecular technique of RT-qPCR. Tyrosinase and TRP-2 were chosen as markers of differentiated melanocytes and of melanocyte precursors respectively. The clinical responses to two different treatment modalities with allegedly different, albeit largely unknown, mechanisms of action were then assessed and correlated to the molecular profile of the vitiligo lesions. It was hoped to utilize our growing understanding of melanocyte biology, in particular the stem cell niche and to relate this to therapeutic options for vitiligo patients. Ultimately, it was hoped to establish a viable prognostic test to help clinicians plan a rational therapeutic approach for their vitiligo patients. In the pursuit of this objective, questions asked in this study were:

a. Can the quantification of melanocyte and melanoblast survival be correlated with patient outcomes?

b. Do lesions with demonstrated melanocyte survival respond differently to different treatments: either potent topical corticosteroid therapy (with immunomodulatory properties) or khellin therapy (with pro-proliferative properties)?

c. Can this quantification be utilized as a feasible prognostic test in terms of its efficiency, reliability, sensitivity and cost-effectiveness (i.e. proof of principle)?

d. Does the epidemiological profile of vitiligo patients contribute to the prediction of likely therapeutic response?

4.1 Development of a more sensitive, quantitative measure of melanocyte/ melanoblast survival in vitiliginous biopsies

The question of whether vitiligo lesions are completely devoid of melanocytes remains in dispute. In 2007, Gottschalk and Kidson published a pilot study analysing melanocyte survival in vitiligo patients utilizing molecular techniques. They found that only 25% of vitiliginous lesions were positive for tyrosinase and/or TRP-2 mRNA expression. However, clinical studies report that 30-75% of vitiligo patients respond to treatment. This suggests that either the presence of melanocytes/melanoblasts is not a good prognostic test and/or that this low detection rate was due to technical limitations of the molecular assay and/or the sample size of this study (n=12) was too small. This study aimed to distinguish between these possibilities and to expand the data pool.

In order to achieve this, RT-qPCR was utilized to quantitate melanocytes and melanoblasts in vitiliginous skin more accurately. To optimize the protocol, lesional and non-lesional skin biopsies were taken from 10 vitiligo patients prior to the initiation of either potent topical corticosteroid or topical khellin therapy in combination with daily sunlight exposure. The accurate analysis of tyrosinase and TRP-2 gene expression of these biopsy samples proved to be challenging owing to a number of reasons.

The first difficulty was the resilience of frozen skin biopsy specimens which proved very tough, resisted homogenization and which were prone to degradation owing to endogenous RNAses. This resulted in incomplete sample disruption and sample loss. These problems were exacerbated by the fact that the punch biopsy specimens were only $4mm^2$ and had an average weight of only 13.8mg. Homogenisation procedures therefore had to be optimized. It was found that three cycles of homogenization of three seconds each with five second intervals of cooling on ice provided optimal results. This protocol combined with stringent RNA protection measures resulted in the satisfactory average RNA yield of 3.3µg per skin biopsy specimen with an average RNA purity of 1.9. More

importantly, all of the skin biopsy specimens yielded usable data utilizing this homogenization technique. This is a significant improvement over prior RNA extraction procedures from skin biopsies such as Cole et al., (2001) where utilizable RNA from breast skin biopsies was extracted in only 56% of the biopsies.

The next technical probem was the formation of non-specific products or primer-dimers during qRT-PCR analysis. At the extension temperature of 72ºC, high levels of primer-dimers were present. RT-qPCR using SYBR Green works on the principle of measurement of fluorescence resulting from the dye binding to dsDNA during extension. Primer-dimer formation therefore results in an increase of the fluorescence, thereby giving incorrect crossing threshold values and thus incorrect quantitation.

