



The
University
Of
Sheffield.

**Identification and Characterisation of Novel Melanocyte Autoantigens in
Alopecia Areata**

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Summary

Background: Alopecia areata is an autoimmune disorder that results in unpredictable hair loss. It is characterised by autoreactive T cells around the hair follicles that release pro-inflammatory cytokines and adversely affect the anagen hair follicle cycle. There is evidence to suggest that hair follicle melanocytes are targeted by T lymphocytes, but major pigment cell autoantigens remain unidentified. Current therapies are predominantly corticosteroids and immuno-modulating agents, but these are often ineffective and new targeted therapies are especially required for children, people with long-term patchy alopecia, and individuals with alopecia totalis or alopecia universalis.

Aims: The aims of this project were 1) to describe the Sheffield alopecia areata patient cohort in terms of its demographic and clinical details; and 2) to identify and characterise novel melanocyte autoantigens using phage-display technology in order to aid a detailed understanding of the pathogenesis of alopecia areata, which is required to inform the development of new treatments.

Methods: Patient recruitment and assessment; phage-display technology to identify melanocyte autoantigens; radioligand binding assays to confirm immuno-reactivity of patient sera to identified autoantigens; ELISAs to characterise autoantibody binding sites and subclass; antibody functional assays.

Results: Biopanning of a melanocyte peptide phage-display library with alopecia areata patient sera, identified several novel melanocyte autoantigens, including OCA2-encoded P protein, the melanocortin 1 receptor (MC1R), and glycoprotein non-metastatic melanoma protein b. The immuno-reactivity of each of these potential autoantigens was tested in radioligand binding assays against 48 alopecia areata patient sera and was detected in 8 (16.7%), 21 (43.8%), and 10 (20.8%) patient serum samples, respectively. Further analysis of MC1R antibodies indicated that their binding sites were on extracellular domains of the receptor, were of the IgG1 subclass and, in two patients, adversely affected the function of the MC1R.

Conclusions: Phage-display technology was useful for identifying novel autoantigens in alopecia areata. Further work is required to ascertain if the novel autoantibody targets are also the targets of autoreactive T lymphocytes in alopecia areata patients.

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 was performed by myself, except where specifically acknowledged in the text. All information sources have been specifically acknowledged by means of references.

Dr Alfituri Alhaslok

August 2020

Dedication

I proudly dedicate this thesis to my beloved mother Masoda Abdualhamid who prayed for me all the time. To my wife, daughter Sara, and both of my sons Sohib and Abdualrhman, and to my father-in-law for his support in caring for my family in my absence.

I dedicate this thesis to my friends and colleagues A. Alabyad, A. Saad, E. Saki, and K. Algaed, and to my MSc supervisor at Cardiff University, Professor Andrew Finlay.

I also dedicate this thesis to the memory of my best friend Khalid who started studying medicine with me at university until he left this world. You are gone and yet your smile and kindness are still there.

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List of Abbreviations

| | |
|----------------|---|
| AA | Alopecia areata |
| ACTH | Adrenocorticotrophic hormone |
| AD | Atopic dermatitis |
| AIRE | Autoimmune regulator protein |
| ANA | Anti-nuclear antibody |
| APS1 | Autoimmune polyendocrine syndrome type 1 |
| AT | Alopecia totalis |
| AU | Alopecia universalis |
| Bp | Basepair/s |
| BMP | Bone morphogenetic protein |
| 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 |
| CLNK | Cytokine-dependent hematopoietic cell linker |
| CPM | Counts per minute |
| CREB | cAMP response 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 |
| DCT | Dopachrome tautomerase |
| DEBR | Dundee Experimental Bald Rat |
| DFS70 | Dense fine speckles |
| DHI | Dihydroxyindole |
| DHICA | Dihydroxyindole-2-carboxylic acid |
| DNCB | Dinitrochlorobenzene |
| DOPA | Dihydroxyphenylalanine |
| DPCP | Diphenylcyclopropenone |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| EBV | Epstein-Barr virus |
| EDN-1 | Endothelin-1 |
| EDTA | Ethylenediaminetetraacetic acid |
| ERK | Extracellular signal-regulated kinases |
| FASLAG | FAS ligand |
| FOXD3 | Forkhead box D3 |
| FcγR | Fc receptors |
| FOXP1 | Forkhead box protein P1 |

| | |
|---------------|--|
| FOXP3 | Forkhead box protein P3 |
| <i>g</i> | Gravity |
| GM-CSF | Granulocyte monocyte-colony stimulating factor |
| GPNMB | Glycoprotein non-metastatic melanoma protein b |
| GZMB | Granzyme B |
| h | Hour/s |
| HEK293 | Human embryonic kidney 293 |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HRP | Horse-radish peroxidase |
| HSP | Heat-shock protein |
| IBMX | 3-Isobutyl-1-methylxanthine |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IFN- α | Interferon- α |
| IFN- β | Interferon- β |
| IFN- γ | Interferon- γ |
| IgG | Immunoglobulin G |
| IgM-RF | IgM-rheumatoid factor |
| IL | Interleukin |
| IL1RN | Interleukin-1 receptor antagonist |
| IL2RA | Interleukin-2 receptor subunit alpha |
| JAK | Janus kinase |
| K16 | Keratin-16 |
| kb | Kilobase/s |
| kDa | Kilodalton |
| LAM | Lamin A |
| LB | Luria Bertani |
| LEDGF | Lens epithelium-derived growth factor |
| m | Metre |
| M | Molar |
| MAGE-A3 | Melanocyte-associated antigen-3 |
| MAS | Multiple autoimmune syndrome |
| MC1R | Melanocortin 1 receptor |
| mg | Milligram/s |
| MHC | Major histocompatibility complex |
| MIF | Macrophage inhibitory factor |
| MITF | microphthalmia-associated transcription factor |
| α -MSH | α -melanocyte-stimulating hormone |
| min | Minute/s |
| miRNAs | MicroRNAs |
| ml | Millilitre/s |
| MTX | Methotrexate |
| NB-UVB | Narrow band-ultraviolet B |
| NFAT | Nuclear Factor of Activated T cells |
| ng | Nanogram |
| NGF | Nerve growth factor |
| NK | Natural killer |
| NKG2D | Natural killer group 2D |
| nm | Nanometre |
| NOTCH4 | Neurogenic locus notch homolog 4 |

| | |
|---------------|--|
| NSB | Non-specific binding |
| OCA2 | OCA2-encoded P protein |
| OD | Optical density |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PMEL | Melanocyte-specific protein PMEL |
| PRP | Platelet rich plasma |
| PRDX5 | Peroxiredoxin 5 |
| PTPN22 | Lymphoid protein tyrosine phosphatase non-receptor type 22 |
| PUVA | Psoralen plus ultraviolet A |
| Rab27A | GTP-binding protein Rab27A |
| RBP-4 | Retinol-binding protein-4 |
| RLBA | Radioligand binding assay |
| RPL24 | Ribosomal protein L24 |
| SADBE | Squaric acid dibutylester |
| SALT | Severity of Alopecia Tool |
| SCID | Severe combined immunodeficient |
| SLE | Systemic lupus erythematosus |
| SNPs | Single nucleotide polymorphisms |
| SOX10 | Transcription factor SOX10 |
| TAE | Tris-acetate-EDTA |
| TCP75 | Transcriptional coactivator p75 |
| TgAb | Thyroglobulin antibody |
| TGFBR2 | Transforming growth factor- β receptor type 2 |
| TGF- β | Transforming growth factor- β |
| TH | Tyrosine hydroxylase |
| Th1 | T helper 1 cells |
| Th17 | T helper 17 cells |
| TH | Tyrosine hydroxylase |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TNF- α | Tumour-necrosis factor- α |
| TPO | Thyroid peroxidase |
| Treg/s | T regulatory cells |
| TSH | Thyroid-stimulating hormone |
| TYR | Tyrosinase |
| TYRP1 | Tyrosinase-related protein-1 |
| TRP2 | Tyrosinase-related protein-2 |
| UV | Ultraviolet |
| VGICC | Vitiligo Global Issues Consensus Conference |

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Chapter 1

Introduction

1 General Introduction

Alopecia areata is defined as an autoimmune disease of the anagen (actively growing) hair follicle which results in the loss of hair most usually from the scalp. The following review will outline the structure and function of the hair, explain the clinical features and epidemiology of alopecia areata, and will consider the factors that have been implicated in the aetiology and pathogenesis of this hair loss disease.

1.1 Hair

1.1.1 Hair function

The main functions of hair are to protect the skin from mechanical insults, irritants, allergens and irradiation by ultra-violet (UV) (Buffoli et al., 2014, de Galvez et al., 2015, Shi et al., 2015), and to regulate body temperature (Tansey and Johnson, 2015). In addition, hair has a sensory function, relaying sensory information from mechanical stimuli at the skin surface to the nervous system (Lechner and Lewin, 2013). As part of the immune response, the hair follicle is also a reservoir of Langerhans cells which re-populate the epidermis layer of the skin following injury and signal to activate other immune cells (Heath and Mueller, 2012). Moreover, the hair is important for an individual's appearance, especially for women. Indeed, patients with hair disorders such as hair loss and hirsutism can suffer from severe psychological stresses (Aghaei and Saki, 2014).

1.1.2 Hair structure

Human hair is a laminar-fibrous tissue mainly composed of the protein keratin which is extremely strong and very difficult to dissolve (Adav et al., 2018). Hair keratins comprise type I and type II keratins encoded by 54 different genes.

Macroscopically, hair has two main parts; the hair shaft and the hair follicle (Figure 1.1) (Schneider et al., 2009). The hair shaft is the external visible part of the hair that projects through the surface of the skin as a fibre and is made of non-nucleated keratinocytes that are biologically dead (Wolfram, 2003). It consists of an outer protective cuticle layer, a middle

cortex layer which contains melanin granules and keratin filaments (Wolfram, 2003), and a central medulla. The hair follicle lies below the surface of the epidermis and is the structure that is required for hair growth (Figure 1.1) (Schneider et al., 2009). Associated with each hair follicle are an arrector pili muscle and a sebaceous gland. In longitudinal section, the hair follicle comprises (a) the infundibulum, which goes from the epidermis to the entrance of the duct of the sebaceous gland, (b) the isthmus which goes from the sebaceous gland duct to the bulge, and (c) the bulb and suprabulb found between the insertion of the arrector pili muscle and the base of the hair follicle.

Within the dermis, the hair fibre is enclosed by the dermal root sheath which protects the growing hair shaft and is made up of two layers (Figure 1.1) (Schneider et al., 2009). The outer root sheath is made of epithelial cells that are arranged in layers (Legue et al., 2010). The bulge is found in the outer root sheath at a point where the arrector pili muscle is inserted. This structure contains multipotent stem cells which contribute new melanocytes and keratinocytes to the hair follicle and which contribute to wound healing and express a unique signature of genes (Ito et al., 2005a, Wang et al., 2012). The inner root sheath is made up of an internal cuticle which adjoins the hair shaft cuticle, the Huxley's layer and the Henle's layer. It is composed of terminally differentiated hair follicle keratinocytes which produce keratins and trichohyalin (Buffoli et al., 2014). These molecules give rigidity to the inner root sheath as it supports the growing hair shaft.

At the base of the hair follicle is the bulb (Figure 1.1) (Schneider et al., 2009). This structure includes the dermal papilla, which is the signalling centre of the hair follicle and consists of specialised mesenchymal fibroblasts, as well as a capillary loop (Buffoli et al., 2014). The bulb also incorporates the germinal matrix, which contains rapidly proliferating epithelial cells that generate the hair fibre and the root sheath by moving upwards and gradually becoming keratinised (Jahoda, 1992, Paus and Foitzik, 2004). Melanocytes are also found in the bulb matrix (Paus et al., 1993, Oshima et al., 2001).

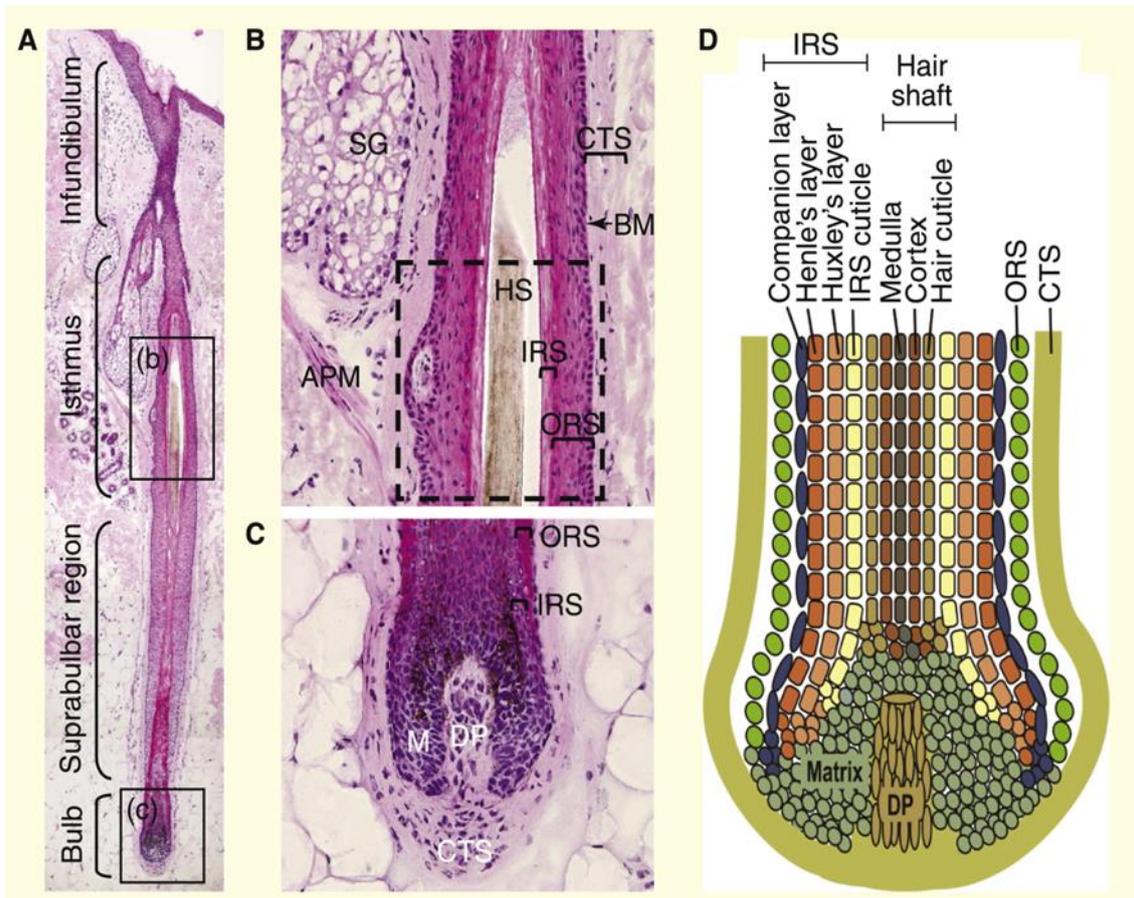


Figure 1.1: Structure of the hair follicle.

(A) Longitudinal section of a human scalp hair follicle. The infundibulum, isthmus, suprabulbar and bulb components of the hair follicle are shown. **(B)** The isthmus is illustrated at a high magnification. The bulge site is defined by the dashed square. **(C)** The bulb at a high magnification. **(D)** The layers of the outer root sheath, inner root sheath, and shaft in the bulb are shown. The inner root sheath consists of the inner root sheath cuticle, the companion layer, the Henle's layer, and the Huxley's layer. APM, arrector pili muscle; BM, basal membrane; CTS, connective tissue sheath; DP, dermal papilla; HS, hair shaft; IRS, inner root sheath; M, matrix; ORS, outer root sheath; SG, sebaceous gland. The figure, taken from a paper by Schneider et al, 2009, is used with kind permission from Elsevier Ltd.

1.1.3 Hair follicle morphogenesis

Hair follicle morphogenesis is governed by the interplay of the Wnt, Hedgehog, and Notch developmental signalling pathways (Schneider et al., 2009), and consists of three main events (Figure 1.2) (Schneider et al., 2009). Firstly, induction forms the hair placode, a thickening of the epidermal basal layer (Paus et al., 1999). This stage is followed by hair follicle organogenesis (Schneider et al., 2009). During this event, the placode stimulates the formation of the dermal papilla and the growth of epidermal hair germ cells which grow downwards to define the orientation of the hair follicle. In addition, the different hair cell lineages develop during organogenesis. Finally, cyto-differentiation (Paus et al., 1999) gives rise to the mature hair follicle with its recognisable parts (Figure 1.1).

1.1.4 The hair cycle

All hairs undergo a hair cycle (Figure 1.2) (Schneider et al., 2009), the duration of which differs from site to site. For example, human scalp hair has long cycles compared to other body sites, with a period that may extend from 2-6 years (Kligman, 1959, Wosicka and Cal, 2010). However, it can be modified or shortened by many external or internal factors such as stress, hormones, internal diseases, exposure to environmental pollution, and smoking, starvation, and cytotoxic drugs (Choi, 2018). The hair growth cycle has three distinct phases; anagen (or active growth stage), catagen (or regression stage), and telogen (or quiescent stage) (Kligman, 1959, Wosicka and Cal, 2010).

1.1.4.1 Anagen

During anagen (Figure 1.2) (Schneider et al., 2009), the hair follicle reaches maximum volume and length, and the hairs tend to have high tensile strength as well as more pigmentation (Stenn and Paus, 2001, Wosicka and Cal, 2010). The phase is split into six stages (I-VI). In stage I, epithelial cells at the bottom of the telogen hair follicle proliferate (Muller-Rover et al., 1998). Stage II-V sees hair stem cells expand, grow downwards into the dermis and envelope the growing dermal papilla (Muller-Rover et al., 1998). In addition, the hair progenitor cells also differentiate into the hair shaft and the inner root sheath, becoming progressively keratinised. In the hair matrix, melanocytes begin to produce melanin (Wosicka and Cal, 2010).

At stage VI, the epithelial hair bulb forms and new hair shaft appears at the skin surface (Stenn and Paus, 2001).

1.1.4.2 Catagen

Catagen (Figure 1.2) (Schneider et al., 2009) is also called the apoptosis-driven phase of the hair cycle. It starts directly after the end of the anagen growth phase. In this phase, melanocytes stop producing pigment, there is a decrease in the proliferation and differentiation of hair matrix keratinocytes, and the production of the hair shaft is completed (Tobin et al., 1999, Stenn and Paus, 2001). The hair follicle diameter reduces to about one-sixth of the normal size and the club hair is formed (Paus and Foitzik, 2004, Wosicka and Cal, 2010). The dermal papilla shrinks and is released from the bulb (Schneider et al., 2009).

1.1.4.3 Telogen

In telogen, hair follicle goes into a phase of quiescence (Figure 1.2) (Schneider et al., 2009), where the hair follicles are characterised by a dearth of pigment cells and lack of an inner root sheath (Paus and Foitzik, 2004, Wosicka and Cal, 2010). At any one time, about 10-15% of all hairs in the telogen phase. This phase may extend for several weeks, in the case of eyelashes, to up to eight months for scalp hair (Wosicka and Cal, 2010). The hair falls out on reaching the end of the telogen phase. After a period of few weeks, the hair follicle growth phase starts again to continue the hair cycle.

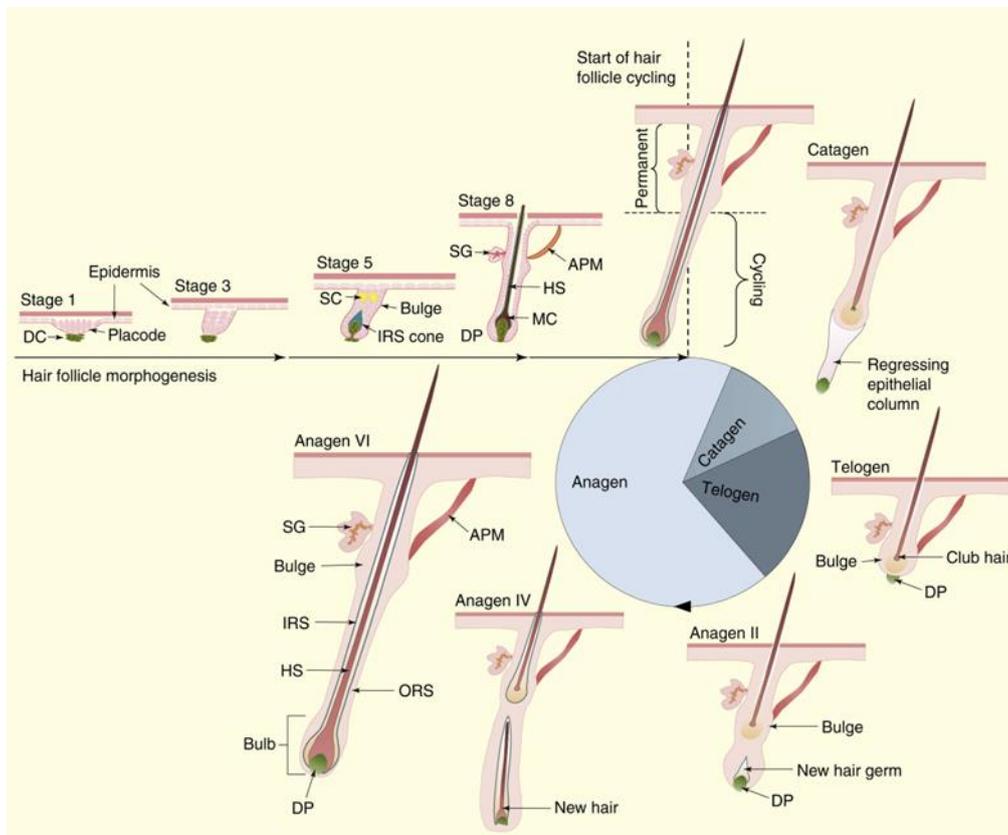


Figure 1.2: Hair follicle morphogenesis and the hair cycle.

Following hair follicle morphogenesis, there are three phases of the hair cycle, anagen (active growth phase), catagen (regression phase) and telogen (resting phase), which are shown in the schematic representation. APM, arrector pili muscle; DC, dermal condensate; DP, dermal papilla; HS, hair shaft; IRS, inner root sheath; MC, melanocytes; ORS, outer root sheath; SC, sebocytes; SG, sebaceous gland. The figure, taken from a paper by Schneider et al, 2009, is used with kind permission from Elsevier.

1.2 Alopecia areata: epidemiology and clinical features

Alopecia areata is an autoimmune disease of the anagen hair follicle that results in non-scarring hair loss, potentially from any area of the body (Messenger et al., 2012b). The condition is believed to result from immune responses against the anagen hair follicle due to the collapse of immune privilege at that site (Gilhar and Kalish, 2006).

1.2.1 Epidemiology

Population-based studies in the United States have reported the incidence of alopecia areata as 0.1% to 0.2% of the population with a lifetime risk of 1.7% to 2.1% (Safavi et al., 1995, Mirzoyev et al., 2014). According to hospital-based studies from India, Singapore, and Mexico, the incidence of alopecia areata ranged from 0.57% to 3.8% (Sharma et al., 1996a, Tan et al., 2002b, Guzman-Sanchez et al., 2007). The disease affects both sexes, although some studies have shown a sex variation with a higher incidence in females; female to male ratios have been reported to range from 2.6:1 to 1.2:1 (Tan et al., 2002b, Guzman-Sanchez et al., 2007, Lundin et al., 2014). In contrast, four studies showed a male predominance ranging from 2:1 to 1.1:1 (Sharma et al., 1996a, Jain and Marfatia, 2003, Yang et al., 2004, Kavak et al., 2008), whilst other studies have reported that male and females are affected with equal frequency (Sobolewska-Wlodarczyk et al., 2016, Strazzulla et al., 2018).

All age groups can be affected by alopecia areata, but the mean age at disease onset is 25-36 years (Tan et al., 2002b, Yang et al., 2004, Mirzoyev et al., 2014). A family history of alopecia areata has been observed in 0-8.6% of patients (Tan et al., 2002b, Yang et al., 2004, Guzman-Sanchez et al., 2007) and, in the children of a disease proband, there is a 10-fold greater risk of alopecia areata compared with that in the general population (van der Steen et al., 1992b). The occurrence of the disease in families, as well as twin studies (Mamelok et al., 1956), suggests that there is an inherited basis to alopecia areata.

1.2.2 Hair loss patterns

Alopecia areata is most often asymptomatic, characterised by the sudden loss of hair at defined areas of the body without atrophy. It can occur at any hairy area, but the scalp is the most common site accounting for approximately 90% of cases seen in the dermatology clinic

(Guzman-Sanchez et al., 2007). The hair loss is usually visible as a round or oval patch with characteristic 'exclamation point' hairs, short hairs tapering towards their base (Figure 1.3) (Alkhalifah et al., 2010a). Re-growing depigmented hairs may also be seen at the edge of the alopecia lesion (Figure 1.3) (Alkhalifah et al., 2010a).

Clinically, alopecia areata can be classified into three common types (Alkhalifah et al., 2010a). Patchy alopecia areata (Figure 1.4a), in which there is a single or multiple patches of hair loss, accounts for about 90% of alopecia areata cases. The skin at the site of the lesion looks normal with no scales, redness, induration or hardness. Alopecia totalis (Figure 1.4b) is characterised by the loss of all scalp hair, while alopecia universalis describes the total loss of body hair including from the scalp. Five percent of alopecia areata patients progress to alopecia totalis or alopecia universalis. Again, the skin at the lesion site usually looks normal in both these clinical types.

Other less common presentations of hair loss can be observed. For example, ophiasis (Alkhalifah et al., 2010a) is a well-defined pattern of alopecia areata when the hair loss is localised to the lower back and sides of the head (Figure 1.4c). Ophiasis inversus (Alkhalifah et al., 2010a) is a rare form characterised by hair loss in the frontal parieto-temporal region of the scalp in a band-like distribution (Figure 1.4d). Sisaipho is another rare form of alopecia areata with hair loss over the scalp but not around the periphery (Pratt et al., 2017).

1.2.3 Associated nail abnormalities

Abnormalities may occur in one or all of the nails of patients with alopecia areata (Figure 1.5) with reported changes ranging from 10% to 39% (Sharma et al., 1996a, Tan et al., 2002b, Goh et al., 2006). Nail pitting with longitudinal rows is the commonest change (Kasumagic-Halilovic and Prohic, 2009), and nail abnormalities are strongly associated with the severity and extent of hair loss (Sharma et al., 1998, Tan et al., 2002b, Goh et al., 2006, Kasumagic-Halilovic and Prohic, 2009)

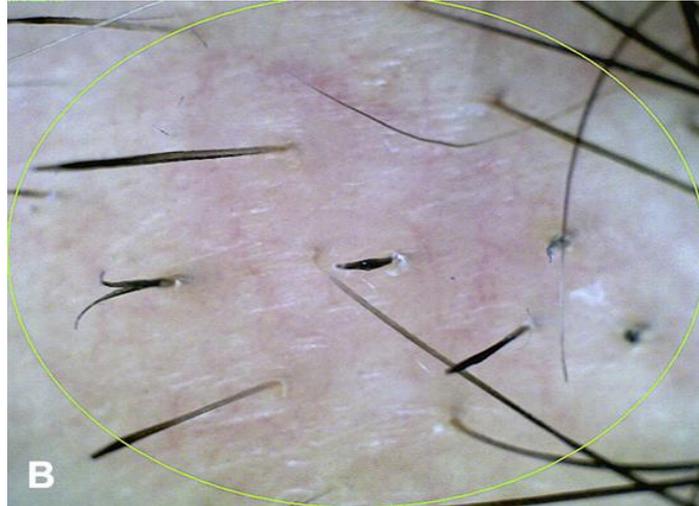


Figure 1.3: Image of an alopecia areata lesion.

The image shows 'exclamation mark' hairs and re-growing depigmented hairs in a hair loss lesion of the scalp of an alopecia areata patient. The image, taken from a paper by Alkhalifah et al, 2010, is used with kind permission from Elsevier.

(A)



(B)



(C)



(D)



Figure 1.4: Clinical patterns of alopecia areata.

(A) Patchy alopecia areata, (B) alopecia totalis, (C) ophiasis and, (D) ophiasis inversus. The images, taken from papers by Madani and Shapiro, 2000, and by Alkhalifah et al, 2010, are used with kind permission from Elsevier.



Figure 1.5: Nail abnormality occurring in a patient with alopecia areata.

The image shows red spotted lunula and proximal trachyonychia of the nails. The image, taken from a paper by Madani and Shapiro, 2000, is used with kind permission from Elsevier.

1.2.4 Associated autoimmune/autoinflammatory diseases

The association of alopecia areata with other autoimmune and autoinflammatory disorders has been reported widely (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Lyakhovitsky et al., 2015). In particular, there are frequent observations of thyroid autoimmunity (hypothyroidism, Graves' disease) occurring in alopecia areata patients, with 2.3% to 14.6% affected depending on the population under study (Table 1.1) (Kasumagic-Halilovic, 2008, Baars et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015). In addition, alopecia areata patients are significantly more likely to have a history of thyroid disease within the family (Kakourou et al., 2007). Other concurrent autoimmune diseases associated with alopecia areata include systemic lupus erythematosus, psoriasis, vitiligo, inflammatory bowel disease, and rheumatoid arthritis (Table 1.1) (Chu et al., 2011, Villasante Fricke and Miteva, 2015)

1.2.5 Associated atopy

There is a high prevalence of atopy amongst patients with alopecia areata, reportedly between 39% and 61% (Barahmani et al., 2009, Tan et al., 2002b). Many studies have provided evidence that a history of atopic diseases, such as atopic dermatitis, asthma, and hay fever, is a risk factor for alopecia areata (Acikgoz et al., 2014). For example, patients with a history of atopic dermatitis specifically have a 70% increased risk of developing alopecia areata (Barahmani et al., 2009).

Table 1.1: Prevalence of autoimmune/autoinflammatory diseases in patients with alopecia areata

| Autoimmune/autoinflammatory disease | Incidence in alopecia areata patients | Reference |
|--|--|---|
| Autoimmune thyroid disease | 2.3–14.6% | (Muller and Winkelmann, 1963, Tan et al., 2002b, Kasumagic-Halilovic, 2008, Chu et al., 2011, Huang et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015, Han et al., 2018, Lee et al., 2019b) |
| Vitiligo | 1.8–7.0% | (Muller and Winkelmann, 1963, Sharma et al., 1996a, Tan et al., 2002b, Chu et al., 2011, Huang et al., 2013) |
| Psoriasis | 1.9–6.3% | (Chu et al., 2011, Huang et al., 2013) |
| Rheumatoid arthritis | 0.9–3.9% | (Chu et al., 2011, Huang et al., 2013) |
| Systemic lupus erythematosus | 1.5% | (Chu et al., 2011) |
| Type 1 diabetes | 0.4–11.1% | (Sharma et al., 1996b, Huang et al., 2013) |
| Inflammatory bowel disease | 2.0% | (Huang et al., 2013) |

1.2.6 Associated psychological conditions

Alopecia areata can cause sufferers a great deal of psychological distress due to the aesthetic repercussions of their hair loss. Reports suggest that the frequency of psychological disorders such as depression, anxiety and neuroticism is significantly greater in alopecia areata patients than in healthy individuals (Aghaei and Saki, 2014). For example, patients with alopecia areata have a high risk of developing major depression with up to 34% of patients affected (Vallerand et al., 2019). Furthermore, the disease has a negative impact on the health-related quality of life of patients with their vitality (energy levels and tiredness) and general mental health scores lower than in healthy people (Gulec et al., 2004, Dubois et al., 2010).

1.2.7 Histopathology

The diagnostic histopathological feature of alopecia areata is an intrafollicular and perifollicular infiltration of T lymphocytes, Langerhans cells and macrophages (Perret et al., 1984, Todes-Taylor et al., 1984), referred to as a 'swarm of bees' (Figure 1.6). Within the lesional skin, there is expression of the inflammatory markers intracellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC) class I and II antigens (Brocker et al., 1987, McDonagh et al., 1993, Ghersetich et al., 1996), indicating the presence of activated immune cells. In addition to lymphocytic cells, mast cells and eosinophils can also be found in acute and chronic alopecia areata lesions (Anzai et al., 2019).

Since the infiltration affects anagen hair follicles or follicles in the early catagen phase of the hair cycle, the number of anagen hair follicles in alopecia lesions is reduced (Whiting, 2003). In the chronically affected scalp, the hair follicles become miniaturised and so-called nanogen follicles are very characteristic of alopecia areata. The histopathological changes during alopecia areata progression are summarised in Table 1.2.

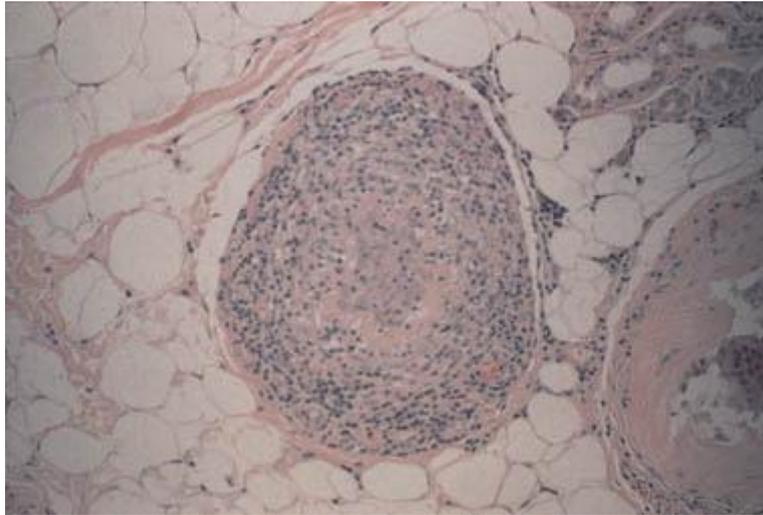


Figure 1.6: Image of lymphocytic infiltrate in an alopecia areata lesion.

The lymphocytic infiltrate is seen around an anagen hair follicle and resembles a 'swarm of bees'. Hematoxylin and eosin stain, 200x magnification. The image, taken from a paper by Madani and Shapiro, 2000, is used with kind permission from Elsevier.

Table 1.2: Histopathological characteristics of alopecia areata

| Acute and subacute alopecia areata¹ | Chronic alopecia areata¹ | Recovery stage¹ |
|---|--|--|
| Peribulbar lymphocytic infiltration of CD4+ and CD8+ T cells in a “swarm of bees” pattern around anagen hair follicles. | Inflammatory cells may or may not be seen. | Few inflammatory cells. |
| Large increase in hair follicles in catagen and telogen phases is seen. | Increase in the number of catagen or telogen hairs, and a prolonged telogen phase without an apparent attempt to return to an anagen growth phase. | Increased anagen (actively growing) hair follicle count. |
| Normal number of hair follicles, but small size without terminal hair fibres. | The hair follicles become miniaturised as so-called nanogen follicles. | Miniaturised hairs grow back. |
| | Terminal-villous ratio is 1:1. | Terminal-villous ratio returned to normal at 1:7. |
| Oedema, pigmentary incontinence, microvesiculation, and macrophage cells. | | |

¹Information taken from (Whiting, 2003, Pratt et al., 2017, Darwin et al., 2018).

1.3 Alopecia areata: diagnosis and treatment

1.3.1 Diagnosis and prognosis

Before starting treatment for alopecia areata, a full assessment of the patient should be performed to exclude other differential diagnosis that may mimic alopecia areata such as tinea capitis, trichotillomania, scarring alopecias, telogen effluvium (Spano and Donovan, 2015a), and other forms of patchy and diffuse hair loss (Table 1.3).

Spontaneous remission of alopecia areata occurs in up to 80% of cases who have a limited, patchy hair loss of not more than a year (Messenger et al., 2012b). It is therefore often appropriate to leave the disease untreated and manage the patients with reassurance that the hair growth will resume in the mid to longer term (Messenger et al., 2012b). However, data suggest that 7-25% of patients and up to 43% of children with alopecia areata progress to alopecia totalis or alopecia universalis and, for these patients, there is a very limited likelihood of disease remission (Safavi et al., 1995), so treatment may be recommended.

Several treatments have been shown to induce hair regrowth in alopecia areata, but these do not have any effect upon the course of the disease in the long-term (Alkhalifah et al., 2010a, Messenger et al., 2012a). Overall, five important factors have been found to influence the prognosis of alopecia areata. These are (a) the type of alopecia, (b) the presence of nail changes, (c) the age at onset, (d) the duration of alopecia areata before treatment, and (e) an association with atopic eczema (Weise et al., 1996).

1.3.2 General management

As the quality of life in patients with alopecia areata is significantly impaired, and anxiety and depression are common findings, patients may benefit from psychological therapy and support. Wearing a hat or sunscreen for exposed scalp skin is recommended to protect from sun damage (Harries et al., 2010). Wigs and interventions such as eyebrow tattooing have given good cosmetic results for patients with alopecia totalis and alopecia universalis (Harries et al., 2010).

Table 1.3: Differential diagnosis of alopecia areata

| Type of alopecia | Clinical features | Laboratory investigations | Reference |
|--|--|--|--|
| Tinea capitis | Children. Scaly patches of hair loss. | Dermoscopy shows comma and corkscrew hairs, zigzag hair, broken hair, and black dots. Positive KOH test. | (Bourezane and Bourezane, 2017, Aqil et al., 2018) |
| Trichotillomania | Broken hairs of different lengths. | Histological examination shows an increased numbers of catagen and telogen hairs without evidence of inflammation. | (Zimova and Zimova, 2016) |
| Scarring alopecias: Folliculitis decalvans; chronic discoid lupus erythematosus; lichen planus follicularis; Brocq's pseudopelade; secondary syphilis | Permanent hair loss. Hair follicle openings are not visible. | Scalp biopsy for histopathology. Serology for syphilis. | (Alkhalifah et al., 2010a) |
| Androgenetic alopecia | Hereditary balding. Positive family history. | Scalp biopsy for histopathology | (Vidal, 2015) |
| Telogen effluvium | History of stress typically occurs 3–4 months prior. | Scalp biopsy for histopathology. | (Vidal, 2015) |
| Marie Antoinette syndrome | Very sudden (overnight) greying followed by alopecia. If associated with vitiligo, it is called canities subita. | | (Navarini et al., 2009, Pratt et al., 2017) |

1.3.3 Topical corticosteroids

Corticosteroids work by decreasing inflammation and reducing the activity of the immune system and so hastening recovery of damaged hair follicles (Charuwichitratana et al., 2000, Pratt et al., 2017). Many clinical studies have been undertaken to evaluate the efficacy of topical corticosteroids with varying results ranging from approximately 25-57% of patients having complete regrowth of hair during the course of treatment (Charuwichitratana et al., 2000).

Tosti and colleagues reported that treatment with 0.05% clobetasol propionate resulted in 8/28 (28.5%) patients with alopecia totalis or alopecia universalis having almost complete hair regrowth. Five of these eight patients had long-term benefits, but three had relapse after cessation of treatment (Tosti et al., 2003). A randomised, double-blind, placebo-controlled trial also using 0.05% clobetasol propionate resulted in clinically significant hair regrowth in up to 25% of patients (Tosti et al., 2006). A similar trial used desoximetasone as a 0.25% cream. The response rates in patients in the treatment and control groups were 57.6% and 39.2%, respectively, a result that was not statistically different (Charuwichitratana et al., 2000). Pascher and co-workers carried out a double-blind, half-head, placebo-controlled study using 0.2% fluocinolone acetonide cream twice daily under occlusion. Hair regrowth on the treated side of the scalp was seen in 54% of patients. No patients had hair regrowth on the vehicle arm (Pascher et al., 1970). In combination with fractional CO₂ laser, topical triamcinolone proved an effective treatment option in resistant alopecia areata; 7/8 (87%) patients, who had not responded to other treatment modalities, gained complete recovery (Majid et al., 2018).

Overall, topical steroids are relatively safe, easy to apply, and quite effective, but have the disadvantages of skin atrophy with long-term use (Pratt et al., 2017), and frequent relapse of between 33% and 75% when treatment is stopped (Kurosawa et al., 2006).

1.3.4 Intra-lesional corticosteroids

The national guidelines from the British Association of Dermatologists recommend intra-lesional corticosteroids as the first-line therapy for localised patchy alopecia areata (MacDonald Hull et al., 2003). Prospective studies have shown that the use of intra-lesional

injections of corticosteroid, in the form of triamcinolone acetonide, stimulate localised hair regrowth at 60-67% of the injection sites (Abell and Munro, 1973, Chang et al., 2009, Kuldeep et al., 2011, Devi et al., 2015). Lower concentrations of triamcinolone acetonide (2.5 mg/ml) have been shown to be as effective as higher concentrations (10 mg/ml), with none of local side-effects such as skin atrophy and telangiectasia that occur with the increased dose (Chu et al., 2015). The side-effects of intra-lesional corticosteroids include pain, skin depigmentation, and localised atrophy, and treatment should be discontinued if there is no improvement after six months (Shapiro, 2013).

1.3.5 Systemic corticosteroids

Systemic corticosteroids have been used for many years to treat patients with extensive alopecia areata with varying results (Shapiro, 2013). In one study, Thi and co-workers evaluated the effectiveness of oral mini-pulse methylprednisolone where patients received 16 mg of methylprednisolone twice weekly (Thi et al., 2019). After six months of treatment, up to 82.2% of patients had recovered well from alopecia areata. The recurrence rate was 2.2%. In contrast, in a study of 32 alopecia areata patients, the response rate to 300-1000 mg of prednisolone daily was much lower at 53% with a high relapse rate of 100% (Sharma, 1996). A study of 30 patients with diffuse alopecia areata treated with 5 mg of dexamethasone twice weekly showed 75-95% hair regrowth in 19 (63.3%) patients with relapse occurring in only one patient (Sharma and Gupta, 1999).

The disadvantages of systemic steroids include a high relapse rate of 14-100% after stopping treatment, and side-effects that include osteoporosis, hyperglycaemia, cataracts, immunosuppression, mood changes, obesity, dysmenorrhea, steroid acne, and Cushing's syndrome (Shapiro, 2013).

1.3.6 Minoxidil

Minoxidil (2, 4-diamino-6-piperidinopyrimidine-3-oxide) was introduced in the early 1970s as a potent vasodilator to treat resistant hypertension. Hypertrichosis was a common side-effect in individuals taking minoxidil tablets, and this observation led to the development of a non-toxic and easy to use topical formulation of minoxidil for the treatment of hair-loss

including alopecia areata (Messenger and Rundegren, 2004). The mechanism by which minoxidil stimulates hair growth is not fully understood, but it may reverse hair loss by stimulating anagen growth of the hair follicle, prolonging the anagen phase, or stimulating anagen recovery from the telogen phase (Suchonwanit et al., 2019).

The evidence for the effectiveness of topical minoxidil is varied. Some half-head studies have failed to report significant treatment effects in alopecia totalis and alopecia universalis (Harries et al., 2010). However, in a double-blind, placebo-controlled trial using 3% minoxidil, hair regrowth was seen in 64% of patients with extensive alopecia areata, compared with 36% in the placebo arm (Price, 1987). In addition, a dose-response efficacy was observed in a study comparing 1% and 5% topical minoxidil in the treatment of patients with extensive alopecia areata. The response rates were 38% in the group treated with 1% minoxidil, and 81% in the group treated with 5% (Fiedler-Weiss, 1987). Furthermore, a double-blind, placebo-controlled study assessing the effectiveness of 1% minoxidil showed new hair growth in 21/26 (81%) patients, compared to no response in the control group (Fenton and Wilkinson, 1983).

Currently, minoxidil is frequently used as a second-line therapy or in combination with other treatments, mainly topical or intra-lesional corticosteroids (Shapiro, 2013). Hypertrichosis and contact dermatitis are the major common adverse effects, which can be minimised by using minoxidil foam that does not contain propylene glycol (Suchonwanit et al., 2019). A recent study showed successful treatment of alopecia areata with minoxidil in eight Chinese patients including those with alopecia totalis and alopecia universalis (Wang et al., 2019). Here, topical minoxidil was combined with non-ablative laser without the occurrence of systemic or local side-effects.

1.3.7 Anthralin

Anthralin is a synthetic tar-like compound that has been used in the treatment of psoriasis and alopecia areata. The mechanism of action is not completely understood. Some studies have demonstrated that anthralin inhibits monocyte secretion of interleukin (IL)-6, IL-8, and tumour-necrosis factor (TNF)- α , decreases expression of epidermal growth factor receptors, and also activates NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) in an *in vitro* murine keratinocyte model (Waller and Sampson, 2018).

Several studies have been carried out to assess the efficacy of anthralin in treating alopecia areata. A prospective randomised control trial showed that 1% anthralin was an effective therapy for alopecia areata as 70% of paediatric-age patients with severe hair loss showed a partial or complete response at 12 months (Ozdemir and Balevi, 2017). Another retrospective study using topical anthralin and 37 patients indicated that 68% reached at least 50% maximal scalp hair regrowth and 32% reached complete scalp hair regrowth (Wu et al., 2018). Another retrospective study of paediatric alopecia areata patients treated with anthralin at 0.5% or 1% revealed 50% hair regrowth within six months (Tan et al., 2002a). Furthermore, a cosmetic response was seen in 17/68 (25%) patients with severe alopecia areata treated with 0.5%–1.0% anthralin cream (Fiedler-Weiss and Buys, 1987). The treatment can be administered at home using a 1-2% anthralin formulation applied weekly for 10-15 minutes before being washed off with shampoo (Spano and Donovan, 2015b). However, the side-effects of anthralin, which are dose-related, are skin irritation, and the staining of lesional and adjoining skin, hair, nails, and clothing (Spano and Donovan, 2015b).

1.3.8 Cyclosporine

Cyclosporine is a calcineurin inhibitor that decreases the production of inflammatory cytokines by T lymphocytes. Several studies in the past 20 years have been conducted to assess the efficacy of oral cyclosporine as a treatment for alopecia areata either as a single therapy or combined with other drugs (Acikgoz et al., 2014). Gupta and colleagues were the first team to report the treatment of alopecia areata with oral cyclosporine (Gupta et al., 1990). After 12 weeks of therapy, 3/6 (50%) patients showed cosmetically acceptable hair growth. However, all patients suffered relapse after cessation of treatment. Unsatisfactory results were also obtained in a double-blinded, randomised, placebo-controlled trial that included 32 alopecia areata patients (Yun Lai et al., 2019); there was no significant difference in the response rate of the treatment and placebo groups. Overall, cyclosporine is not a widely used therapeutic for alopecia areata.

1.3.9 Methotrexate

Methotrexate is a cytotoxic folic acid antagonist found to be effective in the treatment of psoriasis, pemphigoid, pemphigus, and alopecia areata (Yin et al., 2017). In severe cases of

alopecia areata, methotrexate is considered an effective treatment as either a monotherapy or an adjunct therapy in combination with corticosteroids (Phan et al., 2019). Examples of the use of methotrexate in the treatment of alopecia areata are summarised in Table 1.4. However, serious side-effects that limit the use of methotrexate include infections such as septic arthritis and pneumonia, as well as toxicity towards the liver, and the gastrointestinal and respiratory systems (Salliot and van der Heijde, 2009).

