The University Of Sheffield.

Identification and Characterisation of Novel Antibody Targets in Vitiligo

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Summary

Vitiligo is an acquired, non-contagious depigmenting skin disease results from epidermal melanocyte loss. Accumulating evidence suggests a role for autoimmunity in vitiligo aetiology, as antibodies and autoreactive T cells against melanocyte autoantigens can be detected in vitiligo patients. As part of determining the pathomechanisms involved in the development of vitiligo, identifying the specific targets of the immune reactivity in patients is a major goal in the vitiligo research field. A better understanding of vitiligo aetiology may help to develop successful treatment and diagnostic modalities.

One of the main aims of this study was to identify novel autoantigens in vitiligo using phage-display technology; a melanocyte cDNA phage-display library was immuno-screened with IgG from 22 vitiligo patients. Several autoantigens were enriched by this technique, including ones already known (tyrosinase, tyrosinase-related protein 1, melanocyte-specific protein PMEL, L-dopachrome tautomerase, heat-shock protein 90, ribosomal protein L24, and LaminA). Additionally, humoral immune reactivities were identified against previously unreported putative autoantigens glycoprotein non-metastatic melanoma protein b (GPNMB), OCA2-encoded P protein (OCA2 protein), melanocortin-1 receptor, and GTP-binding protein Rab27A.

The frequency of GPNMB, OCA2 protein, and LaminA antibodies in sera from healthy subjects (*n* = 100) and non-segmental (*n* = 231), segmental (n = 4) or focal (*n* = 11) vitiligo patients was investigated using radioligand binding assays (RLBAs). Healthy individuals were all negative for all antibodies tested. In the non-segmental vitiligo patient group, 91/231 (39%) were positive for GPNMB antibodies and this was statistically significant when compared with healthy controls (*P* = < 0.0001). The frequency of GPNMB antibody was significantly higher in the non-segmental vitiligo patient group with active disease compared to those with stable disease (*P* = 0.006). OCA2 protein and LaminA antibodies were detected at an overall frequency of 22%. GPNMB, OCA2 protein, and LaminA were antibody targets in both patient groups with and without accompanying autoimmune disease.

Overall, the study identified GPNMB, OCA2 protein, and LaminA as autoantigens in vitiligo further supporting a role for autoimmunity in vitiligo development.

Declaration

I hereby declare that this thesis has been written by myself and has not been accepted previously in any publication for a higher degree. The work reported in this thesis has been performed by myself, except where specifically acknowledged in the text. All information sources have been specifically acknowledged by means of references.

Safa Faraj

October, 2018

Dedication

I proudly dedicate my thesis to my family; to my beloved parents Hindleeba and Salma, to whom I am truly grateful for their constant tremendous encouragement and prayers, to my loving husband Wisam for all the support, motivation, and love he has always given me unconditionally during the challenges of my whole PhD life, to my lovely son Waqas, to my supportive sisters and brother whom I am thankful for having them in my life. I also dedicate this thesis to the memory of my brother Khalid, who always believed in my ability to be successful in academic research. You are gone and yet your confidence in me has made this journey conceivable.

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List of Abbreviations

°C	Degrees centigrade
µg	Microgram/s
µl	Microlitre
6-BH4	6-tetrahydrobiopterin
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
AIRE	Autoimmune regulator protein
ASP	Agouti signalling peptide
BACH2	Transcription regulator protein BACH2
b-FGF	Basic fibroblast growth factor
bp	Basepair/s
C1QTNF6	Complement C1q tumour necrosis factor-related protein 6
cAMP	Cyclic adenosine monophosphate
CASP7	Caspase-7
CAT	Catalase
CCR6	Chemokine-cytokine receptor 6
CD44	CD44 antigen
CD80	T lymphocyte activation antigen CD80
cfu	Colony-forming units
CGRP	Calcitonin gene-related peptide
CLEC2B	C-type lectin domain family 2 member B
CLNK	Cytokine-dependent hematopoietic cell linker
cpm	Counts per minute
CREB	cAMP-responsive element binding protein
CTLA4	Cytotoxic T lymphocyte antigen-4
CXCL	Chemokine C-X-C motif ligand
CXCR3	C-X-C chemokine receptor type 3
CXCR5	C-X-C chemokine receptor type 5
DAG	Calcium and diacylglycerol
DCT	L-dopachrome tautomerase
E. coli	*Escherichia coli*
EBI-EMBL	European Bioinformatics Institute-European Molecular Biology Laboratory
EDN-1	Endothelin-1
EDTA	Ethylenediaminetetraacetic acid

ERK	Extracellular signal–regulated kinases
F	Forward (primer)
FASLAG	FAS ligand
FOXD3	Forkhead box D3
FOXP1	Forkhead box protein P1
FOXP3	Forkhead box protein P3
g	Gravity
GM-CSF	Granulocyte monocyte-colony stimulating factor
GPCR	G protein-coupled receptors
GPNMB	Glycoprotein non-metastatic melanoma protein b
GZMB	Granzyme B
h	Hour/s
HEK293	Human embryonic kidney 293 cells
HLA	Human leukocyte antigen
HSP	Heat-shock protein
ICAM-1	Intercellular adhesion molecule-1
IFIH1	Interferon-induced helicase C domain 1
IFN-α	Interferon-α
IFN-β	Interferon- β
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IgM-RF	IgM-rheumatoid factor
IKZF4	Zinc finger protein Eos
IL	Interleukin
IL1RN	Interleukin-1 receptor antagonist
IL2RA	Interleukin 2 receptor subunit alpha
kb	Kilobase/s
kDa	Kilodalton
LB	Luria Bertani
L-DOPA	L-dihydroxyphenylalanine
LPP	Lipoma-preferred partner
m	Metre
M	Molar
MAP	Mitogen-activated protein
MC1R	Melanocortin 1 receptor
MCH	Melanin-concentrating hormone

MCHR1	Melanin-concentrating hormone receptor 1
MEK	Mitogen-activated protein kinase
mg	Milligram/s
MHC	Major histocompatibility complex
min	Minute/s
miRNAs	MicroRNAs
MITF	Microphthalmia-associated transcription factor
ml	Millilitre/s
mm	Millimetre
mM	Millimolar
MOPS	3-[N-morpholino]propanesulphonic acid
MTHFR	Methylene tetrahydrofolate reductase
NB-UVB	Narrow band-ultraviolet B
NCBI	National Center for Biotechnology Information
ND	Not determined
ng	Nanogram
NGF	Nerve growth factor
NLRP1	NACHT leucine-rich-repeat protein 1
nm	Nanometer
NPY	Neuropeptide Y
OCA2	OCA2-encoded P protein
PAH	Phenylalanine hydroxylase
PAX3	Paired box gene 3
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pfu	Plaque-forming units
PKA	Protein kinase A
PKC	Protein kinase C
PMEL	Melanocyte-specific protein PMEL
pmol	Picomoles
POMC	Pro-opiomelanocortin
PTPN22	Lymphoid protein tyrosine phosphatase non-receptor type 22
PUVA	Psoralen plus ultraviolet A
R	Reverse (primer)
Rab27A	GTP-binding protein Rab27A
RERE	Arginine–glutamic acid dipeptide repeats protein

RLBA	Radioligand binding assay
RNASET2	Ribonuclease T2
RPL24	39s ribosomal protein L24
rpm	Revolutions per minute
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SH2B3	SH2B adapter protein 3
siRNA	Small interfering RNA
SLA	Src-like-adapter
SNPs	Single nucleotide polymorphisms
SOX10	Transcription factor SOX10
TAE	Tris-acetate-EDTA
TAP1	Transporter associated with antigen processing 1
TEMED	Tetramethylethylenediamine
TGFBR2	Transforming growth factor-beta receptor type 2
TGF-β	Transforming growth factor- β
TH	Tyrosine hydroxylase
Th1	T helper 1 cells
Th17	T helper 17 cells
TNF-α	Tumor necrosis factor-α
TOB2	Protein TOB2
Treg/s	T regulatory cells
TSLP	Thymic stromal lymphopoietin
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein-1
TYRP2	Tyrosinase-related protein-2
UBASH3A	Ubiquitin-associated and SH3 domain–containing A protein
UV	Ultraviolet
V	Vitiligo
v/v	Volume/volume
VGICC	Vitiligo Global Issues Consensus Conference
w/v	Weight/volume
WNT	Wingless-related integration site
XBP1	X box-binding protein 1

ZMIZ1	Zinc finger MIZ domain containing protein 1
α-MSH	α-melanocyte-stimulating hormone

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Chapter 1

General Introduction

1 General Introduction

1.1 The Skin

Human skin is considered the largest organ of the body, comprising approximately 10% of the total body mass with an average surface area of 1.8 m^2 (Sender et al., 2016). Although healthy skin is commonly taken for granted and not thought about in our everyday life, the importance of the skin is frequently underestimated (Menon, 2015). Skin diseases profoundly impact on physical comfort, psychologic well-being of patients and the quality of every aspect of personal and social life, affecting everyday functioning and socioeconomic status (Menon, 2015). One study showed that 64% of patients with skin conditions suffered from experiencing embarrassment, anxiety, a lack of confidence, and depression (Jowett and Ryan, 1985). Moreover, 40% of interviewed patients reported limited employment opportunities and functional and interpersonal difficulties in the workplace (Jowett and Ryan, 1985). Even though not all systemic health problems result in skin diseases, skin is generally a mirror for overall health and provides a window on hereditary disorders of connective tissues (Holbrook and Byers, 1989). Also, the skin is the main surface for medical treatment and nursing care ranging from delivery of transdermal drug to attaching devices to examine physiological functions (Menon, 2015).

1.1.1 Skin functions

The skin has several functions; it provides an effective barrier against external chemical and physical assaults, ultraviolet (UV)-irradiation, and pathogen invasion (Rudikoff, 1998, Proksch et al., 2008, Tay et al., 2014). The skin also regulates trans-epidermal water loss and body temperature, synthesises vitamin D3, and has significant cosmetic, social and sexual functions (Proksch et al., 2008, Menon, 2015). Additionally, the skin plays an important role in immunological surveillance as it encompasses a number of cellular immune components that reside in both the epidermis and dermis (Tay et al., 2014). These immune cells afford protection against a wide range of pathogens while maintaining tolerance to innocuous antigens (Tay et al., 2014). However, they also contribute to the pathology of different skin diseases (Tay et al., 2014).

1.1.2 Structure of the skin

Human skin consists of three structural layers; an outermost cellular avascular layer called the epidermis which arises from the embryonic ectoderm, the dermis, which is a dense vascularised tissue of mesodermal origin and an innermost layer called the subcutaneous layer which is composed primarily of fat and connective tissues (Figure 1.1a). The epidermis comprises five histologically distinct layers, composed mostly of keratinocytes (Figure 1.1b). From the innermost outwards, these layers include the basal cell layer (stratum basale), the spinous cell layer (stratum spinosum), the granular cell layer (stratum granulosum), the transitional cell layer (stratum lucidum) and the horny layer (stratum corneum).

The nomenclature of these layers is based on the differences in keratinocyte morphological appearance according to their stage in keratin maturation. The lower basal layer which is adjacent to the dermis comprises a single layer of columnar cells attached to the basement membrane via hemidesmosomes. The basal layer cells undergo a continual proliferation and differentiation as they move outwards to help form new keratinocytes that can replace the ones shed from stratum corneum. Above the basal cell layer are spinous keratinocytes that appear as prickles due to their protruding desmosomes which are filled with keratin filaments and which provide intracellular bridges. The granular cell layer consists of flattened keratinocytes. Their granular-like appearance is attributed to the aggregation of keratohyalin in their cytoplasm. The transitional keratinocytes constitute a thin layer and following full keratinisation and loss of their cellular contents, these form the outer squamous cell layer and are regularly shed off and substituted with new cells.

As well as keratinocytes, the epidermis also contains Langerhans cells, Merkel cells and melanocytes. Langerhans cells are dendritic cells residing in the basal and suprabasal layers of the epidermis and are capable of antigen presentation to T cells (Friedmann, 1981, Romani et al., 2003, Hunger et al., 2004, Wakim et al., 2008, Heath and Carbone, 2009, Jiang et al., 2012, Seneschal et al., 2012, Doebel et al., 2017). Merkel cells are distributed throughout the epidermis and their function is associated with sensory receptors or transducers (Boulais and Misery, 2007, Maricich et al., 2009, Woo et al.,

2015, Schober et al., 2016). Melanocytes are responsible for the synthesis of skin pigments (Sulaimon and Kitchell, 2003, Goding, 2007, Videira et al., 2013, Reemann et al., 2014, Skoczynska et al., 2017) and are described in Section 1.1.3.

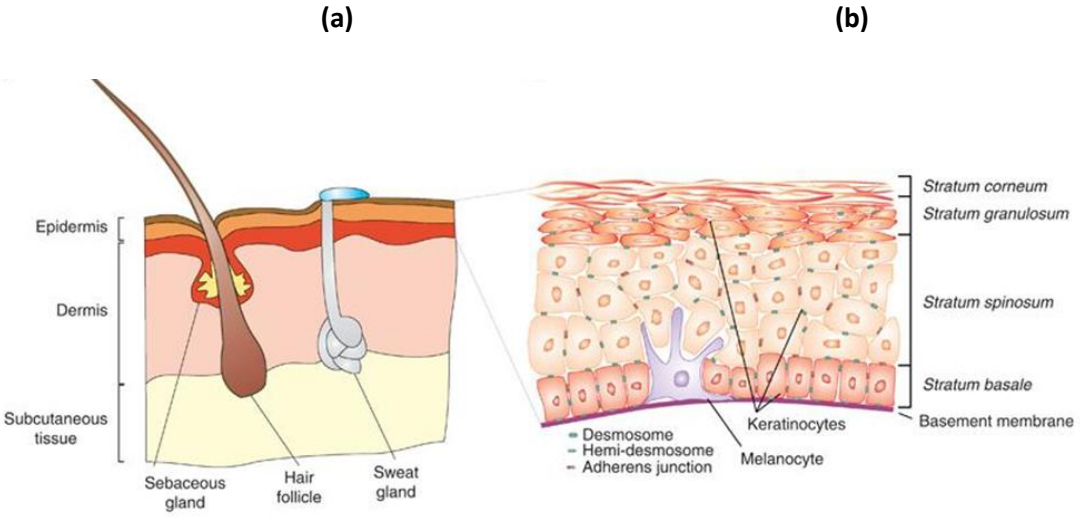


Figure 1.1: Skin structure.

(a) Human skin consists of a subcutaneous layer, the dermis, and the epidermis. The other main features are the sweat glands, sebaceous glands, and the hair follicles. **(b)** The epidermis consists of several layers based on the morphological appearance of the constituent keratinocytes. The image, from a paper by Kern et al. (2011), was used with kind permission from Springer Nature.

1.1.3 Melanocytes

Melanocytes are melanin-producing cells residing mainly in the cutaneous epidermis, hair follicle, uveal tract, inner ear, and leptomeninges, and are responsible for determining skin, hair and eye colour (Sulaimon and Kitchell, 2003, Goding, 2007, Plonka et al., 2009, Nishimura, 2011, Bastian, 2014, Hong et al., 2015, D'Mello et al., 2016). They are pyramidal cells with numerous dendritic projections (Figure 1.2), and arise from melanoblasts that originate in the embryonic neural crest (Fore, 2006, Santiago-Walker et al., 2009, Mull et al., 2015, Zonunsanga, 2015). Undifferentiated melanoblasts in approximately seven-week-old human embryos migrate into the developing epidermis and ultimately reside in the epidermal basal cell layer (Holbrook et al., 1989, Zonunsanga, 2015). In adult skin, melanocytes are found at a density of around 800-2000 cells/mm^2 (Staricco and Pinkus, 1957, Fitzpatrick and Szabo, 1959, Thingnes et al., 2012), and account for approximately 5-10% of the cellular population in the epidermis (Holbrook et al., 1988, Chung et al., 2011, Thingnes et al., 2012, Zonunsanga, 2015).

The principle function of melanocytes is the synthesis of melanin, which protects the genome of proliferating keratinocytes and melanocytes from UV-induced DNA damage (Gasparro, 2000, Kadekaro et al., 2003, Plonka et al., 2009, Abdel-Malek et al., 2010, Hong et al., 2015, D'Mello et al., 2016). Additionally, melanin efficiently absorbs reactive oxygen species (Korytowski et al., 1987, Abdel-Malek et al., 2010, Cichorek et al., 2013), and thus protects metabolically active keratinocytes and melanocytes from oxidative stress (Sulaimon and Kitchell, 2003, Hoogduijn et al., 2004). Melanin is synthesised in bound-membrane organelles called melanosomes (Setaluri, 2003, Barral and Seabra, 2004, Raposo and Marks, 2007, Yamaguchi and Hearing, 2014, Zonunsanga, 2015), which are transported along dendritic projections to surrounding keratinocytes (Scott et al., 2002, Watabe et al., 2008, Ando et al., 2012, Thingnes et al., 2012, Cichorek et al., 2013, Hong et al., 2015).

Keratinocytes act on melanocytes by synthesising several factors that support the growth, melanisation and survival of adjacent melanocytes (Lee et al., 2005, Faria et al., 2014). They generate the essential microenvironment for melanocyte proliferation, differentiation and migration through expression of ligands that interact with the

receptors of melanocytes (Carlson et al., 2007, Faria et al., 2014). Each melanocyte communicates with approximately thirty-six keratinocytes forming an epidermal melanin unit (Fitzpatrick et al., 1967, Seiberg, 2001, Lin and Fisher, 2007, Cichorek et al., 2013). Such close interaction between melanocytes that synthesise melanin and keratinocytes that receive it contribute to skin pigmentation (Westerhof, 2006, Faria et al., 2014).

Melanocytes are situated in the epidermal basal layer at a ratio of 1 to every 5 of basal keratinocytes (Lee et al., 2005, Faria et al., 2014). This balance is maintained via controlled induction of melanocyte division (Haass and Herlyn, 2005, Faria et al., 2014). To proliferate, melanocytes need to separate from the basement membrane and from keratinocytes, pull back their dendritic tips, divide, migrate via the basement membrane and finally attach themselves to the matrix and keratinocytes, forming another epidermal melanin unit (Haass and Herlyn, 2005, Faria et al., 2014). There is no difference in the epidermal melanin unit between ethnic groups (Cichorek et al., 2013), and the difference of skin colour among these groups is attributed to the different rates of melanin synthesis (Iwata et al., 1990, Cichorek et al., 2013), the different type of pigment produced (Cichorek et al., 2013), and the way in which melanin is scattered within keratinocytes (Szabo et al., 1988). Besides melanin synthesis, melanocytes also serve as accessory cells in the skin immune response (Das et al., 2001, Tam and Stepien, 2007, Cichorek et al., 2013). They are capable of expressing cytokines which lead to the maturation of antigen-presenting cells and to the recruitment of immune cells into the skin (Zachariae et al., 1991, Swope et al., 1994, Lu et al., 2002, Cichorek et al., 2013). Moreover, melanocytes are able to behave as antigen-presenting cells due to the expression of intercellular adhesion molecule-1 (ICAM-1) and class I and class II antigens of the major histocompatibility complex (MHC) (Krasagakis et al., 1991, Le Poole et al., 1993b, Das et al., 2001, Schuler et al., 2008, Hong et al., 2015).

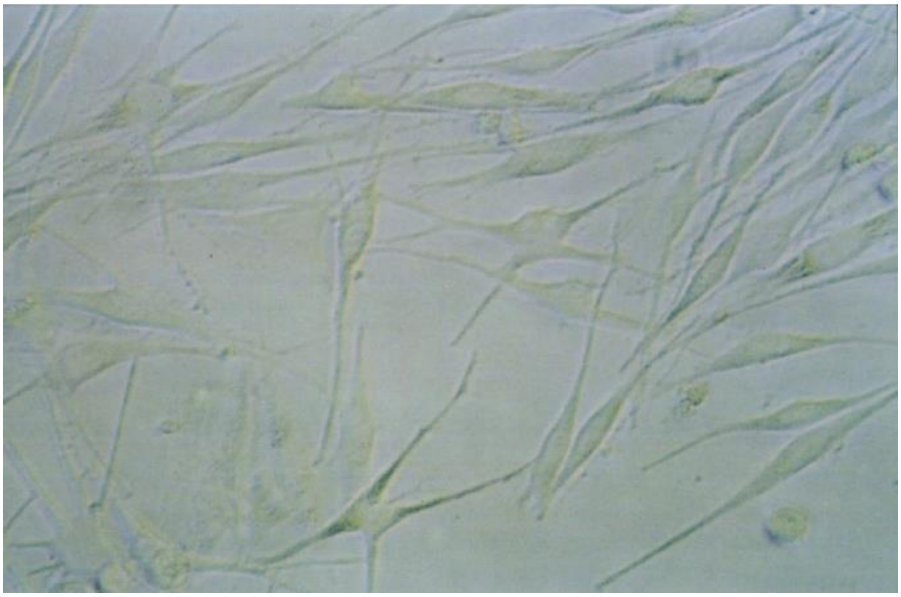


Figure 1.2: Melanocytes growing in culture.

The image shows melanocytes in culture at x200 magnification. The dendritic projections of melanocytes are visible. The image was used with kind permission from Miss Chariklia Balafa and Professor Sheila MacNeil, University of Sheffield, Sheffield, UK.

1.1.4 Melanogenesis

1.1.4.1 Key components of the melanin synthesis pathway

To initiate melanogenesis, melanosomes and the enzymes responsible for melanin synthesis are made independently in the smooth and rough endoplasmic reticulum of melanocytes, respectively (Pawelek et al., 1980, Urabe et al., 1994). Melanogenesis (Figure 1.3) is tightly regulated by a multi-enzyme complex (D'Mello et al., 2016) which includes tyrosinase, tyrosinase-related protein 1 (TYRP1) and L-dopachrome tautomerase (DCT), previously referred to as tyrosine-related protein 2 (TYRP2) (Gillbro and Olsson, 2011, D'Mello et al., 2016). The process begins with the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), which is subsequently oxidised to L-dopaquinone (Gillbro and Olsson, 2011, D'Mello et al., 2016). These first two steps of melanin synthesis are catalysed by tyrosinase (Gillbro and Olsson, 2011, D'Mello et al., 2016). Of particular note, the levels of L-tyrosine for melanin production depends on the conversion of L-phenylalanine by phenylalanine hydroxylase activity (Gillbro and Olsson, 2011, Videira et al., 2013, D'Mello et al., 2016).

Following L-dopaquinone formation, the melanogenesis pathway is divided into the production of brownish black eumelanin and red/yellow pheomelanin (Gillbro and Olsson, 2011, D'Mello et al., 2016). In the pheomelanin pathway, L-dopaquinone reacts with cysteine, if available, to form cysteinyl-dopa, which is the substrate for red-yellow pigment formation (Gillbro and Olsson, 2011, Pillaiyar et al., 2017b). L-dopaquinone can also cyclise to generate DOPAchrome, which can then spontaneously decarboxylate to 5, 6-dihydroxyindole. This product immediately oxidises and polymerises to yield dark brown/black coloured pigment (D'Mello et al., 2016, Pillaiyar et al., 2017b). If DCT is available, DOPAchrome will form 5,6-dihydroxyindole-2-carboxylic acid, which can oxidise and polymerise to produce lighter brown pigment (D'Mello et al., 2016, Pillaiyar et al., 2017b).

1.1.4.2 Regulation of melanin synthesis

Melanin synthesis is regulated by signalling pathways and transcription factors and can be controlled at several levels (Pillaiyar et al., 2017b). During the development of embryos, melanoblasts (melanocyte precursor cells) are generated from the neural crest and migrate to different destinations such as the basal layer of skin epidermis and hair follicles (D'Mello et al., 2016, Pillaiyar et al., 2017b). The migration pattern of melanocytes is the first level of melanogenesis regulation (Pillaiyar et al., 2017b). Subsequently, melanogenesis is regulated at a cellular level through the formation of melanosomes, whose size, quantity and density are dependent on melanin content (Pillaiyar et al., 2017b). Finally, melanin synthesis is regulated at a subcellular level through control of the expression of genes encoding specific melanogenic enzymes such as tyrosinase (Pillaiyar et al., 2017b).

A variety of extrinsic and intrinsic factors can influence the initiation and extension of melanin synthesis (Figure 1.3) (Pillaiyar et al., 2017b). Extrinsic factors encompass UVB radiation and chemicals, whereas intrinsic factors are various molecules secreted by keratinocytes, melanocytes, and fibroblasts, inflammatory, neural and endocrine cells (Videira et al., 2013). In UVB-induced melanogenesis, the paracrine cross-talk between melanocytes and keratinocytes within the epidermis is essential (Pillaiyar et al., 2017b). Following exposure to UVB, keratinocytes potently stimulate or secrete a variety of cytokines such as α-melanocyte-stimulating hormone (α-MSH) and its receptor, melanocortin 1 receptor (MC1R), endothelin-1 (EDN-1), interleukin (IL)-2, basic fibroblast growth factor (b-FGF), and adrenocorticotropic hormone (ACTH) (Pillaiyar et al., 2017a, Pillaiyar et al., 2017b). These factors that have a paracrine effect on melanocytes triggering melanin synthesis via various signalling pathways (Pillaiyar et al., 2017a, Pillaiyar et al., 2017b).

Signalling from MC1R has been established to be the main factor dictating melanin synthesis (D'Mello et al., 2016). There are three main ligands for MC1R, namely, α-MSH, ACTH and agouti signalling peptide (ASP) (Videira et al., 2013, D'Mello et al., 2016). Normally, ASP and α-MSH compete to bind to MC1R (Videira et al., 2013). When ASP binds, pheomelanin is primarily synthesised, whereas eumelanin is synthesised when α-

MSH or ACTH binds (Videira et al., 2013, D'Mello et al., 2016). Both ACTH and α-MSH are derived from a precursor peptide known as pro-opiomelanocortin (POMC) which is synthesised by both the pituitary gland and epidermal keratinocytes permitting local paracrine regulation (Videira et al., 2013, D'Mello et al., 2016, Pillaiyar et al., 2017b). Following MC1R ligand binding, melanogenesis is increased via activation of cyclic adenosine monophosphate (cAMP) pathway (Choi et al., 2016, Pillaiyar et al., 2017b). Production of cAMP leads to the activation of protein kinase A (PKA) and cAMP-responsive element binding protein (CREB), which in turn induces microphthalmia-associated transcription factor (MITF) expression (Choi et al., 2016, Pillaiyar et al., 2017b). The MITF is a master regulator of melanin synthesis that upregulates the expression of the related melanogenic enzymes, tyrosinase, TYRP1 and DCT, and melanocyte development (Choi et al., 2016, Pillaiyar et al., 2017b). Furthermore, the MITF protein regulates the expression of GTP-binding protein Rab27A protein and melanocyte-specific protein (PMEL), previously known as Pmel17 or gp100) (Pillaiyar et al., 2017b). The former is important in melanosome transport, while the latter plays a vital role in the structural organisation of pre-melanosomes (Bissig et al., 2016, Pillaiyar et al., 2017b).

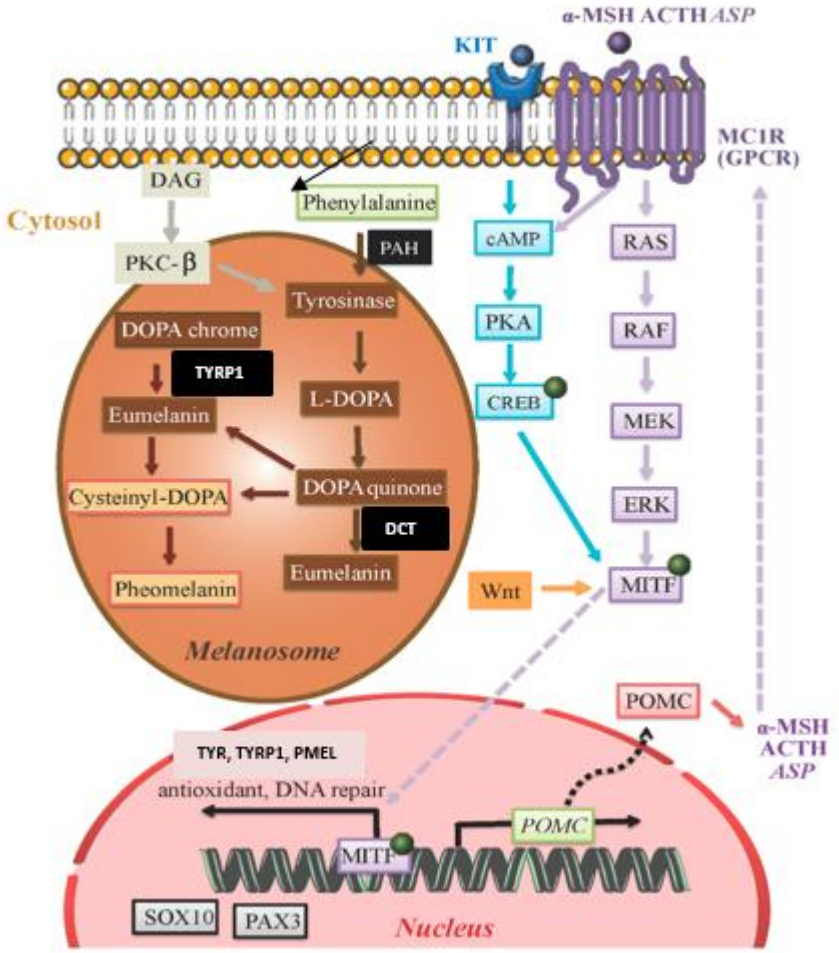


Figure 1.3: Melanin synthesis.

Eumelanin and pheomelanin are produced within melanosomes of melanocytes by a sequence of reactions that are catalysed by melanogenic enzymes. Synthesis of such enzymes is driven by the microphthalmia-associated transcription factor (MITF) whose activity is controlled by a number of signalling pathways such as protein kinase C (PKC), cyclic AMP (cAMP), mitogen-activated protein (MAP) kinase (MEK), and Wingless-related integration site (WNT). These signalling pathways are activated upstream by receptors including KIT with ligand stem cell factor (SCF) and melanocortin 1 receptor (MC1R) with ligands α-melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH) and agouti signalling peptide (ASP). The image, from a paper by D'Mello et al. (2016), was used with kind permission from MDPI-publisher of open access journals, St. Alban-Anlage 66, 4052 Basel, Switzerland. DAG, diacylglycerol; PAH, phenylalanine hydroxylase; PKA, protein kinase A; CREB, cAMP response element-binding protein; DCT, L-dopachrome tautomerase; GPCR, G protein-coupled receptors; ERK, extracellular signal–regulated kinases; POMC, pro-opiomelanocortin; TYR, tyrosinase; TYRP1, tyrosinase-related protein-1; PMEL, melanocyte-specific protein PMEL; SOX10, transcription factor SOX10; PAX3, paired box gene 3.

1.2 Clinical Characteristics, Epidemiology, and Treatment of Vitiligo

1.2.1 Definition of vitiligo

Vitiligo is an acquired depigmenting skin disorder characterised by the development of circumscribed white macules (patches) as a consequence of the loss of functional melanocytes from the epidermis (Taïeb et al., 2007).

1.2.2 Signs of vitiligo

The most apparent sign or symptom of vitiligo is the depigmented patches on the skin. The disease initially occurs as small patches but subsequently these patches are usually enlarged over time. The lesions typically and initially appear on the face, hand and wrist (Ghafourian et al., 2014, Roy, 2017). Rare signs of the disease are depigmentation or greying of scalp hair, eyebrows, eyelashes or other hair-bearing areas (Ezzedine et al., 2012b, Roy, 2017). Also some patients may experience depigmentation on the tissues that line the eye membrane and the mucosal membranes such as the oral and/or genital mucosae (Farajzadeh et al., 2009, Roy, 2017). In addition, a number of vitiligo patients suffer from intense tactile sensation at depigmented areas throughout active stages (Birlea et al., 2008a, Roy, 2017). Patients suffering from vitiligo often suffer from depression (Al'Abadie et al., 1994a, Kent and Al'Abadie, 1996, Ghafourian et al., 2014).

The diagnosis of vitiligo is primarily based on the clinical characteristics of pigmentation loss from the skin (Iannella et al., 2016). A Wood's lamp can be used to optimally evaluate some uncertain patches, by which the specific milky fluorescent aspect of vitiliginous skin can more easily be observed (Picardo et al., 2015, Iannella et al., 2016). Given its high incidence in vitiligo patients, autoimmune thyroiditis should be screened for in patients suffering from vitiligo using thyroid function tests and the measurement of anti-thyroid antibodies (Picardo et al., 2015). In addition, a biopsy or other specific tests may be necessary to differentiate vitiligo from other conditions of decreased pigmentation like pityriasis versicolor, tuberous sclerosis, piebaldism, and hypomelanosis of Ito (Picardo et al., 2015, Iannella et al., 2016). Finally, when vitiligo is

undoubtedly diagnosed, vitiligo clinical type can be determined from the extent and distribution of depigmented patches.

1.2.3 Clinical types of vitiligo

Even now, vitiligo clinical types have neither been uniformly understood nor reliably classified. The simplest classification of vitiligo was proposed by Koga (1977), in which vitiligo divided into segmental and non-segmental types based on the extent and distribution of the lesions. For decades there was no proper global joint effort to create a worldwide consensus on classification of vitiligo, until an article from the Vitiligo Global Issues Consensus Conference (VGICC) was published (Ezzedine et al., 2012b). The VGICC used the classification proposed by Taïeb et al. (2007) in order to achieve a broader international consensus on vitiligo classification. According to the consensus recommendations of the VGICC (Table 1.1) (Ezzedine et al., 2012b), vitiligo is classified in the clinical types which are described below.

1.2.3.1 Non-segmental vitiligo

Non-segmental vitiligo is characterised by symmetrical bilateral white macules (Figure 1.4a) and includes a group of several patterns; acrofacial, mucosal, generalised/symmetrical, universal, and mixed types as well as rare variants (Figure 1.4). Acrofacial vitiligo affects the distal fingers and toes, and facial orifices (Figure 1.4b). Mucosal vitiligo affects the oral and/or genital mucosae involving more than one mucosal site (Figure 1.4 c), and it can be present in patients suffering from acrofacial, generalised, or universal forms. However, when only one mucosal site is affected, this type of vitiligo is classified as undetermined.

Generalised/symmetrical vitiligo, formerly known as vitiligo vulgaris, is characterised by a bilateral, symmetrical distribution of patches (Figure 1.4a). It can affect any site of the body, but mostly the fingers, hands, and face. Universal vitiligo involves 80-90% of the body surface. It mostly occurs in adulthood and is usually preceded by the generalised form. Mixed vitiligo is the concomitant occurrence of segmental and non-segmental

vitiligo (Figure 1.4d). Mostly, the segmental type precedes the non-segmental form (Figure 1.4d).

Rare variants of vitiligo were considered originally as unclassified. They include punctate vitiligo which has sharply demarcated depigmented punctate macules and can involve any site of the body (Figure 1.4e), minor vitiligo which is characterised by partial defects in skin pigmentation (Figure 1.4f), and follicular vitiligo which affects the follicular reservoir and limited skin area (Figure 1.4g).

1.2.3.2 Segmental vitiligo

Segmental vitiligo can affect one or more areas of the skin (Figure 1.4h and i). The most common form of segmental vitiligo is unisegmental which is characterised by one or more white lesions on one side of the body, often respecting the midline of the body, body hair involvement, and rapid development of the condition (Figure 1.4h). Rarely, it can affect more than one area with either a unilateral or bilateral distribution (Figure 1.4i). In contrast to non-segmental vitiligo, segmental vitiligo demonstrates a rapidly progressive but limited course, early onset, and a lesser association with autoimmune diseases. It also shows stable results with autologous grafting and early involvement of the hair compartment after onset. There is often an undistinguishable pattern from nevus depigmentosus in patients with early onset segmental vitiligo (Table 1.2) (Kovacs, 1998, Taieb and Picardo, 2009, Ezzedine et al., 2012b).

1.2.3.3 Unclassifiable or undetermined vitiligo

Unclassifiable or undetermined vitiligo includes focal vitiligo which is characterised by small isolated depigmented macules without a segmental distribution (Figure 1.4j). It can evolve into either segmental vitiligo or non-segmental vitiligo. Also, mucosal when only one mucosal site is affected.

In addition, based on the clinical patterns, there are also some rare morphological variations of vitiligo namely inflammatory vitiligo and trichrome vitiligo. Inflammatory vitiligo presents with erythema at peri-lesional borders and frequent itching (Karelson et al., 2009). Trichrome vitiligo is characterised by complex outline of lesions with partial

or complete depigmentation within each lesion and hyperpigmentation of normally pigmented skin neighbouring the vitiligo lesions (Hann et al., 2000, Karelson et al., 2009). However, these two forms of vitiligo are not present in the vitiligo classification table of the latest international consensus conference (Ezzedine et al., 2012b), possibly because these are regarded as a temporary phenomenon of a restricted area of active spreading vitiligo (Hann et al., 2000).

Table 1.1: Vitiligo classification of the Vitiligo Global Issue Consensus Conference

Clinical type of vitiligo[a]	Clinical subtypes[a]	Remarks[a]
Non-segmental	Mucosal (at least two sites affected) Acrofacial Generalised/Symmetrical Universal Mixed (associated with segmental vitiligo) Rare variants	Subtyping may not reflect discrete nature, but rather valuable data for epidemiologic investigations. Often segmental vitiligo in mixed vitiligo is more extreme.
Segmental	Unisegmental Bisegmental Plurisegmental	Further classification according to lesional distribution pattern is possible, however, not yet standardised.
Undetermined or unclassified	Focal Mucosal (only one mucosal site)	This category is meant to allow time, after sufficient observation to make a conclusive classification.

[a]The data are from Ezzedine et al. (2012b).

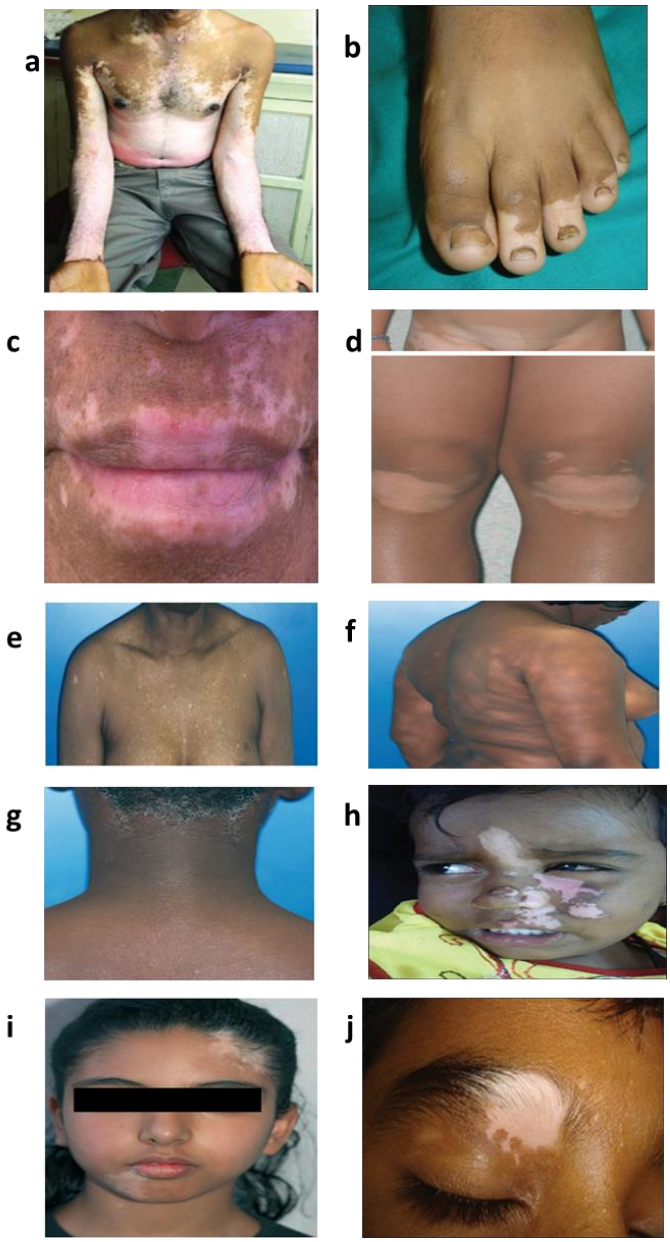


Figure 1.4: Clinical subtypes of vitiligo.

The figure shows vitiligo subtypes (a) generalised non-segmental, (b) acral, (c) mucosal (d) mixed, (e) punctate, (f) minor, (g) follicular, (h) unisegmental, (i) bisegmental, and (j) focal. Figure (a) was used with kind permission from Medknow Publications and Media Pvt. Ltd. (Mumbai, India) from an article by Attili and Attili (2013). Figures (b), (h) and (j) were used with kind permission from Medknow Publications and Media Pvt. Ltd. (Mumbai, India) from an article by Palit and Inamadar (2012). Figures (d), (e), (f), (g), and (i) were used from an article by Ezzedine et al. (2012b) with kind permission from John Wiley and Sons. Figure (c) is used from DermNet NZ, (2018) with kind permission from DermNet New Zealand Trust, Hamilton, New Zealand,.

Table 1.2: Typical characteristics of segmental and non-segmental vitiligo

Segmental vitiligo[a]	Non-segmental vitiligo[a]
Often manifests in childhood	Later onset, at all ages
Rapid onset with limited course	Progressive onset with unpredictable course
Rapid involvement of hair after onset	Later stages of hair involvement
Rarely associated with autoimmune diseases	Common personal and/or family records of autoimmune diseases
Good results with autologous grafting	Usually relapses following autologous grafting
Sometimes undistinguishable from nevus depigmentosus in patients with early onset	-

[a]The data are from Taieb and Picardo, 2009.

1.2.4 Activity status of vitiligo

The course of vitiligo is unpredictable, but is often episodically progressive with phases of stabilised depigmentation (Gawkrodger et al., 2010). Vitiligo is classified as stable if no new patches or growth of old lesions had appeared for the previous 12 months, and as active if existing macules were enlarged or new lesions were developed within the previous 12 months (Gawkrodger et al., 2010, Ezzedine et al., 2012b, Benzekri and Gauthier, 2017). In most cases, the course of segmental vitiligo is rapidly stabilised over a few months following partial or complete skin depigmentation of the affected skin segment, whereas the course of non-segmental vitiligo is usually unpredictable (Picardo et al., 2015).

1.2.5 Associated diseases

Several studies have revealed that patients with vitiligo are more likely to suffer from autoimmune disorders than those in the general population (Table 1.3) (Cunliffe et al., 1968, Tunbridge et al., 1977, Schallreuter et al., 1994b, Alkhateeb et al., 2003, Liu et al., 2005, Birlea et al., 2008b, Narita et al., 2011). A survey of nearly 3,000 vitiligo patients showed one additional autoimmune disease was present in 30% of them, and increased frequencies of Addison's disease, autoimmune thyroid disease, pernicious anaemia, and systemic lupus erythematosus were reported (Alkhateeb et al., 2003). Furthermore, vitiligo patients with a family history of vitiligo have been revealed to have a high frequency of other autoimmune disease (Laberge et al., 2005, Zhang et al., 2009). However, the occurrence of the overall concomitant autoimmune diseases in patients with vitiligo varies, and depends on the population studied. Even within a specific country, pockets of variance exist (Table 1.3). Furthermore, the frequency of an individual autoimmune disease in vitiligo patients is also variable (Table 1.4). Overall, the association of vitiligo with autoimmune disease suggest that in some patients an autoimmune mechanism might be involved in vitiligo pathogenesis (Kemp et al., 2001b). Of note, non-segmental vitiligo shows a greater association with autoimmune diseases compared to segmental vitiligo (Kovacs, 1998, Taieb and Picardo, 2009, Ezzedine et al., 2012b).

Table 1.3: Frequency of concomitant autoimmune diseases in vitiligo patients in different populations

Country	Frequency of concomitant autoimmune disease in vitiligo patients	References
Turkey	55% 35% 14.3%	(Akay et al., 2010) (Oguz Topal et al., 2016) (Arycan et al., 2008)
India	39% 30% 18.5% 2% 1.36	(Abraham and Raghavan, 2015) (Vijayalakshmi et al., 2017) (Singh and Pandey, 2011) (Poojary, 2011) (Shah et al., 2008)
Iran	25.6% 17.4%	(Nejad et al., 2013) (Sedighe and Gholamhossein, 2008)
Estonia	36.7%	(Karelson et al., 2009)
Japan	20% 13.3%	(Narita et al., 2011) (Tanioka et al., 2009)
UK and North America UK USA	30% 34% 31%	(Alkhateeb et al., 2003) (Mason and Gawkrodger, 2005) (Silverberg and Silverberg, 2013)
Nigeria	2%	(Onunu and Kubeyinje, 2003)
Saudi Arabia	1% 43.5%	(Jarallah et al., 1993) (Fatani et al., 2014)
The Netherlands	29%	(Teulings et al., 2016)
Taiwan	14.4%	(Chen et al., 2015)
Belgium	15.4%	(van Geel et al., 2014)

Table 1.4: Frequency of specific autoimmune diseases in vitiligo patients

Autoimmune disease	Percentage of vitiligo patients with autoimmune disease	Country	References
Autoimmune thyroid disease (Hypothyroidism, hyperthyroidism, Graves' disease, Hashimoto's thyroiditis, autoimmune thyroiditis)	12%	India	(Singh and Pandey, 2011)
	34%	UK	(Mason and Gawkrodger, 2005)
	28%	Turkey	(Oguz Topal et al., 2016)
	16.8%	Estonia	(Karelson et al., 2009)
	21.18% 12.3%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	18.4	The Netherlands	(Teulings et al., 2016)
	1.56%	Taiwan	(Chen et al., 2015)
	21%	Iran	(Nejad et al., 2013)
	9.6%	Belgium	(van Geel et al., 2014)
	12%	Japan	(Narita et al., 2011).
Alopecia areata	5%	Turkey	(Oguz Topal et al., 2016)
	5.3%	Japan	(Narita et al., 2011)
	2.4% 3.8%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	1.8%	Taiwan	(Chen et al., 2015)
	1.3%	Belgium	(van Geel et al., 2014)
	0.5%	Saudi Arabia	(Jarallah et al., 1993)
	8.8% 2%	India	(Abraham and Raghavan, 2015) (Singh and Pandey, 2011)
	3.5%	The Netherlands	(Teulings et al., 2016)
	3%	India	(Singh and Pandey, 2011)
Type 1 diabetes	3.9%	Estonia	(Karelson et al., 2009)
	3.7%	Japan	(Narita et al., 2011)
	1% 0.7%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	0.1%	Taiwan	(Chen et al., 2015)
	4.7%	Iran	(Nejad et al., 2013)
	2.4%	The Netherlands	(Teulings et al., 2016)
	2.4%	UK	(Mason and Gawkrodger, 2005)
Pernicious anaemia	1.3%	Estonia	(Karelson et al., 2009)

	3.7%	Japan	(Narita et al., 2011)
	3% 0.5%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	0.06%	Taiwan	(Chen et al., 2015)
	1%	Turkey	(Oguz Topal et al., 2016)
	0%	Iran	(Nejad et al., 2013)
	2.4%	UK	(Mason and Gawkrodger, 2005)
Systemic lupus erythematosus	0.7% 0.3%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	0.5%	The Netherlands	(Teulings et al., 2016)
	0.28%	Taiwan	(Chen et al., 2015)
	0%	Iran	(Nejad et al., 2013)
	1%	India	(Singh and Pandey, 2011)
Rheumatoid arthritis	3.9%	Estonia	(Karelson et al., 2009)
	3.3% 0.5%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
	6.6%	The Netherlands	(Teulings et al., 2016)
	0.6%	Belgium	(van Geel et al., 2014)
	0.4%	Taiwan	(Chen et al., 2015)
	0.8% 0.5%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
Sjogren's syndrome	0.36%	Taiwan	(Chen et al., 2015)
	0.8% 0.2%	USA	(Silverberg and Silverberg, 2013) (Gill et al., 2016)
Addison's disease	0.38% 0.75%	North America and UK	(Alkhateeb et al., 2003) (Laberge et al., 2005)
	0%	USA	(Gill et al., 2016)
	9.6%	The Netherlands	(Zelissen et al., 1995)
	0%	Iran	(Nejad et al., 2013)

1.2.6 Psychological issues

Since vitiligo is not a life threatening disease and does not cause any physical disabilities, it is often perceived as a minor cosmetic disorder (Salzes et al., 2016, Cupertino et al., 2017). However, changes in physical appearance caused by the disease may induce serious psychological harm, particularly in dark-skinned patients in whom the pale patches are very apparent (Linthorst Homan et al., 2009, Ezzedine et al., 2015). In the event of social contact, vitiligo patients commonly experience feelings of stress, unease, embarrassment, negative self-image or self-consciousness (Kent and Al'Abadie, 1996, Ingordo et al., 2012, Picardo et al., 2015, Salzes et al., 2016, Grimes and Miller, 2018). More generally, perception of discrimination, social isolation, loss of self-esteem, and stigmatisation are common, particularly in patients with visible patches affecting areas such as the face and hands, and these can have a major negative impact on patient's quality of life (Dolatshahi et al., 2008, Choi et al., 2010, Salzes et al., 2016, Cupertino et al., 2017, Yang et al., 2017c, Grimes and Miller, 2018).

In some countries such as India, vitiligo is still confused with leprosy and patients are subjected to antagonism, insult, social stigma, or isolation (Parsad et al., 2003a, Ezzedine et al., 2015, Grimes and Miller, 2018). Furthermore, in patriarchal societies, with respect to marriage, for example, women's beauty is of great value in the market of arranged marriages, thus the psychosocial burden on females is greater (Shenoi and Prabhu, 2013, Cupertino et al., 2017, Grimes and Miller, 2018). However, vitiligo can also have a negative impact on the marital status and sexual health in males owing to embarrassment and loss of self-esteem (Dolatshahi et al., 2008, Ezzedine et al., 2015). Moreover, previous reports have demonstrated that vitiligo patients with decreased quality of life at treatment initiation face a lower response rate to a given therapy (Parsad et al., 2003b, Salzes et al., 2016). Therefore, the development of specific psychological intervention and quality of life measures may affect positively the outcome of vitiligo treatment and enhance the patient's self-esteem and confidence (Parsad et al., 2003a, Ezzedine et al., 2015, Whitton et al., 2015, Salzes et al., 2016).

1.2.7 Vitiligo prevalence

Vitiligo is the most common cause of skin depigmentation worldwide (Picardo et al., 2015). It has an estimated worldwide prevalence of less than 0.6% (Howitz et al., 1977, Das et al., 1985, Singh et al., 1985, Boisseau-Garsaud et al., 2000a, Wang et al., 2013). Some studies report a prevalence of 0.4% to 2% in certain population (Figure 1.5) (Kruger and Schallreuter, 2012), with peaks of up to 4% and 8.8% noted in Mexico and India, respectively (Sehgal and Srivastava, 2007). This may be due to an apparent colour contrast and social stigma that may force vitiligo patients to seek an early consultation, as well as to occupational factors where vitiligo may be related to chemically-induced depigmentation by industrial reagents (Sehgal and Srivastava, 2007). In addition, variability in epidemiological data could also be attributable to variances in disease classification, unavailable simple laboratory tests, inconsistent reporting by vitiligo patients, selection bias of hospital-based studies and varied populations with different ethnic backgrounds (Shah et al., 2008, Picardo et al., 2015).

Non-segmental vitiligo is the most frequent type of the disease. The generalised/symmetrical subtype (previously called vitiligo vulgaris) has been the most frequently reported morphological pattern in several published studies, accounting for 39-83% of the subjects (Handa and Kaur, 1999, Dogra et al., 2005, Liu et al., 2005, Mason and Gawkrodger, 2005, Singh and Pandey, 2011, Vora et al., 2014). The second most common subtype is acrofacial, which is reported to be between 17-35% of cases in different clinical studies (Dave et al., 2002, Martis et al., 2002, Nejad et al., 2013, Vora et al., 2014). Universal vitiligo has been shown to present in 0.5-18% of subjects with the disease (Dogra et al., 2005, Wang et al., 2013). Segmental vitiligo is less common than the non-segmental form and its prevalence ranges from 5-16% of overall vitiligo cases (Hann and Lee, 1996, Silverberg, 2010, Ezzedine et al., 2015). Only a few studies have reported cases of mixed vitiligo (Mulekar et al., 2006, Ezzedine et al., 2011, van Geel et al., 2011a). However, longer follow-up of segmental cases might show more examples.

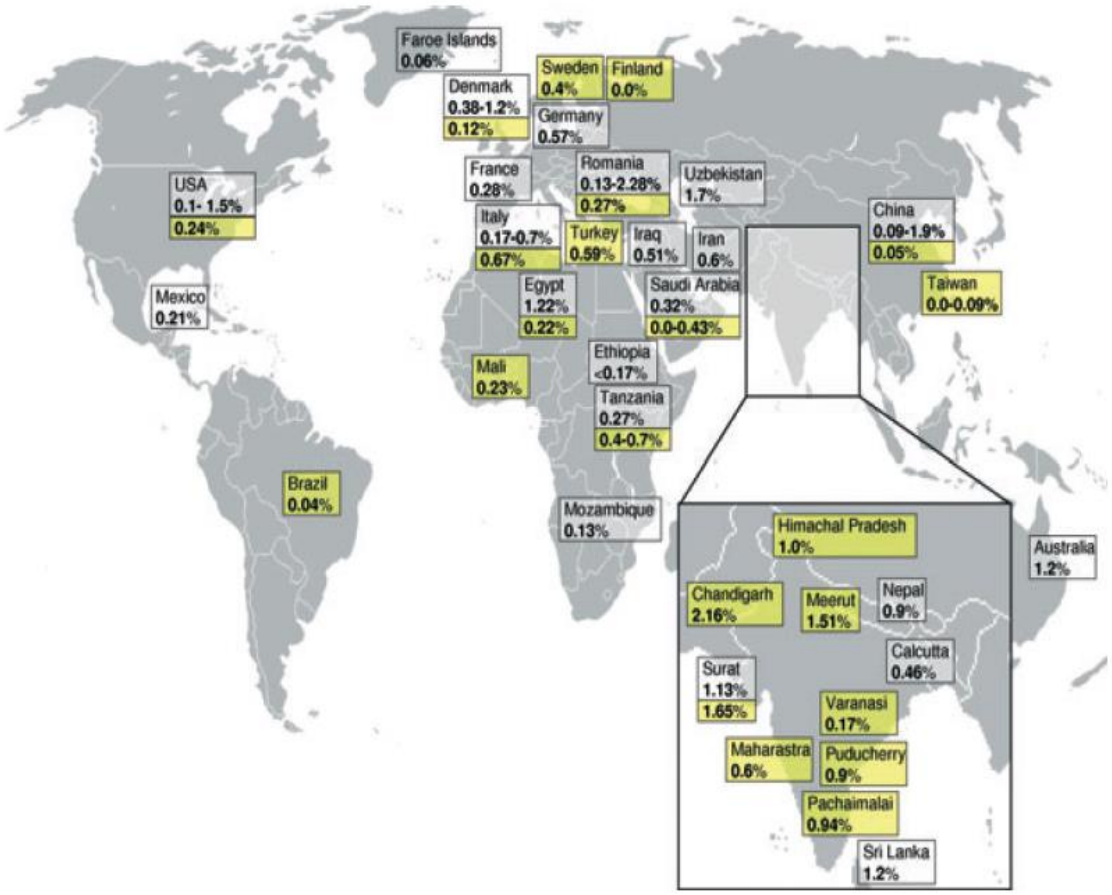


Figure 1.5: Worldwide prevalence of vitiligo.

The white and yellow boxes indicate the prevalence of vitiligo in the general population and children/adolescents, respectively. The image is used with kind permission from John Wiley & Sons Ltd. (Chichester, UK) from an article by Kruger and Schallreuter (2012).

1.2.8 Gender distribution

Individuals of both genders can be affected by vitiligo (Sun et al., 2005, Taïeb and Picardo, 2010, Pradhan et al., 2013). However, epidemiological studies have reported contradictory views of the incidence of vitiligo in both genders. While some studies have demonstrated that vitiligo, like most autoimmune diseases, is more common in females (Shah et al., 2008, Singh and Pandey, 2011, Esfandiarpour and Farajzadeh, 2012, Vora et al., 2014, Zhang et al., 2016), others have shown a greater proportion of the male population is affected (Koranne et al., 1986, Handa and Kaur, 1999, Khaitan et al., 2012). The social stigma associated with vitiligo in women may contribute to the former findings (Patil et al., 2014). However, most studies have reported that both genders are affected almost equally (Dutta and Mandal, 1969, Howitz et al., 1977, Das et al., 1985, Dave et al., 2002, Alkhateeb et al., 2003, Liu et al., 2005, Shajil et al., 2006, Sehgal and Srivastava, 2007, Kyriakis et al., 2009, Taieb and Picardo, 2009, Ezzedine et al., 2015).

1.2.9 Onset age

Vitiligo can appear at any age and disease onset age varies between studies from different countries (Sun et al., 2005, Taïeb and Picardo, 2010, Pradhan et al., 2013). The most common age for the onset of vitiligo is 10-30 years. In approximately half of patients, vitiligo manifests by the age of 20 years and 70-80% of cases develop the disorder before the age of 30 years (Herane, 2003, Zhang et al., 2004, Liu et al., 2005, Sehgal and Srivastava, 2007, Taïeb et al., 2007). However, an epidemiological study in Denmark found that the age of onset of vitiligo most frequently ranged between 40-60 years (Howitz et al., 1977). Segmental type vitiligo has been found to be more characteristic of children and young adults with a median age at onset of 12-16 years in most studies (Barona et al., 1995, Liu et al., 2005, Khaitan et al., 2012). It can account for nearly a third of paediatric cases (Halder et al., 1987, Nicolaidou et al., 2012, Shankar et al., 2012) and only for 0-4.5% of adult patients (Dogra et al., 2005, Mason and Gawkrodger, 2005). However, the segmental form has been reported to affect less than 8% of children with vitiligo in a Jordanian study conducted on 71 children with vitiligo (Al-Refu, 2012). Moreover, late onset of segmental vitiligo (median age at onset 41

years) was reported in China (Wang et al., 2013). In contrast to segmental vitiligo, non-segmental forms develop at all ages (Picardo et al., 2015).

1.2.10 Family history of vitiligo

A number of studies have investigated the effects of a positive family history on the occurrence of vitiligo. These indicate that the familial occurrence of the disease varies from one region of the world to another and lies between 12% and 36% (Pradhan et al., 2013). However, a family history as high as 40% has been found in some areas of India (Sehgal and Srivastava, 2007). Consanguineous marriage may account for an increased incidence of family history of vitiligo in specific regions of the world (Silverberg, 2015). Vitiligo patients who had consanguineous cases affected with vitiligo showed an earlier age of onset of disease (Pajvani et al., 2006, Alzolibani, 2009). Family history and early onset imply a role for a genetic factor in vitiligo pathogenesis (Kumar et al., 2014).

1.2.11 Histopathology

The depigmented area in vitiligo is histologically characterised by melanin loss and reduced numbers of melanocytes in the epidermis (Figure 1.6) (Husain et al., 1982, Tobin et al., 2000, Li et al., 2013, Ezzedine et al., 2015). This decrease varies according to the disease stage (Ezzedine et al., 2015). However, there are reports demonstrating that vitiligo lesions can be completely devoid of melanocytes (Le Poole et al., 1993c, Le Poole et al., 1996, Yaghoobi et al., 2011). Melanocytes from vitiligo patients also show morphological and physiological abnormalities (Li et al., 2009b). Those in peri-lesional borders are seen to be enlarged with longer dendritic ends (Le Poole et al., 1993c, Faria et al., 2014). Moreover, vacuolar degeneration of basal cell layer melanocytes, epidermal and dermal infiltration of lymphocytes (Figure 1.7), and melanin granules in the upper dermis can occur in the uninvolved skin adjacent to vitiligo macules (Hann et al., 1992, Pretti Aslanian et al., 2007, Anbar et al., 2009, Faria et al., 2014). In addition to melanocyte abnormalities, Benzekri et al. (2015) and Picardo et al. (2015) have demonstrated abnormalities in the keratinocyte phenotype in both the peri- lesional and lesional areas of vitiligo patches.

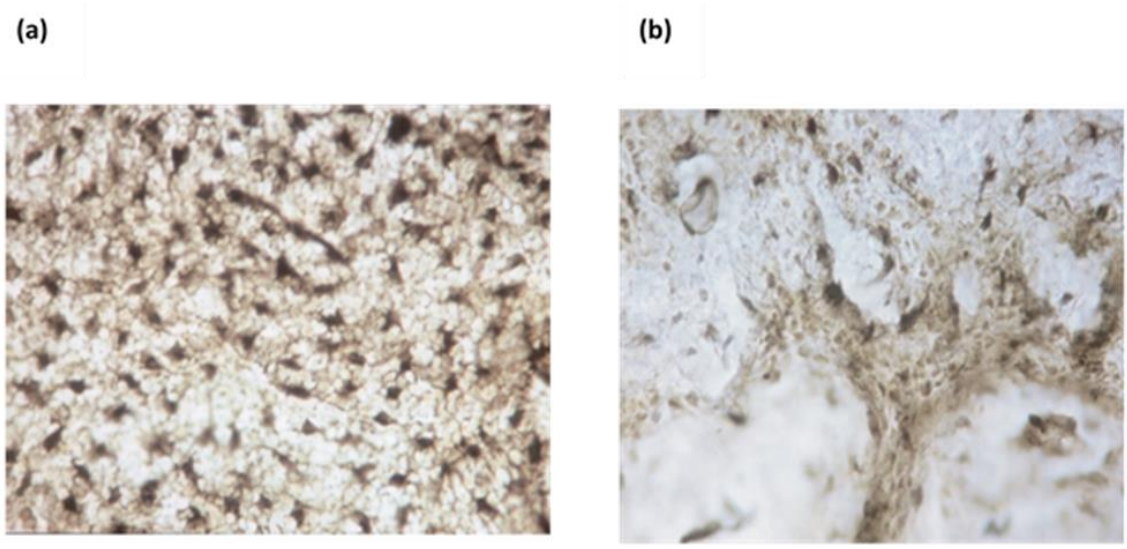


Figure 1.6: Loss of melanocytes in the vitiligo epidermis.

The figure shows **(a)** melanocytes in the normal epidermis, and **(b)** melanocyte loss in the vitiliginous epidermis**.** Both sections had been treated with dihydroxyphenylalanine stain, which identifies tyrosinase activity and reveals melanocytes in brown. Taken with kind permission from Springer Science and Business Media (Dordrecht, The Netherlands) from an article by Gawkrodger (1998).

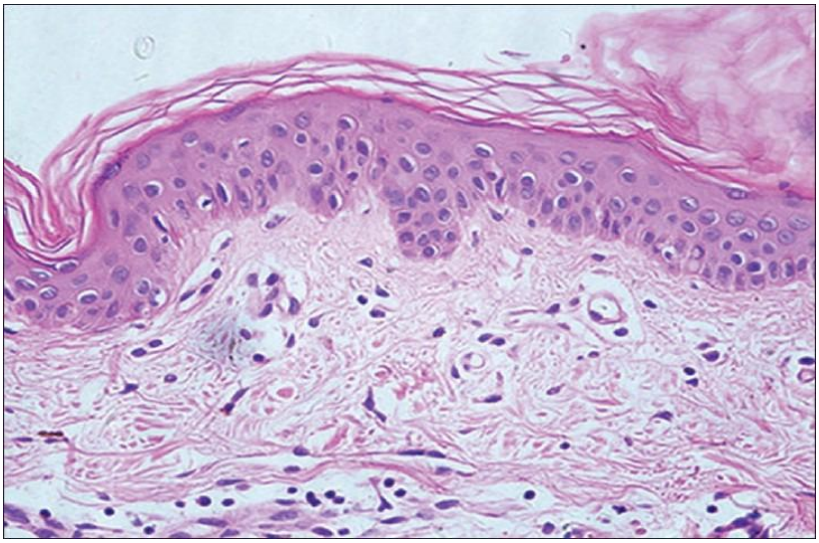


Figure 1.7: Lymphocyte infiltration at the edge of a vitiligo lesion.

The skin section was treated with hematoxylin and eosin stain and is shown at a magnification of x400**.** The image is used with kind permission from Medknow Publications and Media Pvt. Ltd. (Mumbai, India) from an article by Li *et al*. (2013).

1.2.12 Treatment modalities

As the exact aetiology of vitiligo is yet unknown, there are no efficient therapies available to alter the underlying cause of melanocyte loss. Therefore, the positive results of repigmenting treatments are usually unsustained, and patients have to use cosmetics to cover their depigmented patches. However, spontaneous repigmentation has been reported in 1%-25% of vitiligo patients (Iannella et al., 2016). Treatments available for vitiligo include topical and systemic medications, phototherapy and surgical procedures, which together may serve to arrest vitiligo progression, stabilize depigmented macules and obtain skin repigmentation (Rodrigues et al., 2017a) and these are listed in Table 1.5. Often, medical treatments are combined for greater efficacy.

1.2.12.1 Topical therapies

This class of drugs includes topical corticosteroids, topical immunomodulators, and antioxidants (Table 1.5) (Garg et al., 2010, Iannella et al., 2016, Dillon et al., 2017). Topical corticosteroids decrease the activity of T lymphocytes and inhibit the secretion of a wide variety of cytokines by interfering with gene expression (Speeckaert et al., 2015). The use of topical corticosteroids is considered to be the first-line vitiligo treatment in both children and adults with either segmental type or non-segmental type of recent onset (Garg et al., 2010, Iannella et al., 2016, Dillon et al., 2017). Potent corticosteroids such as betamethasone and very potent corticosteroids such as clobetasol can stimulate repigmentation of vitiliginous skin to variable degrees (Lakhani and Deshpande, 2014, Speeckaert et al., 2015, Dillon et al., 2017). However, regardless of corticosteroid efficacy, topical immunomodulators are preferable for treating vitiligo due to the potential for corticosteroids to induce cutaneous atrophy (Garg et al., 2010, Iannella et al., 2016, Dillon et al., 2017).

Topical immunomodulators, also known as calcineurin inhibitors, include tacrolimus and pimecrolimus (Lakhani and Deshpande, 2014, Iannella et al., 2016, Dillon et al., 2017). These reagents also suppress T cell activation (Speeckaert et al., 2015).

Topical antioxidants have also been tried to treat vitiligo patients, as several studies have described the incidence of oxidative stress in the epidermis of vitiliginous skin (Dell'anna

and Picardo, 2006, Taieb et al., 2013). Antioxidants act by decreasing high levels of hydrogen peroxide in melanocytes and keratinocytes to restore the intracellular redox status, resulting in repigmentation of vitiliginous lesions (Schallreuter et al., 2001, Taieb et al., 2013). Antioxidants such as pseudocatalase alone or in combination with ultraviolet light have been used to treat vitiligo lesions and resulted in excellent repigmentation of vitiligo lesions in 90% of 33 vitiligo patients (Schallreuter et al., 1995).

1.2.12.2 Systemic therapy

Oral corticosteroids are very rarely considered as a therapeutic approach for the treatment of vitiligo (Table 1.5) (Iannella et al., 2016). Systemic corticosteroids are mainly used in rapidly progressive cases to arrest disease activity (Dillon et al., 2017). Oral administration of dexamethasone has been reported to halt progressive vitiligo, with some degree of repigmentation being observed (Lee et al., 2010, Manga et al., 2016). However, oral corticosteroids are only used for a short duration due to their potential side effects (Manga et al., 2016). In addition, oral administration of antioxidants has been employed to treat vitiligo (Table 1.5) (Rodrigues et al., 2017a). In one study, vitiligo patients received an oral combination of the antioxidants α-lipoic acid and vitamins C and E before treatment with narrow band-ultraviolet B (NB-UVB). This demonstrated excellent repigmentation in 40% of the vitiligo patients treated (Dell'Anna et al., 2007, Rodrigues et al., 2017a).

1.2.12.3 Phototherapy

Phototherapy as a treatment for vitiligo includes three main types, namely psoralen plus ultraviolet A (PUVA), narrow band-ultraviolet B (NB-UVB), and laser therapy (Table 1.5) (Iannella et al., 2016). PUVA therapy has been reported to help reverse the destruction of melanocytes and keratinocytes around and within vitiligo lesions (Anbar et al., 2012). NB-UVB can trigger the release of EDN-1 and b-FGF from keratinocytes, which then induce melanocyte proliferation. It is also considered to be immunosuppressive (Wu et al., 2004, Rodrigues et al., 2017a, Dillon et al., 2017). Repigmentation rates of 40-100% have been achieved by using NB-UVB irradiation (Njoo et al., 2000a, Kanwar and Dogra,

2005, Arca et al., 2006, Welsh et al., 2009, Rodrigues et al., 2017a) and, compared to other phototherapies, it is more suitable for treatment in pregnancy and in children (Bhatnagar et al., 2007, Rath et al., 2008). Moreover, in combination with tacrolimus, NB-UVB was found to be more efficacious than NB-UVB alone in reducing the size of vitiligo lesions (Nordal et al., 2011, Iannella et al., 2016). In addition, one study showed that vitiligo patients treated with NB-UVB lost their new pigment two years after cessation of treatment (Sitek et al., 2007).

Another effective therapeutic option in vitiligo is the use of excimer laser, which allows targeted treatment of individual vitiligo lesions, cosmetically prominent with a wavelength of 308 nm (Asawanonda et al., 2008, Taieb et al., 2013, Dillon et al., 2017). Excimer laser therapy is thought to induce proliferation and migration of melanocytes and T cell apoptosis (Abrouk et al., 2016). Several studies have reported a greater efficacy of excimer laser over other phototherapies in achieving more rapid and better repigmentation with lesser adverse side-effects (Casacci et al., 2007, Pacifico and Leone, 2011, Taieb et al., 2013, Iannella et al., 2016, Ayob et al., 2018). However, Passeron and Ortonne (2005a) showed that repigmentation relapse occurred in 15% of vitiligo patients 1-3 years following the discontinuation of excimer laser treatment.

1.2.12.4 Depigmenting treatments

In cases of extensive disfiguring vitiligo, where greater than 90% of the body surface area is affected, depigmentation of the remaining normal skin with topical monobenzyl ether of hydroquinone or 4-methoxyphenol may be recommended to render all skin or skin that is socially visible an even colour (Table 1.5) (Ghafourian et al., 2014, Lakhani and Deshpande, 2014, Manga et al., 2016, Grimes and Nashawati, 2017). However, the treatment is irreversible and patients must adhere to sun-safety for life to prevent severe sunburn, ochronosis and melanoma (Phillips et al., 2001, Ghafourian et al., 2014, Rodrigues et al., 2017a).

1.2.12.5 Surgical treatment

The aim of surgical procedures is to replace lost melanocytes with ones obtained from a normally pigmented autologous donor site. These procedures are only performed in patients with lesions refractory to medical treatment (Taieb et al., 2013, Iannella et al., 2016). Variety of surgical techniques have been established (Table 1.5). Surgical treatments offer better repigmentation outcomes for patients with stable segmental vitiligo (Kovacs, 1998, Taieb and Picardo, 2009, Ezzedine et al., 2012b, Rodrigues et al., 2017a). When repigmentation is achieved in these cases, relapse is rare (van Geel et al., 2010a, Gan et al., 2016, Rodrigues et al., 2017a).

1.2.12.6 Camouflage techniques

Given the negative impact of vitiligo on the patient's appearance, camouflaging may be a significant part of disease management and it is offered to every patient (Table 1.5) (Ongenae et al., 2005, Gawkrodger et al., 2010, Taieb et al., 2013, Rodrigues et al., 2017a). Self-tanning agents and highly pigmented creams could be employed to cover aesthetic skin disfigurement and these are easy to apply (Taieb et al., 2013, Rodrigues et al., 2017a). Additionally, cosmetic tattooing is another option to hide vitiligo macules, but particular caution should be considered with this technique because of the unpredictable course of the disease (De Cuyper, 2008, Taieb et al., 2013, Rodrigues et al., 2017a).

Table 1.5: Treatment options for vitiligo

Medical treatments	References
Topical treatment	
Topical steroids e.g., betamethasone	(Kandil, 1974, Taieb et al., 2013, Bilal and Anwar, 2014, Dillon et al., 2017)
Topical immunomodulators e.g., tacrolimus	(Boone et al., 2007, Radakovic et al., 2009, Dillon et al., 2017)
Topical antioxidants e.g., pseudocatalase	(Rodrigues et al., 2017a)
Systemic treatment	
Steroids e.g., dexamethasone	(Taieb et al., 2013, Bilal and Anwar, 2014, Rodrigues et al., 2017a)
Antioxidants e.g., minocycline, vitamin E, vitamin C, and lipoic acid	(Zhang et al., 2004, Dell'Anna et al., 2007, Middelkamp-Hup et al., 2007, Colucci et al., 2015a, Roy, 2017)
Phototherapy	
Narrow-band-ultraviolet B (NB-UVB)	(Westerhof and Nieuweboer-Krobotova, 1997, Brazzelli et al., 2007, Rodrigues et al., 2017a)
Topical or systemic psoralen plus ultraviolet A (PUVA)	(Ortonne et al., 1979, Pathak and Fitzpatrick, 1992, Wu et al., 2007, Taieb et al., 2013, Bilal and Anwar, 2014, Rodrigues et al., 2017a)
Excimer laser	(Passeron and Ortonne, 2005a, Casacci et al., 2007, Pacifico and Leone, 2011, Taieb et al., 2013, Dillon et al., 2017)
Depigmentation therapy	
Depigmenting agents e.g., hydroquinone monobenzyl ether, and 4-methoxyphenol	(Njoo et al., 2000b, AlGhamdi and Kumar, 2011, Taieb et al., 2013, Bilal and Anwar, 2014, Manga et al., 2016, Rodrigues et al., 2017a)
Surgical options	
Tissue grafting e.g., punch grafting, suction blister epidermal grafting, and split-thickness skin grafts	(Falabella, 2003, Taieb et al., 2013, Faria et al., 2014, Manga et al., 2016, Dillon et al., 2017, Rodrigues et al., 2017a)
Cellular grafting technique e.g., transplantation of non-cultured melanocytes	(Parsad and Gupta, 2008, Budania et al., 2012, Manga et al., 2016, Dillon et al., 2017, Rodrigues et al., 2017a)
Skin camouflage	
Cosmetic creams	(Kovacs, 1998, Gawkrodger et al., 2010, Lakhani and Deshpande, 2014, Rodrigues et al., 2017a)
Tattooing	(Taieb et al., 2013, Rodrigues et al., 2017a)

1.3 Aetiology and Pathogenesis of Vitiligo

The exact aetiopathogenesis of vitiligo still remains obscure, but there have been a number of proposed theories and these are discussed in the forthcoming sections.

1.3.1 Psychological stress

Some studies have revealed that vitiligo can develop under conditions of extreme stress (Lerner, 1959, Papadopoulos et al., 1998). However, it has also been shown that vitiligo does not necessarily develop due to the stressing conditions themselves, but to an inability to endure effectively uncontrollable situations (Picardi et al., 2003). In terms of the pathogenic mechanism, physiological stress may elevate the production of neuropeptides Y (Al'Abadie et al., 1994b, Tu et al., 2001, Karia et al., 2015), which could initiate a cascade of immunological reactions leading to melanocyte destruction (Karia et al., 2015).