Since all the RNA from the first patient cohort had been used, it was necessary to recruit a second cohort of patients for further skin biopsy samples and to re-optimise the RT-qPCR methodology in order to eliminate these primer-dimers. To do this, the Light-Cycler's capacity to differentiate between the melting temperatures of the gene product of interest and the incidental primer-dimers was exploited. The strategy used a modification of the RT-qPCR cycling programme to include a primer-dimer melting step which effectively eliminated the primer-dimer fluorescence. This strategy resulted in a 20% decrease in the crossing threshold values and confirmed that the fluorescence of only the desired amplicon was measured.

Using this methodology, it was demonstrated that there was tyrosinase and/or TRP-2 gene expression in 60% of lesional skin biopsies (n=21). In 5/21 patients, both tyrosinase and TRP-2 were expressed, indicating the presence of eumelanin-producing melanocytes. In 4/21 patients, TRP-2 was expressed alone, indicating melanoblast survival, while in 3/21 patients only tyrosinase was expressed. When compared to the result of Gottschalk and Kidson, (2007), where only 25% of samples were positive for tyrosinase and/or TRP-2 gene expression (n=12), the percentage survival found in this study is much higher. This strongly supports the superior sensitivity of the RT-qPCR protocol. When this gene expression in lesional skin was calculated as a percentage of the expression of the pigmented skin of the same patient (using $2^{-\Delta\Delta CT}$) , it was found to range from 8% to 46% for tyrosinase and from 4% to 30% for TRP-2 (n=11). It was hoped to determine whether the level of these lesional gene expression values would correlate with successful repigmentation following treatment and whether a specific cut-off value exists below which treatment always fails.

If so, these values would provide a much needed prognostic tool to guide dermatologists in planning an appropriate therapeutic course of action.

The finding in three patients that only tyrosinase (and not TRP-2) was expressed is surprising. There are two possible reasons for this result: it could represent either a technical inaccuracy or the presence of pheomelanin-producing melanocytes. The more likely explanation is a technical issue as opposed to a biological one. This is supported by the fact that following further fine-tuning of the RT-qPCR protocol for the second cohort of patients, an increased number of patients demonstrated expression of both enzymes together (4/11) as compared to the first cohort of patients (1/10). This also suggests that additional optimization of the RT-qPCR protocol is required to provide a more sensitive and more precise molecular assay and that caution is needed when interpretating this study's results.

4.2 Is there a correlation between melanocyte survival and response to treatment?

After obtaining the biopsies indicated above, the patients were initiated on either potent topical corticosteroid or topical khellin therapy. For both groups, 15 minutes daily sun exposure at noon was stipulated. The percentage repigmentation achieved following three months of treatment was recorded and plotted against the pre-treatment tyrosinase and TRP-2 gene expression values of the vitiliginous lesions. When all the samples were plotted, the results did not show any correlation between melanocyte/melanoblast presence and response to treatment, although a trend can be appreciated.

On closer scrutiny of the scatter graph, two outlying values which influence the correlation curve are conspicuous. A strong correlation becomes clearly apparent upon the exclusion of these two outlying values ($R^2 = 0.77$). This highlights three study design flaws.

1. The first experimental design fault is the fact that the site biopsied was not always the same site that was treated. This was the case for the first outlying value where the biopsy site (the flank area) was not within the area receiving treatment (only the sun-exposed areas). The first critical modification of the study design for future research purposes would therefore be to ensure that the biopsy site is also treated.

2. The second design flaw is that only one treatment modality was attempted for each patient. It is a common and inexplicable finding that vitiligo patients' responses to treatment are heterogenous and that they will respond to one therapeutic modality and not to another. The patient representing the second outlying value, although demonstrating no response to topical corticosteroid therapy in this trial, had in the past responded to a different treatment modality. This raises the issue of the length of follow-up. The second modification of a future study would therefore be to utilize a prospective study design with a longer follow up period in order to allow for various treatment options to be attempted.
3. The final experimental design flaw relates to the small sample size of this study, as is highlighted by the fact that the correlation curve could be so greatly influenced by only two outlying values. The third modification would therefore be to recruit a greater number of patients, in order to attain statistically significant results from which concrete conclusions can be drawn.