Table 1.4: Methotrexate treatment of alopecia areata

| Number of patients | Study design | Outcomes | Reference |
|---|------------------------------------|---|---------------------------------------|
| 14 paediatric | Retrospective non-controlled study | Methotrexate combined with steroids. Duration of treatment was five months. 8/14 (57%) patients experienced good regrowth. | (Landis and Pichardo-Geisinger, 2018) |
| 29 adults | Retrospective non-controlled study | Methotrexate monotherapy. Duration of treatment was range 12-74 weeks. Regrowth was achieved in 26/29 (90%), with 14 patients experiencing total regrowth. | (Lim et al., 2017) |
| 14 paediatric with alopecia universalis or alopecia totalis | Retrospective non-controlled study | Methotrexate and pulsed methylprednisolone. Duration of treatment was 1-3 years. 6/14 (43%) patients reported as good responders. | (Chong et al., 2017) |
| 26 alopecia universalis and 17 alopecia totalis | Retrospective study | Treatment with methotrexate and prednisolone. Duration of treatment was three months. 15/26 (58%) patients had total hair regrowth. 11/15 (73%) of the patients with total hair regrowth experienced a later disease relapse. | (Anuset et al., 2016) |
| 3 paediatric | Case report | Methotrexate monotherapy. Duration of treatment was for four months for one case and 25 months for a second case. Successful outcome reported in these two cases. Third case after 14 months treatment no hair regrowth. | (Batalla et al., 2016) |

1.3.10 Photochemotherapy

Photochemotherapy in the form of psoralen/ultraviolet A (PUVA) has been used in the treatment of alopecia areata (Broniarczyk-Dyla et al., 2006). The mechanism of PUVA treatment is thought to be immunosuppressive. For example, UVA light stimulates the synthesis of prostaglandins and cytokines that play important roles in reducing the number of cutaneous T lymphocytes, Langerhans cells, and mast cells in the dermis (Bulat et al., 2011). In addition, UVA decreases ICAM-1 expression (Norris et al., 1990). Photosensitisation of psoralen has been reported to reduce the expression of cytokines and cytokine receptors (Bulat et al., 2011).

In one study, 75% of patients had hair regrowth after PUVA therapy that involved topical application of psoralen solution (Behrens-Williams et al., 2001). A later study showed 94/124 (76%) patients with patchy alopecia areata and 12/25 (48%) with alopecia totalis and alopecia universalis had complete or more than 50% hair regrowth after treatment with photochemotherapy that combined topical 8-methoxypsoralen with UVA irradiation (Mohamed et al., 2005). Majumdar and colleagues also reported PUVA to be an effective and safe treatment option for alopecia areata (Majumdar et al., 2018). Of 13 patients with at least 70% of scalp hair loss, four (26%) reported a good response, four (26%) a moderate response, three (20%) a mild response and two (13%) had no response. In some cases, PUVA was used to treat patients who had received previous therapies that were unsuccessful. For example, although treatment of alopecia totalis with minoxidil and systemic betamethasone failed, subsequent PUVA resulted in almost complete regrowth of scalp hair (Sornakumar et al., 2010).

1.3.11 Immunotherapy

Topical immunotherapy of alopecia areata has involved the use of three different agents used 2,4-dinitrochlorobenzene (DNCB), squaric acid dibutylester (SADBE), and diphenylcyclopropenone (DPCP), also known as diphenylprone (Zerbinati et al., 2018). However, DNCB is not generally used today because it has mutagenic and genotoxic effects (Nowicka et al., 2018). The mechanism of action topical immunotherapy sensitizers is still unclear, but several theories have been suggested. These include (a) a decrease in the CD4+

to CD8+ lymphocyte ratio from 4:1 to 1:1 (Buckley and Du Vivier, 2001); (b) a decrease in intrabulbar T lymphocytes and Langerhans cells (Singh and Lavanya, 2010); (c) a repeated allergic reaction on the affected area generates suppressor T cells that non-specifically inhibit the autoimmune reaction against the hair follicles which known “antigenic competition” (Singh and Lavanya, 2010); and (d) after repeated applications of immunotherapy, the expression of class I and II major histocompatibility complex molecules that are normally present in areas affected by alopecia areata disappear (Rokhsar et al., 1998).

One of the largest clinical retrospective self-controlled studies with the DPCP was carried out by on 142 patients with alopecia areata (Ohlmeier et al., 2012). The study showed an overall response rate of 71.9%. A complete response (hair regrowth of 90%) was achieved in 37.8% of patients, 14.8% achieved a partial response (hair regrowth of 50-90% re-growth), and 19.3% achieved a minimal response (hair regrowth of 10-50%). Other studies on the immunotherapy of alopecia areata are summarised in Table 1.5. They show variable results. The response rate using either treatment ranges from 9-87% in all types of alopecia areata including alopecia universalis. Both immunotherapies can be safely used in patients of paediatric age. However, DPCP should not be used in pregnancy or in female patients of child-bearing age (Berth-Jones et al., 1994). A longer time interval between treatment sessions was shown to give the same or a higher response rate in studies where this parameter was evaluated (Nowicka et al., 2018). The major side-effects of DPCP and SADBE are severe dermatitis, vitiligo lesions at site of application, erythema multiforme-like reaction, and regional lymphadenopathy (Berth-Jones et al., 1994, Nowicka et al., 2018).

Table 1.5: Studies using immunotherapy for treatment of alopecia areata

| Immunotherapy¹ | Number of patients | Patient age (years) | Alopecia type² | Response rate | Reference |
|----------------------------------|---------------------------|----------------------------|----------------------------------|-------------------------------------|--------------------------------|
| DPCP | 5 | | AA | 60% | (Shapiro, 1993) |
| DPCP | 12 | 5-12 | AA, 4; AT, 8 | 66% all patients; AA, 75%; AT, 62% | (Hull et al., 1991b) |
| DPCP | 78 | | AA, 33; AT 45 | 63% all patients; AA, 78% ; AT, 51% | (Hull and Cunliffe, 1991) |
| DPCP | 35 | 1-14 | AA | 77% | (Tosti et al., 1986) |
| DPCP | 27 | 15-57 | AA, 5; AT, 22 | 85% | (Happle et al., 1983) |
| DPCP | 13 | 4-15 | AA, 10; AT, 16 | 38% | (Shapiro et al., 1995) |
| DPCP | 12 | 5-12 | AA, 4; AT, 8 | 9 % | (Berth-Jones et al., 1994) |
| DPCP | 17 | 5-72 | AT, 8; AU, 9 | 12% | (Ashworth et al., 1989) |
| DPCP | 28 | 18-56 | AA | 30% | (Hull and Norris, 1988) |
| DPCP | 26 | 10-55 | AA, 9; AT, 7; AU, 10 | 15% | (Orecchia and Rabbiosi, 1985) |
| DPCP | 56 | | AA | 51.1% | (Firooz et al., 2005) |
| DPCP | 68 | | AA (> 40% scalp hair loss) | 70.6% | (Pericin and Trueb, 1998) |
| DPCP | 21 | | AA extensive | 71.4% | (Galadari et al., 2003) |
| SADBE | 144 | 5-50 | AA, 129; AT, 13; AU, 2 | 65% | (Micali et al., 1996) |
| SADBE | 19 | 14-54 | AA, 6; AT, 7 AU, 6 | 63% | (Johansson et al., 1986) |
| SADBE | 44 | 14-30 | AA | 64% | (Tosti et al., 1986) |
| SADBE | 38 | 5-61 | AA, 18; AT, 10 AU, 10 | 74% | (Valsecchi et al., 1985) |
| SADBE | 50 | 6-57 | AA, 26; AT, 27 | 87% | (Happle et al., 1980) |
| SADBE | 28 | 6-14 | AA | 32% | (Orecchia et al., 1994) |
| SADBE | 14 | 7-66 | AA, 2; AT, 5; AU, 7 | 29% | (Caserio, 1987) |
| SADBE | 21 | 21-53 | AA, 11; AT, 10 | 38% | (Case et al., 1984) |
| SADBE | 26 | 6-40 | AA, 20; AT, 3; AU, 3 | 46% | (Giannetti and Orecchia, 1983) |

¹DPCP, diphenylcyclopropanone; SADBE, squaric acid dibutylester.

²AA, alopecia areata; AT, alopecia totalis; AU, alopecia universalis.

1.3.12 Janus kinase inhibitors

Janus kinase/signal transducers and activators of transcription (JAK/STATs) are a group of molecules associated with one of the major pathways through which many cytokines exert their function, and as such they are increasingly recognised as playing critical roles in the pathogenesis of various immune-mediated diseases such as rheumatoid arthritis, psoriasis and alopecia areata (Xing et al., 2014, Fragoulis et al., 2019). JAK-based treatments function by inhibiting the activity of one or more of the JAK family of enzymes (JAK1, JAK2, JAK3, TYK2), thereby interfering with the JAK-STAT signalling pathway. JAK inhibitors can be administered orally or used topically, and the first generation of JAK inhibitors tofacitinib and ruxolitinib are the major ones used in dermatology (Damsky and King, 2017).

Studies concerning JAK inhibitor therapy in relation to alopecia areata are summarised in Table 1.6. Some studies showed significant success. For example, 9/13 (70%) adolescent patients aged 12–17 years with alopecia areata experienced clinically significant hair regrowth after six months of treatment with tofacitinib, although there were mild side-effects of (Craiglow et al., 2017). A retrospective study conducted by Lui and colleagues showed that of 90 patients treated by tofacitinib, 77% achieved a clinical response (Liu et al., 2017). More recently, the efficacy, side-effects, and durability of oral tofacitinib and ruxolitinib were analysed in the treatment of two groups of patients with severe (more than 30% scalp involvement) alopecia areata. Thirty-seven were treated with tofacitinib and 38 with ruxolitinib. The results indicated that there was no significant differences between the groups regarding hair regrowth, and concluded that both tofacitinib and ruxolitinib are effective with no reported serious adverse effects (Almutairi et al., 2019).

Table 1.6: Janus kinase inhibitors used in the treatment of alopecia areata

| JAK inhibitor | Number of patients | Study design | Outcome | Reference |
|---------------------------------------|---------------------------|-------------------------------------|---|--------------------------------|
| Oral tofacitinib, 5 mg twice daily | 37 | Open-label | Remarkable hair regrowth | (Almutairi et al., 2019) |
| Oral tofacitinib, 10, 15, 20 mg daily | 12 | Open-label, single-arm trial | Eight patients showed 50% regrowth from baseline. | (Jabbari et al., 2018) |
| Topical 2% tofacitinib | 10 | Open-label, pilot, single-arm trial | Seven patients showed no regrowth; only one patient had significant regrowth. | (Liu et al., 2018) |
| Oral tofacitinib | 32 | Retrospective study | Nine patients had 50%–90% regrowth; six patients had 5%–50% regrowth. | (Park et al., 2017) |
| Oral tofacitinib, 10, 15 mg daily | 13 | Retrospective study | Nine patients (70%) experienced significant hair regrowth. | (Craiglow et al., 2017) |
| Oral tofacitinib, 10, 15, daily | 13 | Retrospective study | Seven patients (53%) achieved regrowth. | (Ibrahim et al., 2017) |
| Oral tofacitinib, 5 mg +/- steroid | 90 | Retrospective study | 20% (13) of patients were complete responders (>90%); 38.4% (25) were intermediate responders (51%–90%); 18.5% (12) were moderate responders; 23.1% (15) had no response. | (Liu et al., 2017) |
| Oral tofacitinib, 5 mg twice daily | 66 | Open-label, single-arm trial | 64% of patients responded to treatment ≥50%; alopecia areata and ophiasis subtypes were more responsive than totalis and universalis subtypes. | (Kennedy Crispin et al., 2016) |
| Oral tofacitinib, 5 mg twice daily | 2 | Case series | Hair growth in both patients was observed in one and three months; both patients had full regrowth in eight months. | (Gupta et al., 2016) |
| Oral ruxolitinib, 20 mg twice daily | 38 | Open-label | Remarkable hair regrowth. | (Almutairi et al., 2019) |
| Oral ruxolitinib, 20 mg twice daily | 1 | Case report | Patient had 50% regrowth of scalp hair and full regrowth of beard. | (Ramot and Zlotogorski, 2018) |
| Oral ruxolitinib, 20 mg twice daily | 12 | Open-label, single-arm trial | 9/12 patients (75%) showed significant scalp hair regrowth. | (Alvarez Otero et al., 2017) |
| Topical 0.6% ruxolitinib | 1 | Case series | No improvement. | (Deeb and Beach, 2017) |
| Topical 0.6% ruxolitinib | 1 | Case series | Growth of about 10% of scalp hair; eyebrows were nearly normal. | (Craiglow et al., 2016) |

1.3.13 Platelet-rich plasma

Platelet-rich plasma (PRP) is defined as the plasma fraction of blood with an above baseline platelet concentration. It contains more than 20 growth factors including fibroblast growth factor, insulin-like growth factors 1 and 2, platelet-derived growth factor, TGF- β , epidermal growth factor, IL-8, and keratinocyte growth factor (Weibrich et al., 2002, Trink et al., 2013, Trueb and Dias, 2018). These growth factors act directly in the bulge area of the follicle and activate the proliferative phase and inhibit apoptosis of the hair, giving rise to the future follicular unit. They also have direct activity in control of immune privilege, in particular insulin-like growth factor 1 (Uebel et al., 2006, Pai et al., 2017).

Platelet-rich plasma has been evaluated as a new treatment modality in medicine for its role in wound healing, orthopaedics, dermatology and dentistry (Rogers, 2012). Recently, clinical improvement of alopecia areata after three months of therapy in a randomised double-blinded, placebo and active-controlled study has been reported (Rinaldi et al., 2019). Another study compared the efficacy of PRP versus 5% topical minoxidil in the treatment of alopecia areata (El Taieb et al., 2017). Ninety patients were divided into three groups; the first was treated with PRP injections, the second was treated with 5% topical minoxidil, and the third with placebo. The study concluded that PRP was more effective in the treatment of alopecia areata than topical minoxidil (El Taieb et al., 2017). A half-head study was carried out by Trink and colleagues to investigate the effects of PRP on alopecia areata (Trink et al., 2013). They reported that PRP increased hair regrowth significantly and decreased hair dystrophy compared with intra-lesional triamcinolone acetonide or placebo. Successful treatment of corticosteroid-resistant ophiasis-type alopecia areata with PRP has also been documented (Donovan, 2015). However, it is still a highly controversial form of therapy and very few studies have evaluated the effects of PRP on hair growth in patients with alopecia areata. So, the effectiveness of PRP warrants further large, randomised blind controlled trials.

1.3.14 Other treatments

Other therapies that have been tried in the treatment of alopecia areata with variable degrees of success and include oral zinc supplementation (Park et al., 2009), a combination of

simvastatin and ezetimibe (Ali and Martin, 2010), topical onion juice (Sharquie and Al-Obaidi, 2002), anti-histamines (Inui et al., 2007), and topical azelic acid (Sasmaz and Arican, 2005).

1.4 Alopecia areata: aetiology and pathogenesis

The aetiology and pathogenesis of alopecia areata are still to be fully resolved, but there has been much research to uncover the details of the cause of the disease. The following sections will consider the factors that may trigger alopecia areata, the variants of specific genes that may render an individual susceptible to developing the disease, and the pathogenic mechanisms that are considered to be involved in hair follicle destruction.

1.4.1 Environmental trigger factors for alopecia areata

In most cases of alopecia areata, no obvious reason for the onset of the disease can be found. However, several trigger factors have been suggested, although these may differ from patient to patient.

1.4.1.1 Psychological stress

A stressful life event has been implicated in triggering the onset of alopecia areata in some studies (Koo et al., 1994, Kakourou et al., 2007, Mulinari-Brenner, 2018). In addition, various psychiatric disorders like neurotic personality, depression, and anxiety have been reported to be associated with alopecia areata (Gupta et al., 1997, Picardi et al., 2003, Chu et al., 2012, Huang et al., 2013, Kutty-Pachecka, 2015, Mulinari-Brenner, 2018). In an early study of patients with alopecia areata, 23% reported that their hair loss had been preceded by acute anxiety or mental shock (Anderson, 1950). Reinhold and co-workers psychologically assessed 52 patients with alopecia areata, and reported all patients were found to have either very stressful life conditions or moderately severe neurotic symptoms, or both, before the onset of the alopecia areata (Reinhold, 1960). More recently, post-traumatic stress disorder in two adolescent boys was reported to be related to their development of alopecia areata (Kara and Topkarci, 2018). However, the connection of emotional stress and hair loss onset has not always been corroborated (van der Steen et al., 1992a, Brajac et al., 2003, Gulec et al., 2004). For example, an investigation of 125 alopecia areata patients documented mental trauma or stress as the cause of hair loss in only 4.8% of cases (Macalpine, 1958).

As well as patient observations, there is biological evidence to indicate a role for the central nervous system and acute stress in the pathology of alopecia areata (Paus, 2016). For example, components of the stress response are upregulated in the skin of patients with alopecia areata including corticotropin-releasing hormone (CRH), CRH receptors, adrenocorticotrophic hormone (ACTH), and cortisol, which may result in localised inflammation (Katsarou-Katsari et al., 2001, Ito et al., 2005b, Kim et al., 2006).

Substance P is a neuropeptide involved in stress-induced neurogenic skin inflammation, which has been observed at increased levels in scalp biopsies from patients with alopecia areata compared with healthy controls (Toyoda et al., 2001). The neuropeptide promotes the collapse of hair follicle immune privilege by upregulating the expression of MHC class I antigens as well as premature catagen development by increasing expression of nerve growth factor and its receptor p75NTR (Peters et al., 2007). Both these events are characteristic of alopecia areata.

Furthermore, abnormalities of the nerve supply to affected hair follicles have been reported in alopecia areata patients, and again support a role for the nervous system in development of the condition (Hordinsky and Ericson, 1996, Alexopoulos and Chrousos, 2016). Finally, immune cells, such as macrophages, and T and B lymphocytes, can interact with the cholinergic nervous system (Kawashima et al., 2012, Fujii et al., 2017). During acute stress, hormones such as acetylcholine are upregulated and these can modulate immune cell function and increase secretion of interferon (IFN)- γ , TNF- α , and IL-6, cytokines that play role in the pathogenesis of alopecia areata (Fujii et al., 2017, Simakou et al., 2019).

1.4.1.2 Infections

Viral infections have been considered as a possible trigger of alopecia areata. Twenty-one patient specimens were analysed by Skinner and co-workers, 10 from patients with alopecia areata, six with androgenetic alopecia, and five with trichotillomania (Skinner et al., 1995). Nine of the 10 patients with alopecia areata were positive for cytomegalovirus (CMV) in their scalp biopsies. Patients with other types of alopecia were negative. In addition, five of 24 alopecia areata patients were CMV seropositive (Jackow et al., 1998). However, in contrast, a study of tissue and serum samples from 14 patients with alopecia areata concluded there was

no correlation between CMV and hair loss (Tosti et al., 1996). Furthermore, in skin biopsies taken from the active margins of alopecia areata patches, there was no evidence of the presence of CMV (Offidani et al., 2000).

Other studies have examined alopecia areata patients for Epstein–Barr virus (EBV) and swine flu virus. One study reported that 12 of 1586 patients with alopecia areata developed hair loss within one week to six months following EBV exposure affected (Rodriguez and Duvic, 2008). However, the suggestion that EBV is a causative factor of alopecia areata in some cases needs much further investigation. Swine flu virus was implicated in alopecia areata in 10 newly occurring or recurring cases (Ito and Tokura, 2012). Again, more studies are required to support or exclude swine flu virus as an aetiological factor in alopecia areata.

1.4.1.3 Vaccinations

Although at low frequency, alopecia areata has been reported to occur shortly after vaccinations against several pathogenic organisms such as hepatitis B virus (Wise et al., 1997) Japanese encephalitis virus (Chu et al., 2016), herpes zoster virus (Lai and Yew, 2015), papillomavirus (Geier and Geier, 2015), and *Clostridium tetani* (Sanchez-Ramon et al., 2011). Specific case reports include a three-year-old boy with a history of atopic dermatitis who experienced two episodes of alopecia areata after vaccination against Japanese encephalitis and influenza (Chu et al., 2016). Both episodes developed within few days after vaccination, followed by complete hair regrowth within six months (Chu et al., 2016). Overall, the evidence suggests that there is a rare association between vaccination and alopecia areata and that hair loss after vaccine application might depend upon the genetic susceptibility of the individual (Chu et al., 2016).

1.4.1.4 Drugs and medication

Alopecia areata has been reported to occur as a side-effect of drug treatment. For example, a 33-year-old female with juvenile-onset arthritis and treated with tocilizumab lost her eye lashes, eye brows, and hair after six months of therapy (Kuet and Goodfield, 2014). Tocilizumab treatment blocks the IL-6 receptor leading to an increase in serum levels of IL-6 (Nishimoto et al., 2008), raised concentrations of which have been found in patients with

patchy alopecia areata (Shohat et al., 2005). The mechanism by which excess IL-6 might lead to hair loss remains obscure. There are many other examples of alopecia areata associated with medication use (Kim and Shin, 2014, Iskandarli and Ozturk, 2016, Barroso-Garcia et al., 2018, Mitchell and Levitt, 2018, Flanagan et al., 2019, Kanda et al., 2019), and several are given in Table 1.7.

A correlation between the use of the IL-4 receptor inhibitor dupilumab and the development of alopecia areata has been described in several patients with atopic dermatitis (Barroso-Garcia et al., 2018, Mitchell and Levitt, 2018, Flanagan et al., 2019, Kanda et al., 2019). The biologic inhibits IL-4-induced responses, including the release of proinflammatory cytokines from T helper type 2 (Th2) cells. It has been suggested that because the Th2 cytokine profile is similar between alopecia areata and atopic dermatitis, dupilumab may actually be of clinical utility in treating hair loss (Renert-Yuval and Guttman-Yassky, 2017). However, the involvement of other immune system mediators, such as T helper type 1 (Th1) cells, combined with the downregulation of Th2 pathways, may amplify the Th1 pathway and promote the development of alopecia areata with dupilumab therapy (Renert-Yuval and Guttman-Yassky, 2017).

Table 1.7: Case reports of associations of alopecia areata with medications

| Study | Reference |
|--|-------------------------------|
| A 31-year-old male with a seven-year history of severe atopic dermatitis was treated with dupilumab. After six weeks of treatment, his atopic dermatitis was significantly improved. However, he developed hair loss in patches on his anterior scalp. | (Barroso-Garcia et al., 2018) |
| A 35-year-old male treated with dupilumab for severe atopic dermatitis. After six weeks of treatment he developed patches of hair loss on the right frontal, occipital, and bilateral temporal scalp. | (Kanda et al., 2019) |
| A 27-year-old male with atopic dermatitis developed drug-induced alopecia after 18 weeks of dupilumab treatment. | (Flanagan et al., 2019) |
| A 29-year-old male presented with a three-year history of poorly controlled atopic dermatitis. After six weeks of treatment with dupilumab, his atopic dermatitis improved significantly. However, he developed several patches of hair loss on his posterior scalp after five weeks of treatment. | (Mitchell and Levitt, 2018) |
| A 33-year-old male patient treated with sulfasalazine, due to lumbar pain, developed vitiligo and alopecia areata. | (Iskandarli and Ozturk, 2016) |
| A 59-year-old HIV-positive male developed alopecia areata three months after starting abacavir treatment. | (Kim and Shin, 2014) |

1.4.1.5 Oxidative stress

Oxidative stress is defined as an imbalance between oxidant and antioxidant mechanisms, and results from inadequate antioxidant protection or excess production of reactive oxygen species (ROS) leading to potential tissue damage (Akar et al., 2002a). It plays a role in the pathogenesis of many dermatological disorders including atopic dermatitis, psoriasis, lichen planus (Sapuntsova et al., 2011, Bacchetti et al., 2013), vitiligo (Akoglu et al., 2013), and pemphigus vulgaris (Naziroglu et al., 2003, Yesilova et al., 2013).

Superoxide dismutases and hydroperoxidases, such as glutathione peroxidase, catalase, and other hemoprotein peroxidases are examples of enzymatic antioxidant defence (Table 1.6) (Prie et al., 2015). In alopecia areata, studies have shown variable results in relation to the presence of superoxide dismutase (SOD) and glutathione peroxidase. There have been reports of decreased SOD activity in the serum of patients with alopecia areata (Koca et al., 2005, Abdel Fattah et al., 2011, Yenin et al., 2015) which may indicate less than adequate protection with regard to oxidative stress. However, increased SOD activity in the scalp of patients with alopecia areata, and no significant difference between SOD activity in alopecia areata patients versus healthy controls have also been documented Equally(Akar et al., 2002a, Motor et al., 2014), both reduced plasma and increased scalp biopsy glutathione peroxidase have been reported in patients with alopecia areata (Naziroglu and Kokcam, 2000, Akar et al., 2002a, Yenin et al., 2015).

During oxidative stress, cell membrane lipids can be modified by ROS exposure (Koca et al., 2005, Yenin et al., 2015) resulting in products such as malondialdehyde and thiobarbituric acid reactive substance (Table 1.8) (Fang et al., 2002, Prie et al., 2015). Significantly high levels of thiobarbituric acid reactive substance have been detected in the plasma and scalp biopsies of patients with alopecia areata compared to healthy subjects (Naziroglu and Kokcam, 2000, Akar et al., 2002a). In addition, high concentrations of tissue and serum malondialdehyde has been found in patients with alopecia areata compared with control subjects (Koca et al., 2005, Abdel Fattah et al., 2011, Bakry et al., 2014, Yenin et al., 2015).

Overall, although there are conflicting results, some studies support an association of alopecia areata aetiology and oxidative stress (Motor et al., 2014). Different patients and stages of hair

loss may account for the variable observations (Prie et al., 2015). Interestingly, antioxidant therapeutics based on the attenuation of oxidative stress may have a role in alopecia areata treatment, although further research in this area is required (Minematsu et al., 2013, Motor et al., 2014, Bakry et al., 2014).

Table 1.8: Components of the oxidative stress response in alopecia areata

| Oxidative stress component | Function | Role in alopecia areata | Reference |
|-----------------------------|--|--|--|
| Superoxide dismutases (SOD) | Enzyme that catalyses the dismutation of superoxide radicals (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), providing cellular defence against reactive oxygen species. | Decreased SOD activity in the serum of patients with alopecia areata. | (Koca et al., 2005, Abdel Fattah et al., 2011, Prie et al., 2015, Yenin et al., 2015) |
| | | Increased SOD activity in the scalp of patients with alopecia areata compared with controls. | (Akar et al., 2002a, Motor et al., 2014) |
| Glutathione peroxidase | Enzyme that catalyses the reduction of hydrogen peroxide to water and oxygen. | Reduced plasma levels of glutathione peroxidase in patients with alopecia areata. | (Naziroglu and Kokcam, 2000, Prie et al., 2015, Yenin et al., 2015) |
| | | Increased glutathione peroxidase activity in scalp biopsies of patients with alopecia areata. | (Akar et al., 2002a) |
| Catalase | Enzyme that destroys H_2O_2 , generating water and oxygen. | No statistically significant difference in patient erythrocyte catalase compared with controls. | (Yenin et al., 2015, Prie et al., 2015) |
| Malondialdehyde | End-product of cell lipid peroxidation. | High concentrations of tissue and serum malondialdehyde in patients with alopecia areata compared with controls. | (Gawel et al., 2004, Koca et al., 2005, Abdel Fattah et al., 2011, Bakry et al., 2014, Yenin et al., 2015) |
| Glutathione | Antioxidant that neutralises reactive oxygen species. | Decreased levels of glutathione in plasma and erythrocytes of alopecia areata patients compared with controls. | (Naziroglu and Kokcam, 2000) |

1.4.1.6 Vitamin D deficiency

Many recent studies have indicated a role for vitamin D deficiency in the development of alopecia areata (Aksu Cerman et al., 2014, Bhat et al., 2017, Ghafoor and Anwar, 2017, Erpolat et al., 2017, Daroach et al., 2018, Gade et al., 2018, Lee et al., 2018b, Unal and Gonulalan, 2018) and some of these are summarised in Table 1.9.

The mechanism by which vitamin D contributes to the onset or progression of alopecia areata is not clear. However, the vitamin does have an impact on an array of immune responses that if adversely affected could contribute to alopecia areata pathogenesis (Lin et al., 2019). Vitamin D reduces the secretion of IFN- γ by peripheral blood mononuclear cells and CD4+ T lymphocytes (Fawzi et al., 2016, Ragab et al., 2016, Paus et al., 2018) inhibits CD8+ and CD4+ T cell proliferation (Cantorna et al., 2015) and T helper 17 (Th17) cell differentiation (Hamzaoui et al., 2014). It also has an inhibitory effect on mast cells (Theoharides, 2017), and enhances the inhibitory function of Tregs (Pietl et al., 2014) that maintain peripheral tolerance. In addition, vitamin D contributes to the prevention of skin NKG2D+/CD8+ T cell activation and trafficking by down-regulating both NKG2D-activating and CXCR3-activating ligands (Dai et al., 2016, Komolmit et al., 2017).

Finally, vitamin D inhibits the JAK/STAT pathway for cytokine production (Zhang et al., 2018), and increases the expression of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein (PD-1) (Jeffery et al., 2015, Sheikh et al., 2018) that negatively regulate the immune response. If any of these processes are adversely affected by vitamin D deficiency, then this could increase the risk of alopecia areata due to an inappropriate or heightened immune response. Interestingly, calcipotriol, a vitamin D analog, has been used topically in treating alopecia areata with promising results (Lin et al., 2019).

Table 1.9: Studies of alopecia areata and vitamin D deficiency

| Number of patients | Study design | Outcome | Reference |
|---|---|---|----------------------------|
| 30 alopecia areata; 30 controls | Prospective case-control study comparative study | Vitamin D receptor expression was reduced in all patients and was normal in controls. Vitamin D levels were significantly decreased in the alopecia areata group, and correlated inversely with disease severity and duration. | (Daroach et al., 2018) |
| 1255 alopecia areata; 784 controls | Retrospective study of 12 studies | Serum 25-hydroxyvitamin D levels were significantly lower in alopecia areata subjects compared to controls. | (Lee et al., 2018b) |
| 20 paediatric alopecia areata; 34 paediatric controls | Prospective case-control study comparative study | No statistically significant difference between the two groups. | (Unal and Gonulalan, 2018) |
| 45 alopecia areata; 45 healthy controls | Cross-sectional study | Significant vitamin D deficiency in alopecia areata, more so with increasing disease severity. | (Gade et al., 2018) |
| 41 alopecia areata; 32 healthy | Prospective case-control comparative study | No statistically significant difference in the serum vitamin D level between alopecia areata patients and healthy controls. | (Erpolat et al., 2017) |
| 30 alopecia areata; 30 healthy controls | Case-control comparative study | Serum vitamin D levels were significantly lower in patients with alopecia areata. | (Ghafoor and Anwar, 2017) |
| 50 alopecia areata; 35 controls | Cross-sectional study | Plasma 25-hydroxyvitamin D were low in alopecia areata patients when compared to healthy controls | (Bhat et al., 2017) |
| 133 alopecia areata | Prospective study | No association between serum vitamin D and risk of alopecia areata. | (Thompson et al., 2016) |
| 86 alopecia areata; 58 healthy controls | Prospective comparative study | Serum 25-hydroxyvitamin D levels in patients with alopecia areata were significantly lower than in healthy controls. | (Aksu Cerman et al., 2014) |
| 23 alopecia areata; 20 healthy controls | Prospective comparative study | Vitamin D levels were significantly decreased in the alopecia areata group. | (Mahamid et al., 2014) |
| 156 alopecia areata | Case control | High vitamin D deficiency prevalence. | (d'Ovidio et al., 2013) |

1.4.2 Genetic susceptibility to alopecia areata

1.4.2.1 Genetic epidemiology

Several pieces of epidemiological evidence support a role for genetic susceptibility to alopecia areata.

1.4.2.1.1 Family history

There may be family history of alopecia areata in probands; 4-28% of alopecia areata patients have a least one affected relative (van der Steen et al., 1992b). A family history of alopecia areata correlates with worse outcomes as characterised by a younger age of onset, lower hair regrowth, severer symptoms, and progression to more severe forms of alopecia (Wang et al., 2018). Many studies have been carried out to estimate the risks of alopecia areata in first- and second-degree relatives of patients. A study conducted by van der Steen and colleagues reported that the parents and siblings of 7% and 2%, respectively, of 348 patients with alopecia areata were affected by the disease (van der Steen et al., 1992b). Another study reported 21.8% of patients had a positive family history of alopecia areata with 7.4% of parents and 5.5% of siblings affected (Blaumeiser et al., 2006). A further report indicated that 8.4% of alopecia areata patients had positive family history; the prevalence of the disease in first- and second-degree relatives was 1.6% and 0.19%, respectively (Yang et al., 2004). Overall, these studies provide support for the role of genetic factors in development of alopecia areata.

1.4.2.1.2 Twin studies

The occurrence of alopecia areata in twins supports the role of genetics in its development, as identical siblings can have similar hair loss patterns and age of disease onset (Stankler, 1979, Scerri and Pace, 1992, Alsaleh et al., 1995). Rodriguez and colleagues reported a 42% alopecia areata concordance rate in monozygotic twins, and 10% in dizygotic twins (Rodriguez et al., 2010).

1.4.2.1.3 Ethnic differences

Recent studies have shown a higher risk of alopecia areata development in African Americans and compared with both Asian and whites (Lee et al., 2019a). The same ethnic difference was found in black and Hispanic women who had an increased risk of alopecia areata compared with white women in the USA (Thompson et al., 2018). In ten years from 1990 to 2000, the number of black and white patients presenting with alopecia areata were 108 and 91 patients, respectively, per 10,000 (McMichael et al., 2007).

1.4.2.1.4 Alopecia areata related to syndromes

Many studies and case reports have documented that alopecia areata is present in different genetic syndromes (Table 1.10). These include Down's syndrome in which an incidence of alopecia areata of 6–11% has been reported in different populations (Daneshpazhooh et al., 2007, Folster-Holst et al., 2018), and autoimmune polyendocrine syndrome type 1 (APS1), where 30% of patients can develop severe early onset hair loss (Gilhar et al., 2007).

Table 1.10: Alopecia areata related to syndromes

| Syndromes | Clinical features | Reference |
|---|---|---|
| Good's syndrome | A rare form of acquired immunodeficiency associated with thymomas, hypogammaglobulinemia, B lymphocyte deficit, and recurrent infections. In addition, alopecia areata has been reported. | (Jimura et al., 2018) |
| Turner syndrome | Turner syndrome is one of the most frequent chromosomal abnormalities characterised by short stature, poor breast development, and a small chin. Two cases of Turner syndrome were reported as affected by alopecia areata, Hashimoto's thyroiditis, celiac disease, and psoriasis. | (Dogruk Kacar et al., 2014, Gianfaldoni et al., 2017) |
| Cronkhite-Canada syndrome | A rare acquired polyposis syndrome, which presents with severe diarrhoea, alopecia areata incognito), hyperpigmentation, and onychomadesis. | (Ong et al., 2017) |
| Paraneoplastic syndrome | Reported case of alopecia areata in patient with gastric adenocarcinoma. | (Alvarez Otero et al., 2017) |
| | Reported case of alopecia areata in patient with Hodgkin's lymphoma. | (Gong and Lim, 2014) |
| | Reported case of alopecia areata in patient with thymoma, vitiligo, myasthenia gravis, and oral lichen planus. | (Qiao et al., 2011) |
| Hirschsprung's disease | A preterm Indian female born with total absence of scalp and body hair. A skin biopsy of the scalp was suggestive of alopecia universalis congenita. | (Malik et al., 2016) |
| Autoimmune polyendocrine syndrome | Can be classified into three groups: type 1, 2, and 3. Genetic, infectious, immunologic and psychological factors have all been implicated in the development of multiple autoimmune syndromes. Patients often have at least one dermatological condition, usually vitiligo or alopecia areata. | (Cojocaru et al., 2010, Samanta et al., 2019) |
| Aicardi-Goutières syndrome | Presence of alopecia areata. | (Samanta et al., 2019) |
| Mayer-Rokitansky-Küster-Hauser syndrome | Characterised by congenital aplasia of the uterus and the upper part of the vagina, and alopecia areata features. | (Al-Awadi et al., 1985, Megarbane et al., 2003, Zaman and Nisar, 2009, Choudhary and Choudhari, 2016) |
| Ring chromosome 18 syndrome | Rare constitutional chromosomal aberration presenting with a dysmorphic face, flat nasal bridge, low set ears, and alopecia areata. | (Kagimoto et al., 2014) |
| Down's syndrome | Many cases of alopecia areata has been reported in patients with Down's syndrome. | (Estefan et al., 2014, Schepis et al., 2017, Rachubinski et al., 2019) |

1.4.2.2 Major histocompatibility complex genes

Many studies have highlighted associations of alopecia areata and molecules of the major histocompatibility complex (MHC) that are integral to the presentation of antigens to T lymphocytes.

1.4.2.2.1 MHC class I antigens

With respect to MHC class I antigens, the human leukocyte antigen (HLA)-A, HLA-B and HLA-C, the evidence supporting an association of specific variants with alopecia areata is inconsistent and differs between populations (Table 1.11). Kianto and colleagues confirmed a significant association of HLA-B12 with alopecia areata especially in patchy types in Finnish people (Kianto et al., 1977), whereas in Turkey, a significantly higher frequency of HLA-A1 and HLA-B62 was found in patients with alopecia areata compared with a healthy control group (Kavak et al., 2000). Other significant associations of MHC class I antigen specificities with alopecia areata have been determined as HLA-B13 and HLA-B27 in Russians, and HLA-B18 in Israelis (Alzolibani, 2011).

1.4.2.2.2 MHC class II antigens

The MHC class II contains many genes that play an important role in the immune system by presenting foreign antigens to T cells (Jabbari et al., 2013). Some relevant studies that show HLA class II status as a primary risk in the development of the alopecia areata are summarised in Table 1.12. Overall associations between HLA class II polymorphisms and alopecia areata risk have been investigated by Ji and colleagues. They made comparisons between case and control groups from twelve relevant publications and concluded that HLA-DRB1*04 and HLA-DRB1*16 polymorphisms might be associated with increased risk of alopecia areata, while HLA-DRB1*0301, HLA-DRB1*09, and HLA-DRB1*13 polymorphisms might decrease the risk (Ji et al., 2018). Other specific studies have suggested that HLA-DR4, HLA-DR5, HLA-DR11, HLA-DQ3, and HLA-DQ7 are associated with more severe forms of alopecia areata like alopecia totalis and universalis (Duvic et al., 1991, Welsh et al., 1994) and that HLA-DRw52a and HLA-DRB1*03 provide relative protection from alopecia areata development (Alzolibani, 2011).

Table 1.11: MHC class I antigen genes associated with susceptibility to alopecia areata

| MHC class I antigen | Population | Reference |
|--|-------------------|-----------------------|
| HLA-B12 | Finnish | (Kianto et al., 1977) |
| HLA-A1; HLA-B62 | Turkish | (Kavak et al., 2000) |
| HLA-C*04:01; HLA-C*07:02; HLA-C*15:02 | Japanese | (Haida et al., 2013) |
| HLA-A*02; HLA-A*03; HLA-B*18; HLA-B*27; HLA-B*52 | Chinese | (Xiao et al., 2006b) |
| HLA-B18 | Israeli | (Alzolibani, 2011) |
| HLA-B18; HLA-B27 | Russian | (Alzolibani, 2011) |

Table 1.12: MHC class II antigen genes associated with susceptibility to alopecia areata

| MHC class II antigen | Population | Reference |
|---|-------------------|---------------------------------|
| HLA-DQ1; HLA-DQ3 | Turkish | (Kavak et al., 2000) |
| HLA-DQB1*0301 | North American | (Welsh et al., 1994) |
| HLA-DQA1; HLA-DQB1 | Chinese | (Xiao et al., 2006a) |
| HLA-DR4; HLA-DRw11 | North American | (Duvic et al., 1991) |
| HLA-DQB1*03 | North American | (Colombe et al., 1999) |
| HLA-DQB1*03 | Turkish | (Akar et al., 2002b) |
| HLA-DQ1-DR6-MICA(*)5.1; HLA-DQB1*0201-DR3- MICA(*)5.1 | North American | (Barahmani et al., 2006) |
| HLA-DQB1*03 | Italian | (Megiorni et al., 2011) |
| HLA-DRB1; HLA-DRB1*11 | Egyptian | (Pratt et al., 2017) |
| HLA-DRB1*1104 | Belgian | (Marques Da Costa et al., 2006) |
| HLA-DQA1; HLA-DQB1; HLA-DPA1 | Danish | (Akar et al., 2002b) |

1.4.2.3 Immunoregulatory genes

In addition to genes of the MHC, other non-MHC loci that are nevertheless involved in the immune response have also been identified as alopecia areata risk factors (Tazi-Ahnini et al., 2003, Petukhova et al., 2010). Several of these are summarised in Table 1.13 and some are discussed below.

The *PTPN22* gene encodes protein tyrosine phosphatase non-receptor 22, which is involved in several signalling pathways associated with the immune response including inhibiting T cell activation (Fousteri et al., 2014). The common *PTPN22* 1858T/Arg620Trp (rs2476601) single nucleotide polymorphism (SNP) adversely affects the function of the enzyme and is associated with susceptibility to autoimmune disease including alopecia areata (Kemp et al., 2006, Betz et al., 2008, Moravvej et al., 2018). In African, Latin American and European populations it is associated with the risk of patchy type alopecia areata (Kemp et al., 2006, El-Zawahry et al., 2013, Salinas-Santander et al., 2015) although other studies in North America and Iran have failed to confirm this (Betz et al., 2008, Moravvej et al., 2018).

The cytotoxic T lymphocyte antigen-4 (CTLA-4), encoded by *CTLA4*, functions as an immune checkpoint downregulating the immune response (Brunet et al., 1987). Variants of *CTLA4* are risk factors for many autoimmune diseases, again including alopecia areata (Petukhova et al., 2010, Jabbari et al., 2013). For example, a genome-wide association study, using 1200 cases and controls, demonstrated a statistically significant association of six different SNPs with alopecia areata (John et al., 2011). One of the SNPs, rs231775, was found in the coding region of *CTLA4*. It results in an amino acid substitution which adversely affects the function of the protein.

The interleukin-2 receptor- α (*IL2RA*) is an important marker of regulatory T cells, which are crucial to maintaining immune tolerance. Many studies have provided strong evidence for the involvement of genetic variants of the receptor in the pathogenesis of alopecia areata, as well as other autoimmunities (Petukhova et al., 2010, Betz et al., 2015, Moravvej et al., 2018). Miao and colleagues concluded that the rs3118470 SNP of *IL2RA* might be a genetic risk marker of alopecia areata in a Chinese population (Miao et al., 2013). Significant association of the *IL2RA*

variant rs706778 with severe types of alopecia areata has also been reported (Redler et al., 2012).

The *AIRE* gene encodes a transcriptional regulator that plays an important role in the development of central tolerance (Zhao et al., 2018). Recessive mutations in *AIRE* are the cause of autoimmune polyendocrine syndrome type 1 (APS1) which manifests with multiple autoimmune disorders (Tazi-Ahnini et al., 2002). Up to 30% of cases develop severe, early-onset alopecia areata (Gilhar and Kalish, 2006, Gilhar et al., 2007). In addition, a significant association between severe forms of alopecia areata in the form of alopecia universalis and the rare *AIRE* allele (C961) has been reported (Tazi-Ahnini et al., 2002). However, Pforr and colleagues in a case-control study of Germans and Belgians did not find a significant association of the same risk allele with alopecia areata (Pforr et al., 2006).

Other immunoregulatory genes associated with alopecia areata reside are those encoding the natural killer (NK) receptor D ligands NKG2DL3, which also known as *ULBP3*, and retinoic acid early transcript protein 1L, also known as *ULBP6* (Petukhova et al., 2010). Expression of *ULBP3* is upregulated within the hair follicle dermal papilla and dermal sheath of patients with alopecia areata (Ito et al., 2008, Petukhova et al., 2010). Whereas, the expression of *ULBP3* is at low levels within the dermal papilla of the normal hair follicle (Petukhova et al., 2010, Ito et al., 2008). These results suggest that the autoimmune destruction in alopecia areata may be mediated in part by CD8+NKG2D+ cytotoxic T cells, whose activation may be induced by upregulation of *ULBP3* in the dermal sheath of the hair follicle (Petukhova et al., 2010).

Table 1.13: Immunoregulatory genes associated with alopecia areata development

| Gene | Function | Reference |
|-----------------------|---|-----------------------------|
| <i>AIRE</i> | Autoimmune regulator. Required for central tolerance. | (Tazi-Ahnini et al., 2002) |
| <i>CTLA4</i> | Cytotoxic T cell antigen-4. Control of T lymphocyte activation. | (Petukhova et al., 2010) |
| <i>IL2</i> | Interleukin-2. Activates lymphocytes. | (Petukhova et al., 2010) |
| <i>IL2RA</i> | Interleukin-2 receptor alpha chain. Receptor for interleukin-2. | (Petukhova et al., 2010) |
| <i>MIF</i> | Macrophage inhibitory factor. Proinflammatory cytokine. | (Shimizu et al., 2005) |
| <i>MICA</i> | MHC class I polypeptide-related sequence A. Activates natural killer cells via the NKG2D receptor. | (Barahmani et al., 2006) |
| <i>NOTCH4</i> | Neurogenic locus notch homolog 4. Maturation of T lymphocytes and differentiation of keratinocytes. | (Tazi-Ahnini et al., 2003) |
| <i>PTPN22</i> | Protein tyrosine phosphatase, non-receptor 22. Control of T lymphocyte activation. | (Kemp et al., 2006) |
| <i>ULBP3</i> | Encodes ligand NKG2DL3 which activates natural killer cells via the NKG2D receptor. | (Martinez-Mir et al., 2007) |
| <i>ULBP6 (RAETIL)</i> | Encodes retinoic acid early transcript protein which activates natural killer cells via the NKG2D receptor. | (Martinez-Mir et al., 2007) |

1.4.2.4 Oxidative stress-related genes

The hair follicle is one of many tissues that expresses peroxiredoxin 5 (*PRDX5*), an antioxidant enzyme induced under conditions of cellular stress (Kakourou et al., 2007), a process that is dysregulated in alopecia areata (Akar et al., 2002a). The rs694739 SNP of *PRDX5* is a risk factor in Crohn's disease (Franke et al., 2010), while the rs574087 variant is associated with alopecia areata (Petukhova et al., 2010). It has been suggested that variants of *PRDX5* may allow an anti-apoptotic phenotype that enables the survival of aberrant cells which harbour danger signals that lead to the presentation of damaged self-antigens to the immune system and as a consequence autoimmune disease (Petukhova et al., 2010).

1.4.2.5 Other genes

Variants of the *STX17* gene have also been reported to be associated with alopecia areata (Jabbari et al., 2013). *STX17* is part of the syntaxin-encoding gene family which are involved in vesicular trafficking and membrane fusion (Jabbari et al., 2013). Although its relation to hair loss has yet to be established, the gene is interesting because a *STX17* insertion with enhancer-like activity was discovered to cause the grey hair phenotype in horses (Rosengren Pielberg et al., 2008, Sundstrom et al., 2012), and grey hairs are spared in alopecia areata.