1.3.2 Koebner's phenomenon

The development of lesions in response to physical trauma of normal skin, also referred to as Koebner's phenomenon, can possibly be an important initiating factor in the the loss of melanocytes in vitiligo (van Geel et al., 2011b, Ezzedine and Silverberg, 2016, Khurrum et al., 2017). It has been noted that the location of initial vitiliginous lesions is related to sites of friction (Gauthier, 1995, van Geel et al., 2011b). The prevalence of Koebner's phenomenon in vitiligo has been reported to range from 21-62% (Barona et al., 1995, Gauthier, 1995, van Geel et al., 2011b, Khurrum et al., 2017).

Currently, the exact pathomechanism of Koebner's phenomenon in causing melanocyte loss is unclear, although several aetiological hypotheses have been suggested including oxidative stress, immunological mechanisms, and deficient adhesion of melanocytes (van Geel et al., 2011b). Indeed, when the non-lesional skin of vitiligo patients is subjected to physical trauma, melanocytes show detachment from the basal cell layer of the epidermis followed by melanocyte death (Gauthier et al., 2003). Two steps of pathophysiology of Koebner's phenomenon have been proposed. Firstly, following skin

injury, inflammatory factors such as tumour necrosis factor-α (TNF-α), IL-1, IL-6, heat-shock protein 70 (HSP70), and ICAM-1 are released (van Geel et al., 2011b). Secondly, these factors may trigger an autoimmune response via activating and recruiting melanocyte-specific T cells in the integument (van Geel et al., 2011b, Sanghavi et al., 2013).

1.3.3 Infections and molecular mimicry

Antigenic structural homology between foreign (microbial) proteins and self-proteins of the human host is a phenomenon referred as molecular mimicry, whereby an immune response to the foreign molecules for defensive purposes can turn against the self-antigens and initiate autoimmune diseases (Cusick et al., 2012, Guarneri and Guarneri, 2013). This mechanism has been suggested as a possible pathogenic factor for vitiligo. It has been speculated that viral or microbial infections may cause vitiligo by triggering immune responses which then adversely affect melanocytes due to their expression of cross-reacting antigens (Le Poole et al., 1993a, Guarneri and Guarneri, 2013). Suggested infectious agents have included cytomegalovirus (Grimes et al., 1996), human immunodeficiency virus (Ivker et al., 1994, Antony and Marsden, 2003, Guarneri and Guarneri, 2013), *Mycobacterium leprae* (Sehgal, 1973), and *Candida albicans* (Howanitz et al., 1981). However, a lack of amino acid homology between known vitiligo autoantigens and microbial or viral agents means there is, as yet, no definitive evidence for molecular mimicry as a possible trigger factor for vitiligo (Gavalas et al., 2009).

1.3.4 Neural factors

The neural theory suggests that certain neurochemical mediators secreted by nearby nerve endings are cytotoxic to melanocytes in vitiligo. This hypothesis is supported by various histological, clinical, and biochemical observations in vitiligo patients.

1.3.4.1 Clinical evidence

Clinically, adverse effects of neurochemical mediators could explain the different patterns of vitiligo, namely the symmetrical appearance of non-segmental vitiligo and

the dermatomal distribution of macules in segmental vitiligo (Lerner, 1959, Choi et al., 2014). According to the neural theory, segmental vitiligo results from dysfunctionally sympathetic nerves that supply the affected dermatome (Lerner, 1959, Choi et al., 2014). Moreover, vitiligo has been noted to spare denervated area of skin, for example, below the neurological damage level in some patients suffering from severe spinal cord injury (Lerner, 1959, Choi et al., 2014). In addition, pathological diseases affecting the nervous system add further evidence to the neural hypothesis in the pathophysiology of vitiligo (Bellei et al., 2013, Choi et al., 2014). Patients with inflammatory diseases that affect the peripheral nervous system such as leprosy can develop vitiligo-like hypopigmented patches (Boisseau-Garsaud et al., 2000b, Choi et al., 2014). Moreover, vitiliginous depigmented lesions can appear with neurodysplasias sush as neurofibromatosis and tuberous sclerosis (Zvulunov and Esterly, 1995, Herane, 2003, Nanda, 2008, Choi et al., 2014).The arrest of such pathological conditions oftentimes results in repigmentation (Choi et al., 2014). Additional evidence for the engagement of nerves in vitiligo is that innervation for transplant skin is required to repigment an area (Lerner, 1959). Moreover, although no sensory abnormalities have been found in vitiliginous areas, autonomic abnormalities such as increased skin temperature and sweating have been reported in lesional skin of vitiligo patients in comparison to normal skin (Al'Abadie et al., 1994a, Nordlund, 2011).

More recently, clinical observational studies of segmental vitiligo patches with respect to their distribution pattern suggest a possible role of cutaneous mosaicism in the aetiology of segmental vitiligo (van Geel and Speeckaert, 2017). van Geel and Speeckaert (2017) showed distinct pattern of segmental vitiligo lesions in some patients, which were not completely corresponding to unilateral or band-shaped dermatomes. In addition, increasing evidence of inflammation has recently been shown in some segmental vitiligo cases (van Geel et al., 2010b, Ezzedine et al., 2012b). Histopathological studies demonstrated an infiltrate of cytotoxic CD8+T lymphocytes around the dermo-epidermal junction suggesting inflammatory or immune-related involvement in the pathogenesis of segmental as well as non-segmental vitiligo (van Geel et al., 2010b).

1.3.4.2 Histological evidence

Histologically, vitiligo patients can have dystrophic and degenerative changes in the dermal nerves and nerve endings (Choi et al., 2014). Electron-microscopic studies have revealed direct contact between nerve endings and melanocytes in vitiligo (Choi et al., 2014). Moreover, melanocytes arise from the neural crest and, in a variety of vitiligo cases, marginal melanocytes seem increasingly more neural in their behaviour, in which they adapt a progressively dendritic morphology and they have the ability to secrete adrenaline (Iyengar and Misra, 1988, Choi et al., 2014). Furthermore, Immunohistochemical studies have shown alterations in the neuropeptide levels in the lesional and peri-lesional skin of vitiligo patients. The levels of neuropeptide Y (NPY) in the plasma and lesional and peri-lesional areas of patients with vitiligo were significantly higher in comparison to healthy controls (Tu et al., 2001, Al'Abadie et al., 1994a, Hristakieva et al., 2000). Additionally, the number of nerve fibres that innervate the skin and secrete neuropeptides e.g., calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) has been exhibited to be elevated in vitiligo lesions as opposed to unaffected and control skin (Al'Abadie et al., 1994a, Liu et al., 1996, Lazarova et al., 2000, Tu et al., 2001, Laddha et al., 2014b, Lotti et al., 2014).

1.3.4.3 Neurotransmitter levels

In addition to the high levels of nerve fibres immunoreactive to NPY, NGF and CGRP, various studies have described elevated amounts of catecholamine neurotransmitters such as adrenaline and noradrenaline in the sera and depigmented skin lesions of patients with vitiligo (Morrone et al., 1992, Schallreuter et al., 1996, Cucchi et al., 2000, Cucchi et al., 2003, Zaki and Elbatrawy, 2009). In addition, increased secretion of catecholamines by keratinocytes has been found in vitiliginous skin (Schallreuter et al., 1992). Increased catecholamines levels can have a direct cytotoxic action on melanocytes. Indirectly, high levels of catecholamines as well as NPY can induce severe vasoconstriction leading to hypoxia with overproduction of oxyradicals, which can result in accumulation of melanocyte-damaging oxidative stress (Lundberg et al., 1983, Morrone et al., 1992, Gillbro et al., 2004, Namazi, 2007, Choi et al., 2014, Laddha et al.,

2014b). The possible mechanisms of action of NGF and CGRP in vitiligo are still unclear. However, it has been shown that lymphocytes express NGF and CGRP receptors, which when stimulated might lead to inflammatory responses (Yu et al., 2012b).

1.3.5 Biochemical factors

1.3.5.1 Accumulation of toxic metabolites

Toxic metabolite accumulation has been suggested to play a part in vitiligo pathogenesis. For example, melanocytes synthesise 6-tetrahydrobiopterin (6-BH4), a phenylalanine hydroxylase cofactor required for the conversion of phenylalanine to tyrosine, the latter being needed for melanin production (Schallreuter et al., 1994a, Schallreuter et al., 1994b, Choi et al., 2014). In vitiligo patient skin, the activity of phenylalanine hydroxylase is low leading to the accumulation of 6-BH4, elevated concentrations of which are toxic to melanocytes (Schallreuter et al., 1994a, Schallreuter et al., 1994b, Choi et al., 2014). In addition, a decrease in the stability of TYRP1, which is required for melanin synthesis (Figure 1.3), has been observed in vitiligo melanocytes (Jimbow et al., 2001, Ghanem and Fabrice, 2011). This defect allows the accumulation of melanin intermediates which are toxic to melanocytes (Jimbow et al., 2001, Ghanem and Fabrice, 2011).

1.3.5.2 Oxidative stress

Oxidative stress is suggested to be a primary event in melanocyte destruction (Sravani et al., 2009, Speeckaert et al., 2015), resulting from an excessive accumulation of reactive oxygen species (Mohammed et al., 2015, Speeckaert et al., 2015, Xie et al., 2016). Reactive oxygen species consist of a number of oxygen-based free radicals such as superoxide and hydrogen peroxide (Sravani et al., 2009, Choi et al., 2014), which are formed during multiple physiological and pathological processes (Knight, 1995, Sravani et al., 2009). Such free radicals are constantly scavenged by antioxidants such as superoxide dismutase, catalase, vitamin C, vitamin E, and other trace elements. In oxidative stress, there is inadequate antioxidant activity leading to the build-up of free

radicals, which cause damage to cellular components such as DNA, proteins, carbohydrates and lipids (Bickers and Athar, 2006, Lubos et al., 2011, Xie et al., 2016).

Under normal circumstances, superoxide dismutase catalyses the first step of dismutation by converting the superoxide anion into oxygen and hydrogen peroxide and then catalase enzyme transforms hydrogen peroxide into water and oxygen (Ines et al., 2006, Lobo et al., 2010). In vitiligo patients, high levels of superoxide dismutase and low levels of catalase have been observed in their skin (Schallreuter et al., 1991, Schallreuter et al., 2001, Sravani et al., 2009). Hydrogen peroxide created from superoxide anion can easily cross melanocyte membranes causing cellular damage (Agrawal et al., 2004, Shajil and Begum, 2006, Sravani et al., 2009, Mohammed et al., 2015).

A decrease in the levels of antioxidants such as vitamin C and vitamin E has also been observed in vitiligo patient skin (Haider et al., 2010, Deo et al., 2013, Korobko and Lomonosov, 2014), and this low antioxidant status could be responsible for increased concentrations of reactive oxygen species (Maresca et al., 1997, Sravani et al., 2009). Furthermore, successful repigmentation of skin with antioxidants such as pseudocatalase adds validity to the theory that oxidative stress is involved in vitiligo development (Schallreuter et al., 2001, Rashighi and Harris, 2017).

Furthermore, there is much evidence suggesting that melanocytes in vitiligo have intrinsic defects that decrease their ability to manage cellular stress (Rashighi and Harris, 2017). Cells in the epidermis including melanocytes, are regularly exposed to various environmental stressors e.g., UV radiation and oxidising chemicals, which can stimulate reactive oxygen species production that has been observed in vitiligo (Rashighi and Harris, 2017). Although normal pigment cells are capable of dealing with such stressors, melanocytes in vitiligo patients appear more vulnerable (Rashighi and Harris, 2017). For example, peri-lesional melanocytes exhibit a dilated endoplasmic reticulum, mitochondrial dysfunction, and an abnormal melanosome structure, all of which are features of augmented cellular stress (Picardo et al., 2015, Xie et al., 2016, Rashighi and Harris, 2017).

1.3.5.3 Chemically-induced vitiligo

Several chemicals, particularly aliphatic or aromatic derivatives of phenols or catechols, have been shown to be preferentially cytotoxic to melanocytes, *in vitro* and *in vivo* (Gellin et al., 1970, Lerner, 1971, Ortonne and Bose, 1993, Fisher, 1994, Cummings and Nordlund, 1995, Wu et al., 2015, Rashighi and Harris, 2017). As these compounds are structurally similar to tyrosine, they compete for hydroxylation via tyrosinase and so interfere with melanin synthesis (Figure 1.3) (Jimbow et al., 1974, Harris, 2016). How this subsequently promoted cells destruction is unclear. However, it has been proposed that semiquinone free radicals and reactive oxygen species formed by tyrosinase catalytic action on derivatives of phenols or catechols can lead to melanocyte damage in vitiligo (Boissy and Manga, 2004, Harris, 2016). In addition, it has recently been reported that melanocytes exposed to bleaching phenols secrete elevated amounts of HSP70 leading to melanocyte sensitisation to dendritic cell-mediated cytotoxicity (Mosenson et al., 2014, Harris, 2016).

1.3.6 Melanocyte growth factors

Melanin synthesis is controlled by a variety of melanogenic cytokines and growth factors including EDN-1, stem cell factor (SCF) and granulocyte monocyte-colony stimulating factor (GM-CSF). Such factors that are synthesised by keratinocytes and fibroblasts have a paracrine action on melanocytes by interacting with their respective receptors to trigger melanogenesis (Pillaiyar et al., 2017b). The expression levels of these melanogenic cytokines have been reported to be altered in vitiligo patients. For instance, SCF mRNA and protein have been found at lower levels in the vitiliginous lesions (Moretti et al., 2002b, Lee et al., 2005, Picardo et al., 2015, Shi et al., 2016). Furthermore, decreased levels of GM-CSF, b-FGF and EDN-1 have been shown in lesions of vitiligo patients (Imokawa et al., 1997, Moretti et al., 2002a, Moretti et al., 2002b, Nada and Rashed, 2012, Seif El Nasr et al., 2013, Mohammed et al., 2015, Shi et al., 2016). A decrease in the levels of such growth factors may contribute to the pathogenesis of vitiligo.

1.3.7 Melanocortin hormones

The melanocortin system in the skin comprises the melanocortin peptides α, β and γ-MSH and ACTH (Kingo et al., 2007, Abdel-Malek et al., 2014, D'Mello et al., 2016). These peptides bind with varying affinities to five different melanocortin receptors (MC1, MC2, MC3, MC4 and MC5R) (Abdel-Malek et al., 2014). The MC1R is the most abundant melanocortin receptor expressed in melanocytes and exhibits the highest affinity for α-MSH, which is shown to be the most prevalent melanocortin in the skin (Kingo et al., 2007, Montero-Melendez, 2015). The α-MSH peptide has been detected at lower levels in the plasma of vitiligo patients compared with normal subjects (Pichler et al., 2006, Khan et al., 2018). Moreover, a reduction in the levels of α-MSH mRNA and peptide has also been reported in lesional and peri-lesional skin of patients with vitiligo compared to normal subjects (Graham et al., 1999, Amin et al., 2013). Moreover, a study of α-MSH-positive melanocytes has revealed that low expression of α-MSH within vitiligo skin results from reduced expression of the peptide rather than a decrease in melanocyte number (Graham et al., 1999). Therefore, low expression levels of α-MSH may be considered a possible explanation for skin depigmentation in vitiligo.

1.3.8 Genetic factors

Approximately 6-38% of vitiligo patients have a positive family history of the disease, suggesting a possible hereditary cause (Nath et al., 1994, Njoo and Westerhof, 2001, Mohammed et al., 2015, Spritz and Andersen, 2017). However, familial cases of vitiligo do not show a Mendelian mechanism of inheritance, indicating the disease is polygenic (Majumder et al., 1993, Alkhateeb et al., 2003, Zhang et al., 2004, Mohammed et al., 2015, Patel et al., 2017, Spritz and Andersen, 2017). Indeed, several studies have shown that multiple loci contribute to vitiligo susceptibility (Table 1.6) (Majumder et al., 1993, Nath et al., 1994, Alkhateeb et al., 2003, Sun et al., 2006). More recently, genome-wide association studies have revealed that approximately half of vitiligo susceptibility genes encode immune-regulatory proteins, while the remainder encode melanocyte-specific proteins (Picardo et al., 2015, Spritz and Andersen, 2017). It must be noted, however, that the concordance rate of vitiligo in monozygotic twins is just 23%, suggesting that

non-genetic factors are also involved in the aetiology of vitiligo (Alkhateeb et al., 2003, Spritz, 2013, Picardo et al., 2015).

1.3.8.1 Major histocompatibility complex genes

In humans, the genes of the MHC encode human leukocyte antigens (Shaker and El-Tahlawi) (Shaker and El-Tahlawi), which are referred to as MHC class I, class II or class III antigens (Singh et al., 1997, Shaker and El-Tahlawi, 2008, Zhang and Xiang, 2014). Molecules encoded by this genetic region are implicated in antigen presentation as well as the regulation of inflammation and the complement system (Shiina et al., 2009, Matzaraki et al., 2017). Variability in the HLA region has been associated with susceptibility to a variety of autoimmune disorders (Choi et al., 2014). In terms of statistical significance and odds ratio, the strongest genetic associations of vitiligo have been found with the MHC class II. Even so, there are differences in the MHC loci and alleles associated with vitiligo when compared between different populations (Table 1.6).

In European-derived white subjects, significant associations of vitiligo are found with allelic variants of HLA class I and II molecules (Jin et al., 2010a, Jin et al., 2010b). Specifically, the HLA class I association is HLA-A*02:01, and the HLA class II gene association is HLA-DRB1*04 (Fain et al., 2006, Jin et al., 2010a, Jin et al., 2011, Jin et al., 2012b, Cavalli et al., 2016, Hayashi et al., 2016). Similar to Europeans, vitiligo in patients from the Indian subcontinent is associated with MHC class II loci, one located upstream of HLA-DRA and the other located between HLA-DRB1 and HLA-DQA1, but no association detected in the MHC class I region (Birlea et al., 2013). Findings in Japanese vitiligo patients resemble those reported for the MHC class I region in vitiligo patients of European decent, specifically, HLA-A*02:01 and HLA-A*02:06 (Jin et al., 2015). In contrast, genome-wide association analysis of Han Chinese has provided evidence for the association of vitiligo susceptibility with HLA specificities in the class III region (Quan et al., 2010). Finally, and interestingly, HLA-A*02:01 is suggested to present the vitiligo autoantigen tyrosinase on the surface of melanocytes, resulting in activation and recruitment of cytotoxic T cells to the skin, which then target and destroy pigment cells (Ostankovitch et al., 2009, Jin et al., 2015).

1.3.8.2 Other immune-regulatory genes

Many immune-regulatory genes have been identified as risk factors for vitiligo which, taken together, provide evidence that the dysregulation of immunity can contribute to vitiligo development (Table 1.6). For example, the *PTPN22* gene, which encodes lymphoid protein tyrosine phosphatase non-receptor type 22, an essential down-regulator of T lymphocyte activation (Hill et al., 2002), is associated with vitiligo in English, North American, Romanian and Mexican populations (Canton et al., 2005, LaBerge et al., 2008a, Laberge et al., 2008b, Jin et al., 2010a, Garcia-Melendez et al., 2014). So too, the NACHT leucine-rich-repeat protein 1 (*NLRP1*) gene which encodes nuclear localization leucine-rich-repeat protein 1, a key regulator of the innate immune system (Spritz et al., 2004, Jin et al., 2007a, Jin et al., 2007b, Martinon et al., 2007, Hoffman and Brydges, 2011, D'Osualdo and Reed, 2012, Levandowski et al., 2013).

A further candidate susceptibility gene encodes the autoimmune regulator (AIRE), which has a role in central tolerance. Single-nucleotide polymorphisms (SNPs) in *AIRE* are associated significantly with vitiligo susceptibility (Tazi-Ahnini et al., 2008, Oftedal et al., 2015). Similarly, SNPs in the gene encoding cytotoxic T lymphocyte antigen-4 (CTLA4) have been shown to associate with vitiligo (Kemp et al., 1999, Blomhoff et al., 2005, Spritz and Andersen, 2017). CTLA4 negatively regulates T cell activation and also controls T cell apoptosis (Das et al., 2001, Al-Shobaili, 2011, Spritz and Andersen, 2017). Recently, many vitiligo susceptibility genes e.g., X-box binding protein 1, forkhead box P1, cytokine-chemokine receptor, thymic stromal lymphopoietin and forkhead box P3, which encode regulating elements of the immune system, have also been identified and confirmed by the application of genome-wide association studies (Cheong et al., 2009, Ren et al., 2009, Jin et al., 2010b, Quan et al., 2010, Birlea et al., 2011).

1.3.8.3 Genes related to melanocyte function

Polymorphisms in non-immune genes such as *OCA2*, the gene encoding P protein (Jin et al., 2012a), *MC1R*, the gene encoding melanocortin 1 receptor (Jin et al., 2012a), *PMEL*, the gene encoding melanocyte-specific protein PMEL(Tang et al., 2013), and *ZMIZ1*, the gene encoding zinc finger MIZ domain containing protein 1 (Sun et al., 2014) have been

identified as vitiligo susceptibility loci (Table 1.6). All of these genes contribute to the development, function and survival of melanocytes (Shen et al., 2016). Of particular interest, is the association found in European-derived whites between vitiligo and SNPs in *TYR*, the gene encoding tyrosinase (Jin et al., 2010a). The causal genetic variation of *TYR* is inversely correlated between vitiligo and malignant melanoma (Spritz, 2010a, Jin et al., 2012a, Jin et al., 2012b), thus suggesting vitiligo may develop from the dysregulation of normal immune surveillance against melanoma (Spritz, 2010a, Shen et al., 2016). Interestingly, a correlation between the minor *TYR* allele, 402Q and predisposition to malignant melanoma has been described (Gudbjartsson et al., 2008, Bishop et al., 2009), suggesting that such a variant may be less accessible to the immune defences. Therefore, in melanoma cases with the 402Q variant, these neoplastic melanocytes perhaps evade immune surveillance (Jin et al., 2010a, Spritz, 2010a). On the other hand, the *TYR* 402R variant possibly presented to the immune defences more effectively than 402Q variant leading to a more efficient immunological responses to neoplastic melanocytes, but equally contributes to susceptibility to vitiligo (Jin et al., 2010a, Spritz, 2010a).

1.3.8.4 Genes encoding metabolic proteins

Many studies have been carried out to identify genes that regulate melanocyte metabolism, development or proliferation, and which could be associated with vitiligo susceptibility (Table 1.6). Such studies have included the genes encoding catalase (Casp et al., 2002, Park et al., 2006, Liu et al., 2010, Birlea et al., 2011, Lv et al., 2011, Lu et al., 2014, Caputo et al., 2017) and catechol-O-methyltransferase (Tursen et al., 2002, Li et al., 2009a, Birlea et al., 2011). Several polymorphisms have been identified in both genes, which might impair the function of the enzymes they encode (Al-Shobaili, 2011, Picardo et al., 2015). Catalase and catechol-O-methyltransferase are critical enzymes that protect cells against oxidative damage via breaking down hydrogen peroxide and catecholamines, respectively (Al-Shobaili, 2011, Ezzedine et al., 2015). Therefore, it is plausible that abnormal expression of such proteins results in toxic radical accumulation and subsequently leads to melanocyte damage, which in turn results in vitiligo (Li et al., 2009a, Al-Shobaili, 2011, Lv et al., 2011).

Polymorphic expression of methylene tetrahydrofolate reductase (*MTHFR*) gene has recently been reported in vitiligo patients (Chen et al., 2014). This enzyme regulates the levels of homocysteine, which has been detected at elevated levels in vitiligo patients (Shaker and El-Tahlawi, 2008, Silverberg and Silverberg, 2011, Singh et al., 2011). Increased levels of homocysteine have been shown to induce cell apoptosis via downregulating enzymes such as catalase leading to increased generation of reactive oxygen species, which in turn can cause damage to melanocyte in vitiligo (Chen et al., 2014, Picardo et al., 2015).

Polymorphisms in the *EDN-1* gene has been correlated with an elevated risk of vitiligo (Kim et al., 2007). EDN-1, produced by keratinocytes, has been shown to activate melanocyte proliferation and stimulates melanogenesis through activating tyorsinase and stimulating expression of TYRP1 (Hara et al., 1995, Imokawa et al., 1997, Funasaka et al., 1998, Mou et al., 2004, Yamaguchi and Hearing, 2009, Aly et al., 2013, Lee et al., 2013b, Bingül et al., 2016).

Allelic variation in *ACE*, the gene encoding angiotensin-converting enzyme (ACE), has been associated with an increased risk of vitiligo (Jin et al., 2004, Deeba et al., 2009, Tippisetty et al., 2011, Patwardhan et al., 2013, Badran et al., 2015, Abdel Azeem et al., 2016). ACE plays a crucial role in converting angiotensin I into angiotensin II which acts as an effective pro-inflammatory modulator (Song et al., 2015). Recent studies have suggested that the catalysis of angiotensin I conversion to angiotensin II by ACE may simultaneously increases the production of reactive oxygen species and secretion of inflammatory cytokines (Gonzalez-Villalobos et al., 2013, Shang et al., 2016).

Table 1.6: A summary of genes associated with susceptibility to vitiligo

Type	Gene	Protein	Function	References
HLA	*HLA–A*	HLA class I histocompatibility antigen, A	Peptide antigen presentation to the immune system	(Liu *et al*., 2007, Jin *et al*., 2010a, Jin *et al*., 2015)
	HLA-DRB1 and DQA1	HLA class II histocompatibility antigen, DRB1 and DQA1	Peptide antigen presentation to the immune system	(Fain *et al*., 2006, Jin *et al*., 2010a)
	HLA class III	HLA class III histocompatibility antigen	Involved in inflammation and cytokine production	(Quan *et al*., 2010)
Immune-regulatory	*AIRE*	Autoimmune regulator	Maintenance of immune tolerance	(Tazi-Ahnini *et al*., 2008, Oftedal *et al*., 2015)
	BACH2	Transcription regulator protein BACH2	B cell transcriptional repressor encodes a transcriptional repressor of B cells, which is a key regulator of CD4+ T-cell differentiation that prevents inflammatory disease by controlling the balance between tolerance and immunity	(Jin *et al.*, 2012a)
	C1QTNF6	Complement C1q tumour necrosis factor-related protein 6	Induces monocyte IL-10 expression	(Jin *et al*., 2010a)

	CASP7	Caspase-7	Executor protein of apoptosis and inflammation	(Jin et al., 2012a)
	CCR6	Chemokine-cytokine receptor 6	Regulates differentiation and function of B and T lymphocytes and dendritic cells	(Jin et al., 2010a, Quan et al., 2010)
	CD44	CD44 antigen	Involves in T cell development	(Jin et al., 2012a)
	CD80	T-lymphocyte activation antigen CD80	Co-stimulates activation of T cells	(Jin et al., 2012a)
	CLNK	Cytokine-dependent hematopoietic cell linker	Positively regulates immune-receptor signalling	(Jin et al., 2012a)
	CTLA4	Cytotoxic T-lymphocyte protein 4	Inhibition of T cells	(Kemp et al., 1999, Birlea et al., 2009, Pehlivan et al., 2009)
	CXCR5	C-X-C chemokine receptor type 5	Involves in B lymphocyte migration	(Tang et al., 2013)
	FASLG	FAS ligand	Regulate immune apoptosis	(Li et al., 2008)
	FOXP1	Forkhead box protein P1	Regulates B cell development	(Jin et al., 2010b)
	FOXP3	Forkhead box protein P3	Regulates development and function of regulatory T cells	(Birlea et al., 2011)

	GZMB	Granzyme B	Mediates the process of immune-induced cell death with contribution of cytotoxic T lymphocytes and natural killer cells	(Jin et al., 2010a, Ferrara et al., 2013)
	IFIH1	Interferon-induced helicase C domain 1	Regulates innate immune response	(Jin et al., 2012a)
	IKZF4	Zinc finger protein Eos	Regulates T cell activation	(Jin et al., 2012a, Tang et al., 2013)
	IL2RA	Interleukin-2 receptor subunit alpha	Regulates regulatory T cells	(Jin et al., 2010a, Tang et al., 2013)
	NLRP1	NACHT leucine-rich-repeat protein 1	Regulates innate immune system	(Jin et al., 2007a, Jin et al., 2007b, Alkhateeb and Qarqaz, 2010, Levandowski et al., 2013)
	PTPN22	Lymphoid protein tyrosine phosphatase non-receptor type 22	Negative regulation of T cell activation	(Canton et al., 2005, LaBerge et al., 2008a, Laberge et al., 2008b, Laddha et al., 2008, Jin et al., 2010a, Song et al., 2013, Garcia-Melendez et al., 2014)

	SH2B3	SH2B adapter protein 3	Development of B and T lymphocytes	(Jin et al., 2012a)
	SLA	Src-like-adapter	Regulates antigen receptor signalling	(Jin et al., 2012a)
	TOB2	Protein TOB2	Involves in T cell tolerance	(Jin et al., 2012a)
	TSLP	Thymic stromal lymphopoietin	Cytokine regulator of maturation of skin dendritic cells and T cells	(Birlea et al., 2011)
	UBASH3A	Ubiquitin-associated and SH3 domain–containing A protein	Suppresses T cell receptor signalling	(Jin et al., 2010a)
	XBP1	X-box-binding protein 1	Regulator of major histocompatibility complex class II	(Ren et al., 2009, Birlea et al., 2011)
Melanocyte function	*ASP*	Agouti signalling protein	Melanogenesis regulator via MC1R	(Na et al., 2003)
	FOXD3	Forkhead box D3	Regulator of melanoblast development	(Alkhateeb et al., 2005)
	MC1R	Melanocortin 1 receptor	Regulator of melanogenesis	(Na et al., 2003, Jin et al., 2012a)
	OCA2	OCA2 gene	Melanosomal transporter	(Jin et al., 2012a)
	PMEL	Melanocyte-specific protein PMEL	Structural organization of premelanosomes	(Tang et al., 2013)
	TYR	Tyrosinase	Regulator of melanogenesis	(Jin et al., 2010a, Jin et al., 2012b)

	ZMIZ1	Zinc finger protein MIZ type 1	Regulates development, function and survival of melanocytes	(Quan et al., 2010, Sun et al., 2014)
Metabolism-related genes	*ACE*	Angiotensin-converting enzyme	Regulator of inflammation and blood pressure	(Jin et al., 2004)
	CAT	Catalase	Protects cells from oxidative stress by breakdown of hydrogen peroxide	(Wood et al., 2008, Liu et al., 2010, Mansuri et al., 2017)
	EDN-1	Endothelin-1	Regulator of melanocyte growth and function	(Kim et al., 2007)
	LPP	Lipoma-preferred partner	Potential co-activator	(Jin et al., 2010a)
	RERE	Arginine–glutamic acid dipeptide repeats protein	Regulates cell apoptosis	(Jin et al., 2010a)
	RNASET2	Ribonuclease T2	Oxidative stress regulator	(Quan et al., 2010)

1.3.9 Autoimmunity

Many studies have indicated a role for autoimmunity in the aetiology and pathogenesis of vitiligo, and the evidence for this will be described and discussed in the next sections.

1.3.9.1 Autoimmune diseases

The association between vitiligo and other autoimmune diseases has been discussed earlier in Section 1.2.5.

1.3.9.2 Immunogenetic factors

The association between vitiligo susceptibility and variability in respect of genes encoding factors that regulate the immune system has been discussed earlier in Section 1.3.8.

1.3.9.3 Animal models of vitiligo

The study of animal models has provided persuasive evidence that immune mechanisms play a role in the pathogenesis of vitiligo. The Smyth line chicken is characterised by the spontaneous loss of pigment from feathers and ocular tissues due to an inherited melanocyte defect (Smyth, 1989). In the Smyth line chicken, abnormal melanocytes are destroyed by autoreactive T cells, and antibodies against TYRP1 constitute the major humoral immune response (Austin et al., 1992, Wang and Erf, 2004, Shi et al., 2012, Essien and Harris, 2014). In addition, the levels of interferon-γ (IFN-γ), IL-10, and IL-21 in growing feathers have been shown to increase at vitiligo onset, reaching a peak during active disease, and decreasing to near pre-vitiligo levels after the complete loss of melanocytes (Shi et al., 2012, Essien and Harris, 2014). These cytokine expression studies suggest that Smyth line chicken vitiligo is a T helper 1 (Th1) cell-polarised autoimmune disease (Shi et al., 2012). Sera from Smyth line chickens and other animal models of vitiligo, for example, horses and dogs also contain anti-melanocyte antibodies that are not present in normal animals (Naughton et al., 1986, Kemp et al., 2001b, Rezaei

et al., 2007, Essien and Harris, 2014), although such antibodies may only be markers of the disease or a secondary phenomenon.

Recent studies using mouse models of vitiligo have provided several lines of evidence which support a vital role for autoimmunity in the development of vitiligo (Chatterjee et al., 2014, Eby et al., 2014, Shi et al., 2014). A recent study of h3TA2 mice, which develop vitiligo spontaneously, showed that the adoptive transfer of T regulatory cells (Tregs) induced a lasting remission of vitiligo when the animals were treated at the onset of or after the establishment of the disease (Chatterjee et al., 2014). Further to this, in IFN-γ-knockout h3TA2 mice, depigmentation was significantly impaired, confirming a central role for IFN-γ in the development of vitiligo. In Vitesse mice, which again develop vitiligo spontaneously, there is an increased level of IL-17-producing cells and a reduced Tregs abundance, although the significance of this has yet to be fully evaluated (Eby et al., 2014). Taken together, these results show mounting evidence implicating autoimmune mechanisms in vitiligo pathogenesis.

1.3.9.4 Immunosuppressive therapies

Repigmentation in vitiligo patients receiving immunosuppressive treatments (Table 1.5) indirectly supports the notion that immune-mediated processes play a part in vitiligo pathogenesis (Parrish et al., 1976, Tsuji and Hamada, 1983, Ongenae et al., 2003, Sandoval-Cruz et al., 2011, Ezzedine et al., 2015). For example, topically-applied tacrolimus, a reagent that suppresses T cells by blocking the action of the cytokine gene-activating cofactor calcineurin (Homey et al., 1998, Lubaki et al., 2010, Speeckaert et al., 2015) has been used to treat vitiligo successfully (Hartmann et al., 2008).

Topical corticosteroids which suppress T lymphocyte activity and B cell antibody responses, are also an effective first line treatment for vitiligo of recent onset (Abu Tahir et al., 2010, Gawkrodger et al., 2010). Corticosteroids inhibit the synthesis of a wide variety of cytokines including IL-1, IL-2, IL-3, IL4, IL-5, IL-6, IL-8, GM-CSF, TNF-α, and IFN-γ by interfering with gene expression (Speeckaert et al., 2015). Although the exact mechanism of action is unclear, it is interesting to note that after treating vitiligo patients with systemic steroids, a decrease in the levels of anti-melanocyte antibodies

and in antibody-mediated anti-melanocyte cytotoxicity has been observed (Takei et al., 1984, Hann et al., 1993a).

With regard to the treatment of vitiligo using photochemotherapy, a decrease in Langerhans cell numbers and in the expression of vitiligo-associated melanocyte antigens has been demonstrated (Viac et al., 1997, Lee et al., 2013a). UV-irradiation itself can also lead to an increase in anti-inflammatory cytokines expression, regulate ICAM-1 expression and evoke apoptosis of skin-infiltrating T lymphocytes (Duthie et al., 1999, Bulat et al., 2011, Lahiri and Malakar, 2012, Lee et al., 2013a).

1.3.9.5 Immunological features of melanocytes

A number of studies have demonstrated abnormal expression of MHC class II antigen HLA-DR and induced expression of ICAM-1 by peri-lesional melanocytes in vitiligo skin (al Badri et al., 1993, Ahn et al., 1994, van den Wijngaard et al., 2000, Dwivedi et al., 2013b, Laddha et al., 2013). These molecules have vital roles in the presentation of antigens and in the activation of T cells (Hedley et al., 1998, Dwivedi et al., 2013b, Benzekri et al., 2015). Their expression by melanocytes may, therefore, contribute to the cellular immune response against pigment cells, as observed in vitiligo (al Badri et al., 1993, Ogg et al., 1998, van den Boorn et al., 2009, Dwivedi et al., 2013b). Both vitiligo and normal melanocytes can also express MHC class I molecules (Hedley et al., 1998) which may allow interaction with cytotoxic T cells (Le Poole et al., 1993b). In addition, peri-lesional melanocytes express CD68 and CD36 which attract macrophages (van den Wijngaard et al., 2000). They also express decreased amounts of membrane regulators of complement activation, such as membrane cofactor protein and decay acceleration factor (van den Wijngaard et al., 2002), implying a vulnerability of such cells to destruction by macrophages and the complement system.

1.3.9.6 Immuno-regulatory micro RNAs

Micro RNAs (miRNAs) are a class of non-coding RNAs that play a role in the negative regulation of gene expression (Ambros, 2003, Lai, 2003). A growing number of studies have demonstrated that miRNAs have a pivotal role in controlling immunity by

regulating cellular differentiation and proliferation of immune cells. Abnormal expression of miRNAs has been reported by several studies on vitiligo (Shi et al., 2013, Shi et al., 2014, Mansuri et al., 2016).

In a mouse model of vitiligo, upregulation of miRNA-146a was observed (Shi et al., 2014). This miRNA is involved in the regulation of Tregs and natural killer cell functions (Lu et al., 2010) and contributes to the development of autoimmune disease in mice (Mueller et al., 2009). Interestingly, increased levels of miRNA-146a in rheumatoid arthritis have been suggested to cause prolonged production of the pro-inflammatory cytokine TNF-α, and significantly upregulate phagocytic activity (Pauley et al., 2008, Shi et al., 2014). As TNF-α, Tregs and natural killer cells play a potential role in vitiligo development, it is plausible to suggest that highly expressed miR-146a in vitiligo could contribute to vitiligo pathogenesis (Shi et al., 2014).

Shi et al. (2013) showed, in patients affected by vitiligo compared to controls, significantly increased expression of miR-19a and miR-19b, both of which have been shown to play a crucial role in Th1 cell responses, through inducing IFN-γ secretion and suppressing inducible Tregs (Jiang et al., 2011). Therefore, miR-19a and miR-19b may be implicated in vitiligo development by regulating Th1 cell responses (Shi et al., 2013). Furthermore, Mansuri et al. (2016) reported a decreased expression of miR-211 which downregulates IL-23A, IL-1B, and IL-1 receptor. The same study reported that lesional skin of vitiligo patients showed increased expression of IL-23, which is mainly synthesised by dendritic cells and macrophages and regulates IL-17A secreted by T helper 17 (Th17) cells (Bettelli et al., 2008, Vaccaro et al., 2015). As IL-23 is important in stimulating the effector function of Th17 cells to produce cytokines and initiate inflammatory responses (Vaccaro et al., 2015), it may contribute to melanocyte destruction in vitiligo. Interleukin-1B and IL-1 receptor are key mediators of the body's cytokine-induced immune and inflammatory responses, and both were shown to be significantly upregulated in vitiligo patients (Mansuri et al., 2016). Interestingly the former was demonstrated to be upregulated in non-lesional skin of vitiligo patients compared to lesional skin, suggesting its vital involvement in the destruction of melanocytes rather than being a consequence of the disorder (Mansuri et al., 2016). Another increased miRNA detected in vitiligo patients was miR-577 which

downregulates PTPN22 expression that encodes a negative regulator of T cell activation (Mansuri et al., 2016). The same study found reduced expression of PTPN22 in the skin of vitiligo patients, suggesting that downregulation of *PTPN22* by miR-577 may result in hyper-responsive T cells and hence an increased susceptibility to autoimmunity (Mansuri et al., 2016). Taken together, the upregulation of IL-1B, IL-1 receptor and IL-23A, and the downregulation of PTPN22 in vitiligo patients indicates a potential role for miRNAs in the autoimmune pathogenesis of vitiligo (Mansuri et al., 2016).

1.3.9.7 Cellular immunity in vitiligo

1.3.9.7.1 Macrophages

Macrophage-infiltration has been observed in vitiligo lesions with an elevated number in peri-lesional skin in comparison to normal skin (Le Poole et al., 1996, van den Wijngaard et al., 2000). Macrophages may be involved in the clearance of melanocytes which have been evoked to apoptosis by cytotoxic T lymphocytes (Rezaei et al., 2007). Serarslan et al. (2010) and Li et al. (2013) showed that serum concentrations of macrophage migration inhibitory factor, which can concentrate macrophages at the sites of inflammation and regulate the activation of macrophages and T cells, are significantly increased in vitiligo patients. Ma et al. (2013) also reported that macrophage migration inhibitory factor mRNA is over-expressed in the peripheral blood mononuclear cells and in the lesional skin of patients with progressive vitiligo. Additionally, the active participation of macrophages in vitiligo development is demonstrated by their expression of immunoglobulin receptors; in a model mouse of vitiligo, macrophages encoding the common γ-chain of the activating Fc receptors are able to mediate melanocyte loss (Trcka et al., 2002). All these data are suggestive of macrophage involvement in melanocyte destruction.

1.3.9.7.2 Dendritic cells

Dendritic cells are antigen-presenting cells which express a high density of MHC class II molecules. Dendritic cells also secrete key inflammatory cytokines such as IL-1, IL-6, IL-12 and IL-23, TNF-α, interferon-α (IFN-α), and interferon-β (IFN-β). Such cytokines are

involved in the activation of B cells and natural killer cells and induce the attraction of other innate immune effector cells (Blanco et al., 2008). Dendritic cell density in lesional skin has been variously observed as normal, reduced, and increased compared with non-lesional skin from the same patients and with control groups (Riley, 1967, Claudy and Rouchouse, 1984, Hatchome et al., 1987, Searle et al., 1993). Such variation may be attributed to vitiligo clinical subtype, techniques of sampling or skin biopsy sites in the different studies.

More recent studies have provided direct evidence that dendritic cells are involved in vitiligo pathogenesis (Wang et al., 2011, Sanchez-Sosa et al., 2013, Yang et al., 2017a). Immunohistochemical studies showed that early vitiligo patches are infiltrated mostly by dendritic cells, whereas later lesions show lower numbers of these cells, but increased proportions of T cells (Sanchez-Sosa et al., 2013). The data imply that the antigen-presentation process occurs at an early stage of vitiligo development (Sanchez-Sosa et al., 2013). Additionally, *in vivo* and *in vitro* studies have shown that melanocyte destruction is mediated by dendritic cells (Kroll et al., 2005). Though the role of dendritic cells in vitiligo is still unclear, they themselves might be cytotoxic or carry and present melanocyte associated antigens to T cells contributing to melanocyte destruction (Rashighi and Harris, 2017).

1.3.9.7.3 Natural killer cells

Natural killer cells are a vital component of the innate immune system. Although they are most noticeably involved in defending against bacterial, viral and parasitic infections, several lines of evidence suggest that natural killer cells play a key role in the initiation of vitiligo. For example, a number of studies have reported an aberrant activation of natural killer cells in vitiligo patients (Basak et al., 2008, Yu et al., 2012a, van den Boorn et al., 2016). In addition, an increase in the number of circulating natural killer cells in the blood of vitiligo patients has been demonstrated by several studies (Ghoneum et al., 1987, Mozzanica et al., 1989, Mozzanica et al., 1992, Hann et al., 1993b, Durham-Pierre et al., 1995, Basak et al., 2008).

More recently, infiltration of GZMB-expressing natural killer cells has been demonstrated in lesional and non-lesional areas of vitiligo patient skin (Yu et al., 2012a). GZMB

expression is a characteristic of activated natural killer cells (Afonina et al., 2010, Yu et al., 2012a) and its role is promoting the destruction of target cells through activation of the caspase pathway as well as generating and presenting autoantigens (Darrah and Rosen, 2010, Yu et al., 2012a).

The upregulation of C-type lectin domain family 2 member B (*CLEC2B*) gene has been observed in vitiliginous skin (Yu et al., 2012a). This gene is mostly expressed by monocytes and macrophages and encodes an activating ligand of natural killer cells which binds to NKp80 receptors (Welte et al., 2006, Yu et al., 2012a). Furthermore, elevated natural killer cell activating receptors (CD16+CD56+ and CD3+CD16+CD56+) and reduced natural killer cell inhibitory receptors (CD16+CD158a+) have been shown in vitiligo patients (Basak et al., 2008). Therefore, such abnormalities in natural killer cells of vitiligo patients, all imply a possible role for natural killer cells in the development of vitiligo.

1.3.9.7.4 CD4+ helper T lymphocytes

The CD4+ helper T cells are crucial components in regulating the immune response. The main function of CD4+ T lymphocytes is the secretion of cytokines that provide signals to other immune cell populations such as B lymphocytes to mature and secrete antibodies, and macrophages to induce phagocytosis of microorganisms. They also play a vital role in suppressing immune responses (Luckheeram et al., 2012). Naïve CD4+ lymphocytes become activated upon the recognition of antigen-MHC class II complexes presented by specific antigen-presenting cells (Luckheeram et al., 2012). Activated CD4+ T lymphocytes can be differentiated into several distinct effector subtypes of T helper cells including Th1, Th2, Th17 and Treg cells, (Muranski et al., 2008), which can contribute to amplifying immune reactions and potentiating the destruction of melanocytes in vitiligo (Lukinovic-Skudar, 2015). With respect to CD4+ T cell-mediated melanocyte destruction, CD4+ T cells are shown to be capable of destroying MHC class II antigen-expressing cells (Rathmell et al., 1995, Lambe et al., 2006) and melanocytes can express MHC class II antigens (al Badri et al., 1993, Lambe et al., 2006). It has been shown that the number of CD4+ T cells is significantly increased in lesional and peri-lesional vitiliginous skin (Le Poole et al., 2004, Abdallah et al., 2014, Yang et al., 2017b).

The subset of Th1 CD4+ T cells and their cytokines IFN-γ, TNF-α, chemokine C-X-C motif ligand 9 (CXCL9), CXCL10, and CXCL11 seem to play a dominant role in vitiligo development (Harris et al., 2012, Antonelli et al., 2015, Lukinovic-Skudar, 2015, Wang et al., 2016), since they can recruit and activate immune cells e.g., macrophages, natural killer cells and CD8+ T cells, stimulating a cellular immune response that results in cytotoxicity to melanocytes (Lukinovic-Skudar, 2015). A predominantly Th1-mediated pattern with a dominant role of CXCL10 in skin depigmentation was revealed in both a vitiligo mouse model and in lesional skin and serum of human patients with vitiligo (Rashighi et al., 2014, Lukinovic-Skudar, 2015, Wang et al., 2016), suggesting the Th1 immune response in vitiligo pathogenesis is significant (Antonelli et al., 2015).

Another subset of CD4+ T lymphocytes, the Th17 cells, has been recently identified as an active participant in the destruction of melanocytes (Lukinovic-Skudar, 2015, Zhou et al., 2015). The Th17 cells play a central role in potentiation of the innate inflammatory response via the production of cytokines e.g., IL-17, IL-21, and IL-22, which in turn recruit neutrophils and macrophages to the antigen site (Lukinovic-Skudar, 2015). Several studies have suggested that Th17 cells and their effector molecule, IL-17, are active participants in the destruction of melanocytes observed in vitiligo (Singh et al., 2016). Studies have shown infiltration of Th17 cells, elevated levels of IL-17 mRNA in vitiligo skin samples, and upregulated levels of IL-17 in vitiligo patient blood (Basak et al., 2009, Wang et al., 2011, Kotobuki et al., 2012, Elela et al., 2013, Zhou et al., 2015), thus implying a potential role for Th17 cells in vitiligo pathogenesis (Zhou et al., 2015).

The predominance of the Th1 or Th17 phenotype in vitiligo may be attributed to a disturbed balance between inflammatory cells and Tregs (Lukinovic-Skudar, 2015). Regulatory T cells are also a subgroup of CD4+ T cells that primarily represent a phenotype of CD4+, CD25+ and forkhead box P3 (Foxp3). These cells have a vital role in controlling autoimmunity by maintaining immunological unresponsiveness to self-antigens, playing a key role in the clearance of self-reactive T cells that have escaped clonal deletion (Jonuleit et al., 2001, Sakaguchi et al., 2001, Kidir et al., 2017). Alterations in Treg number and function can result in autoimmunity (Lukinovic-Skudar, 2015). Both Treg number and function have been shown to be impaired in patients with vitiligo (Dwivedi et al., 2015, Lukinovic-Skudar, 2015, Kidir et al., 2017). Studies have revealed

that the density and the suppressive effects of peripheral Tregs in vitiligo patients are significantly decreased, suggesting a possible failure of Tregs to suppress cytotoxic T cells which target melanocytes (Ben Ahmed et al., 2012, Lili et al., 2012, Dwivedi et al., 2013a).

Notably, the serum concentration of transforming growth factor-β (TGF-β), an immunosuppressive cytokine expressed by Tregs (Bettini and Vignali, 2009) is lower in patients with vitiligo than in controls (Basak et al., 2009, Tu et al., 2011, Khan et al., 2012). In addition, a lower level of TGF-β expression in the skin of patients with vitiligo than in healthy controls has been reported (Moretti et al., 2002b, Klarquist et al., 2010). The reduced TGF-β concentration is also correlated with an increase in the area of skin involvement (Tu et al., 2011). Another suppressor cytokine produced by Tregs, IL-10, has also been found to be decreased in lesional skin of vitiligo patients, suggesting that the IL-10 reduction may be due to Treg-cell insufficiency (Kidir et al., 2017). Furthermore, in a mouse model of vitiligo, repigmentation was accompanied by an elevated infiltration of Tregs into the skin, possibly resulting in the prevention of immune responses against melanocytes (Eby et al., 2014). Interestingly, a previous study found a dramatic increase in Tregs in the peri-lesional skin of vitiligo patients (Ben Ahmed et al., 2012, Lili et al., 2012). However, these Tregs were diminished in their ability to suppress the proliferation and cytotoxic capacity of the elevated levels of peri-lesional CD8+ T cells, suggesting that defective functioning of Tregs and an increase in CD8+ T cells may contribute to melanocyte destruction (Ben Ahmed et al., 2012, Lili et al., 2012).

1.3.9.7.5 CD8+ cytotoxic T lymphocytes

Besides CD4+ T cells, the peri-lesional skin of vitiligo patients can be infiltrated with CD8+ cells, often with an elevated ratio of CD8+/CD4+ lymphocytes (Le Poole et al., 1996, Lili et al., 2012, Abdallah et al., 2014, Furue and Kadono, 2016, Speeckaert et al., 2017, Yang et al., 2017b, Boniface et al., 2018). *In vitro* culture of T cells from peri-lesional skin of vitiligo patients has shown that both CD8+ and CD4+ T cells secrete high levels of the pro-inflammatory cytokines TNF-α and IFN-γ (Wankowicz-Kalinska et al., 2003, van den Boorn et al., 2009, Boniface et al., 2018). In particular, IFN-γ is required for melanocyte-specific autoreactive CD8+ T cell recruitment to the affected skin (Harris et al., 2012,

Rodrigues et al., 2017b). Another important observation is that CD8+ T cells in vitiligo patients were demonstrated to express the skin-homing receptor, cutaneous lymphocyte-association antigen, which can recruit T cells from the peripheral circulation to the area of affected skin (Ogg et al., 1998, Sandoval-Cruz et al., 2011).

Analysis of the specificity of skin-infiltrating CD8+ T cells in vitiligo patients has shown that they recognise specific antigens primarily involved in the melanogenic pathway and these include Melan-A/MART, tyrosinase and PMEL (Palermo et al., 2001, van den Boorn et al., 2009, Rodrigues et al., 2017b). These cytotoxic CD8+ T lymphocytes exhibit *in vitro* anti-melanocyte cytotoxic activity and show skin-homing capacity, which appears to associate with vitiligo extension and severity (Palermo et al., 2001, Sandoval-Cruz et al., 2011, Rashighi et al., 2014, Agarwal et al., 2015). Furthermore, van den Boorn et al. (2009) observed that isolated peri-lesional cytotoxic T lymphocytes could infiltrate explants of autologous healthy pigmented skin and eliminate melanocytes. Further evidence for CD8+ T cells in destroying melanocytes in vitiligo patients is suggested by their expression of the inflammatory cytotoxic markers granzyme B, perforin and cutaneous lymphocyte-association antigen in the lesional and peri-lesional areas of vitiligo patient skin (van den Wijngaard et al., 2000, Oyarbide-Valencia et al., 2006, van den Boorn et al., 2009, Bertolotti et al., 2014, Lukinovic-Skudar, 2015, Yang et al., 2017b).

1.3.9.7.6 Cytokines

Cytokines are small peptides that regulate inflammation and the immune responses and thus can cause an inappropriate immune reaction when imbalanced (Feldmann et al., 1998, Zhang and An, 2007, Yang et al., 2015). Several studies have shown cytokine imbalance in the skin of patients with vitiligo, indicating a role for cytokines in autoimmunity (Yang et al., 2015). A decreased level of GM-CSF has been found in vitiligo patients compared of healthy subjects (Moretti et al., 2002b, Moretti et al., 2009). The cytokine GM-CSF is suggested to serve as melanocyte growth factor, and a lower expression of this molecule might diminish the proliferation of surviving melanocytes in vitiligo patients (Imokawa et al., 1996).

Elevated levels of soluble IL-2 receptor have been revealed in the vitiligo patient skin. Soluble IL-2 receptor is expressed on activated T cells and induces T cell proliferation via binding to its ligand, IL-2 (Grutters et al., 2003) and thus its high expression implies involvement of T cell in vitiligo pathogenesis (Yeo et al., 1999, Vaccaro et al., 2015). Additionally, increased production of IL-6 in vitiligo patients has been identified (Yu et al., 1997, Singh et al., 2012b). This cytokine induces ICAM-1 expression on melanocytes (Kirnbauer et al., 1992), which could facilitate leukocyte–melanocyte interactions and cause melanocyte damage (Kirnbauer et al., 1992, Yu et al., 1997, Sandoval-Cruz et al., 2011). Furthermore, Yu et al. (1997) and Miniati et al. (2014) have reported increased levels of IL-8 in vitiligo patients. This cytokine may recruit neutrophils, T lymphocytes, and basophils to the lesional skin of vitiligo patients, so amplifying inflammation (Luger and Schwarz, 1990, Miniati et al., 2014).

It has also been demonstrated that the expression of pro-inflammatory cytokine TNF-α is significantly elevated in lesional and peri-lesional vitiligo patient skin (Moretti et al., 2002b, Moretti et al., 2009, Wu et al., 2013). This cytokine is secreted by the peri-lesional T cells of the CD4+ and CD8+ subsets in vitiliginous skin and it has an inhibitory influence on melanocyte proliferation and can cause apoptosis (Webb et al., 2015). In addition, TNF-α is implicated in cytotoxic T lymphocyte development and it can induce lymphocytic expression of IFN-γ, a cytokine involved in vitiligo development (Martinez-Esparza et al., 1998, Kim et al., 2007, Chatterjee et al., 2014, Webb et al., 2015).

High levels of IFN-γ, the cytokine required for the recruitment of melanocyte-specific autoreactive CD8+ T lymphocytes to the affected skin, are expressed both in serum and skin of vitiligo patients as well as in a vitiligo mouse model (Harris et al., 2012, Rashighi et al., 2014). The IFN-γ-induced cytokines CXCL9, CXCL10, and their receptor C-X-C chemokine receptor type 3 (CXCR3), which is expressed on autoreactive CD8+ T cells, are also increased in the serum and skin of vitiligo patients as well as in a vitiligo mouse model (Rashighi et al., 2014, Wang et al., 2016). More interestingly, knocking out CXCR3 or blocking CXCL10 action prevents and reverses depigmentation in vitiligo (Harris et al., 2012, Rashighi et al., 2014). These results indicate that CXCL9, CXCL10, and their receptor CXCR3 are required for the accumulation of autoreactive T cells in the skin of vitiligo patients and subsequent melanocyte destruction (Rashighi et al., 2014). Taken

together, these data suggest that cytokines are likely to be involved in vitiligo pathogenesis.

1.3.9.8 Humoral immunity in vitiligo

1.3.9.8.1 Antibodies against melanocytes

Besides cell-mediated and cytokine-mediated mechanisms, humoral (antibody-mediated) immunity adds more supportive evidence for the pathological role of autoimmunity in vitiligo (Mohammed et al., 2015, Singh et al., 2016). Antibodies to melanocytes have been found at significantly elevated levels in the sera of vitiligo patients as opposed to healthy controls (Naughton et al., 1983a, Cui et al., 1992, Hann et al., 1996, Rocha et al., 2000, Farrokhi et al., 2005, Laddha et al., 2014a, Zhu et al., 2015). Initially, immunoprecipitation experiments with melanocyte extracts revealed that 12/12 of vitiligo patient had anti-melanocyte antibodies in their sera versus 0/12 of healthy subjects (Naughton et al., 1983b). Another study observed that 24 (83%) of 29 of patients with vitiligo, and 2 (7%) of 28 control subjects were positive for anti-pigment cell antibodies (Cui et al., 1995).

A direct association has been shown between anti-melanocyte antibody levels and the extent and activity of vitiligo (Naughton et al., 1983b, Uda et al., 1984, Cui et al., 1995, Rocha et al., 2000, Farrokhi et al., 2005, Laddha et al., 2014a, Jimenez-Brito et al., 2016). 93% of patients with greater extent of depigmentation (5-10% of skin area involved) and only 50% of patients with minimal vitiligo lesions (<2% of skin area involved) have anti-melanocyte antibodies (Allam and Riad, 2013), suggesting that antibodies against melanocytes can be markers of disease severity. These anti-melanocyte antibodies belong to the Immunoglobulin G (IgG) class, including subclasses IgG1, IgG2, IgG3 and IgG4 (Rahoma et al., 2012, Colucci et al., 2015b, Li et al., 2016).

Immunoprecipitation studies using melanocyte protein extracts have shown that antibodies in vitiligo patients are most frequently directed to antigens with molecular weights of 35, 40 to 45, 75, 90 and 150-kDa, these being found on the cell surface (Cui et al., 1992, Cui et al., 1995, Zhu et al., 2015). Some of these proteins, including those of 40-45, 75, and 150-kDa, appear to be common tissue antigens, whereas the 35 and 90-

kDa proteins are preferentially expressed on melanocytes (Cui et al., 1995). Additionally, other reports have identified vitiligo patient antibody targets of 45, 65, 70, 88, and 110-kDa, which are specifically expressed in melanocytes (Park et al., 1996).

Various melanocyte-associated autoantigens have been reported (Table 1.7). One of interest is tyrosinase and antibodies against this melanocyte-specific protein have been extensively reported in several independent studies of vitiligo patients (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Kemp et al., 1997b, Merimsky et al., 1998, Pradhan et al., 2013, Speeckaert et al., 2015, Furue and Kadono, 2016). Moreover, antibodies against other proteins of the melanogenic pathway such as DCT, TYRP1, PMEL, and GTP-binding protein Rab38 have been detected in vitiligo patients, albeit at a low prevalence (Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Okamoto et al., 1998, Ram and Shoenfeld, 2007, Waterman et al., 2010, Pradhan et al., 2013, Zhu et al., 2015).

1.3.9.8.2 Other antibody specificities

In addition to the association of vitiligo with autoimmune diseases previously discussed in Section 1.2.5, a variety of organ-specific antibodies, mostly against gastric parietal cells, pancreatic islet cells, thyroid and adrenal glands, are commonly identified in vitiligo patients sera (Table 1.8) (Brostoff, 1969, Betterle et al., 1976, Zauli et al., 1986, Mandry et al., 1996, Rodríguez-Martín et al., 2012, Kasumagic-Halilovic et al., 2013, Hamid et al., 2015). Furthermore, anti-nuclear antibodies and IgM-rheumatoid factor (IgM-RF) have been identified at significantly high levels in vitiligo patients (Farrokhi et al., 2005, Rodríguez-Martín et al., 2012, Hamid et al., 2015). Rheumatoid factor is involved in regulating immunoglobulin secretion by controlling the activation of B cells (Ingegnoli et al., 2013), whereas anti-nuclear antibodies are capable of penetrating cells leading to apoptotic cell death (Farrokhi et al., 2005, Kasumagic-Halilovic et al., 2013).

Anti-keratinocyte antibodies that are associated with the activity and extent of disease have been observed in vitiligo patients (Yu et al., 1993, Pradhan et al., 2013, Hamid et al., 2015). Phage-display of peptides expressed by melanocyte cDNAs has identified, amongst others (Table 1.8), the melanin-concentrating hormone receptor 1 (MCHR1) and tyrosine hydroxylase (TH) as antibody targets in vitiligo (Kemp et al., 2002b,

Waterman et al., 2010, Kemp et al., 2011, Rahoma et al., 2012). The MCHR1 is the receptor for melanin concentrating hormone, and the signalling pathway of MCH/MCHR1 is involved in regulating function of melanocyte along with α-MSH and thus antibodies to MCHR1 may interfere with the normal function of this receptor leading to a change in melanocyte behaviour (Hamid et al., 2015). TH plays a critical role in melanogenesis by catalysing L-tyrosine conversion to L-dopa, a precursor molecule of melanin (Hamid et al., 2015). Taken together, there is obviously strong indication that antibodies are significantly implicated in melanocyte destruction in vitiligo.

1.3.9.8.3 Pathogenic mechanisms of antibodies in vitiligo

The ability of vitiligo-associated antibodies to destroy melanocytes has been demonstrated *in vitro* by both complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (Norris et al., 1988, Fishman et al., 1993, Gottumukkala et al., 2006, Ruiz-Arguelles et al., 2007, Ruiz-Argüelles et al., 2013, Speeckaert et al., 2015). *In vivo*, the administration of IgG from vitiligo patients into human skin grafted onto nude mice has been shown to induce melanocyte destruction (Gilhar et al., 1995). In a reconstructed epidermis model, sera from 9/13 (69%) vitiligo patients induced the detachment of melanocytes, although this was not related to disease extent or activity (Cario-Andre et al., 2007). Vitiligo patient antibodies against MCHR1 were demonstrated to block the function of the receptor (Gottumukkala et al., 2006). However, it is not known if or how this activity could affect the melanocyte function. Moreover, IgG anti-melanocyte antibodies are able to induce the expression of HLA-DR and ICAM-1 on melanocytes and the release of IL-8 from melanocytes (Yohn et al., 1993, Sandoval-Cruz et al., 2011). By enhancing the antigen-presenting activity of melanocytes in this way, they become targets for cytotoxic T cells (Yohn et al., 1993, Sandoval-Cruz et al., 2011).

Table 1.7: Melanocyte-specific antibodies in vitiligo patients

Antibody target	Number of vitiligo patients with antibodies	Reference
Tyrosinase	16/26 (61%) 5/46 (11%)	(Song et al., 1994) (Kemp et al., 1997a, Pradhan et al., 2013)
TYRP1	3/53 (5.7%) 2/46 (4%)	(Kemp et al., 1998b) (Pradhan et al., 2013)
DCT	3/53 (5.7%) 20/30 (67%) 2/46 (4%)	(Kemp et al., 1997b) (Okamoto et al., 1998) (Pradhan et al., 2013)
PMEL	3/53 (5.7%)	(Kemp et al., 1998a)
SOX10 transcription factor	3/93 (3.2%)	(Hedstrand et al., 2001)
SOX9 transcription factor	1/93 (1.1%)	(Hedstrand et al., 2001)
GTP-binding protein Rab38	8/53 (15%)	(Waterman et al., 2010)

Table 1.8: Other antibody specificities identified in vitiligo patients

Antibody target	Number of vitiligo patients with antibodies	Reference
Gastric parietal cells	17/80 (21%) 6/20 (30%) 28/196 (14%)	(Brostoff, 1969) (Mandry et al., 1996) (Rodríguez-Martín et al., 2012)
Thyroid peroxidase	10/20 (50%) 30/196 (15%) 12/51 (24%)	(Mandry et al., 1996) (Rodríguez-Martín et al., 2012) (Pradhan et al., 2013)
Thyroid cytoplasm	22/80 (28%)	(Brostoff, 1969)
Thyroglobulin	8/20 (40%) 9/51(18%) 14/197(7%)	(Mandry et al., 1996) (Pradhan et al., 2013) (Rodríguez-Martín et al., 2012)
Adrenal gland	3/80 (4%)	(Brostoff, 1969)
Pancreatic islet cells	7/96 (7%)	(Betterle et al., 1976)
IgM-rheumatoid factor	6/55(11%)	(Farrokhi et al., 2005)
Lamin A	24/84 (29%) 29/50 (58%)	(Li et al., 2011) (Zhu et al., 2015)
Melanin-concentrating hormone receptor 1	9/55 (16.4%) 24/145 (17%) 0/46 (0%)	(Kemp et al., 2002b) (Zhou et al., 2011) (Pradhan et al., 2013)
γ-enolase	4/53 (8%)	(Waterman et al., 2010)
α-enolase	5/53 (9%)	(Waterman et al., 2010)
Heat-shock protein 90	7/53 (13%)	(Waterman et al., 2010)
Heat-shock protein 70	16/60 (26%)	(Kim et al., 2011)
Osteopontin	5/53 (9%)	(Waterman et al., 2010)
Ubiquitin-conjugating enzyme	8/53 (15%)	(Waterman et al., 2010)
Translation-initiation factor 2	3/53 (6%)	(Waterman et al., 2010)

Presently, it has not been determined if melanocytes are a primary or secondary target of the humoral immune response in vitiligo (Tarle et al., 2014). They might arise from a genetic susceptibility to immune dysregulation at the T or B lymphocyte level, leading to lack of tolerance to pigment cell antigens (Kemp et al., 2001c, Ongenae et al., 2003, Rezaei et al., 2007). Alternatively, vitiligo antibodies might originate from an immune response against melanocytes damaged by some other mechanism (Kemp et al., 2001c, Passeron and Ortonne, 2005b, Rezaei et al., 2007). Interestingly, the normally intracellular melanocyte antigens TYRP1 and PMEL can be expressed on the cell surface and so can be accessible by antibodies (Takechi et al., 1996, Leonhardt et al., 2011). In addition, pigment cell antibodies in vitiligo might be secondary to destruction of the melanocyte from another immune cause such as T-cells, but that once triggered the antibodies are themselves destructive to melanocytes.

1.3.10 The convergence theory

The convergence theory of vitiligo aetiology suggests that several interacting factors contribute to the clinical manifestation of vitiligo (Figure 1.8) (Le Poole et al., 1993a, Le Poole and Luiten, 2008, Dwivedi et al., 2015, Ezzedine et al., 2015). The theory states that the elicitation stage of vitiligo can be due to physical trauma to the skin (van Geel et al., 2011b, Dwivedi et al., 2015), emotional stresses (Papadopoulos et al., 1998, Dwivedi et al., 2015) or imbalances in endogenous neural factors, metabolites, cytokines or hormones (Schallreuter et al., 1996, Yu et al., 1997, Alikhan et al., 2011, Dwivedi et al., 2015). Such factors can result in oxidative stress within melanocytes which subsequently respond by expressing HSP70 and chaperoned melanocyte antigens (Kroll et al., 2005, Hariharan et al., 2010, Mosenson et al., 2012, Dwivedi et al., 2015).

In the stage of immune activation, ‘danger signals’ activate dendritic cells which in turn activate anti-melanocyte autoreactive cytotoxic T cells (van den Boorn et al., 2009, Dwivedi et al., 2015). An absence of functional skin-infiltrating Tregs may also contribute to the on-going immune response (Ben Ahmed et al., 2012, Dwivedi et al., 2015). Antibodies against melanocyte-specific proteins such as tyrosinase (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Pradhan et al., 2013, Zhu et al., 2015) are likely to be generated in response to melanocyte damage. However, it is possible that anti-

melanocyte antibodies can damage pigment cells by activating complement or by antibody-dependent cellular cytotoxicity (Norris et al., 1988, Fishman et al., 1993, Gottumukkala et al., 2006, Hamid et al., 2015). Genetic susceptibility can also influence the development of vitiligo, particularly in regard to the immune activation phase (Spritz, 2013, Picardo et al., 2015).

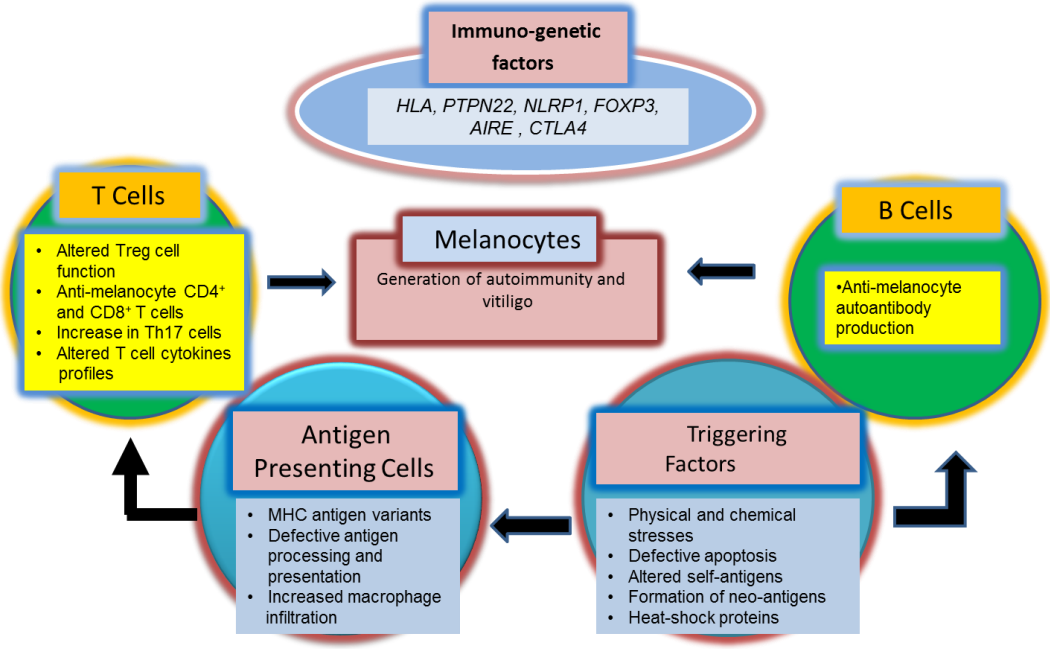


Figure 1.8: A schematic representation of the convergence theory of vitiligo aetiology and pathogenesis.

The convergence theory of vitiligo aetiology suggests that several interacting factors contribute to the clinical manifestation of vitiligo. *HLA*, human leuckocyte antigen; *PTPN22*, protein tyrosine phosphatase non-receptor type 22; *NLRP1*, NACHT leucine-rich-repeat protein 1; *FOXP3*, forkhead box P3; *AIRE*, autoimmune regulator; *CTLA4*, cytotoxic T lymphocyte antigen 4; MHC, major histocompatibility complex; Th17, T helper 17 cells.

1.4 The current project

As discussed earlier in this chapter, there is much evidence to support a role for autoimmune responses in vitiligo pathogenesis (Kemp et al., 2001c, Hamid et al., 2015) and, indeed, antibodies and autoreactive T cells have been reported to be present in vitiligo patients (van den Boorn et al., 2009, Waterman et al., 2010, Hamid et al., 2015, Zhu et al., 2015). As with other autoimmune diseases, identifying and characterising antibodies in vitiligo in terms of their target autoantigen would help in **(a)** developing tests for vitiligo diagnosis and treatment monitoring, and in **(b)** uncovering the pathogenic mechanisms involved in the disease, as antibodies may act as markers of destructive T cell responses against melanocytes. Such knowledge would help define distinct immunological characteristics of the clinical subsets and would help in understanding the aetiological mechanisms of the disease, which is essential for the development of better therapies.

The aims of the current project were:

1. To describe the vitiligo patient group included in this study in relation to their demographic, clinical, and antibody profiles.
2. To identify novel targets of antibodies in vitiligo patients by using phage-display technology (Waterman *et al*., 2010).
3. To confirm the immunoreactivity of individual vitiligo patient sera to any novel autoantigens identified.
4. To determine if antibodies to any novel autoantigens were related to any clinical or demographic features of vitiligo.

Chapter 2

General Materials and Methods

2 General Materials and Methods

2.1 Participant vitiligo patients and healthy controls

Blood samples from 246 patients with vitiligo were obtained from dermatology (Professor David J. Gawkrodger) and endocrinology (Professor Anthony P. Weetman) clinics at the Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK. Specific details of the patients used in this study are given in the relevant chapters. In addition, control blood samples were also collected from 100 healthy volunteers (42 male; 58 female; mean age 34 years; age range 21-59 years) who had no current autoimmune disease, no personal history of autoimmune disease, and no family history of vitiligo or of autoimmune disease. Serum was separated from whole blood samples (10-20 ml) by centrifugation at 3,000 revolutions per minute (rpm) for 10 min in a Sorval® RT6000-D centrifuge. Sera were kept frozen at -80°C until use. The research was approved by the National Research Ethics Service – Sheffield Research Ethics Committee (REC Reference Number 09/H1308/91), and informed consent was gained from all participants. The project STH15257 was under the research governance of the Clinical Research Office, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK.

2.2 Chemicals and reagents

The majority of chemicals and reagents including buffers, solvents, acids, and media constituents were obtained from either Sigma-Aldrich (Poole, UK), Melford Laboratories (Ipswich, UK) or Fisher Scientific UK Ltd. (Loughborough, UK). The source of some chemicals and reagents is indicated in the text where appropriate.

2.3 Plasticware

Plasticware including petri-dishes, 0.5-ml and 1.5-ml Eppendorf tubes, 30-ml universal container tubes, Gilson pipette tips, pipettes, and 50-ml container tubes were from either Sarstedt Ltd. (Numbrecht, Germany), Starlab (UK) Ltd. (Milton Keynes, UK), Corning Incorporated (Corning, NY, USA), Nalgene Nunc International (Rochester, NY, USA), Fisher Scientific UK Ltd. or Bibby Sterilin Ltd. (Bargoed, UK).

2.4 Antibiotics

Antibiotics were prepared as 1000x concentrated stocks. Ampicillin (sodium salt) and kanamycin sulphate was made in deionised water and tetracycline (hydrochloride) in 50% (v/v) ethanol. Antibiotics were sterilised by filtration with a 0.22-micron Millex® Filter Unit (Millipore Corp., Bedford, MA, USA) and stored at -20°C. When required, culture medium and agar plates were supplemented with ampicillin at 100 µg/ml, kanamycin at 10 µg/ml and tetracycline at 10 µg/ml.

2.5 Luria Bertani medium and agar

Luria Bertani (LB) medium was prepared in deionised water and consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride. To sterilise, LB medium was autoclaved at 121°C for 15 min. LB agar was made by adding 2% (w/v) agar to LB medium. Following autoclaving, the agar was left to cool to 40-45°C before the addition of appropriate antibiotics. Subsequently, the agar was poured into 90-mm petri-dishes and allowed to set. Plates were then stored at 4°C until use.

2.6 Bacterial strains

The bacterial strains utilised in this study are derivatives of *Escherichia coli* (*E. coli*) K-12 and are listed in Table 2.1. Bacterial strains harbouring plasmid vectors or recombinant plasmids were constructed by transformation (Section 2.16).

2.7 Growth and storage of bacterial strains

All strains of *E. coli* were grown routinely from frozen stock by streaking onto LB agar plates with the appropriate selective antibiotic(s) (Section 2.4), and incubated at 37°C overnight. A single bacterial colony of the desired *E. coli* strain was then inoculated into LB medium containing the appropriate antibiotics and placed in a rotary incubator shaking at 250 rpm at 37°C overnight. Additionally, 0.7-ml aliquots of an overnight-grown bacterial culture were mixed with 0.3-ml aliquots of 50% (v/v) sterile glycerol, for long-term storage at -80°C.