A significant correlation was found when considering melanocyte survival in the cohort of patients receiving khellin therapy ($R^2 = 0.8919$). This is more likely to be because the two outlying values described above were both in the corticosteroid arm of the trial and thus were not distorting the correlation curve in the khellin group, rather than any significance in these two therapies' presumedly different mechanisms of action in vitiligo.

4.3 Which therapy, topical corticosteroids or topical khellin, combined with sunlight has a better therapeutic response?

Topical corticosteroids have been used as a first-line treatment for vitiligo during the last three decades. The low cost, ease of application and high rate of adherence are the benefits of using this treatment. However, there is a high recurrence rate following cessation of treatment as well as adverse side effects such as skin atrophy, telangiectasia and striae. There is also the risk of increased intraocular pressure, glaucoma and cataracts when using potent topical steroids around the eyes. This is significant as vitiligo often manifests in the peri-ocular region. The advent of KUVA phototherapy therefore heralded great hope for both physicians and patients grappling with vitiligo. It has been lauded as a safe home-based alternative to PUVA therapy which is often unfeasible due to high transport costs and rigid employment hours precluding frequent hospital visits (Valkova et al., 2004). The high solar ultraviolet levels experienced in South Africa make this an especially

exciting therapeutic option. Its safety profile also makes it a good option for the treatment of sensitive areas of the skin such as eyelids. Its efficacy however remains in dispute.

Corticosteroids and khellin are hypothesized to induce repigmentation in vitiligo patients by targeting the auto-immune component or by stimulating melanogenesis respectively. Due to the differences in their alleged mechanisms of actions, it was hoped to determine, in the context of this study, which treatment modality was more effective in vitiligo patients and whether this could provide any further insight into the aetio-pathogenesis of this disorder.

In this study, 15 patients applied topical clobetasol propionate ointment to the hands and fore-arms and betamethosone ointment to the face and neck twice daily while 6 patients applied khellin ointment to these same areas daily for a trial period of three months. In both groups of patients, the treatment was supplemented with 15 minutes daily sun exposure at noon. Following the three months of therapy, the repigmentation of the treated areas was found to be excellent (repigmentation >75%), good (repigmentation 50-75%), average (repigmentation 25-50) or poor (repigmentation <25%).

A good or excellent response of more than 50% repigmentation was seen in 27% of patients (n=15) using corticosteroid therapy. This is markedly lower than that reported in other studies, for example Kumari et al., (1984) where 90-100% repigmentation was achieved in more than 80% of patients with vitiligo of the face (n-75). However it is higher than the low response rate reported by Clayton et al., (1977), (n=23) where only 9% of patients demonstrated >75% repigmentation. In addition, in this study, one would have expected better response rates by virtue of the treatment area including the facial region. It has been reported that the face demonstrates a superior response when compared to other areas of the body as it has a higher concentration of melanocytes and the skin in this area is thinner (Milne et al., Unpublished data). These discrepancies can be explained by study design differences, the smaller sample size of this study as well as the different phenotype of the patients involved. The majority of patients in this study had a Fitzpatrick type IV skin phenotype. As vitiligo in dark-skinned individuals is known to respond better to treatment (Mandel et al., 1997), a superior repigmentation response was anticipated. However, the small patient numbers in this study make any comparisons to larger studies difficult. Other factors limiting comparisons include the fact that in some studies, patients received therapy to small patches in isolation whereas in others, therapy was applied to all affected areas. In addition, the end-points in different studies

vary from the repigmentation of single patches to overall repigmentation. Different compounds have been studied with different potencies and differences in the vehicles chosen may also have effected the penetration and therapeutic efficacy.

With regards to khellin, this study's finding of more than 50% repigmentation occurring in 33% of patients (n=6) applying khellin ointment corroborates the studies of Orecchia et al., (1999) and Valkova et al., (2004) in which 36% and 43.8% of patients respectively experienced a repigmentation of greater than 50%.