1.4.2.6 MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules classified as important translational regulators of gene expression in various tissues and biological processes. They can target immune-regulatory pathways and their action has been associated with the development of autoimmune diseases such as type I diabetes and rheumatoid arthritis (Wang et al., 2017), as well as alopecia areata (Wang et al., 2017, Sheng et al., 2019).

Tafazzoli and colleagues identified three microRNAs (miR-1237, miR-30b/d, and miR-548h-2) that showed reduced expression in the hair follicle of alopecia areata patients (Tafazzoli et al., 2018). Target gene analyses for the three microRNAs showed that the genes under their regulation included the alopecia areata risk genes *IL2RA*, *TNXB* (encodes tenascin), and *ERBB3* (encodes epidermal growth factor receptor (Tafazzoli et al., 2018). An investigation of 2549 circulating miRNAs found that 36 were dysregulated in patients with severe active alopecia

areata (Sheng et al., 2019). These included miR-210 and miR-1246, further analysis of which identified them as potential signatures in the active phase of alopecia areata (Sheng et al., 2019). The dysregulation of miR-210 impairs the immunosuppressive functions of Treg cells by targeting *FOXP3* (Zhao et al., 2014, Bassino et al., 2015), whilst miR-1246 targets p53 to inhibit the expression of *DYRK1A* (encodes dual specificity tyrosine-phosphorylation-regulated kinase 1A) and as a consequence activates nuclear factor of activated T cells (NFAT), which is a transcriptional activator associated with the immune response (Liao et al., 2012).

1.4.3 The role of immune factors in alopecia areata pathogenesis

Many findings have suggested that immune factors are involved in the pathogenesis of alopecia areata (McElwee et al., 1999b). Some of these have been discussed already and include associations between alopecia areata and autoimmune disorders (Section 1.2.4) (Tan et al., 2002b), associations between immunoregulatory gene variants and alopecia areata susceptibility (Section 1.4.2.3) (McDonagh and Tazi-Ahnini, 2002), and the ability of immunosuppressive agents such as corticosteroids, cyclosporine, and immunotherapeutic agents to initiate hair regrowth (Sections 1.3.3, 1.3.4, 1.3.5, 1.3.8, 1.3.11) (Gupta et al., 1990).

Further evidence that the immune response is involved in alopecia areata pathogenesis will be discussed in the next sections and includes animal models of alopecia areata (Michie et al., 1991, Sundberg et al., 1994), the presence of inflammatory cells in and around the hair follicles including CD4+ and CD8+ T lymphocytes, Langerhans cells, and macrophages (Perret et al., 1984, Todes-Taylor et al., 1984), the aberrant expression of MHC class I and II antigens on hair follicle epithelial cells, as well as an elevation of ICAM-1 expression around the hair follicle (Brocker et al., 1987, McDonagh et al., 1993, Ghersetich et al., 1996), increased serum and dermis levels of pro-inflammatory cytokines, which in some cases correlate with disease activity and duration (Tembhre and Sharma, 2013, Ito and Tokura, 2014, Zhang et al., 2015), and the presence of anti-hair follicle autoantibodies in alopecia areata patients (Tobin et al., 1994a).

The major theory to explain the development of alopecia areata is that of the collapse of immune privilege at the hair follicle resulting in aberrant immune responses against it (Paus et al., 2003). This will also be described and discussed in the next sections.

1.4.3.1 Animal models

Several animal models have been established that have helped to clarify the cellular and immunological pathways in alopecia areata (Sun et al., 2008, Gilhar et al., 2016, Shin et al., 2018). These are discussed below and are summarised in Table 1.14.

1.4.3.1.1 Dundee experimental balding rat model

The Dundee experimental balding rat (DEBR) model was the first reported animal model of alopecia areata. However, it is now unavailable and no longer used (Michie et al., 1990, Gilhar et al., 2016). In the past, the DEBR rat provided a potent model for investigating various aspects of the pathogenesis of alopecia areata, as up to 70% of female DEBR rats develop spontaneous lesions and 3% of those affected develop spontaneous remission (Michie et al., 1990, McElwee et al., 1999a). Many studies of DEBR rats have shown perifollicular infiltration of CD4+ and CD8+ T cells and the production of immune response mediators (Michie et al., 1990, Zhang and Oliver, 1994, McElwee et al., 1999a).

1.4.3.1.2 C3H/HeJ mouse model

The C3H/HeJ mouse model (Sundberg et al., 1994, Gilhar et al., 2016, Shin et al., 2018), has a hair-loss phenotype and infiltration of immune cells into the hair follicles that bears a striking resemblance to human alopecia areata (Zhang and Oliver, 1994, McElwee et al., 2003, Sundberg and King, 2003). Experimentally, the affected skin from C3H/HeJ mice can be grafted onto healthy animals with the resulting development of alopecia areata characterised by hair follicle inflammation (McElwee et al., 1998). If grafted onto severe combined immunodeficient (SCID) mice, hair re-growth is observed in alopecia affected skin, indicating the involvement of immune processes in promoting hair loss (McElwee et al., 1998). Furthermore, the inoculation of T cells from C3H/HeJ mice into healthy animals causes the induction of alopecia areata in the recipients with the involvement of both CD8+ and CD4+ T cell subsets (McElwee et al., 2005a).

By further characterising the C3H/HeJ mouse model, IFN- γ was identified as the important cytokine involved in promoting alopecia areata development (Sundberg et al., 1994, Gilhar et al., 2016, Shin et al., 2018). When skin from alopecia areata affected C3H/HeJ mice was grafted onto C3H/HeJ mice with a targeted deletion of IFN- γ , the mice failed to develop alopecia areata, whereas 90% of normal C3H/HeJ mice developed hair-loss (Freyschmidt-Paul et al., 2006). Xing and colleagues also reported that NK-type CD8+NKG2D+ T cells, which produce IFN- γ , are the dominant immune effectors infiltrating the hair follicle C3H/HeJ mice with alopecia areata (Xing et al., 2014). Furthermore, transcriptional profiles of C3H/HeJ mice has

revealed the upregulation of indicators of the IFN- γ response including chemokines CXCL9, CXCL10, and CXCL11, chemokine receptor CXCR3 receptor on CD8+NKG2D+ effector T cells, and cytokines IL-2 and IL-15 that are known to help activate CD8+NKG2D+ T cells (Xing et al., 2014, Dai et al., 2016).

1.4.3.1.3 Humanised mouse models

In humanised mouse models of alopecia areata, hair re-growth can be initiated after transferring alopecia areata-affected skin from human subjects onto SCID mice, and this effect can be nullified by injecting lymphocytes from patients into the grafted skin (Gilhar and Krueger, 1987, Gilhar et al., 2002). In addition, IFN- γ -treatment of skin grafts containing re-grown hair can cause hair-loss as well as up-regulating the expression of MHC class I and class II antigens, and ICAM-1 in the alopecia lesion (Gilhar et al., 1993). More recently, the involvement of natural killer (NK) cells and IL-2 in hair-loss associated with the lesional infiltration of CD8+ and CD4+ T cells has been shown in the humanised SCID mice model (Gilhar et al., 2013).

Table 1.14: Difference between animal model types used in alopecia areata

| Animal model | Dundee experimental balding rat (DEBR) | C3H/HeJ mouse | Humanised mouse | References |
|---------------------------------|--|--|---|--|
| Animal description | Inbred rat. Up to 70% of female DEBR rats develop spontaneous alopecia areata lesions. | Inbred mouse. Up to 20% develop spontaneous alopecia areata lesions. | Normal or lesional human alopecia areata skin transplanted onto severe combined immunodeficiency SCID mice. | (Sun et al., 2008, Gilhar et al., 2016) |
| Skin source | Rat | Mouse | Human | (Gilhar et al., 2016) |
| Useful for study of | Animal nature of alopecia areata in a preclinical <i>in vivo</i> setting. | Animal nature of alopecia areata in a preclinical <i>in vivo</i> setting. Study of both genetic and immune response. | Human nature of alopecia areata in a preclinical <i>in vivo</i> setting. Reflects human disease pathology, but requires a substantial amount of human donor tissue. Mainly used for study of immune response. | (Gilhar et al., 2016, Shin et al., 2018) |
| Pathology and cell infiltration | CD8+ and CD4+ T cell perifollicular infiltration. | CD8+ and CD4+ T cell infiltration above the hair bulb. | As in human alopecia areata, mainly CD8+ T cells around and within the hair bulb. | (Gilhar et al., 2016) |
| Disadvantages | No longer used as expensive. | Does not reflect genetic and SNP markers associated with human alopecia areata. | Has major practical limitations that preclude its widespread use. The model represents a Graft-Versus-Host Disease response that leads to an alopecia areata-like disease phenotype. | (McElwee et al., 1999a, Gilhar et al., 2016, Pratt et al., 2017) |

1.4.3.2 Cellular immune responses in alopecia areata

There are several lines of research that suggest cellular immune responses are involved in the alopecia areata development, and these are described in the next sections.

1.4.3.2.1 Natural killer cells

Natural killer (NK) cells are a type of cytotoxic lymphocyte critical to the innate immune system. They are a major source of IFN- γ (Vivier et al., 2008, Ito et al., 2008), a key cytokine in alopecia areata pathogenesis that is thought to mediate immune privilege collapse of anagen hair follicles (Gilhar and Kalish, 2006, Freyschmidt-Paul et al., 2006). In alopecia areata, there are notable accumulations of activated CD56⁺/NKG2D⁺ NK cells around the hair follicles (Pratt et al., 2017). Studies have shown that, compared with healthy controls, many more NK cell receptors NKG2D and NKG2C, which activate function, and far fewer NK cell immunoglobulin-like receptors 2D2 and 2D3, which suppress activity, are detected on the circulating peripheral blood cells of alopecia areata patients (Ito et al., 2008). Furthermore, the expression of MHC class I polypeptide-related sequence A, which activates NK cells via their NKG2D receptors, is elevated in the anagen hair follicles of alopecia areata patients in comparison with healthy individuals (Ito et al., 2008).

1.4.3.2.2 Langerhans cells

Langerhans cells are specialised antigen-presenting cells of the dendritic cell family and are found in the basal and supra-basal layers of the skin epidermis and in the epithelia of internal organ (Ayala-Garcia et al., 2005, Chomiczewska et al., 2009, Doss and Smith, 2014). Langerhans cells have been shown to be involved in the pathogenesis and pathophysiology of several skin diseases such as atopic dermatitis, alopecia areata, and melanoma (Ayala-Garcia et al., 2005).

In the hair follicle, Langerhans cells are usually found in low numbers in the follicular epithelium, and are not observed in the bulbar epithelium (Moresi and Horn, 1997). However, in alopecia areata patients, activated Langerhans cells expressing CD1a are present in increased numbers in both the peri-vascular and peri-bulbular parts of the hair follicle in the affected scalp (Ghersetich et al., 1996, Zhang et al., 2015). Furthermore, in the deep cutaneous

areas of alopecia areata patients, there is a positive correlation between the infiltration of Langerhans cells and CD8+ T lymphocytes, indicating the possibility of Langerhans cell antigen-presentation and stimulation of CD8+ T cells (Zhang et al., 2015).

1.4.3.2.3 CD8+ T lymphocytes

There is much evidence to support an important role for CD8+ T cells in the pathogenesis of alopecia areata. Early studies estimated that 20-40% of the immune cells around the hair follicles of alopecia areata patients were CD8+ T lymphocytes (Todes-Taylor et al., 1984). Such cytotoxic T cells were also observed to infiltrate into the hair follicles (Ranki et al., 1984, McElwee et al., 2003, Zhang et al., 2015), and exert their effects by producing the cytotoxic molecule granzyme B, which is elevated in the hair follicles of patients with alopecia areata (Ghoreishi et al., 2010). Following immunosuppressive therapy, the recovery of hair growth in some alopecia areata patients, and the reduction of hair follicle-infiltrating cytotoxic T cells in the recovered hair follicles, suggested that T cells were functionally significant in the development of alopecia areata (Van Scott, 1958, Alkhalifah et al., 2010a, Alkhalifah et al., 2010b, Wang et al., 2012). In DEBR rats, hair growth can be restored by *in vivo* depletion of CD8+ T cells (McElwee et al., 1996b), and in C3H/HeJ mice, alopecia areata can be induced by transferring CD8+ T cells from affected to healthy animals (McElwee et al., 2005a, Wang et al., 2012). The transfer of CD8+ T cells to SCID mice grafted with healthy scalp explants has also been shown to induce the development of alopecia areata (Gilhar et al., 2013). More recently, IFN- γ -producing CD8+NKG2D+ T cells have been identified as one of the predominant contributors to hair-loss in alopecia areata (Gilhar et al., 2012, Xing et al., 2014, Pratt et al., 2017, de Jong et al., 2018).

The exact hair follicle targets of cytotoxic T lymphocytes have yet to be determined, but there is evidence that melanocytes may be attacked (Paus et al., 1994). For example, with increasing disease activity, hairs with pigment are lost in preference to hairs without pigment (Guin et al., 1981). Secondly, early re-growing hairs tend to be white (Guin et al., 1981) and, thirdly, melanocytes in the hair bulb are irregular in terms of ultrastructural and histological features (Tobin et al., 1990).

There has also been some research to identify the specific targets of CD8+ T cells. For example, melanocyte-associated peptides from melanocyte proteins Gp100 and MelanA can activate lesional T cells to induce hair loss in SCID-mice grafted with human scalp (Gilhar et al., 2001). In this study, scalps injected with the peptides showed the expected histological changes normally associated with alopecia areata in human patients (Gilhar et al., 2001). Furthermore, T cell-mediated alopecia areata can be induced in mice following immunotherapy for melanoma with melanocyte-associated antigens (Becker et al., 1996), and CD8+ T cells, which are sensitised to melanoma cell antigens, are cytotoxic against normal hair bulb melanocytes (Nagai et al., 2006).

More recently, specific synthetic epitopes derived from hair follicle antigens trichohyalin and tyrosinase-related protein-2 were shown to significantly induce higher frequencies of response in cytotoxic T cells from alopecia areata patients compared with healthy controls (Wang et al., 2016). Increases in melanocyte-associated antigen-3 (MAGE-A3)-reactive CD8+ T cells were found in the serum and skin lesions of patients with alopecia areata and alopecia totalis compared to healthy controls (Ito et al., 2013a). In addition, T cells against keratins K71 and K31 were shown to develop in mice after immunisation (Erb et al., 2013).

1.4.3.2.4 CD4+ T lymphocytes

In alopecia areata affected scalp skin, CD4+ T cells make up between 60% and 80% of the total lymphocytic infiltrate (Todes-Taylor et al., 1984), and there is evidence that these T lymphocytes have an active role in the pathogenesis of alopecia areata. Indeed, the co-operation of CD4+ T cells with cytotoxic T cells in producing the hair loss phenotype occurs in mouse models (Gilhar et al., 2002, McElwee et al., 2005a). For example, in SCID mice with human scalp grafts, alopecia areata development is promoted by a CD4+ T helper 1 (Th1) cell subset response, which is characterised by the production of Th1 cytokines, for example, IFN- γ , TNF- α , and IL-2 (Gilhar et al., 2003, Tojo et al., 2013, Loh et al., 2018)

Less well studied are the exact roles of CD4+ T cells of the Th2 and Th17 subtypes. CD4+ IL-17+ cells have been shown in the lymphocytic infiltrate surrounding hair follicles and hair bulbs (Tanemura et al., 2013, Tojo et al., 2013, Loh et al., 2018). This helper T cell subtype produces IL-17, IL-23, and IL-6. In addition, there are some indications for the involvement of Th2 cells,

which produce cytokines IL-4, IL-5, IL-10, and IL-13, in alopecia areata hair loss (Teraki et al., 1996, Tembhre and Sharma, 2013, Loh et al., 2018).

Regulatory T (Treg) cells are a subpopulation of CD4⁺ T cells that have a suppressive effect on the immune system and are involved in preventing autoimmunity (Sakaguchi et al., 2006). The best understood Treg cell population is identified by CD4⁺CD25⁺FOXP3 markers and the production of several cytokines including TGF- β , IL-10, and IL-35 (Guo et al., 2015). Studies have shown that Treg cells have a protective effect against alopecia areata; in C3H/HeJ mice, the disease can be prevented by the injection of CD4⁺CD25⁺ Treg cells (McElwee et al., 2005a). There are also observations that in C3H/HeJ mice, the number of Treg cells in alopecia areata lesions is very low (Zoller et al., 2004). In alopecia areata patients, circulating Treg cells appear to be normal with regard to numbers, but they are functionally impaired producing significantly less TGF- β than those of healthy individuals (Shin et al., 2013, Tembhre and Sharma, 2013). More recently, Hamed and colleagues investigated the distribution of Treg cell subsets in the lesional skin of patients with alopecia areata and compared this with healthy control skin (Hamed et al., 2019). They reported that actively suppressive CD3⁺FOXP3⁺CD39⁺ Treg cells localised to the hair follicles in normal and non-lesional skin, and infiltrated the outer connective tissue layer (Hamed et al., 2019). However, they were not present in diseased hair follicles, which were infiltrated instead by effector T cells (Hamed et al., 2019). With regard to alopecia areata treatment, hair re-growth was evident in patients where the recruitment of Treg cells was promoted into hair-loss regions using IL-2 (Castela et al., 2014).

1.4.3.2.5 Mast cells

Mast cells are predominantly known in relation to allergy and allergic disease (Rao and Brown, 2008). However, they are also important factors in immunity, inflammation, wound healing, and hair follicle cycling under physiological and pathological conditions (Rao and Brown, 2008, Ito et al., 2010, Weller et al., 2011). Some studies have suggested a role for mast cells in the pathogenesis of alopecia areata (Rao and Brown, 2008, Ito et al., 2010, Bertolini et al., 2014, Azzawi et al., 2018, Carrasco et al., 2019) For example, an increased number and proliferation of perifollicular and perivascular mast cells has been found in alopecia areata patient hair-loss lesions compared with control skin (Bertolini et al., 2014). The mast cells showed a decreased

production of immunosuppressive cytokines IL-10 and TGF- β , and possible interactions with CD8+ T cells. In mouse models, abnormal numbers of mast cells and interactions with CD8+ T lymphocytes were also observed (Bertolini et al., 2014). Overall, the contribution of mast cells to alopecia areata development needs further study to confirm these initial findings.

1.4.3.2.6 Cytokines

Much evidence exists for the role of several cytokines in alopecia areata pathogenesis and several studies are summarised in Table 1.15. Of particular note is the Th1 proinflammatory cytokine IFN- γ , which plays a central role in the development of alopecia areata. It has been shown that injecting IFN- γ into scalp explants on SCID mice causes ICAM-1 and HLA-DR to be expressed on the follicular epithelium (Gilhar et al., 1993). In addition, IFN- γ induces the expression of HLA-DR on cultured dermal papilla cells and keratinocytes (Kalish and Gilhar, 2003), as well as HLA-A, B, and C on follicular epithelial cells and in the lesional skin of alopecia areata patients (Kalish and Gilhar, 2003). Such molecules are then recognised by T lymphocytes with the consequential immune responses.

Other Th1 cytokines such as IL-2, IL-8, and TNF- α have been found at elevated levels in the serum and dermis of patients with alopecia areata (Tembhre and Sharma, 2013, Ito and Tokura, 2014, Zhang et al., 2015). The elevated levels correlated with hair being actively lost, as well as disease duration and extent. Furthermore, serum levels of the Th1 chemoattractants MIG (monokine induced by IFN- γ) and RANTES (regulated on activation, normal T cell expressed and secreted) are increased in alopecia areata patients and their levels are related to alopecia activity (Kuwano et al., 2007).

In the case of Th2 cytokines, serum and dermal levels of IL-13, IL-4, and IL-5 are significantly higher in alopecia areata patients (Teraki et al., 1996, Tembhre and Sharma, 2013, Zhang et al., 2015). However, only in the case of IL-4 did increased serum levels correlate with any clinical phenotype, namely localised hair-loss (Teraki et al., 1996). Overall, the exact role of Th2 cytokines in alopecia areata development remains uncertain (Teraki et al., 1996, Zhang et al., 2015).

Higher serum IL-17A levels, a cytokine produced by CD4+ Th17 cells, have been reported in patients with active alopecia areata (Tojo et al., 2013, Han et al., 2015, El-Morsy et al., 2016, Atwa et al., 2016, Elela et al., 2016) but these levels were not related to the duration or extent of hair-loss (Tembhre and Sharma, 2013). The authors suggest, therefore, that IL-17A may be involved at the start or early active stage of alopecia areata. More recently, circulating levels of other type 17 cytokines, IL-17A, IL-21, and IL-23, have been found at elevated levels in alopecia areata patients (Bain et al., 2019).

The cytokine TGF- β , which is expressed from several immune cell types and influences the development of Tregs and Th17 cells (Eisenstein and Williams, 2009), is at significantly reduced levels in alopecia areata patients in comparison with healthy individuals (Tembhre and Sharma, 2013), although the converse effect has also been reported (Loh et al., 2018).

Overall, IFN- γ , IL-2, and Th17 cytokines seem to play a central role in the pathogenesis of alopecia areata. The parts played by other cytokines show conflicting results (Table 1.13), such that more comparative studies of large multicentre groups are needed to clarify the pathogenic role and clinical significance of many cytokines in the development of alopecia areata.

Table 1.15: The role of cytokines in alopecia areata pathogenesis

| Cytokine | Source of cytokine | Findings in alopecia areata patients | References |
|-----------------|--|--|---|
| IFN- γ | Th1 cells | Higher serum levels of IFN- γ in patients with alopecia areata compared with controls; Higher levels correlate with severity; Significant improvement in hair growth with anti-IFN- γ treatment. | (Teraki et al., 1996, Skurkovich et al., 2005, Kasumagic-Halilovic et al., 2010, Tembhre and Sharma, 2013, Giordano and Sinha, 2013, Gautam et al., 2019) |
| IL-2 | Th1 cells | Increased IL-2 serum levels in patients with initiation of alopecia areata and mainly extensive forms compared with normal controls; Low-dose IL-2 for treating severe alopecia areata by enhancing the recruitment of Treg cells. | (Teraki et al., 1996, Barahmani et al., 2010, Tembhre and Sharma, 2013, Castela et al., 2014, Kasumagić-Halilovic et al., 2018, Gautam et al., 2019) |
| IL-4 | Th2 cells | Differing results; Significantly elevated serum IL-4; Decreased in patients with alopecia areata compared with controls. | (Teraki et al., 1996, Attia et al., 2010, Gautam et al., 2019) |
| IL-17 | Th17 cells | Higher serum IL-17A levels in patients with active alopecia areata and severe forms. | (Tembhre and Sharma, 2013, Han et al., 2015, Atwa et al., 2016, Elela et al., 2016, El-Morsy et al., 2016, Gautam et al., 2019) |
| IL-10 | Th2 cells | Differing results; Serum IL-10 levels higher in patients with alopecia areata compared with controls; No difference reported between alopecia areata patients and controls. | (Barahmani et al., 2010, Tembhre and Sharma, 2013, Giordano and Sinha, 2013, Gautam et al., 2019) |
| IL-6 | Mainly Th17 cells and macrophages | Differing results; Significantly higher IL-6 level in patients with alopecia areata compared with controls; No significant differences between the patient and controls. | (Teraki et al., 1996, Shohat et al., 2005, Barahmani et al., 2010, Bilgic et al., 2016, Atwa et al., 2016) |
| TNF- α | Th1 cells | Differing results; Significantly increased serum TNF- α levels in alopecia areata; No significant relationship between serum TNF- α levels and alopecia areata. | (Teraki et al., 1996, Barahmani et al., 2010, Kasumagic-Halilovic et al., 2010) |
| IL-15 | Mononuclear phagocytes | Significantly increased in children with alopecia areata compared with controls. | (Tabara et al., 2019). |
| IL-13 | Th2 cells | Expression significantly elevated in the serum of patients with alopecia areata with or without atopic dermatitis. | (Tembhre and Sharma, 2013, Wang et al., 2016) |
| TGF- β | Macrophages, dendritic cells, Treg cells | Differing results; Significantly increased serum TGF- β levels in alopecia areata; No difference reported between alopecia areata patients and controls. | (Letterio and Roberts, 1998, Tembhre and Sharma, 2013, Loh et al., 2018) |

1.4.3.3 Autoantibodies in alopecia areata

Several different antibodies against hair follicle structures have been reported in alopecia areata patients, as well as in animal models of the disease (Tobin et al., 1997, Gilhar et al., 2001, Leung et al., 2010, Kemp et al., 2011a). In some cases, anti-hair follicle antibodies can be detected before hair loss is clinically evident (Tobin, 2003, Ito et al., 2013a), and a decrease in both the titre and range of autoantibodies with the concomitant re-growth of hair has been observed following treatment with immunosuppressive agents (Tobin et al., 2002, Ito et al., 2013a). It has also been reported that antibodies against hair follicle components do not prevent the growth of hair in alopecia areata-affected human skin which has been grafted onto SCID mice (Tobin et al., 1997). Overall, the exact role of hair follicle autoantibodies in the pathogenesis of alopecia areata remains obscure. However, they are possible markers for important T cell responses in alopecia areata patients, and autoreactive B cells can also act as antigen-presenting cells, thereby amplifying the autoimmune response.

1.4.3.3.1 Melanocyte-specific autoantibodies

Antibodies in alopecia areata patients can be directed against melanocyte-specific proteins such as tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and melanocyte-specific protein PMEL, but these do not appear to occur at a high frequency (Table 1.16) (Paus et al., 1993, Tobin et al., 1994a, Kemp et al., 2011a, Ito et al., 2013a). So, whether or not there is a dominant melanocyte autoantibody target has not yet been determined.

1.4.3.3.2 Keratin autoantibodies

Hair follicle-specific keratins of molecular weights 44 kD, 46 kD, 50 kD, and 52 kD can be the targets of autoantibodies in alopecia areata (Tobin et al., 1994b, Tobin, 2003). Leung and colleagues used immunoprecipitation of scalp extracts and LC-MALDI-TOF/TOF mass spectrometry to analyse alopecia areata patient sera for anti-hair follicle antibodies (Leung et al., 2010). They reported that 10/10 and 8/10 alopecia areata patients were positive for antibodies against trichohyalin and keratin-16, respectively (Table 1.16) (Leung et al., 2010). Co-localisation studies of patient sera and anti-trichohyalin and anti-keratin-16 antibodies using immunofluorescence revealed that alopecia areata patient sera contained

autoantibodies that co-localised with trichohyalin bound to all three layers of the inner root sheath of the hair follicle. In contrast, co-localised reactivity of patient autoantibodies with keratin-K16 antibody was observed only in the outer root sheath of the hair follicle (Leung et al., 2010).

1.4.3.3.3 Other autoantibody specificities

Kemp and colleagues reported tyrosine hydroxylase antibodies (Table 1.16) (Kemp et al., 2011b), predominantly of the IgG1 subclass, in patients with all variants of alopecia areata except ophiasiform, this antibody subclass response pointing to a Th1-type immune response in alopecia areata pathogenesis (Kemp et al., 2011b, Rahoma et al., 2012). The occurrence of tyrosine hydroxylase antibodies was not associated with age, sex, disease duration or the presence of autoimmune disease (Kemp et al., 2011b). The antibodies were found to recognise two major binding sites located at the amino-terminus of tyrosine hydroxylase (Kemp et al., 2011b, Rahoma et al., 2012).

Retinol-binding protein-4 (RBP-4) is a serum protein that specifically carries retinol from the liver to various target tissues (Tamori et al., 2006, Ahn et al., 2011). Ahn and colleagues demonstrated a positive IgG immunoreactivity against RBP-4 (Table 1.16) amongst patients with alopecia areata 10/15 (67%) compared with healthy controls 2/15 (13%) (Ahn et al., 2011). The RBP-4 autoantibodies co-localised with RBP-4 monoclonal antibody in the outer root sheath and companion layer. Whether the anti-RBP-4 autoantibodies trigger alopecia areata directly or indirectly remains to be explained.

The dense fine speckles 70 kD (DFS70) protein, also known as lens epithelium-derived growth factor protein of 75 kD (LEDGF) and transcriptional coactivator p75, plays an important role in the formation of transcription complexes in active chromatin, transcriptional activation of specific genes, regulation of mRNA splicing, DNA repair, and cellular survival against stress (Ortiz-Hernandez et al., 2020). Immunohistochemical techniques have shown that DFS70 is localised mainly in the outer root sheath cells of the hair follicle (Okamoto et al., 2004). Of 111 alopecia areata patients 22 (20%) of the alopecia patients were shown to be positive for anti-DFS70 antibodies that were of the IgG1 and IgG2 subtype (Okamoto et al., 2004).

Lueking and colleagues identified autoantibody targets in alopecia areata using a protein microarray of a human fetal brain cDNA expression library followed by validation utilising immunoblotting (Lueking et al., 2005). The autoantigens are listed in Table 1.16. Most targets were proteins that had no clear pathophysiological function, although the putative autoantigen FGFR3 showed homology with epidermal growth factor receptor, which plays an important part in the control of the hair cycle. FGFR3 expression was detected in the superbasal layers and the inner layers of hair follicles, and in precuticle cells in the periphery of the hair bulb. However, the relevance of the antibody response to FGFR3 and the other putative autoantigens in alopecia areata remains obscure.

Table 1.16: Examples of autoantibody targets in alopecia areata

| Antigen | Number of antibody-positive patients (%) | Reference |
|--|---|------------------------|
| Trichohyalin | 10/10 (100) | (Leung et al., 2010) |
| Keratin-16 | 8/10 (80) | (Leung et al., 2010) |
| Tyrosine hydroxylase | 6/32 (19) | (Kemp et al., 2011a) |
| Tyrosinase | 3/32 (9) | (Kemp et al., 2011a) |
| Tyrosinase-related protein-1 | 2/32 (6) | (Kemp et al., 2011a) |
| Tyrosinase-related protein-2 | 2/32 (6) | (Kemp et al., 2011a) |
| Melanocyte-specific protein PMEL | 2/32 (6) | (Kemp et al., 2011a) |
| Retinol-binding protein-4 | 10/15 (67) | (Ahn et al., 2011) |
| Dense fine speckles protein 70 kDa protein/transcription coactivator p75/lens epithelium-derived growth factor | 22/111 (20) | (Okamoto et al., 2004) |
| cDNA clone GLCDAC05 4 | 12/24 (50) | (Lueking et al., 2005) |
| EGF-like domain, multiple 3 | 11/24 (46) | (Lueking et al., 2005) |
| cDNA FLJ12693 fis, clone NT2RP1000324 | 19/24 (79) | (Lueking et al., 2005) |
| Endosulfine- α | 15/24 (63) | (Lueking et al., 2005) |
| Signal recognition particle subunit 14 | 12/24 (50) | (Lueking et al., 2005) |
| FGFR3 | 18/24 (75) | (Lueking et al., 2005) |
| Keratinocyte ubiquitin carrier protein | 17/24 (71) | (Lueking et al., 2005) |
| Erythrocyte membrane protein band 4.9 | 20/24 (83) | (Lueking et al., 2005) |
| Neuron-specific growth-associated protein | 12/24 (50) | (Lueking et al., 2005) |
| LMO4 | 18/24 (75) | (Lueking et al., 2005) |

1.4.3.4 Immune privilege and alopecia areata

The amalgamation of animal studies and observations from patients, which have been described in the previous sections, has resulted in a major theory to explain the development of alopecia areata – that of the collapse of immune privilege (Paus et al., 2003), which will be discussed in the following sections.

1.4.3.4.1 Immune privilege

The Dutch ophthalmologist Van Dooremaal was the first to observe and report the idea of immune privilege when he noted prolonged survival of mouse skin grafts placed in the anterior chamber of a dog's eye (Nieder Korn, 2006). In the past 30 years, others have demonstrated that immune privilege is the product of multiple physiological, anatomical, and immunoregulatory processes in the eye that restrict the immune system's recognition of foreign molecules and block the expression of immune-mediated inflammation that might otherwise occur in an immune-privileged site (Streilein, 2003). Many body sites are now recognised for their varying degrees of immune privilege. These include the ocular anterior chamber, brain, testes, pregnant uterus, and the hair follicle (Azzawi et al., 2018).

Evidence such as the ability of black guinea pig melanocytes to survive in hair follicles grafted onto albino recipients (Billingham and Silvers, 1971, Barker and Billingham, 1972, Reynolds et al., 1999) has led to the idea that the hair follicle is a site of immune privilege. This is suggested to be achieved by (i) physical barriers such as a lack of lymphatic drainage and the release of proteoglycans which repel immune cell infiltration (Figure 1.7) (Azzawi et al., 2018); (ii) the non-expression of MHC class I antigens on keratinocytes in the hair follicle (Figure 1.7) (Christoph et al., 2000, Paus et al., 2003, Ito et al., 2004); and (iii) very low numbers of antigen-presenting cells, T cells, and natural killer cells around the hair follicle that are effectively suppressed by Treg cell factors such as IL-10 and TGF- β as well as immunosuppressive molecules ACTH, α -MSH, and insulin-like growth factor-1 (IGF-1) (Figure 1.7) (Table 1.17) (Paus et al., 1999, Christoph et al., 2000).

Table 1.17: Functions of immunosuppressants in maintaining hair follicle immune privilege

| Immunosuppressive molecules | Site of production | Function | References |
|--|---|--|--|
| Transforming growth factor- β 1 | Outer root sheath during late anagen. | Isolates autoantigens associated with anagen and/or melanogenesis from CD8+ T cell-mediated destruction. | (Foitzik et al., 2000, Paus et al., 2003, Azzawi et al., 2018) |
| Transforming growth factor- β 2 | Bulge region of hair follicle. | Helps and protects melanocyte stem cell quiescence. | (Nishimura et al., 2010, Azzawi et al., 2018) |
| α -Melanocyte-stimulating hormone | Hair matrix and outer root sheath. | Suppresses nuclear factor- κ B activation. Upregulates cytokine synthesis inhibitor IL-10. | (Bertolini et al., 2013, Azzawi et al., 2018) |
| Adrenocorticotrophic hormone | Root sheath of the anagen hair follicle. | Powerful immunosuppressant. | (Paus et al., 2003, Azzawi et al., 2018) |
| Insulin-like growth factor-1 | Secreted by liver. | Downregulates ectopic MHC-I expression. | (Paus et al., 2005, Azzawi et al., 2018) |
| Macrophage migration inhibitory factor | Proximal follicular epithelium. | Suppresses natural killer cell attack. | (Meyer et al., 2009, Azzawi et al., 2018) |
| Indoleamine-2,3-dioxygenase | Produced by activated macrophages and other immunoregulatory cells. | Inhibits lymphocyte proliferation. | (Gilhar, 2010) |

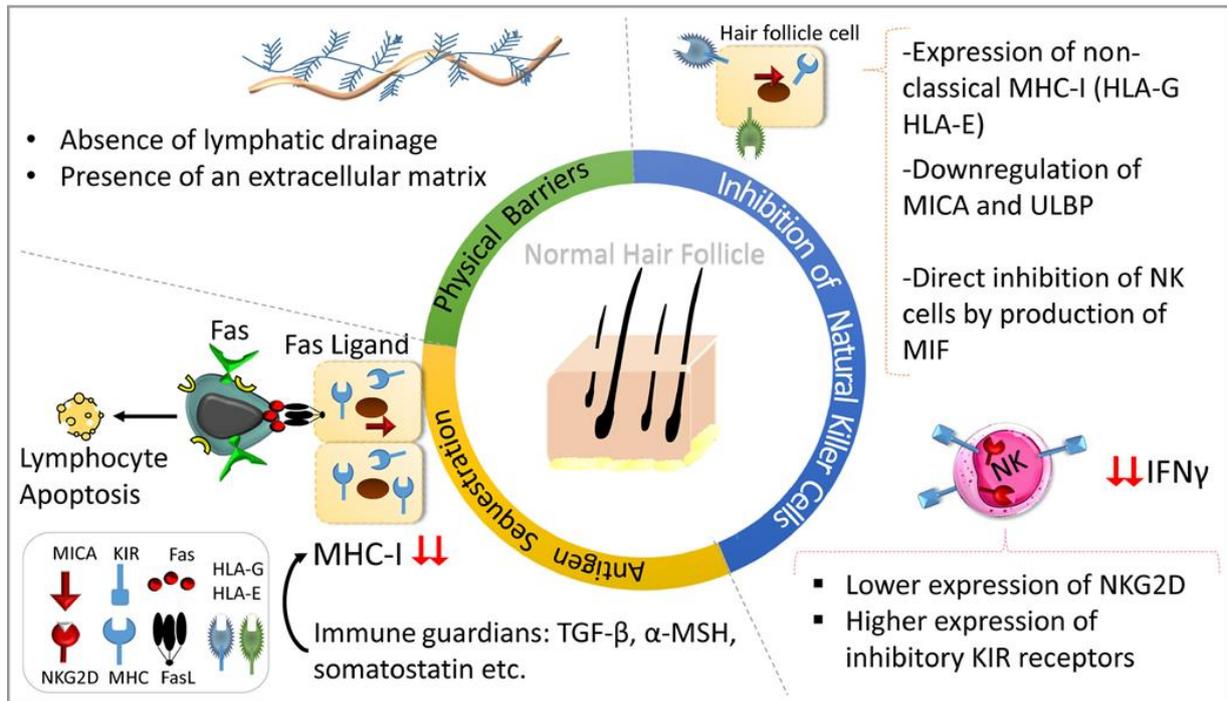


Figure 1.7: The main mechanisms of immune privilege preservation in normal hair follicles.

The main mechanisms of how immune privilege is maintained in the hair follicles are shown and include physical barriers, antigen sequestration, and the inhibition of natural killer (NK) cells that are one of the main cell types involved in alopecia areata pathogenesis. NK cells express a wide range of receptor complexes. The main activating receptor, NKG2D, binds with major histocompatibility complex (MHC) class I chain-related A (MICA) and UL16-binding protein (ULBP), and the major inhibitory receptor, killer cell Ig-like receptor (KIR), binds with human leucocyte antigen (HLA) molecules. The KIR receptors allow the NK cells to be self-tolerated towards normal cells. IFN- γ , interferon- γ ; MIF, macrophage migration inhibitory factor; α -MSH, α -melanocyte-stimulating hormone; TGF- β , transforming growth factor- β . The image, taken from a paper by Rajabi et al, 2019, is used with kind permission from John Wiley & Sons, Inc.

1.4.3.4.2 Immune privilege collapse in alopecia areata

The collapse of immune privilege in alopecia areata was initially proposed by Paus and colleagues (Paus et al., 1993). Since then, many studies have provided evidence of immune privilege breakdown in alopecia areata. These have included the findings of T cells, NK cells, and dendritic cells in the anagen hair follicle's peri-bulbar region (Todes-Taylor et al., 1984, Mounsey and Reed, 2009), as well as increased MHC class I antigen expression in alopecia areata lesions compared with normal hair follicles (Messenger and Bleehen, 1985, Brocker et al., 1987, Khoury et al., 1988). In addition, lower levels of molecules that maintain immune privilege have been found in alopecia areata lesional and peri-lesional areas including TGF- β and α -MSH (Kang et al., 2010b, Subramanya et al., 2010). Other molecules that aid immune responses are upregulated in alopecia areata lesions, for example, MHC class I chain-related A (MICA), chemokines, intercellular adhesion molecules, and IFN- γ , which is the major factor in alopecia areata pathogenesis (Arca et al., 2004, Gilhar et al., 2005, Freyschmidt-Paul et al., 2006, Ito et al., 2008, Subramanya et al., 2010, Ito et al., 2013b, Xing et al., 2014). There have been two main theories put forward to explain the loss of immune privilege: the first theory emphasises local disturbances in the hair follicles whilst the second suggests a dysregulated immune system is responsible.

1.4.3.4.3 Local stress in the hair follicles

This theory of immune privilege collapse in alopecia areata suggests that environmental stresses result in the accumulation of reactive oxygen species in the keratinocytes of hair follicles (Figure 1.8a) (Bakry et al., 2014, Prie et al., 2015). In individuals who are genetically susceptible, such stresses are not managed effectively and lead to the expression of MICA on keratinocytes (Groh et al., 1996, Bauer et al., 1999, Yamamoto et al., 2001, Ito et al., 2008), which then become engaged with NKG2D receptors on NK cells. This interaction results in IFN- γ production, the further accumulation of NK cells, and the expression of MHC class I antigens on hair follicle cells which then present previously hidden antigens to T cells (Ito et al., 2008, Gilhar, 2010, Bakry et al., 2014, Yenin et al., 2015, Prie et al., 2015, Islam et al., 2015). The stressed environment has also been suggested to decrease the expression of molecules that normally protect immune privilege (Ito et al., 2008, Azzawi et al., 2018).

1.4.3.4.4 Immune system dysregulation

The second theory of immune privilege collapse in alopecia areata suggests that the first step is the activation of immune cells in the lymphatic system (Figure 1.8b). CD8+NKG2D⁺ effector T memory cells upregulated in the lymph nodes infiltrate hair follicles producing IFN- γ which induces the expression of MHC antigens and MICA on hair follicle cells making them susceptible to attack by T lymphocytes (Zoller et al., 2002, McElwee et al., 2005a, McElwee et al., 2005b). Again, the expression of immunosuppressive factors such as IL-10 and TGF- β is reduced (Kang et al., 2010a), which perhaps results from the improper functioning of Tregs, and so contributes to the collapse of immune privilege (Gilhar et al., 2007).

1.4.3.4.5 Key immune responses following immune privilege collapse

One of the key events of both mechanisms for immune privilege collapse is the production of IFN- γ around the hair follicles by either NK cells or activated T lymphocytes (Figure 1.8). Importantly, IFN- γ induces the expression of MHC class I antigens, NKG2D, and chemokines which build up an inflammatory cycle around the hair follicle (Ljunggren and Karre, 1990, Ito et al., 2004, Ito et al., 2008, Gilhar, 2010, McPhee et al., 2012, Xing et al., 2014, Dai et al., 2016), with CXCL9, CXCL10 and CXCL11 attracting further immune cells (McPhee et al., 2012, Ito et al., 2013b, Xing et al., 2014, Dai et al., 2016). Expression of cytokines IL-2 and IL-15 from either activated immune cells or stressed keratinocytes, as well as their receptors on CD8⁺ T lymphocytes, is elevated around the hair follicles (Freyschmidt-Paul et al., 2006, Budagian et al., 2006, Colpitts et al., 2012, Xing et al., 2014, Jabri and Abadie, 2015, Fuentes-Duculan et al., 2016). The normally suppressive effect of Tregs is reduced by IL-15 (Marcais et al., 2014, Jabri and Abadie, 2015). This cytokine can also activate NK cells by inducing the expression of NKG2D (Roberts et al., 2001, Tang et al., 2013) and promote the cytotoxic functions of CD8⁺ T lymphocytes (Meresse et al., 2004, Meresse et al., 2006). In addition to cell-mediated immunity, the expression of MHC class II antigens on hair follicle cells and their interaction with CD4⁺ T lymphocytes can promote humoral immune responses and autoantibody production. However, such autoantibodies are unlikely to induce hair loss, though they may be a marker for hair follicle destruction in alopecia areata (Hull et al., 1991a).

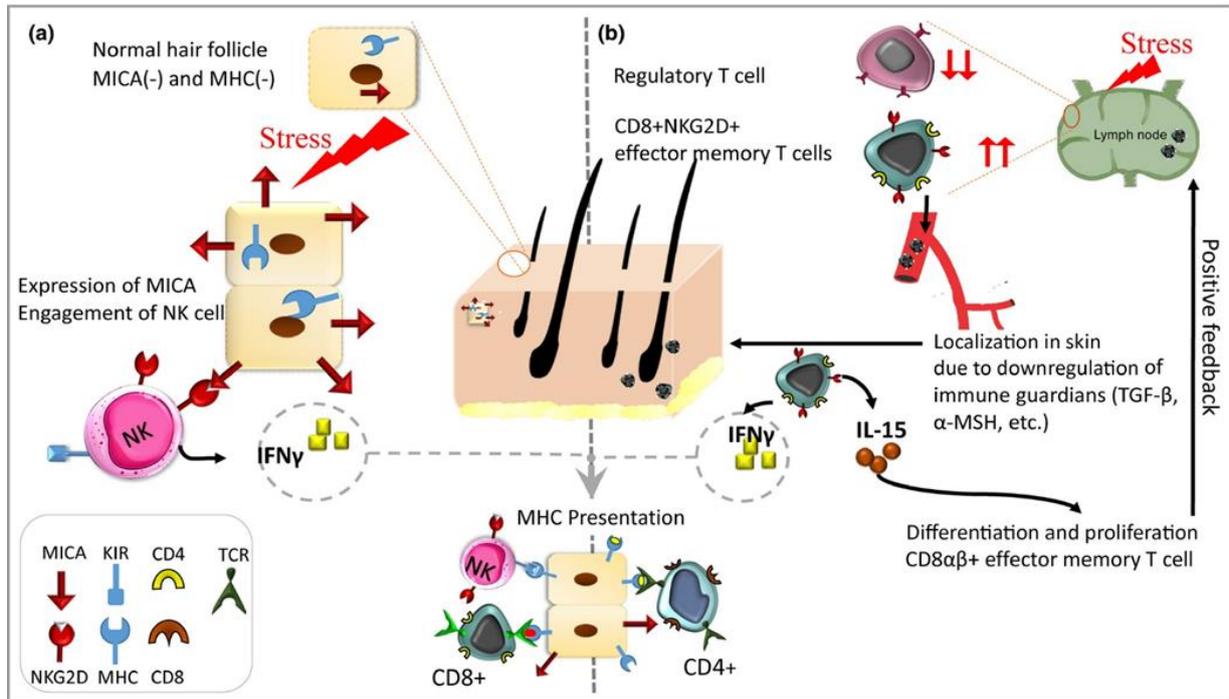


Figure 1.8: The two distinct scenarios for immune privilege collapse in alopecia areata.

The two distinct scenarios for alopecia areata development are shown. **(a)** Immune privilege collapse starting at the hair follicles. Stressors provoke expression of major histocompatibility complex (MHC) class I chain-related A (MICA) proteins on keratinocytes, leading to activation of natural killer (NK) cells and secretion of interferon- γ (IFN- γ). MHC-I protein expression is induced by IFN- γ , revealing the previously obscured antigens to T cells. **(b)** Immune privilege collapse could result from large amounts of CD8+ NKG2D+ effector memory T cells, which upregulate in regional lymph nodes and then invade the hair follicles. These cells produce interleukin-15 (IL-15) and IFN- γ . TCR, T cell receptor; TGF- β , transforming growth factor- β . The image, taken from a paper by Rajabi et al, 2019, is used with kind permission from John Wiley & Sons, Inc.