Table 2.1: Bacterial strains

Strain[1]	Details	Supplier
JM109	A bacterial strain used in the preparation of plasmid vectors and recombinant plasmids.	Promega (Southampton, UK)
JM109 pcDNA3-LaminA	Carries recombinant plasmid pcDNA3-LaminA (Table 2.2).	This study (Chapter 7)
XL1-Blue MRF'	A bacterial strain grown to be infected by phage particles and employed in the phage-display technology	Agilent Technologies, Wokingham, UK (Chapter 4)

[1]All bacterial strains are derivatives of *Escherichia coli* K-12.

2.8 Plasmids

The plasmids used in this study are given in Table 2.2. They were stored at –20°C in sterile TE buffer (10 mM Tris-hydrochloride; 1 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0) (Promega, Southampton, UK). Diagrams of maps of plasmid expression vectors employed in the experiments of DNA cloning are shown in the relevant chapters.

2.9 Small-scale plasmid preparations

The Wizard® Plus SV Minipreps DNA Purification System (Promega) was used to purify plasmid DNA from a 5-ml or 10-ml overnight culture of the desired bacterial strain, according to the manufacturer's protocol. In brief, a single colony of the required bacterial strain was isolated by streaking out 20 µl of frozen bacterial stock on to LB agar containing the appropriate antibiotic(s). A single colony from the selective plate was subsequently inoculated into 10 ml of LB medium containing the relevant antibiotic(s) and placed in a rotary incubator shaking at 250 rpm at 37°C overnight.

A bacterial cell pellets was obtained by centrifugation at 10,000 *g* for 10 min. The pellet was re-suspended in 250 µl of Cell Resuspension Solution (50 mM Tris-hydrochloride, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A) and the cells were then lysed by adding 250 µl of Cell Lysis Solution (0.2 M sodium hydroxide; 1% (w/v) sodium dodecyl sulphate (SDS)). The cell lysate was subsequently neutralised by adding 350 µl of Neutralisation Solution (0.759 M potassium acetate; 4.09 M guanidine hydrochloride; 2.12 M glacial acetic acid; pH 4.2) and mixed gently before centrifugation at 10,000 *g* for 10 min at room temperature. The cleared cell lysate was loaded on to a Wizard® SV Minicolumn which was then centrifuged at 10,000 *g* for 1 min at room temperature. The Minicolumn was washed with 750 µl of Column Wash Solution (60% (v/v) ethanol; 60 mM potassium acetate; 8.3 mM Tris-hydrochloride; 0.04 mM EDTA) and subsequently with 250 µl of Column Wash Solution, followed by centrifugation at 10,000 *g* for 2 min at room temperature.

Table 2.2: Plasmids

Plasmid	Details	Source
pcDNA3	A 5.4-kb plasmid vector with a selectable resistance marker for ampicillin. Comprises promoters for T7 and SP6 polymerases flanking a diverse multiple cloning site (Figure 7.1).	Life Technologies (Paisley, UK)
pDEST15-LaminA	pDEST15 plasmid vector (7.0-kb) carrying LaminA cDNA (2.0-kb) (Figure 7.2). Contains a selectable resistance marker for ampicillin.	imaGene GmbH (Berlin, Germany)
pcDNA3-LaminA	pcDNA3 plasmid vector carrying LaminA cDNA (2.0-kb) cloned between the *Kpn*I and *Xba*I restriction sites (Figure 7.5).	This study (Chapter 7)
pcDNA3-TYR	pcDNA3 vector containing tyrosinase cDNA cloned into the *Kpn*I and *Xba*I restriction sites.	Dr Helen Kemp
pcMCHR1	pcDNA3 vector containing melanin-concentrating hormone receptor-1 cDNA cloned into the *EcoR*I and *Xba*I restriction sites.	Dr Helen Kemp
pcDNA3-TH	pcDNA3 vector containing tyrosine hydroxylase cDNA cloned into the *Hin*dIII and *Bam*HI restriction sites.	Dr Helen Kemp
pCMVDTS	pCMVDTS vector containing L-dopachrome tautomerase cDNA.	Prof. Shigeki Shibahara (Tohoku University School of Medicine, Sendai, Japan)
pcDNA3.1-GPNMB	pcDNA3.1 vector containing glycoprotein non-metastatic melanoma protein b (GPNMB) cDNA cloned into the *Kpn*I and *Hin*dIII restriction sites.	GeneScript (Piscataway, NJ, USA)
pcDNA3.1-OCA2	pcDNA3.1 vector containing OCA2-encoded P protein (OCA2 protein) cDNA cloned into the *Kpn*I and *Hin*dIII restriction sites.	GeneScript (Piscataway, NJ, USA)
pJuFo	A 4.28 bp phage-display vector containing the leucine zippers Jun and Fos, and the viral coat protein, pill, of the filamentous phage.	R. Crameri (Swiss Institute of Allergy 1993 and Asthma Research, Davos, Switzerland)

In order to recover the plasmid DNA, 100 µl of nuclease-free water were added to the column which was then centrifuged at 10,000 *g* for 1 min at room temperature. The concentration of the plasmid DNA was determined by spectrophotometry at 260 nm using a NanoDrop ND-1000 Spectrometer (Labtech, Wilmington, DE, USA) and NanoDrop Software (Labtech). Plasmids were stored at -20°C until required. Plasmid DNA was checked qualitatively using agarose gel electrophoresis (Section 2.11).

2.10 Large-scale plasmid preparations

A large-scale culture of the bacterial strain harbouring the required plasmid was prepared by inoculating of 0.5-1 litre of LB medium containing the relevant antibiotic(s), with a 10-ml starter culture and grown overnight, with shaking at 37°C. The culture was subsequently centrifuged at 4,000 g for 30 min in a 50-ml container tube and plasmid extracted from the cell pellet with the use of a Qiagen Plasmid DNA Maxiprep Kit (Qiagen Ltd., Crawley, UK) according to the kit instructions. The bacterial cell pellet was first re-suspended in 10 ml of P1 Buffer (50 mM Tris-hydrochloride, pH 8.0; 10 mM EDTA; 100 µg/ml RNaseA). Subsequently, 10 ml of P2 Buffer (0.2 M sodium hydroxide; 1% (w/v) SDS) was added to the re-suspended cells and mixed by gentle inversion before incubation at room temperature for 5 min. A volume of 10ml of P3 Buffer (1.32 M potassium acetate, pH 5.5) was added to the lysed cells and incubated on ice for 20 min. The cell lysate was then centrifuged at 20,000 g for 30 min at 4°C and the supernatant collected.

A Qiagen column was equilibrated by the addition of 10 ml of QBT Buffer (750 mM sodium chloride; 50 mM 3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.0; 15% (v/v) isopropanol; 0.15% (v/v) Triton X-100) and was left to empty by gravity flow. The supernatant was loaded onto the equilibrated column and allowed to flow through. The column was then washed twice with 30 ml of QC Buffer (1 M sodium chloride; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). Plasmid DNA was subsequently eluted with 15 ml of QF Buffer (1.25 M sodium chloride; 50 mM Tris-hydrochloride, pH 8.5; 15% (v/v) isopropanol) into a clean tube and then precipitated by adding 10.5 ml of 100% isopropanol and centrifuged at 15,000 g for 30 min at 4°C. The resulting plasmid DNA pellet was washed with 1 ml 70% (v/v) ethanol, and finally re-suspended in 500 µl of TE

buffer. DNA concentration was ascertained using spectrophotometry (Section 2.9). Plasmid DNA was checked qualitatively using agarose gel electrophoresis (Section 2.11).

2.11 Agarose gel electrophoresis

For DNA analysis, agarose gels, 0.8-1% (w/v), were made by boiling molecular biology grade agarose (Sigma-Aldrich) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate; 1 mM EDTA; pH 8.3) (Promega) for 1-2 min in a microwave oven. After it had cooled, 1-2 µl of ethidium bromide solution (10 mg/ml) (Promega) were added for every 50 ml of gel. The molten agarose was then poured into the casting deck of a Sub-Cell® Horizontal Electrophoresis System (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). After it had set, the combs were removed from the gel which was subsequently placed into the electrophoresis tank. The electrophoresis tank was then filled with TAE buffer.

In order to visualise the DNA samples running in agarose gels, Blue/Orange Loading Dye 6x (0.4% (w/v) orange G; 0.03% (w/v) bromphenol blue; 0.03% (w/v) xylene cyanol FF; 15% (w/v) Ficoll® 400; 10 mM Tris-hydrochloride, pH 7.5; 50 mM EDTA, pH 8.0) (Promega) was added to the sample of DNA at 1/6th of the volume, and this was then loaded into a gel slot. In order for DNA products to be sized after they had migrated through the gel, a `marker' lane was always included which contained a 0.5-1.0-µg sample of 1-kb DNA Ladder (500-10,000-bp DNA fragments) (New England Biolabs®, Hitchin, UK). The gels were run in a Sub-Cell® Horizontal Electrophoresis System (Bio-Rad Laboratories Ltd.) at 85 volts using a PowerPac Basic Power Supply (Bio-Rad Laboratories Ltd.), and then viewed and recorded using a GBOX Gel Documentation System (Syngene, Cambridge, UK) and GeneSnap Image Acquisition Software (Syngene).

2.12 Restriction enzyme digests

All restriction enzymes (Table 2.3) and restriction enzyme reaction buffers (Table 2.3) were supplied by Promega. Restriction enzyme digests of plasmids and polymerase chain reaction (PCR) amplification products were performed in 0.5-ml Eppendorf tubes in a volume not normally exceeding 25 µl and contained up to 1 µg of DNA, 10 units of enzyme(s) and 0.1 volumes of the appropriate 10x restriction enzyme buffer. Each

reaction proceeded for 90 min at 37°C, unless otherwise stated. Restriction digests were then analysed by agarose gel electrophoresis (Section 2.11).

2.13 Polymerase chain reaction amplification

Preceding the PCR amplification reaction, forward and reverse oligonucleotide primers were appropriately designed to amplify the desired DNA fragment. Primers were supplied by Eurofins Genetic Services Ltd. (London, UK) and were stored at -40°C in sterile, nuclease-free water at a concentration of 100 pmol/µl. The PCR primers used in this study are listed in Table 2.4, and the exact primers used for PCR amplification of a particular DNA are indicated in the text at the relevant point.

Reactions were carried out in 0.5-ml Eppendorf tubes in 50-µl volumes consisting of, unless indicated, 50 ng of template DNA, 0.01-1 µM of each required (forward and reverse) primer, 1.25 units of GoTaq® Flexi DNA polymerase (Promega), 0.2 mM deoxynucleotides (dATP, dGTP, dCTP and dTTP) (Promega), 1.5 mM magnesium chloride (Promega) and 0.2 volumes of 5x GoTaq® Flexi Buffer (50 mM Tris-hydrochloride, pH 8.5; 0.05% (w/v) gelatin; 250 mM potassium chloride; 0.5% (v/v) Tween 20; and 0.5% (v/v) Nonidet P-40) (Promega). Reactions without template DNA were included as controls. Each reaction was then subjected to PCR amplification in a Techne TC-312 DNA Thermal Cycler (Bibby Scientific Ltd., Stone, UK). The thermocycling conditions were denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min for 35 cycles, unless stated otherwise. The reactions were completed by a final extension at 72°C for 10 min, followed by storage at 4°C. Products of amplification were routinely analysed by agarose gel electrophoresis (Section 2.11).

Table 2.3: Restriction enzymes and buffers

Enzyme[1]	Buffer (10x concentration)[1]	Restriction site
*Kpn*I	Multi-Core™ Buffer (25 mM Tris-acetate, pH 7.5; 100 mM potassium acetate; 10 mM magnesium acetate; 1mM dithiothreitol)	5'-GGTACC-3'
*Xba*I	Multi-Core™ Buffer (25 mM Tris-acetate, pH 7.5; 100 mM potassium acetate; 10 mM magnesium acetate; 1 mM dithiothreitol)	5'-TCTAGA-3'

[1]All restriction enzymes and buffers were purchased from Promega.

Table 2.4: Oligonucleotide PCR amplification primers

Primer	Sequence[1]	Source
LaminA-F	5'-ttggtaccatggagaccccgtcc-3'	Eurofins Genetic Services Ltd. (London, UK)
LaminA-R	5'-aatctagacgcctatcacatgatgctgcagtt-3'	Eurofins Genetic Services Ltd.

[1]The *Kpn*I restriction site is in red in the forward (F) primer. The *Xba*I restriction site is in red in the reverse (R) primer. The ATG translation start codon in the forward primer and the translation stop codons in the reverse primer are in blue.

2.14 Extraction and purification of DNA fragments from agarose gels

DNA produced either from a PCR amplification reaction or a restriction enzyme digest was purified for further use using a Wizard® PCR Preps DNA Purification System (Promega). DNA fragments were initially separated by agarose gel electrophoresis (Section 2.11). The area of the agarose gel containing the relevant band of DNA was then visualised using a Chromato-vue TM-20 transilluminator (UVP, San Gabriel, CA, USA), and then excised using a clean scalpel. Subsequently, the agarose gel slice was placed in a 1.5-ml Eppendorf tube and dissolved by adding 1 ml of Wizard® PCR Preps DNA Purification Resin. The resulting mixture was applied via a 2-ml syringe to a Wizard® Minicolumn, followed by 2 ml of 80% (v/v) isopropanol. The Wizard® Minicolumn was centrifuged at 10,000 *g* for 2 min, to eliminate excess isopropanol, prior to the elution of DNA with 30 µl of sterile TE buffer. The DNA was kept at -20°C until use. Purified DNA fragments were analysed by agarose gel electrophoresis and the concentration of the DNA measured by spectrophotometry (Section 2.9).

2.15 DNA ligations

Ligation of vector and DNA fragments was carried out using T4 DNA ligase (Promega) in a reaction volume of 10-20 µl. Reactions usually contained 20-100 ng of plasmid vector. The amount of insert DNA was calculated as: ng of vector x size of insert (kb)/size of vector (kb) x molar ratio of insert to vector. Vector and insert DNA were usually at a molar ratio of between 1 and 3. The vector and insert DNAs were combined in a 0.5-ml Eppendorf tube, and the required amount of nuclease-free water added prior to the addition of 1-2 units of T4 DNA ligase (Promega) and 0.1 volumes of 10x DNA ligase buffer (300 mM Tris-hydrochloride, pH 7.8; 100 mM magnesium chloride; 100 mM dithiothreitol; 10 mM adenosine triphosphate) (Promega). The reaction was then incubated at 15°C overnight, prior to using to transform bacterial cells (Section 2.16).

2.16 Bacterial transformation

When required, a 50-µl aliquot of chemically competent *E. coli* JM109 (Promega) cells was thawed from storage at -80°C. The desired DNA sample (plasmid DNA or DNA in a

ligation reaction) was gently mixed with the cells and then incubated on ice for 5-10 min. The cells were subsequently heat-shocked at exactly 42°C for 45 sec and returned to ice for 2 min. The cells were then transferred to a sterile plastic universal containing 950 µl of chilled SOC medium (2% (w/v) tryptone; 0.5% (w/v) yeast extract; 10 mM sodium chloride; 2.5 mM potassium chloride; 10 mM magnesium chloride; 10 mM magnesium sulphate; 20 mM glucose) (Life Technologies). The culture was incubated in a rotary shaking at 150 rpm for 1 h to enable expression of the antibiotic resistance genes harboured by the transforming plasmid DNA. A 100-µl aliquot of undiluted transformed cells, and of 1:10 and 1:100 dilutions, were then streaked out on agar plates containing the relevant antibiotic(s), and these plates were subsequently incubated at 37°C overnight. As a control in each transformation experiment, an aliquot of untransformed cells was also spread onto an agar plate. Individual colonies, which had selectively grown from successful transformation, were then purified by streaking onto fresh LB agar plates containing selective antibiotic(s) for growth overnight at 37°C.

2.17 DNA sequencing

Automated DNA sequencing was performed by the Genetics Core Facility at the Medical School, University of Sheffield, Sheffield, UK. DNA templates (plasmids or products of PCR amplification) and oligonucleotide sequencing primers were provided to the service at 50-100 ng/µl and 1 pmol/µl, respectively. Sequencing primers are listed in Table 2.5. Sequencing reactions were carried out using a BigDye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 Capillary Sequencer (Applied Biosystems).

2.18 DNA and protein analyses

Analyses of DNA and protein sequences were performed using Lasergene® Core Suite version 11.0 (DNASTAR, Inc., Madison, WI, USA), and the network facilities of the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (http://www.ebi.ac.uk/) (Cambridge, UK) and the ExPASy Bioformatics Resources Portal (http://web.expasy.org) (SIB Swiss Institute of Bioinformatics, Switzerland). Homology

searches of DNA and proteins against the DNA and protein sequences found in the database of GenBank were carried out by employing the BLAST service of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Bethesda, MD, USA).

Table 2.5: Oligonucleotide sequencing primers

Primer	Sequence	Source
GPNMB-1-Forward	5’-TCCTGACCAGTGACTCACCA-3’	Eurofins Genetic Services Ltd. (London, UK)
GPNMB-2-Forward	5’-TGTCTACAGAAGACATGGA-3’	Eurofins Genetic Services Ltd. (London, UK)
GPNMB-3-Forward	5’-TCAATGGAACCTTCA-3’	Eurofins Genetic Services Ltd. (London, UK)
GPNMB-4-Forward	5’-TTAGAGGTTAACATCATCCA-3’	Eurofins Genetic Services Ltd. (London, UK)
GPNMB-5-Forward	5’-TATTTGTCACTGTGAT-3’	Eurofins Genetic Services Ltd. (London, UK)
OCA2-1-Forward	5’-TGACTGGGAGCGAAGATAC-3’	Eurofins Genetic Services Ltd. (London, UK)
OCA2-2-Forward	5’-ACGCTTTGGGCTCCAGGT-3’	Eurofins Genetic Services Ltd. (London, UK)
OCA2-3-Forward	5’-TAAAGGCATACCGGCTCT-3’	Eurofins Genetic Services Ltd. (London, UK)
OCA2-4-Forward	5’-TCAGCCCGGCCAGCCGCGA-3’	Eurofins Genetic Services Ltd. (London, UK)
OCA2-5-Forward	5’-TAAAGATGGTCCCAGAGGA-3’	Eurofins Genetic Services Ltd. (London, UK)
T7	5’-TAATACGACTCACTATAGGG-3’	Promega (Southampton, UK)
SP6	5’-ATTAACCCTCACTAAAGGGA-3’	Promega (Southampton, UK)
Lamin A-555-Forward	5’-TGAGATGCTGCGGCG-3’	Eurofins Genetic Services Ltd. (London, UK)
Lamin A-700-Revesrse	5’-GCTTCCCATTGTCAA-3’	Eurofins Genetic Services Ltd. (London, UK)
Lamin A-1751-Forward	5’-GCACCGTGCTGTGCG-3’	Eurofins Genetic Services Ltd. (London, UK)
Lamin A-1600-Forward	5’-AGCCGTACGCAGGCT-3’	Eurofins Genetic Services Ltd. (London, UK)
JUFO-1192	5'-CCGCTGGATTGTTATTACTCGCTG-3'	Eurofins Genetic Services Ltd. (London, UK)
JUFO-1500	5’-TGCAAGGCGATTAAGTTGGGTAAC-3'	Eurofins Genetic Services Ltd. (London, UK)

2.19 *In vitro* coupled transcription-translation of cDNA

A TnT® T7-Coupled Reticulocyte Lysate System (Promega, Southampton, UK) was used to produce the protein of interest labelled with [^{35}S]-methionine according to the manufacturer's instructions. Reactions were carried out in 0.5-ml tubes in 50-µl volumes comprising 0.5 µg of plasmid DNA carrying the appropriate cDNA, 0.5 volumes of TnT® Rabbit Reticulocyte Lysate, 10 units of TnT® T7 RNA Polymerase, 0.02 mM amino acid mixture minus methionine, 0.04 volumes of 25x TnT® Reaction Buffer, 40 units of RNasin® Ribonuclease Inhibitor (Promega) and 0.04 volumes of 10 mCi/ml translation-grade [^{35}S]-methionine (1,000 Ci/mmol) (Perkin-Elmer LAS UK Ltd., Beaconsfield, UK). Reactions were incubated for 90 min at 30°C and then stored at -40°C until required. For qualitative analysis, radiolabelled protein products were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Section 2.20).

The percentage incorporation of [^{35}S]-methionine into the translated protein was determined by trichloroacetic acid-precipitation, as detailed by the manufacturer (Promega). In brief, 2 µl of the *in vitro* translation reaction were added to 98 µl of 1 M sodium hydroxide/2% (v/v) hydrogen peroxide and incubated at 37°C for 10 min. Subsequently, 900 µl of ice-cold 25% (w/v) trichloroacetic acid/2% (w/v) casamino acids (Difco, Detroit, MI, USA) were added and the reaction incubated on ice for 30 min. To collect the precipitated translation products, 250 µl of the trichloroacetic acid reaction mixture were vacuum filtered onto a Whatman GF/A glass fibre filter (Whatman International Ltd., Maidstone, UK) pre-wetted with cold 5% (w/v) trichloroacetic acid. The filter was rinsed three times with 1 ml of ice-cold 5% (w/v) trichloroacetic acid, once with 1 ml of acetone and then allowed to dry at room temperature before immersing in 3 ml of Ultima-Gold® XR scintillation fluid (Packard Bioscience, Groningen, The Netherlands) and counting in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA, USA). To determine total counts per min (cpm) present in the reaction, a 5-µl aliquot of the trichloroacetic acid reaction mixture was spotted directly on to a filter. This was dried for 10 min before counting as above. The percent incorporation of [^{35}S]-methionine was determined as: 100 x (cpm of washed filter/cpm of unwashed filter x 50), and this ranged from 15-20%.

2.20 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and autoradiography

The protocol used was that of Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). SDS-PAGE of proteins was carried out in 10% (w/v) polyacrylamide gels. Resolving gels consisted of 10% (v/v) ProtoGel (37.5:1 acrylamide:bisacrylamide) (GeneFlow, Lichfield, UK), 375 mM Tris-hydrochloride (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.1% (v/v) N, N, N, N'-tetramethylethylenediamine (TEMED). Stacking gels contained 4% (v/v) ProtoGel, 125 mM Tris-hydrochloride (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.1% (v/v) TEMED.

The gels were generated using a Bio-Rad Mini-Protean Tetra Cell apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). In brief, glass plates were assembled using the dedicated equipment as per the manufacturer's instructions. The resolving gel solution was poured into the space between the plates and overlaid with 1 ml of butan-1-ol. After the gel had polymerized, the butanol-1-ol was decanted and a gel comb was inserted prior to the solution of the stacking gel was poured in on top. The gel comb was subsequently removed and a full apparatus holding the glass plates with the gel in between was assembled into the gel tank. Running Buffer (25 mM Tris-base; 0.1% (w/v) SDS; 192 mM glycine; pH 8.3) (GeneFlow) was poured into the tank until the bottom of the plates was covered and the top buffer reservoir was full.

Before loading, 5-µl samples of *in vitro* [^{35}S]-labelled proteins were individually mixed with 20 µl of 2x Laemmli sample buffer (2% (w/v) SDS; 25% (v/v) glycerol; 0.01% (w/v) bromophenol blue; 2% (v/v) 2-mercaptoethanol; 62.5 mM Tris-hydrochloride, pH 6.8) (Bio-Rad Laboratories Ltd.) and heated at 100°C for 5 min. Approximately 10 µl of each sample was then loaded into a well. Protein molecular weight markers, either Prestained SDS-PAGE Standards, Low Range (21-103 kDa) (Bio-Rad Laboratories Ltd.) or Precision Plus Protein All Blue Standards (10-250 kDa) (Bio-Rad Laboratories Ltd.) were also run on each gel. Gels were run initially at 44 volts for 20 min up to the resolving gel, and then at 111 volts for 90 min. The apparatus was then disassembled and the gel transferred to a plastic tray and soaked in fixing solution containing 50% (v/v) methanol

and 10% (v/v) glacial acetic acid for 30 min at room temperature with slow shaking on a rocking platform.

Subsequently, the gel was soaked in Amersham Amplify™ Fluorographic Reagent (GE Healthcare Life Sciences, Little Chalfont, UK), for 30 min without shaking. This was followed by soaking the gel in 7% (v/v) methanol/1% (v/v) glycerol for 5 min prior to being dried for 2 h at 60°C on to 3MM Whatman paper (Whatman International Ltd., Maidstone, UK) in a Bio-Rad Gel Dryer 583 (Bio-Rad Laboratories Ltd.). Dried gels were subjected to autoradiography by exposure to Fuji RX x-ray film (Genetic Research Instrumentation Ltd., Dunmow, UK) in a Hypercassette™ (GE Healthcare Life Sciences) at room temperature for 24 h. The film was then developed using Photosol CD18 x-ray developer (Photosol Ltd., Basildon, UK) for 3 min, rinsed with water and subsequently fixed for 3 min in Photosol CF40 fixer (Photosol Ltd.).

2.21 Radioligand binding assays

Antibody-binding to [^{35}S]-labelled antigens was detected in radioligand binding assays (RLBAs) (Figure 2.1). For each RLBA reaction, a 1-2-µl aliquot of the desired *in vitro* coupled transcription-translation reaction was suspended in 50 µl of immunoprecipitation buffer (20 mM Tris-hydrochloride, pH 8.0; 150 mM sodium chloride; 1% (v/v) Triton X-100; 10 mg/ml protease inhibitor aprotinin) in a 1.5-ml Eppendorf tube. Serum (e.g., patient serum, healthy control serum or animal antiserum) was then added to the required final dilution. Following incubation overnight with shaking in a cold room at 4°C, 50 µl of protein G Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences), prepared according to the manufacturer, were added and incubated for a further 90 min at 4°C. The protein G Sepharose™-antibody-antigen complexes were subsequently collected by centrifugation at 5000 rpm for 30 sec and washed six times for 15 min in immunoprecipitation buffer. In each wash, the reaction tube was incubated with shaking for 15-20 min at 4°C. Following each wash, the tubes were spun for 30 sec at 5000 rpm and the supernatant was removed. Fifty microlitres of immunoprecipitation buffer was then added to the reaction tube and mixed gently. The protein G Sepharose™-antibody-antigen complexes were then transferred to a scintillation vial containing 1 ml of scintillation fluid (Molecular Devices, Sunnyvale, CA, USA).

Immunoprecipitated radioactivity was measured in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA, USA). Samples were always tested in duplicate in each experiment and the mean of the cpm was calculated from the two samples.

The binding activity of antibodies to radiolabelled antigen was expressed as an antibody index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the group of healthy control sera. Each serum was tested in at least three experiments and the mean antibody index was calculated from these values. The upper limit of normal for RLBA was calculated using the mean antibody index + 3SD of the population of healthy control subjects. Any patient serum with an antibody index higher than the upper limit of normal was regarded as positive for antibody reactivity.

To determine the titres of antibodies, serum samples from vitiligo patients were analysed in RLBAs at a series of dilutions ranging from 1:100 up to 1:10000. For each dilution of each serum sample, an antibody index was determined as explained above.

SDS-PAGE and autoradiography were performed to qualitatively analyse the immunoprecipitated proteins. The protein G Sepharose-antibody complexes were firstly resuspended in 100 µl of 2x Laemmli sample buffer. Subsequently, they were boiled for 5 min at 100°C. Following centrifugation, the recovered supernatants were subjected to SDS-PAGE and autoradiography as explained in Section 2.20.

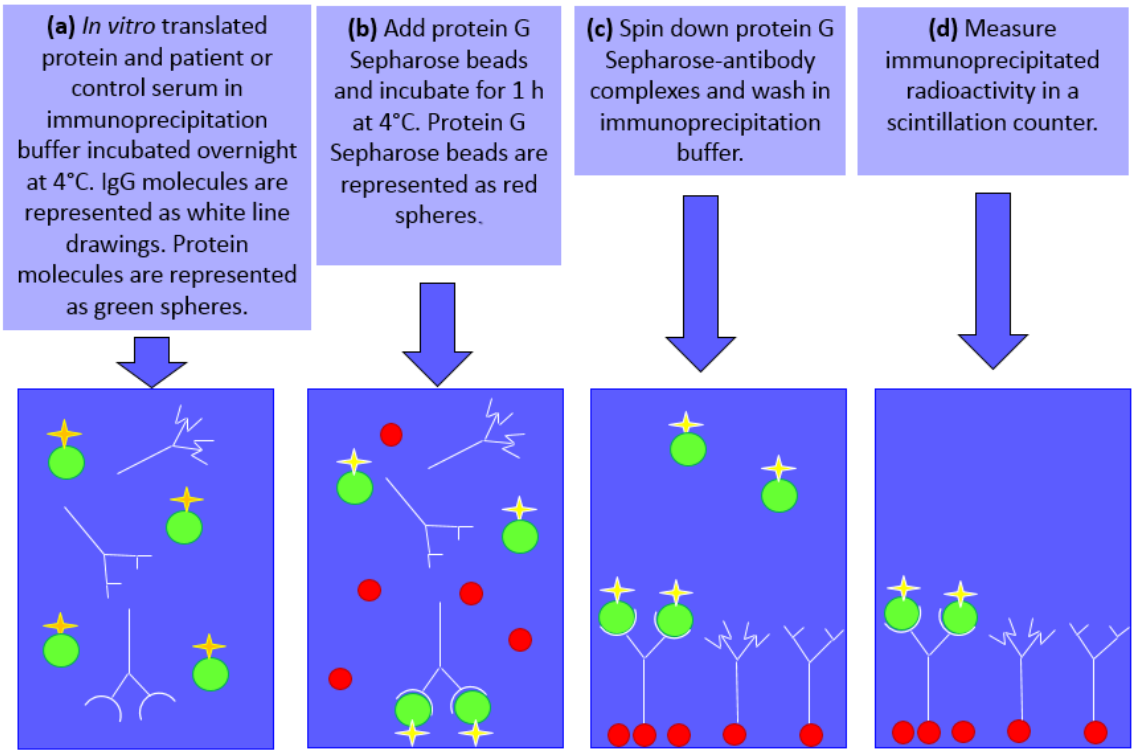


Figure 2.1: Schematic diagram of the radioligand binding assay.

2.22 Antibody absorption experiments using cell extracts

The protocol used was that of Dr Helen Kemp. Cell extracts were a gift from Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). These were made from untransfected human embryonic kidney 293 (HEK293) cells as well as HEK293 cells harbouring the expressed protein (antigen) of interest. To confirm that heterologous proteins were expressed in HEK293 cells, immunoblotting assays with antigen-specific animal antibody (Table 2.6) were carried out. Cell extract samples were kept in extract buffer (150 mM sodium chloride; 10 mM Tris-hydrochloride, pH 7.4; 1% (v/v) Triton X-100; Protease Cocktail Inhibitor) (Sigma-Aldrich) at a concentration of 1 mg/ml total protein. Extract samples were kept at -80°C until use.

In order to assess antibody specificity, absorption experiments were performed. Briefly, samples of sera (100 µl) were incubated with cell extract samples (400 µl) as prepared above. In addition, serum samples without adding any cell extract were incubated in cell extract buffer. Following incubation at 4°C for 16 h, absorbed serum (supernatant) was recovered by centrifugation at 45,000 *g* for 1 h at 4°C. Serum samples of unabsorbed and pre-absorbed were analysed in RLBAs as described earlier (Section 2.21), in order to assess their immunoreactivity against the specific antigen.

2.23 Antibodies

All antibodies employed in this present work were kept as per the manufacturer's instruction at either 4°C or -20°C. These are shown in Table 2.6.

2.24 Statistical analyses

Statistical analyses performed in this study were: Fisher's exact tests for 2 x 2 contingency tables or Chi-square tests to compare categorical data; unpaired t tests to compare two unpaired groups of continuous data; and one-way ANOVA for comparisons of multiple continuous data sets. The tests were carried out as appropriate using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). In all tests, *P* values < 0.05 were regarded as significant.

Table 2.6: Antibodies

Antibody	Details	Source
Polyclonal rabbit anti-GPNMB antiserum sc271435	Raised against a synthetic peptide corresponding to the C-terminal of GPNMB.	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
Polyclonal rabbit anti-OCA2 protein antiserum PA5-23708	Raised against a synthetic peptide corresponding to the C-terminal of OCA2 protein.	Thermo Fisher Scientific, Waltham, MA, USA
Polyclonal rabbit anti-LaminA antiserum ab26300	Raised against a synthetic peptide corresponding to the C-terminal of LaminA.	Abcam, Inc. (Cambridge, MA, USA)
Polyclonal rabbit anti-tyrosinase antiserum α-PEP7	Raised against a synthetic peptide corresponding to the C-terminal of tyrosinase.	Professor Vincent Hearing (National Institutes of Health, Bethesda, MD, USA)
Polyclonal rabbit anti-DCT antiserum α-PEP8	Raised against a synthetic peptide corresponding to the C-terminal of DCT.	Professor Vincent Hearing (National Institutes of Health, Bethesda, MD, USA)
Polyclonal rabbit anti-MCHR1 antiserum MCHR11-S	Raised against a 16-amino acid peptide close to the C-terminal of MCHR1.	Alpha Diagnostics International, Inc. (San Antonio, TX, USA)
Polyclonal rabbit anti-TH antiserum ab59276	Raised against a synthetic peptide close to the N-terminal of TH.	Abcam, Inc. (Cambridge, MA, USA)

Chapter 3

An analysis of the demographic and clinical profile of a Sheffield vitiligo patient cohort

3 An analysis of the demographic and clinical profile of a Sheffield vitiligo patient cohort

3.1 Introduction

As previously detailed (Section 1.2), vitiligo is the most common skin depigmenting condition (Ezzedine et al., 2015, Picardo et al., 2015). Worldwide, the estimated prevalence of vitiligo is 0.4% to 2%, although greater rates of up to 8.8% have been reported in India (Ezzedine et al., 2015, Picardo et al., 2015). The disease affects all races and both genders seem equally vulnerable to vitiligo, although women report to clinic more frequently, probably because of greater social burden (Ezzedine et al., 2015, Picardo et al., 2015). Although vitiligo can occur at any age, almost half of patients develop vitiligo by the age of 20 years, and in approximately 70-80% of cases, vitiligo begins before the age of 30 years (Ezzedine et al., 2015, Picardo et al., 2015). Non-segmental vitiligo can appear at any age, while segmental vitiligo usually occurs at a young age (a median age at onset of 12-16 years) (Khaitan et al., 2012).

The evidence for an autoimmune pathogenesis in vitiligo is supported by its strong association with other autoimmune diseases, with autoimmune thyroid disease being the highest vitiligo-associated disorder (Alkhateeb et al., 2003, Nejad et al., 2013, Lukinovic-Skudar, 2015). The association of vitiligo with other autoimmune disorders and their frequencies are listed in Table 1.3 and Table 1.4. An increased incidence of vitiligo is also observed in patients with vitiligo family records (Alkhateeb et al., 2003, Singh and Pandey, 2011, Nicolaidou et al., 2012, Nejad et al., 2013, Vora et al., 2014). Although this association exists, values vary depending on the population studied, and they range from 12% to 40% (Sehgal and Srivastava, 2007, Nejad et al., 2013, Pradhan et al., 2013). The clinical and demographic characteristics of vitiligo are previously described in further detail in Section 1.2.

3.2 Aim

The aim of the work in this chapter was to describe our Sheffield vitiligo patient cohort with regard to demographic and clinical profiles.

3.3 Methods

3.3.1 Study participants

The study included 246 vitiligo patients referred to the dermatology (Professor David Gawkrodger) and endocrinology (Professor Anthony Weetman) clinics at the Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK. Local ethics approval was in place for the study and all the participants had given written informed consent.

3.3.2 Skin examination and classification of disease status

Clinical diagnosis of vitiligo by these two experienced clinicians was based on the presence of depigmented macules with special focus on lesion morphology, distribution, and comorbidities. The clinical types of vitiligo were categorised as segmental, non-segmental and unclassified/undetermined according to the VGICC classification (Ezzedine et al., 2012b).

Segmental vitiligo was defined as one or a few macules in one area, but in a non-dermatomal pattern. Unclassifiable or undetermined vitiligo included focal vitiligo, and mucosal vitiligo when affecting one site.

Non-segmental vitiligo, defined by a symmetrical distribution of the lesions, was sub-divided into different types. Acral was characterised by several depigmented patches on the extremities of different regions, acrofacial by lesions over the face and acral parts of the extremities, symmetrical (generalised) by scattered lesions extensively distributed, and universal by depigmentation of over 90% to 100% of the body. Mixed vitiligo was considered when there was evidence of the coexistence of segmental and non-segmental types.

Vitiligo was considered active when existing and/or new lesions had spread over the past 12 months (Ezzedine et al., 2012b). Vitiligo was considered stable when there was no history that depigmentation had not progressed during the last 12 months (Ezzedine et al., 2012b).

3.3.3 Data collection

A questionnaire was used for data collection. For this, each patient was interrogated and examined. The results of the history and of a detailed examination were recorded. Special attention was given to gender, age, age at onset, duration of disease, type of vitiligo, progression status, previous treatment, any precipitating factors, history of autoimmune disorders reported in association with vitiligo, family history of vitiligo and/or other autoimmune disease. Associated autoimmune disorders were confirmed by reviewing the medical history of each patient and carrying out a physical examination and, by carrying out relevant laboratory tests.

3.3.4 Statistical analysis

The frequencies of demographic details and clinical features were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

3.4 Results

3.4.1 Age and sex of vitiligo patients

Of the 246 British Caucasian vitiligo patients recruited to the study, 142 (58%) were female and 104 (42%) were male giving a male to female ratio of 1:1.37 (Table 3.1). As shown in Table 3.1, the age of the patients ranged from less than 1 to 88 years with a mean age of 44 ± 17 years. For male patients, the mean age was 43 ± 18 years (range 0-78 years). For female, 45 ± 17 years (range 7-88 years). There was no significant difference in the age profile of the two gender groups (P = 0.38, Unpaired t test).

3.4.2 Age at onset of vitiligo

The age of vitiligo onset varied notably, ranging from birth to 83 years with a mean age of onset of 31 ± 17 years (Table 3.1). Grouping patients by 10-year intervals, the most frequent vitiligo onset age was between 11-20 years of age accounting for 54 of the 246 patients (22%) (Figure 3.1). The majority of patients, 53% (131/246), had their vitiligo develop before the age of 30 years, and 72% (178/246) before 40 years (Figure 3.1).

3.4.3 Duration of vitiligo

The duration of vitiligo at the point of referral ranged from 0-71 years with a mean of 14 ± 13 years (Table 3.1). At the time of inclusion in the study, 55% (136/246) of cases had had vitiligo for 0-10 years, with the 74% (183/246) having a disease duration of less than 20 years (Figure 3.2).

3.4.4 Vitiligo classification

According to the VGICC classification (Ezzedine et al., 2012b), the type of vitiligo in the patient cohort was predominantly non-segmental with 94% (231/246) of patients diagnosed with this type (Table 3.1). Only 4 of 246 (1.6%) patients showed a pattern of segmental vitiligo (Table 3.1). An undetermined classification was attributed to 11/246 (4.5%) cases, which were all focal vitiligo (Table 3.1).

3.4.5 Non-segmental vitiligo subtypes

Amongst 231 non-segmental vitiligo, symmetrical (generalised) was the most common subtype with 157 (68%) cases, followed by acrofacial, 42 (18.2%), acral, 4 (1.7%), peri-orificial, 3 (1.3%), and universal, 1 (0.4%) (Figure 3.3). In addition, there were patients with a combination of symmetrical and peri-orificial, 10 (4.3%), symmetrical and acrofacial, 7 (3.0%), and symmetrical with peri-orificial and acrofacial, 1 (0.4%) (Figure 3.3). One (0.4%) patient had occupational vitiligo (Figure 3.3). Only five (2.2%) patients exhibited mixed (segmental and non-segmental) vitiligo (Figure 3.3).

3.4.6 Vitiligo activity status

Active vitiligo is defined as the spread of existing and/or new lesions within the previous 12 months. Stable vitiligo is defined by depigmentation that had shown no progression for the previous 12 months (Ezzedine et al., 2012b). Of the 246 vitiligo patients included in the study, 226 (92%) were considered to have actively progressing vitiligo at the time of their recruitment (Table 3.1). Only 20 of 246 (8%) patients were classified as having stable vitiligo (Table 3.1).

Of the 226 active vitiligo cases, 216 (95.6%) were non-segmental, 9 (4%) focal, and one (0.4%) segmental (Figure 3.4). In the 20 patients with stable vitiligo, 15 (75%) had non-segmental, two (10%) had focal and three (15%) had segmental vitiligo (Figure 3.4).

Comparing vitiligo activity status in the different disease classifications, the frequency of active vitiligo was significantly increased in the patient group (216/231 = 93.5%) with non-segmental vitiligo when compared with the segmental vitiligo patient cohort (1/4 = 25%) (P = 0.002, Fisher's exact test) (Figure 3.5).

Table 3.1: The demographic and clinical characteristics of 246 vitiligo patients

Characteristic	Detail for vitiligo patients (*n* = 246)
Sex	
Male	104 (42%)
Female	142 (58%)
Age at inclusion in study	
Age, mean ± SD	44 ± 17 years
Age range	0-88 years
Age at onset	
Age at onset, mean ± SD	31 ± 17 years
Age at onset range	0-83 years
Disease duration	
Disease duration, mean ± SD	14 ± 13 years
Disease duration range	0-71 years
Vitiligo clinical type	
Segmental	4 (1.6%)
Non-segmental	231 (94%)
Undetermined (all focal)	11 (4.5%)
Vitiligo activity[1]	
Active	226 (92%)
Stable	20 (8%)
Autoimmune disease	
Absent	162 (66%)
Present (at least one disease)	84 (34%)
One disease	70 (28%)
Two diseases	9 (3.7%)
Three diseases	5 (2.0%)
Family history of vitiligo or autoimmune disease	
No	135 (55%)
Yes (vitiligo and/or autoimmune disease)	111 (45%)
Vitiligo only	19 (7.7%)
Vitiligo with autoimmune disease	26 (10.6%)
Autoimmune disease only	66 (26.8%)

[1]Active vitiligo is defined as the spread of existing and/or new lesions within the previous 12 months. Stable vitiligo is defined by depigmentation that had shown no progression for the previous 12 months (Ezzedine et al., 2012b).

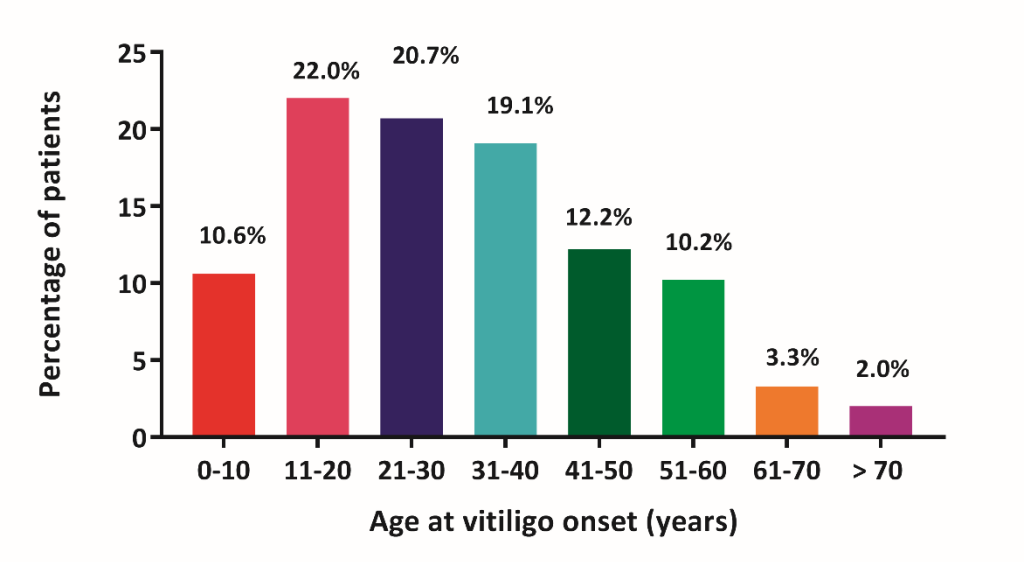


Figure 3.1: Age distribution at vitiligo onset.

The percentage of the 246 vitiligo patients, including segmental, non-segmental and focal types, in each 10-year interval of age of onset is shown.

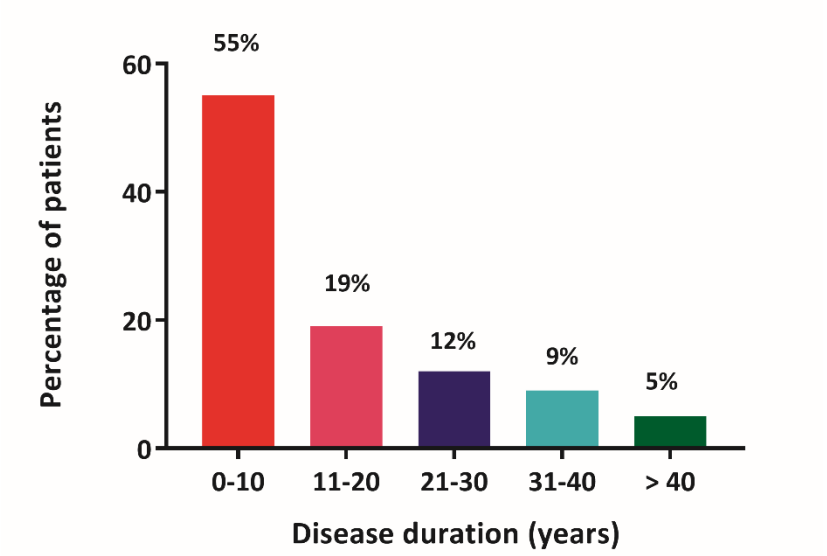


Figure 3.2: Disease duration distribution

The percentage of the 246 vitiligo patients in each 10-year interval of disease duration is shown.

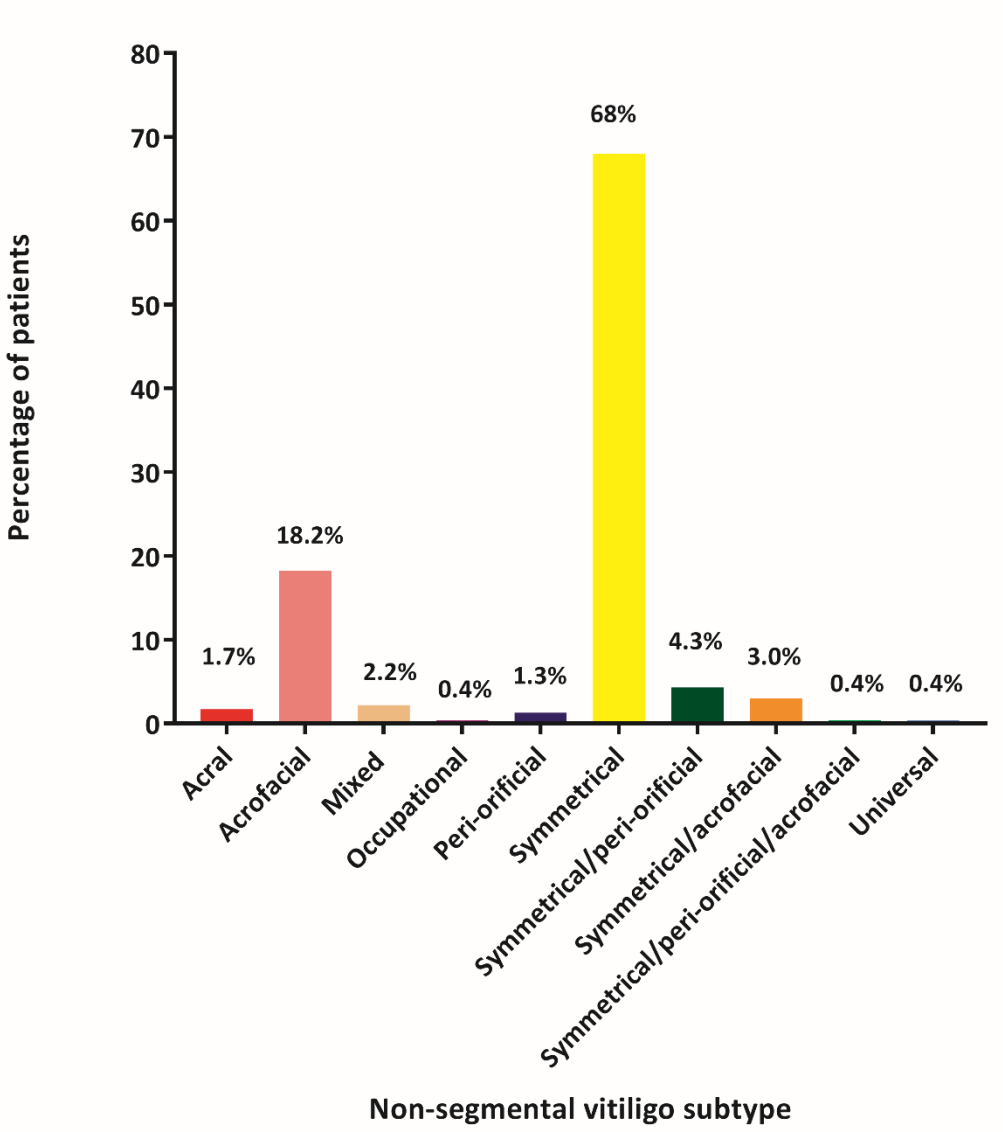


Figure 3.3: Frequency of the non-segmental vitiligo subtypes

The frequency distributions of non-segmental vitiligo subtypes are shown for 231 patients.

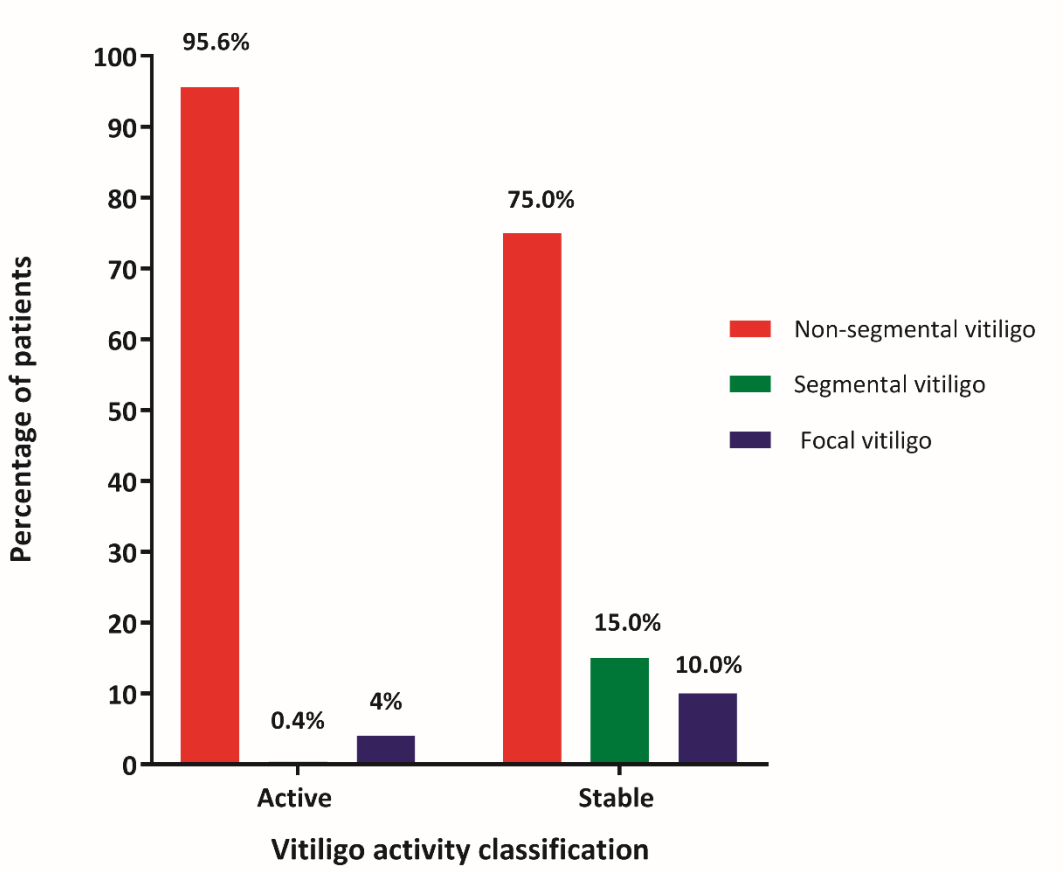


Figure 3.4: The frequency of the three vitiligo subtypes in the active and stable vitiligo classifications.

The frequency of non-segmental, segmental, and focal vitiligo in the active (*n* = 226) and stable (*n* = 20) disease classifications.

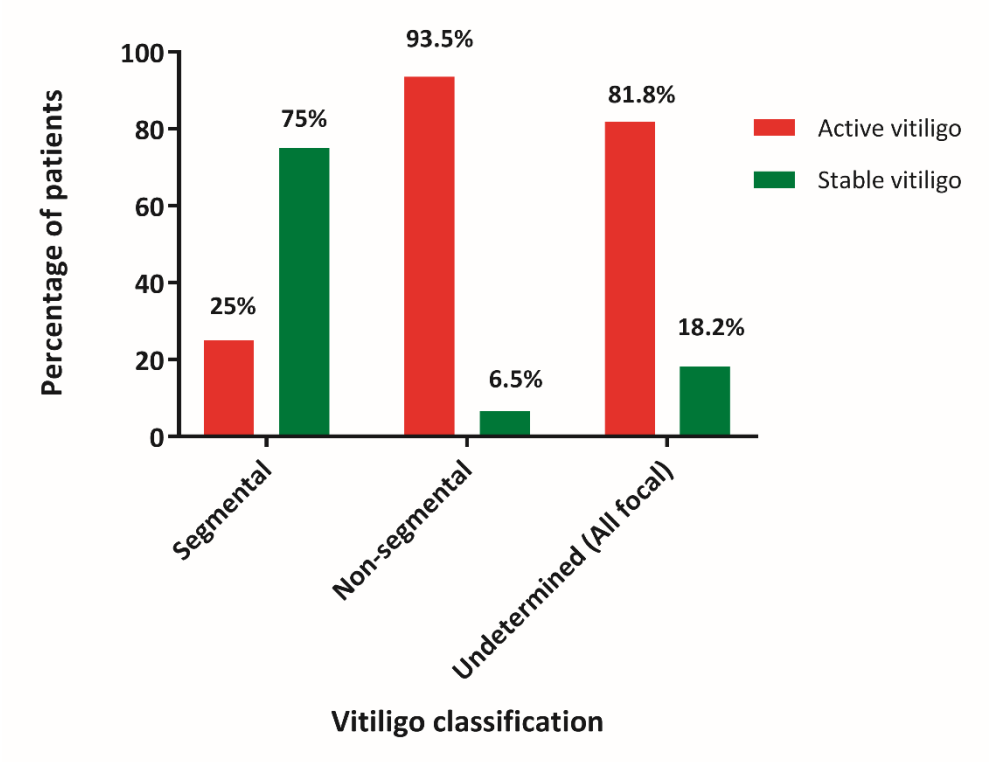


Figure 3.5: The proportion of active and stable vitiligo in the three vitiligo classifications.

The distribution of active and stable vitiligo is shown for the three different vitiligo types. Non-segmental (*n* = 231), segmental (*n* = 4), and focal (*n* = 11).

3.4.7 Comorbid diseases

3.4.7.1 Frequency and type of autoimmune diseases

The frequency of different comorbid diseases in the vitiligo patient cohort is illustrated in Figure 3.6. Autoimmune hypothyroidism, including Hashimoto's thyroiditis, was the most common autoimmune disease being present in 41/246 (16.7%) patients with vitiligo. Other associated autoimmune diseases included Graves' disease (hyperthyroidism), 15 (6.1%), alopecia areata (4.1%), type 1 diabetes, 8 (3.3%), psoriasis, 3 (1.2%), lichen sclerosus, 4 (1.6%), pernicious anaemia, 5 (2.0%), rheumatoid arthritis, 4 (1.6%), systemic lupus erythematosus, 3 (1.2%), Sjögren's syndrome, 1 (0.4%), Addison's disease (adrenal insufficiency), 3 (1.2%), chronic urticaria, 2 (0.8%), Crohn's disease, 1 (0.4%), hypopituitarism, 1 (0.4%), angioedema, 1 (0.4%), and hyperparathyroidism, 1 (0.4%).

Overall, 84/246 (34%) of the vitiligo patients had at least one comorbid autoimmune or inflammatory disease (Table 3.1). Fourteen of the 246 (5.7%) vitiligo patients had more than one concomitant disease (Table 3.1). Nine of the 246 (3.7%) patients had two comorbid autoimmune diseases (Table 3.1). These included two patients each with autoimmune hypothyroidism and type 1 diabetes, two patients each with autoimmune hypothyroidism and Addison's disease, two patients each with autoimmune hypothyroidism and pernicious anaemia, one patient with hypopituitarism and alopecia areata, one patient with chronic idiopathic urticaria and angioedema, and one patient with hyperthyroidism and systemic lupus erythematosus.

Five of the 246 (2.0%) patients were affected by three concomitant autoimmune disorders (Table 3.1). One patient suffered from autoimmune hypothyroidism, type 1 diabetes, and alopecia areata, the second had type 1 diabetes, pernicious anaemia, and rheumatoid arthritis, and the third patient had Addison's disease, autoimmune hypothyroidism and type 1 diabetes. A fourth patient had hyperparathyroidism, autoimmune hypothyroidism, and lichen sclerosus, and a fifth suffered from alopecia areata, autoimmune hypothyroidism, and rheumatoid arthritis.

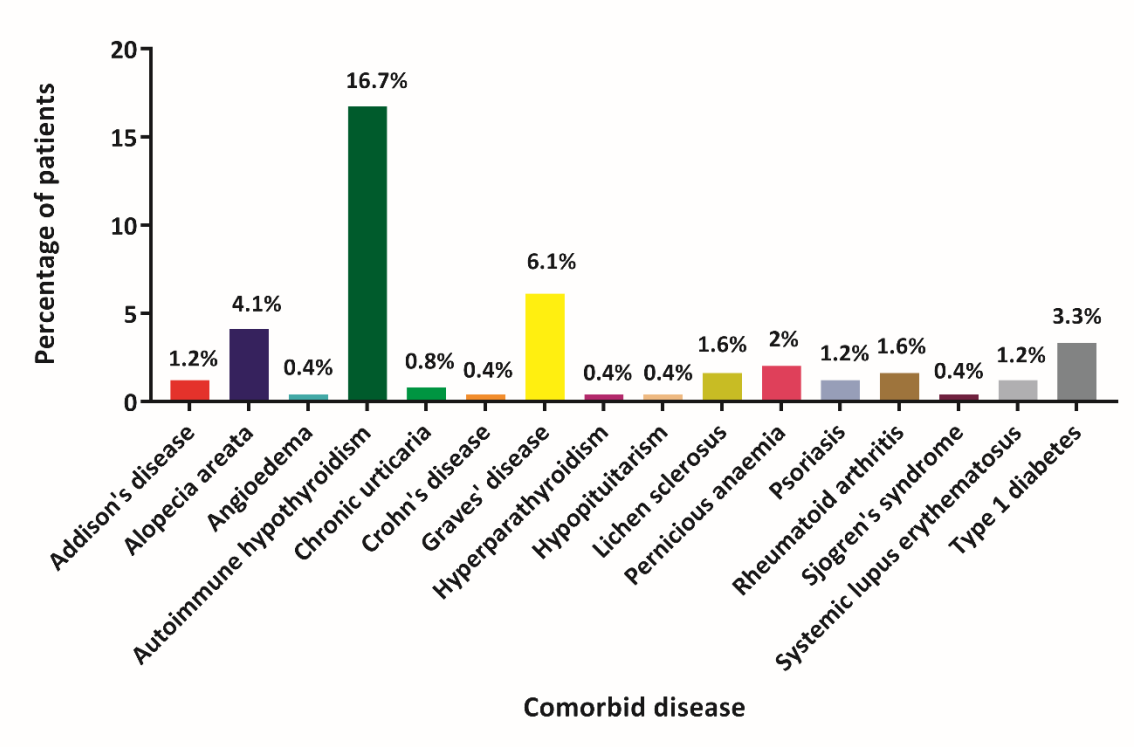


Figure 3.6: Frequency of associated comorbid diseases in vitiligo patients.

The figure shows the percentage of autoimmune and auto-inflammatory diseases observed in the 246 vitiligo patients. In total, 84 of the 246 patients had a comorbid disease.

3.4.7.2 Comparison of frequency of autoimmune diseases with general population

The frequency of the various autoimmune diseases are compared with those in the general population in Table 3.2. Amongst the vitiligo patients, there was a twelve -fold increase over the 1.9% population frequency of self-reported clinical autoimmune thyroid disease in Caucasians (Jacobson et al., 1997). The frequency of pernicious anaemia at 2% was elevated in the study population compared with the frequency in the general population of 0.15% (Jacobson et al., 1997), a fold increase of 13. The frequency of Addison's disease in the general population is extremely low at 0.005% (Jacobson et al., 1997). In the current study, the frequency was 1.2%, a very large fold increase in our vitiligo group.

3.4.7.3 Association with gender

Of the 84 patients with an associated autoimmune disease, 21 (25%) were male and 63 (75%) were female. In respect of the total of 142 female patients with vitiligo, 44% had an associated disorder, while 20% of the 104 male patients with vitiligo suffered from a comorbid disease. Comparing male with female patients, the frequency of an associated autoimmune disease was significantly increased in females ($P < 0.0001$, Fisher's exact test).

3.4.7.4 Association with vitiligo type

With respect to vitiligo classification, 81 of the 84 (96%) patients with autoimmune disease had non-segmental vitiligo, three (4%) had focal, but none had segmental. With respect to patients with non-segmental, focal, and segmental vitiligo, 81/231 (35%), 3/11 (27%), and 0/4 (0%), respectively, had at least one autoimmune disease. Comparisons indicated there was no significant statistical difference in the frequency of comorbid diseases amongst the three vitiligo classifications ($P = 0.39$, Chi-square test).

Table 3.2: Autoimmune disorders in our vitiligo patients compared with the general population

Disease	Frequency in vitiligo patients in this study (%)	Frequency in the general population (%)	Reference for frequency in the general population
Addison's disease	1.2	0.005	(Jacobson et al., 1997)
Autoimmune thyroid disease	22.8	1.9	(Jacobson et al., 1997)
Alopecia areata	4.1	0.2-2.0	(Safavi, 1992, Safavi et al., 1995)
Psoriasis	1.2	0.5-1.6	(Farber and Nall, 1998)
Pernicious anaemia	2.0	0.15	(Jacobson et al., 1997)
Rheumatoid arthritis	1.6	0.86	(Jacobson et al., 1997)
Systemic lupus erythematosus	1.2	0.024	(Jacobson et al., 1997)
Sjogren's syndrome	0.4	0.014	(Jacobson et al., 1997)
Type 1 diabetes	3.3	0.48	(Jacobson et al., 1997)

3.4.8 Family history of vitiligo and/or autoimmune disorders

A positive family history of vitiligo and/or other autoimmune disorders was present in 111/246 (45%) patients, whereas 135 (55%) cases had no family history of vitiligo and/or other autoimmune diseases (Table 3.1). Nineteen (7.7%) patients reported a positive family history of vitiligo alone, 26 (10.6%) reported a family history of vitiligo and autoimmune disease, and 66 (26.8%) reported a family history of autoimmune disease alone (Table 3.1).

The frequency of autoimmune diseases reported in vitiligo patient familial histories is shown in Figure 3.7. Type 1 diabetes was the most common, at 19% (47/246). Autoimmune hypothyroidism was the second most common at 18.7% (46/246). Less common in the family history were pernicious anaemia at 3.3% (8/246), psoriasis and systemic lupus erythematosus each at 1.2% (3/246), Addison's disease and Graves' disease each at 0.8% (2/246), and alopecia areata at 0.4% (1/246).

Of the 45 patients reporting a positive family history of vitiligo (Table 3.1), 26 also reported coexistence of autoimmune disorders in their family history. The autoimmune diseases reported as the only comorbid disorder were: autoimmune hypothyroidism, 9; type 1 diabetes, 7; systemic lupus erythematosus, 2; and pernicious anaemia, 1. In addition, multiple autoimmune diseases reported were: autoimmune hypothyroidism and type 1 diabetes, 3; autoimmune hypothyroidism and pernicious anaemia, 1; autoimmune hypothyroidism and alopecia areata, 1; type 1 diabetes and pernicious anaemia, 1; and type 1 diabetes and psoriasis, 1.

The diseases, reported in the 66 vitiligo patients who had a family history of autoimmunity alone (Table 3.1) were: autoimmune hypothyroidism, 22; type 1 diabetes, 24; pernicious anaemia, 2; psoriasis, 2; Graves' disease, 1; hyperthyroidism, 1; Addison's disease and autoimmune hypothyroidism, 2; Graves' disease and pernicious anaemia, 1; type 1 diabetes and pernicious anaemia, 2; type 1 diabetes and systemic lupus erythematosus, 1; and type 1 diabetes and autoimmune hypothyroidism, 8.

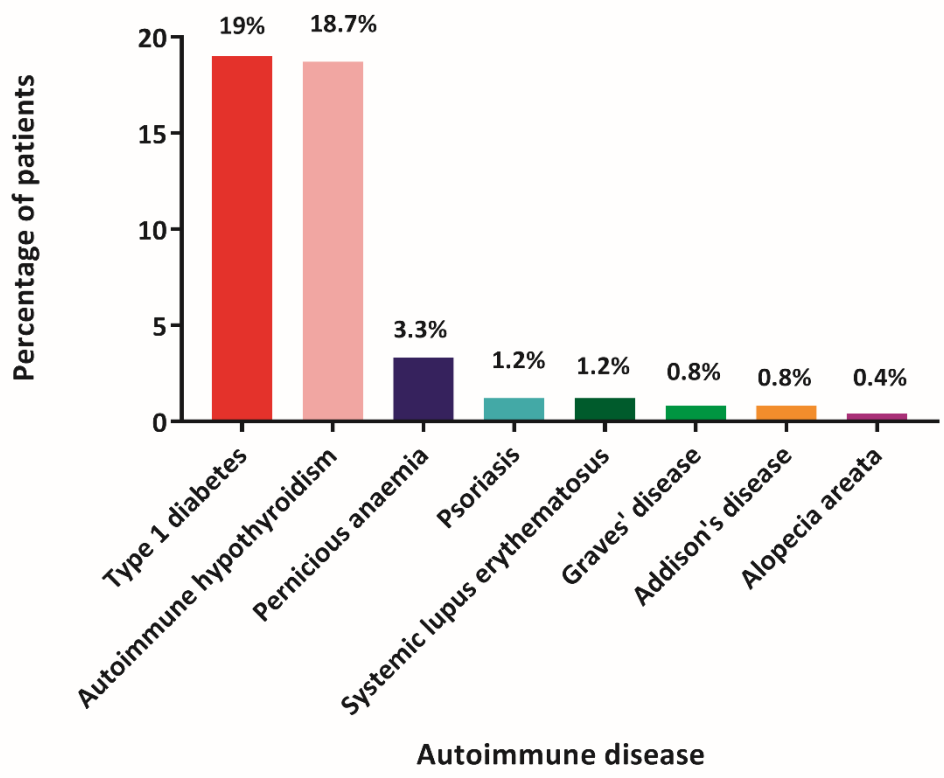


Figure 3.7: Frequency of associated comorbid diseases in vitiligo patient family history.

The frequency of autoimmune diseases reported in the family history of 246 patients with vitiligo. In total, 92 of the 246 vitiligo patients had a family history of autoimmune disease.

3.5 Discussion

Vitiligo is the most prevalent disease of skin depigmentation. It affects 0.4-2% of the world population (Kruger and Schallreuter, 2012), not showing any difference between races. In spite of the high frequency of vitiligo, few studies have been made on its epidemiology, especially longitudinal studies. The aim of this part of the study was to describe our vitiligo patient cohort in terms of demographic details and clinical features.

3.5.1 Gender distribution

Some previous studies have reported both genders are equally affected with vitiligo (Sarin and Ajit, 1977, Taieb and Picardo, 2009, Ezzedine et al., 2012b, Shankar et al., 2012, Picardo et al., 2015). However, a slightly greater prevalence in females have been reported by most studies (Martis et al., 2002, Dogra et al., 2005, Singh and Pandey, 2011, Vora et al., 2014, Lee et al., 2015), although some have observed a substantially higher incidence of vitiligo in women (McBurney, 1979, Shah et al., 2008). In contrast, a higher frequency of vitiligo in men has also been recorded (McBurney, 1979, Wang et al., 2013).

The female to male ratio in our sample of vitiligo patients was 1.37:1. The excess of females observed in our study and other studies could result from greater cosmetic awareness which prompts women to present themselves for treatment. Inconsistency in the ratio of male to female vitiligo patients may also be attributed to differences in sample sizes and ascertainment and referral patterns included in different studies.

3.5.2 Age of onset

Though vitiligo can present at any age from birth to senescence, our study showed that the most common onset age of vitiligo was between 11-20 years, accounting for 22% of patients, and this is consistent with previous studies (Shajil et al., 2006). More than half (53%) of the cases in our study claimed onset occurred before 30 years of age, which agreed with studies reported by Shajil et al. (2006) and Karelson et al. (2009), but differed from another report which showed that the disease appeared before the age of 20 in almost 50% of patients (Sehgal and Srivastava, 2007). In addition, the latter study

(Sehgal and Srivastava, 2007) also reported that around 70-80% of vitiligo patients had onset of the disease below 30 years of age. However, our study observed that a similar percentage of patients (72%) came within an onset age of under 40 years. Interestingly, an epidemiological study in Denmark reported that the age of onset of vitiligo most frequently ranged between 40 to 60 years (Howitz et al., 1977).

Non-segmental vitiligo can occur at all ages, and this was apparent in our study with an onset age range of 0-83 years. In contrast, segmental vitiligo usually presents before 30 years of age in 87% of cases and before 10 years of age in 41.3% (Hu et al., 2006, Picardo et al., 2015). However, the four patients with segmental vitiligo in this study had onset ages of 4, 31, 32, and 54 years.

3.5.3 Disease duration

The mean disease duration we observed of approximately 14 years is a finding almost identical to those shown in some studies (Jarallah et al., 1993, Karelson et al., 2009), but different to most series, which most often report a mean duration of less than five years (George, 1989, Handa and Kaur, 1999, Dave et al., 2002, Martis et al., 2002, Shajil et al., 2006, Khaitan et al., 2012).

3.5.4 Vitiligo classification

Non-segmental vitiligo was the most common clinical type observed in our study at a frequency of 94%, which is consistent with other studies where this vitiligo pattern accounted for 39-83% of the subjects (Handa and Kaur, 1999, Dogra et al., 2005, Liu et al., 2005, Mason and Gawkrodger, 2005, Singh and Pandey, 2011, Vora et al., 2014). Of the non-segmental vitiligo subtypes, symmetrical (generalised) was the most frequent type (68%) followed by acrofacial 18.2%, which is consistent with other studies (Karelson et al., 2009, Esfandiarpour and Farajzadeh, 2012, Vora et al., 2014). Acrofacial has been reported to be seen in between 17-35% of cases in different clinical studies (Dave et al., 2002, Martis et al., 2002, Nejad et al., 2013, Vora et al., 2014). Acral without facial involvement (1.7%), peri-orificial alone (1.3%), and universal (0.4%) were rare in our study, again in agreement with previous reports (Pajvani et al., 2006, Esfandiarpour and

Farajzadeh, 2012). In one series, though, the universal subtype was been recorded in 18% of patients (Dogra et al., 2005, Wang et al., 2013). The mixed subtype of non-segmental vitiligo is a relatively recent description, made in a few studies only (Ezzedine et al., 2011, Ezzedine et al., 2012a). Its frequency in the current study was 2.2%.

Segmental vitiligo reportedly accounts for approximately 5-16% of all vitiligo cases (Koranne and Sachdeva, 1988). Also documented is that a third of paediatric cases (Halder et al., 1987, Nicolaidou et al., 2012, Shankar et al., 2012) but only 0-4.5% of the adult population have this vitiligo type (Dogra et al., 2005, Mason and Gawkrodger, 2005). In the present study, segmental vitiligo was seen in only 1.6% of our patients, perhaps because this pattern generally presents in children below 10 years of age (Palit and Inamadar, 2012), and only 10.6% of our patients belonged to this age group. Indeed, only one of our cases of segmental vitiligo had an onset age below 10 years. Late onset (median age at onset 41 years) of segmental vitiligo has been reported in Chinese (Wang et al., 2013). In this study, the disease started in three of the four segmental vitiligo patients later than 30 years of age. The frequency of segmental vitiligo might be abnormally low in this series since patients were recruited from adult clinics and not from paediatric clinics, in which the incidence of segmental vitiligo might be presumed to be higher.

Focal and mucosal vitiligo are now assigned to the category of undetermined/unclassified vitiligo until a more definite classification can be made on clinical description (Ezzedine et al., 2012b). Focal vitiligo was present in 4.5% of patients. No cases of mucosal vitiligo were reported in our vitiligo patient cohort. Mucosal vitiligo has also been reported as a rarer clinical type at 2.2% (Dogra et al., 2005).

3.5.5 Vitiligo activity

The vast majority (92%) of patients included in this study had active vitiligo at the time of presentation and this is in agreement with previous studies (Chun and Hann, 1997, Dave et al., 2002, Karelson et al., 2009). One study has shown that out of 318 patients with non-segmental vitiligo, 289 (91%) exhibited active vitiligo and the same study concluded that non-segmental vitiligo was significantly associated with disease progression (Chun and Hann, 1997). Similarly, in the current study, 93.5% of non-

segmental vitiligo patients had active disease and the frequency of active disease was significantly increased compared with patients with segmental vitiligo where only 1 of 4 (25%) patients had active disease.

3.5.6 Associated autoimmune disease

Based on autoimmunity as the leading hypothesis in the pathogenesis of vitiligo, studies on vitiligo-associated autoimmune conditions in vitiligo patients and in their families has also been a subject of great interest. Various authors have proposed that vitiligo patients have a higher risk of developing autoimmune disorders than the general population (Safavi, 1992, Safavi et al., 1995, Jacobson et al., 1997, Farber and Nall, 1998, Alkhateeb et al., 2003, Nejad et al., 2013, Lukinovic-Skudar, 2015, Gill et al., 2016), though the findings have been variable in different populations. From this study, we found that many of the associated autoimmune diseases were seen at a higher frequency than in the general population.

In our study, 84 of 246 (34%) patients suffered from at least one other concomitant autoimmune disease. Thyroid disease (22.8%) was the most frequent autoimmune disease reported by our subjects, a finding similar to previous studies in which the association of vitiligo with thyroid disease varied between 12% (Narita et al., 2011, Singh and Pandey, 2011, Gill et al., 2016), 15.4% (Huang et al., 2013), 21% (Karelson et al., 2009, Nejad et al., 2013) and 34% (Mason and Gawkrodger, 2005). Autoimmune hypothyroidism 16.7% was the most prevalent thyroid disease among our vitiligo patients, and this corresponds with previous studies (Nejad et al., 2013, Gill et al., 2016). This was followed by Graves' disease (6.1%), a proportion that is in line with a previous study (Prindaville and Rivkees, 2011).