Comparable degrees of repigmentation were therefore seen in the corticosteroid and khellin groups of this study, however it is difficult to make any concrete analyses due to discrepancies in numbers between the two treatment arms as well as the small sample size. Another important limitation of this study is the fact that patients received corticosteroid or khellin therapies in combination with sunlight. Ultraviolet radiation is a distinct treatment modality which alone can stimulate melanogenesis, melanocyte proliferation and subsequent repigmentation in vitiligo patients. In view of the cost-effectiveness and convenience of sunlight as a treatment modality for vitiligo patients, the role of sunlight, as well as its potential risk of increased skin carcinogenesis, requires further investigation. A future experimental design should therefore include control groups receiving either sunlight alone or no treatment at all as separate arms of the study.

Another consideration when planning a future design study would be to investigate the efficacy of a combination corticosteroid/khellin therapy to ascertain the benefits of their respective immunosuppressive and proliferative effects together. Westerhof et al., (1999) compared topical fluticasone ointment alone or combined with UVA in 135 adults. They found that the potent topical corticosteroid fluticasone used alone for nine months induced a mean repigmentation of only 9%, UVA alone a mean repigmentation of 8% while the combination of fluticasone and UVA induced a mean repigmentation of 31%. Conversely, another study by Lotti et al., (2008) demonstrated a lower response rate of vitiligo patients to a combination therapy of betamethasone dipropionate 0.05% cream twice a day plus 311-nm narrow-band microphototherapy (71.2% demonstrating >75% repigmentation after six months therapy) compared to betamethasone dipropionate cream alone (90.2% demonstrating >75% repigmentation). The small number of randomized controlled trials comparing combination therapies to monotherapies makes it difficult to draw any conclusions. However, one could hypothesize that the synergistic effects of a topical immunosuppressive agent –

i.e. a topical corticosteroid and a photosensitizer – i.e. khellin would provide a greater repigmentation response than either therapy alone, particularly in patients demonstrating melanocyte survival.

4.4 Is there a correlation between the epidemiological profile of vitiligo patients and their response to therapy?

Vitiligo is heterogeneous in its aetiopathogenesis, in its natural history and in its response to therapy. It has been proposed that different pathogeneses might underlie the different clinical phenotypes thus explaining their variable responses to treatment. This study hoped to establish whether any characteristics in the epidemiological profile of vitiligo patients could have prognostic implications.

The clinical profile of the 21 vitiligo patients included in this study is that of generalized vitiligo (one with vitiligo universalis) with a mean duration of 14 years. The finding that all patients have a generalized form of vitiligo in this study can be explained by the fact that children, in whom segmental vitiligo is a common presentation, were not included in this study. Nevertheless, in comparison with the study of Handa et al., (1999) who reported 70% of patients with vitiligo vulgaris, 15% of patients with focal vitiligo and 15% with segmental vitiligo, the lack of cases of localized vitiligo is striking. This is a significant factor as patients with segmental vitiligo have been reported to have a superior response to therapy (Khalid and Muftaba, 1998). This could explain the relatively low percentage (27%) of patients experiencing >50% repigmentation in this study.

Another important prognostic factor is that of the activity of the vitiligo. This is determined by the progression of existing vitiligo lesions, the development of new lesions as well as by repigmentation within existing lesions. In this study, it was found that 62% of patients experienced progressive disease while 38% classified their disease activity as stable. It could be hypothesized that melanocyte survival is more unlikely in stable lesions of long duration and that these patients should be counseled to anticipate a poor response to treatment. This was not found to be the case, however with no correlation between disease activity, melanocyte survival and thus response to therapy being observed.

The finding in this study that 44% of patients reported concomitant auto-immune disorders is higher than the statistics in the literature which report that 13% of vitiligo patients have an underlying auto-immune diathesis. This provided an additional rationale for the choice of topical corticosteroids as a treatment modality. However, an auto-immune diathesis appears to have no prognostic implications in predicting treatment outcome.