1.5 Aims of the project

From the review of the literature, it can be concluded that the exact pathogenesis of alopecia areata remains to be fully elucidated, although the evidence from research strongly indicates that hair loss results in part from targeting of the hair follicles by CD4+ and CD8+ T cells. Current therapies for alopecia areata, which are predominantly topical or systemic corticosteroids and topical immuno-modulating agents, are often ineffective and new targeted therapies are especially required for children, for people with long-term patchy alopecia, and for individuals with alopecia totalis or alopecia universalis. Crucially, a detailed understanding of the pathogenesis of alopecia areata is required to inform the development of new treatments, and this includes the identification of the hair follicle autoantigens that are targeted by T cells.

With the characterisation of dominant alopecia areata-associated autoantigens, it may be possible to develop tolerising therapies whereby injection with antigen/peptide/encoding cDNA is used to induce tolerance to autoimmune responses. Such a protocol has been developed to combat autoimmune encephalomyelitis (Brocke et al., 1996, Robinson et al., 2003). In addition, identification of the autoantigen profile in alopecia areata may enable the therapeutic use of T lymphocytes directed against specific autoreactive T cells. This type of therapy has also been applied to the treatment of autoimmune encephalomyelitis (Jyothi et al., 2002).

Previously, a major hair follicle target trichohyalin was identified using patient sera to precipitate hair follicle autoantigens that were then identified by liquid chromatography and mass spectrometry (Leung et al., 2010). Subsequently, trichohyalin was also found to be targeted by pathogenic T lymphocytes (Wang et al., 2016). Likewise, melanocyte targets such as PMEL, tyrosinase, and tyrosinase-related protein-2 have been identified as autoantibody targets in alopecia areata patients, albeit at a low frequency (Kemp et al., 2011b), as well as being recognised by pathogenic T lymphocytes (McElwee et al., 1996a, Gilhar et al., 2001, Nagai et al., 2006). However, a major melanocyte autoantibody target has not been reported.

Therefore, the main aim of this project was to identify a major melanocyte autoantibody target in alopecia areata patients as this would also likely be targeted by T cells (McElwee et

al., 1996a, Gilhar et al., 2001, Nagai et al., 2006). In addition, autoreactive B cells can act as antigen-presenting cells, thereby amplifying the autoimmune response. Further to the useful information gained with respect to developing novel therapeutics, the identification and characterisation of autoantibody targets in alopecia areata patients may also be helpful in disease diagnosis, prognosis, clinical classification, and as a means of monitoring treatment efficacy.

The specific objectives of the project were to:

1. Describe the alopecia areata patients included in this study with respect to their demographic and clinical details including autoantibodies.
2. Identify novel melanocyte targets of autoantibodies in alopecia areata using phage-display technology.
3. Confirm the humoral immune response against any novel melanocyte autoantigens in a panel of alopecia areata patients
4. Ascertain any correlations between the presence of specific autoantibodies and the clinical features of alopecia areata.

Chapter 2

General Materials and Methods

2 General Materials and Methods

All the written protocols in this thesis were provided by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK).

2.1 Study approval

The Sheffield Research Ethics Committee, Sheffield, UK, approved this study (Reference: 09/H1308/128), and informed written consent was obtained from all participants. The Research Department of the Sheffield Teaching Hospitals NHS Trust, Sheffield, UK, was responsible for the research governance of the study (Reference: STH15125).

2.2 Participants

The study included 48 patients with alopecia areata who attended dermatology clinics at the Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Trust, Sheffield, UK. As controls, 50 healthy individuals (20 males; 30 females; mean age 36 years; age range: 22-53 years) with no personal or family history of alopecia areata or of any other autoimmune disease also participated in the study.

2.3 Preparation of patient sera and immunoglobulin G

Blood samples of 10-20 ml were collected from patients and controls. To prepare sera, blood samples were centrifuged at 4°C at 3,000 *g* for 10 min, and the supernatants removed to a clean sterile tube. Serum samples were stored at –80°C until required for analysis. According to the manufacturer's protocol, immunoglobulin G (IgG) was prepared from serum samples using protein G Sepharose 4 Fast Flow affinity column chromatography (GE Healthcare Life Sciences, Little Chalfont, UK). The IgG samples were dialysed extensively against phosphate-buffered saline pH 7.4 (PBS) at 4°C. The IgG samples were then concentrated using an Amicon Concentrator (Amicon Inc., Beverly, MA, USA), and sterilised using a Millex Filter Unit (Merck Millipore Ltd., Carrigtwohill, Ireland). IgG concentrations were estimated by spectrophotometry at 280 nm using NanoDrop Software with a NanoDrop ND-1000 Spectrometer (Labtech, Wilmington, DE, USA). All IgG samples were kept at 10 mg/ml at –20°C. The IgG preparations were carried out by Dr Helen Kemp.

2.4 Plasticware

Universal tubes, Oak Ridge tubes, tips for Gilson pipettes, Eppendorf tubes (0.5-ml and 1.5-ml), 5-ml and 10-ml pipettes, and petri-dishes were obtained from either Bibby Sterilin Ltd. (Bargoed, UK), Nalgene Nunc International (Rochester, NY, USA), Sarstedt Ltd. (Numbrecht, Germany), Starlab (UK) Ltd. (Milton Keynes, UK) or Corning Incorporated (Corning, NY, USA).

2.5 Chemicals and reagents

Most chemicals, reagents, buffers, and media constituents were obtained from either Melford Laboratories (Ipswich, UK), from Sigma-Aldrich (Poole, UK) or Promega (Southampton, UK). Acids and solvents were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). The suppliers of certain other reagents and kits are given in the text as appropriate.

2.6 Bacterial strains

The *Escherichia coli* (*E. coli*) K-12 bacterial strains used in this study are listed in Table 2.1. Bacterial strains carrying plasmid vectors or recombinant plasmids were constructed by transformation. All strains of *E. coli* were grown from frozen stocks by streaking onto LB agar with appropriate antibiotic/s added, and incubating overnight at 37°C. A single bacterial colony of the required *E. coli* strain was then inoculated into Luria Bertani (LB) medium containing appropriate antibiotics and grown in a shaking incubator at 250 revolutions per minute (rpm) and 37°C overnight. For long-term storage at -80°C, 0.7-ml aliquots of an overnight-grown bacterial culture were mixed with 0.3-ml aliquots of 50% sterile glycerol.

2.7 Luria Bertani medium

For growing bacterial cells, LB medium was made in deionised water with 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. The LB medium was sterilised by autoclaving for 15 min at 120°C. Luria Bertani agar was made by adding agar to LB medium to 1.5%. After sterilisation by autoclaving, LB agar was left to cool to 45°C. The required antibiotics were added to the cooled LB agar, which was then poured into 90-mm petri-dishes. Once set and dried, the LB agar plates were kept at 4°C until required.

2.8 Antibiotics

Ampicillin (sodium salt) and kanamycin sulphate were prepared in deionised water as concentrated stocks to 100 mg/ml and 50 mg/ml, respectively. Tetracycline hydrochloride was made to 10 mg/ml in 50% ethanol to water. All antibiotics were sterilised using a 0.22- μ m Millex[®]-GP Filter Unit (Merck Millipore Ltd.) and were stored at -20°C.

2.9 Bacterial transformation

For transformation, a 50- μ l sample of chemically competent *E. coli* JM109 (Promega, Southampton, UK) cells was thawed from the -80°C freezer. The required plasmid DNA was mixed with the thawed competent cells and left for 5-10 min on ice. Then, the cells were heat-shocked by putting them in a 42°C waterbath for 45 seconds. The cells were then returned to ice for 2 min. Afterwards, the cells were put into a sterile universal tube that contained 950 μ l of SOC medium that consisted of 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, and 20 mM glucose (Life Technologies, Paisley, UK). The cells were incubated for 1 h at 37°C so that the antibiotic resistance genes on the plasmid DNA could be expressed. Aliquots of 100- μ l of the transformed cells, as well as 1:10 and 1:100 dilutions, were streaked onto LB agar plates containing the required antibiotic(s). The plates were incubated overnight at 37°C. A sample of untransformed cells was spread onto a LB agar plate as a control. Individual colonies were purified by streaking onto a fresh LB agar plate with the required antibiotic(s) and incubated overnight at 37°C.

Table 2.1: Bacterial strains

| Strain | Details | Supplier |
|------------------------------|--|---|
| <i>E. coli</i> JM109 | Used in the preparation of plasmid vectors and recombinant plasmids. | Promega (Southampton, UK) |
| <i>E. coli</i> XL1-Blue MRF' | Used in the phage-display technology. Carries episome with tetracycline resistance. | Agilent Technologies (Wokingham, UK) |

2.10 Plasmids

All plasmids are listed in Table 2.2. The original plasmids into which cDNAs were cloned are shown in Figures 2.1-2.3. Plasmids were stored at -20°C in TE (Tris-ethylenediaminetetraacetic acid) buffer that contained 10 mM Tris-hydrochloride and 1 mM ethylenediaminetetraacetic acid (EDTA) and was at pH 8.0.

2.11 Mini plasmid preparations

The Wizard[®] Plus SV Minipreps DNA Purification System (Promega) was used to purify plasmid DNA from a 5-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 pellet 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 $\mu\text{g}/\text{ml}$ RNase A) and the cells were then lysed by adding 250 μl of Cell Lysis Solution (0.2 M sodium hydroxide; 1% 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% 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. In order to recover 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. Plasmid DNA was checked qualitatively using agarose gel electrophoresis and stored at -20°C until required.

Table 2.2: Plasmids

| Plasmid | Details | Source |
|----------------|---|---|
| pDNA3-TYR | pcDNA3 (Figure 2.1) containing tyrosinase cDNA cloned into the <i>KpnI</i> and <i>XbaI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Helen Kemp (University of Sheffield, Sheffield, UK) |
| pDNA3-TRP1 | pcDNA3 containing tyrosine-related protein-1 cDNA cloned into the <i>XhoI</i> and <i>XbaI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Helen Kemp |
| pCMVDTS | pRc/CMV (Figure 2.2) containing tyrosine-related protein-2 cDNA cloned into the <i>HindIII</i> restriction site. Selectable resistance marker for ampicillin. | Professor Shigeki Shibahara (Tohoku University School of Medicine, Sendai, Japan) |
| pMEL | pcDNA3 containing melanocyte-specific protein PMEL cDNA cloned into the <i>EcoRI</i> and <i>XhoI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Paul Robbins (National Institutes of Health, Bethesda, MD, USA) |
| pDNA3-TH | pcDNA3 containing tyrosine hydroxylase cDNA cloned into the <i>HindIII</i> and <i>BamHI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Helen Kemp |
| pMelanA | pcDNA3 containing MelanA cDNA cloned into the <i>EcoRV</i> restriction site. Selectable resistance marker for ampicillin. | Dr Helen Kemp |
| pMCHR1 | pcDNA3 containing melanin-concentrating hormone receptor 1 cDNA cloned into the <i>EcoRI</i> and <i>XbaI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Helen Kemp |
| pGPNMB | pcDNA3.1 (Figure 2.3) containing glycoprotein non-metastatic melanoma protein b cDNA cloned into the <i>KpnI</i> and <i>HindIII</i> restriction sites. Selectable resistance marker for ampicillin. | GeneScript (Piscataway, NJ, USA) |
| pOCA2 | pcDNA3.1 containing OCA2-encoded P protein cDNA cloned into the <i>KpnI</i> and <i>HindIII</i> restriction sites. Selectable resistance marker for ampicillin. | GeneScript (Piscataway, NJ, USA) |
| pMC1R | pcDNA3 containing melanocortin 1 receptor cDNA cloned into the <i>HindIII</i> and <i>XhoI</i> restriction sites. Selectable resistance marker for ampicillin. | Dr Helen Kemp |
| pJuFo | A 4.3-kb phage-display vector containing the leucine zippers Jun and Fos, and the filamentous phage coat protein, pIII. Selectable resistance marker for ampicillin. | Professor Reto Crameri (Swiss Institute of Allergy and Asthma Research, Davos, Switzerland) |

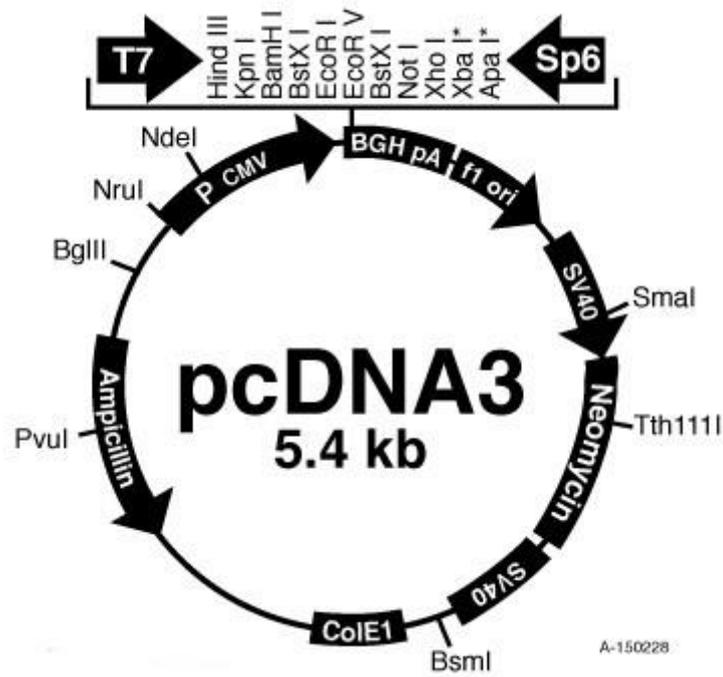


Figure 2.1: Plasmid pcDNA3.

The plasmid restriction sites are illustrated as well as the T7 and SP6 promoters.

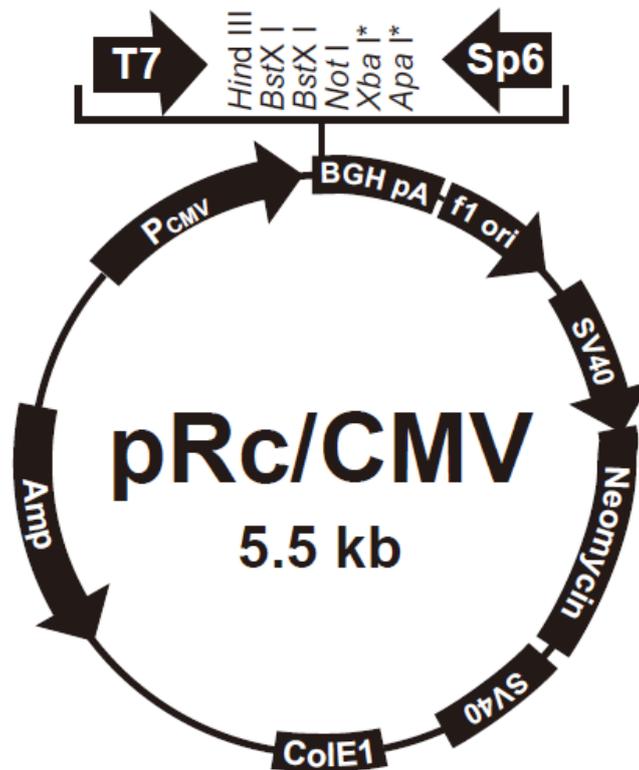


Figure 2.2: Plasmid pRc/CMV.

The plasmid restriction sites are illustrated as well as the T7 and SP6 promoters.

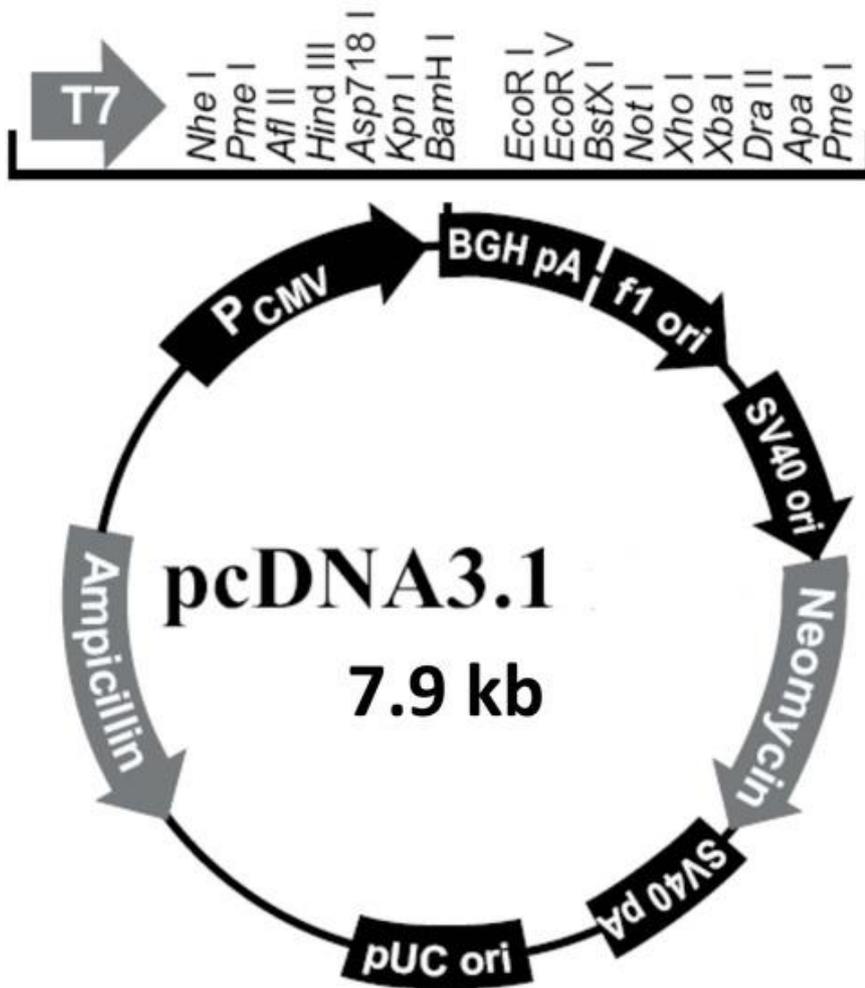


Figure 2.3: Plasmid pcDNA3.1.

The plasmid restriction sites are illustrated as well as the T7 promoter.

2.12 Maxi plasmid preparations

For large-scale plasmid preparation, a bacterial culture carrying the appropriate plasmid was made by inoculating 1 litre of LB medium containing the required antibiotic(s), with a 10-ml starter culture. The large-scale culture was incubated overnight at 37°C in a shaking incubator. The cells were collected by centrifugation at 4,000 *g* for 30 min and plasmid prepared using a Qiagen Plasmid DNA Maxiprep Kit (Qiagen Ltd., Crawley, UK). The cell pellet was re-suspended in 10 ml of P1 Buffer that contained 50 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA and 100 µg/ml RNaseA. Then, 10 ml of P2 Buffer that contained 0.2 M sodium hydroxide and 1% SDS were added to the re-suspended cells in order to lyse them. The cells were gently mixed before being incubated for 5 min at room temperature. Ten millilitres of P3 Buffer that contained 1.32 M potassium acetate (pH 5.5) were added to the cell lysate and incubated for 20 min on ice. The lysate was centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant collected by decanting.

Subsequently, a Qiagen column from the kit was equilibrated by adding 10 ml of QBT Buffer that contained 750 mM sodium chloride, 50 mM 3-[N-morpholino]propanesulphonic acid (MOPS) (pH 7.0), 15% isopropanol and 0.15% Triton X-100. The column was left to stand to empty by gravity. The cell lysate (supernatant) was put onto the column and the flow through discarded. The column was washed with 30 ml of QC Buffer that contained 1 M sodium chloride, 50 mM MOPS (pH 7.0) and 15% isopropanol. The plasmid was eluted from the column with 15 ml of QF Buffer that contained 1.25 M sodium chloride, 50 mM Tris-hydrochloride (pH 8.5) and 15% isopropanol. The collected solution containing the plasmid was precipitated by adding 10.5 ml of 100% isopropanol. The plasmid was harvested by centrifugation at 4°C at 15,000 *g* for 30 min. The plasmid was washed with 70% ethanol and re-suspended in 500 µl of TE buffer. Plasmid DNA was checked qualitatively using agarose gel electrophoresis, and quantitatively using spectrophotometry at 260 nm with a NanoDrop ND-1000 Spectrometer and NanoDrop Software (Labtech).

2.13 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis. The gels were 0.8-1% agarose and were prepared by microwaving agarose for 1-2 min in Tris-acetate-EDTA (TAE) buffer that contained

40 mM Tris-acetate and 1 mM EDTA (pH 8.3) (Promega). After cooling, 2 µl of ethidium bromide solution (10 mg/ml) (Promega) were added per 50 ml of molten agarose. This was poured into a Sub-Cell® Horizontal Electrophoresis System casting deck with a well-former/comb in place (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Once set, the comb was removed and the gel placed into the electrophoresis tank containing TAE buffer.

For visualisation purposes, 6x Blue-Orange Loading Dye that contained 0.4% orange G, 0.03% bromphenol blue, 0.03% xylene cyanol FF, 10 mM Tris-hydrochloride (pH 7.5) and 50 mM EDTA (pH 8.0) and 15% Ficoll® 400 (Promega) was added to DNA samples. These were then loaded into the gel wells. For sizing purposes, DNA markers were included on the gel. These were a 0.5-µg sample of a 1-kb DNA Ladder (500-10,000-bp DNA fragments) (New England Biolabs®, Hitchin, UK). Gels were electrophoresed in a Sub-Cell® Horizontal Electrophoresis System tank (Bio-Rad Laboratories Ltd.) at 85 volts using a PowerPac Basic Power Supply (Bio-Rad Laboratories Ltd.). For viewing and recording of the gels, a GBOX Gel Documentation System and GeneSnap Image Acquisition Software (Syngene, Cambridge, UK) was used.

2.14 DNA sequencing

DNA was sequenced using the University of Sheffield Medical School's Genetics Core Facility. Plasmids and sequencing primers (Table 2.3) were provided to the service at a concentration of 50-100 ng/µl and 1 pmol/µl, respectively. Both the T7 and SP6 primers (Table 2.3) were used check plasmids were as expected before use in experiments. pJuFo primers (Table 2.3) were used to analyse plasmids obtained from phage-display panning experiments. Sequencing reactions were done using an Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI 3730 Capillary Sequencer (Applied Biosystems, Foster City, CA, USA).

2.15 DNA and protein analyses

To analyse DNA and protein sequences, the Lasergene® Core Suite version 11.0 (DNASTAR, Inc., Madison, WI, USA) was used along with the online facilities of the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (<http://www.ebi.ac.uk/>) (Cambridge, UK) and the ExPASy Bioinformatics Resources Portal (<http://web.expasy.org>) (SIB Swiss Institute of Bioinformatics, Switzerland). When needed,

searches for homology between DNAs and between proteins were done using the GenBank database and the BLAST online facility of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Bethesda, MD, USA).

Table 2.3: Oligonucleotide sequencing primers

| Primer | Sequence | Source |
|---------------|--------------------------------|---|
| T7 | 5'-TAATACGACTCACTATAGGG-3' | Promega (Southampton, UK) |
| SP6 | 5'-ATTAACCCTCACTAAAGGGA-3' | Promega |
| JUFO-1192 | 5'-CCGCTGGATTGTTATTACTCGCTG-3' | Eurofins Genetic Services Ltd. (London, UK) |
| JUFO-1500 | 5'-TGCAAGGCGATTAAGTTGGGTAAC-3' | Eurofins Genetic Services |

2.16 *In vitro* coupled transcription-translation of cDNA

To produce the required [³⁵S]-methionine-labelled protein for radioligand binding assays, the appropriate encoding cDNA (in a plasmid) was translated in a TnT[®] T7-Coupled Reticulocyte Lysate System (Promega), according to the manufacturer's instructions. A reaction was set up in a 50- μ l total volume and consisted of 0.5 μ g of plasmid DNA (Table 2.2), 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 [³⁵S]-methionine (1,000 Ci/mmol) (Perkin-Elmer LAS UK Ltd., Beaconsfield, UK). The reaction was incubated at 30°C for 1.5 h. It was kept at -40°C until needed for experiments. The radiolabelled protein was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography to check the size was as expected (Section 2.17).

Trichloroacetic acid-precipitation was used to estimate the percentage of [³⁵S]-methionine that had been incorporated into the translated protein. This work was carried out as directed by the manufacturer (Promega) and was done by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). The *in vitro* translation reaction (2 μ l) was added to 98 μ l of mix of 2% hydrogen peroxide and 1 M sodium hydroxide. The reaction was incubated for 10 min at 37°C. Cold 25% trichloroacetic acid and 2% casamino acids (900 μ l) (Difco, Detroit, MI, USA) were added followed by 30 min further incubation on ice. Precipitated translation products were collected vacuum filtering 250 μ l of the reaction onto a Whatman GF/A filter (Whatman International Ltd., Maidstone, UK). The filter was pre-wetted with 5% trichloroacetic acid. The filter was washed with cold 5% trichloroacetic acid and then with 1 ml of acetone. The filter was dried at room temperature before counting in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA, USA). For analysing the total counts per min (cpm) in the reaction, a sample (5- μ l) was spotted on to a filter, which was dried before counts were determined. The percent incorporation of [³⁵S]-methionine was determined as: $100 \times (\text{cpm of washed filter} / \text{cpm of unwashed filter} \times 50)$, and this ranged from 17-25%.

2.17 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and autoradiography

SDS-PAGE was used to analyse radiolabelled proteins from the *in vitro* translation reactions and was done using 10% gels. The resolving gel was 10% ProtoGel (37.5:1 acrylamide:bisacrylamide) (GeneFlow, Lichfield, UK) in 375 mM Tris-hydrochloride (pH 8.8), 0.1% ammonium persulphate 0.1% SDS and 0.1% N, N, N, N'-tetramethylethylenediamine (TEMED). The stacking gel was 4% ProtoGel in 125 mM Tris-hydrochloride (pH 6.8), 0.05% ammonium persulphate, 0.1% SDS and 0.1% TEMED.

The gel was made using the glass plates of a Bio-Rad Mini-Protean Tetra Cell system (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). It was allowed to set after overlaying with butanol-1-ol. Once set, the butanol-1-ol was removed from the gel and a gel comb placed between the glass plates. The stacking gel was placed on top of the resolving gel and allowed to set. The gel was then assembled into the gel tank and the gel comb removed. The gel running buffer that contained 25 mM Tris-base, 0.1% SDS and 192 mM glycine (pH 8.3) (GeneFlow) was added to the gel tank.

A sample (5- μ l) of radiolabelled protein was mixed with 20 μ l of 2x Laemmli sample buffer that contained 2% SDS, 25% glycerol, 0.01% bromophenol blue, 2% 2-mercaptoethanol and 62.5 mM Tris-hydrochloride (pH 6.8) (Bio-Rad Laboratories Ltd.). The sample was kept at 100°C for 5 min. A 10- μ l sample was then loaded onto the SDS-PAGE gel. Prestained SDS-PAGE Standards, Low Range (21-103 kDa) or Precision Plus Protein All Blue Standards (10-250 kDa) (Bio-Rad Laboratories Ltd.) were also applied to the gel as markers. The gel was run for 20 min at 44 volts and subsequently for 1.5 h at 111 volts. Afterwards, the gel was removed from the gel tank and glass plates and transferred to fixing solution (50% methanol and 10% glacial acetic acid) at room temperature for 30 min.

The gel was then placed in Amersham Amplify™ Fluorographic Reagent (GE Healthcare Life Sciences, Little Chalfont, UK), for 30 min and then in 7% methanol/1% glycerol for 5 min. The gel was dried at 60°C for 2 h onto 3MM Whatman paper (Whatman International Ltd., Maidstone, UK) using a Bio-Rad Gel Dryer 583 (Bio-Rad Laboratories Ltd.). The dried gel was autoradiographed by exposing to x-ray film (Genetic Research Instrumentation Ltd., Dunmow,

UK) overnight at room temperature. The x-ray film was developed using Photosol CD18 developer (Photosol Ltd., Basildon, UK) and fixed by Photosol CF40 fixer (Photosol Ltd.).

The SDS-PAGE gels were performed by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK).

2.18 Radioligand binding assays

The binding of antibodies to radiolabelled antigens (Table 2.4) was determined using in radioligand binding assays (RLBAs). For the assay, a 1-2- μ l aliquot (equivalent to 50,000 cpm of trichloroacetic acid-precipitable material) of the required *in vitro* translation reaction was added to 50 μ l of immunoprecipitation buffer that contained 20 mM Tris-hydrochloride (pH 8.0), 150 mM sodium chloride, 1% Triton X-100 and 10 mg/ml protease inhibitor aprotinin. A sample of serum from a patient or from a healthy control or a positive control antiserum (Table 2.5) was added at the dilution that was required. After overnight incubation at 4°C, a 50- μ l aliquot of protein G Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences) that had been prepared according to the supplier, was added. The reaction was incubated at 4°C for a further 1.5 h.

During the incubation, protein G Sepharose bead-antibody-antigen complexes formed. These were harvested by centrifuging for 30 seconds at 5000 rpm. The beads were washed thoroughly in immunoprecipitation buffer; each of six washes was for 15 min at 4°C with shaking. After the final wash, 50 μ l of immunoprecipitation buffer were added to the beads. The protein G Sepharose bead-antibody-antigen complexes were then put into a scintillation vial with 1 ml of scintillation fluid (Molecular Devices, Sunnyvale, CA, USA). The amount radioactivity that had been immunoprecipitated due to interaction of beads, antibody and radiolabelled antigen was determined in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA, USA). Samples were tested in duplicate and the mean of the cpm was calculated.

The binding of antibodies to a radiolabelled antigen was expressed as an antibody index. The antibody index was: $\text{cpm immunoprecipitated by serum sample} / \text{mean cpm immunoprecipitated by 50 healthy control serum samples}$. Each serum sample was analysed

in three experiments. The mean antibody index was calculated. The upper limit of normal for each RLBA was: the mean antibody index + 3SD of the 50 healthy control subjects. Patients with an antibody index over the upper limit of normal were regarded as positive for antibody reactivity.

2.19 Antibodies

The antibodies (Table 2.5) used in the study were stored as directed by the manufacturers and used at the the dilution recommended by the manufacturers.

Table 2.4: Antigens used in radioligand binding assays

| Antigen required in radioligand binding assay | Plasmid for <i>in vitro</i> translation | Reference for radioligand binding assay |
|--|--|--|
| Tyrosinase | pDNA3-TYR | (Kemp et al., 1997a) |
| Tyrosinase-related protein-1 | pDNA3-TRP1 | (Kemp et al., 1998a) |
| Tyrosinase-related protein-2 | pCMVDTS | (Kemp et al., 1997b) |
| Melanocyte-specific protein PMEL | pMEL | (Kemp et al., 1998b) |
| Tyrosine hydroxylase | pDNA3-TH | (Kemp et al., 2011a) |
| MelanA | pMelanA | (Kemp et al., 2002a) |
| Melanin-concentrating hormone receptor 1 | pMCHR1 | (Kemp et al., 2002b) |
| Glycoprotein non-metastatic melanoma protein b | pGPNMB | This study |
| OCA2-encoded P protein | pOCA2 | This study |
| Melanocortin 1 receptor | pMC1R | This study |

Table 2.5: Positive control antibodies for radioligand binding assays

| Antibody | Details of immunogen | Source |
|---|---|--|
| Rabbit anti-tyrosinase antibody α -PEP7 | Synthetic peptide matching to the carboxy-terminal of tyrosinase. | Prof Vincent Hearing (National Institutes of Health, Bethesda, MD, USA) |
| Mouse anti- tyrosinase-related protein-1 (TYRP1) antibody TA99 | Recombinant full-length human TYRP1 protein. | Prof Sheila MacNeil (University of Sheffield, Sheffield, UK) |
| Rabbit anti-tyrosinase-related protein-2 (TRP2) antibody α -PEP8 | Synthetic peptide matching the carboxy-terminal of TRP2. | Prof Vincent Hearing (National Institutes of Health, Bethesda, MD, USA) |
| Rabbit anti-melanocyte-specific protein PMEL antibody AZN-LAM | Recombinant human full-length PMEL | Dr Marco Schreurs (University Hospital Nijmegen, Nijmegen, the Netherlands). |
| Rabbit anti-tyrosine hydroxylase (TH) antibody ab59276 | Synthetic peptide close to the amino-terminal of TH. | Abcam, Inc. (Cambridge, MA, USA) |
| Mouse anti-MelanA antibody A103 | Recombinant full-length MelanA protein | Novocastra Laboratories (Newcastle-upon-Tyne, UK). |
| Rabbit anti-melanocyte concentrating hormone receptor 1 (MCHR1) antibody MCHR11-S | A 16-amino acid peptide close to the carboxy-terminal of MCHR1. | Alpha Diagnostics International, Inc. (San Antonio, TX, USA) |
| Rabbit anti- glycoprotein non-metastatic melanoma protein b (GPNMB) antibody sc271435 | Synthetic peptide matching the carboxy-terminal of GPNMB. | Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) |
| Rabbit anti-OCA2-encoded P protein (OCA2 protein) antibody PA5-23708 | Synthetic peptide matching the carboxy-terminal of OCA2 protein. | Thermo Fisher Scientific, (Waltham, MA, USA) |
| Rabbit anti-melanocortin 1 receptor (MC1R) antibody PA5-21911 | Synthetic peptide matching a region at amino acids 253 and 317 of MC1R. | Thermo Fisher Scientific, (Waltham, MA, USA) |

2.20 Antibody titres

In order to determine the titres of specific antibodies, RLBA (Section 2.18) were used to test patient serum samples at a series of dilutions that ranged from 1:100 to 1:10000. An antibody index was determined at each dilution. Antibody titres were estimated as the serum dilution at which antibodies were detectable above the upper limit of normal for the RLBA.

2.21 Antibody absorption

Absorption experiments were performed to analyse antibody specificity. Cell extracts were a gift from Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). The cell extracts had been prepared from HEK293 (human embryonic kidney 293) cells that were expressing the required antigen. Extracts of HEK293 cells were also available. Cell extracts were in buffer that contained 150 mM sodium chloride, 10 mM Tris-hydrochloride (pH 7.4), 1% Triton X-100 and Protease Cocktail Inhibitor (Sigma-Aldrich). The extracts were at a protein concentration of 1 mg/ml and were kept at -80°C. To carry out the absorption experiments, a 100- μ l sample of sera was pre-incubated with a 100 μ l-sample of the cell extract of choice. After incubating at 4°C for 16 h, the absorbed serum was recovered by centrifuging for 1 h at 4°C at 45,000 *g*. Unabsorbed and pre-absorbed serum samples were then analysed in RLBA (Section 2.18), to test their immunoreactivity against a specific antigen.

2.22 Statistical analyses

The statistical analyses performed in this study were for comparing categorical data - Fisher's exact tests or Chi-square tests. For comparing two unpaired groups of continuous data - Unpaired t tests. For comparing multiple continuous data sets - one-way ANOVA. The tests were carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). *P* values < 0.05 were regarded as significant.

Chapter 3

Demographic and Clinical Profiles of a Sheffield Alopecia Areata Patient Cohort

3 Demographic and Clinical Profiles of a Sheffield Alopecia Areata Patient Cohort

3.1 Introduction

In several studies, the demographic profile and clinical features of alopecia areata patient cohorts have been described (Safavi et al., 1995, Sharma et al., 1996a, Tan et al., 2002b, Jain and Marfatia, 2003, Yang et al., 2004, Guzman-Sanchez et al., 2007, Kavak et al., 2008, Lundin et al., 2014, Mirzoyev et al., 2014, Sobolewska-Wlodarczyk et al., 2016, Strazzulla et al., 2018), and they are summarised in Section 1.2. In brief, clinically, alopecia areata can be divided according to the area of involvement and is classified as patchy alopecia, alopecia totalis and alopecia universalis (Alkhalifah et al., 2010a, Finner, 2011). Patchy type is the most prevalent form, occurring in up to 90% of patients (Alkhalifah et al., 2010a, Finner, 2011). Other less common presentations of alopecia areata can be observed, for example, ophiasis, ophiasis inversus, and sisaipho (Alkhalifah et al., 2010a, Finner, 2011).

The disease affects all races and both sexes, and likely affects males and females equally (Wasserman et al., 2007, Sobolewska-Wlodarczyk et al., 2016, Strazzulla et al., 2018). However, many studies reported sex variation with a higher incidence in females; male to female ratios have been reported to range from 1:2.6 to 1:1.2 (Tan et al., 2002b, Guzman-Sanchez et al., 2007, Lundin et al., 2014). In contrast, four studies showed a male predominance ranging from 2:1 to 1.1:1 (Sharma et al., 1996a, Jain and Marfatia, 2003, Yang et al., 2004, Kavak et al., 2008). Many studies have reported that all age groups can be affected, but the mean age at disease onset is 25-36 years (Tan et al., 2002b, Yang et al., 2004, Finner, 2011, Mirzoyev et al., 2014, Villasante Fricke and Miteva, 2015).

Several autoimmune and autoinflammatory disorders are associated with alopecia areata at a high frequency, including autoimmune thyroid disease and vitiligo (Muller and Winkelmann, 1963, Tan et al., 2002b, Kasumagic-Halilovic, 2008, Chu et al., 2011, Huang et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015, Han et al., 2018, Lee et al., 2019b). In addition, atopy is especially prevalent in alopecia areata patients (Tan et al., 2002b, Barahmani et al., 2009, Acikgoz et al., 2014), and nail abnormalities are frequent (Sharma et al., 1998, Tan et

al., 2002b, Goh et al., 2006, Kasumagic-Halilovic and Prohic, 2009). Furthermore, a family history of alopecia areata has been observed in 0-8.6% of patients (Tan et al., 2002b, Yang et al., 2004, Guzman-Sanchez et al., 2007).

3.2 Aim

The aim of this chapter was to describe the Sheffield alopecia areata patient cohort in terms of its demographic and clinical details.

3.3 Materials and Methods

3.3.1 Clinical assessment of patients

Details recorded of the 48 recruited alopecia areata patients were sex, age at inclusion in the study, age at onset of alopecia areata, duration of disease, alopecia areata severity and activity, and the presence of nail abnormalities and atopy. Also noted was the occurrence of autoimmune and autoinflammatory diseases that were confirmed by reviewing the medical history of each patient and by carrying out a physical examination as well as relevant laboratory tests when required.

Initially, types of non-cicatricial alopecia including androgenetic alopecia, tinea capitis, and telogen effluvium were excluded, along with cicatricial alopecias such as chronic discoid lupus erythematosus, lichen planus follicularis, alopecia of secondary syphilis, Brocq's pseudopelade, and folliculitis decalvans. Genetic conditions associated with hair loss were also excluded.

The severity of alopecia areata was assessed using the Severity of Alopecia Tool (SALT) score based on the combination of extent and severity of scalp hair loss (Guttikonda et al., 2016). From this assessment, alopecia areata was classified as patchy as defined by one, multiple separate or conjoined (reticular) patches of hair loss; totalis as defined by total or near-total loss of hair on the scalp; universalis as defined by total to near-total loss of hair on all haired surfaces of the body; ophiasis as defined by hair loss in a band-like shape along the circumference of the head, more specifically along the border of the temporal and occipital bones; or sisaipho as defined by extensive alopecia except around the periphery of the scalp. The assessment of disease activity was based on the subjective history of progression and an objective evaluation of a hair pull test at the margins of each individual patch (Finner, 2011, McDonald et al., 2017).

Atopy, defined as allergic disease with a very high serum IgE level in response to common environmental proteins or to a skin prick test to any food or inhaled allergen, includes diseases such as atopic dermatitis, rhinoconjunctivitis, asthma, and hayfever. The diagnosis of atopic

dermatitis was made clinically using the validated UK diagnostic criteria (Flohr et al., 2004, Brenninkmeijer et al., 2008).

Nail abnormalities were diagnosed by visual examination.

Autoimmune hypothyroidism was diagnosed by the combination of elevated thyroid-stimulating hormone (TSH) (normal range, 0.4-4.0 mU/l), low thyroxine (T4) (normal range, 1.1-3.0 nmol/l), and positive thyroid peroxidase (TPO) antibodies (normal < 9 IU/ml) (Chaker et al., 2017, Almutairi et al., 2019).

Hypopituitarism was diagnosed by low pituitary hormone levels. Hormones tested included growth hormone (normal level, > 5.0 µg/l), prolactin (normal range, 3.0-27.0 ng/ml), luteinising hormone (normal range, 2–8 U/l), follicle-stimulating hormone (normal range, 1–8 U/l), TSH (normal range, 0.4-4.0 mU/l), and adrenocorticotrophic hormone in the morning fasting status and by performing stimulation tests if necessary (normal range, 1.1–11.0 pmol/l) (van Aken and Lamberts, 2005, Kim, 2015, Almutairi et al., 2019).

Lichen sclerosus was diagnosed by physical examination of the affected area, and if required, a biopsy of the affected tissue taken for examination under a microscope (Fistarol and Itin, 2013).

Rheumatoid arthritis was diagnosed by a combination of medical history, physical examination, blood tests for rheumatoid factor and cyclic citrullinated peptide antibodies, and measurement of erythrocyte sedimentation rate and C-reactive protein levels (Wasserman, 2011).

Vitiligo diagnosis was based on the presence of depigmented macules on the skin. According to the Vitiligo Global Issues Consensus Conference (VGICC) classification (Ezzedine et al., 2012), vitiligo was categorised as segmental, non-segmental, or unclassified/undetermined. Segmental vitiligo was defined as one or a few macules in one area, but in a non-dermatomal pattern. Unclassified vitiligo included focal vitiligo, and mucosal vitiligo when affecting one site. Non-segmental vitiligo was defined by a symmetrical distribution of the depigmented macules, and included acral, acrofacial, symmetrical, and universal subtypes. Mixed vitiligo

was considered when there was evidence of the coexistence of segmental and non-segmental types.

3.3.2 Antibody testing

The presence of autoantibodies against tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL, tyrosine hydroxylase, MelanA, and melanin-concentrating hormone receptor 1 was analysed using radioligand binding assays as detailed in Materials and Methods Section 2.18.

3.4 Results

A summary of the demographic and clinical details of the 48 British Caucasian alopecia areata patients recruited to the study is given in Table 3.1.

3.4.1 Alopecia areata severity and activity

Patchy alopecia areata was predominant with 68.8% (33/48) of patients diagnosed with this type (Table 3.1; Figure 3.1). Ten of 48 (20.8%) patients showed a pattern of alopecia totalis, 4 (8.3%) a pattern of alopecia universalis, and only one (2.1%) showed a pattern of ophiasis alopecia areata (Table 3.1; Figure 3.1). Overall, 15/48 (31.3%) patients had a severe form of alopecia areata presenting as either totalis, universalis or ophiasis. No cases of sisaipho pattern alopecia areata were evident. In all patients with severe forms of alopecia areata, the disease was active. Of the patients with patchy alopecia areata, all but one case had active disease.

Table 3.1: Overall summary of alopecia areata patient details

| Detail | Alopecia areata patients (n = 48) |
|--|--|
| Demography | |
| Male | 13 (27.1%) |
| Female | 35 (72.9%) |
| Mean age \pm SD in years (range in years) | 46 \pm 16 (18-87) |
| Mean age \pm SD at disease onset in years (range in years) | 36 \pm 16 (7-74) |
| Mean disease duration \pm SD in years (range in years) | 11 \pm 11 (1-50) |
| Alopecia areata type | |
| Patchy | 33 (68.8%) |
| Totalis | 10 (20.8%) |
| Universalis | 4 (8.3%) |
| Ophiasis | 1 (2.1%) |
| Sisaipho | 0 (0%) |
| Alopecia areata activity | |
| Stable | 1 (2.1%) |
| Active | 47 (97.9%) |
| Atopy | |
| None | 12 (25.0%) |
| Present | 36 (75.0%) |
| Family history | 13 (27.1%) |
| Autoimmune/autoinflammatory disease | |
| None | 29 (60.4%) |
| Present | 19 (39.6%) |
| Family history | 16 (33.3%) |
| Nail abnormalities | |
| None | 31 (64.6%) |
| Present | 17 (35.4%) |
| Autoantibodies | |
| None | 30 (62.5%) |
| Present | 18 (37.5%) |

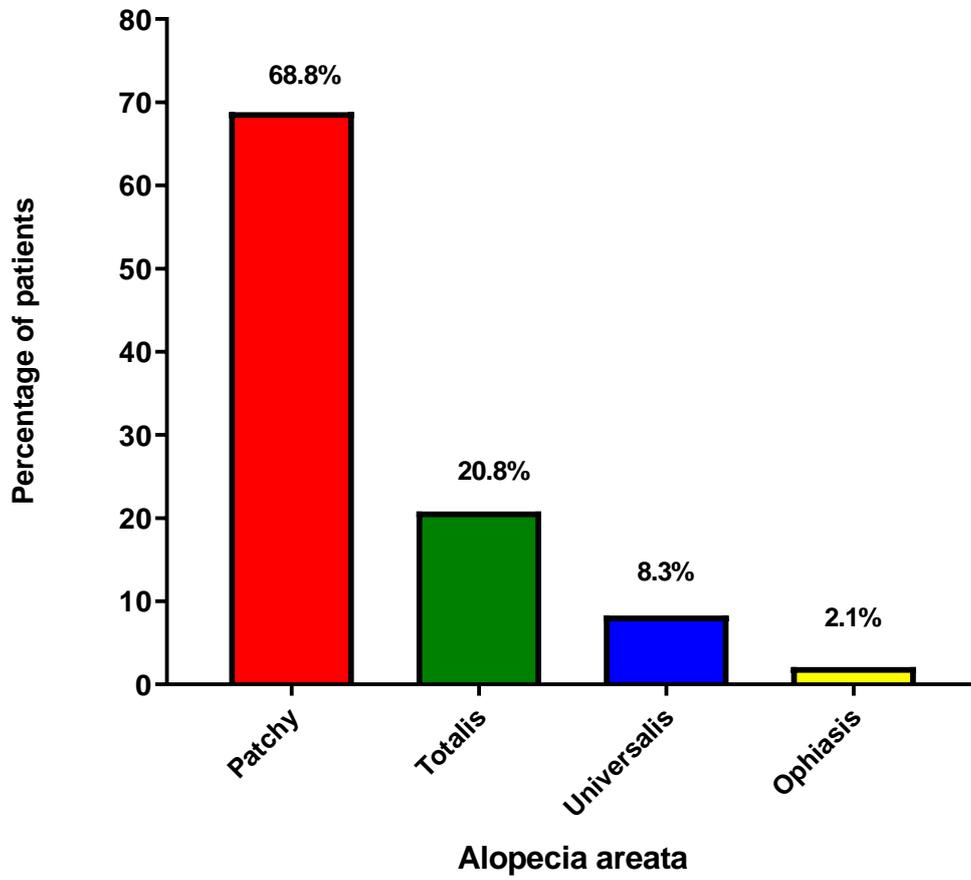


Figure 3.1: Distribution of types of alopecia areata.

The percentage of the 48 alopecia areata patients with patchy, totalis, universalis, and ophiasis type alopecia areata is illustrated.

3.4.2 Age and sex of alopecia areata patients

The ratio of males to females was 1:2.7 with 13 (27.1%) male and 35 (72.9%) female patients (Table 3.1). The age range of the patients was 18-87 years with a mean age of 46 ± 16 years (Table 3.1).