Alopecia areata also was quite frequent occurring in 4.1% of our patients. This was similar to previous findings of 7.4% (Gopal et al., 2007) and 5.3% (Narita et al., 2011). Rheumatoid arthritis, type 1 diabetes and psoriasis were all rarer, each seen in only four (1.6%), eight (3.3%) and three (1.2%) of our patients, respectively. This observation was consistent with data for vitiligo patients in the Caucasian population (Alkhateeb et al., 2003). In contrast, however, Karelson et al. (2009) reported high frequencies of type 1 diabetes (13.6%), psoriasis (8.4%) and rheumatoid arthritis (4.5%) in patients with

vitiligo reported in Estonia. The risk of the presence of coexisting autoimmune diseases in our vitiligo patient group was significantly greater in females compared with males, a finding that is similar to previous studies (Karelson et al., 2009, Amerio et al., 2010, Narita et al., 2011, Vijayalakshmi et al., 2017).

With respect to vitiligo type, none of the four patients with segmental vitiligo, 35% of patients with non-segmental vitiligo, and 27% of patients with focal vitiligo had an associated autoimmune disease. This is in agreement with autoimmune diseases being more frequently associated with the non-segmental classification of the disease (Ezzedine et al., 2011), although our study only contained a small number of segmental vitiligo patients.

Differences in the frequency of vitiligo-associated autoimmune diseases between different studies may be due to the sample sizes, genetic factors and demographic variations. However, altogether, our findings further verify the previous evidence of association between vitiligo and other autoimmune disorders (Majumder et al., 1993, Zelissen et al., 1995, Alkhateeb et al., 2003, Nejad et al., 2013, Gill et al., 2016) and highlight the magnitude of vitiligo-associated diseases in Caucasians.

3.5.7 Family history of vitiligo and autoimmune disease

Our study showed a family history of vitiligo in 18.3% of subjects, a finding midway between 8% (Dave et al., 2002, Shankar et al., 2012) and 30% (Hann et al., 1997, Pajvani et al., 2006) previously noted. These results suggests a genetic component is likely to be involved in the manifestation of vitiligo (Gopal et al., 2007). Moreover, a positive family history of other autoimmune diseases was found in 37.4% of cases, which is in line with a previous study by Mason and Gawkrodger (2005). This finding further indicated that vitiligo aetiology involves susceptibility genes shared with other autoimmune disorders. At least 17 confirmed vitiligo susceptibility genes have been identified recently by genome wide association studies (Jin et al., 2007b, Birlea et al., 2011). Most of these genes have also been involved in other autoimmune disorders e.g., autoimmune thyroid disease, rheumatoid arthritis, and type 1 diabetes (Narita et al., 2011). Therefore, these shared autoimmune disorder susceptibility genes may underlie our detected associations in vitiligo patients and their families.

3.5.8 Limitations of the study

The main limitation of our study was that it was based on adult clinics. Like any investigation based on selectively referred cases, some of the results may not be representative of the population at large. A population-based sample is desirable to understand the exact prevalence and the clinical characteristics of vitiligo in the general population. Additionally, in spite of having information on the family history of vitiligo patients, further definition of the exact relationship of family members, such as whether they were siblings, parents or extended relatives, and so forth would have been helpful. Thus, this is an area for further exploration. Furthermore, the lacking of consensus on disease classification may affect the results of disease classification. Other limitations include the fact that most patients were seen in adult clinics, perhaps missing out presentations in children, the sample size, and possible recall errors by patients, particularly in those whose condition appeared many years prior to presentation to our clinic.

3.6 Conclusion

In conclusion, the demography and comorbidities at presentation for patients with vitiligo in Sheffield were generally in accordance with results from elsewhere in the world. The results of the analysis support the autoimmune hypothesis of vitiligo aetiology since co-existing autoimmune diseases, particularly thyroid diseases, were common in our subjects. In addition, vitiligo and autoimmune diseases, principally again thyroid diseases, were prevalent in patient families indicating that genetic factors may predispose to the disease. Patients with vitiligo should be investigated for and followed up for thyroid dysfunction especially if there is a positive family history of thyroid disease.

Chapter 4

Investigation of phage-display to identify novel antibody targets (autoantigens) in vitiligo

4 Investigation of phage-display to identify novel antibody targets (autoantigens) in vitiligo

4.1 Introduction to phage-display technology

Filamentous bacteriophages are thin, cylindrical particles. They infect strains of *E. coli* which bear the F conjugative episome expressing the primary receptor of the phage; the F pilus. Each phage particle contains a circular single-stranded DNA molecule packaged into a coat composing of several thousands of copies of major coat protein pVlll, which covers the length of the particle (Marvin, 1998, Wilson and Finlay, 1998, Rami et al., 2017). Low copy numbers of the minor coat proteins (pIII, pVI, pVII, and pIX) are located at either extremity of the phage particle (Figure 4.1) (Marvin, 1998, Wilson and Finlay, 1998, Appenzeller et al., 2000, Rami et al., 2017).

The development of phage-display methodology arose from the breakthrough that insertion of DNA molecules into the phage gene gIII leads to the expression of encoded polypeptides as coat-protein fusions with pIII protein which are displayed on the phage particle surface (Figure 4.2) (Smith, 1985, Hairul Bahara et al., 2013). As such, large-scale libraries of fusion proteins that are displayed on the phage can be screened for binding peptides by panning against the ligand/s of choice. Since the selected peptides are "physically" linked to the genetic materials required for their expression, i.e., inside the phage particle, their identity can be determined by sequencing the DNA fragments that encode them.

In order to express cDNA-encoded peptides on phage surfaces, a strategy based on the high affinity binding of Jun and Fos heterodimers combined with elements of phagemid pComb3 was developed (Barbas and Lerner, 1991, Pernelle et al., 1993, Crameri and Walter, 1999, Appenzeller et al., 2000, Waterman et al., 2010). The resulting construct, the pJuFo phagemid vector allows the expression of full-length cDNA products on the phage surface (Figure 4.2) (Crameri and Suter, 1993, Crameri and Walter, 1999). In this cloning system, the Jun protein is expressed as an N-terminal fusion to phage coat protein pIII. The Fos protein is also expressed as an N-terminal fusion with the cDNA-encoded peptide. The Fos-cDNA fusion proteins and the Jun-pIII fusions are secreted to

the bacterial periplasmic space where Jun-Fos heterodimerisation occurs and the formation of disulphide bonds takes place. This provides the phage surface with a covalent link for cDNA products during phage morphogenesis. The physical phenotype-genotype coupling makes the technology a feasible platform for isolating and identifying novel binding peptides based on the powerful panning procedures by which pJuFo phage-displayed proteins are selectively enriched by consecutive rounds of panning against an appropriate ligand (Figure 4.3). Subsequently, enriched proteins can be identified by sequencing the cDNA that encodes them.

Phage-display has been very successfully used to isolate novel ligands for different targets including receptors (Bass et al., 1990) and transcription factors (Butteroni et al., 2000, Lawson and Bleris, 2017). It has also been employed to study antibody-antigen interactions (Barbas, 1993, Burgoon et al., 2001, Kemp et al., 2002b, Rami et al., 2017), and enzyme-substrate interactions (Redl et al., 1999, Cheok and Jaworski, 2016).

For the identification of autoantigens recognised by antibodies, the major advantage of phage-display lies in utilising the process of enrichment which enables efficient isolation of desired target peptides even if the titre of antibodies is relatively low in the patients under study. Moreover, the phage-display technique can identify conformational epitopes, unlike the classical phage lambda-based system which allows only the identification of linear binding domains; enrichment of phage in a fluid phase avoids the effects of denaturation due to protein adsorption to nitrocellulose membranes. In addition, panning enables large phage-displayed cDNA libraries to be screened and characterised more rapidly (Appenzeller et al., 2000, Hairul Bahara et al., 2013, Rami et al., 2017). Due to lower labour intensity and quantifiable analysis, the technique is a more powerful procedure for antigen discovery than other screening systems.

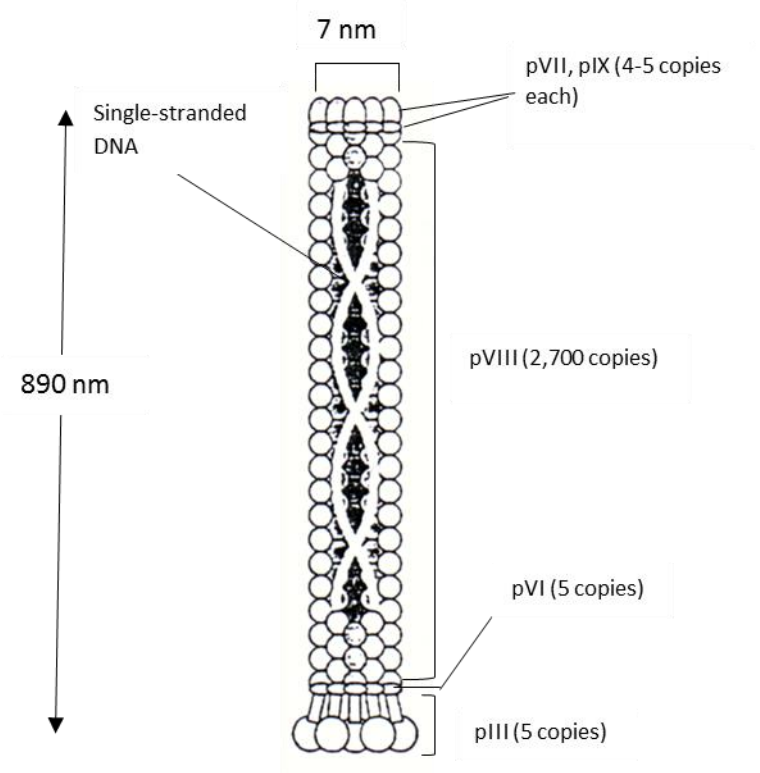


Figure 4.1: Schematic representation of the structure of a filamentous phage particle.

A single-stranded circular DNA is covered by 2,700 copies of pVIII protein (the major coat protein), and 4-5 copies of each of four types of minor coat proteins, including pIII. The figure was used from an article by Wilson and Finlay (1998) with kind permission from NRC Research Press.

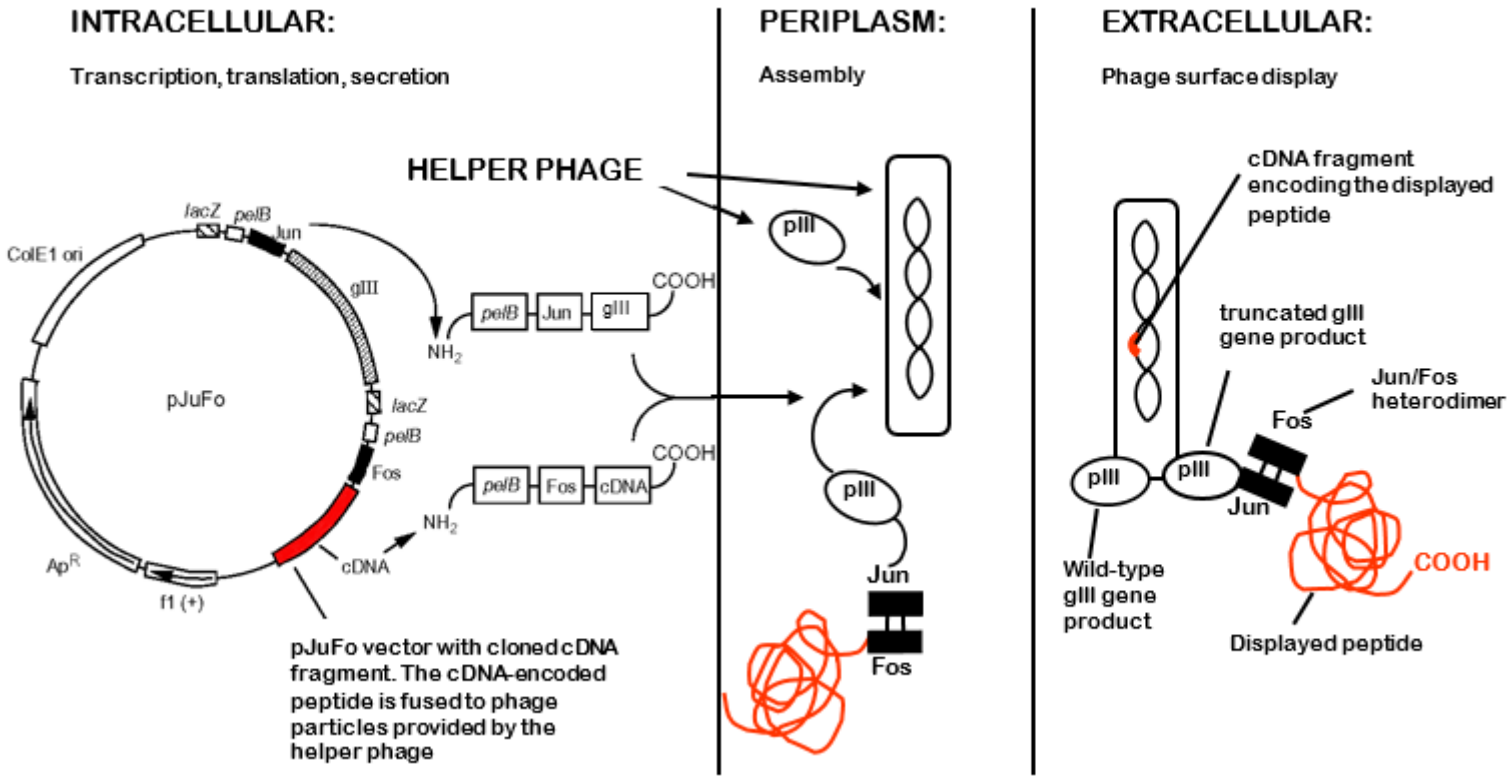


Figure 4.2: The pJuFo phage-display system.

The Jun-coat protein pIII fusion protein and the Fos-cDNA fusion protein are expressed and exported to the periplasm. During assembly of the phage, there is heterodimerisation of Jun-pIII/Fos-cDNA fusion proteins and incorporation of the heterodimers into the surface of the phage particles along with the wild-type phage coat protein pIII which is provided by the helper phage. The figure is used from a paper by Crameri and Suter (1993) with kind permission from Elsevier.

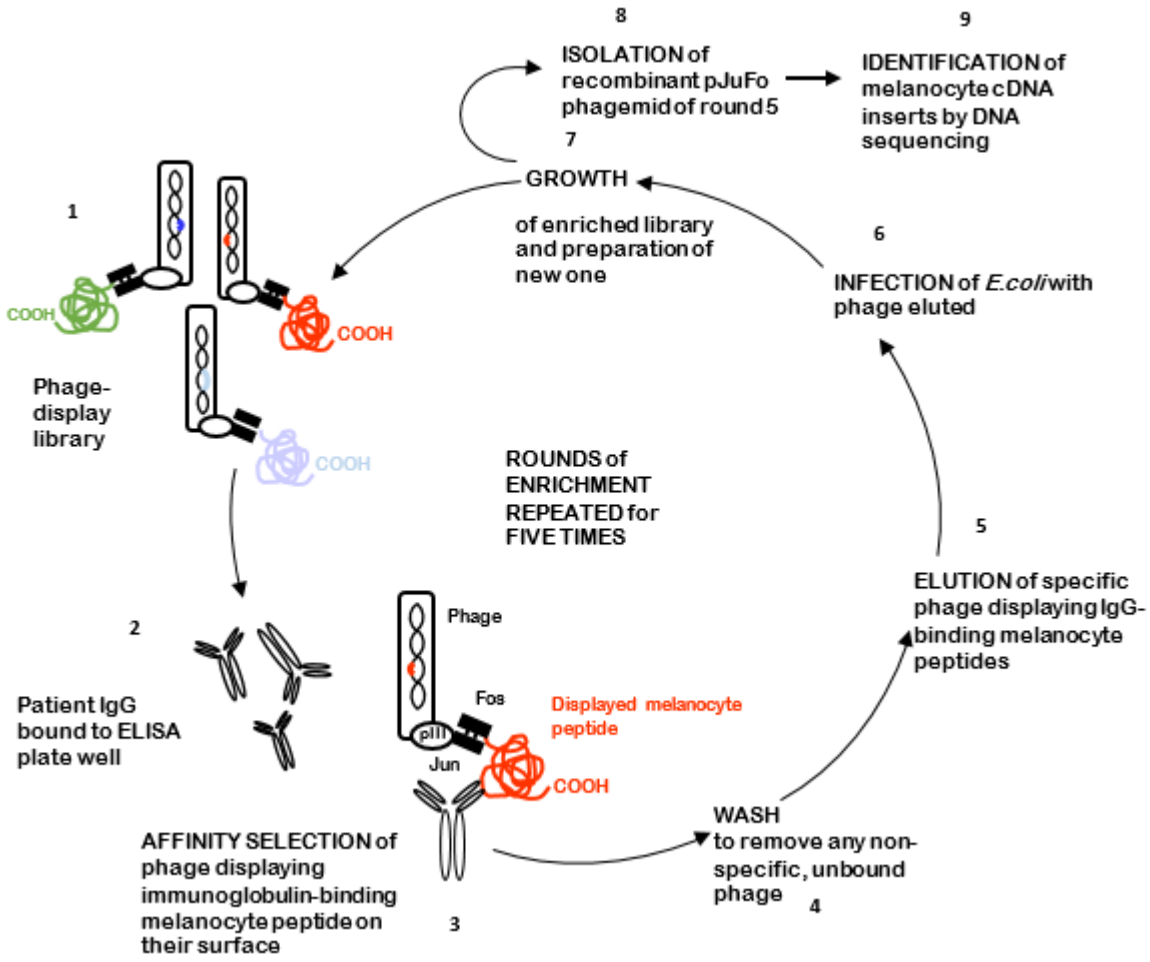


Figure 4.3: Enrichment of phage displaying melanocyte peptides that bind IgG molecules.

1. Phage displaying melanocyte peptides from melanocyte cDNA. **2.** Patient IgG. **3.** Phage expressing immunoreactive melanocyte peptides bind to patient IgG. **4.** Unbound phage are removed by washing. **5.** Phage displaying IgG-binding peptides are eluted with glycine-hydrochloride. **6.** Phage eluted are enriched by the infection into *E. coli* XL1-Blue MRF' **7.** Phage eluted are grown to prepare a new library for further rounds of panning. **8**. In the fifth round, phagemid DNA is extracted from infected cells. **9.** Isolated phagemid DNA is sequenced to identify melanocyte cDNA inserts. The figure was adapted from a paper by Waterman et al. (2010) with kind permission from Elsevier.

4.2 Aim

For this part of the project, the aim was to identify novel antibody targets (autoantigens) in patients suffering from vitiligo by utilising the benefits of phage-display methodology. Antibody targets in vitiligo are important to identify as the autoantigens themselves may also be T cell targets, as is the case with tyrosinase and PMEL (Ogg et al., 1998, Lang et al., 2001, Palermo et al., 2001, Mandelcorn-Monson et al., 2003, van den Boorn et al., 2009).

4.3 Materials and Methods

4.3.1 Vitiligo patient and healthy controls

The details of the clinical and demographic characterstics of the 22 vitiligo patients included in this part of the study are given in Table 4.1. The two controls were one male (age at sample, 27 years) and one female (age at sample, 43 years).

4.3.2 Phage-displayed melanocyte cDNA-encoded peptide library

The melanocyte peptide phage-display library was constructed in the vector pJuFo (Figure 4.2) and was constructed and verified by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). In brief, the phage-display library contained melanocyte cDNAs cloned into the *Xba*I-*Kpn*I site of the pJuFo vector (Figure 4.4). The phage-display library had a titre of 10^{11} colony-forming units (cfu)/ml and the frequency of recombinants (i.e. pJuFo carrying a cDNA insert) in the library had been estimated at 90%. The size of the cloned cDNA inserts ranged from 0.5-2.5 kb with an average of 1.5 kb. In addition, DNA hybridisation experiments had shown that the frequency of β-actin and tyrosinase cDNA inserts were 0.04% and 0.1%, respectively.

4.3.3 Titre of the phage-display library

The phage-display library was stored at -80°C until needed. The titre of the phage-display library was determined by infecting *E. coli* XL1-Blue MRF' cells (Table 2.1). Briefly, *E. coli* XL1-Blue MRF' cells were grown with shaking to log phase in LB medium (10 ml) (Section 2.5) supplemented with 10 µg/ml tetracycline (Section 2.4) at 37°C. An aliquot of 0.5 ml of the culture was subsequently infected with an aliquot of 2 µl of a 10^{-4} phage dilution made in phosphate-buffered saline (PBS) (pH 7.4) and incubated for 15 min at room temperature. From the infected culture, 1, 10 and 100-µl samples were spread on LB agar plates (Section 2.5) supplemented with 100 µg/ml of ampicillin and 10 µg/ml of tetracycline (Section 2.4). A negative control of a 10-µl sample of uninfected culture was also spread onto agar plates. After incubation overnight at 37°C, bacterial colonies were

counted. Subsequently, the titre of the phage which was expressed as cfu/ml was calculated.

4.3.4 IgG purification

Purification of IgG was performed by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). Briefly, IgG molecules were isolated from samples of serum using protein G Sepharose 4 Fast Flow affinity column chromatography (GE Healthcare, Little Chalfont, UK). To elute IgG molecules, 1-2 ml of each serum sample was passed through a protein G sepharose column with 0.01 M sodium phosphate buffer (pH 7.0). UV absorption at 280 nm with a 2138 UVIcord (LKB Wallac, Turku, Finland) was employed to monitor the IgG eluate with 0.1 M glycine-hydrochloride (pH 2.7). Eluted IgGs were subsequently neutralised with 1 M Tris-hydrochloride (pH 9.0). The IgG fractions were then pooled. Following dialysis overnight against PBS at 4°C, samples of IgG were concentrated by an Amicon Concentrator (Amicon Inc., Beverley, MA, USA) and filter-sterilised with a Millex® Filter Unit (Millipore Corp., Bedford, MA, USA). The IgG concentration was determined by spectrophotometry at 280 nm using a NanoDrop ND-1000 Spectrophotometer (Labtech, Wilmington, DE, USA) and NanoDrop Software (Labtech). IgG samples were stored at -20°C with a concentration of 10 mg/ml until needed.

Table 4.1: Demographic and clinical details of the vitiligo patients whose IgG was used in the panning experiments

Vitiligo patient	Sex	Age at sample (years)	Age at onset (years)	Disease duration (years)	Vitiligo clinical type	Vitiligo activity	Autoimmune disease	Family history of vitiligo or of autoimmune disease
V6-1	Female	41	21	20	Non-segmental	Active	Absent	Vitiligo
V11-2	Female	36	31	5	Non-segmental	Active	Absent	Absent
V12-3	Male	76	72	4	Non-segmental	Stable	Absent	Absent
V13-4	Male	70	20	50	Non-segmental	Active	Hypopituitarism and alopecia areata	Vitiligo
V15-5	Female	33	32	1	Segmental	Stable	Absent	Autoimmune hypothyroidism
V20-6	Male	47	5	42	Non-segmental	Active	Psoriasis	Absent
V21-7	Male	46	32	14	Non-segmental	Active	Absent	Type 1 diabetes
V22-8	Female	31	31	1	Segmental	Active	Alopecia areata	Vitiligo
V23-9	Female	28	20	8	Non-segmental	Active	Absent	Vitiligo and systemic lupus erythematosus
V24-10	Female	55	50	5	Non-segmental	Active	Absent	Absent
V224-11	Male	29	15	14	Non-segmental	Active	Absent	Type 1 diabetes
V38-12	Male	21	20	1	Non-segmental	Active	Absent	Psoriasis
V56-13	Male	74	58	16	Non-segmental	Active	Absent	Absent
V73-14	Male	38	26	12	Non-segmental	Active	Alopecia areata	Type 1 diabetes
V102-15	Female	41	0	41	Non-segmental	Active	Absent	Vitiligo
V169-16	Male	36	31	5	Non-segmental	Active	Absent	Type 1 diabetes
V189-17	Female	16	13	3	Non-segmental	Active	Absent	Absent
V216-18	Male	27	17	10	Non-segmental	Active	Absent	Absent
V219-19	Female	36	19	17	Non-segmental	Active	Graves' disease	Autoimmune hypothyroidism
V234-20	Female	48	22	26	Non-segmental	Active	Absent	Vitiligo
V45-21	Male	43	40	3	Non-segmental	Active	Absent	Type 1 diabetes
V46-22	Male	23	19	5	Non-segmental	Active	Absent	Type 1 diabetes

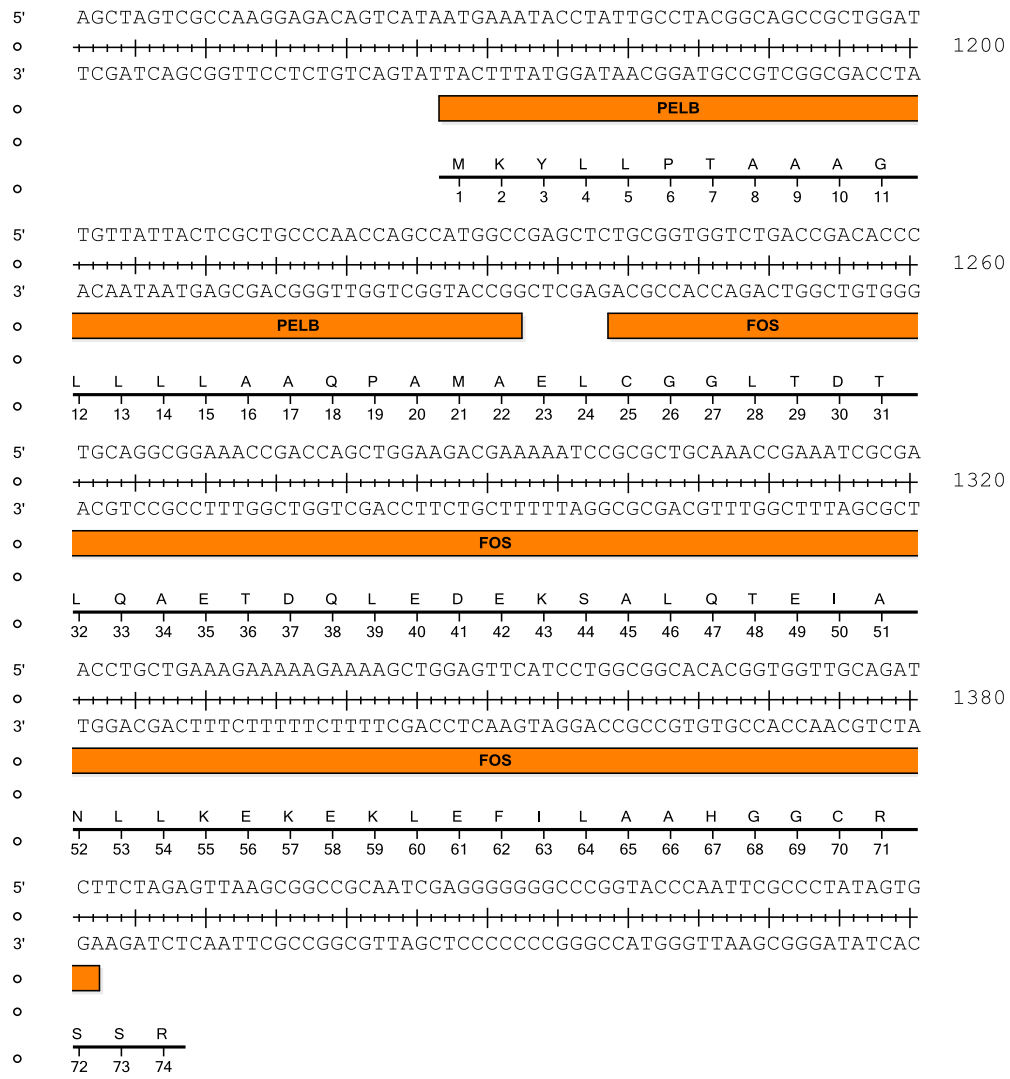


Figure 4.4: Detail of the cloning site of pJuFo.

The sequence shows the PelB and Fos sequences (DNA and amino acid) that form fusions with cDNAs cloned in the *Xba*I (TCTAGA)-*Kpn*I (GGTACC) cloning site. The *Xba*I site starta at nucleotide 1383. The *Kpn*I site starts at nucleotide 1418.

4.3.5 Enrichment of the melanocyte peptide phage-display library (panning)

In panning experiments, a 10-µl aliquot of patient or control IgG was immobilised on the well of a 96-well microtitre plate in 50 µl of coating buffer composed of 3.5 mM sodium hydrogen carbonate and 1.5 mM sodium carbonate (pH 9.2). In addition, two specific antibodies α-PEP7 (anti-tyrosinase antibody) and α-PEP8 (anti-L-dopachrome tautomerase (DCT)) were used as positive controls. Prior to washing with PBS/0.05% (w/v) Tween 20 (PBS/Tween), plates were incubated for 2 h at room temperature to allow the binding of patient IgG to the well surface. The plate wells were then blocked using 400 µl of 2% (w/v) bovine serum albumin prepared in PBS in order to prevent non-specific phage from binding later in the process, and plates were subsequently incubated for a further 2 h at room temperature. The wells were then rinsed once with PBS/Tween prior to adding a 100-µl sample of phage-display library containing an estimation of 10^{11} phage. To permit the interaction of immobilised patient IgG with proteins displayed on phage surfaces, plates were kept at 4°C overnight. Wells were washed 10 times with PBS/Tween in order to remove unbound phage. Following elution of bound phage with 100 µl of acidified glycine (pH 2.2), eluted phage were neutralised with 9 µl of 2 M Tris-base and then used to infect a 2 ml sample of *E. coli* XL1-Blue MRF'. After, the infected cultures were kept for 15 min at room temperature to allow the phage to infect the cells. To assess the total number of eluted phage, infected culture samples (10 µl and 100 µl) were spread onto LB agar supplemented with tetracycline (10 µg/ml) and ampicillin (100 µg/ml), and plates were then incubated at 37°C overnight.

To prepare a phage-display library to use in the subsequent round of panning, 8 ml of LB medium was added to the sample of infected *E. coli* XL1-Blue MRF' cells with the eluted phage to make it up to 10 ml and then incubated shaking at 37°C for 1 h. The culture was subsequently superinfected with 10^{11} plaque-forming units (pfu) of VCS-M13 helper phage (Agilent Technologies) and left to incubate for 15 min at room temperature. The culture was then inoculated into 100 ml of LB medium supplemented with 10 µg/ml tetracycline and 100 µg/ml ampicillin. After further incubation with shaking at 37°C for 1 h, kanamycin at 10 µg/ml (Section 2.4) was added and culture was grown overnight at 37°C with shaking.

The bacterial culture was centrifuged for 15 min at 2,000 *g* to pellet cell debris. For phage precipitation from the culture supernatant, 40% (w/v) polyethylene glycol 4000 (15 ml) and 5 M sodium chloride (15 ml) were added and culture was then incubated overnight at 4°C. To harvest phage particles, the culture was centrifuged at 10,000 *g* for 20 min and subsequently the pellet of phage was resuspended in PBS (1-2 ml) and stored at -20°C until use. This first round library was titrated and used subsequently in panning round 2 following the same way as the original library. For each sample of IgG, five panning rounds in total were carried out.

4.3.6 Analysis of the panned phage-display library

Following the fifth panning round with patient IgG, randomly selected individual colonies of bacterial cells, isolated by plating out the infected *E. coli* XL1-Blue MRF' culture with eluted phage particles, were streaked onto fresh LB agar supplemented with ampicillin at 100 µg/ml and tetracycline at 10 µg/ml. The bacterial clones were then grown shaking overnight at 37°C in 10 ml of LB medium to which 100 µg/ml ampicillin and 10 µg/ml tetracycline were added. Subsequently, phagemid DNA preparation was made from each culture employing a Wizard® Plus SV Minipreps DNA Purification System (Section 2.14).

To identify melanocyte peptides enriched during the panning process, 30 phagemid DNAs carrying peptide-encoding cDNAs were sequenced. Sequencing primers used were JUFO-1192 (5'-CCGCTGGATTGTTATTACTCGCTG-3') and JUFO-1500 (5'-TGCAAGGCGATTAAGTTGGGTAAC-3'), which bind to the pJuFo cloning vector upstream of the cDNA cloning site and downstream of the cDNA cloning site, respectively (Figure 4.2). The cDNAs were identified by comparison with the international genetic sequence database GenBank employing the BLAST service of the NCBI (Bethesda, MD, USA).

4.4 Results

4.4.1 Screening of the melanocyte peptide phage-display library with vitiligo patient IgG, IgG from healthy controls, and positive control antibodies

The melanocyte peptide phage-display library in the vector pJuFo (Figure 4.2) was screened in panning experiments with IgG prepared from 22 vitiligo patients and two healthy controls. In addition, two specific antibodies α-PEP7 (anti-tyrosinase antibody) and α-PEP8 (anti-DCT antibody) were used as positive controls in panning experiments. Five rounds of panning were performed for each individual IgG and postive control antibody. In each round, the number of phage applied to the microtitre plate well and the number of phage eluted were calculated and the ratio of eluted to applied phage determined. The ratio at each round was normalised against the ratio in round one of panning.

The results for the two positive control antibodies α-PEP7 and α-PEP8 are given in Figure 4.5. An increase in the normalised ratio was seen through the panning rounds, indicating the enrichment of phage expressing specific IgG-binding proteins on their surfaces. The results for each of the 22 vitiligo patients and two healthy controls are shown in Figure 4.6. In experiments with 13 of the vitiligo patient IgGs, the normalised ratio was higher by the fifth round of panning, indicating enrichment of the phage-display library. However, in the remaining cases, including both healthy control IgGs, there was either a decrease or no change in the normalised ratio.

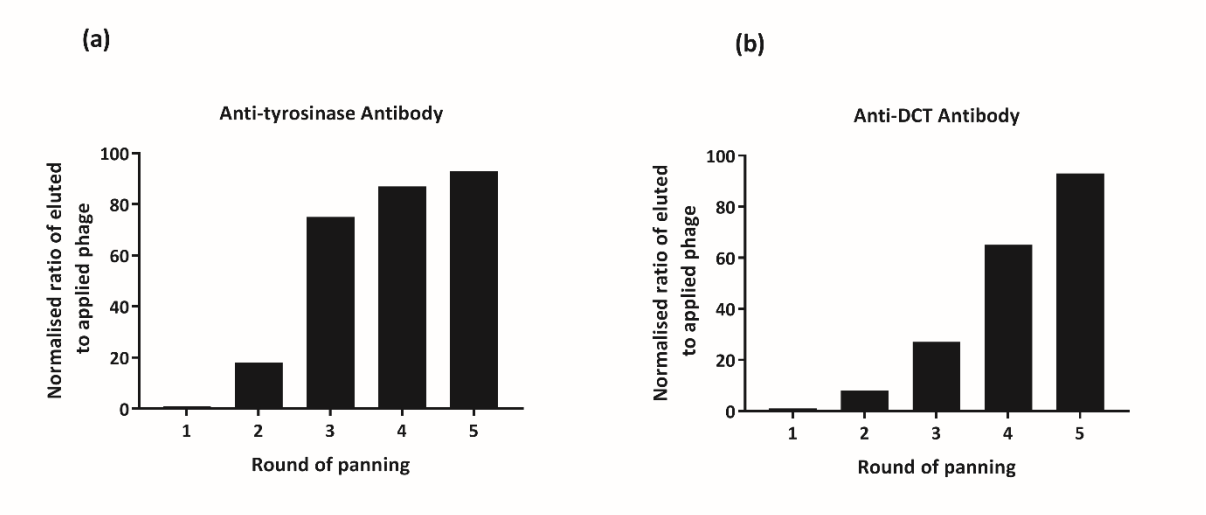
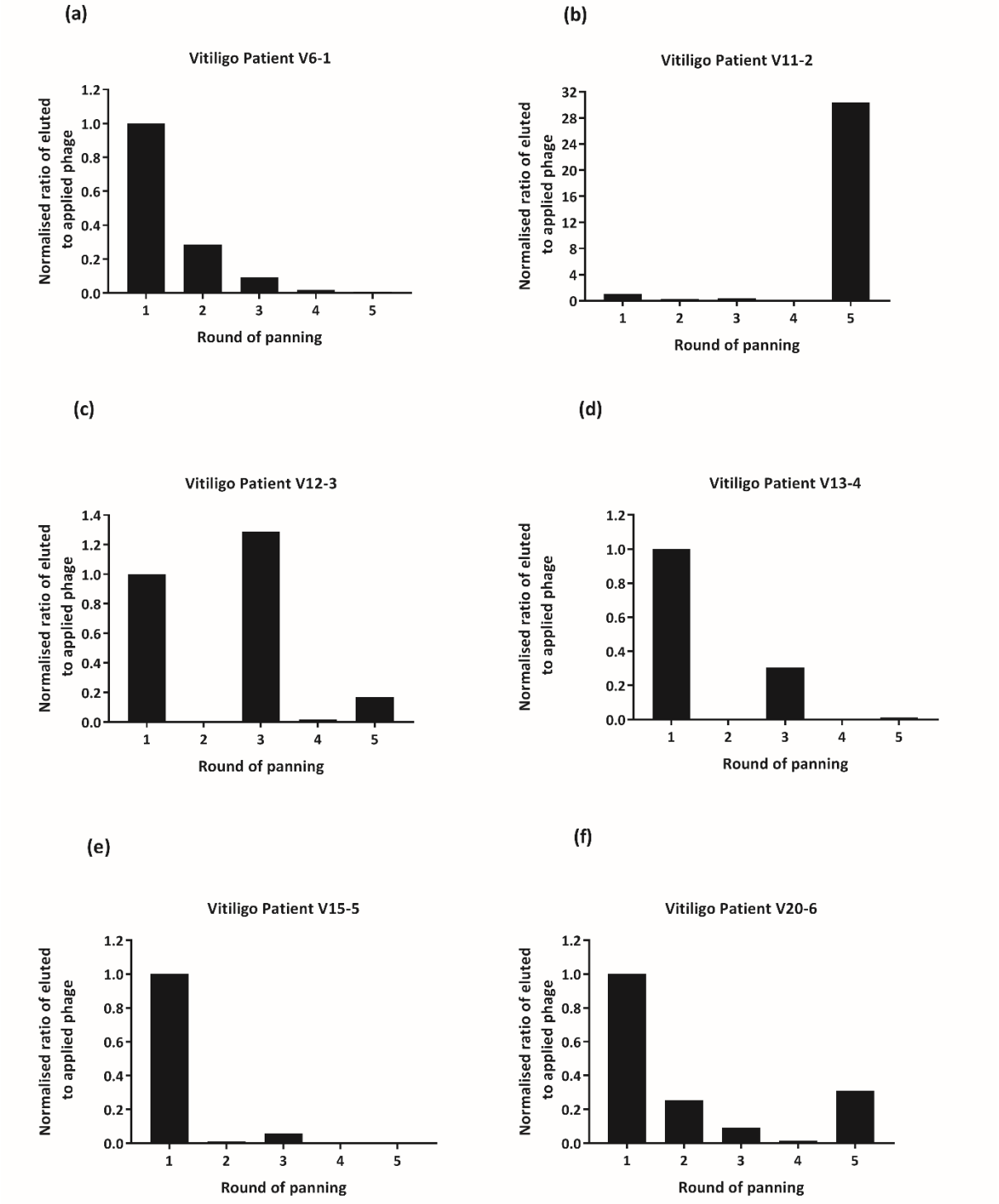
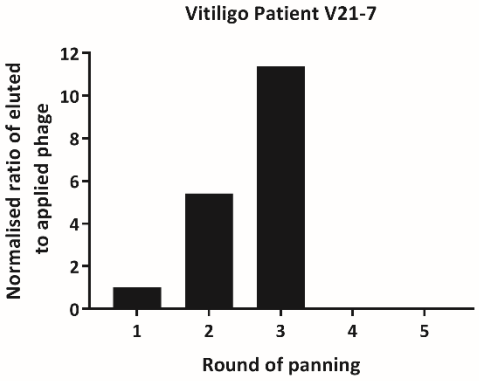


Figure 4.5: Enrichment of the melanocyte peptide phage-display library with anti-tyrosinase and anti-DCT antibodies.

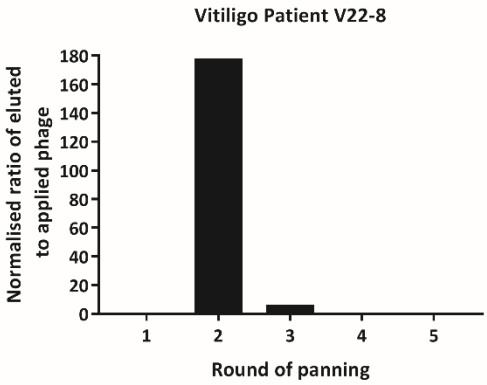
The melanocyte cDNA-encoded peptide phage-display library was used in five rounds of enrichment with anti-tyrosinase antibody α-PEP7 and anti-DCT antibody α-PEP8. The results are given as a fold increase in the ratio of eluted to applied phage in each subsequent panning round with respect to panning round 1. DCT, L-dopachrome tautomerase.


(a)
Vitiligo Patient V6-1
Normalised ratio of eluted to applied phage
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
Round of panning
(b)
Vitiligo Patient V11-2
Normalised ratio of eluted to applied phage
32
28
24
20
16
12
8
4
0
1
2
3
4
5
Round of panning
(c)
Vitiligo Patient V12-3
Normalised ratio of eluted to applied phage
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
Round of panning
(d)
Vitiligo Patient V13-4
Normalised ratio of eluted to applied phage
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
Round of panning
(e)
Vitiligo Patient V15-5
Normalised ratio of eluted to applied phage
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
Round of panning
(f)
Vitiligo Patient V20-6
Normalised ratio of eluted to applied phage
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
Round of panning

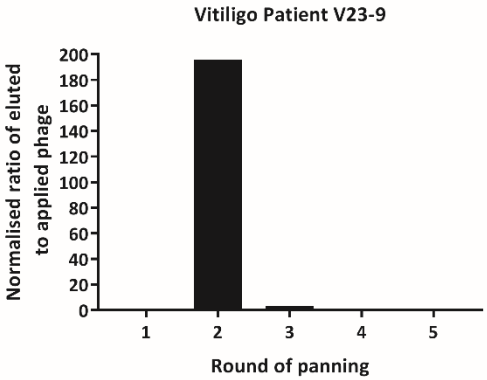
(g)


Vitiligo Patient V21-7
Normalised ratio of eluted to applied phage
0
2
4
6
8
10
12
1
2
3
4
5
Round of panning

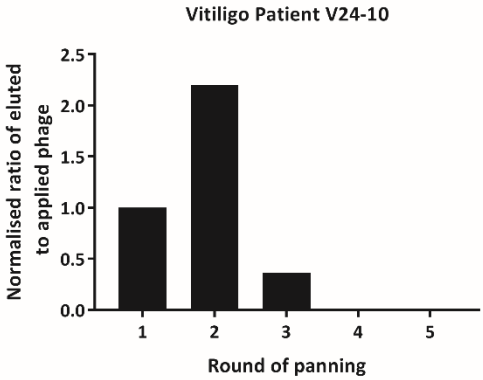
(h)


Vitiligo Patient V22-8
Normalised ratio of eluted to applied phage
0
20
40
60
80
100
120
140
160
180
1
2
3
4
5
Round of panning

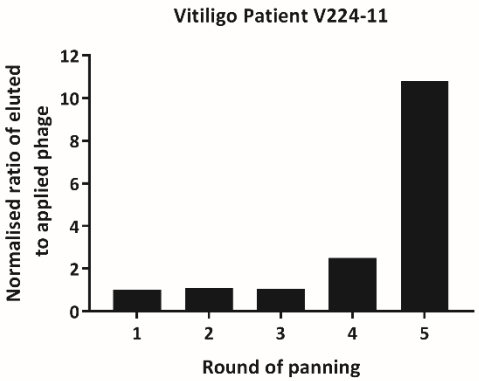
(i)


Vitiligo Patient V23-9
Normalised ratio of eluted to applied phage
0
20
40
60
80
100
120
140
160
180
200
1
2
3
4
5
Round of panning

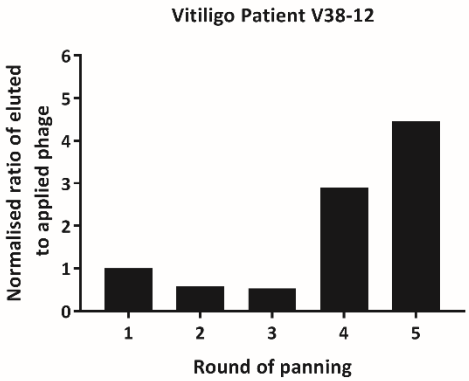
(j)


Vitiligo Patient V24-10
Normalised ratio of eluted to applied phage
0.0
0.5
1.0
1.5
2.0
2.5
1
2
3
4
5
Round of panning

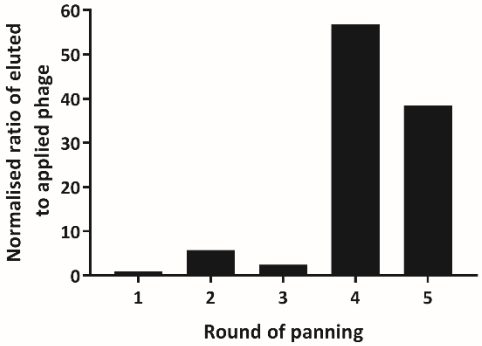
(k)


Vitiligo Patient V224-11
Normalised ratio of eluted to applied phage
0
2
4
6
8
10
12
1
2
3
4
5
Round of panning

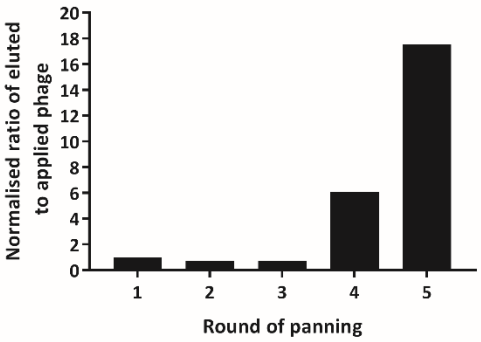
(l)


Vitiligo Patient V38-12
Normalised ratio of eluted to applied phage
0
1
2
3
4
5
6
1
2
3
4
5
Round of panning

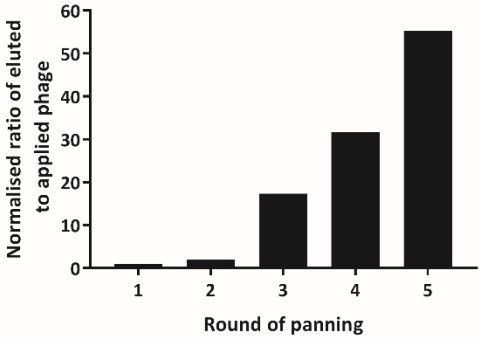
(m)


Vitiligo Patient V56-13
Normalised ratio of eluted to applied phage
0
10
20
30
40
50
60
1
2
3
4
5
Round of panning

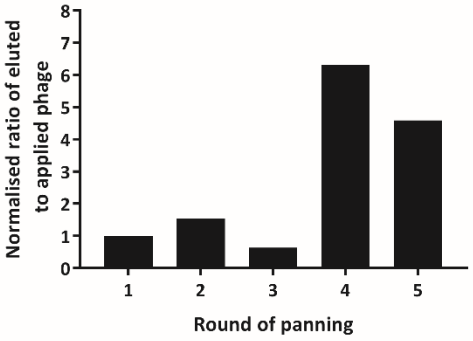
(n)


Vitiligo Patient V73-14
Normalised ratio of eluted to applied phage
0
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5
Round of panning

(O)


Vitiligo Patient V102-15
Normalised ratio of eluted to applied phage
0
10
20
30
40
50
60
1
2
3
4
5
Round of panning

(p)


Vitiligo Patient V169-16
Normalised ratio of eluted to applied phage
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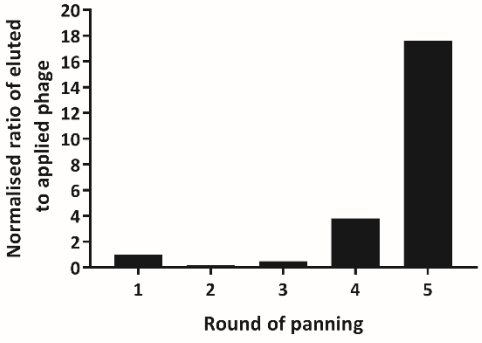
1
2
3
4
5
6
7
8
1
2
3
4
5
Round of panning

(q)


Vitiligo Patient V189-17
Normalised ratio of eluted to applied phage
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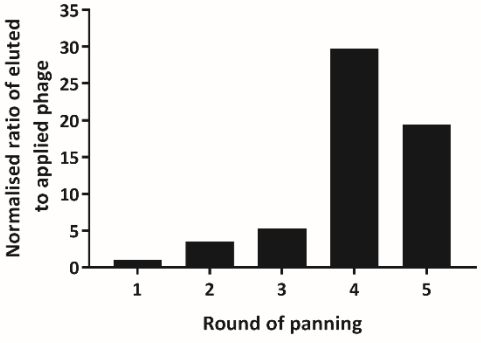
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5
Round of panning

(r)


Vitiligo Patient V216-18
Normalised ratio of eluted to applied phage
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5
10
15
20
25
30
35
1
2
3
4
5
Round of panning

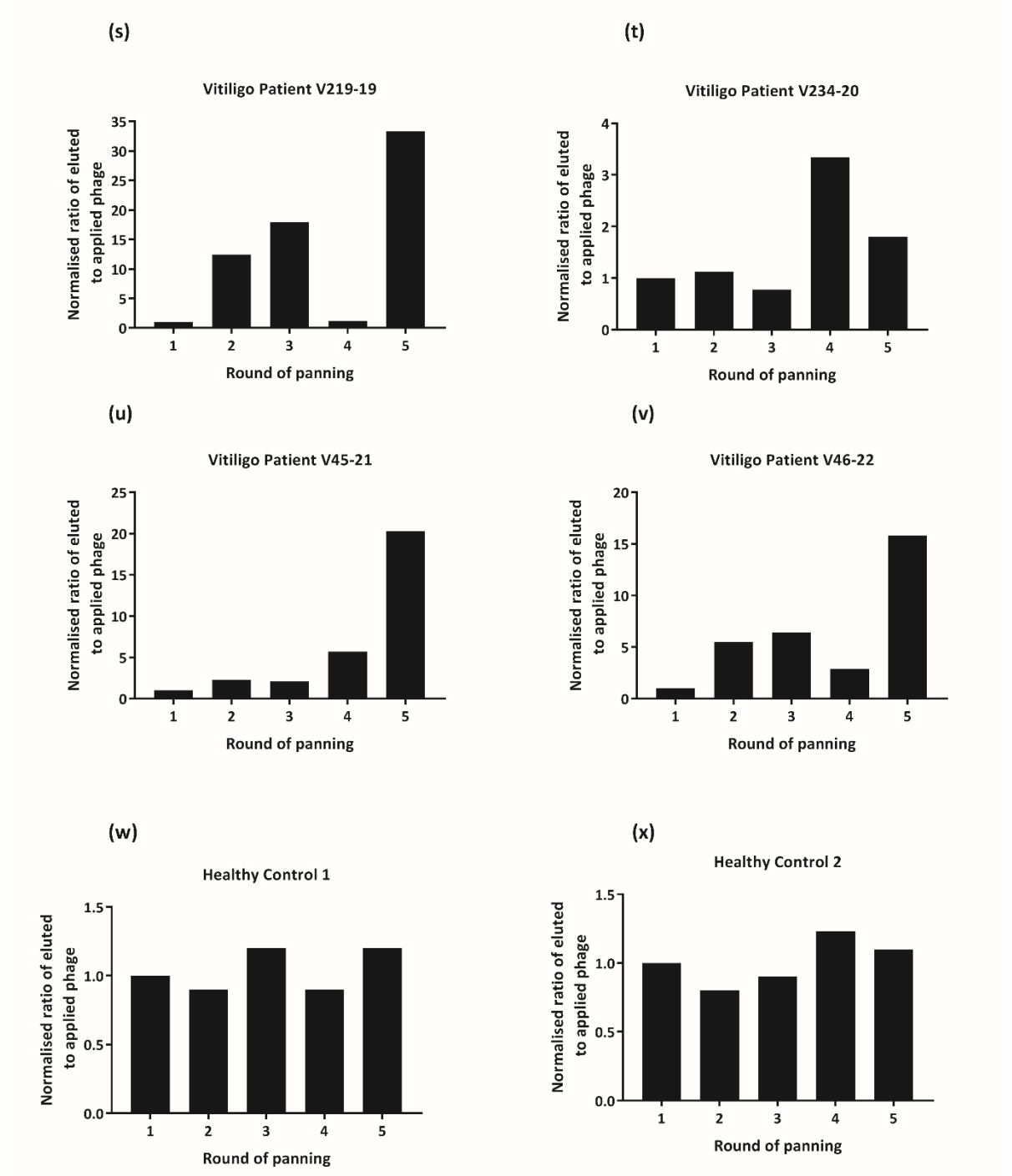


Figure 4.6: Enrichment of the melanocyte peptide phage-display library with vitiligo patient and healthy control IgG samples.

The melanocyte cDNA-encoded peptide phage-display library was used in five rounds of enrichment with IgG from 22 vitiligo patients and from two controls individually. The results are given as a fold increase in the ratio of eluted to applied phage in each subsequent panning round with respect to panning round 1.

4.4.2 Identification of IgG-binding peptides enriched by screening of the melanocyte peptide phage-display library

Following five rounds of panning with each of the 22 vitiligo patient IgG, two healthy control IgG, and two positive control antibodies α-PEP7 (anti-tyrosinase antibody) and α-PEP8 (anti-DCT antibody), 64 randomly selected bacterial clones were grown on LB agar supplemented with antibiotics. Subsequently, 30-50 of the bacterial clones were inoculated into 10 ml of fresh LB media containing 100 μg/ml of ampicillin and tetracycline at 10 μg/ml, and grown overnight in the shaking incubator at 37°C. pJuFo phagemid DNA was prepared from the 30-50 clones and each phagemid subjected to DNA sequencing.

Using BLAST searches of the acquired DNA sequences against the GenBank database, the results of panning experiments with the two positive control antibodies indicated that α-PEP7 (anti-tyrosinase antibody) and α-PEP8 (anti-DCT antibody) enriched tyrosinase and DCT peptides, respectively. In-frame, tyrosinase peptides represented 48/50 sequences (96%) after panning the phage-display library with antibody α-PEP7. In-frame, DCT peptides represented 49/50 sequences (98%) after enrichment of the phage-display library using α-PEP8 as the antibody. The sequences enriched by the two healthy control IgGs were in for the most part out of frame or no significant matches. For one healthy control IgG, none of the peptide sequences in the final panning round were in-frame peptides and 28/30 (93%) had no significant matches to sequences in the GenBank database. For the second healthy control IgG, only one of the peptide sequences was in-frame and identifiable as HSP70.

In the case of the vitiligo patient IgG samples, the results showed that cDNAs encoding several different peptides had been isolated from the panning experiments. These are detailed in the next sections, and in Figure 4.7 and Tables 4.2 to 4.23.

4.4.2.1 Vitiligo patient V6-1

The BLAST analysis of the 30 cDNAs encoded by the enriched phage revealed that 24 of them (80%) showed homology with peptides present in the database and these were all in-frame with the Fos peptide encoded by the pJuFo vector such that they could be

expressed and displayed on the phage surface (Table 4.2). Of the 24, eight were found to encode tyrosinase and seven were identified as PMEL. The other detected IgG-binding peptides were GTP-binding protein Rab27A (Rab27A), heat-shock protein 90 (HSP90), and 39s ribosomal protein L24 (RPL24) which were represented five, two, and two times, respectively (Figure 4.7a). Of the remainder, four cDNAs encoded Rab27A, but these were not in-frame for display on the phage surface, and two of the encoded sequences showed no significant matches with DNA sequences in the database (Figure 4.7a).

4.4.2.2 Vitiligo patient V11-2

For vitiligo patient V11-2, the DNA sequences of 87% (26/30) of the analysed clones encoded identifiable peptides (Table 4.3). These included tyrosinase (4/30), TYRP1 (16/30), PMEL (2/30), HSP90 (3/30), and RPL24 (1/30) (Figure 4.7b). The remaining four cDNA sequences matched Rab27A, but each of these was in an alternative reading frame with respect to the Fos peptide in the pJuFo vector (Figure 4.7b).

4.4.2.3 Vitiligo patient V12-3

For vitiligo patient V12-3, 26/30 (87%) of the peptide sequences analysed were homologous with DNA sequences in the Genbank database and were in-frame with the pJuFo encoded Fos protein required for peptide expression on the phage surface (Table 4.4). These were glycoprotein non-metastatic melanoma protein b (GPNMB) (13/30), MC1R (4/30), tyrosinase (5/30), HSP90 (3/30), and Rab27A (1/30) (Figure 4.7c). One of the remaining four DNAs encoded Rab27A but this could not be expressed and displayed as fusion proteins on the phage particles due to an incorrect reading frame with the Fos protein encoded by the vector (Figure 4.7c). A further three had sequences without significant similarity to database sequences (Figure 4.7c).

4.4.2.4 Vitiligo patient V13-4

For vitiligo patient V13-4, 26 of 30 (87%) of the phage clones encoded identifiable peptides (Table 4.5). These were tyrosinase (11/30), TYRP1 (2/30), PMEL (1/30), HSP90 (3/26), DCT (3/26), Rab27A (3/10), and GPNMB (3/30) (Figure 4.7d). All 26 peptide

sequences were in-frame with the Fos protein expressed by the pJuFo vector. The remaining four phagemid carried DNA insert which did not show significant matches to any sequence in the database.

4.4.2.5 Vitiligo patient V15-5

For vitiligo patient V15-5, of the 30 clones sequenced, 26 (87%) cDNAs encoded peptides that matched to human sequences present in the database (Table 4.6).These were containing OCA2-encoded P protein (OCA2 protein) (12/30), tyrosinase (7/30), HSP90 (3/30), PMEL (2/30), Rab27A (1/30), and RPL24 (1/30) (Figure 4.7e). All 26 identified proteins were in-frame with Fos protein encoded by pJuFo that it is essential for expression of the proteins on the phage surface. The remaining 4 phagemid carried cDNA fragments that had no significant matches to sequences in the database.

4.4.2.6 Vitiligo patient V20-6

For vitiligo patient V20-6, 29/30 (97%) phage clones selected had identifiable peptide inserts except for one clone which did not have significant match to sequences in the database (Table 4.7). Peptide inserts of 15 phage clones matched tyrosinase, six contained HSP90 cDNA, four TYRP1, three Rab27A, and one RPL24 (Figure 4.7f). All 29 identified proteins were in correct frame with the Fos protein encoded by pJuFo vector.

4.4.2.7 Vitiligo patient V21-7

For vitiligo patient V21-7, all 30 (100%) clones selected for sequence analysis had a cloned cDNA insert in-frame with the pJuFo-encoded Fos protein (Table 4.8). These included TYRP1 (16/30), tyrosinase (4/30), HSP90 (5/30), and PMEL (5/ 30) (Figure 4.7g).

4.4.2.8 Vitiligo patient V22-8

For vitiligo patient V22-8, all 30 (100%) clones sequenced encoded identifiable peptides (Table 4.9). These were tyrosinase (6/30), HSP90 (4/30), RPL24 (2/30), OCA2 protein

(10/30), and GPNMB (8/30) (Figure 4.7h). All these identified DNA inserts were in-frame with Fos protein encoded by the vector pJuFo.

4.4.2.9 Vitiligo patient V23-9

For vitiligo patient V23-9, analysis of the 30 cDNA inserts showed that 24 (80%) encoded proteins of known function (Table 4.10). These included DCT (9/30), HSP90 (4/30), RPL24 (4/30), tyrosinase (2/30), MC1R (5/30) (Figure 4.7i). These proteins were all in-frame with the Fos protein encoded by the pJuFo. The remaining six phage clones had no significant matches to DNA sequences present in the database.

4.4.2.10 Vitiligo patient V24-10

For vitiligo patient V24-10, database matches were present for all 30 (100%) sequenced clones (Table 4.11). Eighteen clones showed homology with tyrosinase and the remaining 12 clones had homology with TYRP1 (Figure 4.7j). All 30 peptide sequences were in-frame with the Fos peptide encoded by the pJuFo.

4.4.2.11 Vitiligo patient V224-11

For vitiligo patient V224-11, of the 30 cloned sequences, 26 (87%) encoded proteins of known function that were all in-frame with the pJuFo-encoded Fos protein (Table 4.12). These proteins were PMEL (7/30), tyrosinase (4/30), HSP90 (4/30), Rab27A (2/30), and GPNMB (9/30) (Figure 4.7k). Four phagemid clones carried cDNA inserts that had no significant matches to sequences in the database.

4.4.2.12 Vitiligo patient V38-12

For vitiligo patient V38-12, 24 of 30 (80%) of the clones sequenced showed homology with cDNA sequences in the Genbank database (Table 4.13). Of these homologies, 7/30 corresponded to TYRP1, 5/30 to tyrosinase, 5/30 to HSP90, 3/30 to Rab27A, 2/30 to RPL24, and 2/30 to OCA2 protein (Figure 4.7l). All these 24 identified peptides were in-frame with the Fos protein encoded by the vector pJuFo. The remaining six phage clones

harboured cloned DNA which did not show any significant matches to sequences in the database.

4.4.2.13 Vitiligo patient V56-13

For vitligo patient V56-13, database homologies were found for all clones sequenced except for three (Table 4.14). Fourteen clones matched the coding sequence of PMEL, 6/30 the coding sequence of DCT, 4/30 HSP90, and 3/30 RPL24 (Figure 4.7m). All these peptides were in-frame with pJuFo encoded Fos peptide for expression on the phage surface.

4.4.2.14 Vitiligo patient V73-14

For vitiligo patient V73-14, of the 30 clones sequenced, 25 (83%) had homology with known proteins (Table 4.15). These were TYRP1 (10/30), tyrosinase (7/30), Rab27A (5/30), and HSP90 (3/30) (Figure 4.7n). All these were in the correct frame with the Fos peptide encoded by the pJuFo. The remaining five clones did not have significant matches with sequences in the database.

4.4.2.15 Vitiligo patient V102-15

For vitiligo patient V102-15, of the 30 phage clones sequenced, 25 (83%) carried DNA fragments which matched known sequences in the database (Table 4.16). The remaining five plasmid had cloned DNA which did not show significant homology with sequences in the database. Seven of the IgG-binding proteins were identified as TYRP1 (Figure 4.7o). Other proteins represented more than one time in the analysed sequences included tyrosinase (4/30), HSP90 (4/30), RPL24 (4/30), Rab27A (3/30), and OCA2 protein (3/30) (Figure 4.7o).

4.4.2.16 Vitiligo patient V169-16

For vitiligo patient V169-16, 24/30 (80%) of the sequenced insert cDNAs showed homology with DNA sequences in the database (Table 4.17). These isolated cDNAs

encoded several different proteins and each identified protein was represented more than once. IgG-binding peptides identified here were GPNMB (4/30), HSP90 (4/30), MC1R (4/30), Rab27A (4/30), RPL24 (3/30), tyrosinase (3/30), and DCT (2/30) (Figure 4.7p). All these proteins were in-frame with the Fos protein encoded by the vector. The remaining six phagemid clones contained DNA that had no significant matches to sequences in the database.

4.4.2.17 Vitiligo patient V189-17

For vitiligo patient V189-17, 27 of the 30 (90%) clones sequenced carried DNA fragments which had homology with known peptides (Table 4.18). These homologies corresponded to PMEL (10/30), HSP90 (6/30), tyrosinase (4/30), RPL24 (4/27), and OCA2 protein (3/30) (Figure 4.7q). Again, all these peptides were in frame with the Fos protein encoded by the pJuFo. Three of the 30 clones analysed showed no matches with sequences in the Genbank database.

4.4.2.18 Vitiligo patient V216-18

For vitiligo patient V216-18, 27/30 (90%) sequenced clones encoded identifiable proteins (Table 4.19). These included TYRP1 (8/30), Rab27A (6/30), HSP90 (5/30), tyrosinase (3/30), GPNMB (3/30) and MC1R (2/30) (Figure 4.7r). All 27 peptides were in-frame with the Fos protein encoded by the vector. The remaining three clones did not show any significant matches in the sequence analysis.

4.4.2.19 Vitiligo patient V219-19

For vitiligo patient V219-19, 25 of 30 (83%) clones identified as IgG-binding peptides (Table 4.20) were tyrosinase (12/30), DCT (5/30), Rab27A (3/30), RPL24 (2/30), HSP90 (2/30), and TYRP1 (1/30) (Figure 4.7s). All 25 proteins were in-frame with the Fos protein encoded by the pJuFo. The remaining five phagemid contained DNA which did not significantly match any sequence in the database.

4.4.2.20 Vitiligo patient V234-20

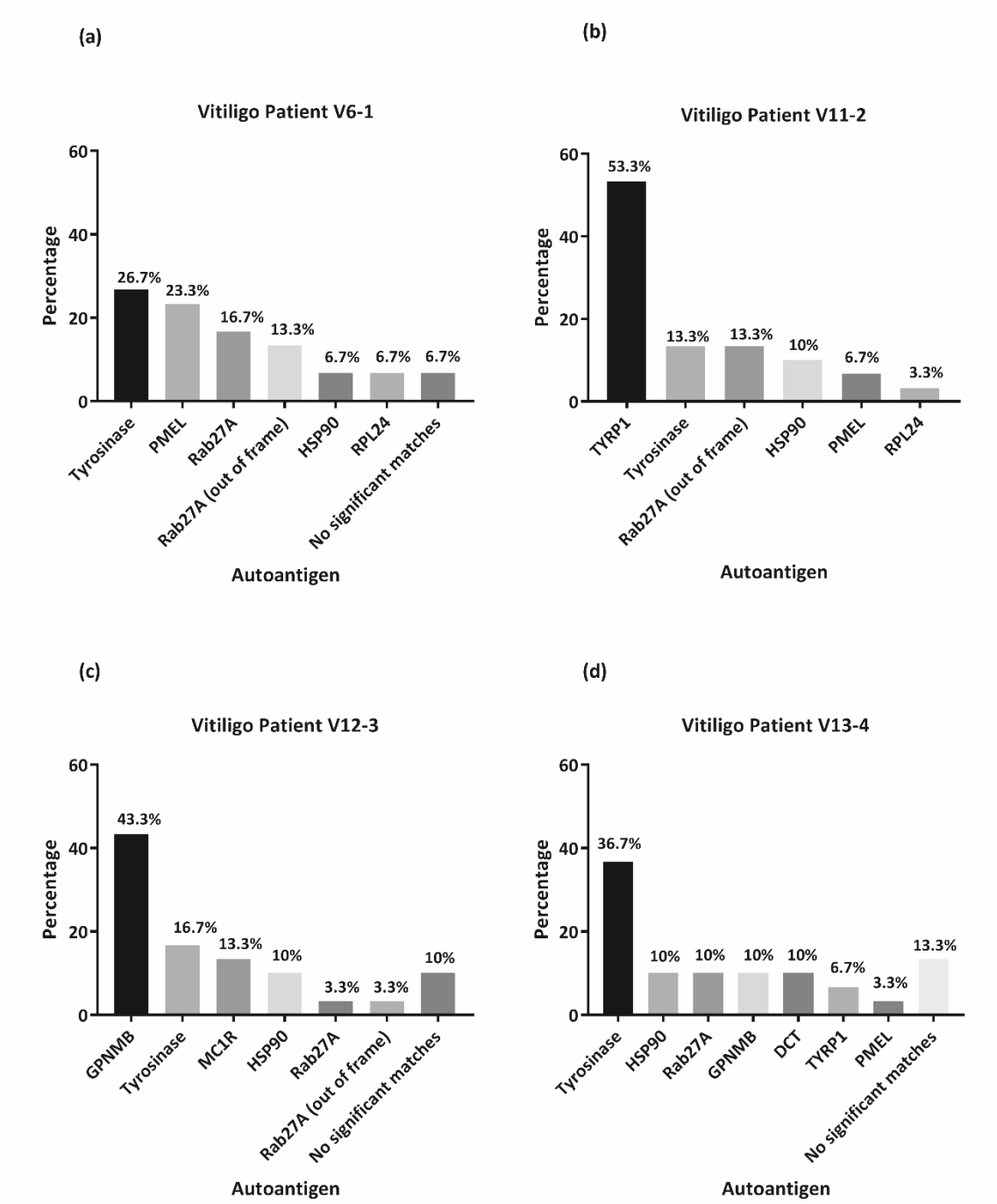
For vitiligo patient V234-20, of the 30 phage clones sequenced, 27/30 (90%) contained cloned DNA which matched identifiable proteins in the database (Table 4.21). These included GPNMB (10/30), OCA2 protein (8/30), MC1R (2/30), Rab27A (4/30), and HSP90 (3/30) (Figure 4.7t). All the 27 peptides were in-frame with the Fos protein encoded by the vector pJuFo. The remaining three clones contained DNA inserts with no homology to sequences in the database.

4.4.2.21 Vitiligo patient V45-21

For vitiligo patient V45-21, 26 of the 30 (87%) clones sequenced demonstrated homology with DNA sequences present in the database (Table 4.22). These were HSP90 (4/30), LaminA (10/30), OCA2 protein (8/30), and GPNMB (4/30) (Figure 4.7u). All these proteins were in-frame with the Fos protein encoded by the vector. Four clones harboured cDNA which had no significant matches with sequences in the Genbank database.

4.4.2.22 Vitiligo patient V46-22

For vitiligo patient V46-22, several different proteins were encoded by 27 out of the 30 (90%) clones (Table 4.23). These peptides included tyrosinase (9/30), TYRP1 (8/30), LaminA (4/30), HSP90 (3/30), and OCA2 protein (3/30) (Figure 4.7v). All these were in-frame with the Fos protein encoded by the pJuFo vector. The remaining three clones did not show any significant matches with DNA sequences in the database.