Despite a thorough history-taking and exploration of all epidemiological factors, the epidemiological profile of vitiligo patients proved unhelpful in predicting their responses to treatment.

4.5 Practical implementation of the RT-qPCR analysis and additional prognostic indicators

A summary of the tyrosinase and TRP-2 mRNA expression in vitiliginous skin lesions with their respective subsequent repigmentation outcomes can be seen in Table 6 below. It can be seen that >50% repigmentation (a good response) was found in 3/5 patients demonstrating both tyrosinase and TRP-2 gene expression, in only 1/4 patients demonstrating TRP-2 gene expression alone and in no patients demonstrating only tyrosinase gene expression (n=3). The presence of both tyrosinase and TRP-2 mRNA expression is therefore a significant positive prognostic indicator.

		Repigmentation			
Gene expression	**Treatment**	**Poor**	**Average**	**Good**	**Excellent**
Tyrosinase and TRP-2 (n=5)	**Corticosteroids**	**1**		**2**	
	Khellin		**1**	**1**	
TRP-2 only (n=4)	**Corticosteroid**	**2**			
	Khellin	**1**			**1**
Tyrosinase only (n=3)	**Corticosteroids**	**2**	**1**		
	Khellin				

Table 6. Summary of tyrosinase and TRP-2 mRNA expression as determined by RT-qPCR and the response to either topical corticosteroid or khellin treatment following three months of therapy.

It is also significant that a good correlation can be seen between melanocyte survival and response to khellin therapy ($R^2 = 0.8919$ as shown in Fig. 16 Chapter 3.6) which is a mitotic and melanogenic agent (Carlie et al., 2003). Out of the 4 patients using khellin who demonstrated melanocyte survival, a good or excellent response to treatment was seen in 2/4, an average response was seen in 1 patient and a lack of repigmentation was seen in 1 patient. This suggests that therapeutic modalities stimulating melanocyte proliferation and migration should be employed in patients demonstrating melanocyte survival.

Conversely in the two patients using khellin therapy in whom melanocyte markers were absent, a poor response to therapy was seen in both. This key finding that an absence of melanocyte survival appears to predict a poor response to treatment is recapitulated in the overall findings: In 7/9 patients (78%) of patients demonstrating a lack of lesional tyrosinase or TRP-2 mRNA expression, there was <25% repigmentation at the conclusion of a three month treatment period. It appears that these therapeutic modalities are ineffective in the absence of a melanocyte/melanoblast reservoir that can be stimulated. These patients should be counseled against expensive, inconvenient therapies with noteworthy side effects and should rather be encouraged to use alternative options such as cosmetic camouflage techniques. This would avoid the inevitable heartbreak which goes hand-in-hand with treatment failure.

An important epidemiological factor to emerge from this study is the presence of a co-existing auto-immune condition in 44% of vitiligo patients. As corticosteroid therapy allegedly exerts its effects in vitiligo by means of its immunosuppressive and anti-inflammatory properties (Boumpas et al., 1993; Ortonne, 1998) it could be hypothesized to best benefit patients with an auto-immune diathesis. This reasoning seems to be flawed for our population as 100% (6/6) vitiligo patients with an associated auto-immune condition using corticosteroid therapy showed <25% repigmentation while 2/3 (66%) patients with a co-morbid auto-immune condition receiving khellin demonstrated >50% repigmentation. This unexpected result is validated by the fact that overall, vitiligo patients showed similar response rates to both treatment modalities. It appears that an auto-immune diathesis alone cannot be used as a justification in the decision to use topical corticosteroid therapy which should be employed only in patients with recent onset or active vitiligo where the melanocyte destruction stimulating this auto-immunity is ongoing. It could be postulated that these auto-immune mechanisms may be quiescent in stable vitiligo rendering corticosteroids a less effective treatment option for these patients. New evidence is emerging that immunomodulators, in particular calcineurin inhibitors, can induce repigmentation in vitiligo patients through non-immune mechanisms, particularly in segmental vitiligo. Recent in vitro evidence of a direct interaction between tacrolimus and keratinocytes creating a favourable milieu for melanocytic growth and migration has been reported (Lan CC et al., 2005; Kang and Choi 2006). Tacrolimus-treated keratinocyte supernatants resulted in melanoblast and melanocyte proliferation as well as enhanced stem cell factor and metalloproteinase-9 activity which is involved in cell migration. It is possible that corticosteroids also have non-immune targets in the treatment of vitiligo and perhaps, should not be utilized only in the setting of patients with an autoimmune diathesis, active vitiligo or micro-inflammatory vitiligo. Further large-scale epidemiological studies evaluating the clinical settings in which corticosteroid therapy would be most appropriate need to be conducted. Studies elucidating the predominant mechanism of corticosteroids in vitiligo – immune vs non-immune- are also needed. Until such time, logic dictates that corticosteroids should have superior efficacy in patients where immune mechanisms are at play.