The female patients had a mean age of 46 ± 17 years with a range of 18-87 years, and the male patients, 47 ± 13 years with a range of 34-78 years. No significant difference was noted between the female and male groups in terms of age profile ($P > 0.05$, Unpaired t test).

Of the 35 female patients, 14 (40.0%) had severe alopecia areata and 21 (60%) had patchy hair loss. With respect to the 13 male cases, severe alopecia areata was diagnosed in one (7.7%) and patchy in 12 (92.3%). The frequency of severe alopecia areata was significantly higher in female patients ($P = 0.039$, Fisher's exact test).

3.4.3 Age at onset of alopecia areata

The onset age for alopecia areata in the 48 patients ranged from 7-74 years (Table 3.1). The mean age of disease onset was 36 ± 16 years (Table 3.1). On grouping cases into early-onset (1-16 years), adult-onset (17-50 years), and late-onset (> 50 years), the majority of patients, 39/48 (81.3%) had developed alopecia areata by the age of 50 years (Figure 3.2).

The sex distribution, in relation to onset age, is given in Table 3.2. No significant difference was evident between the female and male groups, with respect to onset age distribution ($P > 0.05$, Chi-square test).

In the majority of patients with patchy and severe alopecia areata, the disease started in adulthood with 29/33 (87.9%) and 15/15 (100%) cases, respectively (Table 3.2). All four early-onset (1-16 years) cases of alopecia areata presented with the patchy type with no severe forms reported (Table 3.2). The distribution of patchy and severe cases of alopecia areata between the three onset age categories did not differ significantly ($P > 0.05$, Chi-square test).

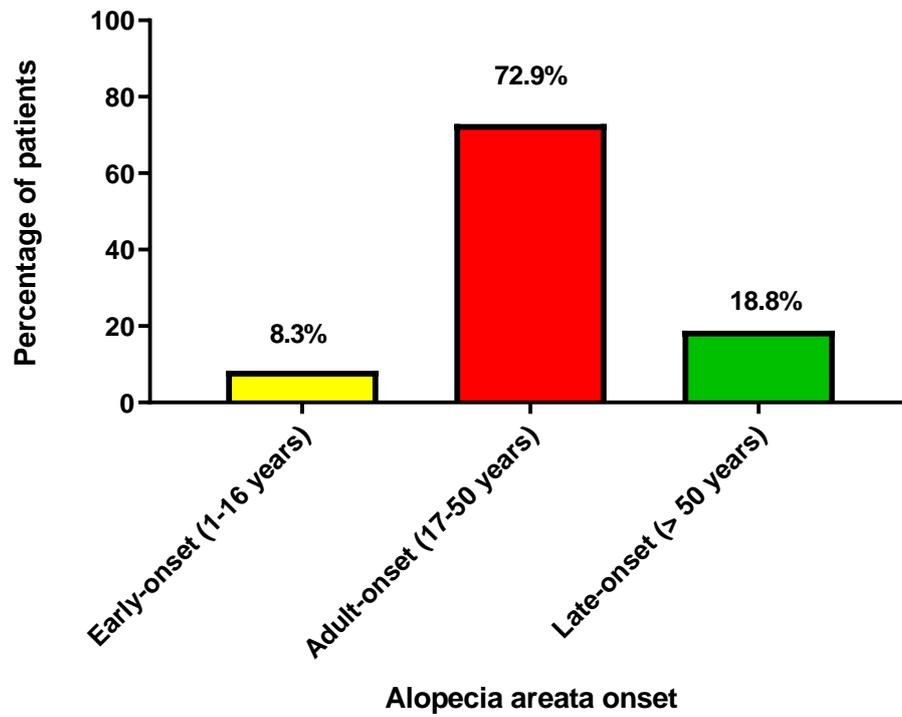


Figure 3.2: Age distribution at onset of alopecia areata.

The percentage of the 48 alopecia areata patients with early-onset (1-16 years), adult-onset (17-50 years), and late-onset (> 50 years) alopecia areata is illustrated.

Table 3.2: Age of onset in relation to sex and alopecia areata type

| Alopecia areata onset age (years) | Sex distribution (male <i>n</i> = 13; female <i>n</i> = 35) | Patchy type alopecia areata (<i>n</i> = 33) | Severe types of alopecia areata¹ (<i>n</i> = 15) |
|--|--|--|--|
| Early-onset (1-16) | 1 (7.7%) male 3 (8.6%) females | 4 (12.1%) | 0 (0%) |
| Adult-onset (17-50) | 10 (76.9%) males 25 (71.4%) females | 24 (72.7%) | 11 (73.3%) |
| Late-onset (> 50) | 2 (15.4%) males 7 (20.0%) females | 5 (15.2%) | 3 (26.7%) |

¹Includes alopecia totalis, universalis, and ophiasis.

3.4.4 Duration of alopecia areata

At the time of referral to the clinic, the duration of alopecia areata in the 48 patients ranged from 1-50 years (Table 3.1). The mean disease duration was 11 ± 11 years (Table 3.1). Most patients, 19/48 (39.6%), had had alopecia areata for 5 years or less at the time of inclusion in the study (Figure 3.3). In all, 32/48 (66.7%) patients had suffered from alopecia areata for 10 years or less (Figure 3.3).

The sex distribution, in relation to disease duration, is given in Table 3.3. No significant difference was evident between the female and male groups, with respect to disease duration ($P > 0.05$, Chi-square test).

The majority of patients with patchy, 24/33 (72.7%), and severe, 8/15 (53.3%), alopecia areata had had the disease for years 10 years or less when they were recruited to the study (Table 3.3). The distribution of patchy and severe cases of alopecia areata between the three disease duration categories did not differ significantly ($P > 0.05$, Chi-square test).

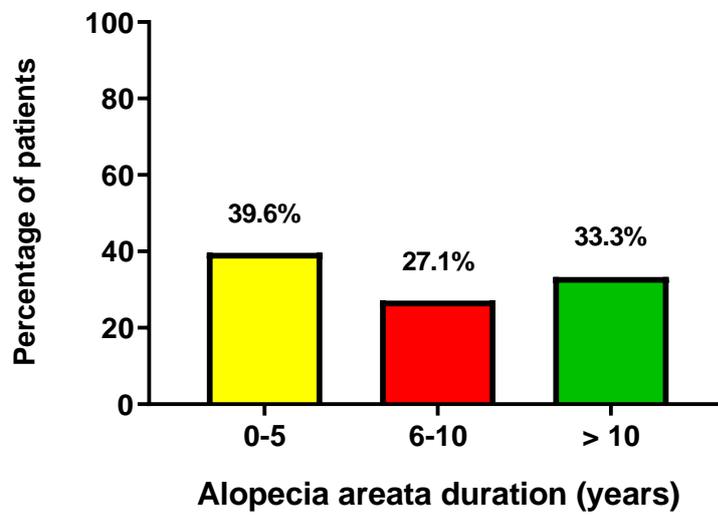


Figure 3.3: Distribution of alopecia areata duration.

The percentage of the 48 alopecia areata patients in each interval of disease duration is illustrated.

Table 3.3: Disease duration in patchy and severe alopecia areata

| Disease duration (years) | Sex distribution (male <i>n</i> = 13; female <i>n</i> = 35) | Patchy type alopecia areata (<i>n</i> = 33) | Severe types of alopecia areata¹ (<i>n</i> = 15) |
|---------------------------------|--|--|--|
| 0-5 | 6 (46.2%) male 13 (37.1%) females | 15 (45.5%) | 4 (26.7%) |
| 6-10 | 3 (23.1%) males 10 (28.6%) females | 9 (27.3%) | 4 (26.7%) |
| > 10 | 4 (30.8%) males 12 (34.3%) females | 9 (27.3%) | 7 (46.7%) |

¹Includes alopecia totalis, universalis, and ophiasis.

3.4.5 Alopecia areata and atopy

Atopy was present in 36/48 (75.0%) of the alopecia areata patients (Table 3.1). Of the 36 patients with atopy, 27 (75.0%) and nine (25.0%) had patchy and severe alopecia areata, respectively. Comparing patchy and severe alopecia areata cases, 27/33 (81.8%) and 9/15 (60.0%), respectively, had atopy. However, there was no significant statistical difference in the overall frequency of concurrent atopy amongst the different types of alopecia areata ($P > 0.05$, Fisher's exact test).

A positive family history of atopy was evident in 13/48 (27.1%) of patients (Table 3.1). Regarding types of alopecia areata, 10/33 (30.3%) with patchy and 3/15 (20.0%) with severe forms had a family history of atopy. However, comparisons showed there was no significant difference in the frequency of a family history of autoimmunity amongst patchy and severe types of alopecia areata ($P > 0.05$, Fisher's exact test).

3.4.6 Alopecia areata and nail abnormalities

Seventeen of the 48 (35.4%) patients had nail changes including nail pitting, trachyonychia and longitudinal ridging (Table 3.4). There were no cases of distal notching or Beau's lines (Table 3.4). Of the nine patients with nail pitting, seven had nail pitting only and two had nail pitting and longitudinal ridging. Of the six patients with longitudinal ridging, four had this abnormality alone. Four patients had nail changes in form of trachyonychia.

Of the 17 patients with nail abnormalities, 12 (70.6%) had a severe form of alopecia areata and 5 (29.4%) had patchy alopecia areata. Comparing patchy and severe alopecia areata cases, 5/33 (15.2%) and 12/15 (80.0%), respectively, had vitiligo. Comparisons showed there was a significant difference in the frequency of concurrent nail abnormalities amongst patchy and severe types of alopecia areata ($P < 0.0001$, Fisher's exact test).

Table 3.4: Nail changes in the alopecia areata patients

| Nail abnormality | Number of patients (%) (<i>n</i> = 48) |
|-------------------------|---|
| Any abnormality | 17 (35.4) |
| Pitting | 9 (18.8) |
| Trachyonychia | 4 (8.3) |
| Longitudinal ridging | 6 (12.5) |
| Distal notching | 0 (0) |
| Beau's lines | 0 (0) |

3.4.7 Alopecia areata and autoimmune/autoinflammatory disease

3.4.7.1 Personal autoimmunity

Vitiligo of the symmetrical type was recorded in 12/48 (25.0%) patients and autoimmune hypothyroidism in 8/48 (16.7%) (Figure 3.4). Lichen sclerosus, rheumatoid arthritis, and hypopituitarism were found in two (4.2%), one (2.1%), and one (2.1%) patients, respectively (Figure 3.4). No cases of psoriasis, type 1 diabetes, Graves' disease, systemic lupus erythematosus, or inflammatory bowel disease were evident.

Of the 48 patients, 19 (39.6%) had at least one concomitant autoimmune/autoinflammatory disease in addition to their alopecia areata (Table 3.1). Fifteen (31.2%) patients had only one concomitant disease that included nine with vitiligo, one with lichen sclerosus, and five with autoimmune hypothyroidism. Three (6.3%) patients had two concomitant diseases. These were a patient with vitiligo and autoimmune hypothyroidism, one with vitiligo and hypopituitarism, and one with autoimmune hypothyroidism and lichen sclerosus. Only one (2.1%) patient had three concomitant diseases that were vitiligo, autoimmune hypothyroidism, and rheumatoid arthritis.

Of the 19 patients with at least one other autoinflammatory or autoimmune disorder, 14 (73.7%) and five (26.3%) had patchy and severe alopecia areata, respectively. Comparing patchy and severe alopecia areata cases, 14/33 (42.4%) and 5/15 (33.3%), respectively, had at least one autoinflammatory/autoimmune disease. However, comparisons showed there was no significant difference in the overall frequency of concurrent autoimmune or autoinflammatory diseases amongst patchy and severe types of alopecia areata ($P > 0.05$, Fisher's exact test).

Of the eight patients with autoimmune hypothyroidism, four (50.0%) had patchy and four (50.0%) had severe alopecia areata. Comparing patchy and severe alopecia areata cases, 4/33 (12.1%) and 4/15 (26.7%), respectively, had autoimmune hypothyroidism. However, there was no significant difference in the frequency of concurrent autoimmune hypothyroidism amongst patchy and severe types of alopecia areata ($P > 0.05$, Fisher's exact test).

Of the 12 patients with vitiligo, 11 (91.7%) had patchy and one (8.3%) had severe alopecia areata. Comparing patchy and severe alopecia areata cases, 11/33 (33.3%) and 1/15 (6.7%), respectively, had vitiligo. However, comparisons showed there was no significant difference in the frequency of concurrent vitiligo amongst patchy and severe types of alopecia areata ($P = 0.0729$, Fisher's exact test).

3.4.7.2 Family history of alopecia areata and other autoimmunity

There was a family history of alopecia areata and/or other autoimmune disease in 16/48 (33.3%) of patients (Table 3.1). No patients had a family history of alopecia areata alone. Three patients (6.3%) had a family history of alopecia areata and at least one other autoimmune disorder, and 13 (27.1%) reported only autoimmune disease in their family history. Of the three patients with a family history of alopecia areata, all of them also had a family history of autoimmune disease with autoimmune thyroid disease in two, and autoimmune thyroid disease and vitiligo in one. The diseases reported in the 13 alopecia areata patients who had a family history of only autoimmune disease were: autoimmune thyroid disease, 4; type 1 diabetes, 1; vitiligo, 2; pernicious anaemia, 1; type 1 diabetes and autoimmune thyroid disease, 1; vitiligo and autoimmune thyroid disease, 4.

Figure 3.5 shows the frequency of autoimmune diseases including alopecia areata recorded in the family histories of the alopecia areata patient cohort. Autoimmune thyroid disease was the most common at 22.9% (11/48 patients) followed by vitiligo at 16.7% (8/48 patients), alopecia areata at 6.25% (3/48), type 1 diabetes at 4.2% (2/48 patients), and pernicious anaemia at 2.1% (1/48 patients).

Regarding types of alopecia areata, 12/33 (36.4%) with patchy and 4/15 (26.7%) with severe forms had a family history of autoimmune diseases inclusive of alopecia areata. However, comparisons showed there was no significant difference in the frequency of a family history of autoimmunity amongst patchy and severe types of alopecia areata ($P > 0.05$, Fisher's exact test).

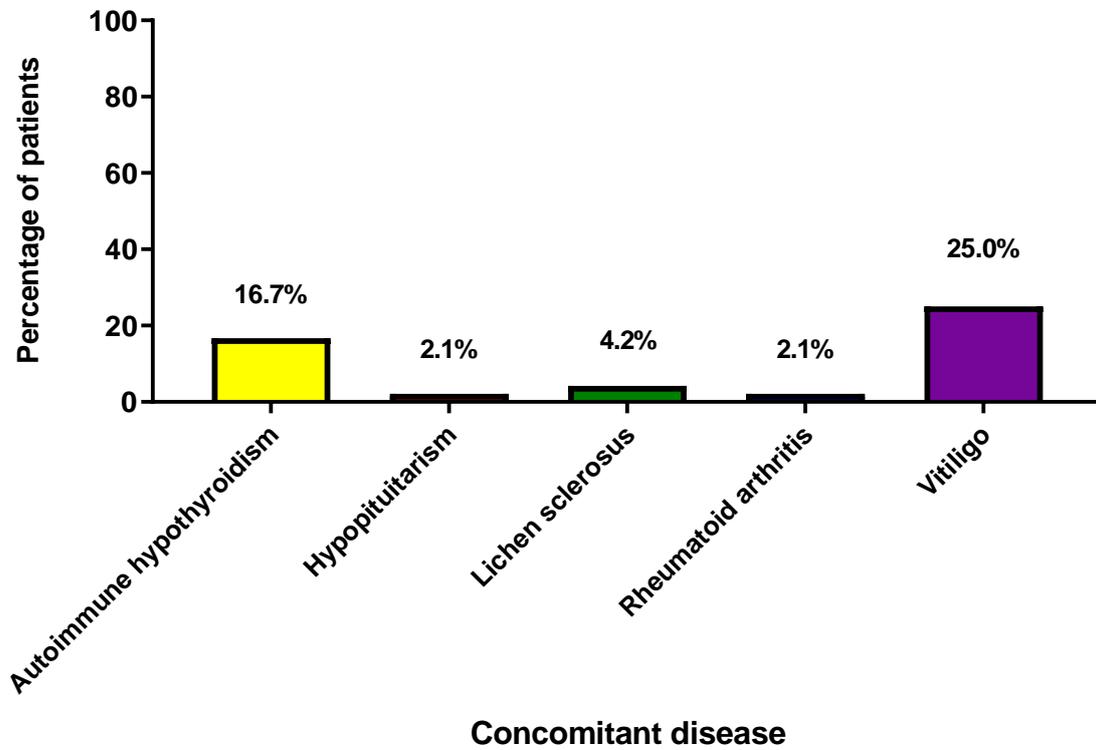


Figure 3.4: Frequency of concomitant autoinflammatory and autoimmune diseases.

The percentage of the 48 alopecia areata patients with each concomitant autoinflammatory and autoimmune disease is illustrated.

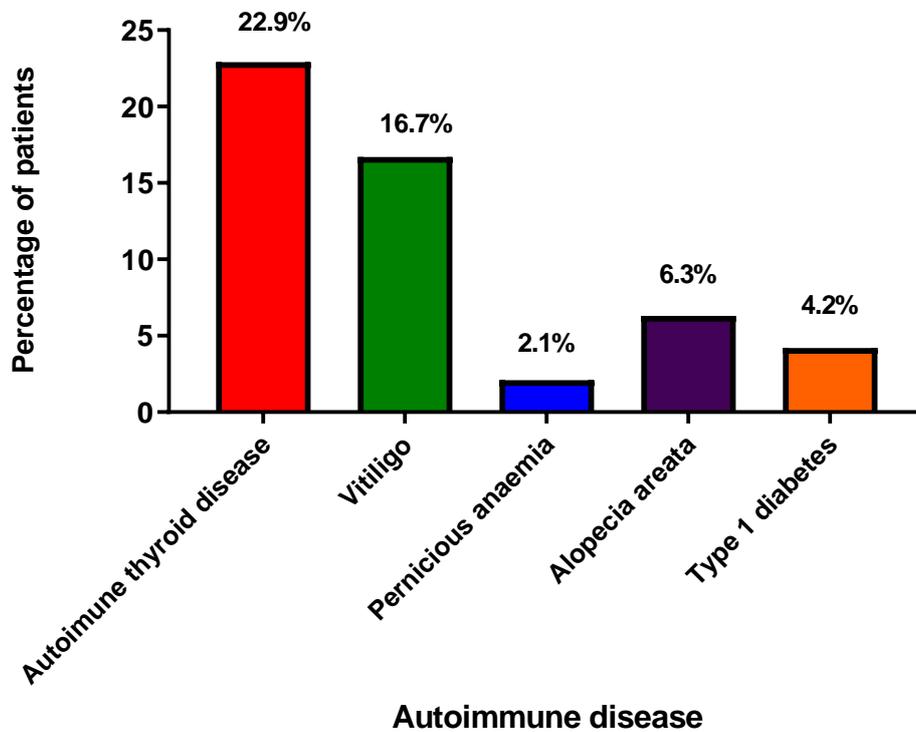


Figure 3.5: Frequency of alopecia areata and autoimmune diseases in patient family history.

The frequency of autoimmune diseases in the family history of 48 patients with alopecia areata.

3.4.8 Presence of antibodies

The sera of the alopecia areata patients were analysed for the presence of several antibodies that had previously been reported in individuals with the disease, including tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL, and tyrosine hydroxylase (Kemp et al., 2011b). In addition, sera were investigated for antibodies to the vitiligo-associated autoantigen melanin-concentrating hormone receptor 1 (Kemp et al., 2002b) as well as to MelanA, a melanocyte antigen that has been reported to stimulate cytotoxic T cell reactivity in alopecia areata patients (Wang et al., 2016). Radioligand binding assays to test for antibodies against tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL, tyrosine hydroxylase, MelanA, and melanin-concentrating hormone receptor 1 were carried out as previously been described (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Kemp et al., 2002a, Kemp et al., 2002b, Kemp et al., 2011a).

Initially, in order to produce [³⁵S]-labelled antigens for use in the RLBA, the appropriate cDNA carried by the relevant plasmid (Table 2.4) was translated using a TnT[®] T7-Coupled Reticulocyte Lysate System as detailed in Section 2.16. The radiolabelled ligands were then used to investigate alopecia areata patient ($n = 48$) and control ($n = 50$) sera for antibodies in RLBA. These were undertaken as described in Section 2.18. Sera were tested at 1:100 final dilution and in duplicate. An appropriate positive control antiserum (Table 2.5) was also tested in the assays at a dilution of 1:100.

The levels of antibody in each serum sample were calculated as an antibody index: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 50 healthy control sera. Sera were analysed in three assays and the mean antibody index was derived. The upper limit of normal for each RLBA was calculated as: mean antibody index + 3SD of the population of 50 healthy controls. Sera with an antibody index above the normal upper limit were classed as positive for the antibody specificity under analysis.

The results of the RLBA indicated that 18/48 (37.5%) patients were positive for at least one of the antibodies tested with antibody indices above the upper limits of normal (Table 3.5). Twelve (66.7%) patients had more than one antibody specificity. All healthy controls were

negative for all tested antibodies. Antibodies against tyrosine hydroxylase were the most frequent being detected in 13 (27.1%) patients. No antibody reactivity was found against MelanA or the melanin-concentrating hormone receptor 1.

The demographic, clinical and serological details of the 18 antibody-positive patients are given in Table 3.6. Of the 18 patients with antibodies, 8 (44.4%) had no autoimmune disease and 10 (55.6%) had at least one form of autoimmunity (Table 3.6). There was a greater frequency of antibodies in the patients who also had concomitant autoimmune disease; 10/19 (52.6%) with autoimmunity *versus* 8/29 (27.6%) without. However, statistical analysis showed that this difference was not statistically significant ($P > 0.05$, Fisher's exact test).

In addition, 14 (77.8%) had patchy and four (22.2%) had severe alopecia areata (Table 3.6). Regarding alopecia areata severity, 14/33 (42.4%) with patchy and 4/15 (26.7%) with severe forms had at least one antibody-positive test. However, comparisons showed there was no significant difference in the frequency of antibody-positivity amongst patchy and severe types of alopecia areata ($P > 0.05$, Fisher's exact test).

Table 3.5: Results of radioligand binding assays

| Antibody target | Upper limit of normal in the radioligand binding assay¹ | Number of patients positive (%) (n = 48) |
|--|---|---|
| Any antibody | - | 18 (37.5) |
| Tyrosinase | 1.29 | 9 (18.8) |
| Tyrosinase-related protein-1 | 1.31 | 6 (12.5) |
| Tyrosinase-related protein-2 | 1.48 | 4 (8.3) |
| Melanocyte-specific protein PMEL | 1.63 | 11 (22.9) |
| Tyrosine hydroxylase | 1.49 | 13 (27.1) |
| MelanA | 1.52 | 0 (0) |
| Melanin-concentrating hormone receptor 1 | 1.85 | 0 (0) |

¹The upper limit of normal for the RLBA was: the mean antibody index + 3SD of the population of 50 control sera. A serum sample with an antibody index above the upper limit of normal was classed as positive for the antibody specificity under analysis.

Table 3.6: Clinical, demographic and serological details of alopecia areata patients positive for specific antibodies

| Patient | Sex | Autoimmune disease ¹ | Alopecia areata severity | Active alopecia areata | Atopy | Age at sample (years) | Onset age (years) | Disease duration (years) | Antibodies ² | | | | |
|---------|--------|---------------------------------|--------------------------|------------------------|-------|-----------------------|-------------------|--------------------------|-------------------------|----------|----------|----------|----------|
| | | | | | | | | | TYR | TYRP1 | TRP2 | PMEL | TH |
| AA01 | Female | ATD, LS | Patchy | Yes | Yes | 53 | 50 | 3 | + (5.78) | + (9.32) | + (4.51) | + (7.66) | - |
| AA03 | Female | None | Totalis | Yes | No | 60 | 50 | 10 | - | - | - | - | + (11.9) |
| AA05 | Male | None | Patchy | Yes | No | 71 | 63 | 8 | - | - | - | - | + (4.94) |
| AA10 | Female | None | Universalis | Yes | No | 33 | 28 | 5 | - | - | - | - | + (6.38) |
| AA15 | Female | None | Patchy | Yes | Yes | 18 | 14 | 4 | + (8.19) | + (4.11) | + (5.29) | + (3.92) | - |
| AA17 | Female | None | Patchy | Yes | Yes | 26 | 23 | 3 | - | - | - | - | + (8.59) |
| AA21 | Male | None | Totalis | Yes | Yes | 41 | 36 | 5 | + (3.64) | - | - | - | + (7.12) |
| AA30 | Female | ATD | Universalis | Yes | No | 63 | 57 | 6 | + (4.51) | - | - | + (2.95) | + (2.87) |
| AA34 | Male | None | Patchy | Yes | Yes | 53 | 49 | 4 | + (11.6) | - | - | + (10.5) | - |
| AA37 | Female | None | Patchy | Yes | Yes | 23 | 20 | 3 | + (18.9) | - | - | - | + (9.26) |
| AA38 | Male | HP, Vitiligo | Patchy | Yes | Yes | 70 | 20 | 50 | - | - | - | + (8.39) | + (5.15) |
| AA39 | Male | Vitiligo | Patchy | Yes | Yes | 45 | 7 | 38 | + (6.32) | + (5.67) | - | + (10.9) | + (12.8) |
| AA40 | Male | Vitiligo | Patchy | Yes | Yes | 43 | 41 | 2 | - | - | - | + (4.42) | - |
| AA43 | Female | ATD, Vitiligo | Patchy | Yes | Yes | 46 | 38 | 8 | + (2.98) | + (6.15) | - | + (3.88) | + (16.2) |
| AA44 | Male | Vitiligo | Patchy | Yes | Yes | 62 | 60 | 2 | - | - | - | + (11.9) | + (9.31) |
| AA45 | Male | Vitiligo | Patchy | Yes | Yes | 44 | 42 | 2 | - | + (8.01) | + (3.20) | - | - |
| AA47 | Female | Vitiligo | Patchy | Yes | Yes | 34 | 32 | 2 | + (10.2) | + (4.98) | + (3.22) | + (14.2) | + (15.8) |
| AA48 | Female | Vitiligo | Patchy | Yes | Yes | 36 | 32 | 4 | - | - | - | + (5.32) | + (17.3) |

¹ATD, Autoimmune hypothyroidism; HP, hypopituitarism; LS, lichen sclerosis.

²+, positive for antibody reactivity; -, negative for antibody reactivity. The antibody index is shown for each antibody-positive patients. PMEL, melanocyte-specific protein PMEL; TH, tyrosine hydroxylase; TYR, tyrosinase; TYRP1, tyrosinase-related protein-1; TRP2, tyrosinase-related protein-2.

3.5 Discussion

The aim of this part of the study was to describe the demographic, clinical forms, and associated diseases in the present cohort of 48 alopecia areata patients.

3.5.1 Alopecia areata severity

Clinically alopecia areata can be classified broadly as patchy or severe, the latter including totalis, universalis, and ophiasiform (Alkhalifah et al., 2010a, Hordinsky, 2013). Patchy type alopecia areata is the most common form occurring in 75-90% of total cases (Nanda et al., 2002, Alkhalifah et al., 2010a, Finner, 2011). In this study, there was a comparable frequency to other reports in the case of patchy alopecia areata, which presented in 68.8% of patients. Rarer forms of alopecia areata including totalis, universalis, and ophiasis have been documented to represent 10–25% of total cases (Finner, 2011). The current study revealed a frequency of 20.8%, 8.3%, and 2.1% for alopecia totalis, universalis, and ophiasis, respectively. The four childhood (1-16 years) cases had patchy type alopecia areata with no severe forms reported, which is consistent with the findings of previous studies (Nanda et al., 2002, Tan et al., 2002b).

3.5.2 Sex distribution

In this study, the ratio of affected males to females was 1:2.7 with 13 (27%) males and 35 (73%) female patients. The result is compatible with previous studies showing a higher incidence of alopecia areata in females, with a male to female ratio ranging from 1:2.6 to 1:2.1 (Tan et al., 2002b, Guzman-Sanchez et al., 2007, Lundin et al., 2014). In contrast, some studies have reported a male predominance with a male to female ratio ranging from 2:1 to 1.1:1 (Sharma et al., 1996a, Jain and Marfatia, 2003, Yang et al., 2004, Kavak et al., 2008). An equal frequency between the sexes has also been noted (Villasante Fricke and Miteva, 2015, Sobolewska-Wlodarczyk et al., 2016, Strazzulla et al., 2018). Interestingly, epidemiological data from Israel indicated that the male to female ratio of alopecia areata frequency changed with age (Lyakhovitsky et al., 2019). Childhood-onset alopecia had a male to female ratio of 1:1.1 compared with an adult-onset ratio of 1:4 (Lyakhovitsky et al., 2019). The excess of

females observed in this and previous studies could result from greater cosmetic awareness, such that women present themselves for treatment more readily. Differences in sample sizes and in the ascertainment and referral patterns of alopecia areata might also influence the inconsistent ratios of male to female alopecia areata patients that have been reported.

3.5.3 Age of onset

Many previous studies have reported that all age groups can be affected by alopecia areata, but that the mean age at disease onset is 25-36 years (Tan et al., 2002b, Yang et al., 2004, Finner, 2011, Mirzoyev et al., 2014, Villasante Fricke and Miteva, 2015). In the current study, the mean age of onset was 36 years, which is comparable. Additionally, here, the majority of patients (81.3%) had developed alopecia areata before the age of 50 years, which is closely compatible with several earlier studies (Kavak et al., 2008, Kyriakis et al., 2009, Trueb and Dias, 2018, Lyakhovitsky et al., 2019). However, some studies have stated that in 20%-50% of cases, alopecia areata develops during childhood between 1-16 years (Muller and Winkelmann, 1963, Sharma et al., 1996a). In this study, only four childhood cases were included.

3.5.4 Disease duration

Reports of disease duration of alopecia areata are variable. Kavak and colleagues reported 82% of patients presented with alopecia areata with a duration of less than one year (Kavak et al., 2008). Another study reported 55% of cases presented with a disease duration less than one year (Lyakhovitsky et al., 2019). In our study, the duration of alopecia areata ranged from one to 50 years, and 19/48 (39.6%) cases had had the disease for less than 5 years.

3.5.5 Associated atopy

There are frequent observations of a high prevalence of atopic diseases such as asthma, rhinitis and atopic dermatitis amongst patients with alopecia areata, reportedly between 39% and 61% (Table 3.8) (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Chu et al., 2011). In the current study, a comparable frequency of atopy was noted, this being found in 75% of the alopecia areata patients (Table 3.8). Atopy was three times as frequent in the present alopecia patient group as in the general population at 75% and 20%, respectively (Table 3.9) (Zheng et al., 2011).

Previously, atopic disease, specifically atopic dermatitis, was significantly associated with severe forms of alopecia areata, totalis and universalis (Goh et al., 2006, Barahmani et al., 2009). However, in our study, there was no significant association of atopy with alopecia areata type; atopy was present in 27 of 33 (81.8%) of patients with patchy alopecia areata, and nine of 15 (60%) of patients with severe forms. A similar result was also reported by Tan and colleagues who found no significant association between atopy and the severity of alopecia areata (Tan et al., 2002b). In addition, atopy was reported to be significantly more common in first-degree relatives of patients with alopecia areata at 19.0% compared to 7.3% of the general population (Agre et al., 2020). The present study reported a comparable frequency at 27% of patients with a family history of atopy.

3.5.6 Associated nail changes

Nail changes are a common feature of alopecia areata and a significant source of cosmetic disfigurement in the disease. The changes can include diffuse fine pitting, trachyonychia, longitudinal ridging, onychorrhexis, Beau's lines, and onychodystrophy (Tosti et al., 1994, Chelidze and Lipner, 2018). Nail pitting and trachyonychia are the most common manifestations in alopecia areata (Chelidze and Lipner, 2018). Nail involvement has a reported incidence ranging from 19-64% (Table 3.7) (Tosti et al., 1994, Sharma et al., 1996a, Gandhi et al., 2003, Kasumagic-Halilovic and Prohic, 2009, Roest et al., 2018) and the results of the current study of 35.4% are in agreement.

Several studies have reported that nail changes in alopecia areata are an important prognostic indicator of disease severity (Sharma et al., 1996a, Gandhi et al., 2003). The results of the current study are compatible with this, as 70.6% of the patients with severe forms of alopecia areata had nail involvement. Interestingly, Lee and colleagues concluded that nail involvement was not a poor prognostic factor in relation to hair regrowth following tofacitinib treatment; 11/15 (73.3%) patients with nail involvement showed improvement within five months regardless of type of nail change. (Lee et al., 2018a).

Table 3.7: Nail changes in the current study compared with the previous studies

| Study design and patient number | Nail changes | Percentage of patients | References |
|--|----------------------|-------------------------------|--|
| Prospective; 272 children | Any | 46 | (Tosti et al., 1994) |
| | Pitting | 33.8 | |
| | Trachyonychia | 11.7 | |
| | Longitudinal ridging | 0 | |
| | Distal notching | 0 | |
| | Beau's lines | 0 | |
| Prospective; 1000 | Any | 19.8 | (Sharma et al., 1996a) |
| | Pitting | 13.9 | |
| | Trachyonychia | 8.3 | |
| | Longitudinal ridging | 7.8 | |
| | Distal notching | 0 | |
| | Beau's lines | 0 | |
| Prospective; 100 | Any | 44 | (Gandhi et al., 2003) |
| | Pitting | 28 | |
| | Trachyonychia | 9 | |
| | Longitudinal ridging | 10 | |
| | Distal notching | 2 | |
| | Beau's lines | 2 | |
| Retrospective; 200 | Any | 24.5 | (Kasumagic-Halilovic and Prohic, 2009) |
| | Pitting | 19.5 | |
| | Trachyonychia | 3.5 | |
| | Longitudinal ridging | 1.5 | |
| | Distal notching | 0 | |
| | Beau's lines | 0 | |
| Questionnaire-based survey; 256 | Any | 64.1 | (Roest et al., 2018) |
| | Pitting | 29.7 | |
| | Trachyonychia | 18 | |
| | Longitudinal ridging | 9 | |
| | Distal notching | 3.5 | |
| | Beau's lines | 0.8 | |

3.5.7 Associated autoinflammatory and autoimmune disease

The association of alopecia areata with other inflammatory and autoimmune disorders has been reported widely (Table 3.8) (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Lyakhovitsky et al., 2015, Villasante Fricke and Miteva, 2015). According to the National Alopecia Areata Registry, 12-25% of alopecia areata patients have other coexisting inflammatory or autoimmune disorders (Goh et al., 2006, Barahmani et al., 2009).

There are frequent observations of autoimmune thyroid disease occurring in alopecia areata cases, with 2.3-14.6% of patients affected depending on the population under study (Table 3.8) (Muller and Winkelmann, 1963, Tan et al., 2002b, Kasumagic-Halilovic, 2008, Chu et al., 2011, Huang et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015, Han et al., 2018, Lee et al., 2019b). Analysis of the current study population showed a similar frequency of autoimmune thyroid disease (hypothyroidism) at 16.7% (Table 3.8). The presence of autoimmune thyroid disease has been reported to occur at a significantly higher frequency in patients with severe forms of alopecia areata, alopecia totalis and alopecia universalis (Goh et al., 2006, Barahmani et al., 2009). In our study, 26.7% of patients with severe alopecia areata had hypothyroidism, whilst 12.1% of cases with patchy type alopecia areata were diagnosed with autoimmune thyroid disease. However, although at a higher prevalence in severe alopecia areata, there was no significant association with autoimmune thyroid disease.

Vitiligo has been documented widely to accompany alopecia areata (Nanda et al., 2002, Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Kos and Conlon, 2009, Alkhalifah et al., 2010a), although a single clinical study in India revealed no relationship between the two diseases (Thomas and Kadyan, 2008). In the current study, the frequency of vitiligo in alopecia areata patients was 25%, an approximate four-fold increase when compared with the frequency of 1.8-7% found in other studies (Table 3.8) (Muller and Winkelmann, 1963, Sharma et al., 1996a, Tan et al., 2002b, Chu et al., 2011, Huang et al., 2013).

Rheumatoid arthritis has been reported in alopecia areata patients with a frequency of 0.9–3.9% (Chu et al., 2011, Huang et al., 2013). In this study, the frequency was similar at 2.1% (Table 3.6). Other disorders reported in the current study were lichen sclerosus and hypopituitarism with a frequency of 4.2% and 2.1%, respectively (Table 3.8). However, no

cases of these two diseases were available in the literature. No cases of psoriasis, type 1 diabetes, Graves' disease, systemic lupus erythematosus, or inflammatory bowel disease were apparent in this alopecia areata patient cohort, although reported previously (Table 3.8) (Sharma et al., 1996b, Chu et al., 2011, Huang et al., 2013).

In Table 3.9, the frequencies of the autoinflammatory and autoimmune diseases found in this alopecia areata study population are compared with those reported in the general population. There was an approximate nine-fold increase over the 1.9% population frequency of autoimmune thyroid disease (Jacobson et al., 1997). The frequency of vitiligo at 25% was at least 12-fold higher than in the general population at 0.5-2.0% (Jacobson et al., 1997). Cases of rheumatoid arthritis and lichen sclerosus in this study were not significantly more frequent than in the general population (Jacobson et al., 1997, Silman and Pearson, 2002, Kirtschig et al., 2017). Hypopituitarism is very rare in the general population, and only one case was found in the present study (Fernandez-Rodriguez et al., 2013).

Some studies have described a positive family history of alopecia areata at a low frequency of less than 10% (Muller and Winkelmann, 1963, Sharma et al., 1996a, Tan et al., 2002a, Agre et al., 2020), and these results are compatible with the present study data at 6.25%. However, other studies reported a positive family history of alopecia areata at rates ranging from 20-51.6% (Shellow et al., 1992, Nanda et al., 2002, Goh et al., 2006, Arousse et al., 2019).

In previous studies, a family history of alopecia areata was not significantly different between patchy and severe forms (Chu et al., 2011, You and Kim, 2017). In contrast, other reports have indicated an association of family history of alopecia areata with severe forms like alopecia totalis and universalis (Sharma et al., 1996a, Goh et al., 2006). From the present study, the numbers of patients with a family history of alopecia areata was too small to compare the different disease types. Patients with a family history of alopecia areata have been reported to have a younger age of onset of the disease (Ranawaka, 2014, Wang et al., 2018). However, in the present study, the three patients with a family history of alopecia areata had onset ages of eight, 39 and 42 years. Interestingly, patients with a positive family history of vitiligo were more frequently affected with severe forms of alopecia areata (Sharma et al., 1996a).

However, in the present study most patients with a positive family history of vitiligo had patchy alopecia areata.

Table 3.8: Atopy, autoinflammatory and autoimmune diseases in this study's alopecia areata patients compared with the previous studies

| Disease | Frequency of alopecia areata patients in this study (%) | Frequency of alopecia areata in previous studies (%) | Reference for frequency in previous studies |
|------------------------------|--|---|---|
| Atopy | 75.0 | 39.0-61.0 | (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Chu et al., 2011). |
| Autoimmune thyroid disease | 16.7 | 2.3-14.6 | (Kasumagic-Halilovic, 2008, Baars et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015). |
| Hypopituitarism | 2.1 | No data | - |
| Inflammatory bowel disease | 0 | 2.0 | (Huang et al., 2013) |
| Lichen sclerosus | 4.2 | No data | - |
| Psoriasis | 0 | 1.9-6.3 | (Chu et al., 2011, Huang et al., 2013) |
| Rheumatoid arthritis | 2.1 | 0.9-3.9 | (Chu et al., 2011, Huang et al., 2013) |
| Systemic lupus erythematosus | 0 | 1.5 | (Chu et al., 2011) |
| Type 1 diabetes | 0 | 0.4-11.1 | (Sharma et al., 1996b, Huang et al., 2013) |
| Vitiligo | 25.0 | 1.8-7.0 | (Muller and Winkelmann, 1963, Sharma et al., 1996a, Tan et al., 2002b, Huang et al., 2013) |

Table 3.9: Atopy, autoinflammatory and autoimmune diseases in this study's alopecia areata patients compared with the general population

| Disease | Frequency in alopecia areata patients in this study (%) | Frequency in the general population (%) | Reference for frequency in the general population |
|------------------------------|--|--|--|
| Atopy | 75.0 | 20.0 | (Zheng et al., 2011) |
| Autoimmune thyroid disease | 16.7 | 1.9 | (Jacobson et al., 1997) |
| Hypopituitarism | 2.1 | 0.004 | (Fernandez-Rodriguez et al., 2013) |
| Inflammatory bowel disease | 0 | 0.1 | (Knigge, 2002) |
| Lichen sclerosus | 4.2 | 0.1-3.0 | (Kirtschig et al., 2017) |
| Psoriasis | 0 | 1-3 | (Ayala, 2007) |
| Rheumatoid arthritis | 2.1 | 0.3-1.2 | (Jacobson et al., 1997, Silman and Pearson, 2002) |
| Systemic lupus erythematosus | 0 | 0.024 | (Jacobson et al., 1997) |
| Type 1 diabetes | 0 | 0.48 | (Jacobson et al., 1997) |
| Vitiligo | 25.0 | 0.5-2.0 | (Jacobson et al., 1997, Kruger and Schallreuter, 2012) |

3.5.8 Presence of antibody responses

Unlike other autoimmune diseases such as vitiligo, only a few attempts have previously been made to identify alopecia areata relevant autoantibodies in the hair follicle structures (Tobin et al., 1997, Gilhar et al., 2001, Leung et al., 2010, Kemp et al., 2011a). Autoantibodies against hair follicle keratins and trichohyalin have been reported at a high frequency of 80-100% (Leung et al., 2010), but those against melanocyte-specific proteins (tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and melanocyte-specific protein PMEL) appear to be infrequent at 6-9% (Kemp et al., 2011b). Other proteins that are not specifically expressed by melanocytes such as tyrosine hydroxylase and retinol-binding protein-4 have also been found as antibody targets at a mid-range frequency of 19% and 67%, respectively (Ahn et al., 2011, Kemp et al., 2011b).

In the present study, antibody-positivity for melanocyte-specific proteins (tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and melanocyte-specific protein PMEL) ranged between 8-23%, so were slightly raised compared with a previous study (Kemp et al., 2011b). Tyrosine hydroxylase antibodies were present at a similar frequency to that previously reported; 27% *versus* 19% (Kemp et al., 2011b). No antibodies against the vitiligo-associated autoantigen melanin-concentrating hormone receptor 1 (Kemp et al., 2002b) were found. In addition, MelanA antibodies were not detected in the current cohort of alopecia areata patients, a result that was the same as that found with vitiligo patients (Kemp et al., 2002b).

3.5.9 Limitations of the study

The main limitation of this study was the number of patients available. In addition, although there was information on the family history of alopecia areata patients, the exact relationship of family members, such as whether they were parents, siblings or extended relatives, would have been useful. This is an area that could be explored further. The patient history could have been further detailed with treatments of associated diseases such as atopy and rheumatoid arthritis, and any history of stress or psychological illness

3.5.10 Conclusions

In conclusion, the demographic and clinical profiles of the Sheffield alopecia areata cohort were in general similar to previously reported studies. Co-existing autoimmune diseases, particularly vitiligo and autoimmune thyroid disease, were common in the study participants supporting the theory that autoimmune responses are involved in the pathogenesis of alopecia areata. In addition, vitiligo and autoimmune thyroid diseases were common in the families of the patients suggesting that in some cases there may be a genetic susceptibility to these autoimmune diseases.

Chapter 4

**Use of Phage-Display to Identify Novel Melanocyte Autoantigens in
Alopecia Areata**

4 Use of Phage-Display to Identify Novel Melanocyte Autoantigens in Alopecia Areata

4.1 Introduction

4.1.1 Filamentous bacteriophage

Filamentous bacteriophages (phage) are a type of virus that are capable of infecting a variety of Gram-negative bacteria without lysing or killing cells. The F pilus-specific filamentous phage of *E. coli* are the most well-studied (Rakonjac et al., 2011). They are cylindrical particles enclosing a single-stranded, closed DNA genome molecule, which includes 11 genes (Figure 4.1) (Tikunova and Morozova, 2009). Each phage particle has a diameter of seven nm and a length of 800–2000 nm, and is composed of several thousand copies of major coat protein pVIII. There are also four minor coat proteins pIII, pVI, pVII, and pIX that are present at five copies per phage particle (Marvin, 1998, Garg, 2019). The largest and most complex protein is pIII, which is essential for the host infection because of its role in the binding to the bacterial pilus and is necessary for the termination of phage assembly (Peltomaa et al., 2019).

4.1.2 Phage-display technology

The principle of phage-display came from experiments that showed insertion of a DNA molecule into the gene encoding phage coat protein pIII could lead to the expression of the encoded polypeptide as a pIII fusion on the surface of the phage particle (Figure 4.1) (Smith, 1985, Hairul Bahara et al., 2013). Consequently, this enabled the screening of libraries of phage-display fusion proteins for their binding capacity to the required ligand (Figure 4.2) (Wu et al., 2016). Importantly, any enriched peptide was linked to the DNA required for its expression so its identity could be determined by sequencing of the DNA fragment that encoded it. The initial use of phage-display was to identify antibodies, expressed as pIII fusion proteins in the phage-display vector pComb3, which could bind to specific antigens (Barbas and Lerner, 1991, Winter et al., 1994). Since then, phage-display has been utilised to identify novel ligands for receptors (Bass et al., 1990) and transcription factors (Butteroni et al., 2000, Lawson and Bleris, 2017), as well as to evaluate antibody-antigen and enzyme-substrate

interactions (Barbas, 1993, Burgoon et al., 2001, Kemp et al., 2002b, Rami et al., 2017, Redl et al., 1999, Cheok and Jaworski, 2016).

The main advantage of phage-display technology for the identification of autoantigens is the very efficient enrichment process. This allows the isolation of autoantibody targets even if the titre of the autoantibody in the patient's serum is relatively low. In addition, phage-display can enable the identification of conformational epitopes since the process is carried out in a fluid phase. This is unlike the phage λ -based protocol used for identifying antibody-binding peptides that only allows linear epitopes to be identified due to protein denaturation. Furthermore, the panning protocol enables the screening of large cDNA autoantigen libraries along with a quantifiable analysis of how well the technique has worked (Appenzeller et al., 2000, Hairul Bahara et al., 2013, Rami et al., 2017).