(a)
Vitiligo Patient V6-1
Percentage
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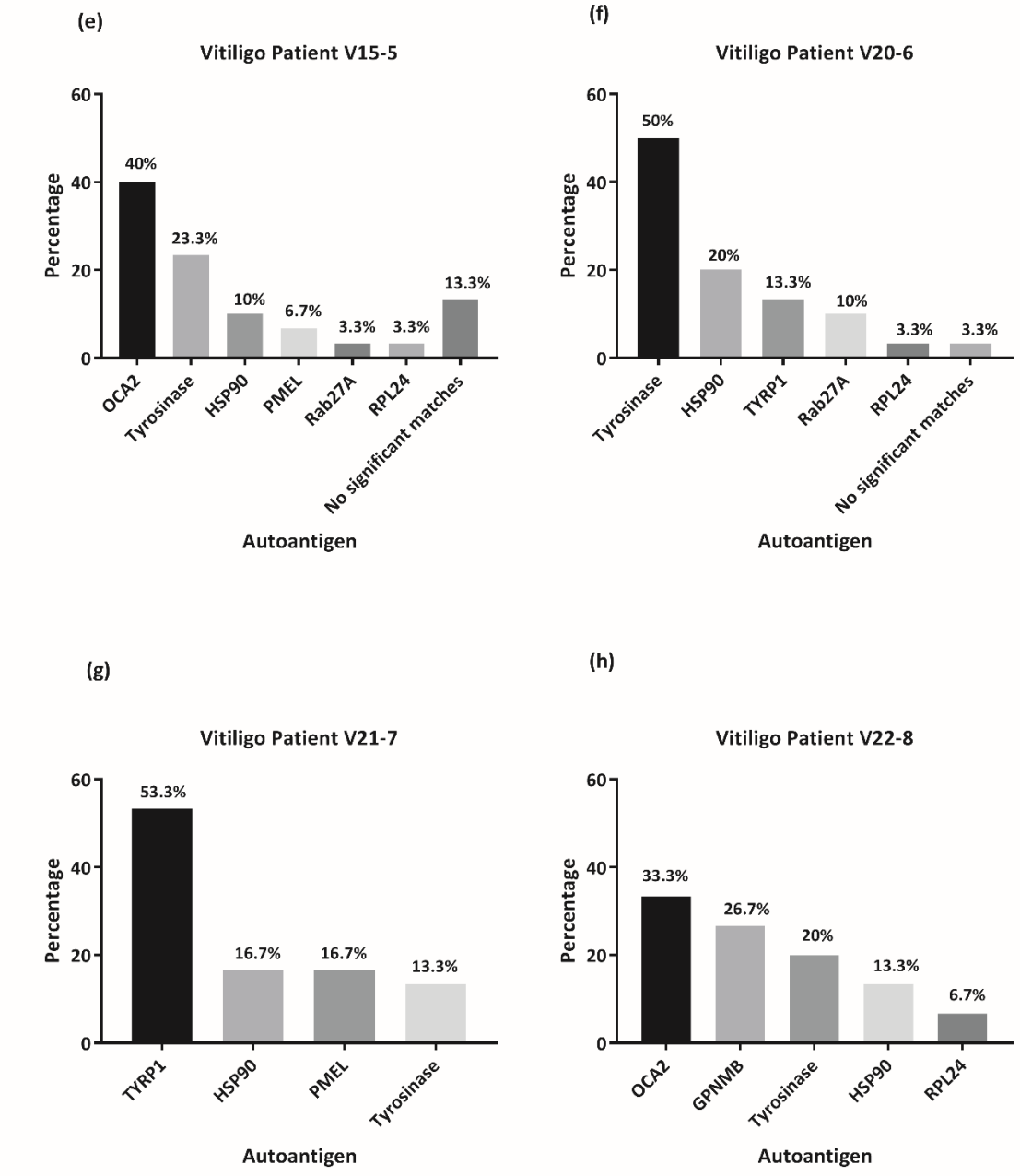
40
20
0
26.7%
23.3%
16.7%
13.3%
6.7%
6.7%
6.7%
Tyrosinase
PMEL
Rab27A
Rab27A (out of frame)
HSP90
RPL24
No significant matches
Autoantigen
(b)
Vitiligo Patient V11-2
Percentage
60
40
20
0
53.3%
13.3%
13.3%
10%
6.7%
3.3%
TYRP1
Tyrosinase
Rab27A (out of frame)
HSP90
PMEL
RPL24
Autoantigen
(c)
Vitiligo Patient V12-3
Percentage
60
40
20
0
43.3%
16.7%
13.3%
10%
3.3%
3.3%
10%
GPNMB
Tyrosinase
MC1R
HSP90
Rab27A
Rab27A (out of frame)
No significant matches
Autoantigen
(d)
Vitiligo Patient V13-4
Percentage
60
40
20
0
36.7%
10%
10%
10%
10%
6.7%
3.3%
13.3%
Tyrosinase
HSP90
Rab27A
GPNMB
DCT
TYRP1
PMEL
No significant matches
Autoantigen


(e)
Vitiligo Patient V15-5
Percentage
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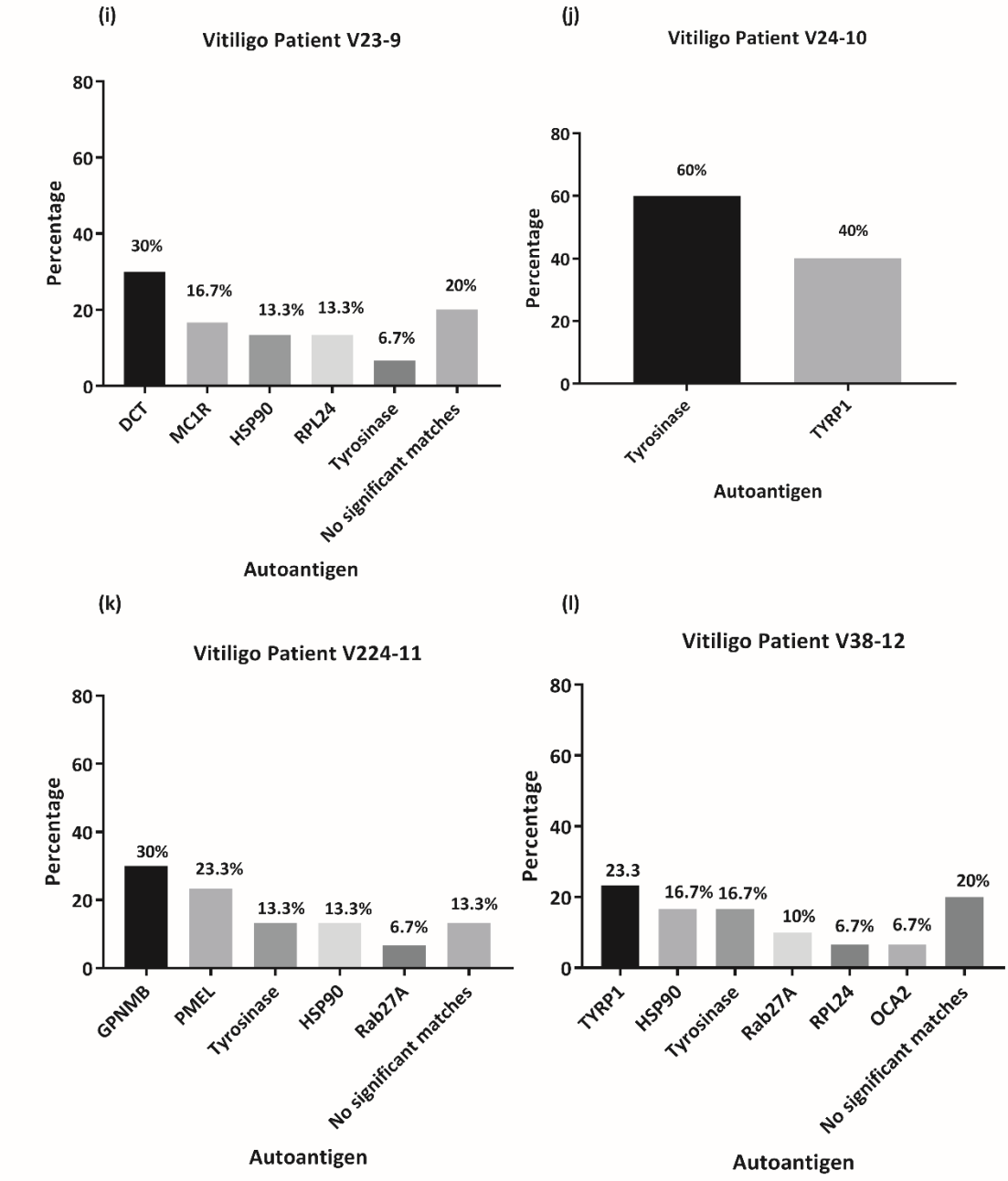
20
0
40%
23.3%
10%
6.7%
3.3%
3.3%
13.3%
OCA2
Tyrosinase
HSP90
PMEL
Rab27A
RPL24
No significant matches
Autoantigen
(f)
Vitiligo Patient V20-6
Percentage
60
40
20
0
50%
20%
13.3%
10%
3.3%
3.3%
Tyrosinase
HSP90
TYRP1
Rab27A
RPL24
No significant matches
Autoantigen
(g)
Vitiligo Patient V21-7
Percentage
60
40
20
0
53.3%
16.7%
16.7%
13.3%
TYRP1
HSP90
PMEL
Tyrosinase
Autoantigen
(h)
Vitiligo Patient V22-8
Percentage
60
40
20
0
33.3%
26.7%
20%
13.3%
6.7%
OCA2
GPNMB
Tyrosinase
HSP90
RPL24
Autoantigen


(i)
Vitiligo Patient V23-9
Percentage
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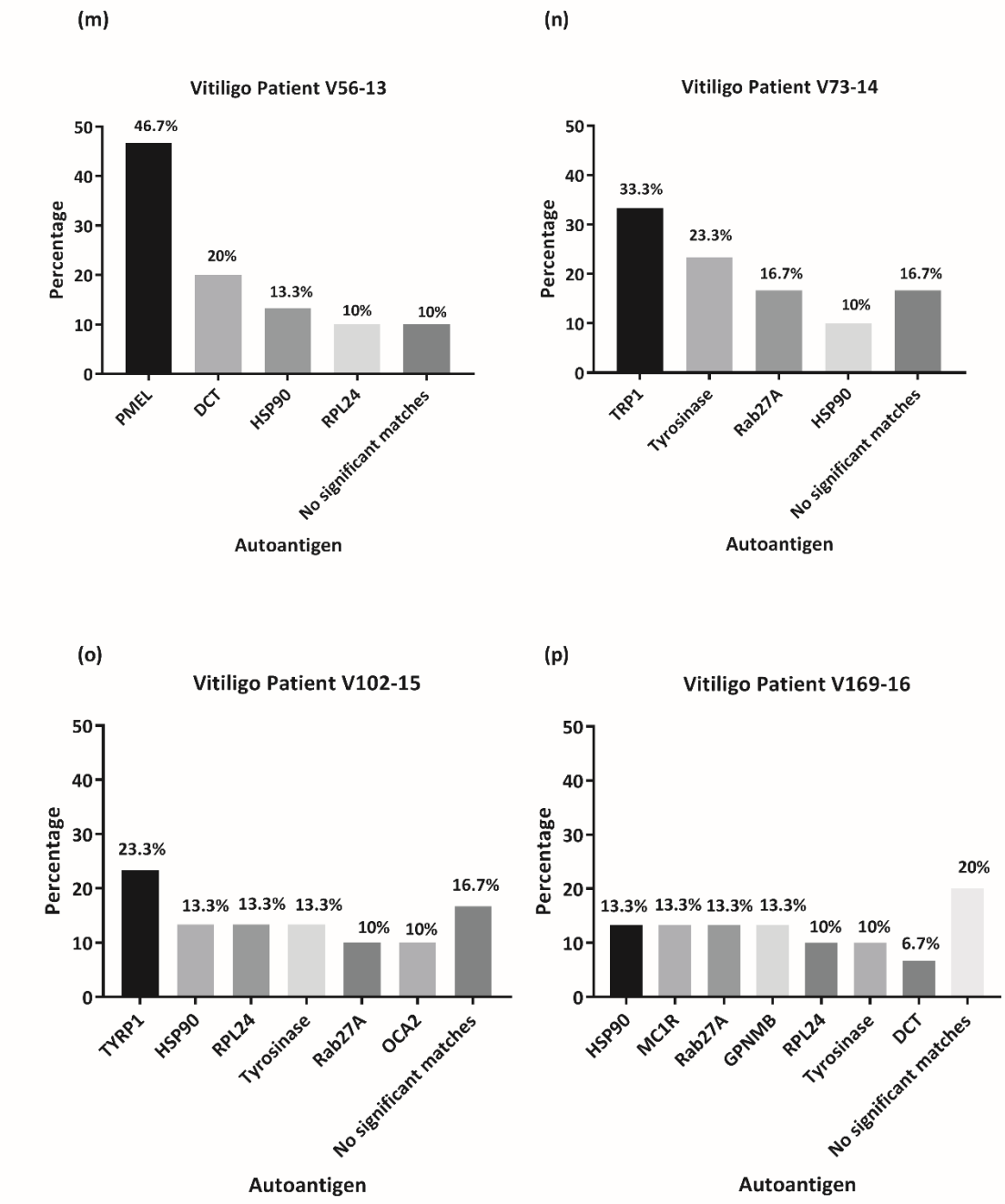
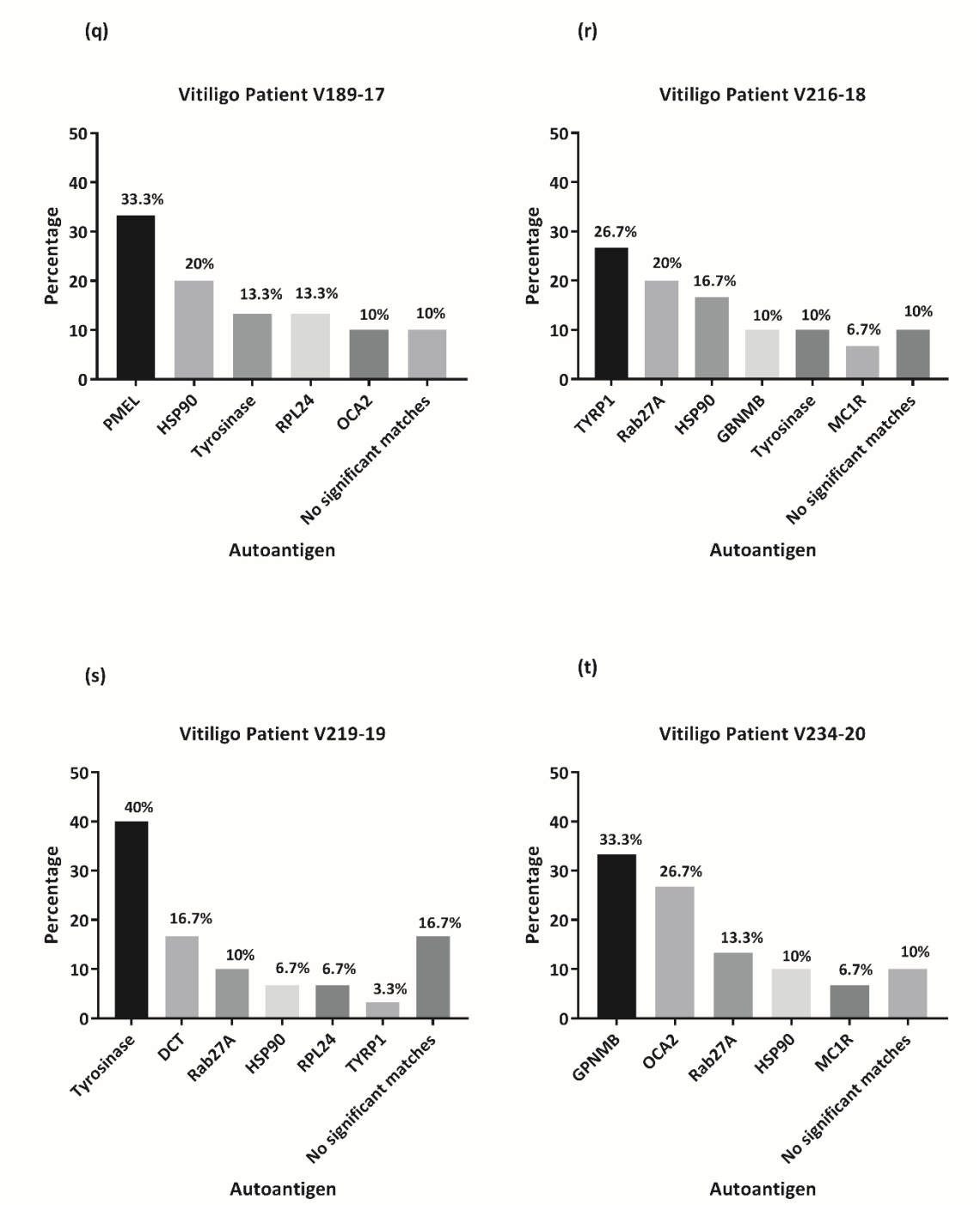
30%
16.7%
13.3%
13.3%
6.7%
20%
DCT
MC1R
HSP90
RPL24
Tyrosinase
No significant matches
Autoantigen
(j)
Vitiligo Patient V24-10
Percentage
80
60
40
20
0
60%
40%
Tyrosinase
TYRP1
Autoantigen
(k)
Vitiligo Patient V224-11
Percentage
80
60
40
20
0
30%
23.3%
13.3%
13.3%
6.7%
13.3%
GPNMB
PMEL
Tyrosinase
HSP90
Rab27A
No significant matches
Autoantigen
(l)
Vitiligo Patient V38-12
Percentage
80
60
40
20
0
23.3
16.7%
16.7%
10%
6.7%
6.7%
20%
TYRP1
HSP90
Tyrosinase
Rab27A
RPL24
OCA2
No significant matches
Autoantigen


(m)
Vitiligo Patient V56-13
Percentage
46.7%
20%
13.3%
10%
10%
PMEL
DCT
HSP90
RPL24
No significant matches
Autoantigen
(n)
Vitiligo Patient V73-14
Percentage
33.3%
23.3%
16.7%
10%
16.7%
TRP1
Tyrosinase
Rab27A
HSP90
No significant matches
Autoantigen
(o)
Vitiligo Patient V102-15
Percentage
23.3%
13.3%
13.3%
13.3%
10%
10%
16.7%
TYRP1
HSP90
RPL24
Tyrosinase
Rab27A
OCA2
No significant matches
Autoantigen
(p)
Vitiligo Patient V169-16
Percentage
13.3%
13.3%
13.3%
13.3%
10%
10%
6.7%
20%
HSP90
MC1R
Rab27A
GPNMB
RPL24
Tyrosinase
DCT
No significant matches
Autoantigen


(q)
Vitiligo Patient V189-17
Percentage
50
40
30
20
10
0
33.3%
20%
13.3%
13.3%
10%
10%
PMEL
HSP90
Tyrosinase
RPL24
OCA2
No significant matches
Autoantigen
(r)
Vitiligo Patient V216-18
Percentage
26.7%
20%
16.7%
10%
10%
6.7%
10%
TYRP1
Rab27A
HSP90
GBNMB
Tyrosinase
MC1R
No significant matches
Autoantigen
(s)
Vitiligo Patient V219-19
Percentage
40%
16.7%
10%
6.7%
6.7%
3.3%
16.7%
Tyrosinase
DCT
Rab27A
HSP90
RPL24
TYRP1
No significant matches
Autoantigen
(t)
Vitiligo Patient V234-20
Percentage
33.3%
26.7%
13.3%
10%
6.7%
10%
GPNMB
OCA2
Rab27A
HSP90
MC1R
No significant matches
Autoantigen

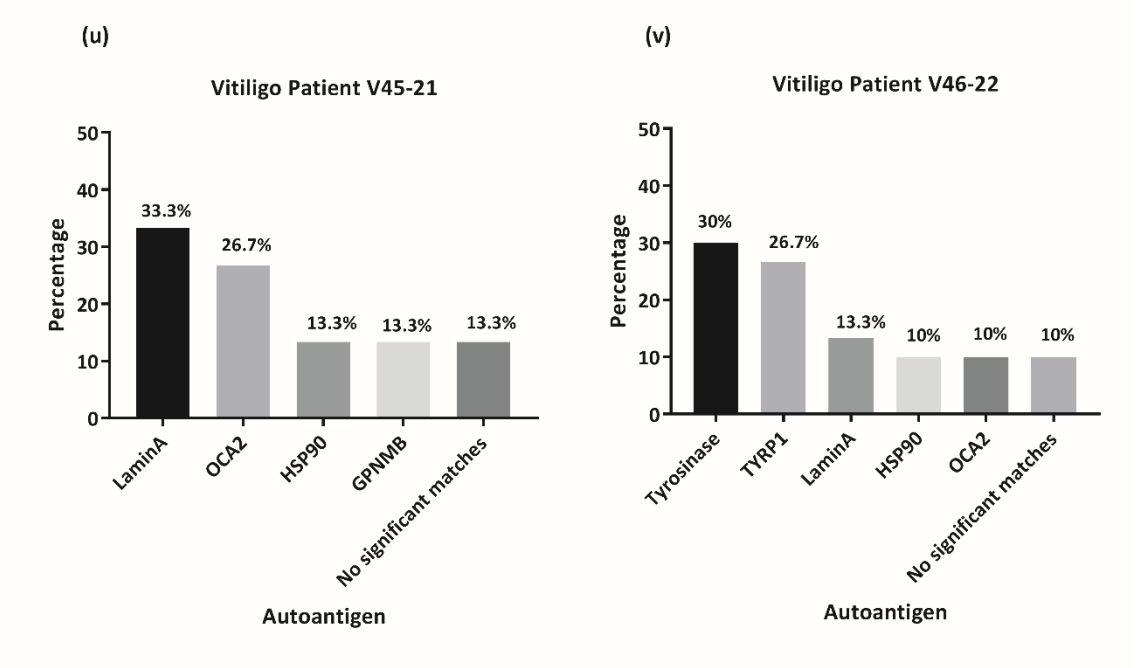


Figure 4.7: Enriched cDNA-encoded proteins isolated from panning of the melanoctye cDNA phage-display library with vitiligo patient IgG.

Phagemid DNA from 30 randomly selected bacterial clones were isolated from the fifth panning round with 22 vitiligo patient IgG individually. To identify if particular proteins had been enriched during the panning process, the DNA samples were subjected to DNA sequencing. GPNMB, glycoprotein non-metastatic melanoma protein b; HSP90, heat-shock protein 90; MC1R, melanocortin 1 receptor; PMEL, melanocyte-specific protein PMEL; OCA2, OCA2-encoded P protein; Rab27A, GTP-binding protein Rab27A; RPL24, 39s ribosomal protein L24; TYRP1, tyrosinase-related protein 1; DCT, L-dopachrome tautomerase.

Table 4.2: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V6-1

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V6-1.R5.1	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V6-1.R5.2	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V6-1.R5.3	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V6-1.R5.4	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V6-1.R5.5	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V6-1.R5.6	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V6-1.R5.7	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V6-1.R5.8	GTP-binding proteinRab27A	NM_004580.4	104-221	104-221	No
V6-1.R5.9	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V6-1.R5.10	No significant similarity found	-	-	-	-
V6-1.R5.11	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V6-1.R5.12	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V6-1.R5.13	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V6-1.R5.14	GTP-binding protein Rab27A	NM_004580.4	104-221	104-221	No
V6-1.R5.15	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V6-1.R5.16	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V6-1.R5.17	GTP-binding protein Rab27A	NM_004580.4	104-221	104-221	No
V6-1.R5.18	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V6-1.R5.19	Melanocyte-specific protein PMEL	NM_001200054.1	28-2004	10-668	Yes
V6-1.R5.20	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V6-1.R5.21	Tyrosinase	NM_000372.4	46-1587	16-529	Yes
V6-1.R5.22	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V6-1.R5.23	GTP-binding protein Rab27A	NM_004580.4	10-663	4-221	Yes
V6-1.R5.24	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V6-1.R5.25	No significant similarity found	-	-	-	-
V6-1.R5.26	Melanocyte-specific protein PMEL	NM_001200054.1	31-2004	11-668	Yes
V6-1.R5.27	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V6-1.R5.28	GTP-binding protein Rab27A	NM_004580.4	104-221	104-221	No
V6-1.R5.29	Tyrosinase	NM_000372.4	55-1587	19-529	Yes
V6-1.R5.30	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes

[1]Patient number.panning round.clone number.

Table 4.3 Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V11-2

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V11-2.R5.1	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V11-2.R5.2	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V11-2.R5.3	Tyrosinase-related protein 1	NM_000550.2	19-1611	7-537	Yes
V11-2.R5.4	Tyrosinase-related protein 1	NM_000550.2	43-1611	15-537	Yes
V11-2.R5.5	Heat-shock protein 90	NM_005348.3	2260-2562	754-854	Yes
V11-2.R5.6	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V11-2.R5.7	Tyrosinase-related protein 1	NM_000550.2	28-1611	10-537	Yes
V11-2.R5.8	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V11-2.R5.9	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V11-2.R5.10	Heat-shock protein 90	NM_005348.3	2260-2562	754-854	Yes
V11-2.R5.11	Melanocyte-specific protein PMEL	NM_001200054.1	31-2004	11-668	Yes
V11-2.R5.12	Tyrosinase-related protein 1	NM_000550.2	31-1611	11-537	Yes
V11-2.R5.13	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V11-2.R5.14	GTP-binding protein Rab27A	NM_004580.4	316-663	106-221	No
V11-2.R5.15	Melanocyte-specific protein PMEL	NM_001200054.1	25-2004	9-668	Yes
V11-2.R5.16	Tyrosinase-related protein 1	NM_000550.2	37-1611	13-537	Yes
V11-2.R5.17	Tyrosinase-related protein 1	NM_000550.2	37-1611	13-537	Yes
V11-2.R5.18	GTP-binding protein Rab27A	NM_004580.4	322-663	108-221	No
V11-2.R5.19	Heat-shock protein 90	NM_005348.3	2260-2562	754-854	Yes
V11-2.R5.20	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V11-2.R5.21	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V11-2.R5.22	Tyrosinase-related protein 1	NM_000550.2	34-1611	12-537	Yes
V11-2.R5.23	Tyrosinase-related protein 1	NM_000550.2	49-1611	17-537	Yes
V11-2.R5.24	Tyrosinase	NM_000372.4	76-1587	26-529	Yes
V11-2.R5.25	Tyrosinase-related protein 1	NM_000550.2	64-1611	22-537	Yes
V11-2.R5.26	GTP-binding protein Rab27A	NM_005348.3	2272-2562	758-854	No
V11-2.R5.27	Tyrosinase-related protein 1	NM_000550.2	7-1611	3-537	Yes
V11-2.R5.28	Tyrosinase-related protein 1	NM_000550.2	31-1611	11-537	Yes
V11-2.R5.29	GTP-binding protein Rab27A	NM_004580.4	331-663	111-221	No
V11-2.R5.30	Tyrosinase	NM_000372.4	40-1587	14-529	Yes

[1]Patient number.panning round.clone number.

Table 4.4: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V12-3

Clone numbe[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V12-3.R5.1	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V12-3.R5.2	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V12-3.R5.3	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V12-3.R5.4	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V12-3.R5.5	Melanocortin 1 receptor	NM_002386.3	34-951	12-317	Yes
V12-3.R5.6	Melanocortin 1 receptor	NM_002386.3	37-951	13-317	Yes
V12-3.R5.7	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	37-1716	13-527	Yes
V12-3.R5.8	Melanocortin 1 receptor	NM_002386.3	1-951	1-317	Yes
V12-3.R5.9	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V12-3.R5.10	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	16-1716	6-572	Yes
V12-3.R5.11	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V12-3.R5.12	No significant similarity found	-	-	-	-
V12-3.R5.13	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V12-3.R5.14	Tyrosinase	NM_000372.4	13-1587	7-529	Yes
V12-3.R5.15	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V12-3.R5.16	No significant similarity found	-	-	-	-
V12-3.R5.17	Heat-shock protein 90	NM_005348.3	1895-2196	632-732	Yes
V12-3.R5.18	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V12-3.R5.19	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	46-1716	16-572	Yes
V12-3.R5.20	No significant similarity found	-	-	-	-
V12-3.R5.21	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V12-3.R5.22	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V12-3.R5.23	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	28-1719	10-572	Yes
V12-3.R5.24	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V12-3.R5.25	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V12-3.R5.26	Melanocortin 1 receptor	NM_002386.3	1-951	1-317	Yes
V12-3.R5.27	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	37-1716	13-572	Yes
V12-3.R5.28	GTP-binding protein Rab27A	NM_004580.4	310-663	104-221	No
V12-3.R5.29	Glycoprotein non-metastatic melanoma protein b	NP_001005340.1	124-1719	42-572	Yes
V12-3.R5.30	Tyrosinase	NM_000372.4	61-1587	21-529	Yes

[1]Patient number.panning round.clone number.

Table 4.5: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V13-4

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V13-4.R5.1	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V13-4.R5.2	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V13-4.R5.3	No significant similarity found	-	-	-	-
V13-4.R5.4	Tyrosinase-related protein 1	NM_000550.2	7-1611	3-537	Yes
V13-4.R5.5	Tyrosinase	NM_000372.4	139-1587	47-529	Yes
V13-4.R5.6	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V13-4.R5.7	Tyrosinase	NM_000372.4	49-1587		Yes
V13-4.R5.8	L-dopachrome tautomerase	NM_001129889.2	1-1656	1-552	Yes
V13-4.R5.9	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V13-4.R5.10	No significant similarity found	-	-	-	-
V13-4.R5.11	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V13-4.R5.12	Tyrosinase	NM_000372.4	28-1587	10-529	Yes
V13-4.R5.13	Tyrosinase	NM_000372.4	82-1590	28-529	Yes
V13-4.R5.14	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V13-4.R5.15	Tyrosinase	NM_000372.4	49-1587	17-529	Yes
V13-4.R5.16	Tyrosinase	NM_000372.4	49-1587	17-529	Yes
V13-4.R5.17	L-dopachrome tautomerase	NM_001129889.2	10-1656	4-552	Yes
V13-4.R5.18	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V13-4.R5.19	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V13-4.R5.20	L-dopachrome tautomerase	NM_001129889.2	22-1659	8-552	Yes
V13-4.R5.21	Heat shock protein 90	NM_005348.3	1900-2196	634-732	Yes
V13-4.R5.22	Melanocyte-specific protein PMEL	NM_001200054.1	31-2004	11-668	Yes
V13-4.R5.23	Ras-related protein Rab27A	NM_004580.4	1-663	1-221	Yes
V13-4.R5.24	No significant similarity found	-	-	-	-
V13-4.R5.25	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V13-4.R5.26	No significant similarity found	-	-	-	-
V13-4.R5.27	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V13-4.R5.28	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V13-4.R5.29	Tyrosinase	NM_000372.4	25-1587	9-529	Yes
V13-4.R5.30	Tyrosinase	NM_000372.4	46-1587	16-529	Yes

[1]Patient number.panning round.clone number.

Table 4.6: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V15-5

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V15-5.R5.1	Heat-shock protein 90	NM_005348.3	1906-2196	636-732	Yes
V15-5.R5.2	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V15-5.R5.3	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V15-5.R5.4	Tyrosinase	NM_000372.4	46-1587	16-529	Yes
V15-5.R5.5	No significant similarity found	-	-	-	-
V15-5.R5.6	OCA2-encoded P protein	NM_001300984.1	22-2442	8-814	Yes
V15-5.R5.7	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V15-5.R5.8	OCA2-encoded P protein	NM_001300984.1	28-2442	10-814	Yes
V15-5.R5.9	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V15-5.R5.10	No significant similarity found	-	-	-	-
V15-5.R5.11	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V15-5.R5.12	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V15-5.R5.13	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V15-5.R5.14	No significant similarity found	-	-	-	-
V15-5.R5.15	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V15-5.R5.16	Melanocyte- specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V15-5.R5.17	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V15-5.R5.18	OCA2-encoded P protein	NM_001300984.1	10-2442	4-814	Yes
V15-5.R5.19	No significant similarity found	-	-	-	-
V15-5.R5.20	OCA2-encoded P protein	NM_001300984.1	13-2442	5-814	Yes
V15-5.R5.21	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V15-5.R5.22	OCA2-encoded P protein	NM_001300984.1	22-2442	8-814	Yes
V15-5.R5.23	Heat-shock protein 90	NM_005348.3	1900-2196	634-732	Yes
V15-5.R5.24	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V15-5.R5.25	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V15-5.R5.26	Tyrosinase	NM_000372.4	58-1587	20-529	Yes
V15-5.R5.27	Tyrosinase	NM_000372.4	31-1587	11-529	Yes
V15-5.R5.28	OCA2-encoded P protein	NM_001300984.1	10-2443	4-814	Yes
V15-5.R5.29	OCA2-encoded P protein	NM_001300984.1	16-2442	6-814	Yes
V15-5.R5.30	Heat-shock protein 90	NM_005348.3	1906-2196	636-732	Yes

[1]Patient number.panning round.clone number.

Table 4.7: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V20-6

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V20-6.R5.1	Tyrosinase-related protein 1	NM_000550.2	13-1611	5-537	Yes
V20-6.R5.2	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V20-6.R5.3	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.4	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V20-6.R5.5	Tyrosinase	NM_000372.4	43-1587	15-529	Yes
V20-6.R5.6	No significant similarity found	-	-	-	-
V20-6.R5.7	Tyrosinase	NM_000372.4	28-1587	10-529	Yes
V20-6.R5.8	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V20-6.R5.9	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V20-6.R5.10	Heat-shock protein 90	NM_005348.3	1894-1196	632-732	Yes
V20-6.R5.11	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V20-6.R5.12	Heat-shock protein 90	NM_005348.3	1894-1196	632-732	Yes
V20-6.R5.13	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V20-6.R5.14	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.15	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V20-6.R5.16	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.17	Tyrosinase-related protein 1	NM_000550.2	1-1611	1-537	Yes
V20-6.R5.18	Tyrosinase	NM_000372.4	28-1587	10-529	Yes
V20-6.R5.19	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.20	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V20-6.R5.21	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.22	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V20-6.R5.23	Tyrosinase	NM_000372.4	37-1587	13-529	Yes
V20-6.R5.24	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V20-6.R5.25	Tyrosinase	NM_000372.4	42-1587	15-529	Yes
V20-6.R5.26	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V20-6.R5.27	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.28	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.29	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V20-6.R5.30	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes

[1]Patient number.panning round.clone number.

Table 4.8: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V21-7

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V21-7.R5.1	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V21-7.R5.2	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V21-7.R5.3	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V21-7.R5.4	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V21-7.R5.5	Melanocyte-specific protein PMEL	NM_001200054.1	21-2004	8-668	Yes
V21-7.R5.6	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.7	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.8	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.9	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.10	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V21-7.R5.11	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.12	Melanocyte-specific protein PMEL	NM_001200054.1	21-2004	8-668	Yes
V21-7.R5.13	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.14	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.15	Melanocyte-specific protein PMEL	NM_001200054.1	21-2004	8-668	Yes
V21-7.R5.16	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V21-7.R5.17	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V21-7.R5.18	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.19	Melanocyte-specific protein PMEL	NM_001200054.1	21-2004	8-668	Yes
V21-7.R5.20	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.21	Heat-shock protein	NM_005348.3	1894-2196	632-732	Yes
V21-7.R5.22	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.23	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.24	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.25	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V21-7.R5.26	Melanocyte-specific protein PMEL	NM_001200054.1	21-2004	8-668	Yes
V21-7.R5.27	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.28	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.29	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V21-7.R5.30	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes

[1]Patient number.panning round.clone number.

Table 4.9: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V22-8

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V22-8.R5.1	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.2	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	y
V22-8.R5.3	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.4	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.5	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.6	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V22-8.R5.7	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V22-8.R5.8	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.9	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V22-8.R5.10	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.11	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V22-8.R5.12	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.13	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.14	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.15	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.16	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.17	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.18	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.19	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.20	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.21	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.22	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V22-8.R5.23	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.24	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.25	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.26	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.27	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V22-8.R5.28	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V22-8.R5.29	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V22-8.R5.30	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes

[1]Patient number.panning round.clone number.

Table 4.10: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V23-9

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V23-9.R5.1	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V23-9.R5.2	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.3	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.4	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V23-9.R5.5	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V23-9.R5.6	No significant similarity found	-	-	-	-
V23-9.R5.7	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V23-9.R5.8	No significant similarity found	-	-	-	-
V23-9.R5.9	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V23-9.R5.10	No significant similarity found	-	-	-	-
V23-9.R5.11	No significant similarity found	-	-	-	-
V23-9.R5.12	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.13	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.14	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V23-9.R5.15	No significant similarity found	-	-	-	-
V23-9.R5.16	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V23-9.R5.17	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V23-9.R5.18	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V23-9.R5.19	No significant similarity found	-	-	-	-
V23-9.R5.20	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V23-9.R5.21	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.22	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.23	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V23-9.R5.24	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V23-9.R5.25	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V23-9.R5.26	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V23-9.R5.27	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V23-9.R5.28	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V23-9.R5.29	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V23-9.R5.30	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes

[1]Patient number.panning round.clone number.

Table 4.11: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V24-10

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V24-10.R5.1	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.2	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.3	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.4	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.5	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.6	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.7	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.8	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.9	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.10	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.11	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.12	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.13	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.14	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.15	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.16	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.17	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.18	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.19	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.20	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.21	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.22	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.23	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.24	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.25	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.26	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V24-10.R5.27	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.28	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V24-10.R5.29	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V24-10.R5.30	Tyrosinase	NM_000372.4	40-1587	14-529	Yes

[1]Patient number.panning round.clone number.

Table 4.12: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V224-11

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V224-11.R5.1	No significant similarity found	-	-	-	-
V224-11.R5.2	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.3	No significant similarity found	-	-	-	-
V224-11.R5.4	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.5	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.6	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.7	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.8	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.9	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V224-11.R5.10	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.11	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.12	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.13	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.14	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V224-11.R5.15	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V224-11.R5.16	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.17	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V224-11.R5.18	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V224-11.R5.19	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.20	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V224-11.R5.21	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.22	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V224-11.R5.23	No significant similarity found	-	-	-	-
V224-11.R5.24	No significant similarity found	-	-	-	-
V224-11.R5.25	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	64-1716	22-572	Yes
V224-11.R5.26	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.27	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V224-11.R5.28	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V224-11.R5.29	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V224-11.R5.30	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes

[1]Patient number.panning round.clone number.

Table 4.13: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V38-12

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V38-12.R5.1	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.2	No significant similarity found	-	-	-	-
V38-12.R5.3	No significant similarity found	-	-	-	-
V38-12.R5.4	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V38-12.R5.5	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V38-12.R5.6	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V38-12.R5.7	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.8	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V38-12.R5.9	No significant similarity found	-	-	-	-
V38-12.R5.10	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V38-12.R5.11	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.12	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.13	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V38-12.R5.14	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V38-12.R5.15	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V38-12.R5.16	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V38-12.R5.17	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.18	No significant similarity found	-	-	-	-
V38-12.R5.19	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V38-12.R5.20	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V38-12.R5.21	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V38-12.R5.22	No significant similarity found	-	-	-	-
V38-12.R5.23	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V38-12.R5.24	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V38-12.R5.25	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V38-12.R5.26	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V38-12.R5.27	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.28	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V38-12.R5.29	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V38-12.R5.30	No significant similarity found	-	-	-	-

[1]Patient number.panning round.clone number.

Table 4.14: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V56-13

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V56-13.R5.1	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.2	No significant similarity found	-	-	-	-
V56-13.R5.3	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V56-13.R5.4	No significant similarity found	-	-	-	-
V56-13.R5.5	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V56-13.R5.6	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.7	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.8	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V56-13.R5.9	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.10	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.11	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.12	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.13	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.14	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.15	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V56-13.R5.16	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.17	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V56-13.R5.18	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.19	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.20	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.21	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.22	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V56-13.R5.23	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.24	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.25	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.26	No significant similarity found	-	-	-	-
V56-13.R5.27	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.28	Melanocyte-specific protein PMEL	NM_001200054.1	13-2004	5-668	Yes
V56-13.R5.29	L-dopachrome tautomerase	NM_001129889.2	1-1651	1-552	Yes
V56-13.R5.30	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes

[1]Patient number.panning round.clone number.

Table 4.15: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V73-14

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V73-14.R5.1	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V73-14.R5.2	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.3	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.4	No significant similarity found	-	-	-	-
V73-14.R5.5	No significant similarity found	-	-	-	-
V73-14.R5.6	No significant similarity found	-	-	-	-
V73-14.R5.7	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.8	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes
V73-14.R5.9	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.10	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.11	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.12	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.13	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V73-14.R5.14	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V73-14.R5.15	GTP-binding proteinRab27A	NM_004580.4	1-663	1-221	Yes
V73-14.R5.16	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.17	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.18	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.19	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.20	No significant similarity found	-	-	-	-
V73-14.R5.21	No significant similarity found	-	-	-	-
V73-14.R5.22	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.23	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V73-14.R5.24	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V73-14.R5.25	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.26	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.27	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.28	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V73-14.R5.29	Tyrosinase	NM_000372.4	37-1987	13-529	Yes
V73-14.R5.30	Heat-shock protein 90	NM_001017963.2	2260-2562	754-854	Yes

[1]Patient number.panning round.clone number.

Table 4.16: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V102-15

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V102-15.R5.1	No significant similarity found	-	-	-	-
V102-15.R5.2	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V102-15.R5.3	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.4	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.5	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.6	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V102-15.R5.7	No significant similarity found	-	-	-	-
V102-15.R5.8	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.9	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.10	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V102-15.R5.11	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V102-15.R5.12	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V102-15.R5.13	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V102-15.R5.14	No significant similarity found	-	-	-	-
V102-15.R5.15	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V102-15.R5.16	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V102-15.R5.17	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V102-15.R5.18	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V102-15.R5.19	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V102-15.R5.20	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.21	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V102-15.R5.22	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V102-15.R5.23	No significant similarity found	-	-	-	-
V102-15.R5.24	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V102-15.R5.25	OCA2-encoded P protein	NM_001300984.1	1-2442	1-814	Yes
V102-15.R5.26	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V102-15.R5.27	No significant similarity found	-	-	-	-
V102-15.R5.28	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V102-15.R5.29	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V102-15.R5.30	Tyrosinase	NM_000372.4	19-1587	7-529	Yes

[1]Patient number.panning round.clone number.

Table 4.17: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V169-16

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V169-16.R5.1	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V169-16.R5.2	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V169-16.R5.3	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V169-16.R5.4	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V169-16.R5.5	No significant similarity found	-	-	-	-
V169-16.R5.6	No significant similarity found	-	-	-	-
V169-16.R5.7	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V169-16.R5.8	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V169-16.R5.9	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V169-16.R5.10	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V169-16.R5.11	No significant similarity found	-	-	-	-
V169-16.R5.12	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V169-16.R5.13	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V169-16.R5.14	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V169-16.R5.15	Heat-shock protein 90	NM_005348.3	1894-2196	632-732	Yes
V169-16.R5.16	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V169-16.R5.17	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V169-16.R5.18	No significant similarity found	-	-	-	-
V169-16.R5.19	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V169-16.R5.20	L-dopachrome tautomerase	NM_001129889.2	22-1657	8-552	Yes
V169-16.R5.21	Tyrosinase	NM_000372.4	19-1587	7-529	Yes
V169-16.R5.22	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V169-16.R5.23	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes
V169-16.R5.24	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V169-16.R5.25	No significant similarity found	-	-	-	-
V169-16.R5.26	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V169-16.R5.27	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V169-16.R5.28	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V169-16.R5.29	No significant similarity found	-	-	-	-
V169-16.R5.30	Melanocortin 1 receptor	NM_002386.3	13-951	5-317	Yes

[1]Patient number.panning round.clone number.

Table 4.18: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V189-17

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V189-17.R5.1	Heat-shock protein 90	NM_005348.3	1906-2196	616-732	Yes
V189-17.R5.2	Tyrosinase	NM_000372.4	46-1587	36-529	Yes
V189-17.R5.3	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.4	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V189-17.R5.5	No significant similarity found	-	-	-	-
V189-17.R5.6	OCA2-encoded P protein	NM_001300984.1	22-2442	16-814	Yes
V189-17.R5.7	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.8	Tyrosinase	NM_000372.4	46-1587	16-529	Yes
V189-17.R5.9	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	22-668	Yes
V189-17.R5.10	Heat-shock protein 90	NM_005348.3	1906-2196	636-732	Yes
V189-17.R5.11	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.12	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V189-17.R5.13	Heat-shock protein 90	NM_005348.3	1906-2196	624-732	Yes
V189-17.R5.14	Heat-shock protein 90	NM_005348.3	1906-2196	636-732	Yes
V189-17.R5.15	No significant similarity found	-	-	-	-
V189-17.R5.16	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.17	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V189-17.R5.18	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	22-668	Yes
V189-17.R5.19	OCA2-encoded P protein	NM_001300984.1	22-2442	8-814	Yes
V189-17.R5.20	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V189-17.R5.21	No significant similarity found	-	-	-	-
V189-17.R5.22	Tyrosinase	NM_000372.4	46-1587	36-529	Yes
V189-17.R5.23	Tyrosinase	NM_000372.4	46-1587	16-529	Yes
V189-17.R5.24	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.25	Heat-shock protein 90	NM_005348.3	1906-2196	636-732	Yes
V189-17.R5.26	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	22-668	Yes
V189-17.R5.27	Heat-shock protein 90	NM_005348.3	1906-2196	616-732	Yes
V189-17.R5.28	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.29	Melanocyte-specific protein PMEL	NM_001200054.1	22-2004	8-668	Yes
V189-17.R5.30	OCA2-encoded P protein	NM_001300984.1	22-2442	16-814	Yes

[1]Patient number.panning round.clone number.

Table 4.19: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V216-18

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V216-18.R5.1	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.2	No significant similarity found	-	-	-	-
V216-18.R5.3	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V216-18.R5.4	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V216-18.R5.5	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V216-18.R5.6	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.7	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.8	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V216-18.R5.9	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.10	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V216-18.R5.11	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.12	No significant similarity found	-	-	-	-
V216-18.R5.13	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.14	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.15	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V216-18.R5.16	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.17	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.18	No significant similarity found	-	-	-	-
V216-18.R5.19	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.20	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V216-18.R5.21	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.22	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V216-18.R5.23	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.24	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V216-18.R5.25	Melanocortin 1 receptor	NM_002386.3	16-951	6-317	Yes
V216-18.R5.26	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V216-18.R5.27	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V216-18.R5.28	GTP-binding protein Rab27A	NM_004580.4	1-663	1-221	Yes
V216-18.R5.29	Melanocortin 1 receptor	NM_002386.3	16-951	6-317	Yes
V216-18.R5.30	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes

[1]Patient number.panning round.clone number.

Table 4.20: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V219-19

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V219-19.R5.1	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.2	No significant similarity found	-	-	-	-
V219-19.R5.3	L-dopachrome tautomerase	NM_001129889.2	22-1656	8-552	Yes
V219-19.R5.4	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V219-19.R5.5	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.6	No significant similarity found	-	-	-	-
V219-19.R5.7	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.8	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V219-19.R5.9	L-dopachrome tautomerase	NM_001129889.2	22-1656	8-552	Yes
V219-19.R5.10	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.11	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V219-19.R5.12	No significant similarity found	-	-	-	-
V219-19.R5.13	Heat-shock protein 90	NM_005348.3	1900-2196	633-732	Yes
V219-19.R5.14	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.15	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V219-19.R5.16	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.17	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V219-19.R5.18	L-dopachrome tautomerase	NM_001129889.2	22-1656	8-552	Yes
V219-19.R5.19	L-dopachrome tautomerase	NM_001129889.2	22-1656	8-552	Yes
V219-19.R5.20	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.21	No significant similarity found	-	-	-	-
V219-19.R5.22	No significant similarity found	-	-	-	-
V219-19.R5.23	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.24	39S ribosomal protein L24	NM_024540.3	1-648	1-216	Yes
V219-19.R5.25	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.26	L-dopachrome tautomerase	NM_001129889.2	22-1656	8-552	Yes
V219-19.R5.27	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.28	Heat-shock protein 90	NM_005348.3	1900-2196	633-732	Yes
V219-19.R5.29	Tyrosinase	NM_000372.4	40-1587	14-529	Yes
V219-19.R5.30	Tyrosinase	NM_000372.4	40-1587	14-529	Yes

[1]Patient number.panning round.clone number.

Table 4.21: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V234-20

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V234-20.R5.1	GTP-binding protein Rab27A	NM_004580.4	278-663	96-221	Yes
V234-20.R5.2	No significant similarity found	-	-	-	-
V234-20.R5.3	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V234-20.R5.4	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.5	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V234-20.R5.6	Heat-shock protein 90	NM_005348.3	1903-2196	635-732	Yes
V234-20.R5.7	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.8	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V234-20.R5.9	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.10	Heat-shock protein 90	NM_005348.3	1903-2196	635-732	Yes
V234-20.R5.11	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.12	GTP-binding protein Rab27A	NM_004580.4	278-663	96-221	Yes
V234-20.R5.13	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.14	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.15	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.16	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.17	Heat-shock protein 90	NM_005348.3	1903-2196	635-732	Yes
V234-20.R5.18	GTP-binding protein Rab27A	NM_004580.4	277-663	93-221	Yes
V234-20.R5.19	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.20	No significant similarity found	-	-	-	-
V234-20.R5.21	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.22	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.23	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.24	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.25	Melanocortin 1 receptor	NM_002386.3	34-951	12-317	Yes
V234-20.R5.26	No significant similarity found	-	-	-	-
V234-20.R5.27	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.28	OCA2-encoded P protein	NM_001300984.1	60-2442	20-814	Yes
V234-20.R5.29	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	1-1716	1-572	Yes
V234-20.R5.30	Melanocortin 1 receptor	NM_002386.3	34-951	12-317	Yes

[1]Patient number.panning round.clone number.

Table 4.22: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V45-21

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V45-21.R5.1	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.2	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V45-21.R5.3	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V45-21.R5.4	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.5	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.6	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.7	No significant similarity found	-	-	-	-
V45-21.R5.8	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.9	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.10	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.11	No significant similarity found	-	-	-	-
V45-21.R5.12	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V45-21.R5.13	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V45-21.R5.14	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V45-21.R5.15	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.16	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.17	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.18	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V45-21.R5.19	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.20	No significant similarity found	-	-	-	-
V45-21.R5.21	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.22	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V45-21.R5.23	Glycoprotein non-metastatic melanoma protein b	NM_001005340.1	13-1716	5-572	Yes
V45-21.R5.24	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.25	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.26	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.27	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.28	Lamin A	NM_170707	1-1992	1-664	Yes
V45-21.R5.29	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V45-21.R5.30	No significant similarity found	-	-	-	-

[1]Patient number.panning round.clone number.

Table 4.23: Identification of cDNA-encoded peptides enriched in the panning experiments of the melanocyte cDNA phage-display library with IgG from vitiligo patient V46-22

Clone number[1]	Protein identity	Database accession number	Base pairs of cDNA encoded	Amino acids encoded	In-frame with Fos
V46-22.R5.1	No significant similarity found	-	-	-	-
V46-22.R5.2	Lamin A	NM_170707	1-1992	1-664	Yes
V46-22.R5.3	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.4	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.5	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V46-22.R5.6	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V46-22.R5.7	No significant similarity found	-	-	-	-
V46-22.R5.8	Lamin A	NM_170707	1-1992	1-664	Yes
V46-22.R5.9	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.10	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V46-22.R5.11	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.12	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.13	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.14	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.15	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V46-22.R5.16	OCA2-encoded P protein	NM_001300984.1	36-2442	12-814	Yes
V46-22.R5.17	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.18	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.19	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.20	Lamin A	NM_170707	1-1992	1-664	Yes
V46-22.R5.21	Lamin A	NM_170707	1-1992	1-664	Yes
V46-22.R5.22	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.23	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.24	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.25	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.26	Heat-shock protein 90	NM_005348.3	1894-2197	632-732	Yes
V46-22.R5.27	No significant similarity found	-	-	-	-
V46-22.R5.28	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes
V46-22.R5.29	Tyrosinase	NM_000372.4	19-1586	7-529	Yes
V46-22.R5.30	Tyrosinase-related protein 1	NM_000550.2	1-1601	1-537	Yes

[1]Patient number.panning round.clone number.

4.4.3 Summary of autoantigens from the panning experiments with vitiligo patient IgG

The results for the panning of 22 vitiligo patient IgG against the melanocyte peptide phage-display library revealed that several different proteins were enriched during the process (Table 4.24). These included peptides already described as autoantigens in vitiligo, including, HSP90 (Waterman et al., 2010), tyrosinase (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Pradhan et al., 2013), RPL24 (Waterman et al., 2010), TYRP1 (Kemp et al., 1998b, Li et al., 2011, Pradhan et al., 2013), PMEL (Kemp et al., 1998a, Pradhan et al., 2013), DCT (Kemp et al., 1997b, Okamoto et al., 1998, Pradhan et al., 2013), and LaminA (Li et al., 2011). In addition, previously unreported putative autoantigens were recognised by some vitiligo patient IgGs and these included GPNMB, OCA2 protein, MC1R, and Rab27A.

Table 4.24: Summary of cDNA-encoded peptides isolated from selective enrichment of the melanocyte cDNA phage-display library with vitiligo patient IgG

cDNA-encoded peptide[1]	Number of patients with the identified antigen (%) (*n* = 22)	Previously identified as an autoantigen
HSP90	21 (95.5)	(Waterman et al., 2010)
Tyrosinase	19 (86.4)	(Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Pradhan et al., 2013)
Rab27A	13 (59.1)	Not previously identified
RPL24	12 (54.6)	(Waterman et al., 2010)
TYRP1	11 (50)	(Kemp et al., 1998b, Li et al., 2011, Pradhan et al., 2013)
PMEL	8 (36.4)	(Kemp et al., 1998a, Pradhan et al., 2013)
GPNMB	8 (36.4)	Not previously identified
OCA2 protein	8 (36.4)	Not previously identified
MC1R	5 (22.7)	Not previously identified
DCT	5 (22.7)	(Kemp et al., 1997b, Okamoto et al., 1998, Pradhan et al., 2013)
LaminA	2 (9.1)	(Li et al., 2011)

[1]GPNMB, glycoprotein non-metastatic melanoma protein b; HSP90, heat-shock protein 90; MC1R, melanocortin 1 receptor; OCA2, OCA2-encoded P protein; PMEL, melanocyte-specific protein PMEL; Rab27A, GTP-binding protein Rab27A; RPL24, 39s ribosomal protein L24; TYRP1, tyrosinase-related protein 1; DCT, L-dopachrome tautomerase.

4.5 Discussion

The identification of autoantigens is key to a full understanding of the role of autoimmunity in the pathogenesis of vitiligo. Autoantigens can be the targets of antibodies and/or T cells. Of course, antibodies might just be passive markers of disease without being involved in the depigmentation process (Zhang et al., 2005), but they can suggest the presence of autoreactive anti-melanocyte T lymphocytes (Norris et al., 1988, Oyarbide-Valencia et al., 2006, van den Boorn et al., 2009). Indeed, the autoantigens tyrosinase and PMEL were initially identified as antibody targets in vitiligo (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Kemp et al., 1998a, Pradhan et al., 2013) and were then studied further as cytotoxic T lymphocyte targets; the T cells were able to destroy melanocytes *in vitro* leading to depigmentation (Lang et al., 2001, Palermo et al., 2001, Mandelcorn-Monson et al., 2003).

Phage-display technology is a powerful tool that offers a sensitive and effective approach to identify novel antibody targets in an unbiased fashion by combining high-affinity selection with biological amplification. Large numbers of phage (10^{11}) can be screened in a one experiment, thus increasing the possibility of recovering autoantigens (Kemp et al., 2002a). Even if expressed rarely in the original library, IgG-binding proteins can become highly enriched through repeated rounds of panning. This can increase the chance of detection and identification of autoantigens. In addition, peptides displayed on the phage surface are assumed to retain native structure (Skerra and Pluckthun, 1988) as they are screened in a fluid-phase which avoids polypeptides denaturation. This could be important to detect antibody binding to conformational epitopes (Morgenthaler et al., 1999).

Previously, a phage-display strategy has been utilised widely to identify antigen-antibody interactions (Germaschewski and Murray, 1995, Grihalde et al., 1995). Employing phage-display, novel autoantigens have been identified in chronic inflammatory central nervous system disease (Burgoon et al., 2001), systemic lupus erythematosus (Kemp et al., 2002a), type 1 diabetes (Fierabracci et al., 1999), rheumatoid arthritis (Mewar et al., 2005) and vitiligo (Kemp et al., 2002b, Waterman et al., 2010). Several autoantigens in vitiligo have already been identified by using phage-

display technology including TH, MCHR1, γ-enolase, α-enolase, ubiquitin-conjugating enzyme, and osteopontin (Kemp et al., 2002b, Waterman et al., 2010). However, the IgG sample used in enrichment experiments by Kemp et al (2002b) and Waterman et al (2010) was a pool from 10 vitiligo patients so using the panning of individual patient IgG samples might have the advantage of antibody titres being less dilute.

In the current project, phage-display based on the pJuFo cDNA expression system (Crameri and Suter, 1993) was utilised to identify novel autoantigens in vitiligo. The results showed that during the panning procedure with vitiligo patient IgG samples, there was a demonstrable increase in the normalised ratio of phage eluted to phage applied, indicating that IgG-binding phage were being enriched through each panning round. However, it was notable that in some panning experiments there was a decrease in this normalised ratio indicating that enrichment might not have occurred. Despite this, sequencing of clones from the final panning rounds in these experiments did show enrichment of autoantigens. Although the reason for this is not clear, it may have been due to low antibody titres in some of the patients.

Sequence analysis revealed that several putative vitiligo-associated autoantigens had been enriched from the melanocyte cDNA phage-display library. Several of the autoantigens identified here had already been characterised as autoantigens in vitiligo including HSP90 (Waterman et al., 2010), tyrosinase (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Pradhan et al., 2013), RPL24 (Waterman et al., 2010), TYRP1 (Kemp et al., 1998b, Li et al., 2011, Pradhan et al., 2013), PMEL (Kemp et al., 1998a, Pradhan et al., 2013), DCT (Kemp et al., 1997b, Okamoto et al., 1998, Pradhan et al., 2013) and LaminA (Li et al., 2011), or had previously been described as autoantigens in other disorders (Conroy et al., 1994, Kiniwa et al., 2001, Zippelius et al., 2007). However, several novel autoantigens were also identified including GPNMB, OCA2 protein, MC1R, and Rab27A.

4.5.1 Previously identified autoantigens

4.5.1.1 Melanocyte-specific enzymes and PMEL

The melanocyte-specific proteins tyrosinase, TYRP1, DCT, and PMEL were enriched by 19, 11, five, and eight vitiligo patient IgGs, respectively. Tyrosinase, TYRP1 and DCT are key enzymes involved in melanin biosynthesis, and PMEL plays an important role in the structural organisation of premelanosomes (Yamaguchi et al., 2007, Tang et al., 2013, D'Mello et al., 2016). These melanocyte-specific proteins are localised to the melanosomes and so are considered as cytoplasmic proteins. However, different studies have shown that tyrosinase, TYRP1 and PMEL can be expressed transiently on the surface of the cell (Takechi et al., 1996, Calvo et al., 1999, Le Borgne et al., 2001, Lepage and Lapointe, 2006, Theos et al., 2006), which would make them available as targets for the immune system. Antibodies against these four melanogenic proteins have also been detected in earlier studies (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Okamoto et al., 1998, Li et al., 2011, Pradhan et al., 2013).

4.5.1.2 LaminA

IgG from two individual vitiligo patients enriched the antibody target, Lamin A. In 2011, Li and colleagues (Li et al., 2011) demonstrated that a previously undefined melanocyte protein with a molecular weight of 75-kD, and that was defined as a vitiligo-associated autoantigen (Cui et al., 1995), was LaminA. LaminA exists in the cell nucleus and can be expressed also on the surface of melanocytes since it can be detected using lactoperoxidase-labelling (Cui et al., 1995). LaminA plays a significant role in maintaining cellular morphological structure along with other nuclear laminar proteins B and C (Zhu et al., 2015). Antibodies to nuclear laminar proteins have been previously detected in patients suffering from other autoimmune disorders such as autoimmune liver disease (Wesierska-Gadek et al., 1988) and systemic lupus erythematosus (Senecal et al., 1999), so such antibodies may not be specifically associated with vitiligo.

4.5.1.3 Heat-shock protein 90

Our experiments revealed an enrichment of HSP90 by IgG from 95.5% (21/22) of the vitiligo patients. This protein has been previously described as an autoantigen in vitiligo by Waterman et al. (2010) as well as in systemic lupus erythematosus (Conroy et al., 1994, Stephanou et al., 1998). Interestingly, antibody reactivity to HSP70 and HSP90 has been identified also in a single patient with melanoma-associated hypopigmentation (Kiniwa et al., 2001). HSP90 has been found to play a vital role in antigen presentation, activation of lymphocytes and macrophages, maturation and activation of dendritic cells, and in inflammation induction (Srivastava, 2002). The high levels of HSP90 in the serum of patients suffering from systemic lupus erythematosus have been correlated with elevated levels of IL-6 and the existence of antibodies against HSP90 (Shukla and Pitha, 2012). Albeit not normally expressed on the surface of the cell, HSP90 has been observed on the surface of peripheral blood monocytes in all patients with moderate or severe systemic lupus erythematosus (Conroy et al., 1994) where it could be targeted by the immune system (Conroy et al., 1994). In addition, more recent studies have shown the secretion of HSP70 from stressed melanocytes (Denman et al., 2008).

4.5.2 Novel putative antibody targets in vitiligo

Four novel putative antibody targets associated with epidermal and melanocyte function were enriched by vitiligo patient IgG.

4.5.2.1 Melanocortin 1 receptor

The MC1R which is expressed specifically on the integumental melanocyte membrane (Hadley et al., 1981, Suzuki et al., 1996) was identified from the panning of the phage-display library with IgG from five different vitiligo patients. The receptor's ligand α-MSH regulates melanin production by acting through MC1R to increase melanin synthesis (Valverde et al., 1995). It could be argued that the function of MC1R might be adversely affected by antibodies that recognise the receptor, although this would need investigation.

4.5.2.2 Glycoprotein non-metastatic melanoma protein b

The GPNMB, a membrane-bound glycoprotein expressed in numerous types of cells including melanocytes, antigen presenting cells, macrophages, and retinal pigment epithelial cells (Owen et al., 2003, Kumagai et al., 2015), was a putative autoantigen in eight patients. In melanocytes, GPNMB is specifically expressed in late stage melanosomes, and thus it is speculated to play a role in late melanogenesis, possibly trafficking of melanosomes to keratinocytes (Hoashi et al., 2010). It has also been demonstrated that GPNMB promotes melanocyte adhesion to keratinocytes (Tomihari et al., 2009). The same study revealed the expression of GPNMB on the surface of pigment cells, albeit to a lesser degree than intracellularly where it is preferentially localised within melanosomes. In addition, the melanocyte surface-expression of GPNMB was noted to be upregulated by the pro-inflammatory cytokines IFN-γ and TNF-α, suggesting that GPNMB-mediated adhesion between melanocyte and keratinocyte maybe modulated by these cytokines (Tomihari et al., 2009).

4.5.2.3 OCA2-encoded P protein

The third previously unreported antigen detected in this study was OCA2 protein, a melanosome-specific membrane protein which regulates pH providing an optimal environment to facilitate tyrosine transportation and folding and consequent melanin production (Puri et al., 2000, Sitaram et al., 2009, Bellono et al., 2014). Additionally, OCA2 protein is suggested to play a critical role in determining the colour of skin, hair and eyes (Jin et al., 2012a). Cheng et al. (2013) found that polymorphisms in the *OCA2* gene were correlated with a high risk of developing vitiligo and melanoma. Moreover, previous studies showed that mutations in *OCA2* result in its mislocalisation to the cell surface and this impairs OCA2 protein function in melanin synthesis (Sitaram et al., 2009).

4.5.2.4 GTP-binding protein Rab27A

A further novel candidate was Rab27A, a small GTP-binding protein which belongs to the Rab family (Yoshida-Amano et al., 2012). A previous study showed a clear positive

correlation between the levels of Rab27A expression and the melanin content of melanocytes (Yoshida-Amano et al., 2012). Melanosome transfer to keratinocytes was decreased with lower levels of Rab27A expression and the intensity of skin colour was less when Rab27A expression was driven down by Rab27A-specific small interfering RNA (siRNA) (Yoshida-Amano et al., 2012). These data indicate consistently that Rab27A plays a vital role in trafficking melanosomes to the melanocyte outer edges and in consequent cutaneous pigmentation in humans. In terms of its cellular location, Rab27A localises with melanosomes but not with the cell membrane (Bahadoran et al., 2001). Interestingly, antibodies to the melanocyte-expressed GTP-binding protein Rab38, which is involved in the transport of melanogenic enzymes from the Golgi apparatus to melanosomes (Wasmeier et al., 2006), have been revealed in vitiligo patients (Waterman et al., 2010).

4.5.3 Limitations of phage-display in autoantigen discovery

Despite the encouraging results, it is important to be aware that phage-display does have disadvantages in its application related to antigen discovery. Expression of proteins on the phage surface may be prevented by different factors, hence limiting the available target autoantigens. The proteins displayed on the phage must be effectively secreted through *E. coli* inner membrane, fold into the correct structure in the periplasm, and retain their native configuration on the phage surface following exposure to an oxidising environment (Sambrook and Russell, 2001). Additionally, proteins normally associated with membranes may not have the ability to be transferred to the periplasm, and proteins with charged residues may not be expressed (Wilson and Finlay, 1998). However, phage-display has identified the MCHR1 as an autoantigen (Kemp et al., 2002b) and, in this study, the receptor MC1R was panned from the phage-display library. A further issue is that the diversity of the library is limited by its source mRNA and the quality of the inserts of cDNA, which may not contain open reading frames.

4.6 Conclusions

Overall, our results illustrate that phage-display technology is applicable to the identification of novel autoantigens in vitiligo. However, further experiments are required to prove the immunoreactivity of the potential autoantigens against a panel of vitiligo patient sera. This can be accomplished by testing the binding activity of patient IgG to [^{35}S]-labelled autoantigen, produced in an *in vitro* transcription-translation system (Section 2.19), in RLBAs (Section 2.21). Due to their reported effects on melanogenesis, the putative novel autoantigens GPNMB and OCA2 protein as well as the previously identified autoantigen LaminA were chosen for further analysis in the subsequent chapters.

Chapter 5

Analysis of antibody reactivity against glycoprotein non-metastatic melanoma protein b in vitiligo patients

5 Analysis of antibody reactivity against glycoprotein non-metastatic melanoma protein b in vitiligo patients

5.1 Introduction

Glycoprotein non-metastatic melanoma protein b (GPNMB) is a type I transmembrane protein, secreted by different phagocytic cell types such as macrophages, dendritic cells, and osteoclasts as well as melanocytes and keratinocytes (Tomihari et al., 2009, Kramer et al., 2016). In melanocytes, GPNMB is a vital structural protein of melanosomes, which mostly resides intracellularly within melanosomes, but with a small fraction on the pigment cell surface (Tomihari et al., 2009). GPNMB is enriched in late-stage (III and IV) melanosomes in contrast to its close homologue, PMEL, which is abundant in early-stage (I and II) melanosomes (Yamaguchi and Hearing, 2014). PMEL protein is a key element in early-melanosome maturation; thus, GPNMB was speculated to likely play an important role in the function of late-stage melanosomes, perhaps their transport to keratinocytes (Yamaguchi and Hearing, 2014). Indeed, pigment dispersion in the iris in mouse pigmentary glaucoma was shown to result from mutations in the gene GPNMB (Anderson et al., 2001, Tomihari et al., 2010). In addition, GPNMB expression on melanocytes has been shown to contribute to melanocyte/keratinocyte adhesion by binding to integrins (Hoashi et al., 2010, Maric et al., 2013), indicating it may be involved in melanin transfer (Tomihari et al., 2009, Zhang et al., 2013).

Of particular note, GPNMB expressed on antigen presenting cells has been shown to serve as an inhibitor of T cell activation through binding to syndecan-4 on the surface of activated T cells (Tomihari et al., 2009). Therefore, it has been suggested that the GPNMB/syndecan-4 pathway is involved in the regulation of autoimmune responses (Kramer et al., 2016). The ability of GPNMB to modulate immune responses has also been documented in graft-versus-host disease, where GPNMB engaged with its respective T cell ligand suppressed the activity of T lymphocytes, diminished IL-2 secretion, and T cell proliferation (Chung et al., 2007). Furthermore, expression of the GPNMB gene associated inversely with the metastatic capacity of human melanoma (Weterman et al., 1995, Tomihari et al., 2010).

This would indicate that GPNMB may play a role in the spread of cancerous melanocytes.

In this project, GPNMB was identified as a potential antibody target following rounds of panning against the melanocyte cDNA phage-display library with vitiligo patient IgG (Chapter 4).

5.2 Aims

The main objectives of this part of the project were:

a) To develop a radioligand binding assay (RLBA) to confirm antibody reactivity against GPNMB.

b) To investigate GPNMB antibody frequencies in 246 vitiligo patients and 100 healthy controls using the RLBA.

c) To determine if antibodies to GPNMB were related to any clinical, demographic or serological features of vitiligo patients.

5.3 Experiments and Results

5.3.1 Development of an GPNMB RLBA

In order to study the prevalence of antibodies against GPNMB in vitiligo patient sera, it was first necessary to have GPNMB cDNA in a suitable plasmid vector for expression of the GPNMB, which could then be used as the target antigen to test antibody binding in RLBAs. Plasmid pcDNA3.1 (Figure 5.1) was chosen as it contains a T7 polymerase promoter binding site from which cloned cDNA can be transcribed and translated *in vitro* with concomitant incorporation of radiolabelled [^{35}S]-methionine. The [^{35}S]-GPNMB produced in this way can then be used in RLBAs to detect GPNMB antibodies in vitiligo patient and healthy control serum samples.

5.3.1.1 GPNMB cDNA clone confirmation

The GPNMB cDNA cloned into the *Kpn*I-*Hin*dIII sites of pcDNA3.1 was obtained from GeneScript (Piscataway, NJ, USA). As a check that the recombinant plasmid pcDNA3.1-GPNMB was correct, the plasmid was subjected to DNA sequencing (Section 2.17) using primers T7, SP6, GPNMB-1-Forward, GPNMB-2-Forward, GPNMB-3-Forward, GPNMB-4-Forward, and GPNMB-5-Forward (Table 2.5). The results verified that the cloned GPNMB cDNA was correct when searched against the GenBank database using the network facilities of the NCBI (Bethesda, MD, USA) (Figure 5.2). The amino acid sequence of GPNMB is given in Figure 5.3.

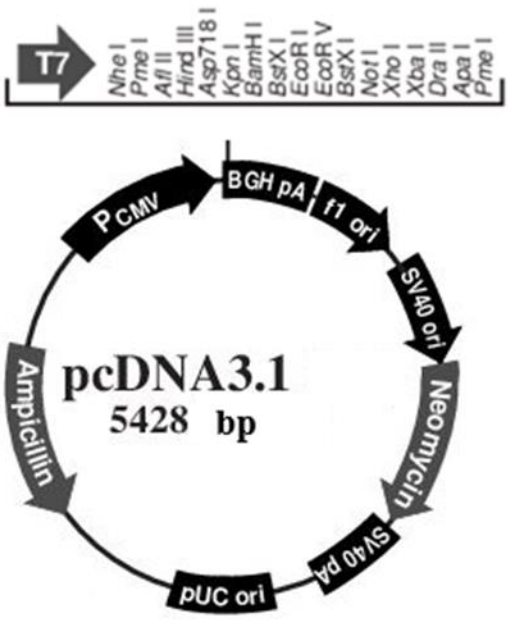


Figure 5.1: A map of the pcDNA3.1 plasmid vector.

The map shows restriction enzyme sites and the direction of T7 promoter in the pcDNA3.1 plasmid vector. The 1.7-kb GPNMB cDNA was cloned between the *Kpn*I and *Hin*dIII restriction enzyme sites.

atggaatgtctctactatttcctgggatttctgctcctggctgcaagattgccacttgatgccgccaa
acgatttcatgatgtgctgggcaatgaaagaccttctgcttacatgagggagcacaatcaattaaatg
gctggtcttctgatgaaaatgactggaatgaaaaactctacccagtgtggaagcggggagacatgagg
tggaaaaactcctggaagggaggccgtgtgcaggcggtcctgaccagtgactcaccagccctcgtggg
ctcaaatataacatttgcggtgaacctgatattccctagatgccaaaaggaagatgccaatggcaaca
tagtctatgagaagaactgcagaaatgaggctggtttatctgctgatccgtatgtttacaactggaca
gcatggtcagaggacagtgacggggaaaatggcaccggccaaagccatcataacgtcttccctgatgg
gaaaccttttcctcaccaccccggatggagaagatggaatttcatctacgtcttccacacacttggtc
agtatttccagaaattgggacgatgttcagtgagagtttctgtgaacacagccaatgtgacacttggg
cctcaactcatggaagtgactgtctacagaagacatggacgggcatatgttcccatcgcacaagtgaa
agatgtgtacgtggtaacagatcagattcctgtgtttgtgactatgttccagaagaacgatcgaaatt
catccgacgaaaccttcctcaaagatctccccattatgtttgatgtcctgattcatgatcctagccac
ttcctcaattattctaccattaactacaagtggagcttcggggataatactggcctgtttgtttccac
caatcatactgtgaatcacacgtatgtgctcaatggaaccttcagccttaacctcactgtgaaagctg
cagcaccaggaccttgtccgccaccgccaccaccacccagaccttcaaaacccacccttctttagca
actactctaaaatcttatgattcaaacaccccaggacctgctggtgacaaccccctggagctgagtag
gattcctgatgaaaactgccagattaacagatatggccactttcaagccaccatcacaattgtagagg
gaatcttagaggttaacatcatccagatgacagacgtcctgatgccggtgccatggcctgaaagctcc
ctaatagactttgtcgtgacctgccaagggagcattcccacggaggtctgtaccatcatttctgaccc
cacctgcgagatcacccagaacacagtctgcagccctgtggatgtggatgagatgtgtctgctgactg
tgagacgaaccttcaatgggtctgggacgtactgtgtgaacctcaccctgggggatgacacaagcctg
gctctcacgagcaccctgatttctgttcctgacagagacccagcctcgcctttaaggatggcaaacag
cgccctgatctccgttggctgcttggccatatttgtcactgtgatctccctcttggtgtacaaaaaac
acaaggaatacaacccaatagaaaatagtcctgggaatgtggtcagaagcaaaggcctgagtgtcttt
ctcaaccgtgcaaaagccgtgttcttcccgggaaaccaggaaaaggatccgctactcaaaaaccaaga
atttaaaggagtttct**taa**

Figure 5.2: The cDNA sequence encoding the GPNMB.

The cDNA sequence matched that of GPNMB cDNA Accession Number BC032783 when searched against the GenBank database using the network facilities of the NCBI (Bethesda, MD, USA). The ATG translation start codon and the TAA translation termination codon are underlined and given in bold-type. The cDNA has 1719 base pairs.

MECLYYFLGFLLLAARLPLDAAKRFHDVLGNERPSAYMREHNQLNGWSSDENDWNEKLYPVWKRGDMR
WKNSWKGGRVQAVLTSDSPALVGSNITFAVNLIFPRCQKEDANGNIVYEKNCRNEAGLSADPYVYNWT
AWSEDSDGENGTGQSHHNVFPDGKPFPHHPGWRRWNFIYVFHTLGQYFQKLGRCSVRVSVNTANVTLG
PQLMEVTVYRRHGRAYVPIAQVKDVYVVTDQIPVFVTMFQKNDRNSSDETFLKDLPIMFDVLIHDPSH
FLNYSTINYKWSFGDNTGLFVSTNHTVNHTYVLNGTFSLNLTVKAAAPGPCPPPPPPPRPSKPTPSLA
TTLKSYDSNTPGPAGDNPLELSRIPDENCQINRYGHFQATITIVEGILEVNIIQMTDVLMPVPWPESS
LIDFVVTCQGSIPTEVCTIISDPTCEITQNTVCSPVDVDEMCLLTVRRTFNGSGTYCVNLTLGDDTSL
ALTSTLISVPDRDPASPLRMANSALISVGCLAIFVTVISLLVYKKHKEYNPIENSPGNVVRSKGLSVF
LNRAKAVFFPGNQEKDPLLKNQEFKGVS

Figure 5.3: GPNMB amino acid sequence.

The protein has 572 amino acid residues which are shown by their one letter symbol.

5.3.1.2 *In vitro* transcription-translation of GPNMB cDNA

Plasmid pcDNA3.1-GPNMB was transcribed and translated in a TnT® T7-Coupled Reticulocyte Lysate System, as described in Section 2.19. In order to analyse the radiolabelled product qualitatively, a 5-µl aliquot of the *in vitro* transcription-translation reaction was added to 20 µl of 2x Laemmli sample buffer (Section 2.20) and heated to 100°C for 5 min. A 10-µl sample was then applied to a 10% (w/v) SDS-polyacrylamide gel and subjected to electrophoresis (Section 2.20). After drying, the gel was exposed to x-ray film, which was developed after 24 h (Section 2.20).

The result showed a protein product with an estimated molecular weight of 64 kDa (Figure 5.4). This agrees well with the molecular weight of 63 kDa calculated from the amino acid sequence of the protein with 572 residues (Figure 5.3).

5.3.1.3 Analysis of the immunoreactivity of [^{35}S]-GPNMB

The immunoreactivity of *in vitro* translated [^{35}S]-GPNMB was analysed in a RLBA (Section 2.21) with an anti-GPNMB antibody and a panel of antibodies raised against proteins unrelated to GPNMB (Table 2.6). The antibodies used were sc271435 (anti-GPNMB), α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH)), and α-PEP8 (anti-DCT). They were all used at a 1:100 dilution. An immunoprecipitation reaction without any antibodies was also included in each assay set, as a background control. From each RLBA, a GPNMB antibody index was calculated for each of the antibodies tested as: cpm immunoprecipitated by tested antibody/cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean GPNMB antibody index was calculated from these values. The GPNMB antibody indices of the different antibodies were compared using one-way ANOVA, with P values < 0.05 considered significant.

The results are shown in Figure 5.5, and indicated that the mean GPNMB antibody index ± SD for each antibody was 19.6 ± 3.44 for sc271435, 1.02 ± 0.15 for α-PEP7, 1.12 ± 0.13 for MCHR11-S, 1.07 ± 0.11 for ab59276, and 1.01 ± 0.10 for α-PEP8. In one-way ANOVA, the GPNMB antibody index of GPNMB antibody sc271435 was statistically significantly higher in comparison to those for α-PEP7, MCHR11-S, ab59276, and α-PEP8 antibodies;

P < 0.0001. The result demonstrated a specific immunoprecipitation of [^{35}S]-GPNMB by anti-GPNMB antibody, and indicated that [^{35}S]-GPNMB could be used as a ligand in a RLBAs to detect antibodies against GPNMB.

The GPNMB immunoprecipitated in the RLBA by antibody sc271435 was also analysed quantitatively. Following the RLBA using [^{35}S]-GPNMB and antibody sc271435, the protein G Sepharose-GPNMB-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged, and the supernatant analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel (Section 2.20). The gel was then subjected to autoradiography (Section 2.20). The results indicated that the antibody sc271435 immunoprecipitated a protein band equivalent in size (64 kDa) to that of *in vitro* translated [^{35}S]-GPNMB, and this is shown in Figure 5.6.

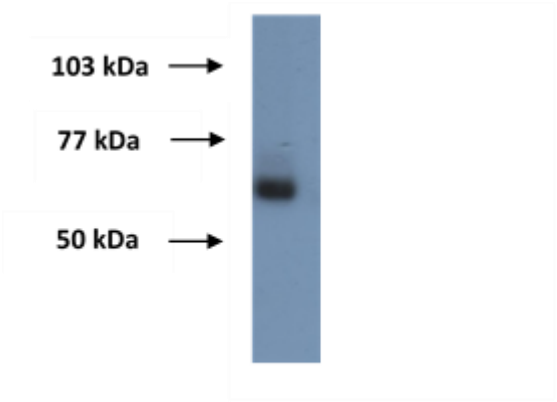


Figure 5.4: SDS-PAGE and autoradiography of [^{35}S]-GPNMB.