The epidemiological profile of each vitiligo patient in combination with the results of the RT-qPCR analysis of their lesional skin biopsy samples, can therefore be used to plan an individualised therapeutic approach as is shown in Figure 26. This algorithm should apply only to the repigmenting stage of therapy in patients with stable vitiligo. There is now evidence that active

vitiligo is a micro-inflammatory disease. Micro-inflammatory vitiligo is recognized clinically by depigmented patches surrounded by a raised erythematous border with variable scale and marginal hyperpigmentation and histologically by a superficial perivascular lymphohistiocytic infiltrate and variable vacuolar change, lichenoid inflammation, epidermal spongiosis and psoriasiform epidermal hyperplasia. Lichenoid infiltrates have also been demonstrated in 23% of common macular vitiligo lesions where the inflammation was subclinical. Therapeutic modalities for vitiligo should therefore take this stage into account more specifically (Swick and Walling, 2008; Attili and Attili, 2008)."

Positive tyrosinase and/or TRP-2 mRNA expression shown during molecular profiling of vitiligo lesions by RT-qPCR indicates a melanocyte/melanoblast reservoir. Should these patients have no history of an auto-immune diathesis and should they have stable disease (as defined in Chapter 2.6), they would benefit best from topical khellin therapy in combination with daily sunlight exposure in order to stimulate melanogenesis. It would appear that topical corticosteroids would not provide any additional benefit in this patient group. The presence of an auto-immune diathesis, inflammatory vitiligo or active disease, however, suggests that the auto-immune mechanisms hypothesized to initiate/propogate the disease process, are still active. In these patients, the topical khellin and sunlight therapy should be used in combination with topical corticosteroids to dampen down the ongoing immune response targeting the melanocytes while simultaneously stimulating melanogenesis. Should these therapies have failed following a three month trial period, dermatologists, knowing the heterogenous response of vitiligo patients to various therapeutic modalities, should persevere in exploring alternative therapeutic options.

In the absence of a melanocyte/melanoblasts reservoir, as indicated by the RT-qPCR analysis of lesional skin samples, khellin should not be prescribed. The chance of repigmentation is poor in this patient group. Patients should be counseled and alternative treatments such as camouflage cosmetics or surgical treatment modalities should be advocated. However, in the presence of an auto-immune diathesis, inflammatory vitiligo or active disease, topical corticosteroid therapy should still be used in order to stop the further progression of the disease process.