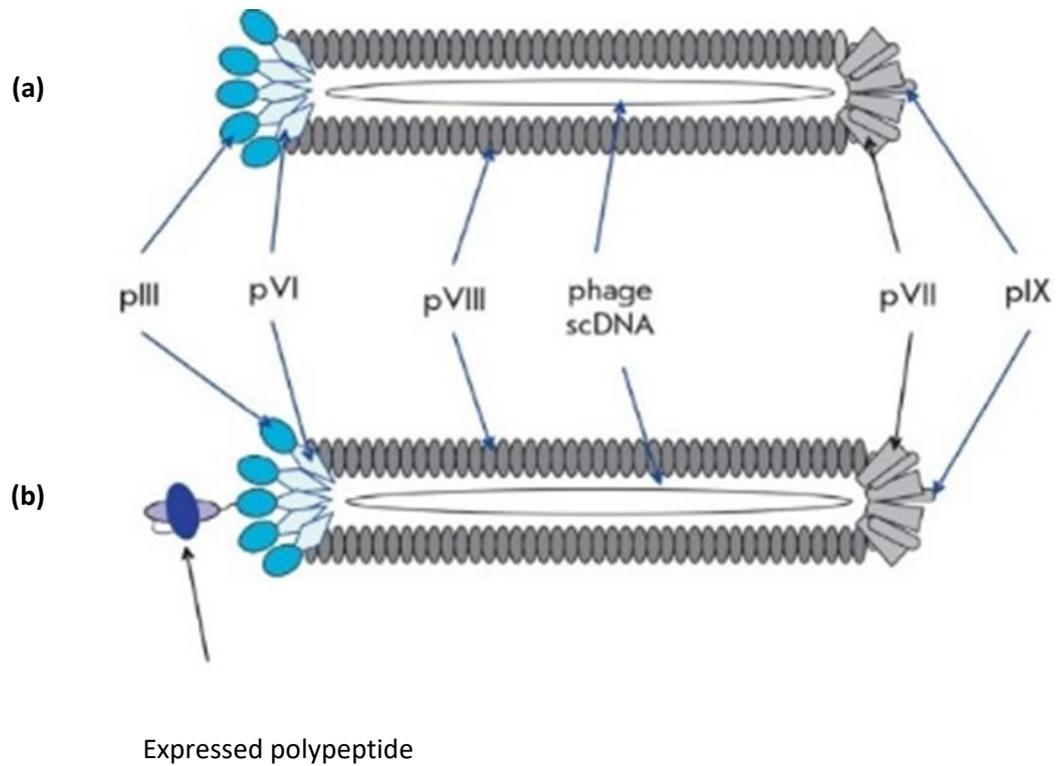


Figure 4.1: A schematic representation of a filamentous phage.

The diagram shows (a) a wild-type filamentous phage and (b) a filamentous phage with peptide or antibody attached to coat protein pIII. The figure, taken from a paper by Tikunova and Morozova, 2009, is used with kind permission from Park-Media Ltd.

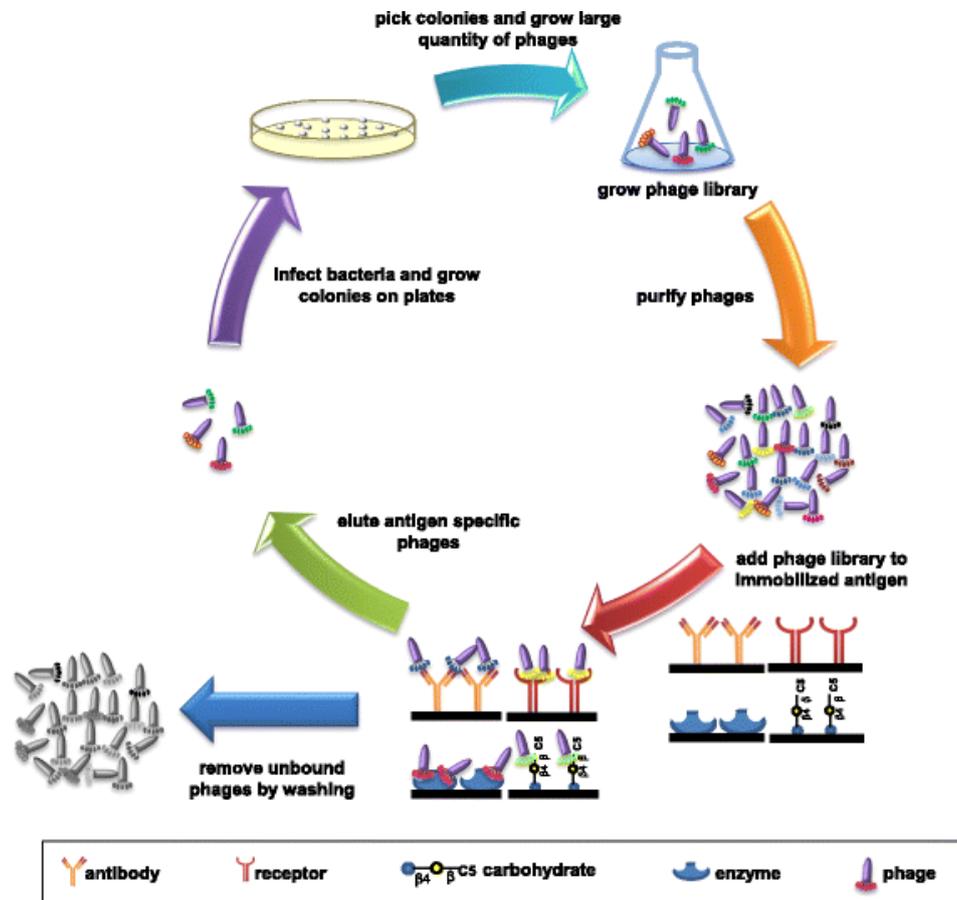


Figure 4.2: The principle of phage-display screening to identify binding peptides.

The figure, taken from a paper by Wu et al, 2016, with kind permission from Springer Nature Ltd.

4.1.3 The pJuFo phage-display system

The pJuFo phagemid vector allows the expression of full-length cDNA-encoded polypeptides on the phage surface (Figure 4.3) (Cramer and Suter, 1993, Cramer and Walter, 1999). It is based on the affinity binding qualities of Jun and Fos heterodimers together with elements of the pComb3 phage-display vector (Barbas and Lerner, 1991, Pernelle et al., 1993, Cramer and Walter, 1999, Appenzeller et al., 2000, Waterman et al., 2010). In this cloning system, the Jun protein is expressed as an amino-terminal fusion to the pIII phage coat protein. Similarly, the Fos protein is expressed as an amino-terminal fusion with the cDNA-encoded polypeptide. Following expression, both the Jun-pIII and Fos-cDNA fusion proteins are secreted to the bacterial periplasm. Here, the Jun and Fos proteins heterodimerise and are held together by disulphide bonds providing a covalent link for cDNA products with the phage surface during phage morphogenesis. During rounds of panning of the cDNA phage library, pJuFo phage-displayed polypeptides can be selectively enriched for binding against an appropriate ligand such as patient IgG (Figure 4.4). Subsequently, the enriched polypeptides can be identified by sequencing the cDNA that encodes them. The system has previously been used to identify autoantigens in systemic lupus erythematosus and vitiligo (Barbas and Lerner, 1991, Pernelle et al., 1993, Cramer and Walter, 1999, Appenzeller et al., 2000, Waterman et al., 2010).

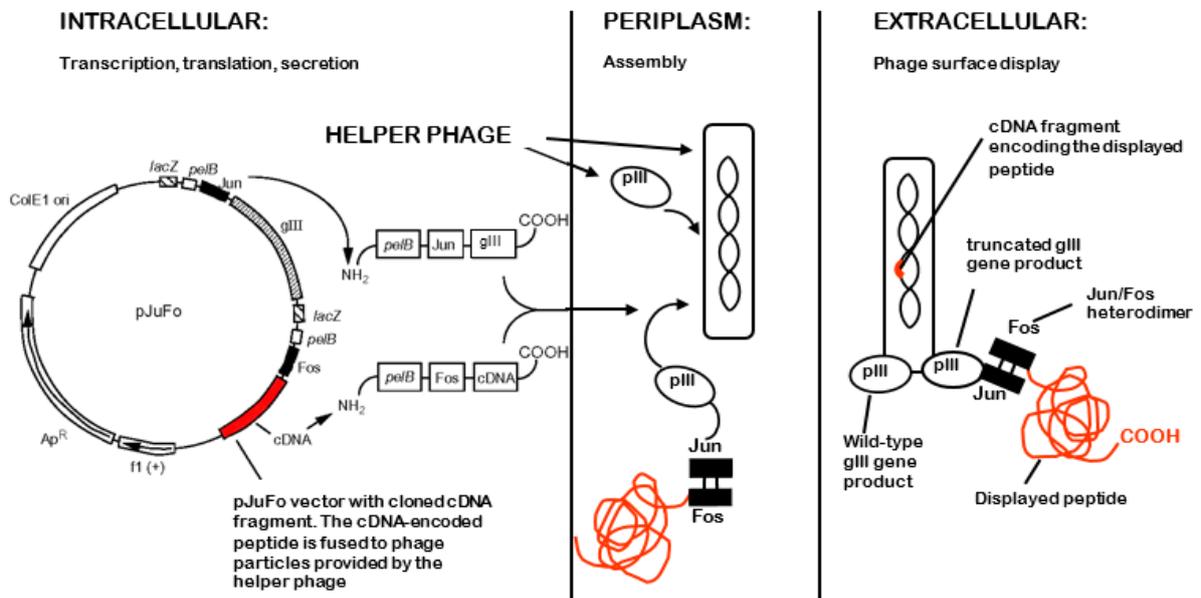


Figure 4.3: The pJuFo phage-display system.

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, from a paper by Cramer and Suter, 1993, is used with kind permission from Elsevier.

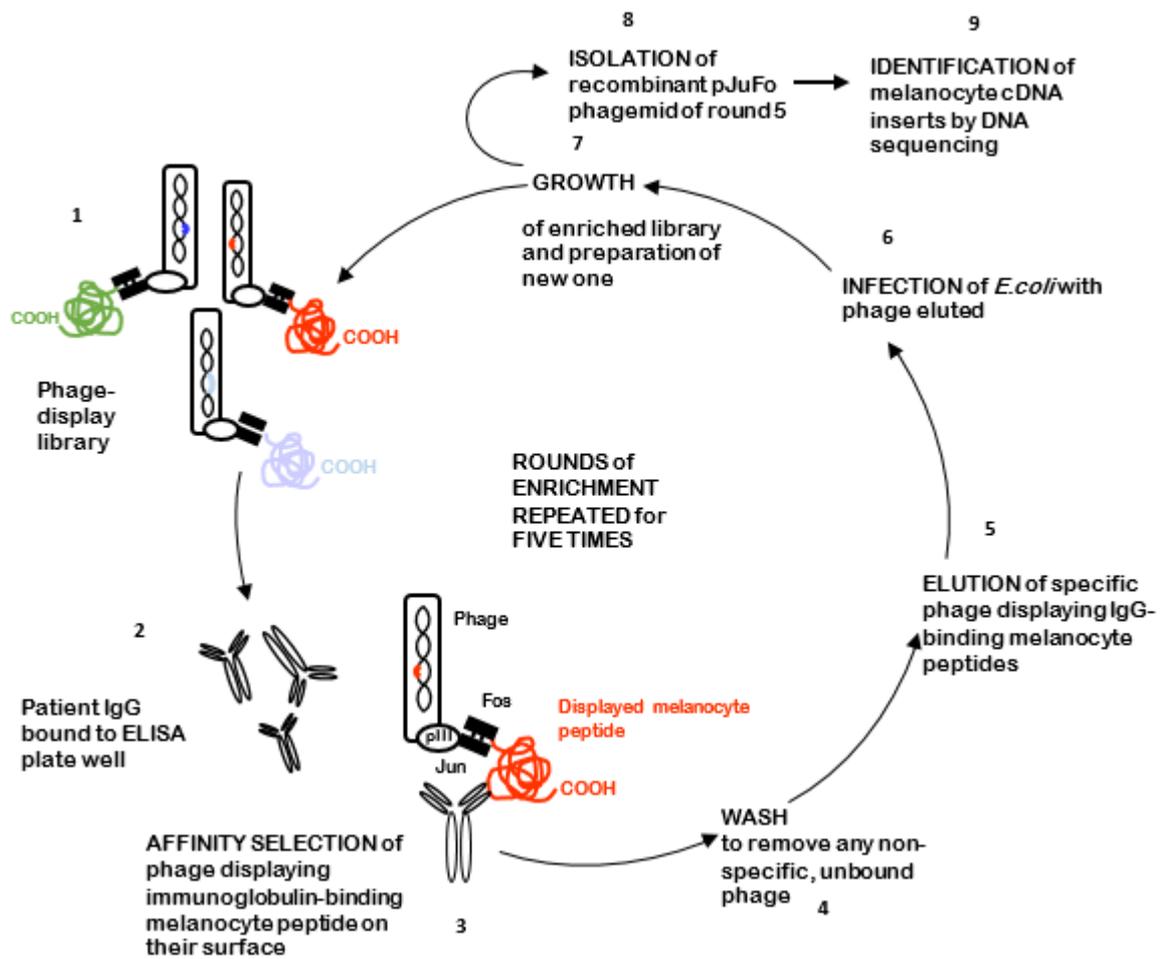


Figure 4.4: Enrichment of phage displaying peptides that bind IgG molecules.

The diagram shows (1) phage displaying peptides from cDNA, (2) patient IgG, (3) phage expressing immunoreactive 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, and (9) isolated phagemid DNA is sequenced to identify cDNA inserts. The figure, from a paper by Waterman et al, 2010, is used with kind permission from Elsevier.

4.2 Aim

In this section of the research project, the aim was to try to identify novel melanocyte autoantibody targets (autoantigens) in patients suffering from alopecia areata using the advantages of phage-display as a method. Autoantigens in alopecia areata are important to identify as they themselves may also be T cell targets, as with tyrosinase, trichohyalin, and melanocyte-specific protein PMEL (Leung et al., 2010, Ahn et al., 2011, Kemp et al., 2011b). In addition, autoreactive B cells can also act as antigen-presenting cells, thereby amplifying the autoimmune response. The identified targets would add to the knowledge base with respect to alopecia areata in terms of its pathogenesis and treatments.

4.3 Materials and Methods

4.3.1 Phage-display library

The melanocyte cDNA-encoded peptide phage-display library and the phage-display methodologies were provided by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). The library was stored at -80°C. The phage-display library contained human melanocyte cDNAs that had been cloned into the *XbaI-KpnI* site of the pJuFo vector shown Figure 4.5. The frequency of recombinant pJuFo phagemid (those carrying a cDNA insert) in the library had been estimated as 95%. It had been shown that the cloned cDNA inserts ranged in size from 0.5-2.5 kb. The average insert size was 1.5 kb. The frequency of β -actin cDNA inserts in the library had been estimated at 0.04%.

The library was initially titred as follows. *Escherichia coli* XL1-Blue MRF' (Agilent Technologies, Wokingham, UK) was grown in 10 ml of LB medium with tetracycline at 10 μ g/ml in a shaking incubator at 37°C to an OD600 of 0.5. A 0.5-ml aliquot of the bacterial culture was then inoculated with a 2- μ l sample of a 1×10^{-4} dilution of a phage-display library. The culture was left for 15 min at room temperature to allow phage infection. Samples of 1- μ l, 10- μ l and 100- μ l of the culture were plated out onto LB agar containing 100 μ g/ml of ampicillin and tetracycline at 10 μ g/ml. An uninfected 100- μ l aliquot of the culture was plated out as a control to ensure that the culture was not contaminated with phage. The plates were incubated overnight at 37°C overnight and then the bacterial colonies were counted. The phage titres were determined from the number of colonies and expressed as colony-forming units (cfu) per ml.

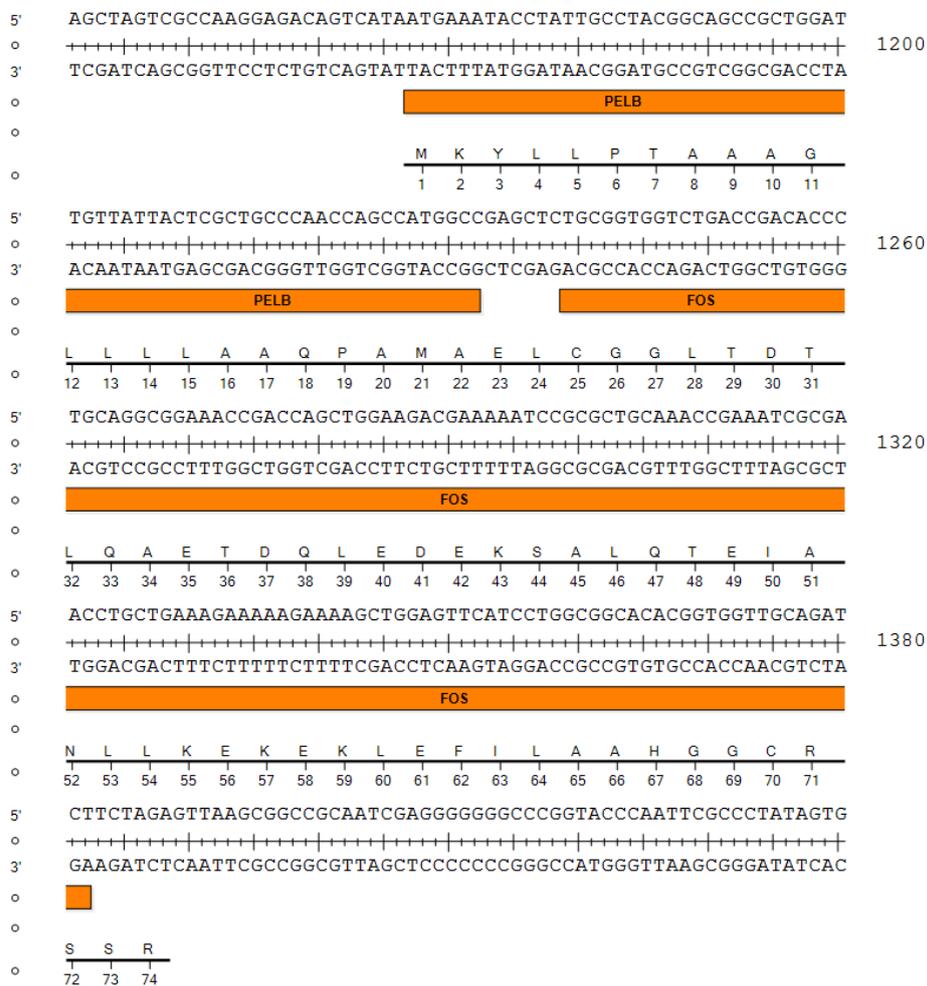


Figure 4.5: Details of the cloning site of pJuFo.

The PelB and Fos sequences (DNA and amino acid) are shown. They form fusions with cDNAs cloned in the *Xba*I (TCTAGA)-*Kpn*I (GGTACC) cloning site. The *Xba*I site begins at nucleotide number 1383. The *Kpn*I site begins at nucleotide number 1418.

4.3.2 Panning experiments

For panning experiments, a 10- μ l aliquot of alopecia areata patient IgG, control IgG, or a positive control antibody (Table 2.5) was applied to the well of a 96-well microtitre plate in 50 μ l of coating buffer (3.5 mM sodium hydrogen carbonate; 1.5 mM sodium carbonate; pH 9.2). The plates were left for 2 h at room temperature so that binding of the IgG to the well could take place. The plate was then washed with PBS (pH 7.4)/0.05% Tween 20 (PBS/Tween). In order to stop any binding of phage to the plate well later in the process, 400 μ l of 2% bovine serum albumin made in PBS were added to the wells. The plates were then incubated at room temperature for a further 2 h. Subsequently, the wells were washed once with PBS/Tween and then a 100- μ l sample of a phage-display library containing approximately 1×10^{10} phage was added to the well. The number of phage was estimated from the titre of the phage-display library. To allow the interaction of patient IgG with peptides displayed on the surface of the phage particles, the plates were incubated overnight at 4°C.

To remove unbound phage, the well was washed ten times with PBS/Tween. The phage still bound to the well were then eluted with 100 μ l of acidified glycine (pH 2.2) and then neutralised with 9 μ l of 2 M Tris-base. The eluted phage were added into 2 ml of *E. coli* XL1-Blue MRF' and left for 15 min at room temperature so that the phage could infect the bacteria. Ten and 100- μ l aliquots of the infected cells were then plated out onto LB agar containing 10 μ g/ml tetracycline and 100 μ g/ml ampicillin. After incubation overnight at 37°C, the colonies on the plates were counted to estimate of the total phage eluted from the microtitre plate well.

To make a phage-display library for the next round of panning, the *E. coli* XL1-Blue MRF' bacterial cell culture infected with the eluted phage was made up to 10 ml with LB medium and incubated with shaking for 1 h at 37°C. The culture was then superinfected with of 1×10^{11} plaque-forming units of VCS-M13 helper phage (Agilent Technologies). After incubating at room temperature for 30 min, the bacteria were inoculated into 100 ml of LB medium to which 10 μ g/ml of tetracycline and 100 μ g/ml of ampicillin were added. Following further incubation for 1 h at 37°C with shaking, kanamycin was added to a final concentration of 10 μ g/ml. The infected culture was then left at 37°C in the shaking incubator overnight.

Subsequently, the 100-ml culture was spun at 2,000 *g* for 15 min to remove bacterial cells and the supernatant recovered to a clean Oak Ridge tube. To precipitate phage particles, 15 ml of 40% polyethylene glycol 4000 and 15 ml of 5 M sodium chloride were added to the culture supernatant. After incubation at 4°C for 16 h, the phage were collected by centrifuging at 10,000 *g* for 30 min. The pellet of phage was resuspended in 1-2 ml of PBS. This first round (R1) phage-display library, which was enriched in phage particles with surface-expressed IgG-binding peptides, was titrated and then kept at -80°C.

The phage-display library from round one was used in a second round of panning in the same way as the original library. Five rounds of panning (R1-R5) were undertaken for each patient IgG sample. In each round of panning, the ratio of the total phage eluted to the total phage applied to the microtitre plate well was determined. The fold increase in the ratio in each subsequent panning round was calculated by comparing to the ratio in round one.

4.3.3 Identification of enriched peptides

Following panning round five, bacterial colonies were randomly selected. They were streaked out onto LB agar containing 100 µg/ml of ampicillin and 10 µg/ml of tetracycline. The purified bacterial clones were cultured in 10 ml of LB medium containing 100 µg/ml ampicillin and 10 µg/ml tetracycline overnight at 37°C. Plasmid was prepared from the cultures using a Wizard® Plus SV Minipreps DNA Purification System.

In order to identify the melanocyte peptides that had been enriched during the panning process, plasmids carrying the peptide-encoding cDNAs were subjected to DNA sequencing. The primers used for sequencing were JUFO-1192 and JUFO-1500 (Table 2.3) that bind to the pJuFo cloning vector upstream of the cDNA cloning site and downstream of the cDNA cloning site, respectively, as shown in Figure 4.5. The cDNAs were identified by comparison with the GenBank database and employing the BLAST online facility of the NCBI.

4.4 Results

4.4.1 Panning of alopecia areata patient IgG against the melanocyte cDNA-encoded peptide phage-display library

The melanocyte cDNA-encoded peptide phage-display library was used in five panning rounds with IgG from 20 alopecia areata patients (Table 4.1), two healthy control IgGs, and four positive control antibodies. Following each round of panning, the total phage eluted was calculated by infecting the phage into *E. coli* XL1-Blue MRF', and plating out the infected culture onto LB agar containing antibiotics. The ratio of the total phage eluted to total phage applied to the microtitre plate well was also determined. The fold-increase (compared with panning round 1) of this ratio in each subsequent panning round was also calculated.

The results for the positive control antibodies and the healthy IgG samples are shown in Figures 4.6 and 4.7, respectively. The results for the four positive control antibodies against tyrosinase, tyrosinase-related protein-2, tyrosinase hydroxylase, and melanocyte-specific protein PMEL (Table 2.5) showed an increase in the normalised ratio of phage eluted:phage applied phage throughout the panning rounds, up to 100 to 140-fold by round five, indicating the enrichment of phage expressing specific IgG-binding proteins on their surfaces. For the two healthy control IgG samples, there was an increase of five-fold and 13-fold in the normalised ratio by round five.

The results for each of the 20 patients are illustrated in Figure 4.8. For the 20 alopecia areata patient IgGs, the results after five rounds of panning showed there was an increase in the normalised ratio in all cases. For the majority of patient IgG samples, the increase was above that of the control IgG and ranged from 15-fold to 491-fold, depending on the IgG sample used in the panning experiment. However, in the results for three patient IgG samples AA08, AA11, and AA12, the increase was only five-fold, 13-fold, and seven-fold, respectively, similar to the healthy control IgG samples.

Table 4.1: Details of the alopecia areata patients from whom IgG was used in phage-display panning experiments

| Patient | Sex | Age at sample (years) | Age at onset (years) | Disease duration (years) | Alopecia areata severity | Atopy | Autoimmune disease |
|----------------|------------|------------------------------|-----------------------------|---------------------------------|---------------------------------|--------------|---|
| AA01 | Female | 53 | 50 | 3 | Patchy | Yes | Autoimmune hypothyroidism; lichen sclerosus |
| AA02 | Female | 24 | 16 | 8 | Patchy | Yes | None |
| AA03 | Female | 60 | 50 | 10 | Totalis | No | None |
| AA04 | Female | 66 | 53 | 13 | Totalis | Yes | Lichen sclerosus |
| AA05 | Male | 71 | 63 | 8 | Patchy | No | None |
| AA06 | Male | 38 | 18 | 20 | Patchy | Yes | None |
| AA07 | Female | 64 | 63 | 1 | Patchy | No | None |
| AA08 | Female | 34 | 26 | 8 | Patchy | No | None |
| AA09 | Female | 26 | 25 | 1 | Patchy | Yes | None |
| AA10 | Female | 33 | 28 | 5 | Universalis | No | None |
| AA11 | Female | 30 | 23 | 7 | Ophasiform | Yes | None |
| AA12 | Female | 87 | 74 | 13 | Universalis | No | Autoimmune hypothyroidism |
| AA13 | Female | 61 | 53 | 8 | Patchy | No | Autoimmune hypothyroidism |
| AA14 | Female | 37 | 20 | 17 | Patchy | No | None |
| AA15 | Female | 18 | 14 | 4 | Patchy | Yes | None |
| AA16 | Male | 42 | 35 | 7 | Patchy | Yes | None |
| AA17 | Female | 26 | 23 | 3 | Patchy | Yes | None |
| AA18 | Male | 34 | 25 | 9 | Patchy | Yes | None |
| AA19 | Female | 45 | 39 | 6 | Patchy | Yes | Autoimmune hypothyroidism |
| AA20 | Male | 35 | 23 | 12 | Patchy | No | None |

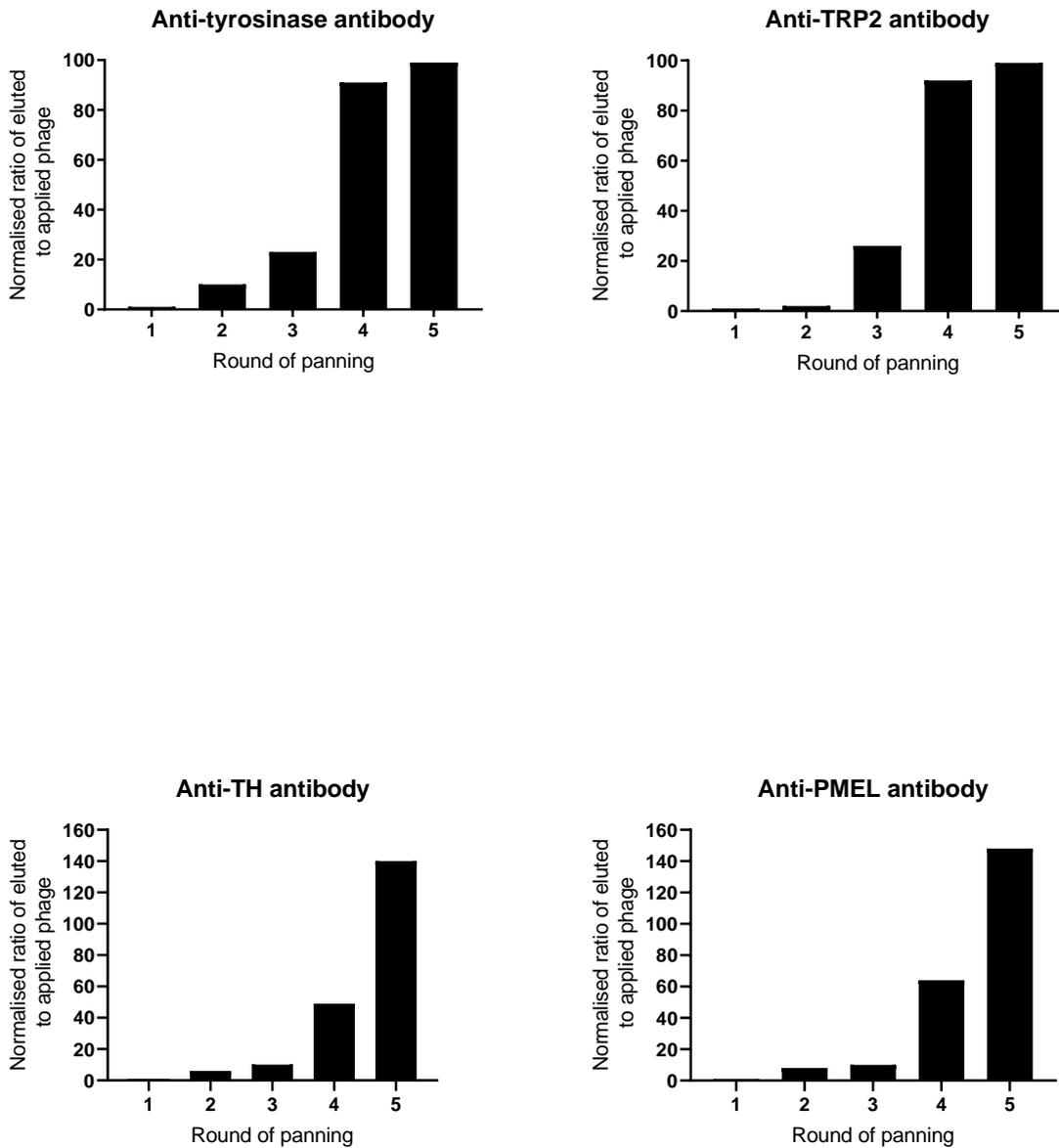


Figure 4.6: Rounds of panning with positive control antibodies.

The phage-display library of melanocyte cDNA-encoded peptides was used in five rounds of panning with four positive control antibodies. The graphs show the fold increase in the ratio of phage eluted:phage applied in each panning round. TRP2, tyrosinase-related protein-2; TH, tyrosine hydroxylase; PMEL, melanocyte-specific protein PMEL.

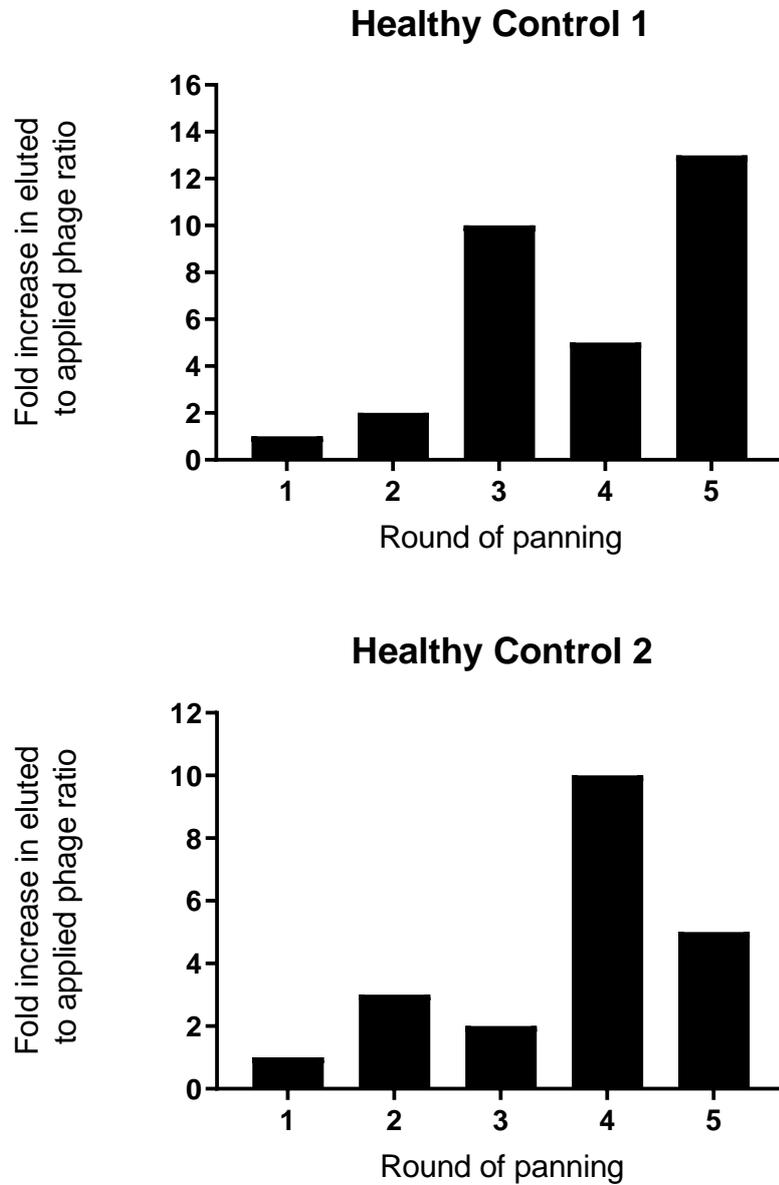
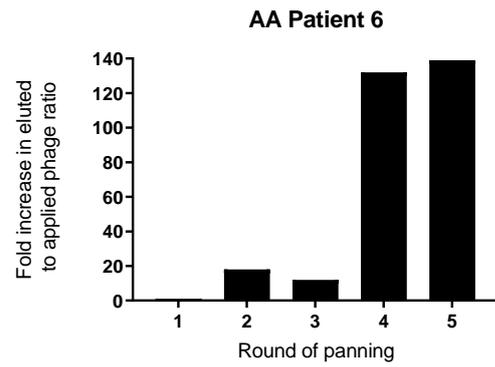
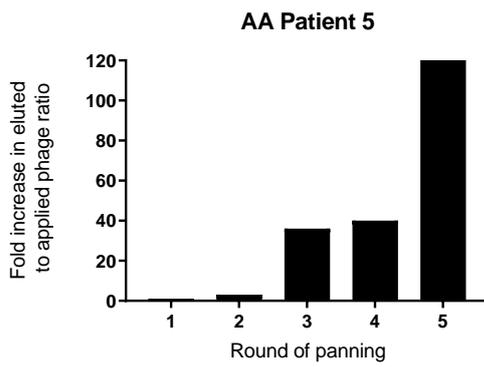
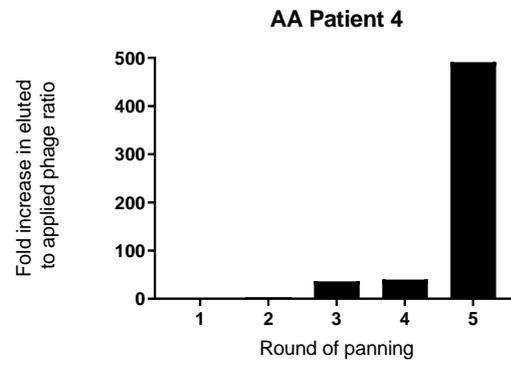
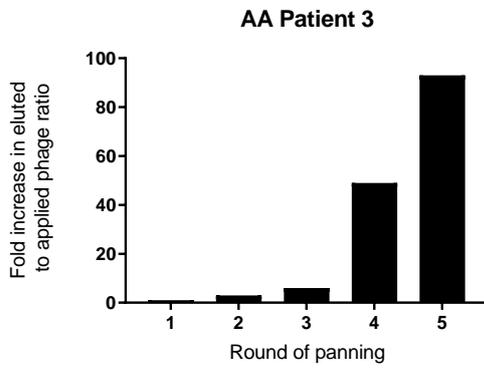
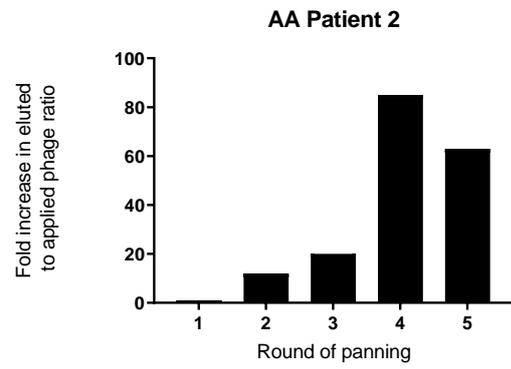
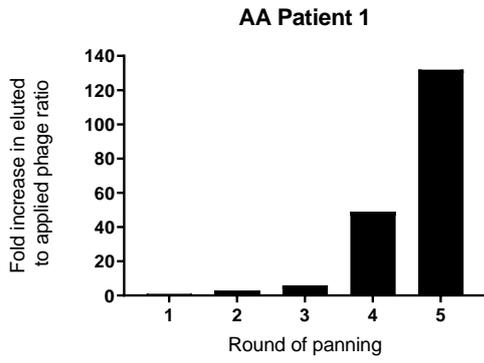
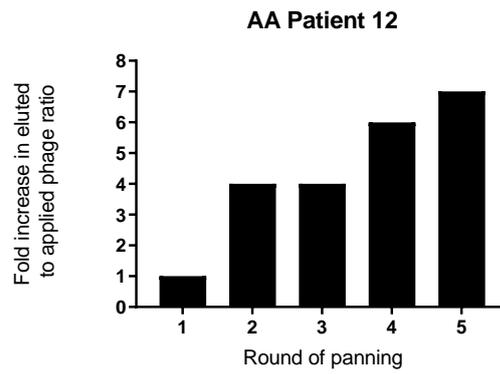
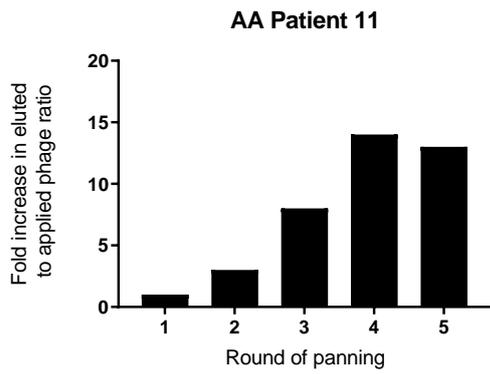
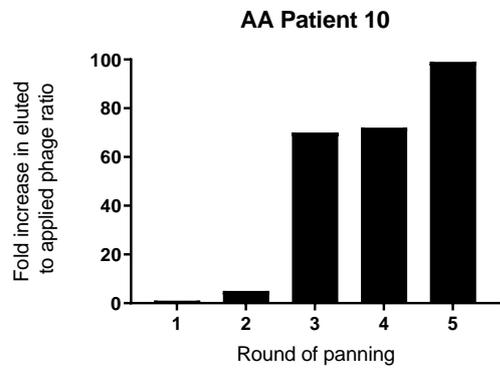
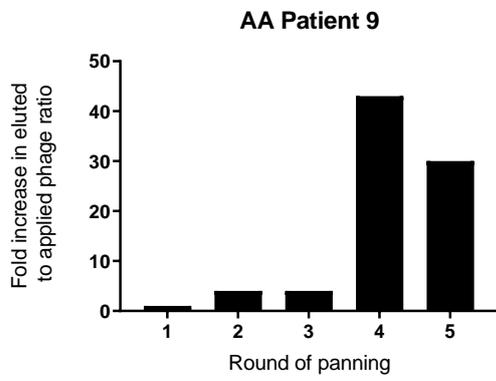
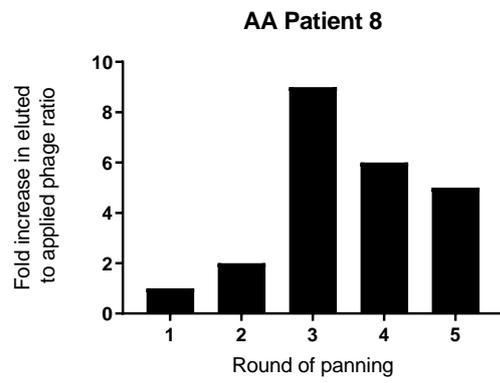
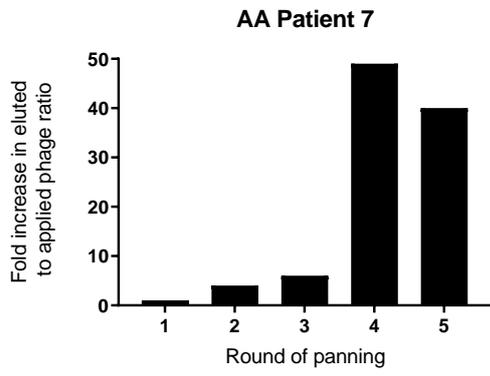
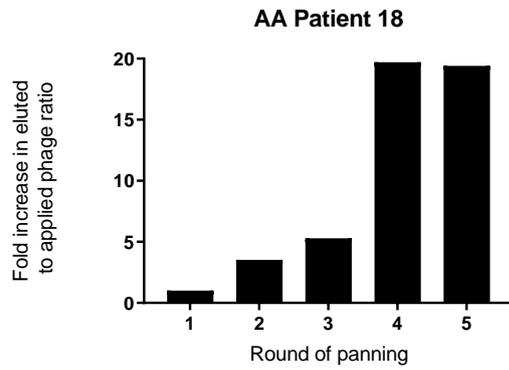
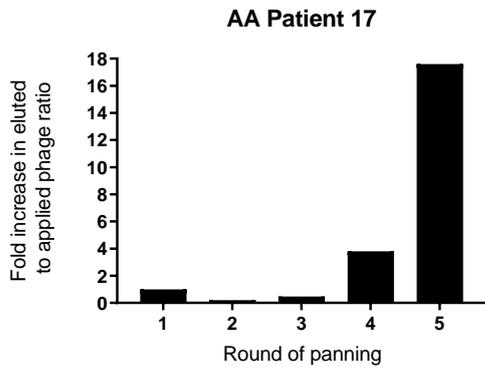
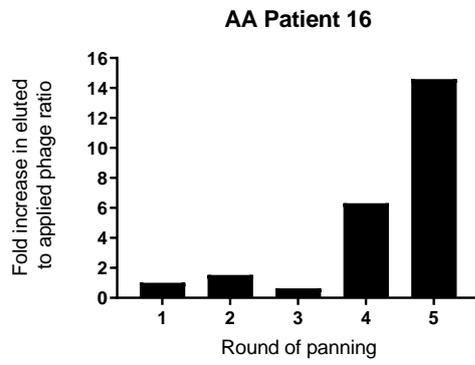
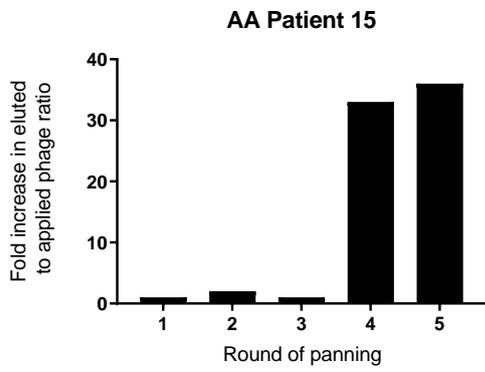
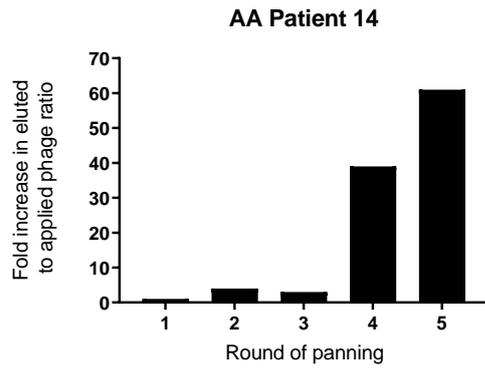
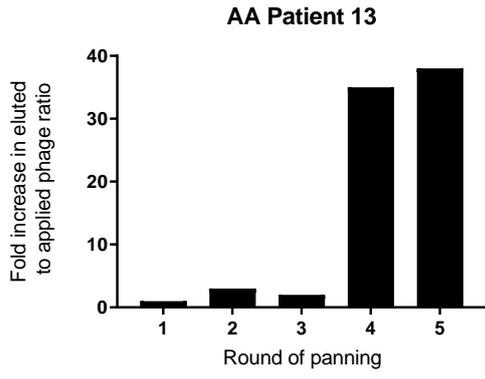


Figure 4.7: Rounds of panning with healthy control IgG.

The phage-display library of melanocyte cDNA-encoded peptides was used in five rounds of panning with two healthy control IgGs. The graphs show the fold increase in the ratio of phage eluted:phage applied in each panning round.







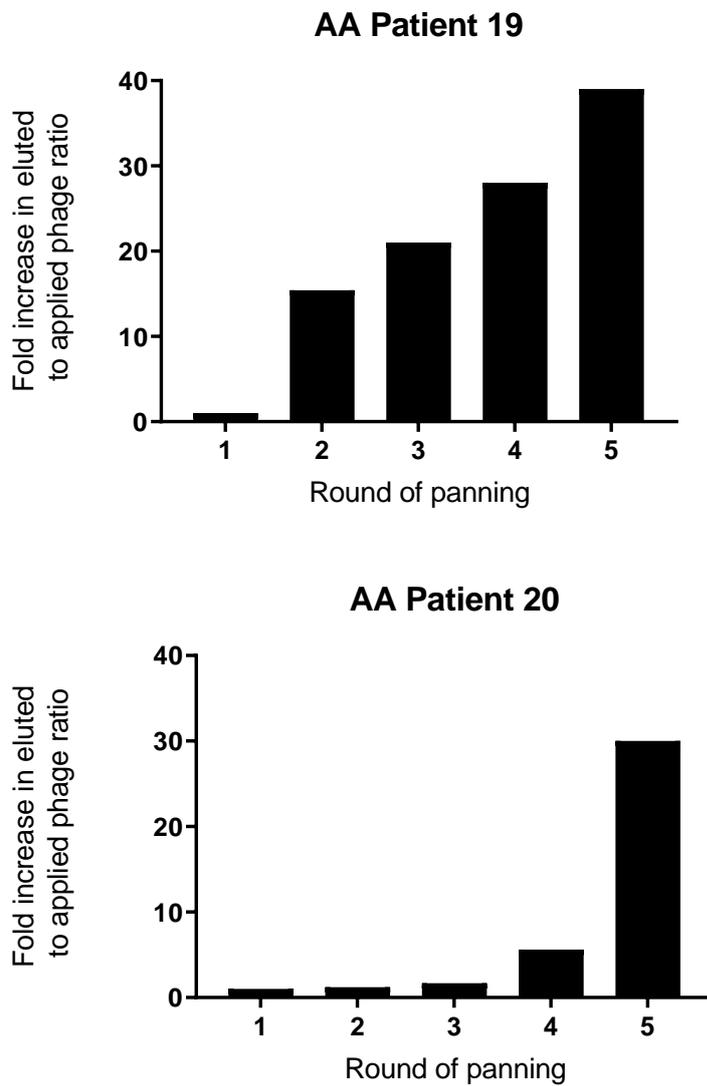


Figure 4.8: Rounds of panning with alopecia areata patient IgG samples.

The phage-display library of melanocyte cDNA-encoded peptides was used in five rounds of panning with 20 alopecia areata patient IgGs. The graphs show the fold increase in the ratio of phage eluted:phage applied in each panning round.