[^{35}S]-GPNMB was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3.1-GPNMB as a template for transcription and translation of GPNMB cDNA. For analysis, the radiolabelled protein was run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The GPNMB is visible at 64 kDa.

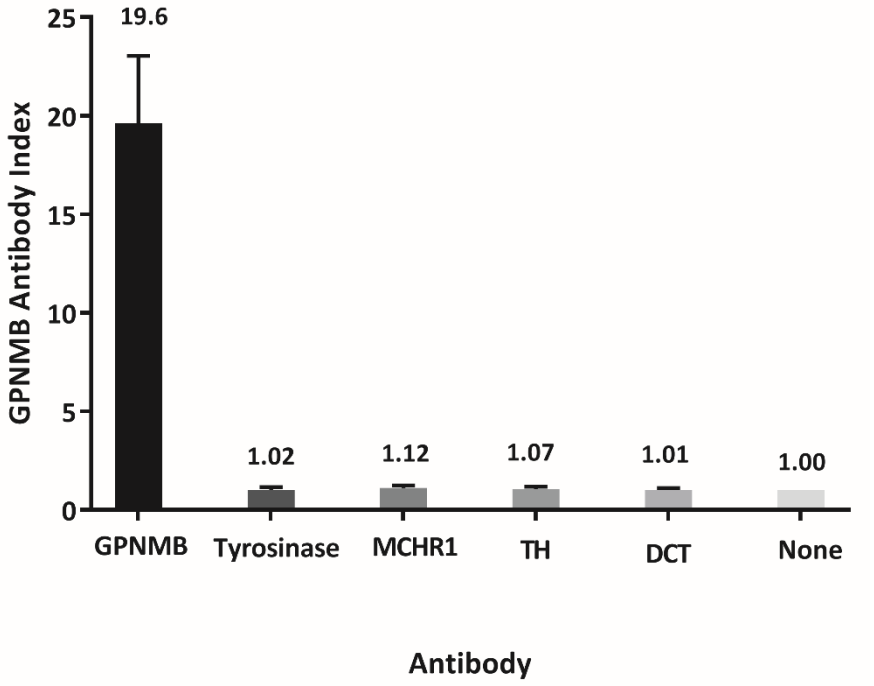


Figure 5.5: GPNMB RLBA using different antibodies.

The immunoreactivity of *in vitro* translated [^{35}S]-GPNMB was tested in a RLBA with antibodies anti-GPNMB (sc271435), anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276) and anti-DCT (α-PEP8). An immunoprecipitation reaction without serum was included as a background control. The mean GPNMB antibody index ± SD of four experiments is given for each antibody: sc271435, 19.6 ± 3.44; α-PEP7, 1.02 ± 0.15; MCHR11-S, 1.12 ± 0.13; ab59276, 1.07 ± 0.11; and α-PEP8, 1.01 ± 0.10. $P < 0.0001$ (One-way ANOVA).

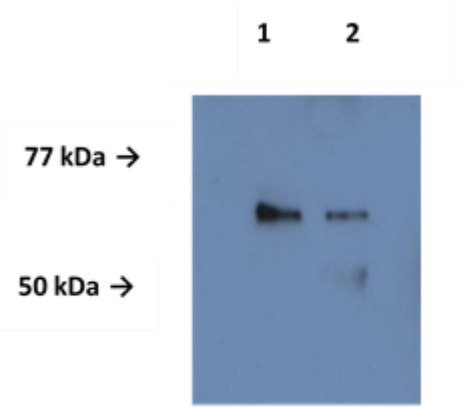


Figure 5.6: SDS-PAGE and autoradiography of [^{35}S]-GPNMB and [^{35}S]-GPNMB immunoprecipitated by anti-GPNMB antibody sc271435

[^{35}S]-GPNMB was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3.1-GPNMB. Subsequently, radiolabelled GPNMB was immunoprecipitated in a RLBA with anti-GPNMB antibody sc271435. For analysis, immunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results show: *In vitro* translated, non-immunoprecitated [^{35}S]-GPNMB (lane 1); [^{35}S]-GPNMB immunoprecipitated with anti-GPNMB antibody sc271435 (lane 2). The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The GPNMB protein is visible at 64 kDa.

5.3.1.4 Analysis of the specificity of anti-GPNMB antibody

In order to determine the anti-GPNMB antibody sc271435s specificity, sc271435s was used in RLBAs with other radiolabelled antigens and these included tyrosinase, MCHR1, TH and DCT.

Radiolabelled [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT were produced in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.19) from plasmids pcDNA3-TYR, pcMCHR1, pcDNA3-TH and pCMVDTS (Table 2.2), respectively. RLBAs were carried out as detailed in (Section 2.21), using these labelled antigens with antibody sc271435 at a 1:100 dilution. Appropriate positive control antibodies were included at a 1:100 dilution in each RLBA: α-PEP7 for tyrosinase, MCHR11-S for MCHR1, ab59276 for TH, and α-PEP8 for DCT (Table 2.6). An immunoprecipitation reaction without antibody was also included in each RLBA, as a background control. In each RLBA, an antibody index was calculated for each antibody as: cpm immunoprecipitated by tested antibody/cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean antibody index was calculated from these values. Antibody indices were compared using one-way ANOVA with *P* values < 0.05 considered significant.

The mean antibody index ± SD for anti-GPNMB antibody in RLBAs against different antigens was 19.5 ± 4.14 against GPNMB, 0.99 ± 0.17 against tyrosinase, 1.09± 0.19 against MCHR1, 1.12 ± 0.15 against TH, and 1.09 ± 0.11 against DCT (Figure 5.7). In one-way ANOVA, the antibody index of the anti-GPNMB antibody in the different RLBAs was compared; $P < 0.0001$, indicating there was no significant immunoprecipitation of other unrelated proteins. Anti-GPNMB antibody sc271435 could be used therefore as a positive control antibody in GPNMB RLBAs.

Additionally, the mean antibody index ± SD for the antibodies α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH), and α-PEP8 (anti-DCT) in RLBAs against their cognate antigens was 12.5 ± 1.83, 10.1 ± 1.42, 16.2 ± 1.86 and 15.1 ± 1.36, respectively (Figure 5.7).

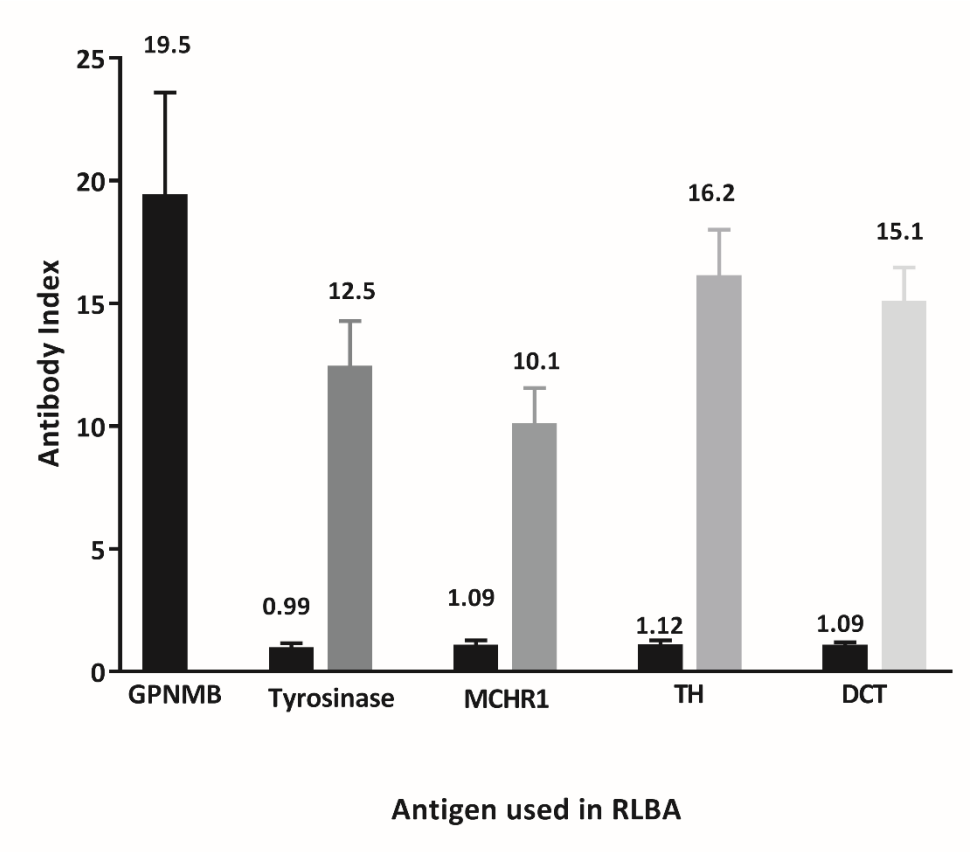


Figure 5.7: RLBAs with anti-GPNMB antibody sc271435 and different antigens.

The immunoreactivity of the anti-GPNMB antibody sc271435 was tested against *in vitro* translated [^{35}S]-GPNMB, [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT in RLBAs. Antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) were included in the relevant RLBA as positive controls for immunoprecipitation of their respective antigens. An immunoprecipitation reaction without any serum was included as a background control in each RLBA. The mean antibody index ± SD of four experiments is shown for the antibody sc271435 in RLBAs against the different antigens; 19.5 ± 4.14 against GPNMB, 0.99 ± 0.17 against tyrosinase, 1.09 ± 0.19 against MCHR1, 1.12 ± 0.15 against TH, and 1.09 ± 0.11 against DCT. $P < 0.0001$ (One-way ANOVA). The mean antibody index ± SD of four experiments is also shown for the antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) in RLBAs against their cognate antigens; 12.5 ± 1.83, 10.1 ± 1.42, 16.2 ± 1.86 and 15.1 ± 1.36, respectively.

5.3.2 Use of the GPNMB RLBA to analysis vitiligo patients and control sera for GPNMB antibodies

To analyse vitiligo patient and control sera for the presence of GPNMB antibodies, GPNMB RLBAs were carried out using [^{35}S]-GPNMB as the radiolabelled antigen. The sera analysed were from patients with vitiligo (*n* = 246) and from healthy controls (*n* = 100). All sera were assayed in duplicate at a final dilution of 1:100. The positive control anti-GPNMB antibody sc271435 was also included in each set of the RLBAs at a 1:100 dilution. GPNMB antibody index that express antibody levels was assigned to each serum sample, and this was the mean GPNMB antibody index of three experiments at least. The GPNMB antibody index was determined for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 100 healthy control sera. The upper limit of normal for the RLBA with GPNMB was determined using the mean GPNMB antibody index + 3SD of the population of 100 healthy controls. Serum samples with a GPNMB antibody index higher than the upper limit of normal were considered as positive for GPNMB antibodies.

The results of the GPNMB RLBAs are given in Figure 5.8. The mean GPNMB antibody index ± SD for the control group (*n* = 100) was 1.03 ± 0.12 with a range of 0.71 to 1.31. The mean GPNMB antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo (*n* = 4), and undetermined vitiligo (*n* = 11) was 2.46 ± 2.42 (range of 0.56 to 11.09), 0.99 ± 0.04 (range of 0.96 to 1.05), and 1.04 ± 0.16 (range of 0.78 to 1.42), respectively. The upper limit of normal for the GPNMB RLBA was a GPNMB antibody index of 1.38.

Healthy controls were all GPNMB antibody-negative with GPNMB antibody indices below 1.38. Of the 231 non-segmental vitiligo patients, 91 (39%) were considered GPNMB antibody-positive (Table 5.1), as their GPNMB antibody index was above the upper limit of normal value of 1.38. GPNMB antibodies were not detected in any patient with segmental vitiligo and only one patient with undetermined vitiligo sub-type was GPNMB antibody-positive (Table 5.1), as their GPNMB antibody index was above the upper limit of normal value of 1.38.

The prevalence of GPNMB antibodies was compared between vitiligo patients and controls in Fisher's exact test for 2 x 2 contingency tables (Section 2.24). *P* values < 0.05 were regarded as significant. The results are given in Table 5.1. In comparison with controls, a statistically significant increase in the prevalence of GPNMB antibodies was found in the patient group with non-segmental vitiligo; the *P* value was < 0.0001. In addition, there was a statistically significant increase in the frequency of GPNMB antibodies in patients with symmetrical or acrofacial vitiligo (Table 5.1). However, in most vitiligo sub-type groups, there were too few patients to make statistical analysis robust (Table 5.1). A statistically significant increase in GPNMB antibody frequency was also noted in the active vitiligo patient cohort in comparison to the stable disease patient group: *P* was 0.001 (Table 5.1). However, there was no difference in GPNMB antibody prevalence in patients with autoimmune diseases compared to those without (Table 5.1).

To analyse qualitatively, the proteins immunoprecipitated in the GPNMB RLBA by vitiligo patient and control sera, protein G Sepharose-GPNMB-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged and the supernatant recovered for SDS-PAGE in 10% (w/v) SDS-polyacrylamide gels and subsequent autoradiography (Section 2.20).

The results indicated that all 92 of the GPNMB antibody-positive vitiligo patient sera analysed immunoprecipitated a protein band of 64 kDa, comparable to the size of GPNMB in SDS-polyacrylamide gels. The results for six GPNMB antibody-positive vitiligo patient sera and for two healthy controls sera are illustrated in Figure 5.9.

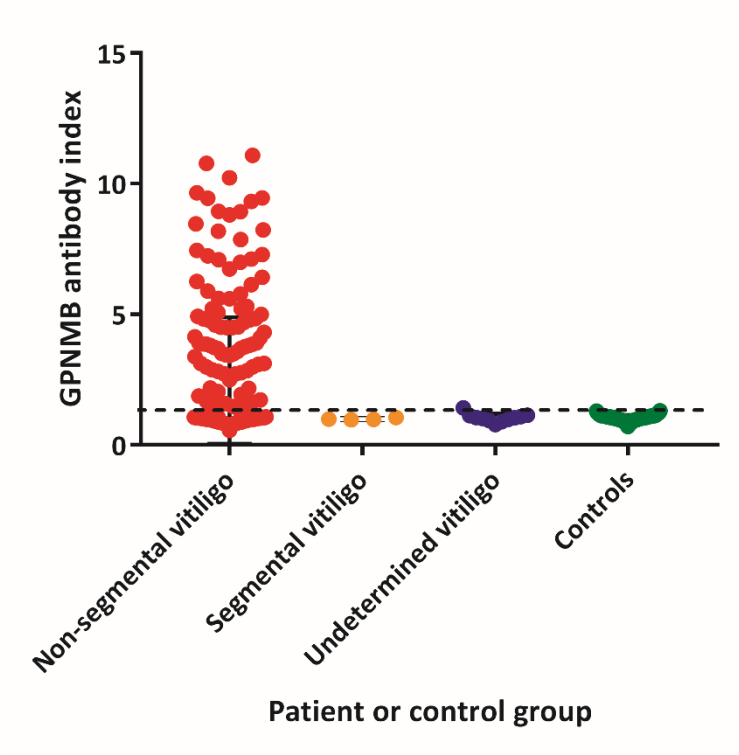


Figure 5.8: GPNMB RLBA results for vitiligo patient and healthy control sera.

Vitiligo patient and healthy control sera were evaluated for GPNMB antibodies in the GPNMB RLBA. The GPNMB antibody indices are shown for sera from vitiligo patients (*n* = 246) and healthy controls (*n* = 100). The GPNMB antibody index shown for each serum is the mean of three experiments. The mean GPNMB antibody index ± SD for the control group was 1.03 ± 0.12 with a range of 0.71 to 1.31. The mean GPNMB antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo (*n* = 4), and undetermined vitiligo (*n* = 11) was 2.46 ± 2.42 (range of 0.56 to 11.09), 0.99 ± 0.04 (range of 0.96 to 1.05), and 1.04 ± 0.16 (range of 0.78 to 1.42), respectively. The upper limit of normal (mean GPNMB antibody index + 3SD of 100 healthy controls) for the GPNMB RLBA was a GPNMB antibody index of 1.38, and this is shown by the dotted line.

Table 5.1: GPNMB antibody prevalence in vitiligo patients and healthy controls

Vitiligo patient or control group	GPNMB antibody-positive samples	*P* value[1]	*P* value[2]
Vitiligo patient or control group			
Non-segmental vitiligo	91/231 (39%)	< 0.0001	-
Segmental vitiligo	0/4 (0%)	ND	-
Undetermined vitiligo (all focal)	1/11 (9%)	ND	-
Healthy controls	0/100 (0%)	ND	-
Non-segmental vitiligo sub-type			
Symmetrical (generalised)	82/157 (52%)	< 0.0001	-
Acrofacial	7/42 (17%)	0.0001	-
Acral	0/4 (0%)	ND	-
Peri-orificial	0/3 (0%)	ND	-
Universal	0/1 (0%)	ND	-
Symmetrical and peri-orificial	1/10 (10%)	ND	-
Symmetrical and acrofacial	1/7 (14.3%)	ND	-
Symmetrical with peri-orificial and acrofacial	0/1 (0%)	ND	-
Occupational	0/1 (%)	ND	-
Mixed	0/5 (0%)	ND	-
Vitiligo activity			
Active	91/226 (40%)	< 0.0001	0.001
Stable	1/20 (5%)	0.17	
Autoimmune disease			
Present	37/84 (44%)	< 0.0001	0.13
Absent	55/162 (34%)	< 0.0001	

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of GPNMB antibodies in vitiligo patients and controls. $P < 0.05$ was considered statistically significant.

[2]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of GPNMB antibodies in vitiligo patient groups. $P < 0.05$ was considered statistically significant.

ND, not determined due to small sample size or no GPNMB antibody-positive patients in the group.

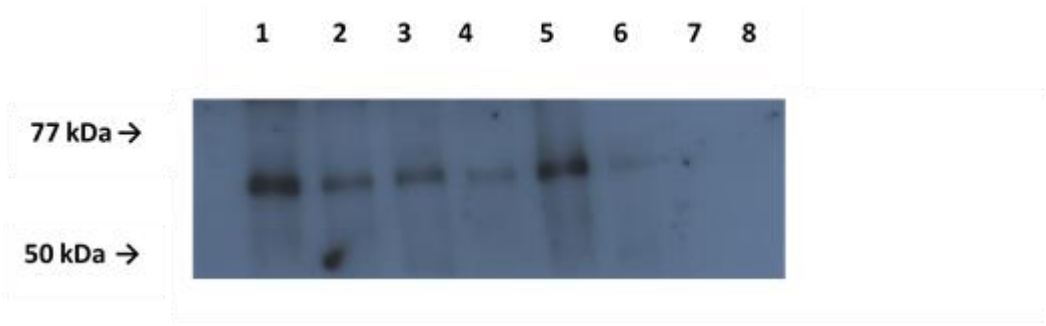


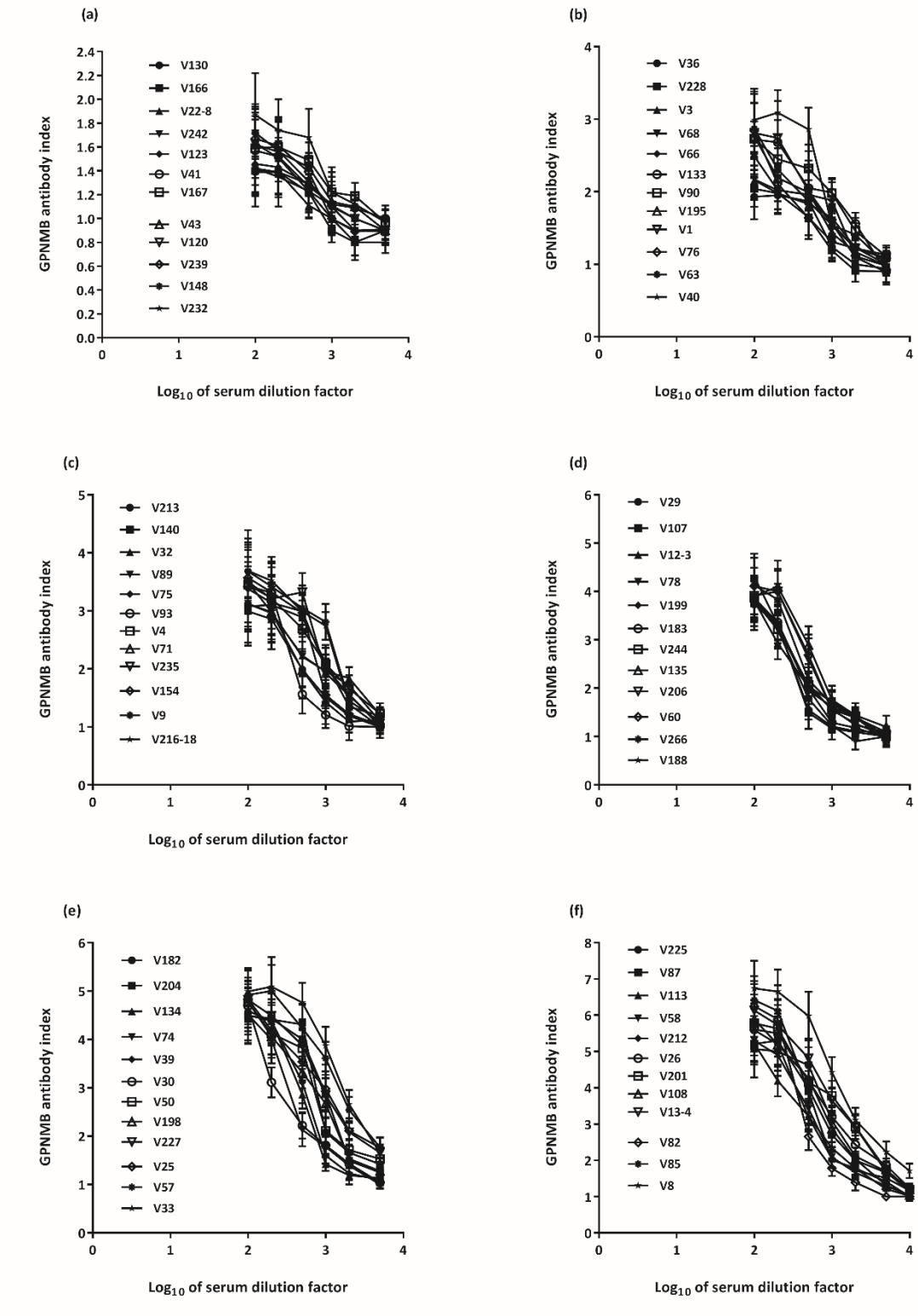
Figure 5.9: SDS-PAGE and autoradiography of [^{35}S]-GPNMB following immunoprecipitation in GPNMB RLBAs with vitiligo patient and healthy control sera.

Radiolabelled GPNMB was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System and then immunoprecipitated in GPNMB RLBAs with either vitiligo or healthy control sera. For analysis, immunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results are shown for six vitiligo patient and two healthy control sera: [^{35}S]-GPNMB immunoprecipitated with sera from GPNMB antibody-positive vitiligo patients (lanes 1-6); [^{35}S]-GPNMB immunoprecipitated with sera from healthy controls (lanes 7-8).

5.3.3 Determination of GPNMB antibody titres in vitiligo patients

To determine the titres of GPNMB antibodies, sera from all GPNMB antibody-positive vitiligo patients (*n* = 92) and healthy controls (*n* = 10) were analysed in the GPNMB antibody RLBA at different dilutions including 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10000. For each serum sample tested, a GPNMB antibody index (cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by 10 healthy control sera at each dilution) was calculated and this was the mean GPNMB antibody index of three experiments.

The results of the antibody titration experiments are given in Figure 5.10. The titration results are also summarised in Table 5.2, and show the serum dilution at which GPNMB antibodies were still detectable at levels above the upper limit of normal for the GPNMB antibody RLBA. Although the titres of GPNMB antibodies varied between the different vitiligo patients, the majority, 72/92 (78%), had a titre at 1:1000 or above, and 28/92 (30%) had a titre at 1:5000 or above.


(a)
GPNMB antibody index
V130
V166
V22-8
V242
V123
V41
V167
V43
V120
V239
V148
V232
Log10 of serum dilution factor
(b)
GPNMB antibody index
V36
V228
V3
V68
V66
V133
V90
V195
V1
V76
V63
V40
Log10 of serum dilution factor
(c)
GPNMB antibody index
V213
V140
V32
V89
V75
V93
V4
V71
V235
V154
V9
V216-18
Log10 of serum dilution factor
(d)
GPNMB antibody index
V29
V107
V12-3
V78
V199
V183
V244
V135
V206
V60
V266
V188
Log10 of serum dilution factor
(e)
GPNMB antibody index
V182
V204
V134
V74
V39
V30
V50
V198
V227
V25
V57
V33
Log10 of serum dilution factor
(f)
GPNMB antibody index
V225
V87
V113
V58
V212
V26
V201
V108
V13-4
V82
V85
V8
Log10 of serum dilution factor

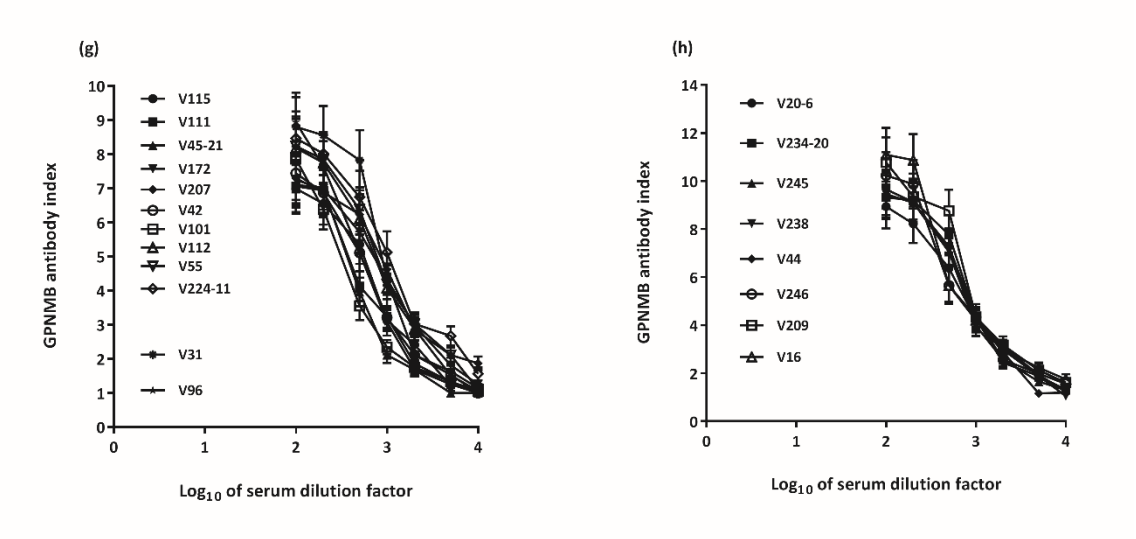


Figure 5.10: GPNMB antibody titres.

The 92 GPNMB antibody-positive vitiligo patient sera were analysed in the GPNMB antibody RLBA at a range of dilutions (1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:5000). The GPNMB antibody index (mean ± SD of three exepriments) of each serum at each dilution is given. (a) The results are shown for 12 vitiligo patients. (b) The results are shown for 12 vitiligo patients. (c) The results are shown for 12 vitiligo patients. (d) The results are shown for 12 vitiligo patients. (e) The results are shown for 12 vitiligo patients. (f) The results are shown for 12 vitiligo patients. (g) The results are shown for 12 vitiligo patients. (h) The results are shown for eight vitiligo patients.

Table 5.2: Summary of GPNMB antibody titres

Patients	GPNMB antibody titre[1]
V242	1:100
V130, V166, V22-8, V123, V41, V120, V232	1:200
V167, V43, V239, V148, V36, V228, V76, V93, V29, V107, V78, V183	1:500
V3, V68, V66, V195, V1, V63, V213, V140, V71, V154, V12-3, V206, V134, V39, V82	1:1000
V133, V90, V40, V32, V89, V75, V4, V235, V9, V216-18, V199, V244, V135, V60, V226, V188, V204, V74, V30, V198, V58, V108, V85, V115, V111, V45-21, V101, V96, V44	1:2000
V225, V87, V113, V212, V26, V201, V13-4, V172, V42, V112, V55, V31, V20-6, V234-20, V245, V238	1:5000
V182, V50, V227, V25, V57, V33	>1:5000
V8, V207, V224-11, V246, V209, V16	>1:10000

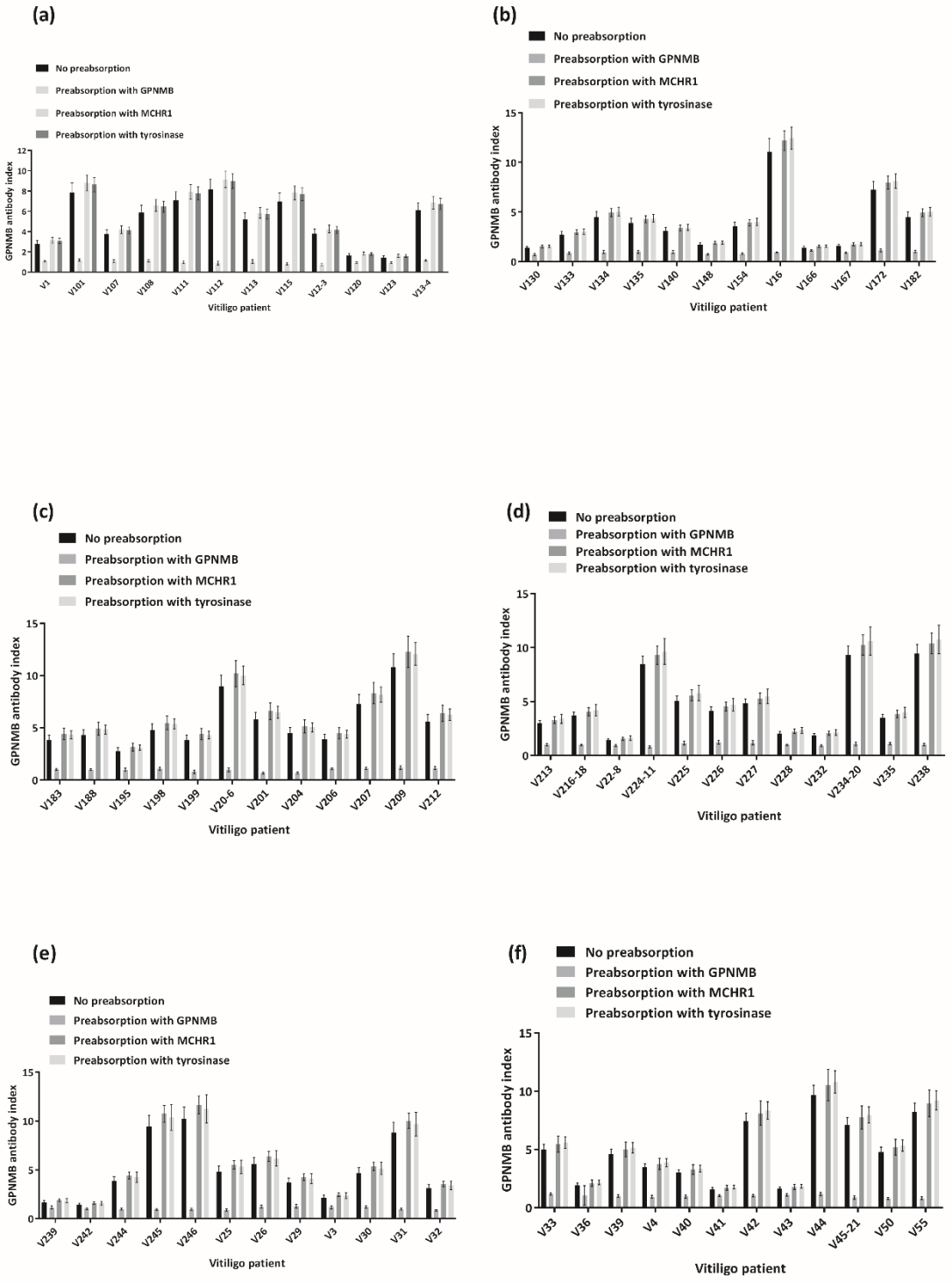
[1]The titre of GPNMB antibody is shown as the dilution at which antibody reactivity in the sample of serum could still be detectable above the upper limit of normal in the GPNMB antibody RLBA.

5.3.4 Assessment of the specificity of GPNMB antibodies

In order to determine that GPNMB antibodies in vitiligo patient sera reacted specifically against the protein GPNMB, absorption experiments were performed as detailed in Section 2.22. Briefly, serum samples from GPNMB antibody-positive vitiligo patients (*n* = 92) and healthy individuals (*n* = 10) were absorbed with extracts prepared from untransfected HEK293 cells or from HEK293 cells transfected with GPNMB, MCHR1 or tyrosinase. Subsequently, duplicate samples of pre-absorbed sera at a 1:100 dilution (equivalent to the original sera dilution) were evaluated in the GPNMB RLBA. For each serum sample, a GPNMB antibody index (cpm immunoprecipitated by tested serum/the cpm immunoprecipitated by 10 healthy control sera) was calculated. Serum samples were all tested in triplicate. To compare the GPNMB antibody index of absorbed against unabsorbed vitiligo patient sera, one-way ANOVA was used (Section 2.24). *P* values < 0.05 were considered as significant in all tests.

The absorption experiment results are given in Figure 5.11. There was a significant reduction in the GPNMB antibody indices by pre-absorption of GPNMB antibody-positive vitiligo patient sera with the GPNMB protein expressed in the extracts prepared from HEK293 cells: all *P* values were < 0.05. In contrast, GPNMB antibody indices were not significantly decreased after pre-absorption of GPNMB antibody-positive vitiligo patient sera with extracts made from untransfected HEK293 cells or HEK293 cells harbouring expressed MCHR1 or tyrosinase: *P* values were > 0.05.

Overall, these findings indicated that GPNMB antibodies in all GPNMB antibody-positive patient sera specifically recognised their target autoantigen without cross-reactivity with vitiligo-associated autoantigens MCHR1 and tyrosinase.


(a)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V1
V101
V107
V108
V111
V112
V113
V115
V12-3
V120
V123
V13-4
Vitiligo patient
(b)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V130
V133
V134
V135
V140
V148
V154
V16
V166
V167
V172
V182
Vitiligo patient
(c)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V183
V188
V195
V198
V199
V20-6
V201
V204
V206
V207
V209
V212
Vitiligo patient
(d)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V213
V216-18
V22-8
V224-11
V225
V226
V227
V228
V232
V234-20
V235
V238
Vitiligo patient
(e)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V239
V242
V244
V245
V246
V25
V26
V29
V3
V30
V31
V32
Vitiligo patient
(f)
No preabsorption
Preabsorption with GPNMB
Preabsorption with MCHR1
Preabsorption with tyrosinase
GPNMB antibody index
V33
V36
V39
V4
V40
V41
V42
V43
V44
V45-21
V50
V55
Vitiligo patient

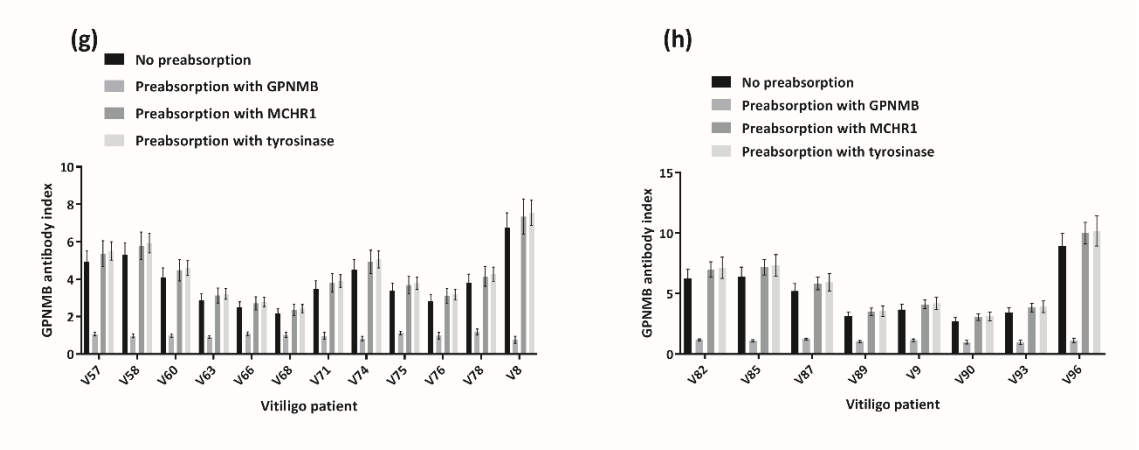


Figure 5.11: GPNMB antibody absorption experiments.

The 92 GPNMB antibody-positive vitiligo patient sera were preabsorbed with extract prepared from either untransfected HEK293 cells or from HEK293 cells expressing glycoprotein non-metastatic melanoma protein b (GPNMB), tyrosinase or melanin-concentrating hormone receptor 1 (MCHR1). Unabsorbed and preabsorbed sera were subsequently analysed in the GPNMB RLBA. The results demonstrate the mean GPNMB antibody index (± SD) of three experiments for unabsorbed and preabsorbed sera. The results are given for the 92 vitiligo patients. Comparing the antibody indices of GPNMB-absorbed sera with those without preabsorption; *P* values were < 0.0001 (One-way ANOVA).

5.3.5 Analysis of vitiligo patient sera for other vitiligo-associated antibodies

To determine if antibodies to GPNMB are related to the presence of any antibodies directed against other vitiligo-associated autoantigens, RLBAs for tyrosinase, DCT, TYRP1, PMEL, TH, and MCHR1 antibodies (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011) were performed on all the 246 vitiligo patient sera.

In order to produce radiolabelled ligands for use in RLBAs, transcription and translation of plasmids carrying the relevant cDNA (Table 2.2), were carried out using a TnT® T7-Coupled Reticulocyte Lysate System as described in Section 2.19. Sera from either patients (*n* = 246) or healthy controls (*n* = 100) were tested for the presence of antibodies to the radiolabelled antigens in RLBAs (Section 2.21). Serum samples were all tested in duplicate at 1:100 dilutions. As a positive control, each assay set included an appropriate antigen-specific antibody (Table 2.6). In each assay set, antibody levels were expressed as an antibody index (Section 5.3.2).

The results of the RLBAs are given in Table 5.3. All healthy controls were negative for all the antibodies tested. Of the 231 of the non-segmental vitiligo patient sera, 42 (18%), 51 (22%), 35 (15%), 44 (19%), 43 (19%), and 53 (23%) were regarded as tyrosinase, MCHR1, PMEL, TYRP1, DCT, and TH antibody-positive, respectively, since their antibody indices were above the upper limit of normal for the specific antibody RLBA (Table 5.3). In the group of four segmental vitiligo patients, 1 (25%), 0 (0%), 1 (25%), 0 (0%), 0 (0%) and 2 (50%) were regarded as tyrosinase, MCHR1, PMEL, TYRP1, DCT, and TH antibody-positive, respectively, as they had antibody indices above the upper limit of normal for the specific antibody RLBA (Table 5.3). In the group of 11 undetermined vitiligo patients, 1 (9%), 0 (0%), 0 (0%), 0 (0%), 0 (0%) and 2 (18%) were regarded as tyrosinase, MCHR1, PMEL, TYRP1, DCT, and TH antibody-positive, respectively (Table 5.3).

Table 5.3: Results of RLBAs for antibodies in vitiligo patients

Patient or control group → / Antibody RLBA ↓	Non-segmental vitiligo patients (*n* = 231)	Segmental vitiligo patients (*n* = 4)	Undetermined vitiligo patients (*n* = 11)	Healthy controls (*n* = 100)
Tyrosinase				
Tyrosinase antibody index: mean ± SD	1.61 ± 1.60 (0.61-9.76)	1.25 ± 0.52 (0.96-2.03)	1.06 ± 0.30 (0.67-1.85)	0.92 ± 0.27 (0.23-1.40)
Number positive for tyrosinase antibodies[1]	42 (18%)	1 (25%)	1 (9%)	0 (0%)
MCHR1				
MCHR1 antibody index: mean ± SD	1.96 ± 2.21 (0.12-11.2)	0.94 ± 0.08 (0.84-1.01)	0.91 ± 0.16 (0.67-1.11)	1.02 ± 0.15 (0.64-1.37)
Number positive for MCHR1 antibodies[1]	51 (22%)	0 (0%)	0 (0%)	0 (0%)
PMEL				
PMEL antibody index: mean ± SD	1.33 ± 1.41 (0.01-11.36)	0.83 ± 0.76 (0.07-1.88)	0.90 ± 0.14 (0.76-1.2)	0.99 ± 0.10 (0.79-1.25)
Number positive for PMEL antibodies[1]	35 (15%)	1 (25%)	0 (0%)	0 (0%)
TYRP1				
TYRP1 antibody index: mean ± SD	1.43 ± 1.28 (0.44-10.52)	1.07 ± 0.06 (0.98-1.11)	1.01 ± 0.09 (0.81-1.12)	1.00 ± 0.19 (0.51-1.50)
Number positive for TYRP1 antibodies[1]	44 (19%)	0 (0%)	0 (0%)	0 (0%)
DCT				
DCT antibody index: mean ± SD	1.39 ± 1.34 (0.2-11.72)	1.02 ± 0.04 (0.97-1.05)	0.98 ± 0.10 (0.78-1.12)	0.99 ± 0.12 (0.44-1.40)
Number positive for DCT antibodies[1]	43 (19%)	0 (0%)	0 (0%)	0 (0%)
TH				
TH antibody index: mean ± SD	1.58 ± 1.63 (0.26-10.77)	2.51 ± 2.86 (0.76-6.78)	1.69 ± 2.33 (0.65-8.65)	1.01 ± 0.08 (0.87-1.29)
Number positive for TH antibodies[1]	53 (23%)	2 (50%)	2 (18%)	0 (0%)

[1]Patient sera with an antibody index greater than the upper limit of normal (mean antibody index + 3SD of 100 healthy controls), were regarded as positive for antibodies. The upper limits of normal were: 1.72, 1.49, 1.29, 1.57, 1.36, and 1.24 for the tyrosinase, MCHR1, PMEL, TYRP1, DCT, and TH antibody RLBAs, respectively.

5.3.6 Comparison of the demographic, clinical and serological details of the GPNMB antibody-positive and GPNMB antibody-negative non-segmental vitiligo patients

In order to compare the details of the demographic, clinical and serological features of the GPNMB antibody-positive (*n* = 91) and antibody-negative (*n* = 140) non-segmental vitiligo patient groups, Fisher's exact tests for 2 x 2 contingency tables or unpaired t tests were used as appropriate (Section 2.24). *P* values < 0.05 were considered as significant.

The results are shown in Table 5.4. A statistically significant difference was not evident between the two cohorts in terms of sex, patient age, duration of disease, vitiligo onset age, the occurrence of other autoimmune disorders or the prevalence of tyrosinase, PMEL, TYRP1, DCT, and TH antibodies; *P* values were all > 0.05, indicating that GPNMB antibodies were not associated with any of these analysed parameters.

A significantly increased incidence of patients with the symmetrical vitiligo sub-type was obvious in the group of GPNMB antibody-positive patients: *P* value was < 0.0001, whereas an elevated prevalence of patients with the acrofacial sub-type was demonstrated in the cohort of GPNMB antibody-negative patients: *P* value was 0.001. In addition, there was an increased prevalence of patients who had active disease in the cohort of GPNMB antibody-positive patients; *P* value was 0.006. Also, a statistically significant difference was observed in the patient group who were positive for GPNMB antibodies compared to patients in the GPNMB antibody-negative cohort in terms of the presence of antibodies against MCHR1; *P* value was 0.03.

Table 5.4: Comparison of the demographic, clinical, and serological details in non-segmental vitiligo patients positive and negative for GPNMB antibodies

Patient detail	GPNMB antibody-positive non-segmental vitiligo patients (*n* = 91)	GPNMB antibody-negative non-segmental vitiligo patients (*n* = 140)	*P* value
Demographic details			
Male	40 (44%)	57 (41%)	0.68[1]
Female	51 (56%)	83 (59%)	
Mean age ± SD (years)	46 ± 17 (13 - 77)	45 ± 18 (0 - 88)	0.88[2]
Mean onset age ± SD (years)	32 ± 18 (0 - 73)	30 ± 17 (0 - 83)	0.33[2]
Mean disease duration ± SD (years)	13 ± 13 (0.5 - 51)	15 ± 14 (0 - 71)	0.29[2]
Non-segmental vitiligo sub-type			
Symmetrical	82 (90%)	75 (54%)	< 0.0001[1]
Acrofacial	7 (8%)	35 (25%)	0.001[1]
Vitiligo activity			
Active vitiligo	90 (99%)	126 (90%)	0.006[1]
Stable vitiligo	1 (1%)	14 (10%)	
Autoimmune disease			
Present	36 (40%)	45 (32%)	0.26[1]
Absent	55 (60%)	95 (68%)	
Serology tests			
Tyrosinase antibody-positive	19 (21%)	23 (16%)	0.39[1]
MCHR1 antibody-positive	27 (30%)	24 (17%)	0.03[1]
PMEL antibody-positive	12 (13%)	23 (16%)	0.58[1]
TYRP1 antibody-positive	21 (23%)	23 (16%)	0.23[1]
DCT antibody-positive	19 (21%)	24 (17%)	0.49[1]
TH antibody-positive	25 (27%)	28 (20%)	0.20[1]

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of demographic, clinical, and serological details in the GPNMB antibody-positive and the GPNMB antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

[2]*P* value calculated using unpaired t tests (Section 2.24) for comparing the age, onset age, and disease duration in the GPNMB antibody-positive and GPNMB antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

5.4 Discussion

Expression of GPNMB in the epidermis, particularly within melanocyte, has been well-documented (Loftus et al., 2009, Hoashi et al., 2010, Zhang et al., 2012, Maric et al., 2013). It preferentially localises to mature (stage III and IV) melanosomes, which are characterised by the build-up of the melanin pigment, signifying a critical role for GPNMB in melanosome maturation (Tomihari et al., 2009, Hoashi et al., 2010, Maric et al., 2013). GPNMB has been confirmed to mediate melanocyte adhesion to keratinocytes, indicating its involvement in the transport of mature melanosome to keratinocytes (Tomihari et al., 2009, Hoashi et al., 2010, Maric et al., 2013). As other pigmentation proteins such as tyrosinase, TYRP1, DCT, and PMEL, the expression of GPNMB is regulated by the MITF, a major transcriptional factor involved in cell growth, differentiation, and survival of melanocytes (Loftus et al., 2009, Hoashi et al., 2010).

In this study, GPNMB was identified as a novel target of antibody responses in patients suffering from vitiligo by using phage-display technology (Chapter 4). In this chapter, a RLBA was employed to study the prevalence of immunoreactivity to GPNMB in a series of vitiligo patients, and also to find out if there were any associations of GPNMB antibodies with the demographic (e.g., age, gender), clinical features (e.g., vitiligo activity, autoimmune disease) or serological details of vitiligo patients.

5.4.1 Frequency of GPNMB antibodies

Firstly, a RLBA was developed which could be utilised to detect and measure GPNMB antibodies in human sera. After confirming the specificity of the GPNMB RLBA, it was used to analyse 246 vitiligo patient sera for GPNMB antibodies. Of 246 vitiligo patient sera analysed by RLBAs, GPNMB antibodies were detected in 37%, while healthy control sera demonstrated no antibody reactivity, suggesting a high disease-related specificity. The frequency of GPNMB antibodies was relatively high compared to antibody frequencies reported for other pigmentation-related proteins, including tyrosinase (11%) (Kemp et al., 1997a), TYRP1 (6%) (Kemp et al., 1998b), DCT (6%) (Kemp et al., 1997b), MCHR1 (16%) (Kemp et al., 2002b), PMEL (6%) (Kemp et al., 1998a), GTP-binding protein Rab38 (15%) (Waterman et al., 2010), and TH (23%) (Kemp et al., 2011).

This finding suggests that GPNMB might be a useful serum marker for vitiligo diagnosis and evaluation.

5.4.2 GPNMB antibody titres and specificity

In the current work, titration experiments were also performed in order to measure GPNMB antibody titres in the 92 patient sera designated as positive for GPNMB antibodies. The majority (72/92 = 78%) had GPNMB antibodies at a titre of 1:1000 or above. In contrast, previous studies showed much lower titres of antibodies against other melanogenic proteins. For example, TH, PMEL, TYRP1 and MCHR1 antibody titres ranged from 1:50 to 1:200 (Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011).

Also, in order to ensure that GPNMB antibody reactivity was specific to the GPNMB, absorption experiments were carried out. The absorption experiments showed that all the 92 GPNMB antibody-positive patient sera contained GPNMB antibodies that reacted specifically with their target autoantigens without cross-reactivity with other vitiligo-associated autoantigens MCHR1 and tyrosinase, indicating high specificity of GPNMB antibodies. Of note, GPNMB exhibits a high amino acid sequence homology with the melanosomal structural protein PMEL (Hoashi et al., 2010, Zhang et al., 2013). Therefore, GPNMB epitopes may share sequence homology with the antibody binding sites on PMEL(Kemp et al., 2001a), although this was not analysed in the current project.

5.4.3 Prevalence of GPNMB antibodies in vitiligo sub-types

Autoimmunity has been strongly proposed to play a potential role in the aetiology of non-segmental vitiligo, since this type of vitiligo is frequently correlated with autoimmune diseases (Taieb, 2000, Alkhateeb et al., 2003), responds poorly to autologous melanocyte grafting (Taieb, 2000), and is well-responsive to immunosuppressive therapies (Lepe et al., 2003). Non-autoimmune aetiological mechanisms have been suggested to cause segmental vitiligo (Taieb, 2000, Allam and Riad, 2013). In keeping with this, GPNMB antibody reactivity was identified in non-segmental vitiligo and focal vitiligo but not in segmental vitiligo. However, since only

four segmental vitiligo patient samples were available for analysis, a definite conclusion cannot be achieved regarding the frequency of GPNMB antibody reactivity in this form of vitiligo.

5.4.4 Associations of GPNMB antibodies

Importantly, a comparison of GPNMB antibody-positive and GPNMB antibody-negative non-segmental vitiligo patients demonstrated that GPNMB was an antibody target in those with and those without concomitant autoimmune disease. This finding is consistent with previous studies in which TH antibodies were detected in patients with or without accompanying autoimmune disorder (Kemp et al., 2011). However, contradictory results have been reported in other studies, in which antibodies to melanin-related proteins (TYRP1, DCT and PMEL) were only identified in vitiligo patients with other autoimmune diseases (Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b). In addition, Kemp et al. (1997a) and Hedstrand et al. (2001) reported a predominant prevalence of tyrosinase and SOX10 antibody reactivity in vitiligo cases with additional autoimmune disorders.

Additionally, when comparing GPNMB antibody-positive and GPNMB antibody-negative non-segmental vitiligo patients, the frequency of GPNMB antibodies was not significantly correlated with either patient gender, patient age at sampling time, disease onset age, disease duration or the occurrence of antibodies against either tyrosinase, PMEL, TYRP1, DCT or TH. Such results have also been reported for TH antibodies in vitiligo (Kemp et al., 2011).

In contrast, the findings revealed that the prevalence of GPNMB antibodies was significantly increased in the non-segmental vitiligo patient group with active disease. However, only 20 cases with stable vitiligo were available for testing, thus further verification of this finding is required. These results are consistent with a previous study of TH antibodies which were associated with active non-segmental vitiligo (Kemp et al., 2011). Serological analysis revealed association of MCHR1 antibodies with the presence of GPNMB antibodies. Immune reactivity against the receptor had not previously been associated with other antibodies in vitiligo (Kemp et al., 2011).

5.4.5 Development of GPNMB antibodies and potential pathogenicity

The stimulus for the production of GPNMB antibodies might arise from a genetic predisposition to autoimmunity or they may represent a secondary phenomenon emerging as a result of damage to pigment cells by other causal factors, albeit they might then act to further aggravate vitiligo. However, perhaps, GPNMB antibodies play no part in the pathogenesis of vitiligo, but might indicate the existence of autoreactive anti-GPNMB T lymphocytes that are capable of destroying melanocytes, a scenario that merits further investigation.

Antibody reactivity in patients with vitiligo is most often directed against antigens expressed on melanocyte surface (Kemp et al., 2002b). GPNMB primarily localises intracellularly mostly within melanosomes, although it can also be expressed on the surface of melanocytes (Tomihari et al., 2009) making the protein accessible as a target for antibodies that might adversely affect the protein function. Vitiligo-associated antibodies can also destroy melanocytes *in vitro* and *in vivo* following the injection of patient IgG into nude mice grafted with human skin (Gilhar et al., 1995, Essien and Harris, 2014). Similarly, in order to ascertain the potential pathogenicity of GPNMB antibodies, studies on isolated GPNMB-specific antibodies could be carried out.

Since GPNMB expression is unrestricted to pigment cells and the protein is detected, for instance, in dendritic cells, macrophages and keratinocytes (Tomihari et al., 2009, Hoashi et al., 2010, Kramer et al., 2016), any selective destruction of melanocytes in vitiligo might occur as the result of the fact that pigment cells are relatively susceptible to immune injury, as opposed to, for instance, fibroblasts and keratinocytes (Norris et al., 1988).

5.5 Conclusions

To conclude, GPNMB has been found as a novel antibody target in vitiligo, and this further supports the involvement of autoimmunity in vitiligo aetiology. To the best of our knowledge, this finding has not previously been reported. Our finding of significantly increased frequency of GPNMB antibodies in patients with actively progressing vitiligo lesions warrants further investigations regarding its applicability as a useful serum

marker for evaluating disease activity as well as its role in the pathogenesis of the disease.

Chapter 6

Analysis of antibody reactivity against OCA2-encoded P protein in vitiligo patients

6 Analysis of antibody reactivity against OCA2-encoded P protein in vitiligo patients

6.1 Introduction

Human skin colour is primarily determined by melanin, the pigment that is formed from the amino acid precursor L-tyrosine in the intracellular lysosome-like organelles; melanosomes, which are contained in melanocytes (Andrade et al., 2017). In addition to the numerous enzymatic proteins, the melanogenic process also requires other membrane-bound proteins such as OCA2-encoded P protein (OCA2 protein) (Yamaguchi et al., 2007, Andrade et al., 2017). The OCA2 protein is encoded by the *OCA2* gene and is expressed exclusively in melanocytes as an integral constituent of the melanosome membrane (Rimoldi et al., 2014). However, the specific function of OCA2 protein is not fully clear. A previous study showed that in OCA2 protein mutant melanocytes, tyrosinase was properly glycosylated and functionally active but most of the enzyme was never delivered to melanosomes, hence causing disruption in melanin synthesis (Kondo et al., 2015). Therefore, the OCA2 protein was hypothesised to be involved in tyrosinase trafficking to melanosomes (Kondo et al., 2015). In addition, several studies have suggested that the OCA2 protein may be involved in the regulation of pH within melanosomes (Puri et al., 2000, Kondo et al., 2015, Andrade et al., 2017), since an appropriate gradient of pH is necessary for protein trafficking to their destined organelles (Mindell, 2012, Ishida et al., 2013, Kondo et al., 2015).

In addition to causing disruption in tyrosinase transport to melanosomes, mutations in the *OCA2* gene are also associated with genetic predisposition to melanoma, type II oculocutaneous albinism in humans, and pink-eye dilution in mice (Oetting et al., 2005, Ibarrola-Villava et al., 2010, Wang et al., 2015, Andrade et al., 2017). Furthermore, non-pathological polymorphisms in this gene are associated with normal pigmentation variation in different populations, and thus the gene has been considered as a major determinant of skin colour (Frudakis et al., 2007, Yuasa et al., 2011, Andrade et al., 2017). Normal polymorphisms of *OCA2* also influence hair and eye colour (Cook et al., 2009). Interestingly, *OCA2* has been identified as a susceptibility gene for vitiligo (Jin et

al., 2012a), and single nucleotide polymorphic alleles of *OCA2* that are of low-risk for vitiligo are strongly correlated with a high risk of malignant melanoma (Jannot et al., 2005, Amos et al., 2011, Jin et al., 2012a). In this respect, *OCA2* is analogous to the *TYR* gene (Jin et al., 2012a). Both genes also encode melanocyte-specific proteins that can be presented to the immune system by HLA-A*02 (Jin et al., 2012a).

In this project, the OCA2 protein was identified as a potential antibody target following rounds of panning against a melanocyte cDNA phage-display library with vitiligo patient IgG (Chapter 4).

6.2 Aims

The main objectives of this part of the project were:

a) To develop a RLBA to confirm antibody reactivity against OCA2 protein.

b) To investigate OCA2 protein antibody frequencies in 246 vitiligo patients and 100 healthy controls using the RLBA.

c) To determine if antibodies to OCA2 protein were related to any clinical, demographic or serological features of vitiligo patients.

6.3 Experiments and Results

6.3.1 Development of an OCA2 protein RLBA

In order to study the prevalence of antibodies against OCA2 protein in vitiligo patient sera, it was first necessary to have OCA2 protein cDNA in a suitable plasmid vector for expression of the OCA2 protein, which could then be used as the target antigen to test antibody binding in RLBAs. Plasmid pcDNA3.1 (Figure 6.1) was chosen as it contains a T7 polymerase promoter binding site from which cloned cDNA can be transcribed and translated *in vitro* with the concomitant incorporation of radiolabelled [^{35}S]-methionine. The [^{35}S]-OCA2 protein produced in this way can then be used in RLBAs to detect OCA2 protein antibodies in vitiligo patient and healthy control serum samples.

6.3.1.1 OCA2 protein cDNA clone confirmation

The OCA2 protein cDNA cloned into the *Kpn*I-*Hin*dIII restriction enzyme sites of pcDNA3.1 was obtained from GeneScript (Piscataway, NJ, USA). As a check that the recombinant plasmid pcDNA3.1-OCA2 was correct, the plasmid was subjected to DNA sequencing (Section 2.17) using primers T7, SP6, OCA2-1-Forward, OCA2-2-Forward, OCA2-3-Forward, OCA2-4-Forward, and OCA2-5-Forward (Table 2.5). The results verified that the cloned OCA2 cDNA was correct when searched against the GenBank database using the network facilities of the NCBI (Bethesda, MD, USA) (Figure 6.2). The amino acid sequence of the OCA2 protein is illustrated in Figure 6.3.

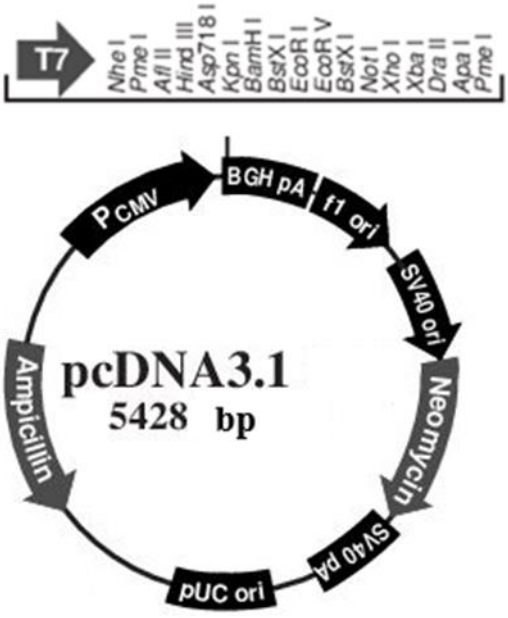


Figure 6.1: A map of the pcDNA3.1 plasmid vector.

The map shows restriction enzyme sites and the direction of the T7 promoter in the pcDNA3.1 plasmid vector. The 2.5-kb OCA2 protein cDNA was cloned between the *Kpn*I and *Hin*dIII restriction enzyme sites.

atgcatctggagggcagagacggcaggcggtaccccggcgcgccggcggtggagctcctgcagacgtc
cgtgcccagcggactcgctgaacttgtggccggcaagcgcaggcttcctcggggagccggtggagctg
acccctcgcactcctgccccaggggggctgccgggcagagctcttgggctcctgcaggccaggagttt
gcttcattcctcacaaaagggaggtctcactcttctttgccccagatgtccagctccaggtctaaaga
ttcctgctttacagaaaacactcctttgctgaggaattccttacaggagaaagggtcacggtgcatac
ctgtttaccatccagagttcatcactgctgaagagtcttgggaagacagctctgctgactgggagcga
agatacctgctaagcagggaggtgtctggtctgtctgcatctgcctcctccgagaagggagaccttct
ggacagcccgcacatccgactccgtctttccaagctgaggcgctgtgtgcagtggctgaaagtcatgg
gcctgtttgcctttgtggtgctgtgttctattttgttcagcctatatccggatcaaggaaagctctgg
cagctgttggccttatcaccgctggagaactactccgtgaaccttagcagccacgtggactccacgct
gctgcaggtggacctggcaggggccctagtggccagtgggccgagtcgtcctgggagggaagagcaca
tcgtggtggagctgacccaggctgacgctttgggctccaggtggcggcggccacagcaggtcactcac
aactggacggtgtatttaaatccgaggagaagcgagcactcagtgatgagcaggacctttgaggtact
gaccagagagacggtgtccatcagcatccgggcctccctgcagcagacccaggctgtccctcttttga
tggctcatcagtacctccgcggaagtgtagaaacccaggtgaccatcgcgacggccatcctcgcgggc
gtctacgcgctgatcatatttgagagacccagcctgacccatgtggtggagtggattgattttgagac
gctggccctgctgtttggcatgatgatcttagtagccatattttcagaaacgggatttttcgattatt
gtgctgtaaaggcataccggctctcccggggacgggtgtgggccatgatcatcatgctctgtctcatc
gcggccgtcctctctgccttcttggacaacgtcaccaccatgctcctcttcacgcctgtgaccataag
gttgtgtgaggtgctcaaccttgatccaagacaagtcctgattgcagaagtgatcttcacaaacattg
gaggagctgccactgccatcggggaccctccaaatgtcattattgtttccaaccaagagctgaggaag
atgggcctggactttgccggattcactgcacacatgttcattgggatttgtcttgttctcctggtctg
ctttccgctcctcagactcctttactggaacagaaagctttataacaaggaacccagtgagattgttg
aactgaagcacgagattcacgtctggcgcctgactgctcagcgcatcagcccggccagccgcgaggag
acagctgtgcgccgcctgctgctggggaaggtgctggcactggagcacctgctcgcccggaggctgca
caccttccacagacagatctcacaggaggacaaaaattgggagaccaatatccaagaactccaaaaaa
agcataggatatctgacgggattctgctcgccaaatgcctgacagtgttgggatttgttatcttcatg
tttttcctcaattcgtttgtccctggcattcatcttgatcttggatggattgctattctgggtgccat
ctggttgctaattttagctgatattcatgattttgagataattctacacagagtggaatgggcaaccc
ttctgtttttgcagcgctctttgttctgatggaggcattggcacatctccacttaatagaatatgtt
ggagaacaaactgctttgctaataaagatggtcccagaggagcagcgcctcatagccgccattgtcct
ggtggtgtgggtctcagccctggcgtcgtccctgattgacaacatcccgttcactgctaccatgattc
ccgtgctcctgaacctgagccacgaccctgaggttggcctgcccgcaccgccgctcatgtatgccctg
gccttcggtgcttgcctgggaggtaacgggacactgattggcgcgtcagcaaacgtcgtgtgtgcagg
gattgcagaacagcatggatatgggttctccttcatggaatttttcaggctgggcttcccaatgatgg
ttgtgtcctgcactgttgggatgtgttatctccttgtggctcatgtggtggtgggatggaat**taa**

Figure 6.2: The cDNA sequence encoding the OCA2 protein.

The cDNA sequence matched that of the OCA2 protein cDNA Accession Number NM_000275 when searched against the GenBank database using the network facilities of NCBI (Bethesda, MD, USA). The ATG translation start codon and the TAA translation termination codon are underlined and given in bold-type. The cDNA has 2,445 base pairs.

MHLEGRDGRRYPGAPAVELLQTSVPSGLAELVAGKRRLPRGAGGADPSHSCPRGAAGQSSWAPAGQEF
ASFLTKGRSHSSLPQMSSSRSKDSCFTENTPLLRNSLQEKGSRCIPVYHPEFITAEESWEDSSADWER
RYLLSREVSGLSASASSEKGDLLDSPHIRLRLSKLRRCVQWLKVMGLFAFVVLCSILFSLYPDQGKLW
QLLALSPLENYSVNLSSHVDSTLLQVDLAGALVASGPSRPGREEHIVVELTQADALGSRWRRPQQVTH
NWTVYLNPRRSEHSVMSRTFEVLTRETVSISIRASLQQTQAVPLLMAHQYLRGSVETQVTIATAILAG
VYALIIFERPSLTHVVEWIDFETLALLFGMMILVAIFSETGFFDYCAVKAYRLSRGRVWAMIIMLCLI
AAVLSAFLDNVTTMLLFTPVTIRLCEVLNLDPRQVLIAEVIFTNIGGAATAIGDPPNVIIVSNQELRK
MGLDFAGFTAHMFIGICLVLLVCFPLLRLLYWNRKLYNKEPSEIVELKHEIHVWRLTAQRISPASREE
TAVRRLLLGKVLALEHLLARRLHTFHRQISQEDKNWETNIQELQKKHRISDGILLAKCLTVLGFVIFM
FFLNSFVPGIHLDLGWIAILGAIWLLILADIHDFEIILHRVEWATLLFFAALFVLMEALAHLHLIEYV
GEQTALLIKMVPEEQRLIAAIVLVVWVSALASSLIDNIPFTATMIPVLLNLSHDPEVGLPAPPLMYAL
AFGACLGGNGTLIGASANVVCAGIAEQHGYGFSFMEFFRLGFPMMVVSCTVGMCYLLVAHVVVGWN

Figure 6.3: The OCA2 protein amino acid sequence.

The protein has 815 amino acid residues which are shown by their one letter symbol.

6.3.1.2 *In vitro* transcription-translation of OCA2 protein cDNA

Plasmid pcDNA3.1-OCA2 was transcribed and translated in a TnT® T7-Coupled Reticulocyte Lysate System, as described in Section 2.19. In order to analyse the radiolabelled product qualitatively, a 5-µl aliquot of the *in vitro* transcription-translation reaction was added to 20 µl of 2x Laemmli sample buffer (Section 2.20) and heated to 100°C for 5 min. A 10-µl sample was then applied to a 10% (w/v) SDS-polyacrylamide gel and subjected to electrophoresis (Section 2.20). After drying, the gel was exposed to x-ray film, which was developed after 24 h (Section 2.20).

The result showed a protein product with an estimated molecular weight of 88 kDa (Figure 6.4). This agrees well with the molecular weight of 89 kDa calculated from the amino acid sequence of the protein with 815 residues (Figure 6.3).

6.3.1.3 Analysis of the immunoreactivity of [^{35}S]-OCA2 protein

The immunoreactivity of the *in vitro* translated [^{35}S]-OCA2 protein was analysed in a RLBA (Section 2.21) with an anti-OCA2 protein antibody and a panel of antibodies raised against proteins unrelated to the OCA2 protein (Table 2.6). The antibodies used were PA5-23708 (anti-OCA2 protein), α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH), and α-PEP8 (anti-DCT). They were all used at a 1:100 dilution. An immunoprecipitation reaction without any antibodies was also included in each assay set, as a background control. From each RLBA, an OCA2 protein antibody index was calculated for each of the antibodies tested as: cpm immunoprecipitated by tested antibody/cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean OCA2 protein antibody index was calculated from these values. The OCA2 protein antibody indices of the different antibodies were compared using one-way ANOVA, with P values < 0.05 considered significant.

The results are shown in Figure 6.5, and indicated that the mean OCA2 protein antibody index ± SD for each antibody was 28.1 ± 4.98 for PA5-23708, 1.12 ± 0.12 for α-PEP7, 1.09 ± 0.11 for MCHR11-S, 1.15 ± 0.09 for ab59276, and 1.06 ± 0.08 for α-PEP8. In one-way ANOVA, the OCA2 protein antibody index of the OCA2 protein antibody PA5-23708 was statistically significantly higher in comparison to those for α-PEP7, MCHR11-S, ab59276,

and α-PEP8; $P < 0.0001$. The result demonstrated a specific immunoprecipitation of [^{35}S]-OCA2 protein by anti-OCA2 protein antibody, and indicated that [^{35}S]-OCA2 protein could be used as a ligand in a RLBAs to detect antibodies against OCA2 protein.

The OCA2 protein immunoprecipitated in the RLBA by antibody PA5-23708 was also analysed quantitatively. Following the RLBA using [^{35}S]-OCA2 protein and antibody PA5-23708, the protein G Sepharose-OCA2 protein-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged, and the supernatant analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel (Section 2.20). The gel was then subjected to autoradiography (Section 2.20). The results indicated that the antibody PA5-23708 immunoprecipitated a protein band of 88 kDa, equivalent in size to that of *in vitro* translated [^{35}S]-OCA2 protein, and this is shown in Figure 6.6.

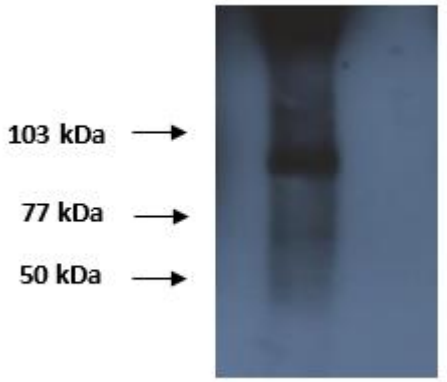


Figure 6.4: SDS-PAGE and autoradiography of [^{35}S]-OCA2 protein.

[^{35}S]-OCA2 protein was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3.1-OCA2 as a template for transcription and translation of OCA2 cDNA. For analysis, the radiolabelled protein was run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The OCA2 protein is visible at 88 kDa.

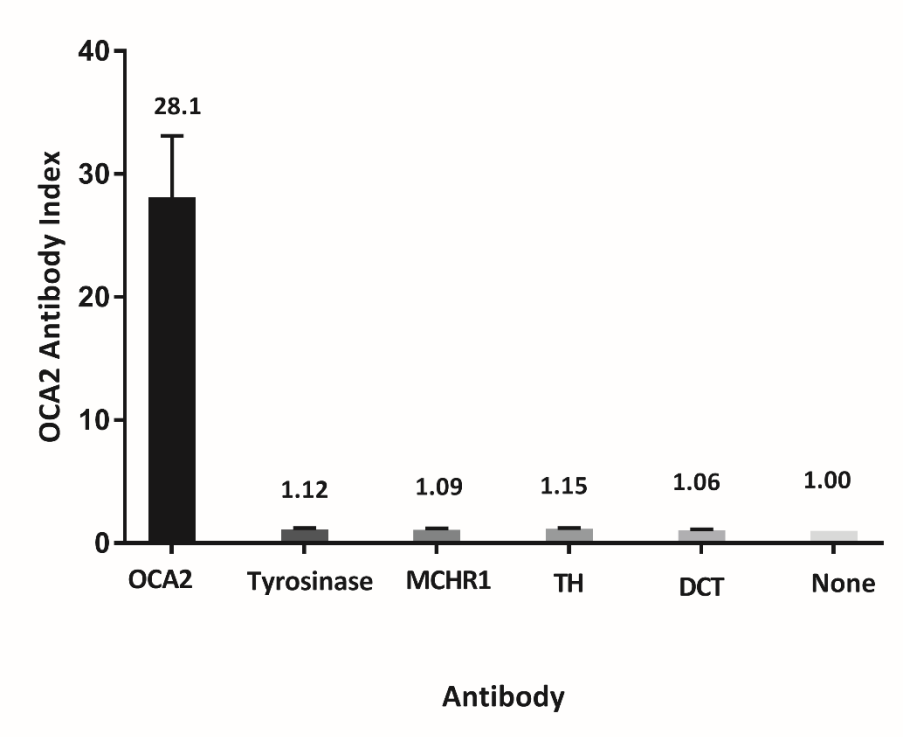


Figure 6.5: OCA2 protein RLBA using different antibodies.

The immunoreactivity of *in vitro* translated [^{35}S]-OCA2 protein was tested in a RLBA with antibodies anti-OCA2 protein (PA5-23708), anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276) and anti-DCT (α-PEP8). An immunoprecipitation reaction without serum was included as a background control. The mean OCA2 protein antibody index ± SD of four experiments is given for each antibody: PA5-23708, 28.1 ± 4.98; α-PEP7, 1.12 ± 0.12; MCHR11-S, 1.09 ± 0.11; ab59276, 1.15 ± 0.09; and α-PEP8, 1.06 ± 0.08. $P < 0.0001$ (One-way ANOVA).

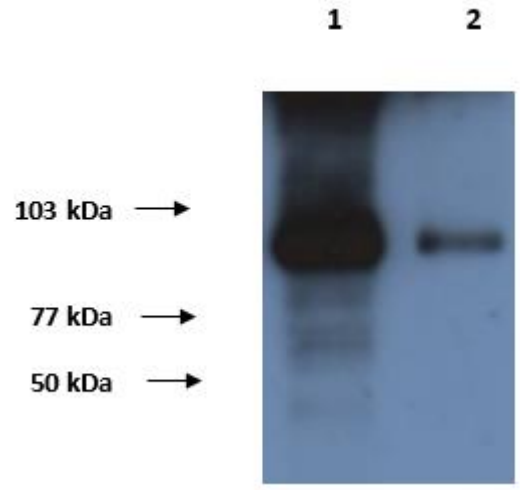


Figure 6.6: SDS-PAGE and autoradiography of [^{35}S]-OCA2 protein and [^{35}S]-OCA2 protein immunoprecipitated by anti-OCA2 protein antibody PA5-23708.

[^{35}S]-OCA2 protein was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3.1-OCA2. Subsequently, radiolabelled OCA2 protein was immunoprecipitated in a RLBA with anti-OCA2 protein antibody PA5-23708. For analysis, Iimmunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results are shown for: *In vitro* translated, non-immunoprecitated [^{35}S]-OCA2 protein (lane 1); [^{35}S]-OCA2 protein immunoprecipitated with anti-OCA2 protein antibody PA5-23708 (lane 2). The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The OCA2 protein is visible at 88 kDa.

6.3.1.4 Analysis of the specificity of the anti-OCA2 protein antibody

In order to determine the anti-OCA2 antibody PA5-23708 specificity, PA5-23708 was used in RLBAs with other radiolabelled antigens and these included tyrosinase, MCHR1, TH and DCT.

Radiolabelled [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT were produced in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.19) from plasmids pcDNA3-TYR, pcMCHR1, pcDNA3-TH and pCMVDTS (Table 2.2), respectively. RLBAs were carried out as detailed in (Section 2.21), using these labelled antigens with antibody PA5-23708 at a 1:100 dilution. Appropriate positive control antibodies were included at a 1:100 dilution in each RLBA: α-PEP7 for tyrosinase, MCHR11-S for MCHR1, ab59276 for TH, and α-PEP8 for DCT. An immunoprecipitation reaction without antibody was also included in each RLBA, as a background control. In each RLBA, an antibody index was calculated for each antibody as: cpm immunoprecipitated by tested antibody/cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean antibody index was calculated from these values. Antibody indices were compared using one-way ANOVA with *P* values < 0.05 regarded as significant.