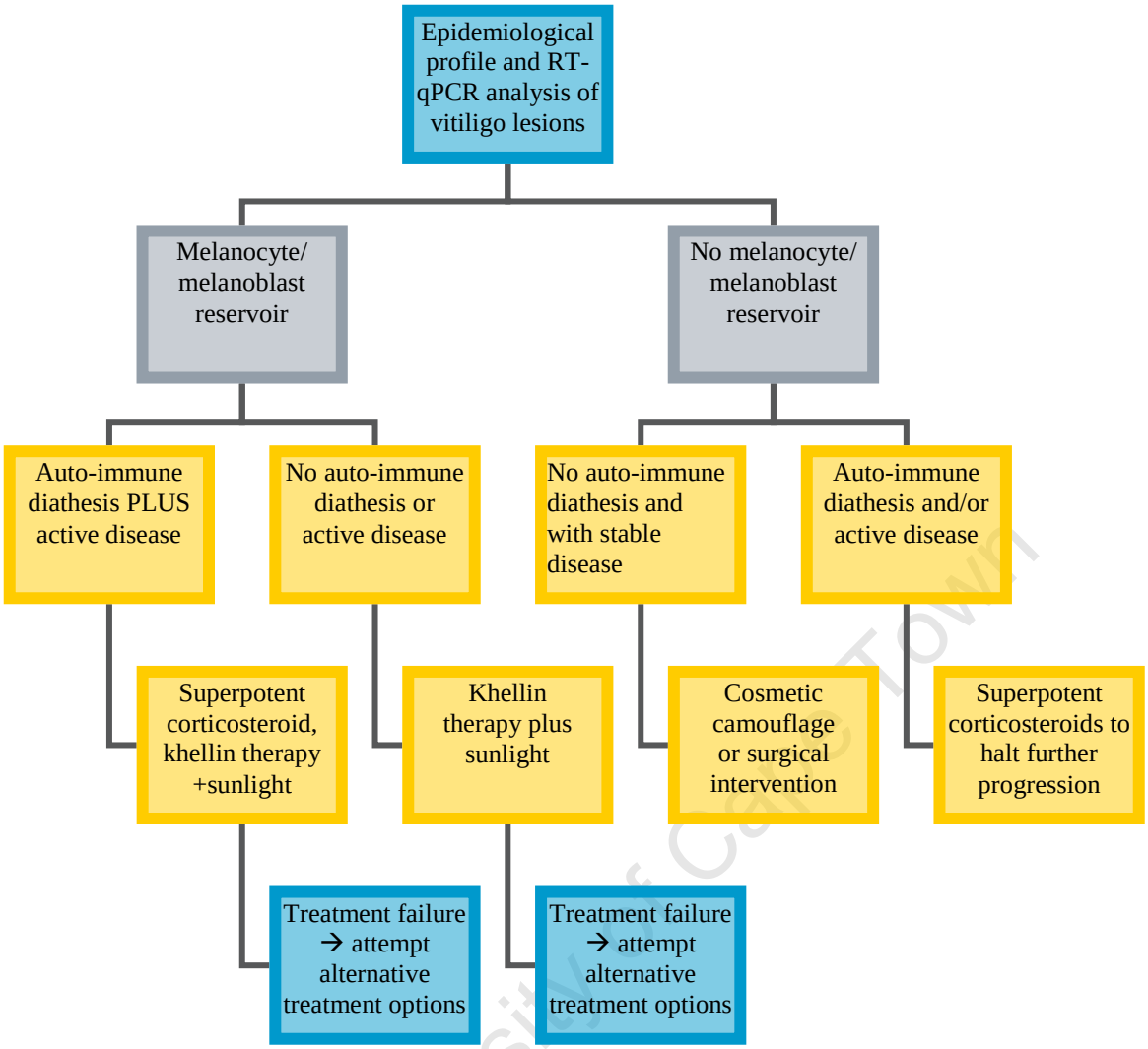


Fig. 26 Proposed algorithm of management of vitiligo patients based on their epidemiological profile and results of the molecular profiling of their vitiligo lesions by means of RT-qPCR

4.6 Future endeavours

Although the evidence presented in this study gives clear priorities for research, it is not robust enough to provide comprehensible guidelines in terms of the prognosis or treatment of vitiligo patients. The algorithm presented in Fig. 26 above on the management of vitiligo patients based on their epidemiological profile and the molecular profile of their lesional skin will need further fine-tuning. This would depend on an expanded dataset with an increase in the sample size as well as a more exacting study design. Inclusion of patients with segmental vitiligo, stringent selection of

biopsies from only areas to be treated and a longer study period of one year to allow for trials of different therapeutic modalities would provide more meaningful results.