4.4.2 Preparation of recombinant pJuFo plasmid DNA from bacterial clones isolated after round five of panning

After the fifth panning round of 20 alopecia areata patient IgG, two healthy control IgG and four positive control antibodies, at least 50 randomly selected bacterial colonies, isolated by plating out the culture infected with eluted phage, were streaked out onto LB agar containing ampicillin at 100 µg/ml and tetracycline at 10 µg/ml. The bacterial clones were then grown overnight with shaking at 37°C in 10 ml of LB medium containing ampicillin at 100 µg/ml and tetracycline at 10 µg/ml. Subsequently, recombinant pJuFo plasmid DNA encoding IgG-binding peptides was prepared from each culture employing a Wizard® Plus SV Minipreps DNA Purification System. The recombinant pJuFo plasmids were electrophoresed in agarose gels to check that the plasmid preparations had been successful.

The results of the plasmid preparations from one panning experiment are illustrated in Figures 4.9 and 4.10, which show recombinant pJuFo plasmid DNA prepared from a total of 68 individual bacterial colonies from round five of panning with IgG from alopecia areata patient AA01. The isolated recombinant pJuFo plasmids were of a range of sizes and the majority were larger than the pJuFo vector which did not contain any cloned cDNA insert. This indicated the presence of a cloned cDNA fragment in most of the recovered plasmids. However, the recombinant pJuFo plasmids that were of a similar size to the pJuFo vector, these were still analysed further in case there was just a small insert of cDNA present.

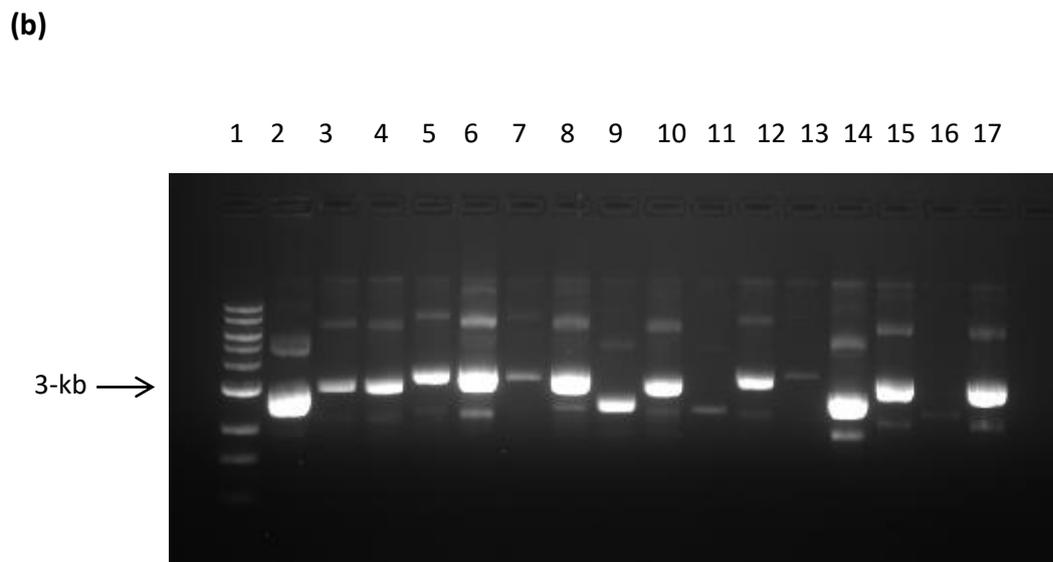
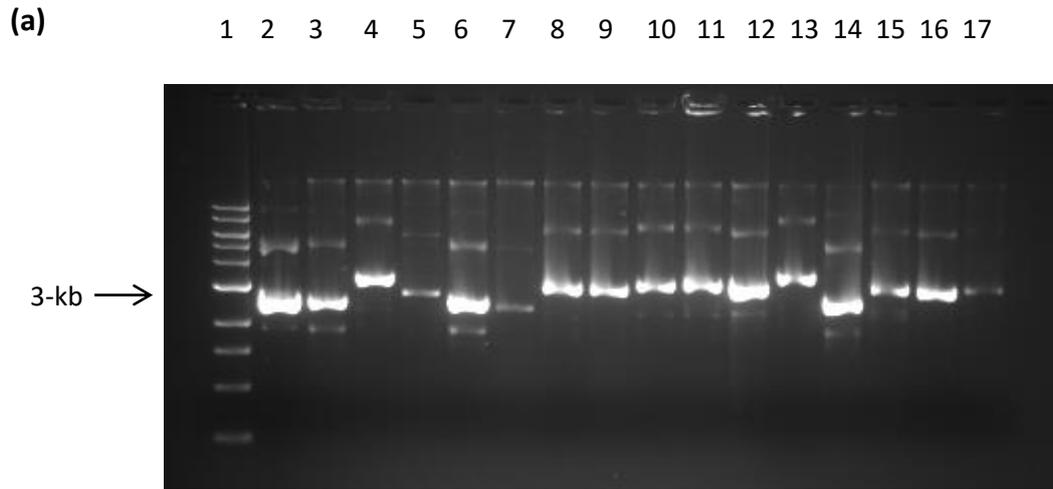
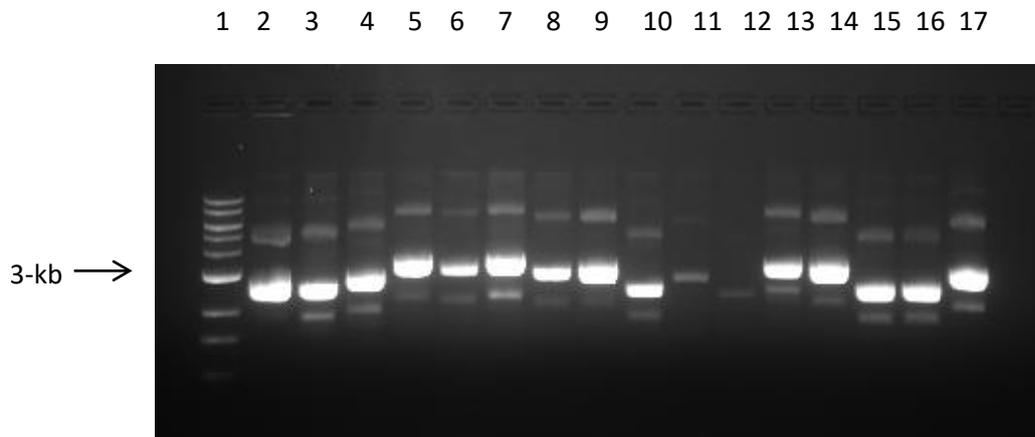


Figure 4.9: Agarose gels showing recombinant pJuFo plasmid DNA made from bacterial clones isolated in round five of panning with IgG from alopecia areata patient AA01.

Recombinant plasmid pJuFo DNA was prepared from 34 individual ampicillin and tetracycline resistant colonies from round five of panning with alopecia areata patient IgG (AA01) were electrophoresed in a 0.8% (w/v) agarose gel. (a) 1-kb DNA Markers with DNA fragments from 500-10,000 bp (lane 1); pJuFo plasmid vector with no cDNA insert (lane 2); plasmid pJuFo isolated from an individual bacterial clone (lanes 3-17). (b) 1-kb DNA Markers with DNA fragments from 500-10,000 bp (lane 1); pJuFo plasmid vector with no cDNA insert (lane 2); and plasmid pJuFo isolated from an individual bacterial clone (lanes 3-17).

(a)



(b)

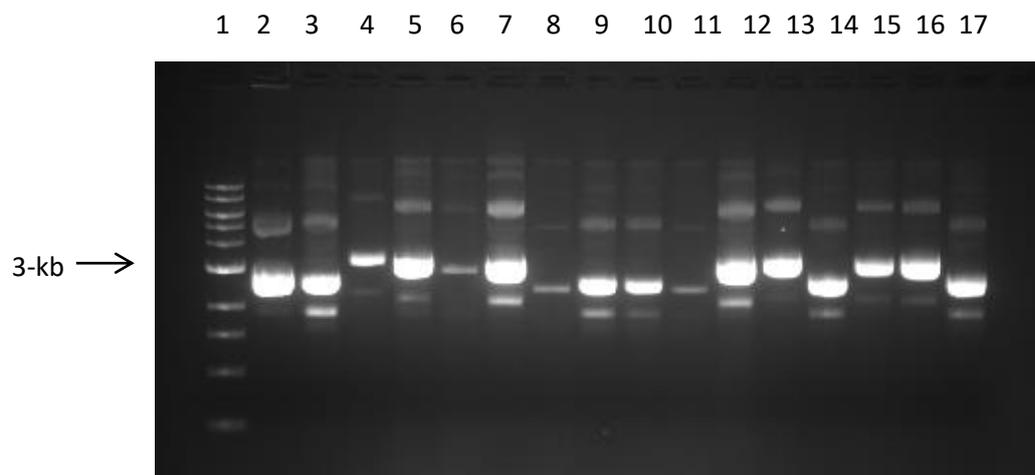


Figure 4.10: Agarose gels showing recombinant pJuFo plasmid DNA made from bacterial clones from round five of panning with IgG from alopecia areata patient AA01.

Recombinant plasmid pJuFo DNA was prepared from 34 individual ampicillin and tetracycline resistant colonies from round five of panning with alopecia areata patient IgG (AA01) were electrophoresed in a 0.8% (w/v) agarose gel. (a) 1-kb DNA Markers with DNA fragments from 500-10,000 bp (lane 1); pJuFo plasmid vector with no cDNA insert (lane 2); plasmid pJuFo isolated from an individual bacterial clone (lanes 3-17). (b) 1-kb DNA Markers with DNA fragments from 500-10,000 bp (lane 1); pJuFo plasmid vector with no cDNA insert (lane 2); and plasmid pJuFo isolated from an individual bacterial clone (lanes 3-17).

4.4.3 Identification of IgG-binding peptides enriched by panning of the melanocyte peptide phage-display library

Recombinant pJuFo plasmids from the panning experiments with alopecia areata patient IgG ($n = 20$), healthy control IgG ($n = 2$), and positive control antibodies ($n = 4$) were subjected to DNA sequencing using JUFO-1192 and JUFO-1500 sequencing primers (Table 2.3). Following DNA sequencing, BLAST searches against the GenBank database were made to identify the cDNAs carried by the plasmids. The sequences were also analysed to see if they were in-frame with the Fos peptide encoded by pJuFo which is needed for surface expression of peptides (Figure 4.3 and 4.5).

4.4.3.1 Panning with positive control antibodies

The results of panning experiments with four positive control antibodies against tyrosinase, tyrosinase-related protein-2, tyrosinase hydroxylase, and melanocyte-specific protein PMEL showed that they enriched the cDNAs encoding their respective antigens and that in at least 96% of cases, the peptides were in-frame with the pJuFo Fos peptide for expression on the phage surface (Table 4.2).

4.4.3.2 Panning with healthy control IgG

The majority of cDNA sequences carried by 50 plasmids from the fifth round of the panning process using IgG from two healthy controls were either out-of-frame with the pJuFo Fos peptide or had no significant matches with identifiable DNA sequences in the GenBank database. One healthy control IgG had enriched two peptides that were in-frame with the Fos peptide and were identified as heat-shock protein 90 (HSP90) (NM_001017963.2).

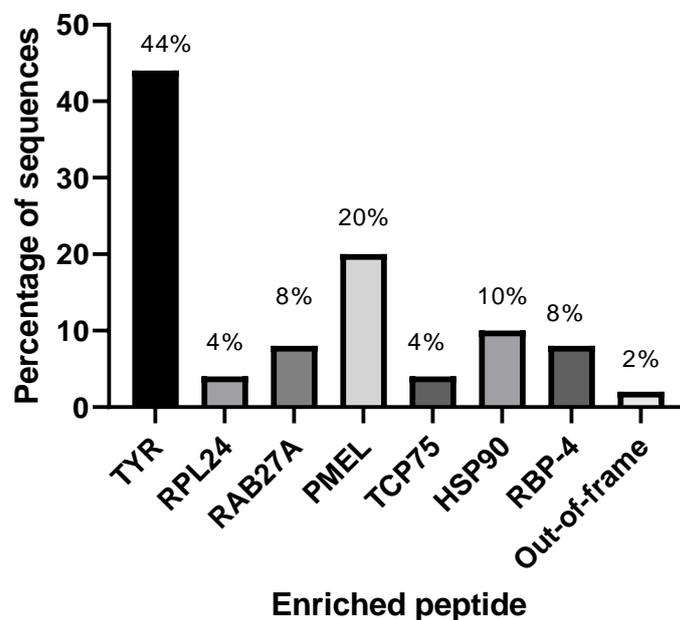
Table 4.2: Peptides enriched by positive control antibodies

| Antibody used in panning | Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---------------------------------|---|---|----------------------------|---|
| Anti-tyrosinase | Tyrosinase (25/50 = 50%) | NM_000372.4 | 13-529 | Yes |
| Anti-tyrosinase | Tyrosinase (13/50 = 26%) | NM_000372.4 | 7-529 | Yes |
| Anti-tyrosinase | Tyrosinase (11/50 = 22%) | NM_000372.4 | 16-529 | Yes |
| Anti-tyrosinase | Out-of-frame tyrosinase (1/50 = 2%) | NM_000372.4 | 2-529 | No |
| Anti-tyrosine hydroxylase | Tyrosine hydroxylase (48/50 = 96%) | NM_000360.1 | 1-497 | Yes |
| Anti-tyrosine hydroxylase | Out-of-frame tyrosine hydroxylase (2/50 = 4%) | NM_000360.1 | 2-497 | No |
| Anti-TRP2 | Tyrosinase-related protein-2 (40/50 = 80%) | NM_001129889.2 | 10-552 | Yes |
| Anti-TRP2 | Tyrosinase-related protein-2 (9/50 = 18%) | NM_001129889.2 | 19-552 | Yes |
| Anti-TRP2 | Out-of-frame tyrosinase-related protein-2 (1/50 = 2%) | NM_001129889.2 | 2-552 | No |
| Anti-PMEL | Melanocyte-specific protein PMEL (11/50 = 22%) | NM_001200054.1 | 19-668 | Yes |
| Anti-PMEL | Melanocyte-specific protein PMEL (13/50 = 26%) | NM_001200054.1 | 11-668 | Yes |
| Anti-PMEL | Melanocyte-specific protein PMEL (16/50 = 32%) | NM_001200054.1 | 31-668 | Yes |
| Anti-PMEL | Melanocyte-specific protein PMEL (5/50 = 10%) | NM_001200054.1 | 4-668 | Yes |
| Anti-PMEL | Melanocyte-specific protein PMEL (5/50 = 10%) | NM_001200054.1 | 22-668 | Yes |

4.4.3.3 Panning with alopecia areata IgG

The results of the panning experiments with alopecia areata patient IgG indicated that several different peptides had been enriched during the panning process. For each of the 20 patient IgGs, the enriched peptides encoded by 100 pJuFo plasmids are given in Figures 4.11 to 4.30. The majority of the identified peptides were in-frame with the pJuFo Fos peptide so that their expression on the phage surface would be expected. A summary of the peptides enriched by all 20 patient IgGs in the panning experiments is given in Figure 4.31.

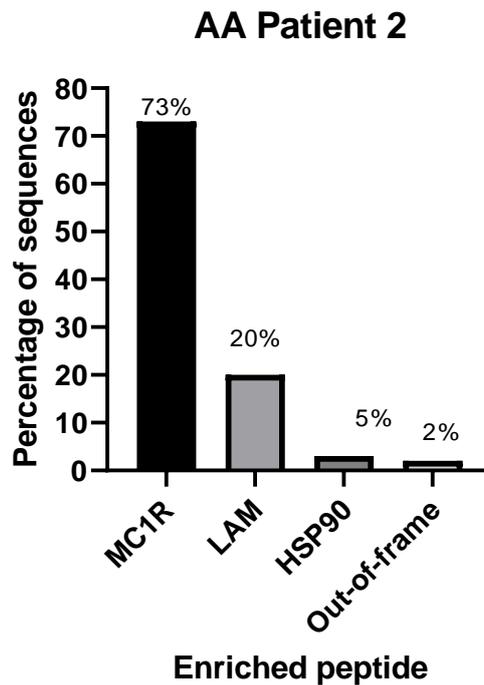
AA Patient 1



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosinase (TYR) | NM_000372.4 | 7-529 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 1-216 | Yes |
| GTP-binding protein Rab27A (RAB27A) | NM_004580.4 | 1-221 | Yes |
| Melanocyte-specific protein PMEL (PMEL) | NM_001200054.1 | 5-668 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 1-530 | Yes |
| Heat-shock protein 90 (HSP90) | NM_001017963.2 | 753-854 | Yes |
| Retinol-binding protein-4 (RBP-4) | NM_006744.3 | 1-201 | Yes |

Figure 4.11: IgG-binding peptides enriched in the panning process for alopecia areata patient AA01.

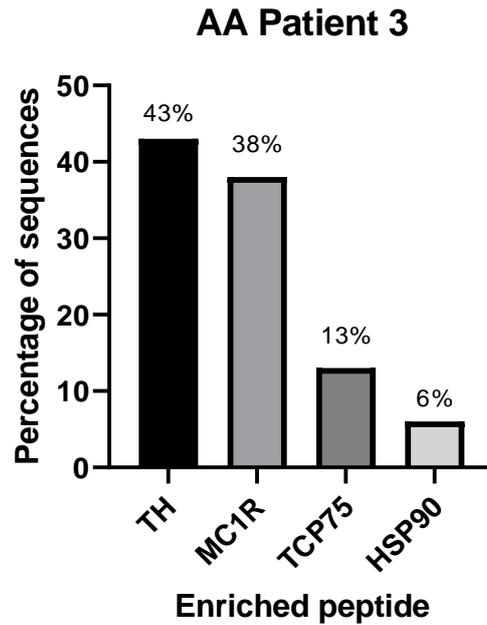
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning using IgG from alopecia areata patient AA01. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 16-317 | Yes |
| Lamin A (LAM) | NM_170707 | 4-664 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |

Figure 4.12: IgG-binding peptides enriched in the panning process for alopecia areata patient AA02.

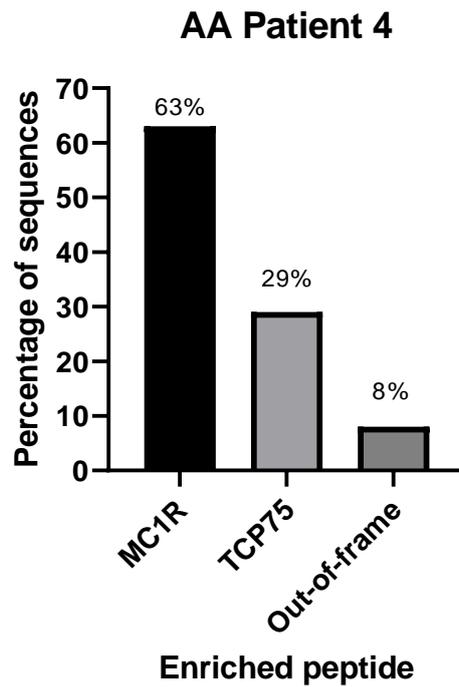
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning using IgG from alopecia areata patient AA02. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosine hydroxylase (TH) | NM_000360.1 | 1-497 | Yes |
| Tyrosine hydroxylase (TH) | NM_000360.1 | 16-497 | Yes |
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 1-530 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |

Figure 4.13: IgG-binding peptides enriched in the panning process for alopecia areata patient AA03.

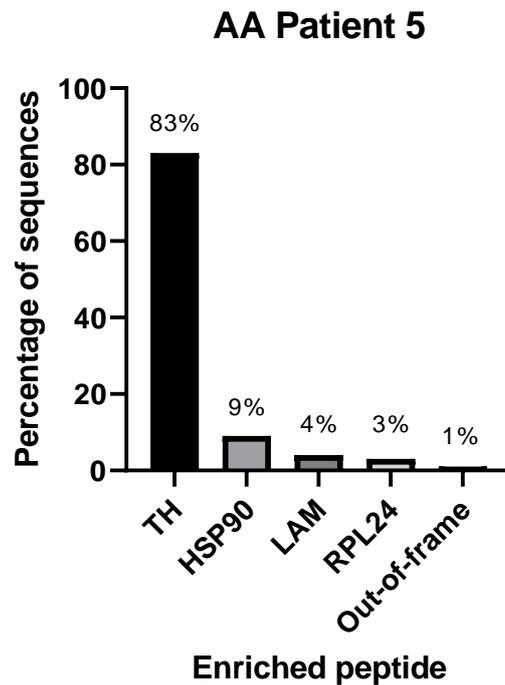
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning using IgG from alopecia areata patient AA03. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 1-530 | Yes |

Figure 4.14: IgG-binding peptides enriched in the panning process for alopecia areata patient AA04.

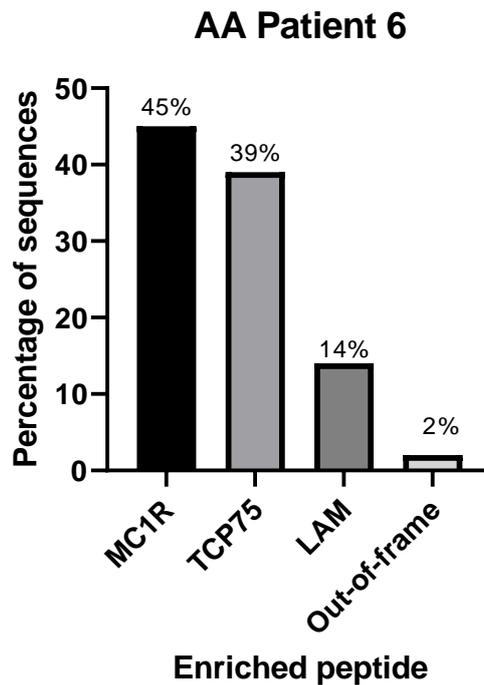
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning using IgG from alopecia areata patient AA04. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosine hydroxylase (TH) | NM_000360.1 | 16-497 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |
| Lamin A (LAM) | NM_170707 | 4-664 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 13-216 | Yes |

Figure 4.15: IgG-binding peptides enriched in the panning process for alopecia areata patient AA05.

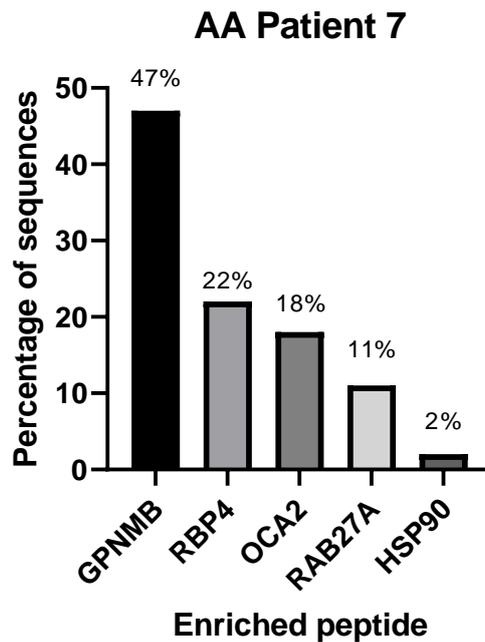
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA05. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 1-530 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 19-530 | Yes |
| Lamin A (LAM) | NM_170707 | 4-664 | Yes |
| Lamin A (LAM) | NM_170707 | 13-664 | Yes |

Figure 4.16: IgG-binding peptides enriched in the panning process for alopecia areata patient AA06.

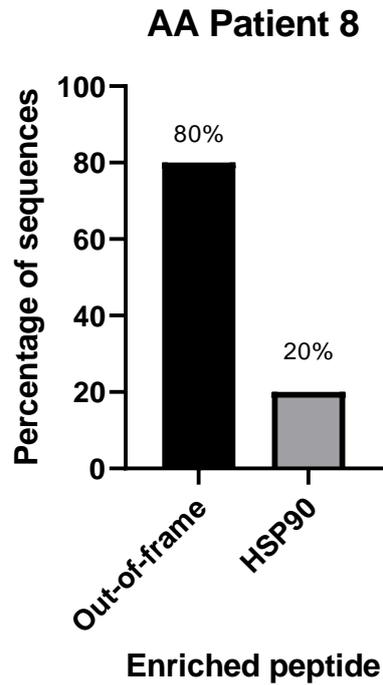
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA06. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|--|--------------------------------------|---------------------|--|
| Glycoprotein non-metastatic melanoma protein b (GPNMB) | NM_001005340.1 | 13-572 | Yes |
| Retinol-binding protein-4 (RBP-4) | NM_006744.3 | 1-201 | Yes |
| OCA2-encoded P protein (OCA2) | NM_001300984.1 | 15-814 | Yes |
| GTP-binding protein Rab27A (RAB27A) | NM_004580.4 | 1-221 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |

Figure 4.17: IgG-binding peptides enriched in the panning process for alopecia areata patient AA07.

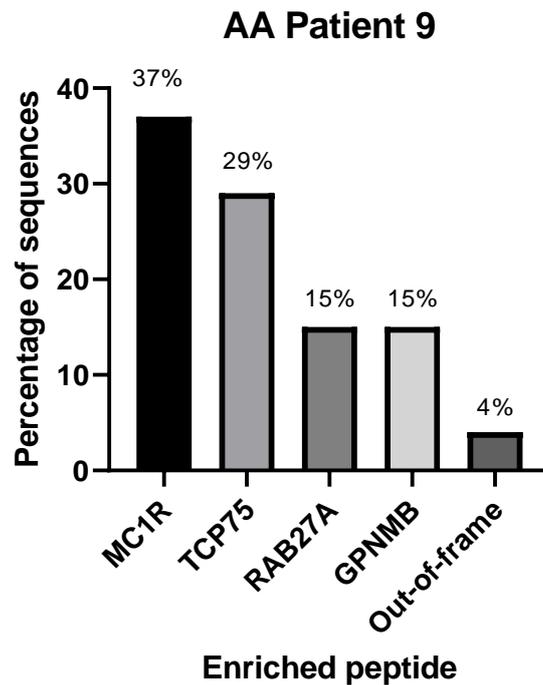
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA07. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |

Figure 4.18: IgG-binding peptides enriched in the panning process for alopecia areata patient AA08.

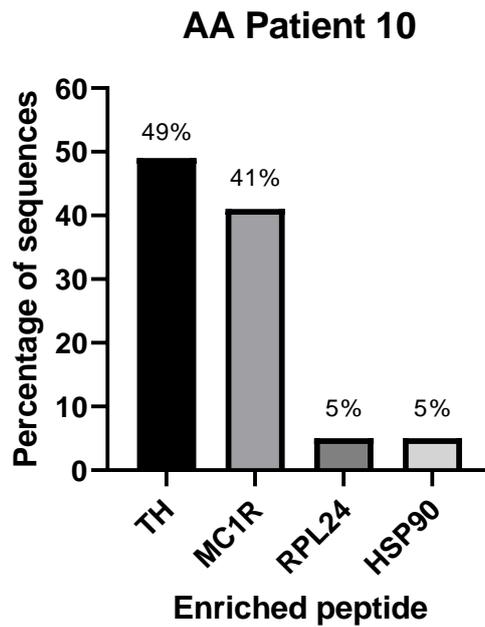
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA08. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|--|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 19-530 | Yes |
| GTP-binding protein Rab27A (RAB27A) | NM_004580.4 | 1-221 | Yes |
| Glycoprotein non-metastatic melanoma protein b (GPNMB) | NM_001005340.1 | 13-572 | Yes |

Figure 4.19: IgG-binding peptides enriched in the panning process for alopecia areata patient AA09.

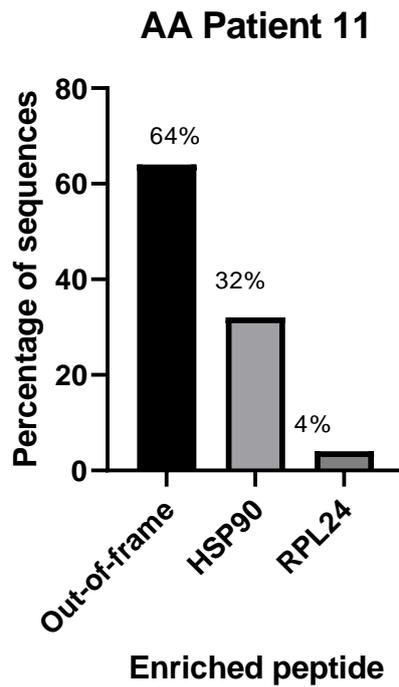
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA09. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosine hydroxylase (TH) | NM_000360.1 | 16-497 | Yes |
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 13-216 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 7-732 | Yes |

Figure 4.20: IgG-binding peptides enriched in the panning process for alopecia areata patient AA10.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA10. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

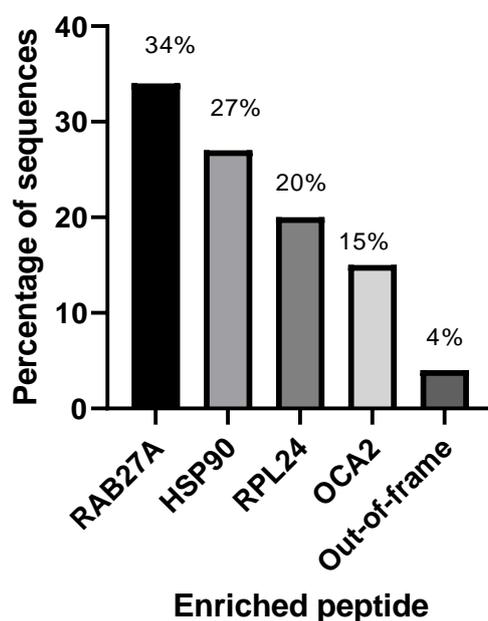


| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 10-216 | Yes |

Figure 4.21: IgG-binding peptides enriched in the panning process for alopecia areata patient AA11.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA11. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

AA Patient 12

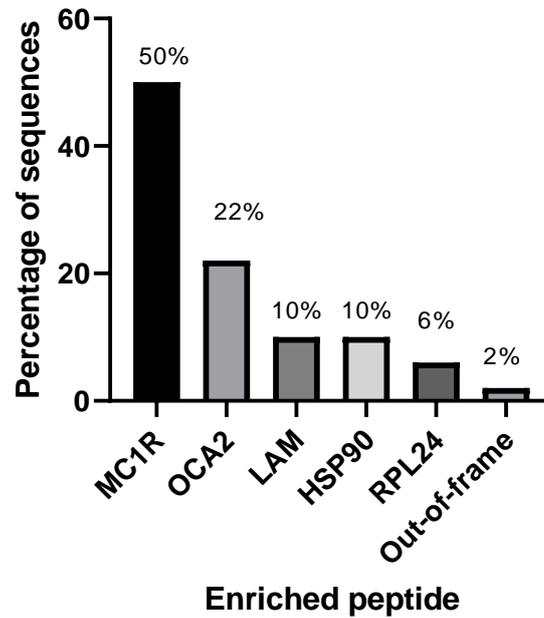


| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| GTP-binding protein Rab27A (RAB27A) | NM_004580.4 | 1-221 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 10-216 | Yes |
| OCA2-encoded P protein (OCA2) | NM_001300984.1 | 15-814 | Yes |

Figure 4.22: IgG-binding peptides enriched in the panning process for alopecia areata patient AA12.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA12. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

AA Patient 13

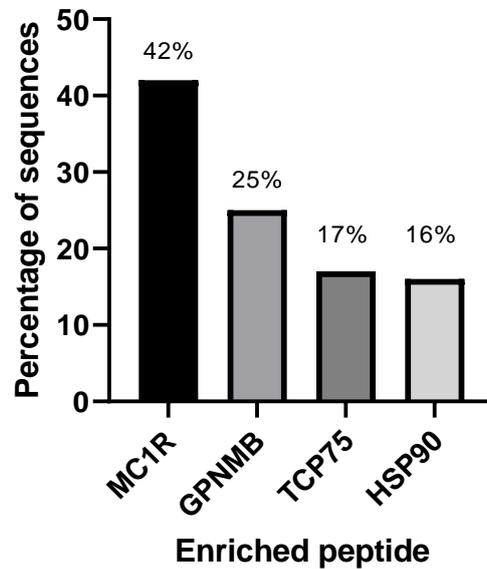


| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| OCA2-encoded P protein (OCA2) | NM_001300984.1 | 15-814 | Yes |
| Lamin A (LAM) | NM_170707 | 16-664 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |
| Ribosomal protein L24 (RPL24) | NM_145729.2 | 10-216 | Yes |

Figure 4.23: IgG-binding peptides enriched in the panning process for alopecia areata patient AA13.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA13. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

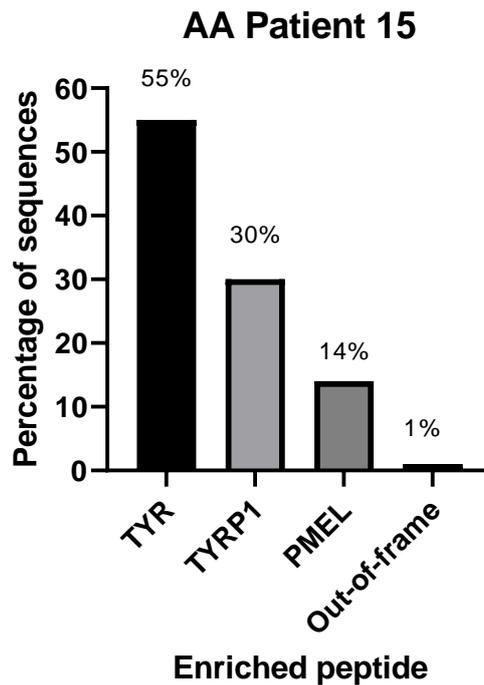
AA Patient 14



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|--|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Glycoprotein non-metastatic melanoma protein b (GPNMB) | NM_001005340.1 | 13-572 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 19-530 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |

Figure 4.24: IgG-binding peptides enriched in the panning process for alopecia areata patient AA14.

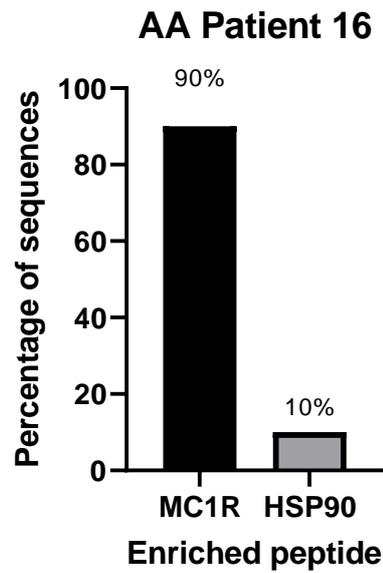
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA14. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosinase (TYR) | NM_000372.4 | 19-529 | Yes |
| Tyrosinase-related protein-1 (TYRP1) | NM_000550.2 | 4-537 | Yes |
| Melanocyte-specific protein PMEL (PMEL) | NM_001200054.1 | 5-668 | Yes |

Figure 4.25: IgG-binding peptides enriched in the panning process for alopecia areata patient AA15.

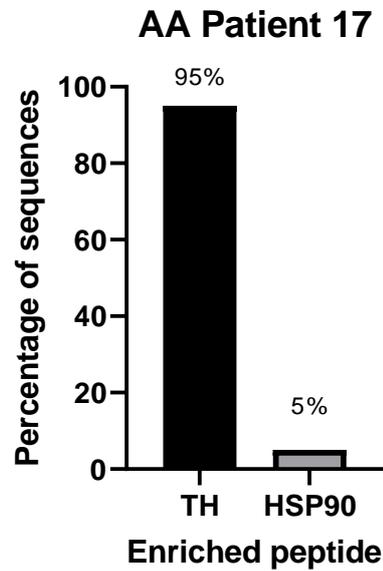
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning using IgG from alopecia areata patient AA15. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |

Figure 4.26: IgG-binding peptides enriched in the panning process for alopecia areata patient AA16.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA16. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

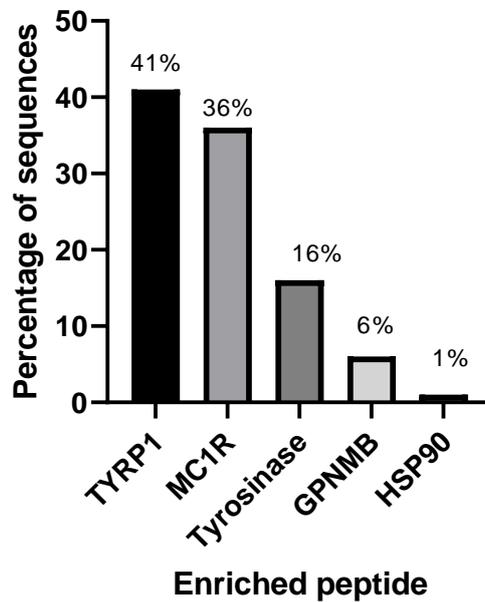


| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Tyrosine hydroxylase (TH) | NM_000360.1 | 22-497 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |

Figure 4.27: IgG-binding peptides enriched in the panning process for alopecia areata patient AA17.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA17. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.

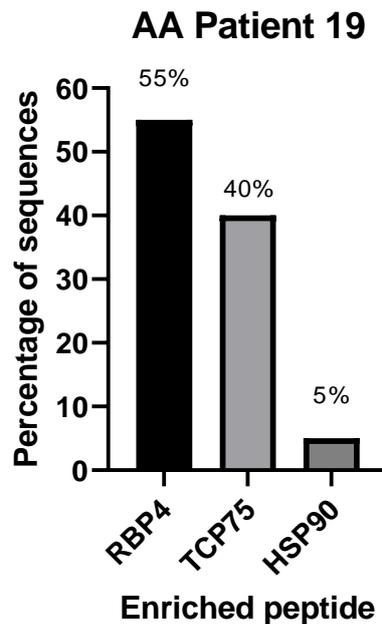
AA Patient 18



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|--|--------------------------------------|---------------------|--|
| Tyrosinase-related protein-1 (TYRP1) | NM_000550.2 | 4-537 | Yes |
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| Tyrosinase (TYR) | NM_000372.4 | 19-529 | Yes |
| Glycoprotein non-metastatic melanoma protein b (GPNMB) | NM_001005340.1 | 13-572 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |

Figure 4.28: IgG-binding peptides enriched in the panning process for alopecia areata patient AA18.

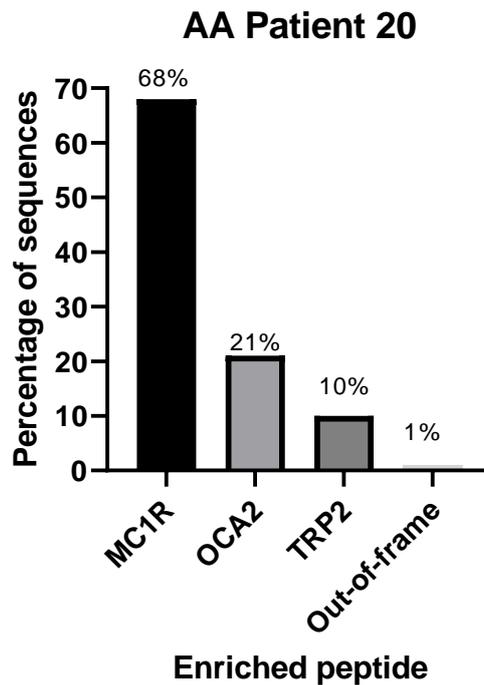
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA18. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Retinol-binding protein-4 (RBP-4) | NM_006744.3 | 1-201 | Yes |
| Transcriptional coactivator p75 (TCP75) | NM_001128217.1 | 19-530 | Yes |
| Heat-shock protein 90 (HSP90) | NM_005348.3 | 13-732 | Yes |

Figure 4.29: IgG-binding peptides enriched in the panning process for alopecia areata patient AA19.

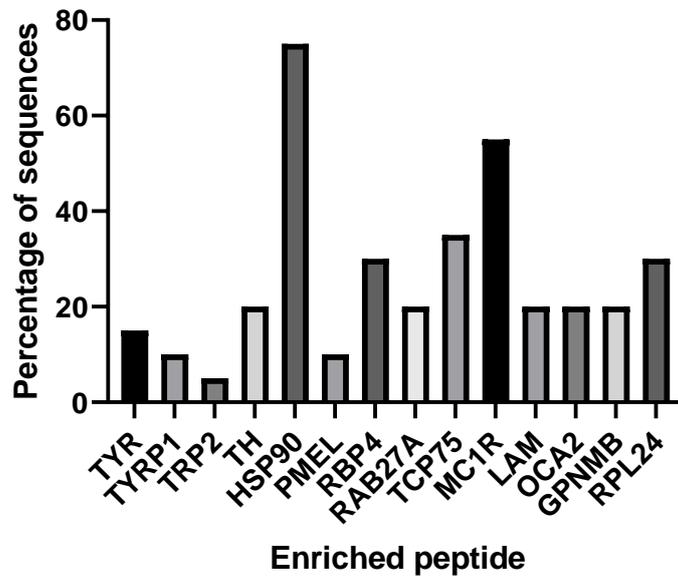
Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA19. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Identity of peptide in sequenced clones | Accession number in Genbank database | Amino acids encoded | In-frame fusion with pJuFo Fos protein |
|---|--------------------------------------|---------------------|--|
| Melanocortin 1 receptor (MC1R) | NM_002386.3 | 22-317 | Yes |
| OCA2-encoded P protein (OCA2) | NM_001300984.1 | 15-814 | Yes |
| Tyrosinase-related protein-2 (TRP2) | NM_001129889.2 | 10-552 | Yes |

Figure 4.30: IgG-binding peptides enriched in the panning process for alopecia areata patient AA20.

Plasmid DNA from 100 randomly selected bacterial clones were isolated from the fifth round of panning round using IgG from alopecia areata patient AA20. The identify of enriched peptides was enabled by DNA sequencing and database searches using BLAST. The results for each peptide are expressed as a percentage of the total.



| Enriched peptide | Number of patient IgGs that enriched the peptide (%) (n = 20) |
|--|---|
| Tyrosinase (TYR) | 3 (15) |
| Tyrosinase-related protein-1 (TYRP1) | 2 (10) |
| Tyrosinase-related protein-2 (TRP2) | 1 (5) |
| Tyrosine hydroxylase (TH) | 4 (20) |
| Heat-shock protein 90 (HSP90) | 15 (75) |
| Melanocyte-specific protein PMEL (PMEL) | 2 (10) |
| Retinol-binding protein-4 (RBP-4) | 3 (15) |
| Ribosomal protein L24 (RPL24) | 6 (30) |
| GTP-binding protein Rab27A (RAB27A) | 4 (20) |
| Transcriptional coactivator p75 (TCP75) | 7 (35) |
| Melanocortin 1 receptor (MC1R) | 11 (55) |
| Lamin A (LAM) | 4 (20) |
| OCA2-encoded P protein (OCA2) | 4 (20) |
| Glycoprotein non-metastatic melanoma protein b (GPNMB) | 4 (20) |

Figure 4.31: Summary of peptides isolated from panning experiments with 20 alopecia areata patient IgG.

4.5 Discussion

In phage-display technology, combining the affinity of IgG to its cognate peptide with the ability to amplify the selected phage expressing the peptide makes a very useful method to isolate novel autoantigens. Sizable libraries of cDNA, containing up to 10^{11} independent clones, can be screened for IgG-binding peptides in just one series of panning experiments. Sometimes, IgG-binding peptides may be rarely expressed in the original phage-display library, but because repeated rounds of selection are undertaken during phage-display experiments, they may still be identified. Moreover, displayed peptides may sometimes adopt their native structure (Skerra and Pluckthun, 1988) as they are selected in liquid-phase as opposed to conventional immunoscreening which requires adsorption of the peptides onto nitrocellulose membranes. Indeed, a phage-display strategy has been employed recently in the discovery of self-antigens in chronic inflammation of the central nervous system (Burgoon et al., 2001), in systemic lupus erythematosus (Kemp et al., 2002a), and in vitiligo (Kemp et al., 2002b, Waterman et al., 2010). In the current project, the pJuFo phage display system (Cramer and Suter, 1993) was used to identify novel autoantigens in alopecia areata.

4.5.1 Results analysis

The results showed that during the enrichment process with IgG samples from 17/20 alopecia areata patients there was an increase of 15-491-fold in the normalised ratio of phage eluted:phage applied when compared to healthy control IgGs (5-13-fold). This indicated that in most cases, phage carrying IgG-binding proteins were being enriched in each round of panning above what might be considered background levels or non-specific binding. Despite the lack of an increase in the normalised ratio of phage eluted:phage for three IgG samples (AA08, AA11 and AA12), sequencing of the cDNAs from the final panning rounds did show enrichment of some peptides. However, the reason for this is not clear, although it may have been due to low antibody titres in some patients.

Following sequence analysis of clones from the fifth round of panning, several putative autoantigens were identified. Some autoantigens had already been characterised as antibody targets in alopecia areata including tyrosine hydroxylase, tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL (Kemp et al.,

2011b), and retinol-binding protein-4 (Ahn et al., 2011). However, several novel autoantigens were also identified.

4.5.1.1 Previously identified autoantibody targets in alopecia areata

The melanocyte-specific proteins tyrosinase, tyrosinase-related protein-2, tyrosinase-related protein-1, melanocyte-specific protein PMEL were enriched by three (15%), one (5%), two (10%), and two (10%) of the 20 alopecia areata patient IgGs, respectively (Figure 4.30). Melanocyte-specific protein PMEL has an important role in the structural organisation of premelanosomes, while the other three proteins are enzymes required for the production of melanin (Yamaguchi et al., 2007, D'Mello et al., 2016). They are all located intracellularly in the melanosomal compartments of the melanocyte. However, several studies have indicated that tyrosinase, tyrosinase-related protein-1, and melanocyte-specific protein PMEL are sometimes transiently expressed on the cell surface so exposed to the immune system (Takechi et al., 1996, Calvo et al., 1999, Le Borgne et al., 2001, Lepage and Lapointe, 2006, Theos et al., 2006). Indeed, autoantibodies against these melanogenic proteins are found at a low prevalence in alopecia areata (Kemp et al., 2011b), as well as being T cell targets in patients with the disease (Gilhar et al., 2001, Wang et al., 2016). All the aforementioned melanocyte-specific proteins have also been identified as autoantigens in vitiligo (Song et al., 1994, Baharav et al., 1996, Kemp et al., 1997a, Kemp et al., 1998a, Lang et al., 2001, Palermo et al., 2001, Mandelcorn-Monson et al., 2003, Pradhan et al., 2013).

Tyrosine hydroxylase, the enzyme responsible for converting tyrosine to L-dopa in the synthesis of melanin (Lewis et al., 1993) was identified as a B cell autoantigen in patients with autoimmune polyendocrine syndrome type 1, where antibodies against the enzyme were strongly associated with the presence of alopecia areata (Hedstrand et al., 2000). Antibody reactivity against tyrosine hydroxylase has also been detected in patients with vitiligo, a disease in which melanocytes are destroyed by autoimmune mechanisms (Kemp et al., 2011a). In the current study, four (20%) alopecia areata patient IgGs enriched this protein in the final round of panning (Figure 4.30).