The mean antibody index ± SD for anti-OCA2 protein antibody in RLBAs against different antigens was 26.5 ± 3.94 against OCA2 protein, 1.01 ± 0.05 against tyrosinase, 0.99 ± 0.11 against MCHR1, 1.06 ± 0.08 against TH, and 1.02 ± 0.10 against DCT (Figure 6.7). In one-way ANOVA, the antibody index of the anti-OCA2 protein antibody in the different RLBAs was compared; *P* < 0.0001, indicating there was no significant immunoprecipitation of other unrelated proteins. Anti-OCA2 protein antibody PA5-23708 could be used therefore as a positive control antibody in OCA2 protein RLBAs.

Additionally, the mean antibody index ± SD for the antibodies α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH), and α-PEP8 (anti-DCT) in RLBAs against their cognate antigens was 13.6 ± 1.74, 12.7 ± 1.28, 10.5 ± 1.57 and 11.4 ± 1.24, respectively (Figure 6.7).

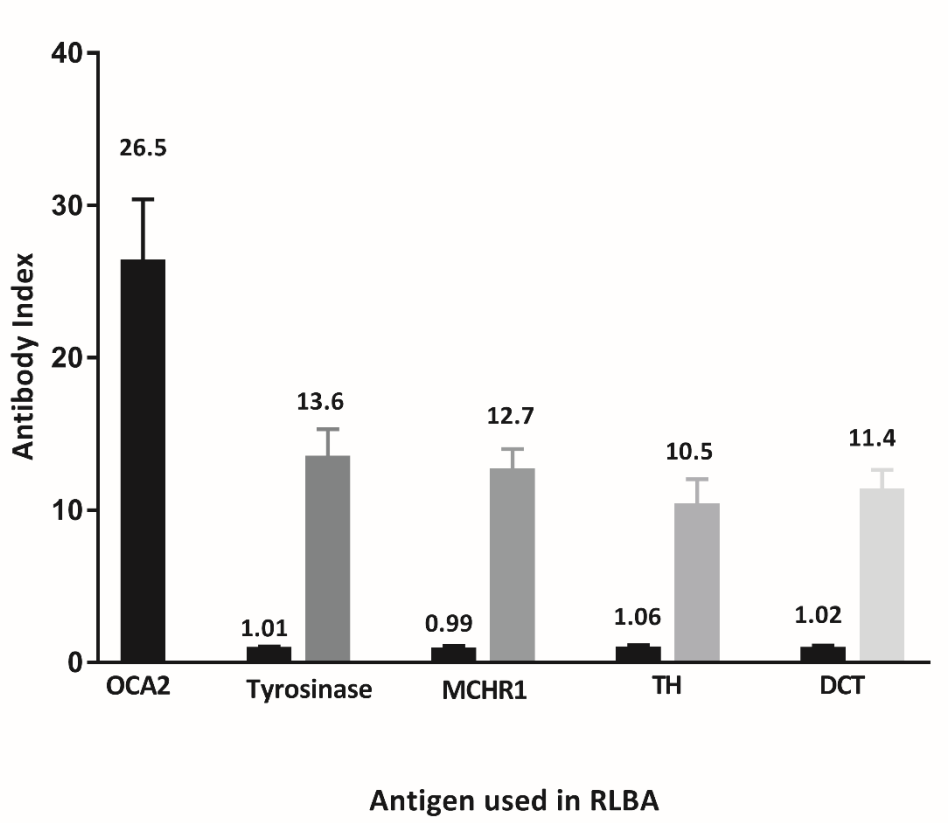


Figure 6.7: RLBAs with anti-OCA2 protein antibody PA5-23708 and different antigens.

The immunoreactivity of the anti-OCA2 protein antibody PA5-23708 was tested against *in vitro* translated [^{35}S]-OCA2 protein, [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT) in RLBAs. Antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) were included in the relevant RLBA as positive controls for immunoprecipitation of their respective antigens. An immunoprecipitation reaction without any serum was included as a background control in each RLBA. The mean antibody index ± SD of four experiments is shown for the antibody PA5-23708 in RLBAs against the different antigens; 26.5 ± 3.94 against the OCA2 protein, 1.01 ± 0.05 against tyrosinase, 0.99 ± 0.11 against MCHR1, 1.06 ± 0.08 against TH, and 1.02 ± 0.10 against DCT. $P < 0.0001$ (One-way ANOVA). The mean antibody index ± SD of four experiments is also shown for the antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) in RLBAs against their cognate antigens; 13.6 ± 1.74, 12.7 ± 1.28, 10.5 ± 1.57 and 11.4 ± 1.24, respectively.

6.3.2 Use of the OCA2 protein RLBA to analyse vitiligo patients and control sera for OCA2 protein antibodies

To analyse vitiligo patient and control sera for the presence of OCA2 protein antibodies, OCA2 protein RLBAs were carried out using [^{35}S]-OCA2 protein as the radiolabelled antigen. The sera analysed were from patients with vitiligo (*n* = 246) and from healthy controls (*n* = 100). All sera were assayed in duplicate at a final dilution of 1:100. Anti-OCA2 protein antibody PA5-23708 was included in each assay set as a positive control at a dilution of 1:100. In each assay set, antibody levels were expressed as OCA2 protein antibody index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 100 healthy control sera. Each serum was tested in at least three experiments and the mean OCA2 protein antibody index was calculated from these values. The upper limit of normal for the assay was calculated using the mean OCA2 protein antibody index + 3SD of the population of 100 healthy controls. Any serum sample with an OCA2 protein antibody index above the upper limit of normal was designated as positive for OCA2 protein antibodies.

The results of the OCA2 protein RLBAs are shown in Figure 6.8. The mean OCA2 protein antibody index ± SD for the control group (*n* = 100) was 1.02 ± 0.11 with a range of 0.75-1.33. The mean OCA2 protein antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo (*n* = 4), and undetermined vitiligo (*n* = 11) was 2.14 ± 2.64 (range, 0.77-13.1), 2.05 ± 2.00 (range, 0.95-5.05), and 1.40 ± 1.18 (range, 0.90-4.95), respectively. The upper limit of normal for the OCA2 protein RLBA was an OCA2 protein antibody index of 1.34.

Healthy controls were all OCA2 protein antibody-negative with OCA2 protein antibody indices below 1.34. Of the 231 non-segmental vitiligo patients, 52 (23%) were considered OCA2 protein antibody-positive (Table 6.1), as their OCA2 protein antibody index was above the upper limit of normal value of 1.34. Anti-OCA2 protein antibodies were detected in one patient with segmental vitiligo and in one patient with undetermined vitiligo sub-type (Table 6.1).

The prevalence of OCA2 protein antibodies was compared between vitiligo patients and controls in Fisher's exact test for 2 x 2 contingency tables (Section 2.24). *P* values < 0.05

were regarded as significant. The results are given in Table 6.1. In comparison with controls, a statistically significant increase in the prevalence of OCA2 protein antibodies was found in the patient group with non-segmental vitiligo; the *P* value was < 0.0001. In addition, there was a statistically significant increase in the frequency of OCA2 protein antibodies in patients with symmetrical or acrofacial vitiligo (Table 6.1). However, in most vitiligo sub-type groups, there were too few patients to make statistical analysis robust (Table 6.1). There was no difference in OCA2 protein antibody prevalence in patients with active or stable vitiligo or in those with or without autoimmune disease (Table 6.1).

To analyse qualitatively, the proteins immunoprecipitated in the OCA2 protein RLBA by vitiligo patient and control sera, protein G Sepharose-OCA2 protein-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged and the supernatant recovered for SDS-PAGE in 10% (w/v) SDS-polyacrylamide gels and subsequent autoradiography (Section 2.20).

The results indicated that all 54 of the OCA2 protein antibody-positive vitiligo patient sera analysed immunoprecipitated a protein band of 88 kDa, comparable to the size of OCA2 protein in SDS-polyacrylamide gels. The results for eight OCA2 protein antibody-positive vitiligo patient sera and for one healthy control sera are illustrated in Figure 6.9.

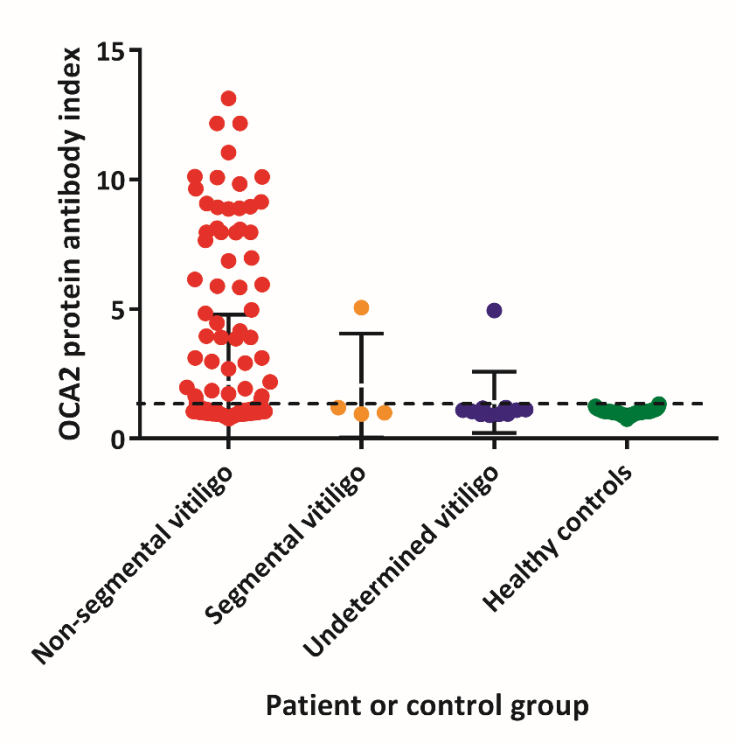


Figure 6.8: OCA2 protein RLBA results for vitiligo patient and healthy control sera.

Vitiligo patient and healthy control sera were evaluated for OCA2 protein antibodies in the OCA2 protein RLBA. The OCA2 protein antibody indices are shown for sera from vitiligo patients (*n* = 246) and healthy controls (*n* = 100). The OCA2 protein antibody index shown for each serum is the mean of three experiments. The mean OCA2 protein antibody index ± SD for the control group was 1.02 ± 0.11 with a range of 0.75-1.33. The mean OCA2 protein antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo (*n* = 4), and undetermined vitiligo (*n* = 11) was 2.14 ± 2.64 (range, 0.77-13.1), 2.05 ± 2.00 (range, 0.95-5.05), and 1.40 ± 1.18 (range, 0.90-4.95), respectively. The upper limit of normal (mean OCA2 protein antibody index + 3SD of 100 healthy controls) for the OCA2 protein RLBA was an OCA2 protein antibody index of 1.34, and this is shown by the dotted line.

Table 6.1: OCA2 protein antibody prevalence in vitiligo patients and healthy controls

Vitiligo patient or control group	OCA2 protein antibody-positive samples	*P* value[1]	*P* value[2]
Vitiligo patient or control group			
Non-segmental vitiligo	52/231 (23%)	< 0.0001	-
Segmental vitiligo	1/4 (25%)	ND	-
Undetermined vitiligo	1/11 (9%)	ND	-
Healthy controls	0/100 (0%)	ND	-
Non-segmental vitiligo sub-type			
Symmetrical (generalised)	38/157 (24%)	< 0.0001	-
Acrofacial	11/42 (26%)	< 0.0001	-
Acral	1/4 (25%)	ND	-
Peri-orificial	0/3 (0%)	ND	-
Universal	0/1 (0%)	ND	-
Symmetrical and peri-orificial	2/10 (20%)	ND	-
Symmetrical and acrofacial	0/7 (0%)	ND	-
Symmetrical, peri-orificial and acrofacial	0/1 (0%)	ND	-
Occupational	0/1 (0%)	ND	-
Mixed	0/5 (0%)	ND	-
Vitiligo activity			
Active	50/226 (22%)	< 0.0001	1.00
Stable	4/20 (20%)	0.006	
Autoimmune disease			
Present	20/84 (24%)	< 0.0001	1.00
Absent	34/162 (21%)	< 0.0001	

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of OCA2 protein antibodies in vitiligo patients and controls. $P < 0.05$ was considered statistically significant.

[2]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of OCA2 protein antibodies in vitiligo patient groups. $P < 0.05$ was considered statistically significant.

ND, not determined due to small sample size or no OCA2 protein antibody-positive patients in the group.

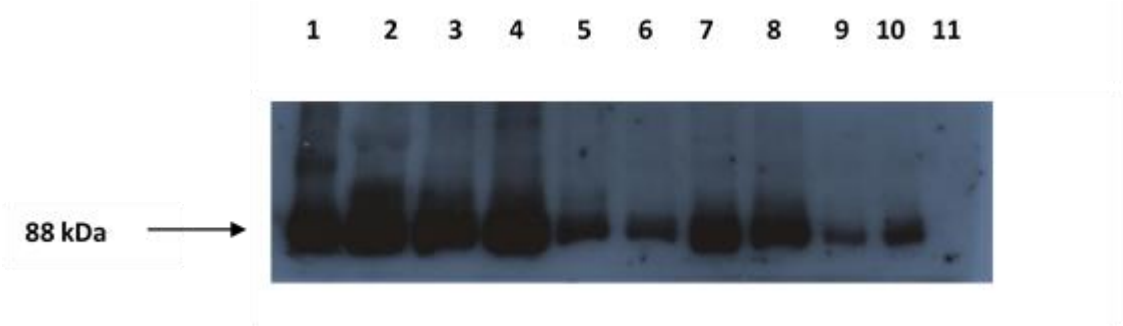


Figure 6.9: SDS-PAGE and autoradiography of [^{35}S]-OCA2 protein following immunoprecipitation in OCA2 protein RLBAs with vitiligo patient and healthy control sera.

Radiolabelled OCA2 protein was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System and then immunoprecipitated in OCA2 protein RLBAs with either vitiligo or healthy control sera. For analysis, immunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results are shown for eight vitiligo patients and one healthy control sera: *In vitro* translated [^{35}S]-OCA2 protein (lane 1); [^{35}S]-OCA2 protein immunoprecipitated with: anti-OCA2 protein antibody PA5-23708 (lane 2); sera from OCA2 protein antibody-positive vitiligo patients (lanes 3-10); sera from healthy control (lane 11). The OCA2 protein is visible at 88 kDa.

6.3.3 Determination of OCA2 protein antibody titres in vitiligo patients

To determine the titres of OCA2 protein antibodies, sera from all OCA2 antibody-positive vitiligo patients (*n* = 54) and healthy controls (*n* = 10) were analysed in the OCA2 protein antibody RLBA at different dilutions including 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:5000. For each serum sample tested, an OCA2 protein antibody index (cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by 10 healthy control sera at each dilution) was calculated and this was the mean OCA2 protein antibody index of three experiments.

The results of the antibody titration experiments are given in Figure 6.10. The titration results are also summarised in Table 6.2, and show the serum dilution at which OCA2 protein antibodies were still detectable at levels higher than the upper limit of normal for the OCA2 protein antibody RLBA. Although the titres of OCA2 protein antibodies varied between the different vitiligo patients, the majority, 43/54 (80%), had a titre at or above 1:1000, and 13/54 (24%) had a titre above 1:5000.

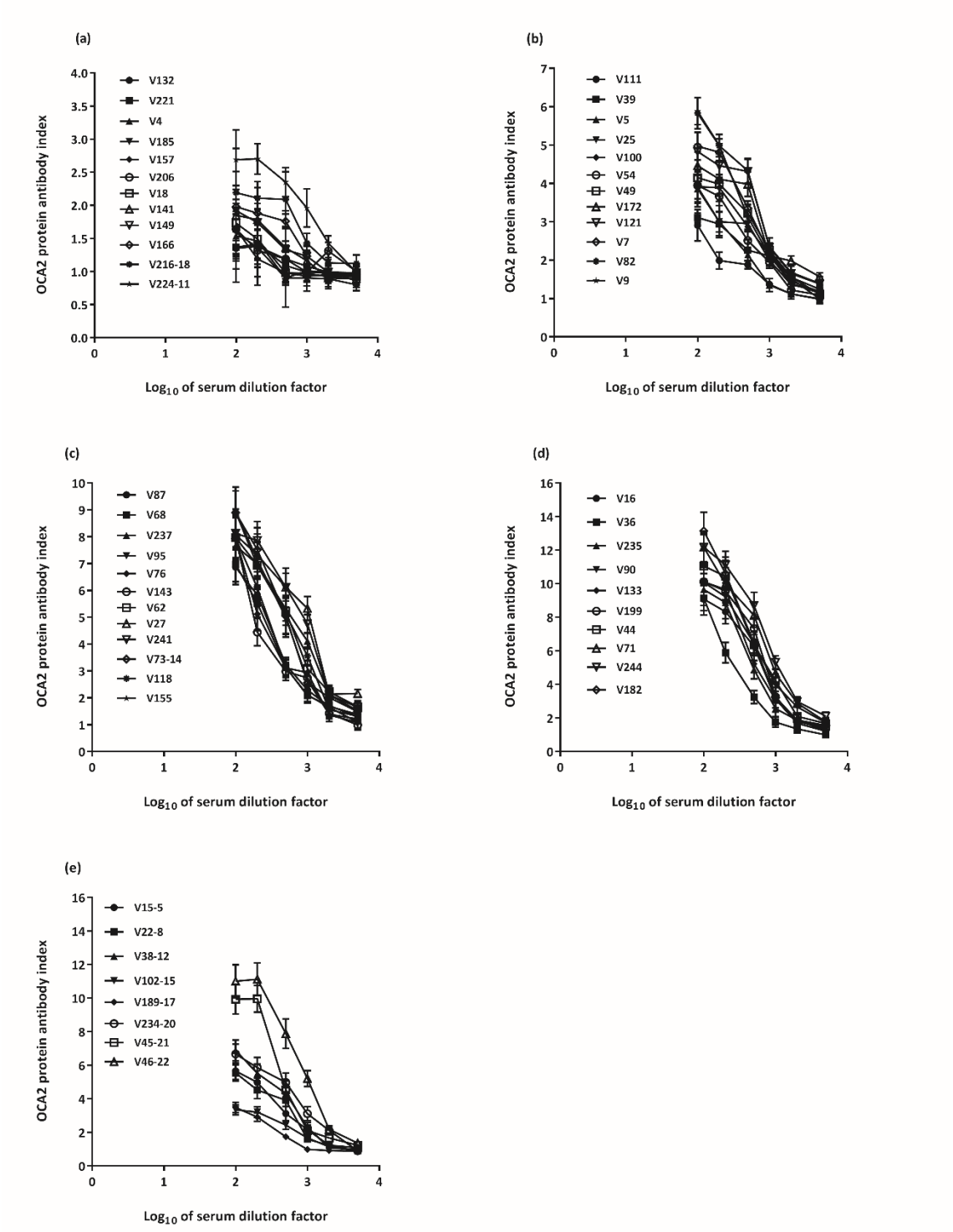


Figure 6.10: OCA2 protein antibody titres.

The 54 OCA2 protein antibody-positive vitiligo patient sera were analysed in the OCA2 protein antibody RLBA at dilutions of 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:5000. The OCA2 protein antibody index (mean ± SD of three exepriments) of each serum at each dilution is shown. (a) The results are shown for 12 vitiligo patients. (b) The results are shown for 12 vitiligo patients. (c) The results are shown for 12 vitiligo patients. (d) The results are shown for 10 vitiligo patients. (e) The results are shown for eight vitiligo patients.

Table 6.2: Summary of OCA2 protein antibody titres

Patients	OCA2 protein antibody titre[1]
V157, V206	1:100
V4, V132, V221, V185, V18, V141, V149	1:200
V166, V189-17	1:500
V216-18, V111, V5, V54, V36, V15-5, V22-8, V38-12, V102-15	1:1000
V224-11, V39, V25, V100, V49, V7, V9, V95, V76, V118, V155, V16, V133, V234-20, V45-21, V87, V68, V143	1:2000
V172, V121, V82	1:5000
V237, V62, V27, V241, V73-14, V235, V90, V199, V44, V71, V244, V182, V46-22	>1:5000

[1]The titre of OCA2 protein antibody is shown as the dilution at which immunoreactivity of the serum sample could still be detectable above the upper limit of normal in the OCA2 protein antibody RLBA.

6.3.4 Assessment of the specificity of OCA2 protein antibodies

In order to determine that OCA2 protein antibodies in vitiligo patient sera reacted specifically against the protein OCA2, absorption experiments were performed as detailed in Section 2.22. Briefly, serum samples from OCA2 protein antibody-positive vitiligo patients (*n* = 54) and healthy individuals (*n* = 10) were absorbed with extracts prepared from untransfected HEK293 cells or from HEK293 cells transfected with OCA2 protein, MCHR1 or tyrosinase. Subsequently, duplicate samples of pre-absorbed sera at a 1:100 dilution (equivalent to the original sera dilution) were evaluated in the OCA2 protein RLBA. For each serum sample, an OCA2 protein antibody index (cpm immunoprecipitated by tested serum/the cpm immunoprecipitated by 10 healthy control sera) was calculated. Serum samples were all tested in triplicate. To compare the OCA2 protein antibody index of absorbed against unabsorbed vitiligo patient sera, one-way ANOVA was used (Section 2.24). *P* values < 0.05 were considered as significant in all tests.

The absorption experiment results are illustrated in Figure 6.11. There was a statistically significant reduction in the OCA2 protein antibody indices by pre-absorption of OCA2 protein antibody-positive vitiligo patient sera with the OCA2 protein expressed in the extracts prepared from HEK293 cells: all *P* values were < 0.05. In contrast, OCA2 protein antibody indices were not significantly decreased after pre-absorption of OCA2 protein antibody-positive vitiligo patient sera with extracts made from untransfected HEK293 cells or HEK293 cells harbouring expressed MCHR1 or tyrosinase: *P* values were > 0.05.

Overall, these findings indicated that OCA2 protein antibodies in all OCA2 protein antibody-positive patient sera specifically recognised their target autoantigen without cross-reactivity with vitiligo-associated autoantigens MCHR1 and tyrosinase.

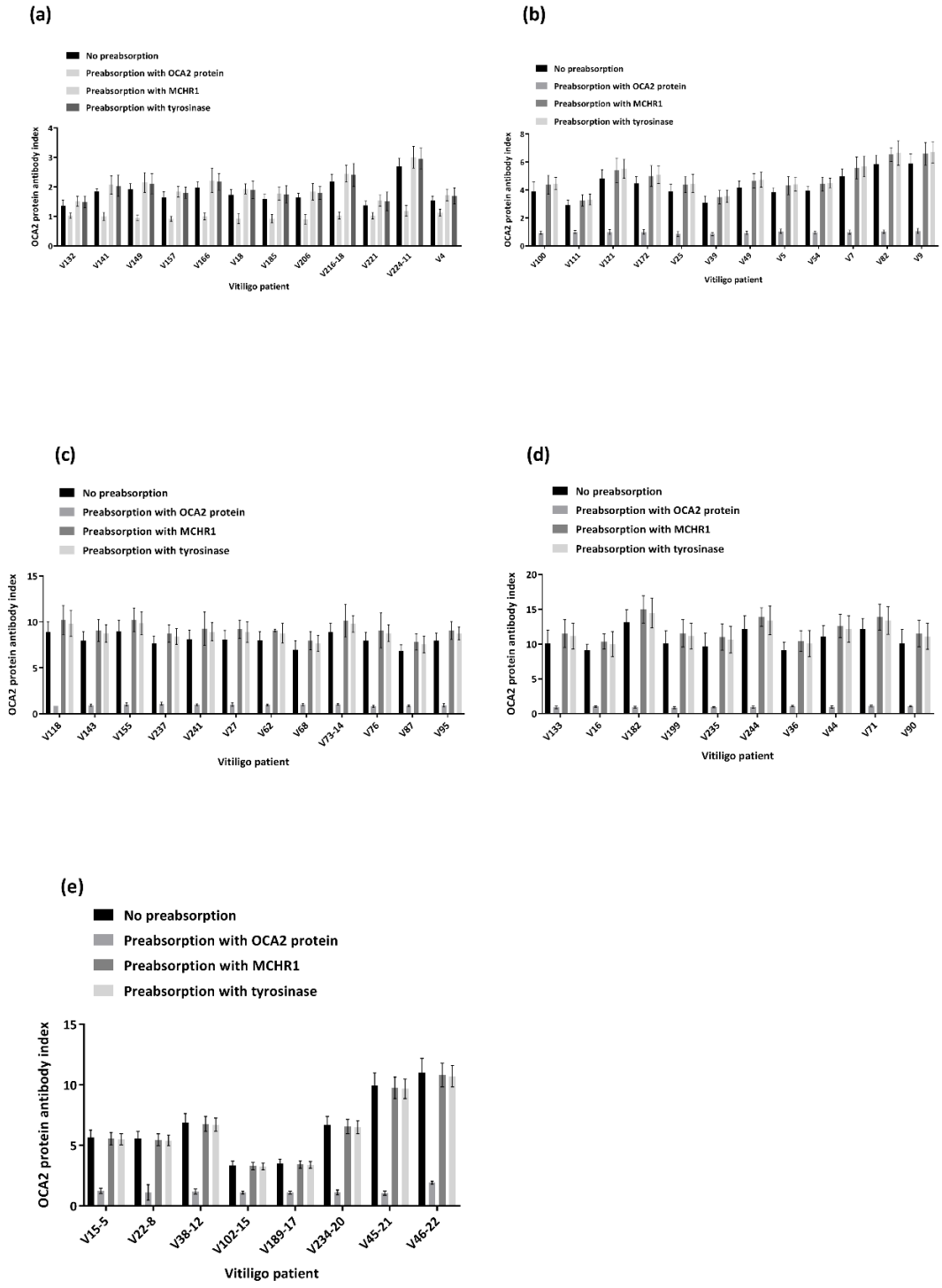


Figure 6.11: OCA2 protein antibody absorption experiments.

Serum samples from the 54 OCA2 protein antibody-positive vitiligo patients and 10 healthy controls were preabsorbed with extract made from either untransfected HEK293 cells or from HEK293 cells expressing OCA2-encoded P protein (OCA2 protein), tyrosinase or melanin-concentrating hormone receptor 1 (MCHR1). Unabsorbed and preabsorbed sera were then analysed in the OCA2 protein RLBA. The results demonstrate the mean OCA2 protein antibody index (± SD) of three experiments for unabsorbed and preabsorbed sera. The results are given for the 54 vitiligo patients. Comparing the antibody indices of OCA2 protein-absorbed sera with those without preabsorption; *P* values were < 0.0001 (One-way ANOVA).

6.3.5 Analysis of vitiligo patient sera for other vitiligo-associated antibodies

As detailed earlier in Section 5.3.5, RLBAs for tyrosinase, DCT, TYRP1, PMEL, TH, and MCHR1 antibodies (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011) were performed on all the 246 vitiligo patient sera. The results are shown in Table 5.3, and used to determine if antibodies to OCA2 protein were related to the presence of any antibodies directed against other vitiligo-associated autoantigens (Section 6.3.6).

6.3.6 Comparison of the demographic, clinical, and serological details of the OCA2 protein antibody-positive and OCA2 protein antibody-negative non-segmental vitiligo patients

In order to compare the details of the demographic, clinical and serological features of the OCA2 protein antibody-positive (*n* = 52) and antibody-negative (*n* = 179) non-segmental vitiligo patient groups, Fisher's exact tests for 2 x 2 contingency tables or unpaired t tests were used as appropriate (Section 2.24). *P* values < 0.05 were considered as significant.

The results are given in Table 6.3. A statistically significant difference was not evident between the two cohorts in terms of sex, age of patient, duration of disease, vitiligo onset age, activity of vitiligo, the occurrence of other autoimmune disorders, the clinical sub-type of non-segmental vitiligo or the prevalence of MCHR1, PMEL, TYRP1, DCT, and TH antibodies; *P* values were all > 0.05, indicating that OCA2 protein antibodies were not associated with any of these analysed parameters. However, a statistically significant difference was observed between the two groups in terms of the presence of antibodies against tyrosinase; *P* value was 0.004 (Table 6.3).

Table 6.3: Comparison of the demographic, clinical, and serological details in non-segmental vitiligo patients positive and negative for OCA2 protein antibodies

Patient detail	OCA2 protein antibody-positive non-segmental vitiligo patients (*n* = 52)	OCA2 protein antibody-negative non-segmental vitiligo patients (*n* = 179)	*P* value
Demographic details			
Male	27 (52%)	70 (39%)	0.11[1]
Female	25 (48%)	109 (61%)	
Mean age ± SD (years)	46 ± 17 (14 - 77)	45 ± 17 (0 - 88)	0.70[2]
Mean onset age ± SD (years)	29 ± 16 (0 - 72)	32 ± 18 (0 - 83)	0.35[2]
Mean disease duration ± SD (years)	17 ± 14 (0.5 - 51)	13 ± 13 (0 - 71)	0.09[2]
Non-segmental vitiligo sub-type			
Symmetrical	38 (73%)	119 (66%)	0.40[1]
Acrofacial	11 (21%)	31 (17%)	0.54[1]
Vitiligo activity			
Active	49 (94%)	167 (93%)	1.00[1]
Stable	3 (6%)	12 (7%)	
Autoimmune disease			
Present	19 (37%)	62 (35%)	0.87[1]
Absent	33 (63%)	117 (65%)	
Serology tests			
Tyrosinase antibody-positive	17 (33%)	25 (14%)	0.004[1]
MCHR1 antibody-positive	11 (21%)	40 (22%)	1.00[1]
PMEL antibody-positive	10(19%)	25 (14%)	0.38[1]
TYRP1 antibody-positive	14 (27%)	30 (17%)	0.11[1]
DCT antibody-positive	14 (27%)	29 (16%)	0.10[1]
TH antibody-positive	11 (21%)	42 (23%)	0.85[1]

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of demographic, clinical, and serological details in the OCA2 protein antibody-positive and the OCA2 protein antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

[2]*P* value calculated using Unpaired t tests (Section 2.24) for comparing the age, onset age, and disease duration in the OCA2 protein antibody-positive and OCA2 protein antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

6.4 Discussion

The specific function of the OCA2 protein remains to be precisely characterised, however studies using cultured melanocytes from *OCA2* mutant mice propose its involvement in the transport of tyrosinase, the precursor enzyme to melanin biosynthesis, to melanosomes within melanocytes (Chen et al., 2002, Toyofuku et al., 2002, Chen et al., 2004, Cook et al., 2009, Rimoldi et al., 2014), and in the regulation of melanosomal pH (Puri et al., 2000, Suzuki and Tomita, 2008, Rimoldi et al., 2014). Initially in this thesis, OCA2 protein was identified as a novel target of antibody responses in patients suffering from vitiligo by using phage-display technology (Chapter 4). Therefore, in this chapter, a RLBA was employed to study the prevalence of immunoreactivity to the OCA2 protein in a series of vitiligo patients, and also to find out if there were any associations of OCA2 protein antibodies with the demographic features (e.g., age, gender), clinical features (e.g., vitiligo activity, autoimmune disease), or serological details of vitiligo patients.

6.4.1 Frequency of OCA2 protein antibodies

Firstly, a RLBA was developed which could be utilised to detect and measure OCA2 protein antibodies in human sera. The RLBA proved to be specific for the detection of OCA2 protein antibodies. Secondly, the RLBA was used to analyse 246 vitiligo patient sera for OCA2 protein antibodies. OCA2 protein antibodies were detected in 54 (22%) patients. Although the frequency of OCA2 protein antibodies was relatively low (22%) in the vitiligo patients included in the current study, it is not different from other studies which have demonstrated a low frequency of antibodies to tyrosinase (11%) (Kemp et al., 1997a), TYRP1 (6%) (Kemp et al., 1998b), DCT (6%) (Kemp et al., 1997b), MCHR1 (16%) (Kemp et al., 2002b), PMEL (6%) (Kemp et al., 1998a), and TH (23%) (Kemp et al., 2011). Likewise, low frequencies (6-15%) of antibodies against several different autoantigens were detected in vitiligo patients by Waterman et al. (2010).

6.4.2 OCA2 protein antibody titres and specificity

To measure OCA2 protein antibody titres, the 54 patient sera positive for OCA2 protein antibodies were tested in RLBAs at a series of final dilutions. Although the titres of OCA2 protein antibodies varied between the different vitiligo patients, the majority, 43/54 (80%), had a titre at 1:1000 or above, and 13/54 (24%) had a titre above 1:5000. In contrast, titres of TH, PMEL, TYRP1, and MCHR1 antibodies in vitiligo patients were much lower at 1:200, 1:100, 1:100 and 1:50, respectively (Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011).

Previous analysis has shown that vitiligo patient antibodies against tyrosinase are cross-reactive with TYRP1 and DCT (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998b) due to their amino acid sequence homology. In order to ensure that OCA2 protein antibody reactivity was specific to the OCA2 protein, absorption experiments were performed. The absorption experiments showed that all 54 OCA2 protein antibody-positive patient sera contained OCA2 protein antibodies that reacted specifically with their target antigen with no cross-reactivity to other vitiligo-associated antigens MCHR1 and tyrosinase. This would be expected due to lack of amino acid sequence similarity of the three proteins.

6.4.3 Prevalence of OCA2 protein antibodies in vitiligo sub-types

OCA2 protein antibody responses were detected at slightly higher prevalence (25%) in segmental vitiligo patients than in non-segmental vitiligo patients (23%). This contrasts with the hypothesis that segmental vitiligo type has a non-autoimmune aetiology, whereas non-segmental vitiligo type has an autoimmune aetiology (Taieb, 2000). However, samples from only four patients with segmental vitiligo were available for testing. Segmental vitiligo mostly presents in children (Halder et al., 1987, Nicolaidou et al., 2012, Silverberg, 2015), but children were seen rarely in our clinics as they usually present to paediatric dermatology. Additionally, segmental vitiligo less commonly present in adulthood and, due to a usually more confined skin area involved, it is less likely to precipitate a referral to a clinic, than an advancing case of non-segmental

vitiligo. Therefore, a definite conclusion cannot be drawn regarding the frequency of OCA2 protein antibodies in this vitiligo sub-type.

6.4.4 Associations of OCA2 protein antibodies

A comparison of OCA2 protein antibody-positive and OCA2 protein antibody-negative non-segmental vitiligo patients showed that the frequency of OCA2 protein antibodies did not correlate with disease activity, a finding contradictory to previous studies where a significant increase in TH antibodies was found in vitiligo patients with active disease (Kemp et al., 2011).

In addition, comparing the same two vitiligo groups demonstrated no evident correlation between the occurrence of OCA2 protein antibodies and either sex of patient, age at sampling time, disease onset age, or disease duration, results consistent with previous studies in relation to MCHR1 (Kemp et al., 2002b) and TH (Kemp et al., 2011) antibodies in vitiligo patients.

The OCA2 protein was a target of antibodies in vitiligo patients with (24%) and without (21%) a concomitant autoimmune disorder and there was no significant difference in the prevalence of OCA2 protein antibodies between the two groups, a similar finding to a previous study investigated TH antibodies in vitiligo patients (Kemp et al., 2011). However, these results contrast to other previous reports in which antibodies against melanogenic proteins TYRP1, DCT, and PMEL were only found in patients suffering from both vitiligo and other autoimmune diseases (Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b). In addition, predominant antibody responses against tyrosinase and SOX10 were detected in vitiligo patients with accompanying autoimmune diseases (Kemp et al., 1997a, Hedstrand et al., 2001).

In the present work, there was also no apparent correlation between the occurrence of OCA2 protein antibodies and MCHR1, PMEL, TYRP1, DCT or TH antibodies. Similarly, no association between MCHR1, tyrosinase, TYRP1, DCT, TH, and PMEL antibodies has been reported in vitiligo patients (Kemp et al., 2002b, Kemp et al., 2011). However, in this work, an association of OCA2 protein antibodies with the presence of tyrosinase antibodies was noted. Interestingly, the OCA2 protein, being a melanosomal membrane

transporter, is involved in tyrosinase transport and it is analogous to tyrosinase in that both proteins can be presented to the immune system by HLA-A*02 (Zhang and Xiang, 2014).

6.4.5 Development of OCA2 protein antibodies

Owing to the intracellular location of OCA2 protein, OCA2 protein antibodies most likely occur by the antigen exposure after the destruction of melanocytes by oxidative stress (Kemp et al., 2011) or cytotoxic cells (Palermo et al., 2001, van den Boorn et al., 2009). Several hypotheses including neo-antigen formation, exposure of cryptic epitopes and protein modification during apoptosis have been put forward about the emergence of antibody reactivity to cytoplasmic melanocyte antigens (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011). After processing by dendritic cells, antigenic proteins can be presented to either autoreactive T lymphocytes which have escaped clonal deletion or to naïve T cells which have not been tolerised against cryptic epitopes (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011). Antibodies might then be produced following the triggering of autoreactive B cells by activated CD4+ T lymphocytes (Namazi, 2007).

6.4.6 Potential pathogenicity of OCA2 protein antibodies

Generally for autoantigens to be recognised by antibodies, their expression on the cell surface is required. However, the OCA2 protein localises on melanosomes contained within melanocytes (Sitaram et al., 2009). Interestingly, mutant OCA2 protein has been reported to mislocalise to the surface of melanocytes (Sitaram et al., 2009), and could therefore be accessible as an antibody target in such cases. However, with restricted access to the target antigen in normal pigment cells, OCA2 protein antibodies are unlikely to adversely affect the function of the OCA2 protein, and although melanocyte destruction by vitiligo-associated antibodies has been demonstrated by both complement-mediated activation and antibody-dependent cellular cytotoxicity (Norris et al., 1988, Gilhar et al., 1995, Kemp et al., 2011), OCA2 protein antibodies may merely serve as useful markers for melanocyte destruction and/or T cell reactivity against the

OCA2 protein. Indeed, several melanocyte autoantigens that are recognised as antibody targets, for example tyrosinase, are also the target of T cell responses in vitiligo skin (Ogg et al., 1998, Lang et al., 2001, Palermo et al., 2001, van den Boorn et al., 2009).

6.5 Conclusions

The OCA2 protein has been found as a novel antibody target in vitiligo. To the best of our knowledge, this finding has not been previously reported. It requires further characterisation, but the finding further supports the involvement of an autoimmune mechanism in vitiligo development. Interestingly, in this work, an association was evident between OCA2 protein and tyrosinase antibodies. Of particular note, the OCA2 protein functions in the transportation of tyrosinase, the key enzyme required for melanin synthesis, to melanosomes. Therefore, OCA2 protein is a promising antigen candidate for further investigation.

Chapter 7

Analysis of antibody reactivity against LaminA in vitiligo patients

7 Analysis of antibody reactivity against LaminA in vitiligo patients

7.1 Introduction

The nuclear envelope in eukaryotic cells consists of three distinctive membrane domains: the outer nuclear membrane, which is linked with the endoplasmic reticulum; the membranous wall of the nuclear pore complex, which is situated where the outer and inner nuclear membranes fuse to create the pore channel; and the inner nuclear membrane, which is connected with a network of proteins, the nuclear lamina (Schoft et al., 2003). The Lamins, nuclear intermediate filament proteins, are the major structural components of the lamina (Dechat et al., 2000, Wilson et al., 2001, Dittmer and Misteli, 2011). In most mammalian cells, the nuclear lamina is composed of three major proteins, Lamins A, B, and C (Hozak et al., 1995) with molecular weights of 60 to 75-kDa (Heitlinger et al., 1991). The different lamins polymerise to form homopolymeric and heteropolymeric 10-nm diameter filaments in the peripheral nuclear lamina (Lin and Worman, 1993). They may also assemble into intra-nuclear structures (Dechat et al., 2000) forming part of a diffuse skeleton spread throughout the nuclear interior (Hozak et al., 1995, Dittmer and Misteli, 2011). In addition, according to the reported interactions of the lamins with chromatin (Gotzmann and Foisner, 1999), these proteins are suggested to be involved in the post-mitotic nuclear reorganisation, such as DNA replication, chromatin de-condensation and transcription (Schoft et al., 2003, Wilson et al., 2001, Dittmer and Misteli, 2011).

Recently, LaminA was identified as a target of antibodies in vitiligo patients by proteome analysis (Li et al., 2011). Immunoblotting verified that 28.6% of vitiligo patients had antibodies against LaminA and that 3.1% of healthy individuals were positive (Li et al., 2011). Overall, 91.7% of the patients who were positive for LaminA antibodies also had active non-segmental vitiligo, and 50% had at least one autoimmune disease (Li et al., 2011). Furthermore, anti-LaminA, B1, A/C, and ABC antibodies have been reported also in patients with systemic lupus erythematosus, chronic autoimmune hepatitis, rheumatoid arthritis, linear scleroderma, and autoimmune cytopenias (McKeon et al., 1983, Wesierska-Gadek et al., 1988, Senecal et al., 1999, Coppo et al., 2004), suggesting they might be a general marker for autoimmune disease. In this project, LaminA was

identified as potential antibody target following rounds of panning against the melanocyte cDNA phage-display library with vitiligo patient IgG samples (Chapter 4).

7.2 Aims

The main objectives of this part of the project were:

a) To develop a RLBA to confirm antibody reactivity against LaminA.

b) To investigate LaminA antibody frequencies in 246 vitiligo patients and 100 healthy controls using the RLBA.

c) To determine if antibodies to LaminA were related to any clinical, demographic or serological features of vitiligo patients.

7.3 Experiments and Results

7.3.1 Development of an LaminA RLBA

In order to study the prevalence of antibodies against LaminA in vitiligo patient sera, it was first necessary to clone LaminA cDNA into a suitable plasmid vector for expression of the LaminA, which could then be used as the target antigen to test antibody binding in RLBAs. Plasmid pcDNA3 (Figure 7.1) was chosen as it contains a T7 polymerase promoter binding site from which cloned cDNA can be transcribed and translated *in vitro* with the concomitant incorporation of radiolabelled [^{35}S]-methionine. The [^{35}S]-LaminA produced in this way can then be used in RLBAs to detect LaminA antibodies in vitiligo patient and healthy control serum samples.

7.3.1.1 PCR amplification of LaminA cDNA

Plasmid pDEST15-LaminA DNA containing LaminA DNA (Figure 7.2) was obtained from imaGene GmbH (Berlin, Germany). A sample (50 ng) of pDEST15-LaminA DNA was subjected to 35 cycles of PCR amplification in a DNA thermal cycler using the reaction conditions given in Section 2.13 with primers LaminA-F and LaminA-R (Table 2.4). To check for the correctly sized PCR amplification product, a 5-µl aliquot of the PCR amplification reaction was electrophoresed in a 0.8% (w/v) agarose gel (Section 2.11). The results are shown in Figure 7.3. The product was visible at approximately 2,000-bp, in good agreement with the 1,992-bp DNA fragment that encodes LaminA cDNA.

The PCR product was purified from a 0.8% (w/v) agarose gel using a Wizard® PCR Preps DNA Purification System (Section 2.14) and restricted in a 20-µl reaction with *Kpn*I and *Xba*I (Section 2.12). The restricted LaminA cDNA was re-purified from a 0.8% (w/v) agarose gel using a Wizard® PCR Preps DNA Purification System (Section 2.14). Finally, a 3-µl aliquot of the purified product was analysed by agarose gel electrophoresis in a 0.8% (w/v) gel (Section 2.11), quantified by spectrophotometry (Section 2.9), and then stored at -20°C until required for cloning.

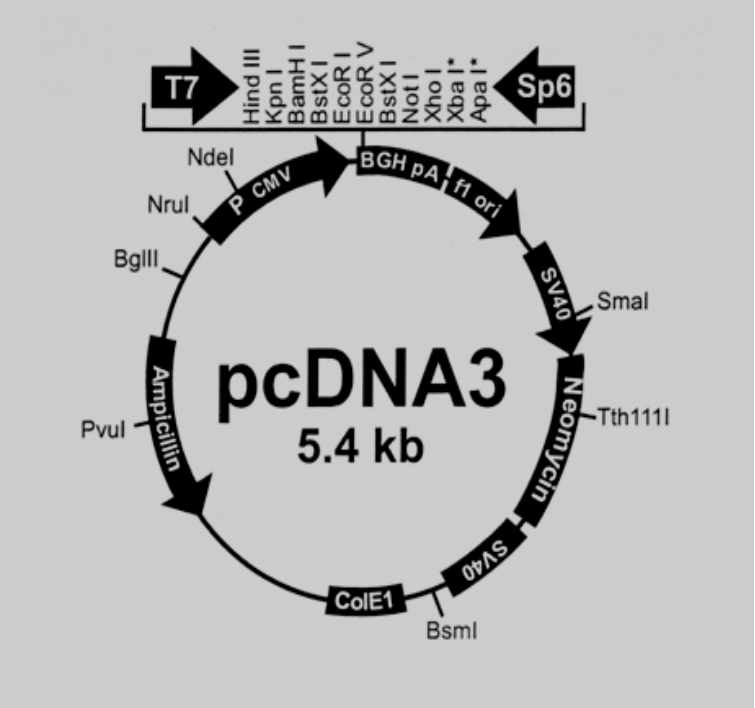


Figure 7.1: A map of the pcDNA3 plasmid vector.

The map shows restriction enzyme sites and the direction of the T7 promoter in the pcDNA3 plasmid vector. The 2.0-kb LaminA cDNA was cloned between the *Kpn*I and *Xba*I restriction enzyme sites.

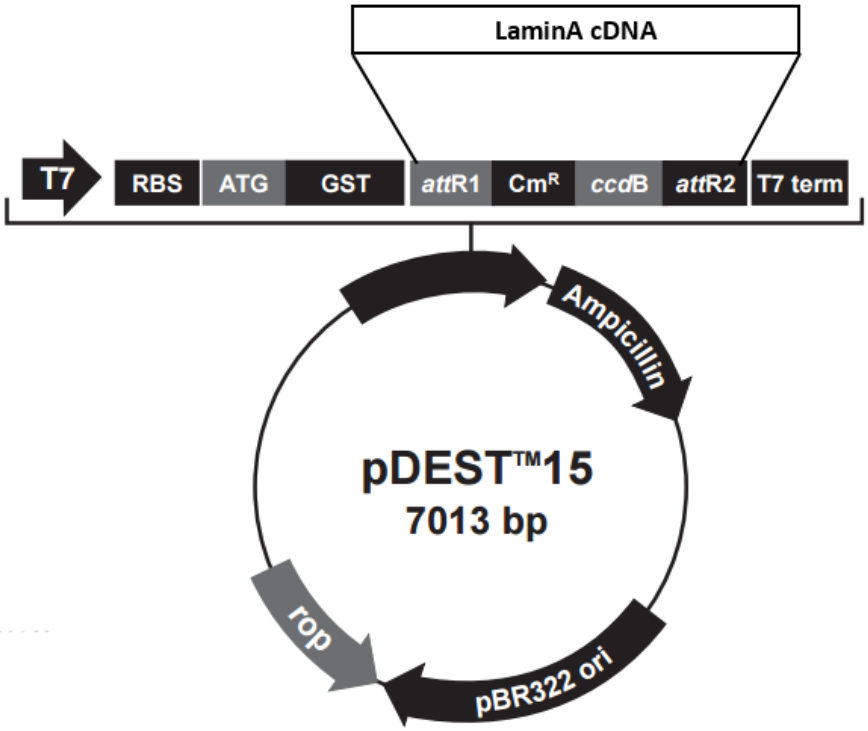


Figure 7.2: A map of the pDEST15-LaminA plasmid.

The map shows restriction enzyme sites and the direction of the T7 promoter in the pDEST15 plasmid vector. The 2.0-kb LaminA cDNA was cloned between the *att*R1 and *att*R2 restriction enzyme sites.

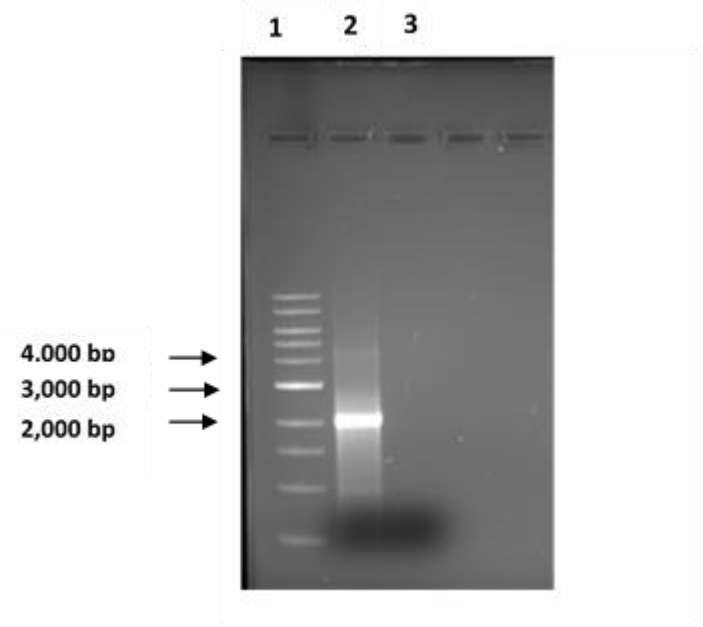


Figure 7.3: Agarose gel electrophoresis of PCR amplification product from pDEST15-LaminA.

Plasmid pDEST15-LaminA was subjected to PCR amplification using oligonucleotide primers LaminA-F and LaminA-R. The PCR amplification product was analysed by electrophoresis in a 0.8% (w/v) agarose gel. The gel image shows: the 1-kb DNA Ladder (500-10,000-bp DNA fragments) (lane 1); plasmid pDEST15-LaminA amplified with primers LaminA-F and LaminA-R (lane 2); no DNA control with LaminA-F and LaminA-R primers (lane 3).

7.3.1.2 Preparation of plasmid vector pcDNA3

For cloning of the PCR product, 1 µg of plasmid vector pcDNA3 was digested with *Kpn*I and *Xba*I in a 25-µl reaction (Section 2.12). The linearised vector was purified from a 0.8% (w/v) agarose gel using a Wizard® PCR Preps DNA Purification System (Section 2.14). A 3-µl aliquot of the purified vector was checked on a 0.8% (w/v) agarose gel (Section 2.11), quantified by spectrophotometry (Section 2.9), and then stored at -20°C until required for cloning experiments.

7.3.1.3 Ligation of LaminA cDNA into pcDNA3

The *Kpn*I and *Xba*I-restricted LaminA cDNA fragment was cloned into *Kpn*I and *Xba*I-digested pcDNA3 by setting up a 20-µl ligation reaction as described in Section 2.15. A 5-µl aliquot of the ligation reaction was then used to transform 50 µl of chemically competent *E. coli* JM109 cells (Section 2.16), with transformants being selected on LB agar (Section 2.5) containing ampicillin at a concentration of 100 µg/ml (Section 2.4). Following overnight incubation at 37°C, eight individual bacterial colonies from the transformation were purified by streaking on to fresh LB agar containing ampicillin at a concentration of 100 µg/ml and subsequently incubating the plates at 37°C overnight.

7.3.1.4 Screening of *E. coli* JM109 transformants for recombinant plasmids

For screening of *E. coli* JM109 transformants for recombinant plasmids that contained the correct LaminA cDNA fragment, eight purified bacterial transformants were inoculated separately into 10 ml of LB with ampicillin at 100 µg/ml, and then incubated at 37°C overnight. Plasmid DNA was purified from each transformant using a Wizard® Plus SV Minipreps DNA Purification System (Section 2.9). Plasmids were then checked for the presence of the correctly sized DNA insert by restriction enzyme digestion of a 0.5-µg sample with *Kpn*I and *Xba*I in a 25-µl reaction (Section 2.12), followed by analysis by electrophoresis in 0.8% (w/v) agarose gels (Section 2.11). The frequency of recombinant plasmids was 100%, with 8/8 plasmids having a DNA insert of the correct size (Figure 7.4).

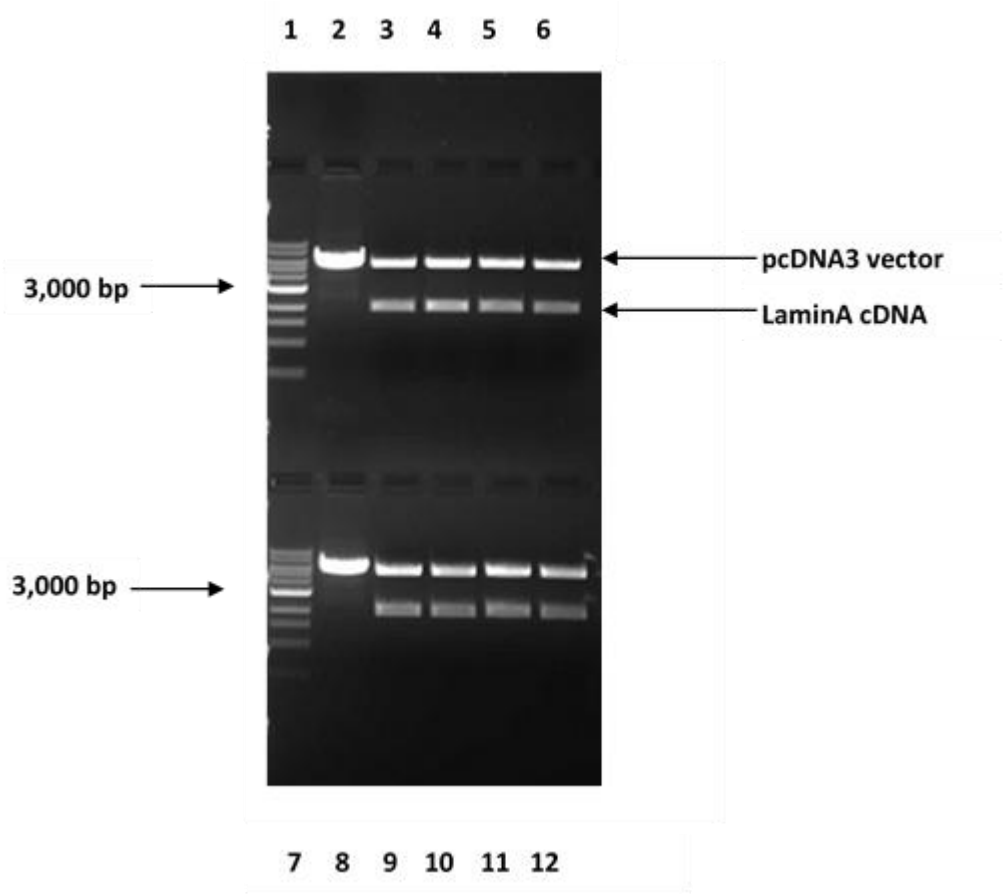


Figure 7.4: Agarose gel electrophoresis of potential recombinant plasmids.

Plasmid DNA was prepared from eight individual bacterial clones isolated from transformation of *E. coli* JM109 with the ligation reaction of pcDNA3 and LaminA cDNA. The plasmids were restricted with *Kpn*I and *Xba*I and then subjected to electrophoresis in a 0.8% (w/v) agarose gel. *Kpn*I and *Xba*I-restricted pcDNA3 was included for comparison. The gel shows: the 1-kb DNA Ladder (500-10,000-bp DNA fragments) (lanes 1 and 7); *Kpn*I and *Xba*I-restricted pcDNA3 (lanes 2 and 8); *Kpn*I and *Xba*I-restricted plasmid DNA prepared from eight bacterial clones (lanes 3-6 and 9-12).

7.3.1.5 Restriction digest analysis of pcDNA3-LaminA

One recombinant plasmid from the cloning experiment was designated as pcDNA3-LaminA (Figure 7.5) and the bacterial strain as JM109 pcDNA3-LaminA. A large-scale plasmid preparation of pcDNA3-LaminA from JM109 pcDNA3-LaminA was carried out (Section 2.10), and the plasmid then analysed by restriction digestion with *Kpn*I and *Xba*I. The results showed the correctly sized cDNA insert (Figure 7.6). The plasmid preparation had a concentration of 1.2 µg/µl and was stored at -20°C until needed.

7.3.1.6 LaminA cDNA clone confirmation

As a final check that the recombinant plasmid pcDNA3-LaminA was correct and to verify that no sequence errors had been introduced to the LaminA cDNA fragment during PCR amplification, the plasmid was subjected to DNA sequencing (Section 2.17) using primers T7, SP6, LaminA-555-Forward, LaminA-700-Revesrse, LaminA-1751-Forward, and LaminA-1600-Forward (Table 2.5). The results verified that the cloned LaminA cDNA fragment was correct on comparison of its sequence with the parent LaminA cDNA in pDEST15-LaminA (Figure 7.7) using the Global Alignment tool at the network facilities of the EBI-EMBL (Section 2.18). The amino acid sequence of LaminA is given in Figure 7.8.

7.3.1.7 *In vitro* transcription-translation of LaminA cDNA

Plasmid pcDNA3-LaminA was transcribed and translated in a TnT® T7-Coupled Reticulocyte Lysate System, as described in Section 2.19. In order to analyse the radiolabelled product qualitatively, a 5-µl aliquot of the *in vitro* transcription-translation reaction was added to 20 µl of 2x Laemmli sample buffer (Section 2.19) and heated to 100°C for 5 min. A 10-µl sample was then applied to a 10% (w/v) SDS-polyacrylamide gel and subjected to electrophoresis (Section 2.20). After drying, the gel was exposed to x-ray film, which was developed after 24 h (Section 2.20).

The results showed a protein product with an estimated molecular weight of 74 kDa (Figure 7.9). This agrees well with the molecular weight of 73 kDa anticipated from the amino acid sequence of the protein with 664 residues (Figure 7.8).

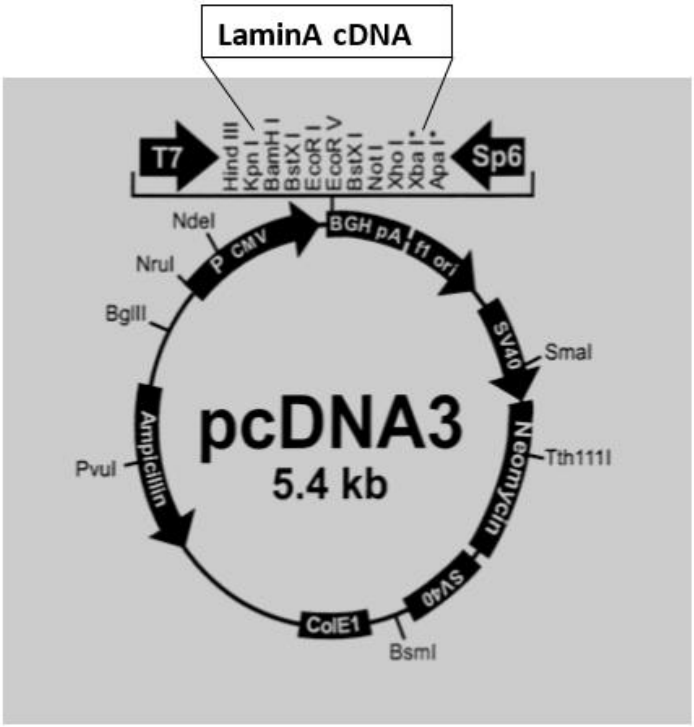


Figure 7.5: A map of the pcDNA3-LaminA plasmid.

The map shows restriction enzyme sites and the direction of the T7 and SP6 promoters in the pcDNA3 plasmid vector. The 2.0-kb LaminA cDNA was cloned between the *Kpn*I and *Xba*I restriction enzyme sites.

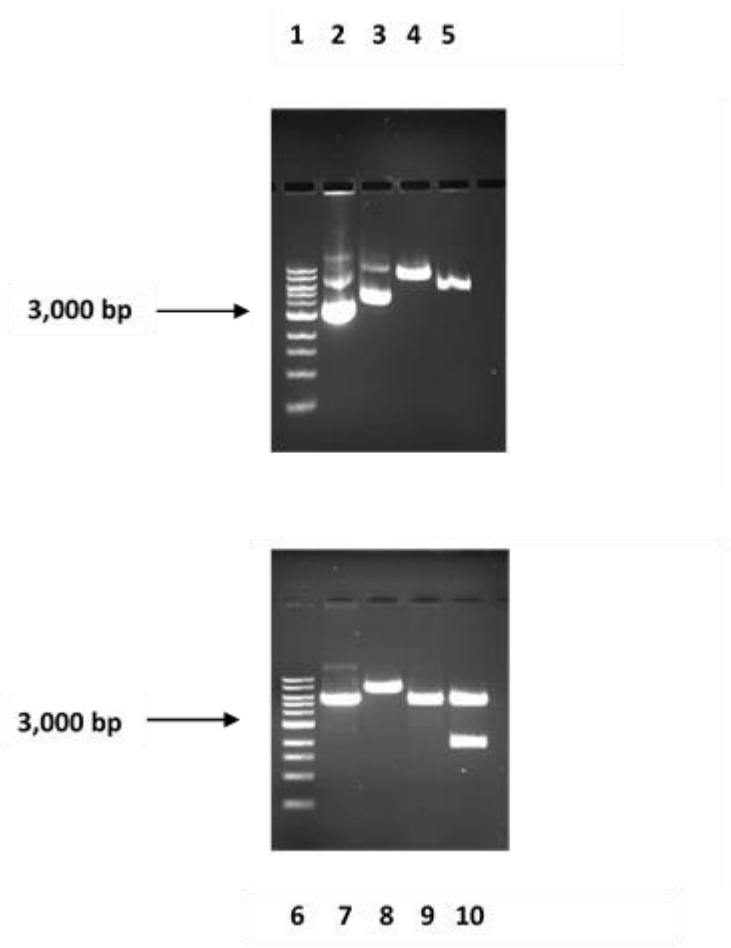


Figure 7.6: Agarose gel electrophoresis of plasmid pcDNA3-LaminA.

Unrestricted pcDNA3-LaminA and plasmid restricted with *Kpn*I and/or *Xba*I were subjected to electrophoresis in a 0.8% (w/v) agarose gel. Unrestricted pcDNA3 and plasmid restricted with *Kpn*I and/or *Xba*I was included for comparison. The gel shows: the 1-kb DNA Ladder (500-10,000-bp DNA fragments) (lanes 1 and 6); unrestricted pcDNA3 (lane 2); unrestricted pcDNA3-LaminA (lane 3); *Kpn*I-restricted pcDNA3-LaminA (lane 4); *Kpn*I-restricted pcDNA3 (lane 5); *Xba*I-restricted pcDNA3 (lane 7); *Xba*I-restricted pcDNA3-LaminA (lane 8); *Kpn*I and *Xba*I-restricted pcDNA3 (lane 9); *Kpn*I and *Xba*I-restricted pcDNA3-LaminA (lane 10).

ATGGAGACCCCGTCCCAGCGGCGCGCCACCCGCAGCGGGGCGCAGGCCAGCTCCACTCCGCTGTCG
CCCACCCGCATCACCCGGCTGCAGGAGAAGGGGACCTGCAGGAGCTCAATGATCGCTTGGCGGTCT
ACATCGACCGTGTGCGCTCGCTGGAAACGGAGAACGCAGGGCTGCGCCTTCGCATCACCGAGTCTG
AAGAGGTGGTCAGCCGCGAGGTGTCCGGCATCAAGGCCGCCTACGAGGCCGAGCTCGGGGATGCCC
GCAAGACCCTTGACTCAGTAGCCAAGGAGCGCGCCCGCCTGCAGCTGGAGCTGAGCAAAGTGCGTG
AGGAGTTTAAGGAGCTGAAAGCGCGCAATACCAAGAAGGAGGGTGACCTGATAGCTGCTCAGGCTC
GGCTGAAGGACCTGGAGGCTCTGCTGAACTCCAAGGAGGCCGCACTGAGCACTGCTCTCAGTGAGA
AGCGCACGCTGGAGGGCGAGCTGCATGATCTGCGGGGCCAGGTGGCCAAGCTTGAGGCAGCCCTAG
GTGAGGCCAAGAAGCAACTTCAGGATGAGATGCTGCGGCGGGTGGATGCTGAGAACAGGCTGCAGA
CCATGAAGGAGGAACTGGACTTCCAGAAGAACATCTACAGTGAGGAGCTGCGTGAGACCAAGCGCC
GTCATGAGACCCGACTGGTGGAGATTGACAATGGGAAGCAGCGTGAGTTTGAGAGCCGGCTGGCGG
ATGCGCTGCAGGAACTGCGGGCCCAGCATGAGGACCAGGTGGAGCAGTATAAGAAGGAGCTGGAGA
AGACTTATTCTGCCAAGCTGGACAATGCCAGGCAGTCTGCTGAGAGGAACAGCAACCTGGTGGGGG
CTGCCCACGAGGAGCTGCAGCAGTCGCGCATCCGCATCGACAGCCTCTCTGCCCAGCTCAGCCAGC
TCCAGAAGCAGCTGGCAGCCAAGGAGGCGAAGCTTCGAGACCTGGAGGACTCACTGGCCCGTGAGC
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AGCAGCAGCTGGACGAGTACCAGGAGCTTCTGGACATCAAGCTGGCCCTGGACATGGAGATCCACG
CCTACCGCAAGCTCTTGGAGGGCGAGGAGGAGAGGCTACGCCTGTCCCCCAGCCCTACCTCGCAGC
GCAGCCGTGGCCGTGCTTCCTCTCACTCATCCCAGACACAGGGTGGGGGCAGCGTCACCAAAAAGC
GCAAACTGGAGTCCACTGAGAGCCGCAGCAGCTTCTCACAGCACGCACGCACTAGCGGGCGCGTGG
CCGTGGAGGAGGTGGATGAGGAGGGCAAGTTTGTCCGGCTGCGCAACAAGTCCAATGAGGACCAGT
CCATGGGCAATTGGCAGATCAAGCGCCAGAATGGAGATGATCCCTTGCTGACTTACCGGTTCCCAC
CAAAGTTCACCCTGAAGGCTGGGCAGGTGGTGACGATCTGGGCTGCAGGAGCTGGGGCCACCCACA
GCCCCCCTACCGACCTGGTGTGGAAGGCACAGAACACCTGGGGCTGCGGGAACAGCCTGCGTACGG
CTCTCATCAACTCCACTGGGGAAGAAGTGGCCATGCGCAAGCTGGTGCGCTCAGTGACTGTGGTTG
AGGACGACGAGGATGAGGATGGAGATGACCTGCTCCATCACCACCACGGCTCCCACTGCAGCAGCT
CGGGGGACCCCGCTGAGTACAACCTGCGCTCGCGCACCGTGCTGTGCGGGACTGCGGGCAGCCTGC
CGACAAGGCATCTGCCAGCGGCTCAGGAGCCCAGGTGGGCGGACCCATCTCCTCTGGCTCTTCTGC
CTCCAGTGTCACGGTCACTCGCAGCTACCGCAGTGTGGGGGGCAGTGGGGGTGGCAGCTTCGGGGA
CAATCTGGTCACCCGCTCCTACCTCCTGGGCAACTCCAGCCCCCGAACCCAGAGCCCCCAGAACTG
CAGCATCATGTAG

Figure 7.7: LaminA cDNA sequence.

The cDNA sequence matched that of LaminA cDNA Accession Number NM170707 when searched against the GenBank database using the network facilities of NCBI (Bethesda, MD, USA). The ATG translation start codon and the TAG translation termination codon are shown in red. The cDNA has 1,992 base pairs.

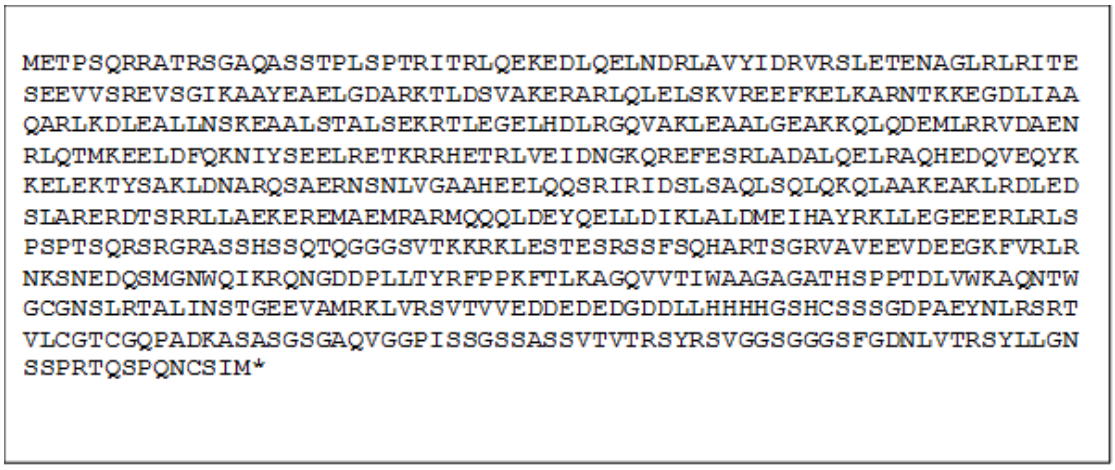

Figure 7.8: LaminA amino acid sequence.

The protein has 664 amino acids residues which are shown by their one letter symbol.

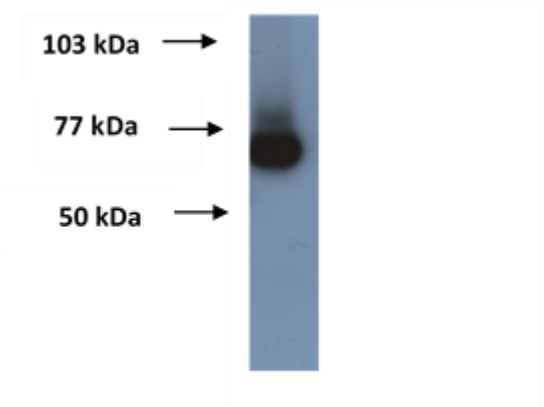

Figure 7.9: SDS-PAGE and autoradiography of [^{35}S]-LaminA.

[^{35}S]-LaminA was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3-LaminA as a template for transcription and translation of LaminA cDNA. For analysis, the radiolabelled protein was run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The LaminA is visible at 74 kDa.

7.3.1.8 Analysis of the immunoreactivity of [^{35}S]-LaminA

The immunoreactivity of *in vitro* translated [^{35}S]-LaminA was analysed in a RLBA (Section 2.21) with an anti-LaminA antibody and a panel of antibodies raised against proteins unrelated to LaminA (Table 2.6). The antibodies were ab26300 (anti-LaminA), α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH) and α-PEP8 (anti-DCT), and were all used at a 1:100 dilution. An immunoprecipitation reaction without any antibodies was also included in each assay set, as a background control. From each RLBA, a LaminA antibody index was calculated for each of the antibodies tested as: cpm immunoprecipitated by tested antibody/cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean LaminA antibody index was calculated from these values. The LaminA antibody indices of the different antibodies were compared using one-way ANOVA, with *P* values < 0.05 considered significant.

The results are shown in Figure 7.10, and indicated that the mean LaminA antibody index ± SD for each antibody was 37.1 ± 8.85 for ab26300, 1.15 ± 0.10 for α-PEP7, 1.16 ± 0.07 for MCHR11-S, 1.10 ± 0.11 for ab59276, and 1.02 ± 0.09 for α-PEP8. In one-way ANOVA, the LaminA antibody index of LaminA antibody ab26300 was statistically significantly higher in comparison to those for α-PEP7, MCHR11-S, ab59276, and α-PEP8; $P < 0.0001$. The result demonstrated a specific immunoprecipitation of [^{35}S]-LaminA by anti-LaminA antibody, and indicated that [^{35}S]-LaminA could be used as a ligand in a RLBAs to detect antibodies against LaminA.

The LaminA immunoprecipitated in the RLBA by antibody ab26300 was also analysed quantitatively. Following the RLBA using [^{35}S]-LaminA and antibody ab26300, the protein G Sepharose-LaminA-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged, and the supernatant analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel (Section 2.20). The gel was then subjected to autoradiography (Section 2.20). The results indicated that the antibody ab26300 immunoprecipitated a protein band equivalent in size (74 kDa) to that of *in vitro* translated [^{35}S]-LaminA, and this is shown in Figure 7.11.

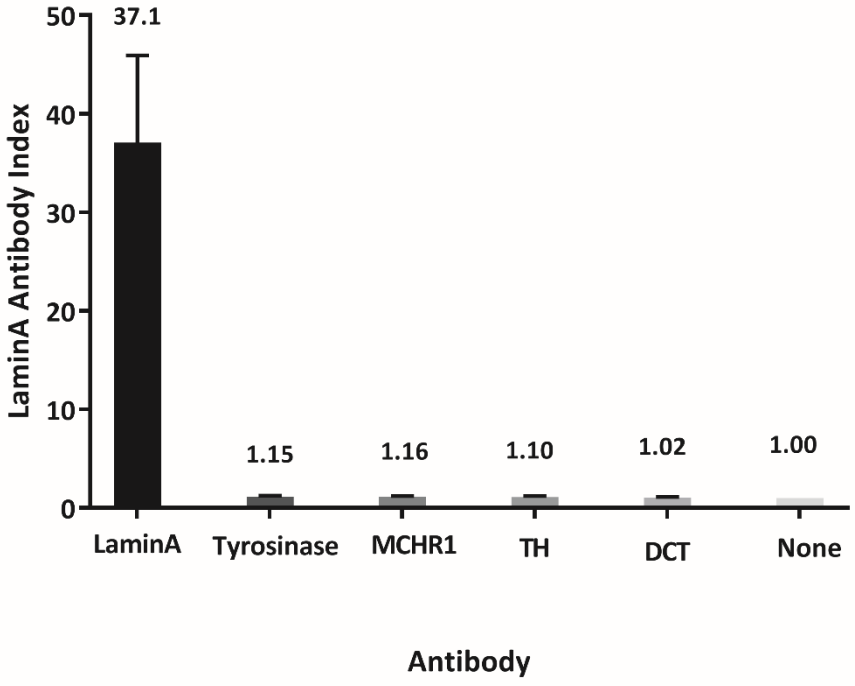

Figure 7.10: LaminA RLBA using different antibodies.

The immunoreactivity of *in vitro* translated [^{35}S]-LaminA was tested in a RLBA with antibodies anti-LaminA (ab26300), anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276) and anti-DCT (α-PEP8). An immunoprecipitation reaction without serum was included as a background control. The mean LaminA antibody index ± SD of four experiments is given for each antibody: ab26300, 37.1 ± 8.85; α-PEP7, 1.15 ± 0.10; MCHR11-S, 1.16 ± 0.07; ab59276, 1.10 ± 0.11; and α-PEP8, 1.02 ± 0.09. $P < 0.0001$ (One-way ANOVA).