Pigment cell research is experiencing an exciting era with new innovations in stem cell biology. Novel cell surface markers have been identified on human follicular bulge cells using microarray analyses and precise laser capture microdissection (Osawa et al., 2005) and the presence of intraepidermal stem cells has been confirmed (Passeron et al., 2007). The ability to differentiate between epidermal and follicular melanocyte stem cells could provide the key to profiling vitiligo lesions. Passeron et al., (2007) explored the antigenic expression patterns of markers for the subpopulations of melanocytes in the epidermis and hair follicles. This study demonstrated that while tyrosinase and TRP-2 were positive markers for melanocytes/melanoblasts in the epidermis, Pmel17, c-KIT and Bcl-2 could prove to be superior markers of melanocyte stem cells in the bulge region of the hair follicle. Analysing vitiligo lesions more widely to include these markers would provide further insight into the targets of current vitiligo therapies.

4.7 Concluding remarks

This study has provided new insights into the fate of melanocytes in vitiligo. The fact that there is melanocyte survival in 60% of vitiligo patients has important therapeutic implications, providing greater motivation to persevere with treatment in these patients. A prognostic test would enable physicians to develop practical management plans and offer some guidance in a condition whose treatment is otherwise rather hit-or-miss. Despite the limitations of this study which have been outlined in 4.2 above, the molecular profile of vitiligo lesions can still be used as a presumptive prognostic aid. Evidence of melanocyte survival should encourage physicians to instigate treatment – in particular melanogenic agents such as KUVA. It would also encourage perseverence with alternative therapeutic options should a lack of repigmentation be observed with one therapy. An absence of melanocyte markers, however, is a poor prognostic sign and medical therapy should be cautioned against. In these cases, melanocyte grafting techniques or camouflage cosmetics should be considered depending on the extent and stability of the patients' lesions as well as their level of distress. The epidemiological profile can also guide dermatologists in planning treatment for their vitiligo patients. A history of an auto-immune diathesis, inflammatory vitiligo or of an active disease process should prompt treatment with topical corticosteroids.

Despite the despondency and frustrations implicit in this condition, physicians should not fall into the trap of dismissing vitiligo as a condition not worth treating. Instead, a drive to invest more research into the nature of this disorder and its treatment and to provide more tangible links between science and clinical experience is long overdue.

5 APPENDIX:

Consent form for punch biopsies

To be filed in the patients folder Patient code number…….

I (full name)……
hereby give consent for a punch biopsy to be taken from my skin. I understand that the doctor/clinician will remove a very small (4mm) piece of my skin from the pigmented skin and from the vitiligo skin and that the risk of infection is minimal. This procedure will be performed under local anaesthesia.

I understand that my participation in this study is absolutely voluntary and that should I refuse to participate or decide to withdraw from participation at any time, it will not affect my subsequent clinical treatment or care.

I hereby understand that my skin will be used in scientific experiments to improve our knowledge about whether any pigment cells that cause colour in the skin survive in vitiligo and whether their survival affects response to treatment.
I understand that any results obtained will be entirely confidential.
I understand that I may be contacted in the future for consent for further studies on these skin samples should :
1) my skin yield results that may impact on potential treatment for me, or
2) should there be a need to use these biopsies for other studies.

I consent/do not consent for photographs of my skin to be taken for the purposes of this study. I understand that it will not be possible to identify me from these photographs.

I have read all of the above and anything I have not understood has been fully explained to me.

Signed:……Date:…….

Place:……

Groote Schuur Hospital folder number:…….

Doctors Full name:…….....Doctors Signature……

Witness (full name):……..…….....Witness's signature…….

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