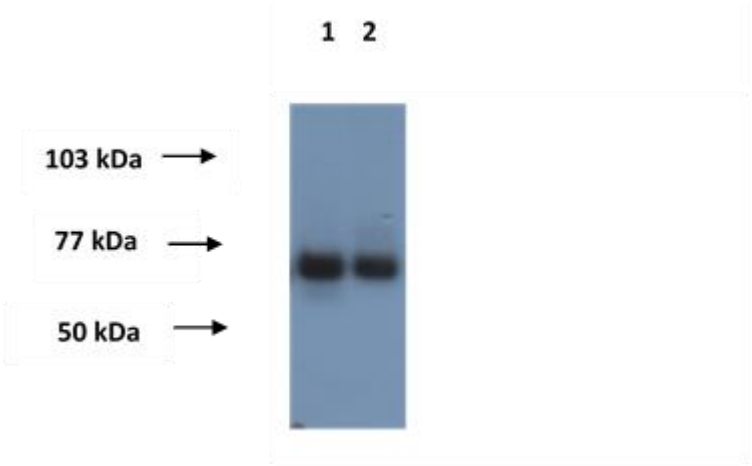

Figure 7.11: SDS-PAGE and autoradiography of [^{35}S]-LaminA and [^{35}S]-LaminA immunoprecipitated by anti-LaminA antibody ab26300

[^{35}S]-LaminA was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3-LaminA. Subsequently, radiolabelled LaminA was immunoprecipitated in a RLBA with anti-LaminA antibody ab26300. For analysis, immunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results are shown for: *In vitro* translated, non-immunoprecitated [^{35}S]-LaminA (lane 1); [^{35}S]-LaminA immunoprecipitated with anti-LaminA antibody ab26300 (lane 2). The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The LaninA protein is visible at 74 kDa.

7.3.1.9 Analysis of the specificity of anti-LaminA protein antibody

In order to analyse the specificity of the anti-LaminA antibody ab26300, it was used in RLBAs with a panel of different radiolabelled antigens including tyrosinase, MCHR1, TH, and DCT.

Radiolabelled [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT were produced in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.19) from plasmids pcDNA3-TYR, pcMCHR1, pcDNA3-TH and pCMVDTS (Table 2.2), respectively. RLBAs were carried out as detailed in (Section 2.21), using these labelled antigens with antibody ab26300 at a 1:100 dilution. Appropriate positive control antibodies were included at a 1:100 dilution in each RLBA: α-PEP7 for tyrosinase, MCHR11-S for MCHR1, ab59276 for TH, and α-PEP8 for DCT. An immunoprecipitation reaction without antibody was also included in each RLBA, as a background control. In each RLBA, an antibody index was calculated for each antibody as: cpm immunoprecipitated by tested antibody /cpm immunoprecipitated by the background control. Each antibody was tested in four experiments and the mean antibody index was calculated from these values. Antibody indices were compared using one-way ANOVA with *P* values < 0.05 regarded as significant.

The mean antibody index ± SD for anti-LaminA antibody in RLBAs against different antigens was 38.9 ± 1.38 against LaminA, 0.95 ± 0.01 against tyrosinase, 1.03 ± 0.04 against MCHR1, 0.97 ± 0.03 against TH, and 0.99 ± 0.02 against DCT (Figure 7.12). In one-way ANOVA, the antibody index of the anti-LaminA antibody in the different RLBAs was compared; $P < 0.0001$, indicating there was no significant immunoprecipitation of other unrelated proteins. Anti-LaminA antibody ab26300 could be used therefore as a positive control antibody in LaminA RLBAs.

Additionally, the mean antibody index ± SD for the antibodies α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), ab59276 (anti-TH), and α-PEP8 (anti-DCT) in RLBAs against their cognate antigens was 13.6 ± 1.74, 12.7 ± 1.28, 10.5 ± 1.57 and 11.4 ± 1.24, respectively (Figure 7.12).

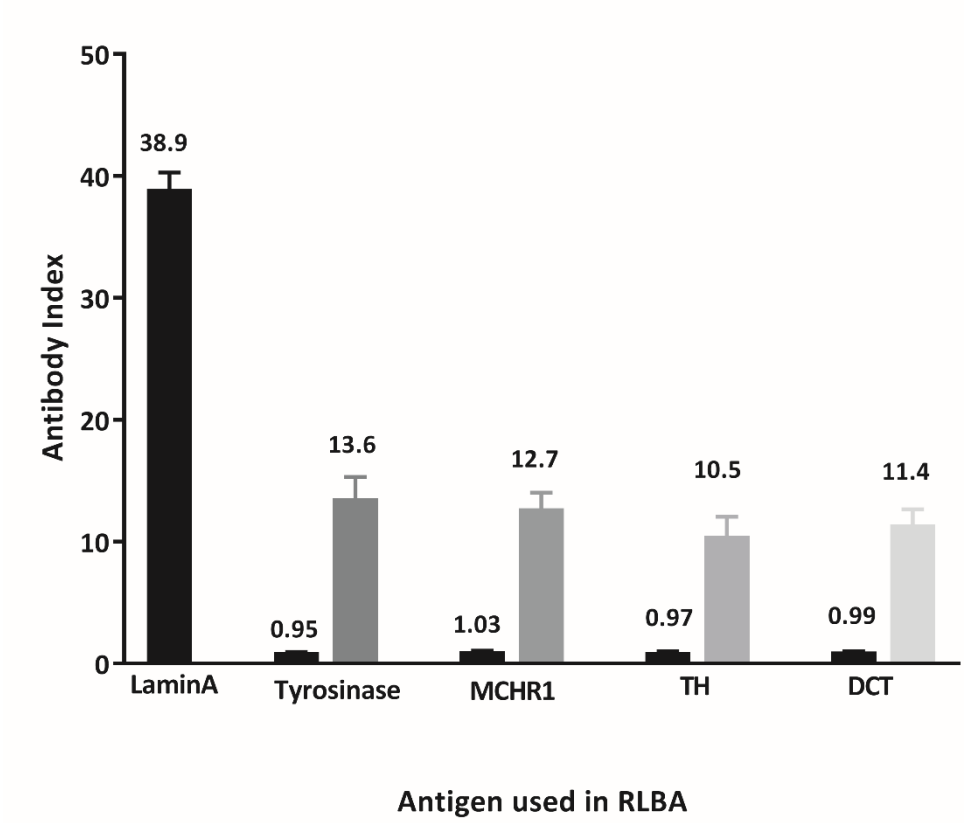

Figure 7.12: RLBAs with anti-LaminA antibody ab26300 and different antigens.

The immunoreactivity of the anti-LaminA antibody ab26300 was tested against *in vitro* translated [^{35}S]-LaminA, [^{35}S]-tyrosinase, [^{35}S]-MCHR1, [^{35}S]-TH, and [^{35}S]-DCT in RLBAs. Antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) were included in the relevant RLBA as positive controls for immunoprecipitation of their respective antigens. An immunoprecipitation reaction without any serum was included as a background control in each RLBA. The mean antibody index ± SD of four experiments is shown for the antibody ab26300 in RLBAs against the different antigens; 38.9 ± 1.38 against LaminA, 0.95 ± 0.01 against tyrosinase, 1.03 ± 0.04 against MCHR1, 0.97 ± 0.03 against TH, and 0.99 ± 0.02 against DCT. $P < 0.0001$ (One-way ANOVA). The mean antibody index ± SD of four experiments is also shown for the antibodies anti-tyrosinase (α-PEP7), anti-MCHR1 (MCHR11-S), anti-TH (ab59276), and anti-DCT (α-PEP8) in RLBAs against their cognate antigens; 13.6 ± 1.74, 12.7 ± 1.28, 10.5 ± 1.57 and 11.4 ± 1.24, respectively.

7.3.2 Use of the LaminA RLBA to analyse vitiligo patients and control sera for LaminA antibodies

To analyse vitiligo patient and control sera for the presence of LaminA antibodies, LaminA RLBAs were carried out using [^{35}S]-LaminA as the radiolabelled antigen. The sera analysed were from patients with vitiligo (*n* = 246) and from healthy controls (*n* = 100). All sera were assayed in duplicate at a final dilution of 1:100. Anti-LaminA antibody ab26300 was included in each assay set as a positive control at a dilution of 1:100. In each assay set, antibody levels were expressed as a LaminA antibody index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 100 healthy control sera. Each serum was tested in at least three experiments and the mean LaminA antibody index was calculated from these values. The upper limit of normal for the assay was calculated using the mean LaminA antibody index + 3SD of the population of 100 healthy controls. Any serum sample with a LaminA antibody index above the upper limit of normal was designated as positive for LaminA antibodies.

The results of the LaminA RLBAs are shown in Figure 7.13. The mean LaminA antibody index ± SD for the healthy control group (*n* = 100) was 1.00 ± 0.09 with a range of 0.77 to 1.23. The mean LaminA antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo *(n* = 4), and undetermined vitiligo (*n* = 11) was 1.54 ± 1.34 (range of 0.67 to 10.3), 1.17 ± 0.32 (range of 0.91 to 1.64), and 1.44 ± 1.43 (range of 0.91 to 5.76), respectively. The upper limit of normal for the LaminA RLBA was a LaminA antibody index of 1.29.

Healthy controls were all LaminA antibody-negative with LaminA antibody indices below 1.29. Of the 231 non-segmental vitiligo patient sera, 51 (22%) were considered LaminA antibody-positive (Table 7.1), as their LaminA antibody index was above the upper limit of normal value of 1.29. Anti-LaminA antibodies were detected in one patient with segmental vitiligo and in one patient with undetermined vitiligo sub-type (Table 7.1).

The prevalence of LaminA antibodies was compared between vitiligo patients and controls in Fisher's exact test for 2 x 2 contingency tables (Section 2.24). *P* values < 0.05 were regarded as significant. The results are given in Table 7.1. In comparison with

controls, a statistically significant increase in the prevalence of LaminA antibodies was found in the patient group with non-segmental vitiligo; the *P* value was < 0.0001. In addition, there was a statistically significant increase in the frequency of LaminA antibodies in patients with symmetrical or acrofacial vitiligo (Table 7.1). However, in most vitiligo sub-type groups, there were too few patients to make statistical analysis robust. There was no difference in LaminA antibody prevalence in patients with active or stable vitiligo or in those with or without autoimmune disease (Table 7.1).

To analyse qualitatively, the proteins immunoprecipitated in the LaminA RLBA by vitiligo patient and control sera, protein G Sepharose-LaminA-antigen complexes were suspended in 50 µl of Laemmli sample buffer, heated at 100°C for 5 min, centrifuged and the supernatant recovered for SDS-PAGE in 10% (w/v) SDS-polyacrylamide gels and subsequent autoradiography (Section 2.20).

The results indicated that all 53 of the LaminA antibody-positive vitiligo patient sera analysed immunoprecipitated a protein band of 74 kDa, comparable to the size of LaminA in SDS-polyacrylamide gels. The results for six LaminA antibody-positive vitiligo patient sera and for four healthy control sera are illustrated in Figure 7.14.

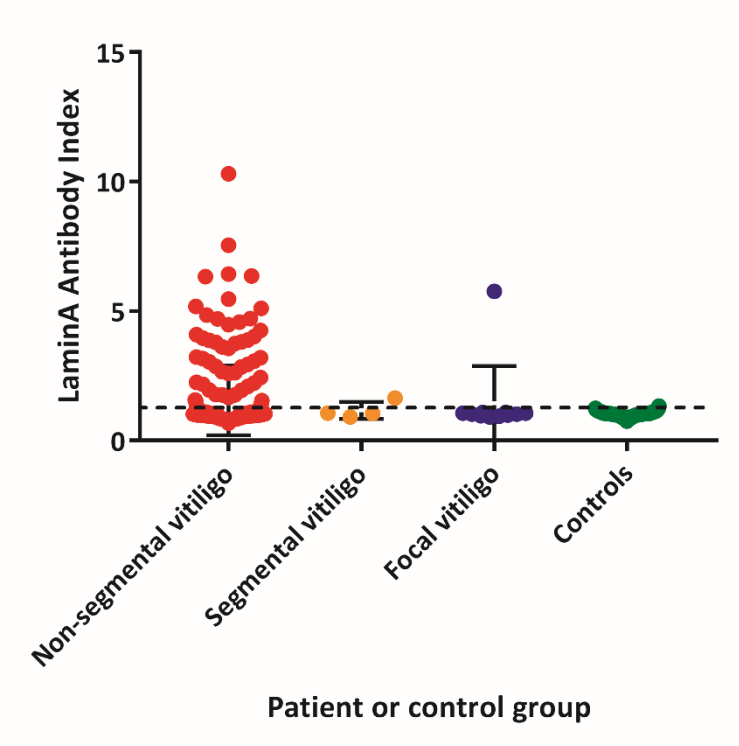

Figure 7.13: LaminA RLBA results for vitiligo patient and control sera.

Patient and control sera were evaluated for LaminA antibodies in the LaminA RLBA. The LaminA antibody indices are shown for sera from vitiligo patients (*n* = 246) and healthy controls (*n* = 100). The LaminA antibody index shown for each serum is the mean of three experiments. The mean LaminA antibody index ± SD for the healthy control group was 1.00 ± 0.09 with a range of 0.77 to 1.23. The mean LaminA antibody index ± SD for patients with non-segmental vitiligo (*n* = 231), segmental vitiligo (*n* = 4), and undetermined vitiligo (*n* = 11) groups was 1.54 ± 1.34 (range of 0.67 to 10.3), 1.17 ± 0.32 (range of 0.91 to 1.64), and 1.44 ± 1.43 (range of 0.91 to 5.76), respectively. The upper limit of normal (mean LaminA antibody index + 3SD of 100 healthy controls) for the LaminA RLBA was a LaminA antibody index of 1.29, and this is shown by the dotted line.

Table 7.1: LaminA antibody prevalence in vitiligo patients and healthy controls

Vitiligo patient or control group	LaminA antibody-positive samples	*P* value[1]	*P* value[2]
Vitiligo patient or control group			
Non-segmental vitiligo	51/231 (22%)	< 0.0001	-
Segmental vitiligo	1/4 (25%)	ND	-
Undetermined vitiligo (all focal)	1/11 (9%)	ND	-
Healthy controls	0/100 (0%)	ND	-
Non-segmental vitiligo sub-type			
Symmetrical (generalised)	34/157 (22%)	< 0.0001	-
Acrofacial	10/42 (24%)	< 0.0001	-
Acral	1/4 (25%)	ND	-
Peri-orificial	0/3 (0%)	ND	-
Universal	0/1 (0%)	ND	-
Symmetrical and peri-orificial	3/10 (30%)	ND	-
Symmetrical and acrofacial	2/7 (29%)	ND	-
Symmetrical, peri-orificial, and acrofacial	0/1 (0%)	ND	-
Occupational	0/1 (0%)	ND	-
Mixed	1/5 (20%)	ND	-
Vitiligo activity			
Active	51/226 (23%)	< 0.0001	0.26
Stable	2/20 (10%)	0.026	
Autoimmune disease			
Present	19/84 (23%)	< 0.0001	0.87
Absent	34/162 (21%)	< 0.0001	

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of LaminA antibodies in vitiligo patients and controls. $P < 0.05$ was considered statistically significant.

[2]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of LaminA antibodies in vitiligo patient groups. $P < 0.05$ was considered statistically significant.

ND, not determined due to small sample size or no LaminA antibody-positive patients in group.

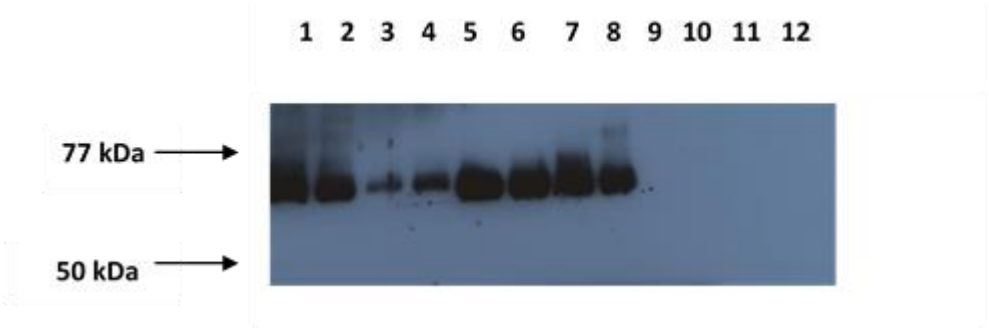

Figure 7.14: SDS-PAGE and autoradiography of [^{35}S]-LaminA following immunoprecipitation in LaminA RLBAs with vitiligo patient and healthy control sera.

Radiolabelled LaminA was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System and then immunoprecipitated in LaminA RLBAs with either vitiligo or healthy control sera. For analysis, immunoprecipitated proteins were run in a 10% (w/v) SDS-polyacrylamide gel and autoradiographed. The results are shown for six vitiligo patient and four healthy control sera: *In vitro* translated [^{35}S]-LaminA (lane 1); [^{35}S]-LaminA immunoprecipitated with: anti-LaminA antibody ab26300 (lane 2); sera from LaminA antibody-positive vitiligo patients (lanes 3-8); sera from healthy controls (lanes 9-12). The protein molecular weight markers on the gel are Prestained SDS-PAGE Standards, Low Range (Bio-Rad Laboratories Ltd.). The LaminA is visible at 74 kDa.

7.3.3 Determination of LaminA antibody titres in vitiligo patients

To determine the titres of LaminA antibodies, sera from all LaminA antibody-positive vitiligo patients (*n* = 53) and healthy controls (*n* = 10) were analysed in the LaminA antibody RLBA at different dilutions including 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10000. For each serum sample tested, a LaminA antibody index (cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by 10 healthy control sera at each dilution) was calculated and this was the mean LaminA antibody index of three experiments.

The results of the antibody titration experiments are given in Figure 7.15. The titration results are also summarised in Table 7.2, and show the serum dilution at which LaminA antibodies were still detectable at levels above the upper limit of normal for the LaminA antibody RLBA. Although the titres of LaminA antibodies varied between the different vitiligo patients, the majority, 36/53 (68%), had a titre at or above 1:500, and 23/53 (43%) had a titre at or above 1:1000.

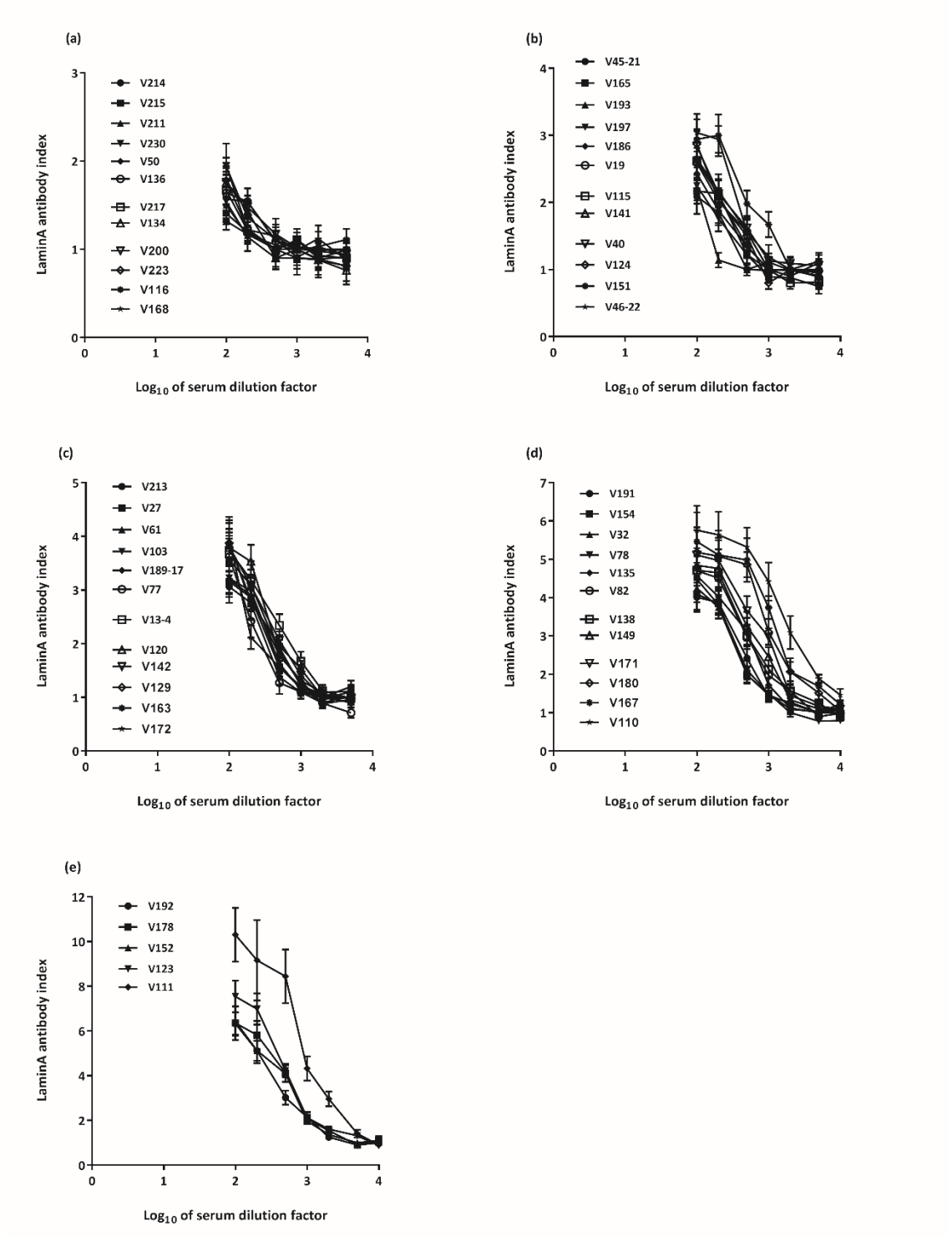

Figure 7.15: LaminA antibody titres.

The 53 LaminA antibody-positive vitiligo patient sera were analysed in the LaminA antibody RLBA at different dilutions including 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:5000. The LaminA antibody index (mean ± SD of three exepriments) of each serum at each dilution is given. (a) The results are shown for 12 vitiligo patients. (b) The results are shown for 12 vitiligo patients. (c) The results are shown for 12 vitiligo patients. (d) The results are shown for 12 vitiligo patients. (e) The results are shown for five vitiligo patients.

Table 7.2: Summary of LaminA antibody titres

Patients	LaminA antibody titre[1]
V214, V215, V211, V230, V217, V223, V168, V193	1:100
V50, V136, V134, V200, V116, V45-21, V165, V197, V77	1:200
V186, V19, V115, V141, V40, V124, V46-22, V27, V103, V189-17, V129, V163, V172	1:500
V151, V213, V61, V13-4, V120, V142, V191, V154, V32, V78, V135, V192	1:1000
V82, V138, V149, V171, V178, V152	1:2000
V180, V167, V123, V111	1:5000
V110	>1:10000

[1]The titre of LaminA antibody is shown as the dilution at which immunoreactivity of the serum sample could still be detectable above the upper limit of normal in the LaminA antibody RLBA.

7.3.4 Assessment of the specificity of LaminA antibodies

In order to determine that LaminA antibodies in vitiligo patient sera reacted specifically against the protein LaminA, absorption experiments were performed as detailed in Section 2.22. Briefly, serum samples from LaminA antibody-positive vitiligo patients (*n* = 53) and healthy individuals (*n* = 10) were absorbed with extracts prepared from untransfected HEK293 cells or from HEK293 cells transfected with LaminA, MCHR1 or tyrosinase. Subsequently, duplicate samples of pre-absorbed sera at a 1:100 dilution (equivalent to the original sera dilution) were evaluated in the LaminA RLBA. For each serum sample, a LaminA antibody index (cpm immunoprecipitated by tested serum/the cpm immunoprecipitated by 10 healthy control sera) was calculated. Serum samples were all tested in triplicate. To compare the LaminA antibody index of absorbed against unabsorbed vitiligo patient sera, one-way ANOVA was used (Section 2.24). *P* values < 0.05 were considered as significant in all tests.

The absorption experiment results are illustrated in Figure 7.16. There was a statistically significant reduction in the LaminA antibody indices by pre-absorption of LaminA antibody-positive vitiligo patient sera with the LaminA protein expressed in the extracts prepared from HEK293 cells: all *P* values were < 0.05. In contrast, LaminA antibody indices were not significantly decreased after pre-absorption of LaminA antibody-positive vitiligo patient sera with extracts made from untransfected HEK293 cells or HEK293 cells harbouring expressed MCHR1 or tyrosinase: *P* values were > 0.05.

Overall, these findings indicated that LaminA antibodies in all LaminA antibody-positive patient sera specifically recognised their target autoantigen without cross-reactivity with vitiligo-associated autoantigens MCHR1 and tyrosinase.

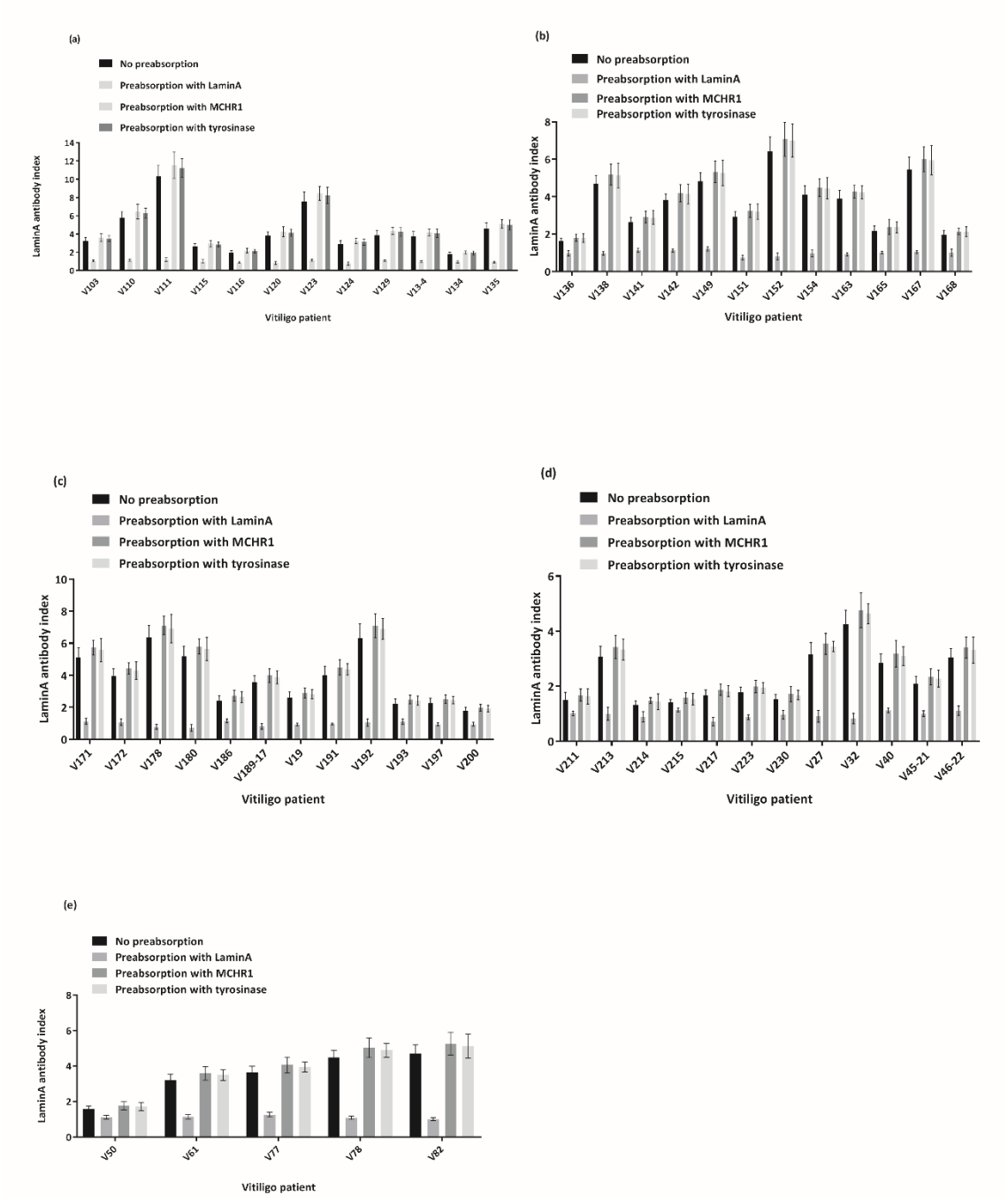

Figure 7.16: LaminA antibody absorption experiments.

Serum samples from the 53 LaminA antibody-positive vitiligo patient and 10 healthy control were preabsorbed with extract made from either untransfected HEK293 cells or from HEK293 cells expressing LaminA, tyrosinase or melanin-concentrating hormone receptor 1 (MCHR1). Unabsorbed and preabsorbed sera were then analysed in the LaminA RLBA. The results demonstrate the mean LaminA antibody index (± SD) of three experiments for unabsorbed and preabsorbed sera. The results are given for the 53 vitiligo patients. Comparing the antibody indices of LaminA absorbed sera with those without preabsorption; *P* values were < 0.0001 (One-way ANOVA).

7.3.5 Analysis of vitiligo patient sera for other antibodies

As detailed earlier in Section 5.3.5, RLBAs for tyrosinase, DCT, TYRP1, PMEL, TH, and MCHR1 antibodies (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011) were performed on all the 246 vitiligo patient sera. The results are shown in Table 5.3, and used to determine if antibodies to LaminA were related to the presence of any antibodies directed against other vitiligo-associated autoantigens (Section 7.3.6).

7.3.6 Comparison of the demographic, clinical and serological details of the LaminA antibody-positive and LaminA antibody-negative non-segmental vitiligo patient groups

In order to compare the details of the demographic, clinical and serological features of the LaminA antibody-positive (*n* = 51) and antibody-negative (*n* = 180) non-segmental vitiligo patient groups, Fisher's exact tests for 2 x 2 contingency tables or unpaired t tests were used as appropriate (Section 2.24). *P* values < 0.05 were considered as significant.

The results are given in Table 7.3. A statistically significant difference was not evident between the two cohorts in terms of sex, age of patient, duration of disease, vitiligo onset age, activity of vitiligo, the occurrence of other autoimmune disorders, the clinical sub-type of non-segmental vitiligo or the prevalence of tyrosinase, MCHR1, PMEL, TYRP1, DCT, and TH antibodies; *P* values were all > 0.05, indicating that LaminA antibodies were not associated with any of these analysed parameters.

Table 7.3: Comparison of the demographic, clinical, and serological details in non-segmental vitiligo patients positive and negative for LaminA antibodies

Patient detail	LaminA antibody-positive non-segmental vitiligo patients (*n* = 51)	LaminA antibody-negative non-segmental vitiligo patients (*n* = 180)	*P* value
Demographic details			
Male	22 (43%)	75 (42%)	0.87[1]
Female	29 (57%)	105 (58%)	
Mean age ± SD (years)	48 ± 18 (16 - 83)	45 ± 17 (0 - 88)	0.17[2]
Mean onset age ± SD (years)	35 ± 18 (6 - 71)	30 ± 17 (0 - 83)	0.11[2]
Mean disease duration ± SD (years)	14 ± 12 (0.5 - 50)	14 ± 14 (0 - 71)	0.79[2]
Non-segmental vitiligo sub-type			
Symmetrical	34 (67%)	123 (68%)	0.87[1]
Acrofacial	10 (20%)	32 (18%)	0.84[1]
Vitiligo activity			
Active	50 (98%)	166 (92%)	0.20[1]
Stable	1 (2%)	14 (8%)	
Autoimmune disease			
Present	18 (35%)	63 (35%)	1.00[1]
Absent	33 (65%)	117 (65%)	
Serology tests			
Tyrosinase antibody-positive	5 (10%)	37 (21%)	0.10[1]
MCHR1 antibody-positive	13 (25%)	38 (21%)	0.57[1]
PMEL antibody-positive	5 (10%)	30 (17%)	0.27[1]
TYRP1 antibody-positive	7 (14%)	37 (21%)	0.32[1]
DCT antibody-positive	10 (20%)	33 (18%)	0.84[1]
TH antibody-positive	13 (25%)	40 (22%)	0.71[1]

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.24) for comparing the prevalence of demographic, clinical, and serological details in the LaminA antibody-positive and the LaminA antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

[2]*P* value calculated using unpaired t tests (Section 2.24) for comparing the age, onset age, and disease duration in the LaminA antibody-positive and LaminA antibody-negative non-segmental vitiligo patient groups. *P* values < 0.05 were considered significant.

7.4 Discussion

In most mammalian cells, the nuclear lamina is composed of three major proteins, Lamins A, B, and C (Hozak et al., 1995). They are structural proteins that provide stability and strength to cells via forming part of a diffuse skeleton spread throughout the nuclear interior (Hozak et al., 1995). These proteins are suggested to be implicated in the post-mitotic nuclear reorganisation like DNA replication and transcription (Wilson et al., 2001, Schoft et al., 2003, Dittmer and Misteli, 2011).

LaminA was identified recently as a putative antibody target in vitiligo by proteome analysis (Li et al., 2011). The aims of the present study were to investigate the prevalence of LaminA antibodies in vitiligo patients, and to find out if there were any associations of LaminA antibodies with the demographic (e.g., age, gender), clinical (e.g., vitiligo activity, autoimmune disease) or serological details of vitiligo patients. Of interest, antibodies against LaminA, B1, A/C, and ABC have been previously reported in patients' sera with rheumatoid arthritis , systemic lupus erythematosus (Senecal et al., 1999), chronic autoimmune hepatitis (Wesierska-Gadek et al., 1988), linear scleroderma (McKeon et al., 1983), and autoimmune cytopenias (Coppo et al., 2004).

7.4.1 Frequency of LaminA antibodies

Firstly, a RLBA was developed which could be utilised to detect and measure LaminA antibodies in human sera. After verifying the specificity of LaminA RLBA for the detection of LaminA antibodies, RLBA was used to analyse 246 vitiligo patient sera for LaminA antibodies. Of 246 patients, LaminA antibodies were found in 22% (51/231) of patients with non-segmental vitiligo, 25% (1/4) with segmental vitiligo, and 9% (1/11) with undetermined vitiligo. Similar findings have been reported by Li and colleagues (Li et al., 2011), where 33% (24/72) of Chinese patients with non-segmental vitiligo had antibody reactivity to LaminA. However, in the same study, none of 12 of segmental vitiligo patients was positive for LaminA antibodies. In contrast to our results, a study conducted by Kim and co-workers (Kim et al., 2011) failed to detect LaminA antibodies in sera of vitiligo patients of Korean descent. These different findings could be attributed to the differences in the ethnicity of the patients included in the three studies.

Although in the current study, the prevalence of LaminA antibodies in vitiligo patients was found to be relatively low at 22%, it is similar to other studies which have reported a low prevalence of antibodies against tyrosinase (11%) (Kemp et al., 1997a), TYRP1 (6%) (Kemp et al., 1998b), DCT (6%) (Kemp et al., 1997b), MCHR1 (16%) (Kemp et al., 2002b), PMEL (6%) (Kemp et al., 1998a), and TH (23%) (Kemp et al., 2011). Likewise, Waterman and colleagues (Waterman et al., 2010) recently showed low frequencies of antibodies in vitiligo patients, ranging from 6 to 15%, against several different autoantigens. These results may indicate that vitiligo represents a disorder with a spectrum of pathologies that result in similar clinical features instead of being a disease with a single pathogenicity (Li et al., 2011).

7.4.2 LaminA antibody titres and specificity

To measure LaminA antibody titres, the 53 patient sera positive for LaminA antibodies were tested in RLBAs at a series of final dilutions. Although the titres of LaminA antibodies varied between the different vitiligo patients, the majority, 36/53 (68%), had a titre at or above 1:500, and 23/53 (43%) had a titre at or above 1:1000. In contrast, lower titres of antibodies against other melanogenic proteins such as TH, PMEL, TYRP1, and MCHR1 were reported and these ranged from 1:50 to 1:200 (Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002b, Kemp et al., 2011).

Previous studies have shown that vitiligo patient antibodies against tyrosinase are cross-reactive with TYRP1 and DCT (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998b) because of their amino acid sequence homology. In order to ensure that LaminA antibody reactivity was specific to the LaminA, absorption experiments were carried out. The absorption experiments showed that all 53 LaminA antibody-positive patient sera contained LaminA antibodies that reacted specifically with their target antigen with no cross-reactivity to vitiligo-associated antigens MCHR1 and tyrosinase. This was as expected since the three proteins lack amino acid sequence similarity.

7.4.3 Prevalence of LaminA antibodies in vitiligo sub-types

An autoimmune involvement has been suggested to contribute to the aetiology and pathogenesis of non-segmental vitiligo, as the disease is characterised by a correlation with autoimmune disorders, unstable results following autologous melanocyte grafting (Taieb, 2000), and a positive response to immunosuppressive treatments (Lepe et al., 2003). In contrast, non-autoimmune pathomechanisms have been suggested to be involved in the development of segmental vitiligo (Taieb, 2000). However, it was found that the LaminA antibody response was detectable at a slightly higher frequency (25%) in patients with segmental vitiligo than in those with the non-segmental disease sub-type (22%). However, only a small number of patients with segmental vitiligo, four in all, was available. This was because segmental vitiligo mostly affects children (Halder et al., 1987, Nicolaidou et al., 2012, Silverberg, 2015), but children present rarely in our clinics as they usually go to paediatric dermatology. Additionally, segmental vitiligo is less common in adulthood and, due to a usually more confined lesions, it is less likely to precipitate a referral to a clinic, than an advancing case of non-segmental vitiligo. Therefore, a definite conclusions cannot be made regarding the prevalence of immunoreactivity against LaminA in this disease sub-type.

7.4.4 Associations of LaminA antibodies

Previously, antibodies in vitiligo have been reported to occur more frequently in patients with active disease (Naughton et al., 1986, Kemp et al., 2011). A recent example of this was the finding of the association of TH antibodies with active vitiligo (Kemp et al., 2011). A comparison of LaminA antibody-positive and LaminA antibody-negative non-segmental vitiligo patients showed no statistically significant difference between the presence of LaminA antibodies and vitiligo activity. Additionally, comparing the same two vitiligo patient groups also demonstrated there was no significant association between the presence of LaminA antibodies and the non-segmental vitiligo clinical sub-type, patient gender, patient age, disease duration or vitiligo onset age, a finding that is consistent with other studies (Li et al., 2011).

Past investigations have reported that several melanocyte-specific proteins, including TYRP1, DCT, and PMEL are antibody targets in vitiligo patients with accompanying autoimmune diseases (Kemp et al., 1997b, Kemp et al., 1998b, Kemp et al., 1998a). In addition, antibodies to tyrosinase and transcription factor SOX10 are found predominantly in vitiligo patients with autoimmune disorders (Kemp et al., 1997a, Hedstrand et al., 2001). However, antibody reactivity to LaminA was detected in patients with (19/84 = 23%) and without (34/162 = 21%) a concomitant autoimmune disease, and there was no significant difference in the prevalence of LaminA antibodies between the two groups. Similar results were found in relation to TH antibodies in vitiligo patients (Kemp et al., 2011).

In the present work, there was also no obvious correlation between the occurrence of LaminA antibodies and the presence of tyrosinase, MCHR1, PMEL, TYRP1, DCT or TH antibodies. Likewise, no correlation between tyrosinase, MCHR1, TYRP1, DCT, TH, and PMEL antibodies has been reported in vitiligo patients (Kemp et al., 2002b, Kemp et al., 2011).

7.4.5 Development of LaminA antibodies and potential pathogenicity

The question concerning the breakage of self-tolerance resulting in the development of antibodies directed to LaminA remains unanswered, although LaminA antibodies have been identified in the sera of patients with a variety of autoimmune disorders (Nesher et al., 2001). Two hypothesises have been put forward as to how LaminA antibodies emerge. One possibility is that many LaminA antibodies are natural antibodies that exist in the sera of normal individuals and can react with autoantigens in the absence of previous apparent specific antigenic contact (Li et al., 2011). Indeed, antibodies against LaminA have been detected in normal sera, albeit at a lower frequency than in vitiligo patient sera (Li et al., 2011). However, LaminA antibodies were not evident in the sera of the healthy individuals included in this study. Another hypothesis is that the emergence of LaminA antibodies in vitiligo patients may be attributed to apoptosis of epidermal melanocytes (Li et al., 2011); it has been shown that Lamin proteins redistribute from the nuclear lamina to apoptotic blebs during apoptosis of endothelial cells in patients with systemic lupus erythematosus (Li et al., 2011). If these apoptotic

microbodies are not cleared effectively, then their accumulation could result in an immune response against the proteins which they contain. Like LaminA, antibodies against several other intracellular proteins such as tyrosinase can be found in vitiligo patients (Kemp et al., 1997a). Again, however, the processes leading to immune responses against such antigens have yet to be clarified. Importantly, although LaminA is located intracellularly, the protein also can be expressed on the surface of melanocytes since it can be labelled by the lactoperoxidase-labelling technique (Cui et al., 1995). This is significant since antibody-mediated melanocyte destruction requires the expression of target antigens on the cell surface. As such LaminA may be available for binding by antibodies, although any specific LaminA antibody-mediated cytotoxicity has yet to be investigated in vitiligo or other autoimmune diseases.

Of interest, is the question as to whether or not LaminA antibodies play any role in skin depigmentation. It is possible that they are an epiphenomenon and do not have a role in melanocyte destruction, but nevertheless, indicate the presence of autoreactive T cells.

7.5 Conclusions

In conclusion, LaminA has been identified as an autoantigen in vitiligo and these results further support the possibility that autoimmunity might play a role in vitiligo development. However, LaminA antibodies were detected at higher frequency in patients with segmental vitiligo, which is suggested to have a non-autoimmune aetiology (Taieb, 2000). In addition, since antibodies to nuclear lamins have been frequently reported in most sera from patients with other autoimmune diseases (Li et al., 2011), the specificity of LaminA antibodies in vitiligo pathogenesis merits further investigation.

Chapter 8

General Discussion

8 General Discussion

8.1 Discussion of the results

Vitiligo is the most common pigmentary disease, with an estimated prevalence of 0.4% to 2% in various populations (Ezzedine et al., 2015, Picardo et al., 2015). To date, the aetiology and pathogenic mechanisms of vitiligo are not completely understood, although various hypotheses have been suggested such as genetic vulnerability, biochemical and neural abnormalities, dysfunctional melanocytes, and aberrant cellular and humoral immune responses (Picardo et al., 2015). Much accumulating and convincing experimental and observational evidence suggests a leading role for autoimmunity in the destruction of melanocytes in vitiligo. The principal reasons for believing autoimmunity is the cause of melanocyte loss in vitiligo are that:

- Vitiligo patients commonly suffer from other autoimmune diseases such as autoimmune thyroid disease, alopecia areata, systemic lupus erythematosus, Addison's disease, rheumatoid arthritis, and type 1 diabetes (Taieb, 2000, Alkhateeb et al., 2003, Ezzedine et al., 2015).
- Autoreactive T lymphocytes and antibodies against melanocyte proteins are found in vitiligo patients (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Hedstrand et al., 2001, Lang et al., 2001, Palermo et al., 2001, Kemp et al., 2002b, van den Boorn et al., 2009, Kemp et al., 2011, Li et al., 2011).
- Repigmentation occurs in vitiligo patients receiving immunosuppressive therapies such as corticosteroids and immunomodulators (Lepe et al., 2003, Rodrigues et al., 2017a).
- Allelic variations in vitiligo patients are present in genes that encode components of the immune system (Jin et al., 2012a, Spritz, 2012, Ezzedine et al., 2015). For instance, the correlation between specific genetic variations at HLA and predisposition to vitiligo (Foley et al., 1983, Yang et al., 2005, Singh et al., 2012a, Jin et al., 2015, Spritz and Andersen, 2017).

Characterising the autoimmune responses against melanocytes in vitiligo may lead to recognising new immune targets that would be useful for developing new therapeutic and diagnostic tools, and, possibly, in unveiling the aetiological mechanisms involved in the disease.

The work in this thesis aimed to: (i) describe our Sheffield vitiligo patient cohort with regard to demographic and clinical profiles, (ii) identify novel disease autoantigens in patients suffering from vitiligo by the application of the powerful tool of phage-display technology, (iii) develop a RLBA to detect antibodies in vitiligo patients and measure their frequencies, (iv) investigate antibody frequencies against three identified autoantigens i.e., GPNMB, OCA2 protein, and LaminA using the RLBAs, and (v) determine if antibodies to either GPNMB, OCA2 protein or LaminA were related to any clinical, demographic or serological features of vitiligo patients.

8.1.1 Vitiligo patient details

Previous epidemiological studies have disagreed as to whether men and women are affected by vitiligo at the same frequency or whether there is a preponderance of females (Alkhateeb et al., 2003). Like most studies based on analysis of patient cohorts (Martis et al., 2002, Alkhateeb et al., 2003, Dogra et al., 2005, Singh and Pandey, 2011, Vora et al., 2014, Lee et al., 2015), an excess of females among our analysed patients was observed. This might result from greater cosmetic awareness that prompts women to consult a doctor for treatment. The disease onset age was under 40 years in most (72%) cases, a finding that differs slightly from most previous studies (Herane, 2003, Zhang et al., 2004, Liu et al., 2005, Sehgal and Srivastava, 2007, Taïeb et al., 2007) that reported 70-80% of vitiligo cases develop the disease before the age of 30 years.

Almost all patients (231/246 = 94%) had the non-segmental form of vitiligo. Segmental vitiligo most often presents in children and can account for almost a third of paediatric cases (Halder et al., 1987, Nicolaidou et al., 2012, Silverberg, 2015). However, children were seen rarely in our clinics as they usually present to paediatric dermatology. In addition, presentation of segmental vitiligo in adulthood is less common and, due to a usually more confined presentation in terms of the area of skin involved, it is less likely to precipitate a referral to a clinic, than an advancing case of non-segmental vitiligo. At

time of examination, vitiligo was active in most (92%) cases, which is consistent with other studies (Chun and Hann, 1997, Dave et al., 2002, Karelson et al., 2009). This finding was as expected since non-segmental vitiligo dominated our patient group, and the non-segmental form is more frequently associated with disease progression than segmental vitiligo (Chun and Hann, 1997).

Our findings confirm previous evidence of correlations between vitiligo and other autoimmune disorders (Majumder et al., 1993, Zelissen et al., 1995, Alkhateeb et al., 2003, Mason and Gawkrodger, 2005, Singh and Pandey, 2011, Vijayalakshmi et al., 2017). In the present study, several autoimmune diseases were at a higher frequency in our vitiligo patient cohort than in the general population. The most frequent autoimmune disease was autoimmune thyroiditis, particularly hypothyroidism, and this agrees well with previous studies (Alkhateeb et al., 2003, Nejad et al., 2013, Gill et al., 2016). With respect to vitiligo sub-type, associated autoimmune diseases were more frequently (35%) found in patients with non-segmental vitiligo. Three of 11 patients (27%) with focal vitiligo had an associated autoimmune disorder, whereas none of the four patients with segmental vitiligo had an associated autoimmune disease. This is consistent with previous reports in which autoimmune diseases correlated with the non-segmental form of vitiligo, although our study only included a small number of patients with segmental vitiligo (Ezzedine et al., 2011).

A family history of vitiligo was present in 18.3% of our subjects, consistent with most previous studies where the percentage frequency lies between 8% and 30% (Hann et al., 1997, Dave et al., 2002, Pajvani et al., 2006, Shankar et al., 2012, Silverberg, 2015). In addition, family history of other autoimmune diseases was positive in approximately a third (37.4%) of our patients, a result that is almost identical to previous studies (Mason and Gawkrodger, 2005). This result further indicates that vitiligo may share common aetiologic factors with other autoimmune diseases. Indeed, genome-wide association studies have identified several susceptibility genes in vitiligo that are also associated with other autoimmune diseases (Jin et al., 2012a).

Collectively, the demographic and clinical profiles of our vitiligo patient group were generally in agreement with results from elsewhere in the world.

8.1.2 Identification of novel autoantigens

Identifying and characterising vitiligo-associated antibody targets accounts for the greater part of this thesis. By defining the targets of antibodies in vitiligo, the hope was that knowledge would be gained about antibody correlation with disease pathogenesis. Furthermore, antibodies in vitiligo may act as potentially useful autoantigen markers for pathologically more important involvement of cellular immune reactivity.

A powerful strategy for identifying vitiligo associated-autoantigens was employed herein. A melanocyte cDNA phage-display library was constructed in the phage-display vector pJuFo and this was used to screen vitiligo patient IgGs that could bind melanocyte peptides. The pJuFo phage-display approach (Crameri and Suter, 1993, Crameri et al., 1994) was validated previously by the successful enrichment of several different autoantigens in vitiligo and other autoimmune diseases (Crameri and Blaser, 1996, Crameri et al., 1996, Lindborg et al., 1999, Kemp et al., 2002b, Waterman et al., 2010). The present study further demonstrated that the phage-display technique was an efficient tool for molecular antigen profiling of the humoral immune response in vitiligo. Several putative autoantigens were successfully enriched from the cDNA melanocyte library in which they were able to interact with IgG molecules from patients with vitiligo. These included proteins that were previously reported as autoantigens in different diseases as well as proteins related to melanocyte function. We report the identification of a panel of 11 different autoantigens. Four were major melanocyte-specific proteins already identified as autoantigens in vitiligo. These were tyrosinase (Baharav et al., 1996, Fishman et al., 1997, Kemp et al., 1997a), TYRP1 (Kemp et al., 1998b, Okamoto et al., 1998), PMEL (Kemp et al., 1998a), and DCT (Kemp et al., 1997b). In addition, HSP90, ribosomal protein RPL24, and LaminA, all formerly characterised autoantigens in vitiligo (Waterman et al., 2010, Li et al., 2011), were also enriched from the melanocyte peptide phage-display library. Most significantly, the screening of the phage-display library led to the discovery of four putative novel autoantigens GPNMB, OCA2 protein, MC1R and Rab27A. The frequency of immunoreactivity in sera from vitiligo patients against three autoantigens, namely GPNMB, OCA2 protein, and LaminA, was investigated using RLBAs specific for GPNMB, OCA2 protein, and LaminA antibodies.

8.1.2.1 GPNMB antibodies

GPNMB is localised abundantly in late-stage melanosomes, which are characterised by the deposition of melanin pigment, thus, GPNMB might be critical in the functions of mature melanosomes, probably their transport to keratinocytes (Hoashi et al., 2010, Maric et al., 2013). In the current work, antibody reactivity to GPNMB was found at a greater frequency (39% in non-segmental vitiligo patients) than vitiligo-specific antibodies against tyrosinase, TYRP1, DCT, PMEL, and MCHR1 (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998b, Kemp et al., 2002b). None of the segmental vitiligo patient sera demonstrated immunoreactivity against GPNMB. This further supports the idea that non-autoimmune involvement contribute to the aetiology and pathogenesis of segmental vitiligo (Taieb, 2000, Kemp et al., 2011). However, due to the availability of only four segmental vitiligo patient sera, definite conclusions were unlikely to be drawn regarding the frequency of GPNMB antibodies in this form of vitiligo. Therefore, the analysis of a greater number of vitiligo patients with segmental sub-type would be necessary to verify this result.

GPNMB antibodies were found at a significantly higher frequency in vitiligo patients with active disease. Such a result was reported for TH antibodies in vitiligo (Kemp et al., 2011). However, due to the availability of only 20 cases with stable disease, it is conceivable that further investigations by including a larger group of patients with stable vitiligo are needed to confirm these findings. Interestingly, serological analysis showed an association of MCHR1 antibodies with the occurrence of GPNMB antibodies; antibody reactivity against the receptor had not been correlated with other antibodies in vitiligo before (Kemp et al., 2011).

8.1.2.2 OCA2 protein antibodies

Another novel autoantigen detected in this study was the OCA2 protein. The precise function of this protein is not entirely clear, although studies suggest its involvement in the transportation of tyrosinase to melanosomes as well as in maintaining the pH of melanosomes. OCA2 protein antibodies were detected in 22% of vitiligo patients, a low frequency comparable to the prevalence of antibodies against several autoantigens

reported in earlier studies (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Waterman et al., 2010, Kemp et al., 2011, Li et al., 2011).

Unlike GPNMB antibodies, OCA2 protein antibodies were detected at slightly greater frequency (25%) in segmental vitiligo patients than in non-segmental vitiligo patients (23%). However, again, the availability of only four segmental vitiligo patient sera means it is unlikely to firmly conclude that OCA2 protein antibodies are prevalent at higher levels in segmental form of vitiligo. Interestingly, an association of OCA2 protein antibodies with the occurrence of tyrosinase antibodies was noted. Importantly, the OCA2 protein, being a melanosomal membrane transporter, is involved in tyrosinase transportation to melanosomes and it is like tyrosinase in that both proteins can be presented to the immune system by HLA-A*02 (Zhang and Xiang, 2014).

8.1.2.3 LaminA antibodies

A further candidate autoantigen identified in this study was LaminA. LaminA was characterised recently as a putative antibody target in vitiligo by proteome analysis (Li et al., 2011). It is a structural protein that provides stability and strength to cells and is proposed to be involved in the post-mitotic nuclear reorganisation along with other nuclear laminar proteins B and C (Hozak et al., 1995, Wilson et al., 2001, Schoft et al., 2003, Zhu et al., 2015). How this particular protein relates to melanocytes has not been reported and the reason for its autoantigenicity remains undetermined, but LaminA as wells as other nuclear laminar proteins; B and C, have previously been reported as autoantigens in other autoimmune conditions (Wesierska-Gadek et al., 1988, Senecal et al., 1999).

LaminA antibodies were detected in 22% of vitiligo patients. Although the frequency of LaminA antibodies is not high, Li et al. (2011) reported similar frequency (28.6%) for LaminA antibodies in vitiligo patients. As with OCA2 protein antibodies, the LaminA antibody response was detected at a slightly higher frequency (25%) in patients with segmental vitiligo than in those with the non-segmental disease sub-type (22%). Again, limited numbers of segmental vitiligo patients limit the conclusions that can be made. No association was evident between vitiligo activity and LaminA immunoreactivity, a finding that is inconsistent with a previous study (Li et al., 2011).

Overall, GPNMB, OCA2 protein, and LaminA were antibody targets in both those with and those without concomitant autoimmune disease. Similarly, TH antibody prevalence did not differ between vitiligo patients with accompanying autoimmune disease and those without (Kemp et al., 2011). However, previous studies reported antibody detection against TYRP1, DCT, and PMEL only in vitiligo patients suffering from other autoimmune disorders (Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b). Antibodies reactive to tyrosinase, SOX10, and LaminA were also identified mostly in patients with both vitiligo and accompanying autoimmune diseases (Kemp et al., 1997a, Hedstrand et al., 2001). However, analysis of a larger group of vitiligo patients is needed in order to determine whether our results reflect a distinct difference in the pattern of GPNMB, OCA2 protein, and LaminA antibody production.

8.1.2.4 Other autoantigens

Further autoantigens of note that were identified by using phage-display technology included HSP90, MC1R, Rab27A, tyrosinase, TYRP1, DCT, RPL24, and PMEL. HSP90 has been characterised as an autoantigen in systemic lupus erythematosus (Conroy et al., 1994). More recently, HSP90 has also been reported as antibody target in vitiligo by using RLBAs, where the majority of vitiligo patients with antibody reactivity to HSP90 also had an autoimmune disease (Waterman et al., 2010). Therefore, HSP90 antibodies are probably markers of autoimmunity, but not specifically associated with vitiligo. Indeed, previous studies showed the secretion of HSP70 from stressed melanocyte (Denman et al., 2008) and thus, anti-HSP antibodies detected in some patients with vitiligo may occur as a result of an immune response to extracellular HSPs (Waterman et al., 2010).

To our knowledge, MC1R is a previously unreported vitiligo autoantigen. The melanocyte-specific surface receptor MC1R regulates the amount of melanin and it is activated upon binding with its agonist α-MSH (Donatien et al., 1992, McRobie et al., 2014, Salinas-Santander et al., 2018). When MC1R interacts with α-MSH, melanin synthesis is induced (Donatien et al., 1992, Dessinioti et al., 2011, Salinas-Santander et al., 2018). Therefore, antibody reactivities to MC1R are likely to affect the receptor function in an adverse way, causing disruption in melanin synthesis. However, a previous

study investigated the ability of IgG extracted from 41 vitiligo patient sera to block the stimulatory effect of α-MSH on the MC1R showed that none of the vitiligo patient IgGs was able to inhibit cAMP production stimulated by α-MSH in the human melanocytes expressing the MC1R (Agretti et al., 2010). Since only 41 vitiligo patient sera were tested for their ability to interfere with α-MSH activity on the MC1R in the study reported by Agretti et al. (2010), further investigations including larger numbers of vitiligo patients are required in order to identify whether or not antibodies to MC1R have function-blocking to the receptor affecting its activity in relation to pigmentation.

The Rab27A, which anchors melanosomes to plasma membrane of melanocytes (Yoshida-Amano et al., 2012), has not previously been identified as autoantigen in vitiligo, but another GTP-binding protein, namely Rab38, has been reported as an autoantigen in vitiligo (Waterman et al., 2010). Rab38 regulates melanogenic enzyme trafficking from Golgi apparatus to melanosomes (Wasmeier et al., 2006).

In the present study, the library screening has provided additional evidence that melanocyte-specific proteins tyrosinase, TYRP1, DCT, and PMEL are B cell autoantigens in vitiligo (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b). Tyrosinase, TYRP1, and DCT play a central role in melanin biosynthesis, while PMEL is involved in the structural organisation of premelanosomes (Yamaguchi et al., 2007, Tang et al., 2013, D'Mello et al., 2016). However, usually, these autoantigens have been detected at a low frequency, for instance, tyrosinase (11%) (Kemp et al., 1997a), TYRP1 (6%) (Kemp et al., 1998b), DCT (6%) (Kemp et al., 1997b), and PMEL (6%) (Kemp et al., 1998a). The low prevalence of different autoantigens may signify that vitiligo represents a disorder with a spectrum of pathologies, which can result in a similar clinical features rather than a disease with a single pathogenicity (Waterman et al., 2010, Li et al., 2011). In this respect, vitiligo could be a symptom complex resulting from multiple causes (Bystryn, 1989). Genetic factors, oxidative stress, humoral and cellular autoimmunity, biochemical and neural abnormalities and melanocyte defect may all contribute to the disease, in which autoimmunity reactivities may contribute to a greater or lesser degree.

Interestingly, genome-wide association analyses have discovered several genetic loci that contribute to risk of vitiligo in European white people (Spritz, 2010b, Spritz and

Andersen, 2017). Almost all of the identified susceptibility genes encode components involved in immunoregulation, supporting the autoimmune basis of vitiligo which can result from dysregulated immune programming, cellular activation and pigment cell target recognition and killing (Jin et al., 2010a, Spritz and Andersen, 2017). Several of these loci such as *HLA* class I and II, *PTPN22*, *GZMB*, *FOXP3*, and *CCR6* suggest a role for the adaptive immune response. Some of them are associated with other autoimmune disorders such as thyroid disease, rheumatoid arthritis, and type 1 diabetes, with which vitiligo is epidemiologically associated (Jin et al., 2010a, Spritz, 2012, Ezzedine et al., 2015, Spritz and Andersen, 2017). In addition, other vitiligo susceptibility genes that have been identified by genome-wide association analyses include *TYR*, *OCA2*, and *MC1R*, which are involved in melanocyte function (Jin et al., 2010a). These proteins were antibody targets in the current study.

8.2 Antibodies in vitiligo: possible implications

8.2.1 GPNMB, OCA2 protein, and LaminA antibodies in diagnosis

It is clear that the occurrence of antibodies is a feature of vitiligo. Antibodies that are highly disease specific do exist in different autoimmune disorders and are likely to reflect unique pathological aspects in each case. Such antibodies have proven to be efficient diagnostic and prognostic markers. For instance, anti-DNA antibody titres in systemic lupus erythematosus patient sera can be correlated with disease activity and the presence of anti-Scl70 and anti-centromere protein B antibodies can be predictive tools in systemic sclerosis (Riboldi et al., 2005, Nihtyanova and Denton, 2010). Therefore, antibodies are likely to be useful diagnostic and prognostic markers in vitiligo, particularly in early disease to differentiate melanoma from vitiligo as patients with melanoma can demonstrate skin whitening before melanoma detection with a clinical manifestation that resembles vitiligo (Picardo et al., 2015), and the significance of early treatment for melanoma is well-established (Bishnoi and Parsad, 2018). The presence of specific antibodies or increased occurrence of such antibodies can be an indication of the involvement of other organs in some autoimmune diseases like antibodies to Sm antigen in systemic lupus erythematosus (Rahman and Isenberg, 2008). Antibody

reactivity seems to occur more frequently in active vitiligo with regard to GPNMB antibodies, but this was not the case for antibody responses against the OCA2 protein or LaminA. However, to verify these findings further investigations of a larger series of patients is required.

8.2.2 Possible origin of GPNMB, OCA2 protein, and LaminA antibodies

One of the principal reasons for supporting the autoimmune theory in vitiligo pathogenesis is that a variety of antibodies are identified in patients with the disorder. However, it is not entirely clear how antibodies originate against cytoplasmic antigens.

Previous studies have demonstrated that intracellular antigens in cells undergoing apoptosis can be contained into apoptotic blebs or microbodies and then released and clustered on the cell surface (Herrmann et al., 1998, Casciola-Rosen et al., 1999). Since deficiency in taking up apoptotic cell materials has been described in different autoimmune diseases, accumulation of cytoplasmic proteins from apoptotic waste inside the microbodies can become source for autoantigens that activate dendritic cells and trigger immune responses by which antibody production could be stimulated and directed against these fragments (Munoz et al., 2010, Mahajan et al., 2016). Additionally, accumulating evidence shows that alteration in epigenetic modification, such as DNA methylation and histone acetylation, can contribute to the development of autoreactive B cells and the induction of antibodies reactive to intracellular antigens (Renaudineau et al., 2010, Mahajan et al., 2016). For example, a systemic lupus erythematosus-like syndrome has been shown to develop in some patients following treatment with drugs such as hydralazine or procainamide, which prevent DNA methylation and hence initiate the disorder (Dubroff and Reid, 1980, Mahajan et al., 2016).

The intracellular location of GPNMB, OCA2 protein, and LaminA, suggests that the development of antibodies to these proteins most likely arises from melanocyte damage instigated mainly by non-antibody-dependent processes like oxidative stress or cytotoxic T cells (Abdel-Naser et al., 1992, Lang et al., 2001, Schallreuter et al., 2005, Dell'anna and Picardo, 2006, Shalbaf et al., 2008, van den Boorn et al., 2009). Several suggestions have been put forward about the emergence of antibody reactivity to

intracellular melanocyte proteins, and these include neo-antigen formation, exposure of cryptic epitopes and protein modification during apoptosis (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011). Following processing by dendritic cells, antigenic proteins can be presented to either autoreactive T cells which evaded clonal deletion or to naïve T cells which have not been tolerised against cryptic epitopes (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011). In consequence, antibodies can be secreted following autoreactive B cell stimulation by activated autoreactive CD4+ T lymphocytes (Namazi, 2007).

Interestingly, GPNMB has been found to be expressed on the surface of melanocytes, albeit to a lesser amount than the intracellular fraction (Tomihari et al., 2009). In addition, surface expression of LaminA on melanocytes has been revealed by lactoperoxidase-labelling technique, a method that labels only molecules exposed on the external surface of cells (Bystryn, 1989, Cui et al., 1995). Also, mislocalisation of mutant OCA2 protein to the surface of melanocytes has been reported (Sitaram et al., 2009). Therefore, these proteins could be accessible as antibody targets in such cases. This is important as antibody-mediated melanocyte destruction requires the expression of target antigens on the cell surface. As such GPNMB, OCA2 protein, and LaminA may be available for binding by antibodies, but antibody-mediated cytotoxicity directed against these proteins has to be investigated in vitiligo.

Antibody production in vitiligo may also arise from other stimuli such as a genetic susceptibility to autoimmunity and cross-reactivity with microorganism antigens (Kemp et al., 2002b). Equally, antibodies against these proteins might represent an epiphenomenon emerging as a result of damage to pigment cells by other causal factors, albeit they might then act to further aggravate vitiligo (Kemp et al., 2002b).

8.2.3 Possible pathogenic effects of GPNMB, OCA2 protein, and LaminA antibodies

The presence of antibodies to melanocytes in vitiligo patients suggests that they may be involved in the disease pathogenesis. This possibility is supported by the observations that most target antigens in vitiligo patients are expressed on the melanocyte surface where it is accessible to interact with immune effector mechanisms and that there is a correlation between the frequency of melanocyte antibodies and disease activity

(Bystryn, 1989, Cui et al., 1992, Cui et al., 1995, Kemp et al., 2011). However, it is presently unknown if antibody abnormalities in vitiligo are a primary or secondary cause of the disease.

GPNMB, OCA2 protein, and LaminA are intracellularly located, although surface expression of GPNMB, LaminA, and mutant OCA2 protein on melanocytes has been reported (Cui et al., 1995, Sitaram et al., 2009, Tomihari et al., 2009). However, a previous study has provided evidence that antibody-mediated protection does not terminate at the cell membrane, for instance, antibodies to the intracellular SSA antigen in systemic autoimmune diseases can exert their cytopathic immune attack inside the cells (Mallery et al., 2010, Racanelli et al., 2011, Yoshimi et al., 2012). Previous studies have shown that antibodies targeting specific proteins can alter the activity of such proteins. In patients with autoimmune polyglandular syndrome type 1, for example, antibodies reactive to TH have been found to have an inhibitory effect on the enzyme activity (Hedstrand et al., 2000). Expression of OCA2 protein and GPNMB have been detected in cutaneous melanocytes particularly in melanosomes, where they have been suggested to play a role in the transportation of tyrosinase to melanosomes and the transfer of melanosomes to keratinocytes, respectively (Yamaguchi and Hearing, 2014). However, to date, the functions of OCA2 protein and GPNMB in melanin synthesis are not clearly characterised and hence conclusions on how antibodies that might affect the functions of OCA2 protein and GPNMB could affect melanogenesis and ultimately melanocyte behaviour are difficult to be made. In the case of LaminA, it is a structural molecule vital in cell stability and post-mitotic nuclear reorganisation (Hozak et al., 1995, Schoft et al., 2003, Dittmer and Misteli, 2011), but how this specific protein relates to melanogensis has not been reported.

Other possible pathogenic effects of GPNMB, OCA2 protein, and LaminA antibodies include complement binding and antibody-dependent cellular cytotoxicity. However, how the interactions between such antibodies and their cytoplasmic antigens occur to destroy melanocytes via a cytoplasmic mechanism is difficult to imagine. Although melanocyte damage by vitiligo-associated antibodies has been demonstrated by both complement-mediated activation and antibody-dependent cellular cytotoxicity (Norris et al., 1988, Gilhar et al., 1995, Kemp et al., 2011), GPNMB, OCA2 protein, and LaminA

antibodies may merely serve as useful markers for melanocyte destruction and/or autoreactive T cells that are cytotoxic to melanocytes. Indeed, several melanocyte autoantigens that are recognised as antibody targets, for example tyrosinase, are also the target of T cell responses in vitiligo skin (Ogg et al., 1998, Lang et al., 2001, Palermo et al., 2001, van den Boorn et al., 2009).

If indeed antibodies to GPNMB, OCA2 protein, and LaminA have cytopathic effects, and this remains to be confirmed, the selective damage of melanocytes in vitiligo might occur as the result of the fact that pigment cells are relatively susceptible to immune injury, as opposed to, for instance, fibroblasts and keratinocytes (Norris et al., 1988). Antibodies in vitiligo were capable of destroying pigment cells *in vitro* (Norris et al., 1988) and *in vivo* following the injection of patient IgG into nude mice grafted with human skin (Gilhar et al. 1995). However, the exact pathogenic role the GPNMB, OCA2 protein, and LaminA antibodies play in vitiligo is still unclear.

8.3 Future Work

This work could be improved by the analysis of more vitiligo patient serum samples for GPNMB, OCA2 protein, and LaminA antibodies. Moreover, in order to extend the preliminary findings, additional cases with segmental form of vitiligo as well as subjects with stable vitiligo should be tested for the occurrence of antibodies reactive to GPNMB, OCA2 protein, and LaminA. Nevertheless, it is unlikely to be able to provide sufficient numbers of patients with these categories in the short-term. Other future studies can also include:

8.3.1 Identification of GPNMB, OCA2 protein, and LaminA antibodies which recognise conformation-dependent epitopes

In the pathogenesis of autoimmune diseases, it is of prime importance for antibodies to interact with conformational epitopes. In Graves' disease and autoimmune hypothyroidism, for instance, it is well established that the most prevalent antibodies to the thyrotropin receptor and thyroid peroxidase, respectively, and which are responsible for disease, only react with native protein since they recognise several

conformational epitopes (Morgenthaler et al., 1999, Gora et al., 2004). It is likely that antibodies that bind to conformational epitopes on the native proteins of GPNMB, OCA2 protein, and LaminA were not detected in the RLBAs that use recombinant GPNMB, OCA2 protein, and LaminA. The prevalence of the humoral immune responses against these proteins in vitiligo may, therefore, be underestimated. Identification of antibodies that recognise conformational epitopes can be an important future goal. This can be undertaken by using GPNMB, OCA2 protein, and LaminA expressed in a mammalian cell line in immunoprecipitation assays. Using a mammalian expression system allows the production of the native structure of these antigens. A previous study in our laboratory identified calcium-sensing receptor antibodies by utilising this technique (Gavalas et al., 2007).

8.3.2 Examination of the possible effects of GPNMB, OCA2 protein, and LaminA antibodies on the function of their respective targets

Antibodies that affect the function of GPNMB, OCA2 protein, and LaminA are more likely to play a part in vitiligo aetiology than just being antibodies that bind GPNMB, OCA2 protein, and LaminA. For instance, in Graves' disease, antibodies to thyroid-stimulating hormone receptor can mimic the action of thyroid-stimulating hormone by binding to the receptor and subsequently stimulate hyperthyroidism (Weetman and McGregor, 1994, Chistiakov, 2003, Davies and Latif, 2015, Giménez-Barcons et al., 2015). Thyroid-stimulating hormone receptor mediates the activation of thyroid-stimulating hormone to thyroid glands, resulting in thyrocytes growth and proliferation and production of thyroid hormone (Chistiakov, 2003, Davies and Latif, 2015, Giménez-Barcons et al., 2015). In addition, function-blocking antibodies to the acetylcholine receptor, affecting the receptor activity have been detected in patients with myasthenia gravis. Antibody reactivity to this receptor can interfere with and block binding sites of acetylcholine causing receptor functional loss and degradation resulting in myasthenia gravis (Hoedemaekers et al., 1997, Meriggioli and Sanders, 2012).

Antibodies to GPNMB, OCA2 protein, and LaminA might have deleterious effects on the functioning of these proteins leading to impaired melanocyte behaviour in vitiliginous skin, a preliminary step in disease development. Although GPNMB, OCA2 protein, and

LaminA are generally located inside the cell, evidence is presented that antibody-mediated protection does not terminate at the cell membrane, for instance, antibodies to the intracellular SSA antigen in systemic autoimmune diseases can exert their cytopathic immune attack inside the cells (Mallery et al., 2010, Racanelli et al., 2011). Moreover, in patients with autoimmune polyglandular syndrome type 1, for example, antibodies reactive to TH have been found to have an inhibitory effect on the enzyme activity (Hedstrand et al., 2000). Thus, the effects of vitiligo IgG on the functions of GPNMB, OCA2 protein, and LaminA should be evaluated in an *in vitro* assay (Daubner and Fitzpatrick, 1993, Lou et al., 2010).

8.3.3 Examination of vitiligo patient lesions for GPNMB, OCA2 protein, and LaminA antibodies

So far, the GPNMB, OCA2 protein, and LaminA antibodies identified have just been circulating antibodies in the sera of vitiligo patient. Therefore, further studies on the determination of whether patient GPNMB, OCA2 protein, and LaminA antibodies are also found *in situ*, in skin from active vitiligo lesions would be of interest. Moreover, the co-localisation of purified vitiligo patient GPNMB, OCA2 protein, and LaminA antibodies and expressed GPNMB, OCA2 protein, and LaminA within the same skin tissue sample could be evaluated using double indirect immunofluorescence technique.

8.3.4 Investigation of cytotoxicity of GPNMB, OCA2 protein, and LaminA antibodies

In vitro studies have demonstrated that anti-melanocyte antibodies in vitiligo patient sera can damage cultured melanocytes, both by complement-mediated damage and antibody-dependent cellular cytotoxicity (Norris et al., 1988). In addition, injection of IgG molecules from vitiligo patient sera have the ability to destroy melanocytes of the human skin grafted onto nude mice (Gilhar et al., 1995). Similar studies would be of interest in order to investigate if GPNMB, OCA2 protein, and LaminA antibodies in sera of vitiligo patients may able to exert damaging effects on melanin-producing cells by either of these processes.

8.3.5 Investigation of T cell responses against GPNMB, OCA2 protein, and LaminA

A number of studies have reported the occurrence of melanocyte-specific CD8+ cytotoxic T lymphocytes circulating in the sera of vitiligo patients. These vitiligo-circulating lymphocytes specifically recognise pigment cell differentiation antigens e. g., tyrosinase , MelanA/MART-1 and PMEL (Ogg et al., 1998, Lang et al., 2001, Le Gal et al., 2001, Palermo et al., 2001, Mandelcorn-Monson et al., 2003, Le Poole et al., 2004, Garbelli et al., 2005, Le Poole and Luiten, 2008, van den Boorn et al., 2009). These cytotoxic T cells are characterised by high expression of the skin-homing receptor cutaneous lymphocyte-associated antigen (Lang et al., 2001). In addition, their numbers are associated with both disease activity and extent supporting a critical role for the involvement of cell-mediated immunity in vitiligo (Lang et al., 2001). Thus, further studies that would be of special importance are to investigate into T cell reactivities to GPNMB, OCA2 protein, and LaminA, since antibodies to GPNMB, OCA2 protein, and LaminA might be just markers of autoreactive anti- GPNMB, OCA2 protein, and LaminA T lymphocytes in vitiligo patients.

8.4 Overall conclusions

The work in this study has concentrated on describing the demographic and clinical profiles of our Sheffield vitiligo patient cohort as well as on identifying and characterising putative antibody targets in vitiligo patients. The demography and comorbidities for vitiligo patients in Sheffield were generally in accordance with finding from elsewhere in the world, supporting a role for autoimmunity in the development and progression of vitiligo. Several novel autoantigens were identified by using the phage-display technology and the immunoreactivity of three potential autoantigens (GPNMB, OCA2 protein, and LaminA) against a panel of vitiligo patient sera were confirmed by using the RLBA.

However, the relationship between antibodies reactive to GPNMB, OCA2 protein, and LaminA, and indeed all vitiligo-associated antibodies, and the disease pathogenesis still remain to be determined. The likelihood that the GPNMB, OCA2 protein, and LaminA antibodies have no aetiological role in vitiligo but rather they indicate the existence of

autoreactive anti-GPNMB, OCA2 protein, and LaminA T lymphocytes ought to be investigated. Regardless of whether or not they are directly pathogenic, antibodies to GPNMB, OCA2 protein, and LaminA may act as potential markers for epidermal melanocyte disruption in vitiligo and, therefore, further characterisation of them will be helpful in further elucidating the causative role of autoimmunity in vitiligo.

As is true for most autoimmune disorders, the answer to several key questions is unknown. Characterising the specificities of antibodies in vitiligo as well as the detection of their expression in vitiligo lesions may help resolve the questions as to whether: (i) the melanocyte antibodies are a cause or a result of the disease, (ii) there is a primary defect in the immune system such as tolerance breakdown that results in autoimmune responses to melanocytes and to the subsequent damage of these cells, (iii) or there is an initial injury to pigment cells that leads to release of melanocyte antigens and to the subsequent stimulation of an immune response to such antigens. In any one of these cases, once pigment cells are injured, release of other antigens could exaggerate whatever immune responses are present and exacerbate the injury.

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