Although it is not clear if retinol-binding protein-4 is expressed in melanocytes, cDNA encoding the protein was found enriched by three (15%) patient IgGs (Figure 4.30). In a

previous study, 67% of alopecia areata patients were found to be positive for antibodies against retinol-binding protein-4 (Ahn et al., 2011).

The transcription coactivator p75 (dense fine speckles 70 kD (DFS70) protein/lens epithelium-derived growth factor (LEDGF) (Ortiz-Hernandez et al., 2020) was reported as an antibody target in 20% of alopecia areata patients (Okamoto et al., 2004), and in 36% of patients with atopic dermatitis (Ochs et al., 2000). In the present study, 7/20 (35%) of patient IgGs enriched this peptide during panning experiments.

4.5.1.2 Novel autoantibody targets in alopecia areata

The melanocortin 1 receptor (MC1R), also known as melanocyte-stimulating hormone receptor, is expressed on the plasma membrane of melanocyte and plays an important role in pigmentation (Hadley et al., 1981, Suzuki et al., 1996). Via the MC1R and the binding of its ligand α -melanocyte-stimulating hormone, the synthesis of melanin is increased in melanocytes (Valverde et al., 1995). In the current study, 11/20 (55%) alopecia areata patient IgGs enriched MC1R in the final round of panning (Figure 4.30). It is possible that the function of the receptor is affected adversely by binding antibodies as in the case of the melanin-concentrating hormone receptor-1 in vitiligo (Gottumukkala et al., 2006), the thyrotropin receptor in Graves' disease (Weetman and McGregor, 1994), and the acetylcholine receptor in myasthenia gravis (Hoedemaekers et al., 1997).

Lamin A is a nuclear protein involved in the maintenance of cellular morphology (Zhu et al., 2015). Antibodies against laminar proteins have been detected in patients with other autoimmunities such as autoimmune liver disease (Wesierska-Gadek et al., 1988), systemic lupus erythematosus (Senecal et al., 1999), and vitiligo (Cui et al., 1995, Li et al., 2011). Although Lamin A is located in the nucleus of the cell, it can be expressed on the surface of melanocytes so may be accessible to the immune system (Cui et al., 1995). In the present study, Lamin A was enriched by phage-display panning experiments in 4/20 (20%) of the alopecia areata patient IgGs investigated.

The glycoprotein non-metastatic melanoma protein b (GPNMB) is expressed in different cell types including melanocytes and retinal pigment epithelial cells as well as immune cells including antigen-presenting cells and macrophages (Owen et al., 2003, Kumagai et al., 2015).

In pigment cells, the protein is expressed in late-stage melanosomal organelles and enables keratinocyte-melanocyte adhesion, and so is thought to play a role in the trafficking of melanosomes to keratinocytes (Tomihari et al., 2009b). It has also been reported to be expressed on the surface of melanocytes (Tomihari et al., 2009b). The current study identified a small number (4/20 = 20%) of alopecia areata patient IgG that enriched the GPNMB protein through the rounds of panning (Figure 4.30).

The OCA2-encoded P protein is also known as melanocyte-specific transporter protein or pink-eyed dilution protein homolog. It is specifically expressed on the surface of melanosomes and is an essential component of a melanosome-specific anion channel that regulates melanosomal pH. This provides the best cellular conditions for the activity of tyrosinase and subsequent melanin production (Puri et al., 2000, Sitaram et al., 2009, Bellono et al., 2014). The OCA2-encoded P protein is essential for the normal coloring of skin, eyes, and hair (Jin et al., 2012), and mutations in the gene cause mislocalisation of the P protein to the cell surface which impairs its function in pigment production (Sitaram et al., 2009). Mutations in the *OCA2* gene are also risk factors for vitiligo and melanoma (Cheng et al. (2013). Previously, the OCA2-encoded P protein has not been identified as an autoantigen in alopecia areata. In the current study, 4/20 (20%) alopecia areata patient IgGs enriched the protein in phage-display panning experiments (Figure 4.30).

The GTP-binding protein Rab27a, which functions in exosome secretion (Ostrowski et al., 2010), was identified as a novel autoantigen that has not been reported before in alopecia areata. Here, 4/20 (20%) alopecia areata patient IgGs enriched GTP-binding protein Rab27a in phage-display panning experiments (Figure 4.30). Notably, antibodies against the GTP-binding protein Rab38, a protein involved in the transport of melanogenic enzymes from the Golgi system to melanosomal organelles have been reported in patients with vitiligo (Waterman et al., 2010). The protein is involved in the transfer of melanosomes to keratinocytes (Yoshida-Amano et al., 2012). It is located within melanosomes and is not associated with the membrane of the cell (Bahadoran et al., 2001).

Heat-shock protein 90 (HSP90) plays an important role in immune responses including antigen presentation, the activation of lymphocytes and macrophages, the maturation and activation of dendritic cells, and in the induction of inflammation (Srivastava, 2002). The protein is not

usually expressed on cell surfaces so would not be expected to be seen by the immune system. However, high serum levels of HSP90 and surface expression of the protein on peripheral blood monocytes has been reported in patients suffering from systemic lupus erythematosus (Conroy et al., 1994), and its secretion has also been observed from melanocytes under stress (Denman et al., 2008). So, HSP90 antibodies have been identified in systemic lupus erythematosus (Conroy et al., 1994, Stephanou et al., 1998), in vitiligo (Waterman et al., 2010), and in melanoma-associated hypopigmentation (Kiniwa et al., 2001). In the current investigation, HSP90 was identified as an antibody target in 15/20 (75%) of alopecia areata patients.

In various autoimmune diseases such as systemic lupus erythematosus, autoantibodies against ribosomal proteins have often been detected (Elkon et al., 1992). So too have CD4+ T cell responses against ribosomal proteins (Donauer et al., 1999). Although the reason for the frequent immunoreactivity against ribosomal proteins is not clear, it has been suggested that B lymphocytes drive the autoimmune response against ribosomal proteins by their efficient uptake of ribonucleoproteins and ribosomes due to their particulate nature (Datta and Kaliyaperumal, 1997). Other reports have proposed that the highly basic nature of ribosomal proteins make them more frequent targets of the autoimmune response (Lee et al., 1997). In the current study, 3/20 (15%) alopecia areata patient IgGs enriched ribosomal protein L24 (RPL24) in the final round of panning.

4.5.2 Limitations of phage-display

Although the results are encouraging, phage-display does have limitations for autoantigen identification. The expression of some proteins on the surface may be hindered by several factors, such that only a proportion of the cDNAs in the library are carried by the phage. The encoded peptides must be secreted through the inner membrane of *E. coli* and fold into their three-dimensional forms in the periplasm. The peptides must also maintain their configuration on the phage particles surface after being exposed to an oxidising environment (Wind et al., 1999). Furthermore, proteins that are usually associated with cell membranes may not pass into periplasm (Wilson and Finlay, 1998). Proteins that contained charged residues might not be secreted (Wilson and Finlay, 1998). However, receptor proteins have been enriched by phage-display (Kemp et al., 2002a). The library complexity may also be

limited by the cloning of the cDNA inserts where they might not possess an open reading frame for expression. The source mRNA may also limit the diversity of the cDNAs expressed in the library.

4.5.3 Conclusions

The results confirm that phage-display is a technique applicable to identifying novel autoantigens in alopecia areata. However, further analysis is needed to verify the immunoreactivity of the potential autoantigens against a group of patients with alopecia areata and this will be detailed in the following chapter.

Identifying autoantigens is key to a full understanding of the role of autoimmunity in the pathogenesis of alopecia areata. Autoantigens can be the targets of both or either of the humoral or cellular immune system, and antibodies might just be markers of disease without any pathogenic activity. However, they may indicate the presence of autoreactive T cells. Indeed, although antibodies against tyrosinase and PMEL have been identified at a low frequency in alopecia areata (Kemp et al., 2011b), these autoantigens are the targets of cytotoxic T lymphocyte targets in alopecia areata patients (Gilhar et al., 2001, Wang et al., 2016).

Chapter 5

**Validation of the Antibody Response Against Novel Autoantigens
Identified by Phage-Display**

5 Validation of the Antibody Response Against Novel Autoantigens Identified by Phage-Display

5.1 Introduction

The phage-display experiments identified several novel peptides as potential autoantigens in alopecia areata. However, these needed to be analysed in further experiments in order to validate them as autoantibody targets. Three of the newly identified autoantigens were chosen for further analysis: glycoprotein non-metastatic melanoma protein b (GPNMB), OCA2-encoded P protein (OCA2), and the melanocortin 1 receptor (MC1R).

5.2 Aims

The aims of this part of the project were to analyse the frequency of antibodies against GPNMB, OCA2, and MC1R in alopecia areata patients using radioligand binding assays and to determine if there were any associations between the presence of novel antibodies and the demographic or clinical details of the alopecia areata patients.

5.3 Results

5.3.1 Radioligand binding assays to detect antibodies against novel autoantigens

Initially, in order to produce [³⁵S]-labelled antigens GPNMB, OCA2, and MC1R for use in the RLBA, the appropriate plasmids (Table 2.4) were translated using a TnT[®] T7-Coupled Reticulocyte Lysate System as described in Section 2.16. The radiolabelled ligands were then used to test alopecia areata patient ($n = 48$) and control ($n = 50$) sera for antibodies in RLBA that were undertaken as detailed in Section 2.18. Sera were tested at a final dilution of 1:100 in duplicate. An appropriate positive control antibody (Table 2.5) was used in the RLBA at a dilution of 1:100.

The levels of antibody were calculated as an antibody index: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 50 healthy control sera. Sera were tested in three RLBA and the mean antibody index was derived. The upper limit of normal for each RLBA was: the mean antibody index + 3SD of the population of 50 healthy controls. Sera with an antibody index above the normal upper limit were classed as positive for the antibody specificity under analysis.

The results of the RLBA for antibodies against GPNMB, OCA2, and MC1R in patients and controls are shown in Figures 5.1-5.3, respectively, and are given in Table 5.1. Control antibodies were positive in the appropriate RLBA (Table 5.1). Healthy controls were negative for antibodies to all three autoantigens with antibody indices below the upper limit of normal for each assay. Of the 48 alopecia areata patients, 10 (20.8%), 8 (16.7%), and 21 (43.8%) were considered positive for antibodies against GPNMB, OCA2, and MC1R, respectively. The prevalence of GPNMB, OCA2, and MC1R antibodies was compared between alopecia patients and healthy controls in Fisher's exact test for 2 x 2 contingency tables. All P values were < 0.05 so that the differences in antibody frequencies were regarded as significant (Table 5.1).

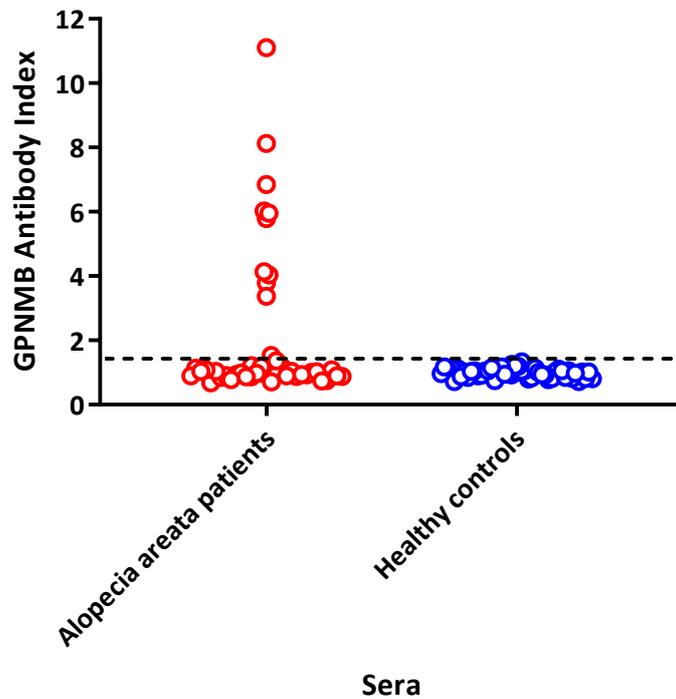
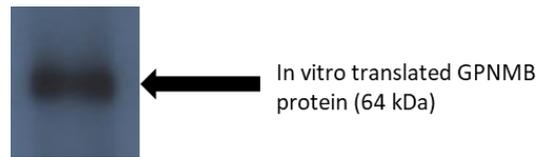


Figure 5.1: Results of GPNMB RLBA for alopecia areata patients and healthy controls.

Alopecia areata patient and healthy controls were analysed for antibodies against GPNMB in RLBAs. The antibody indices are shown for sera from alopecia areata patients ($n = 48$) and healthy controls ($n = 50$). The antibody index shown is the mean of three experiments. The upper limit of normal (1.41) of the RLBA (mean antibody index + 3SD of 50 healthy controls) is shown by the dotted line. The *in vitro* translated GPNMB protein used as the radiolabelled ligand in the RLBA is shown.

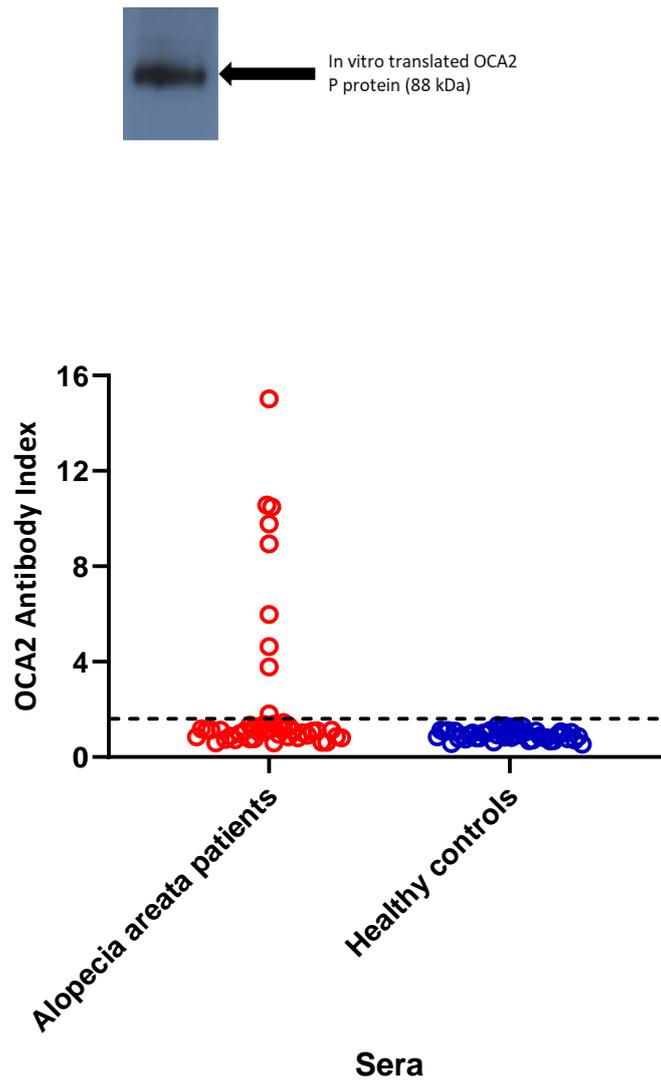


Figure 5.2: Results of OCA2 RLBA for alopecia areata patients and healthy controls.

Alopecia areata patient and healthy controls were analysed for antibodies against OCA2 in RLBA. The antibody indices are shown for sera from alopecia areata patients ($n = 48$) and healthy controls ($n = 50$). The antibody index is the mean of three experiments. The upper limit of normal (1.55) of the RLBA (mean antibody index + 3SD of 50 healthy controls) is shown by the dotted line. The *in vitro* translated OCA2 P protein used as the radiolabelled ligand in the RLBA is shown.

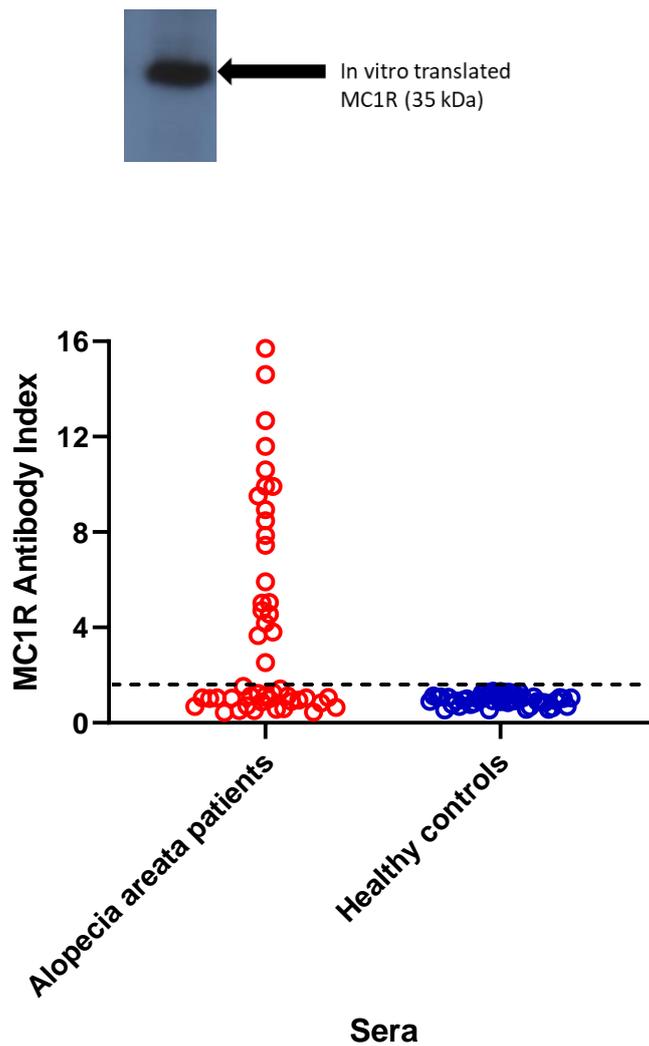


Figure 5.3: Results of MC1R RLBA for alopecia areata patients and healthy controls.

Alopecia areata patient and healthy controls were analysed for antibodies against MC1R in RLBA. The antibody indices are shown for sera from alopecia areata patients ($n = 48$) and healthy controls ($n = 50$). The antibody index shown is the mean of three experiments. The upper limit of normal (1.63) of the RLBA (mean antibody index + 3SD of 50 healthy controls) is shown by the dotted line. The *in vitro* translated MC1R used as the radiolabelled ligand in the RLBA is shown.

Table 5.1: Summary of radioligand binding assay results

| Autoantibody target ¹ | Mean antibody index of healthy control group (n = 50) | Upper limit of radioligand binding assay | Antibody index of positive control antibody | Number of 48 alopecia areata patients positive for antibodies (%) | Number of 50 healthy controls positive for antibodies (%) | P value ² |
|----------------------------------|---|--|---|---|---|----------------------|
| GPNMB | 0.99 ± 0.14 | 1.41 | 24.3 | 10 (20.8) | 0 (0) | 0.0005 |
| OCA2 | 0.95 ± 0.20 | 1.55 | 43.8 | 8 (16.7) | 0 (0) | 0.0024 |
| MC1R | 0.98 ± 0.22 | 1.63 | 50.7 | 21 (43.8) | 0 (0) | < 0.0001 |

¹GPNMB, glycoprotein non-metastatic melanoma protein b; OCA2, OCA2-encoded P protein; MC1R melanocortin 1 receptor.

²P value calculated using Fisher's exact test for 2 x 2 contingency tables for comparing the prevalence of antibodies in alopecia areata patients and healthy controls. P < 0.05 was considered statistically significant.

5.3.2 Determination of GPNMB, OCA2, and MC1R antibody titres

To estimate titres for GPNMB, OCA2, and MC1R antibodies, patients positive for these antibodies were investigated in the appropriate RBLA at final dilutions of ranging from 1:100-1:5000, along with 10 healthy control sera. Antibody indices were calculated as: cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by 10 healthy control sera at each dilution. Sera were tested in three experiments. The mean antibody index was calculated. Titres were estimated as the serum dilution where antibodies could be detected above the normal upper limit of the RLBA.

Antibody titrings are shown in Figures 5.4-5.6 and titres are summarised in Table 5.2. GPNMB antibody titres were from 1:200-1:2000. OCA2 antibody titres were from 1:200-1:2000. MC1R antibody titres were from 1:200 to >1:5000.

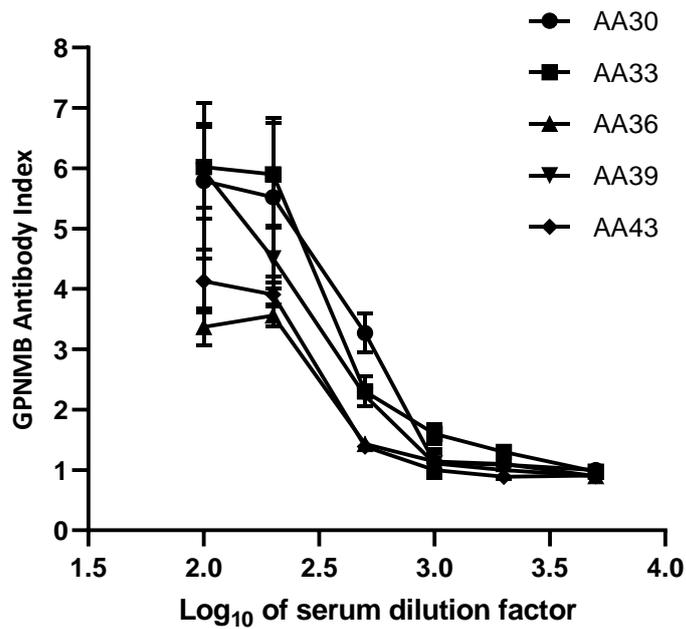
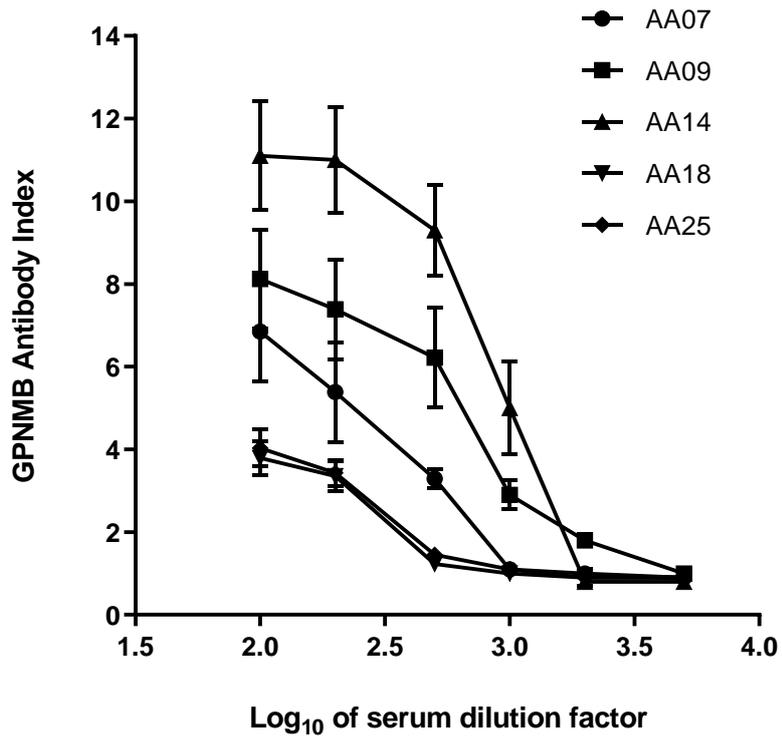


Figure 5.4: GPNMB antibody titres.

Ten GPNMB antibody-positive patient sera were tested in the GPNMB RLBA at dilutions of 1:100 to 1:5000. The GPNMB antibody indices are from the mean \pm SD of three experiments.

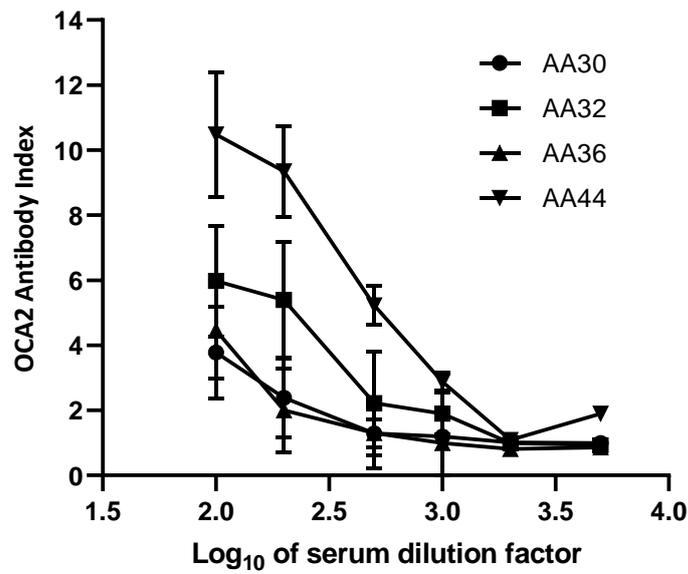
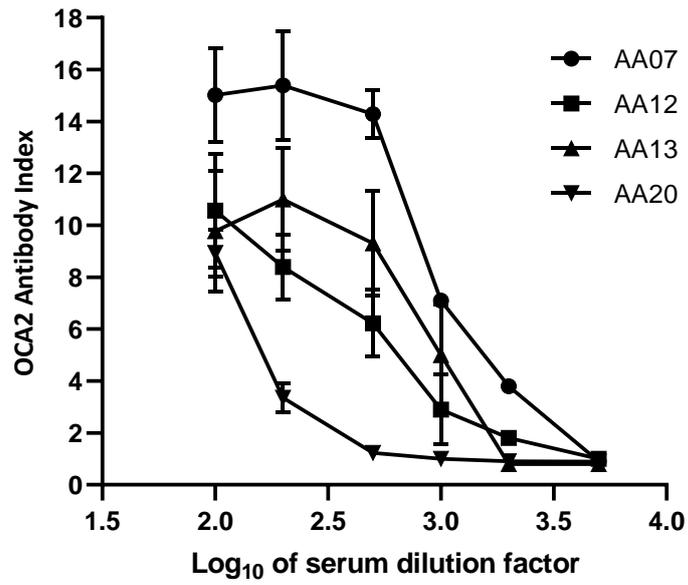


Figure 5.5: OCA2 antibody titres.

The eight OCA2 antibody-positive patient sera were analysed in the OCA2 RLBA at dilutions of 1:100 to 1:5000. The OCA2 antibody indices are from the mean \pm SD of three experiments.

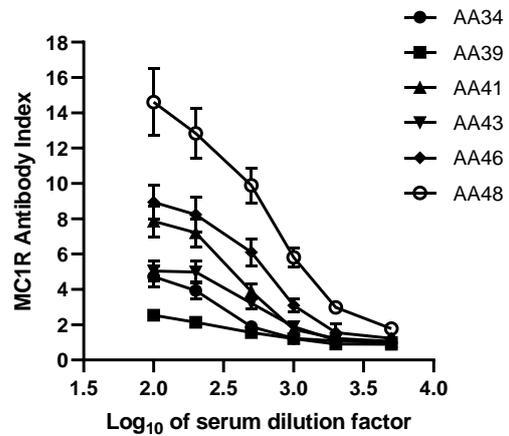
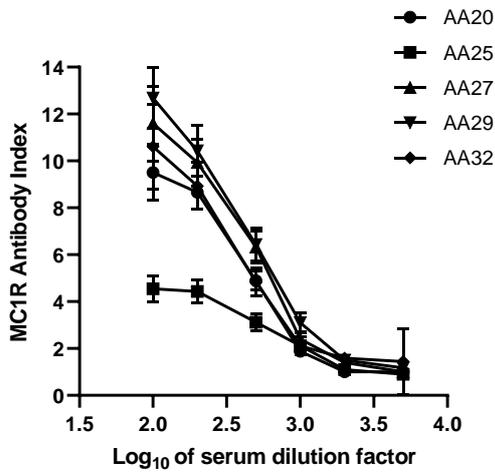
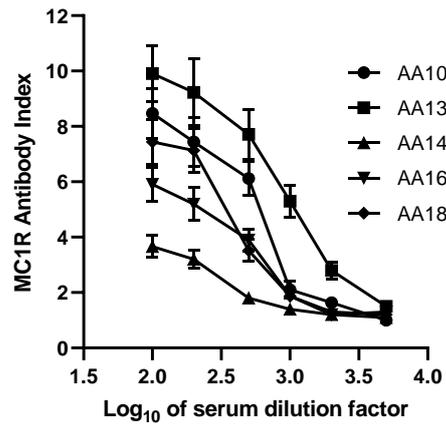
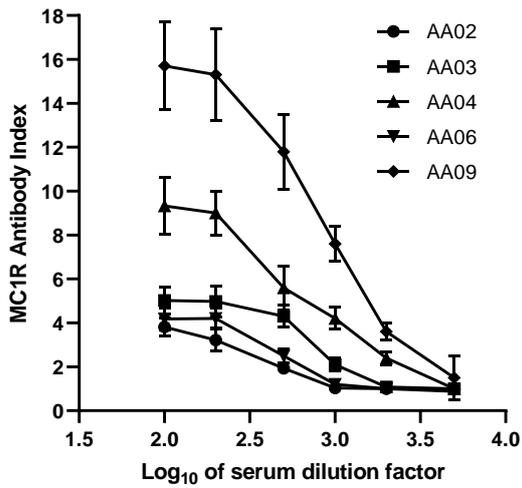


Figure 5.6: MC1R antibody titres.

The 21 MC1R antibody-positive patient sera were analysed in the MC1R RLBA at dilutions of 1:100 to 1:5000. The MC1R antibody indices are from the mean \pm SD of three experiments.

Table 5.2: Summary of GPNMB, OCA2, and MC1R antibody titres

| Antibody titre¹ | Alopecia areata patients with GPNMB antibody titre² | Alopecia areata patients with OCA2 antibody titre² | Alopecia areata patients with MC1R antibody titre² |
|-----------------------------------|---|--|--|
| 1:100 | - | - | - |
| 1:200 | AA18; AA43 | AA20; AA30; AA36 | AA39 |
| 1:500 | AA07; AA25; AA30; AA36 | - | AA02; AA06; AA14; AA34 |
| 1:1000 | AA14; AA33; AA39 | AA13; AA32; AA44 | AA03; AA10; AA16; AA18; AA20; AA25; AA27; AA29; AA32; AA41; AA43; AA46 |
| 1:2000 | AA09 | AA07; AA12 | AA04; AA09; AA13 |
| 1:5000 | - | - | - |
| >1:5000 | - | - | AA48 |

¹The antibody titre is the dilution at which immunoreactivity was still detected in the alopecia areata patient's serum sample above the upper limit of normal in the RLBA.

²GPNMB, glycoprotein non-metastatic melanoma protein b; OCA2, OCA2-encoded P protein; MC1R melanocortin 1 receptor.

5.3.3 Specificity of GPNMB, OCA2, and MC1R antibodies

To determine if GPNMB, OCA2, and MC1R antibodies were specific for their antigen, absorption experiments were carried out. No alopecia areata patient had antibodies against the MCHR1 so it was used as a control antigen and would not be expected to absorb GPNMB, OCA2 or MC1R antibodies from the sera.

First, sera from patients with antibodies and controls were pre-incubated with cell extract made from HEK293 cells containing expressed antigens GPNMB, OCA2, MC1R or MCHR1 as detailed in Section 2.21. Pre-incubation with untransfected HEK293 cell extract was used as a control. Following pre-absorption, duplicate samples of sera were tested in antibody RLBA. An antibody index was calculated as: cpm immunoprecipitated by tested serum/the cpm immunoprecipitated by 10 controls. All sera were tested in three experiments. The antibody indices for the pre-absorbed and non-absorbed patient serum samples were compared using one-way ANOVA (Section 2.22) where P values < 0.05 were considered significant.

The absorption experiments results are in Figures 5.7-5.9. The results show that antibody-indices in RLBA were only decreased significantly when pre-absorbed with extract containing the equivalent expressed antigen. This indicated that GPNMB, OCA2 and MC1R antibodies recognised specifically their target antigen. No cross-reactivity was found with the control protein MCHR1.

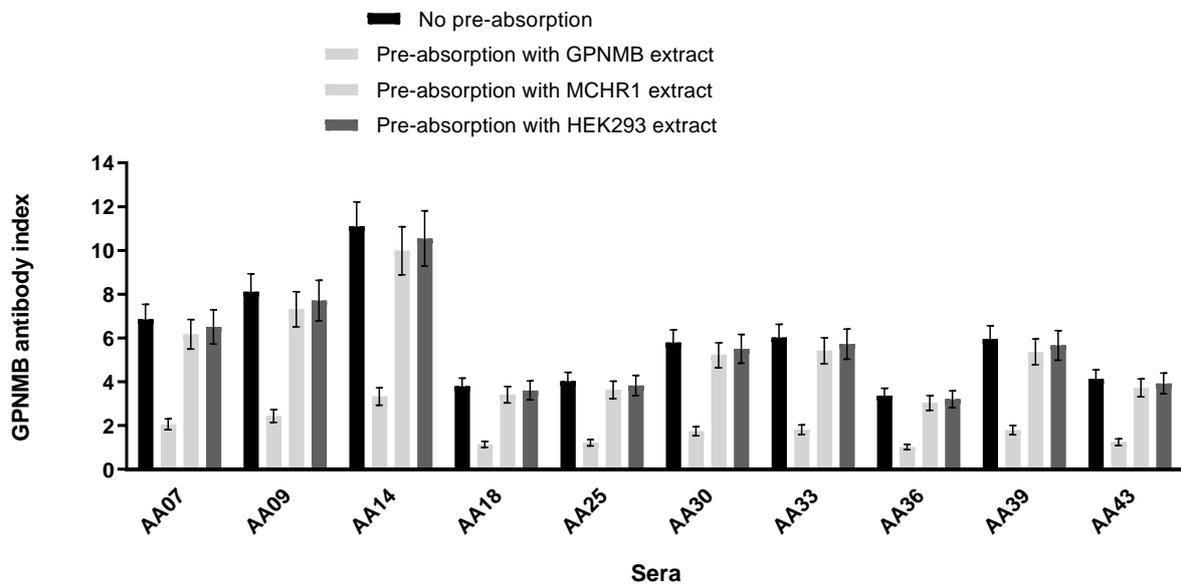


Figure 5.7: Absorption experiments of GPNMB antibodies.

The 10 GPNMB antibody-positive patient sera were pre-absorbed with cell extract from either HEK293 cells or from HEK293 cells containing expressed GPNMB or MCHR1. Subsequently, non-absorbed and pre-absorbed sera were tested in RLBAs. The results are the mean antibody index (\pm SD) of three experiments. The antibody indices of patient sera pre-absorbed with GPNMB were compared with non-absorbed sera; P values were < 0.0001 in a one-way ANOVA.

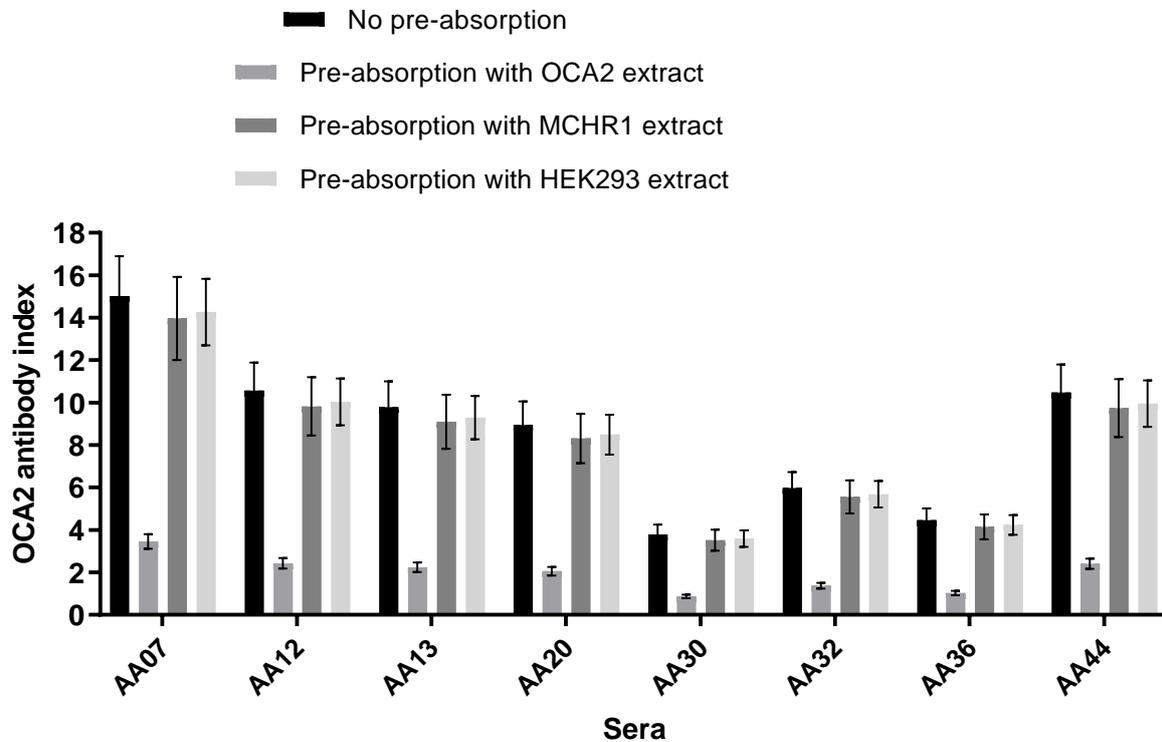


Figure 5.8: Absorption experiments of OCA2 antibodies.

The eight OCA2 antibody-positive patient sera were pre-absorbed with cell extract from either HEK293 cells or from HEK293 cells containing expressed OCA2 or MCHR1. Subsequently, non-absorbed and pre-absorbed sera were tested in RLBAs. The results show the mean antibody index (\pm SD) of three experiments. The antibody indices of patient sera pre-absorbed with OCA2 were compared with non-absorbed sera; P values were < 0.0001 in a one-way ANOVA.

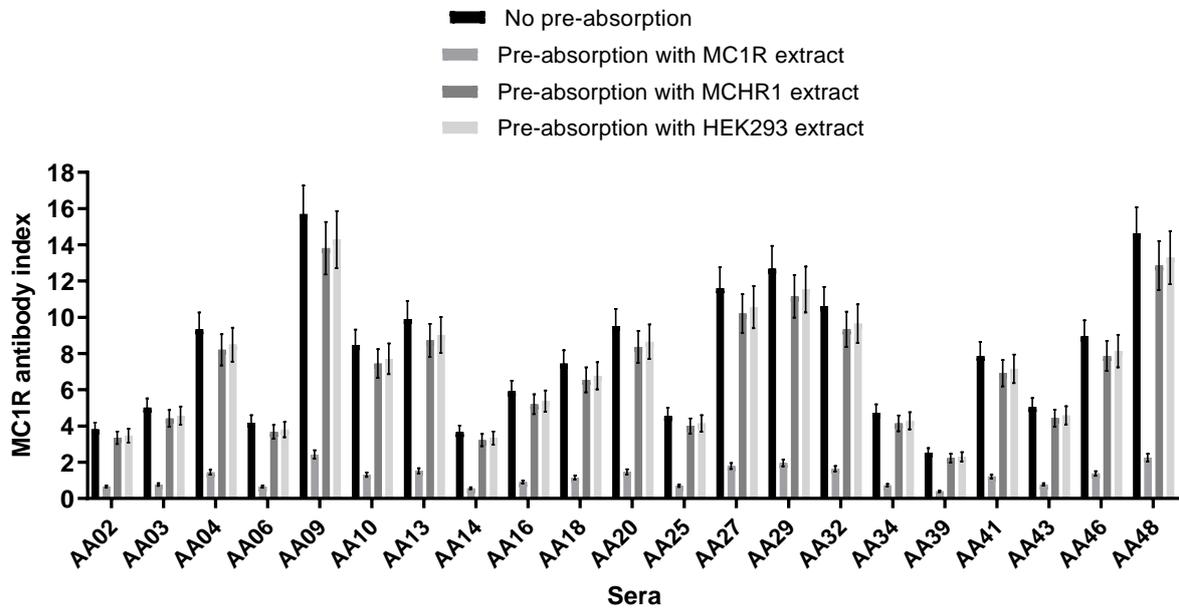


Figure 5.9: Absorption experiments of MC1R antibodies.

The eight MC1R antibody-positive patient sera were pre-absorbed with cell extract from either HEK293 cells or from HEK293 cells containing expressed MC1R or MCHR1. Subsequently, non-absorbed and pre-absorbed sera were tested in RLBAs. The results show the mean antibody index (\pm SD) of three experiments. The antibody indices of patient sera pre-absorbed with MC1R were compared with non-absorbed sera; P values were < 0.0001 in a one-way ANOVA.

5.3.4 Comparison of the demographic, clinical, and serological details of GPNMB, OCA2, and MC1R antibody-positive and antibody-negative patients

The features, including the demography, clinical, and serology of the alopecia areata patients with and without GPNMB, OCA2, and MC1R antibodies were compared in Fisher's exact test or in unpaired t tests (Section 2.23). In Table 5.3 the results are summarised. In the majority of cases, no difference was observed in relation to sex, age, disease duration, onset age of alopecia areata, alopecia areata severity, the presence of autoimmunity, nail changes, atopy, or antibodies against tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL, and tyrosine hydroxylase.

For OCA2 antibodies, antibody-positive patients had a significantly increased age ($P = 0.024$) and age of alopecia areata onset ($P = 0.0059$). OCA2 antibodies were also more prevalent in patients without atopic disease ($P = 0.017$). For MC1R antibodies, antibody-positive patients had a significantly lower age of alopecia areata onset ($P = 0.035$).

Table 5.3: Comparison of the demographic, clinical, and serological details in alopecia areata patients positive and negative for GPNMB, OCA2, and MC1R antibodies

| Patient detail | GPNMB antibody-positive (n = 10) (%) | GPNMB antibody-negative (n = 38) (%) | P value | OCA2 antibody-positive (n = 8) (%) | OCA2 antibody-negative (n = 40) (%) | P value | MC1R antibody-positive (n = 21) (%) | MC1R antibody-negative (n = 27) (%) | P value |
|------------------------------------|--------------------------------------|--------------------------------------|---------|------------------------------------|-------------------------------------|---------------|-------------------------------------|-------------------------------------|--------------|
| Male | 2 (20.0) | 11 (28.9) | 0.71 | 2 (25.0) | 11 (27.5) | 1.00 | 6 (28.6) | 7 (25.9) | 1.00 |
| Female | 8 (80.0) | 27 (71.1) | | 6 (75.0) | 29 (72.5) | | 15 (71.4) | 20 (74.1) | |
| Mean age ± SD (years) | 47.5 ± 13.1 | 46.5 ± 16.9 | 0.86 | 58.3 ± 15.6 | 44.4 ± 15.3 | 0.024 | 43.2 ± 11.8 | 49.4 ± 18.5 | 0.19 |
| Mean onset age ± SD (years) | 32.0 ± 19.9 | 37.2 ± 14.7 | 0.36 | 49.8 ± 17.5 | 33.4 ± 14.1 | 0.0059 | 30.7 ± 13.8 | 40.3 ± 16.2 | 0.035 |
| Mean disease duration ± SD (years) | 15.5 ± 14.1 | 9.3 ± 10.1 | 0.12 | 8.5 ± 5.5 | 11.1 ± 12.0 | 0.55 | 12.5 ± 11.1 | 9.1 ± 11.1 | 0.29 |
| Patchy alopecia areata | 8 (80.0) | 25 (65.8) | 0.47 | 5 (62.5) | 28 (70.0) | 0.69 | 14 (66.7) | 19 (70.4) | 1.00 |
| Severe alopecia areata | 2 (20.0) | 13 (34.2) | | 3 (37.5) | 12 (30.0) | | 7 (33.3) | 8 (29.6) | |
| Autoimmunity present | 3 (30.0) | 16 (42.1) | 0.47 | 4 (50.0) | 15 (37.5) | 0.47 | 7 (33.3) | 12 (44.4) | 0.47 |
| Autoimmunity absent | 7 (70.0) | 22 (57.9) | | 4 (50.0) | 25 (62.5) | | 14 (66.7) | 15 (55.6) | |
| Vitiligo present | 2 (20.0) | 10 (26.3) | 1.00 | 1 (12.5) | 11 (27.5) | 0.66 | 5 (23.8) | 7 (25.9) | 1.00 |
| Vitiligo absent | 8 (80.0) | 28 (73.6) | | 7 (87.5) | 29 (72.5) | | 16 (76.2) | 20 (74.1) | |
| Nail changes present | 4 (40.0) | 13 (34.2) | 0.73 | 3 (37.5) | 14 (35.0) | 1.00 | 8 (38.1) | 9 (33.3) | 0.77 |
| Nail changes absent | 6 (60.0) | 25 (65.8) | | 5 (62.5) | 26 (65.0) | | 13 (61.9) | 18 (66.7) | |
| Atopy present | 7 (70.0) | 29 (76.3) | 0.69 | 3 (37.5) | 33 (82.5) | 0.017 | 16 (76.2) | 20 (24.1) | 0.74 |
| Atopy absent | 3 (30.0) | 9 (23.7) | | 5 (62.5) | 7 (17.5) | | 5 (23.8) | 7 (25.9) | |
| TYR antibody-positive | 3 (30.0) | 6 (15.8) | 0.37 | 1 (12.5) | 8 (20.0) | 1.00 | 3 (14.3) | 6 (22.2) | 0.71 |
| PMEL antibody-positive | 3 (30.0) | 8 (21.1) | 0.68 | 2 (25.0) | 9 (22.5) | 1.00 | 4 (19.0) | 7 (25.9) | 0.73 |
| TYRP1 antibody-positive | 2 (20.0) | 4 (10.5) | 0.59 | 0 (0.0) | 5 (12.5) | 0.57 | 2 (9.5) | 5 (18.5) | 0.45 |
| TRP2 antibody-positive | 0 (0.0) | 4 (10.5) | 0.57 | 0 (0.0) | 4 (10.0) | 1.00 | 0 (0.0) | 4 (14.8) | 0.12 |
| TH antibody-positive | 3 (30.0) | 10 (26.3) | 1.00 | 2 (25.0) | 11 (27.5) | 1.00 | 5 (23.8) | 8 (29.6) | 0.75 |

¹P value calculated using Fisher's exact test for 2 x 2 contingency tables for comparing the frequency of demographic, clinical, and serological details in antibody-positive and antibody-negative alopecia areata patients. P values < 0.05 were considered significant.

²P value calculated using unpaired t test for comparing the age, onset age, and disease duration in antibody-positive and antibody-negative alopecia areata patients. P values < 0.05 were considered significant.

5.4 Discussion

In this part of the study, RLBA were used to investigate the frequency of antibody reactivity in alopecia areata patients against potential antigens GPNMB, OCA2, and MC1R, previously identified by phage-display (Chapter 4). In addition, to identify any associations of the antibodies with the demographic, clinical, and serological features of the patients.

5.4.1 Results summary

Of the 48 alopecia areata patients analysed, 10 (20.8%), 8 (16.7%), and 21 (43.8%) were positive for GPNMB, OCA2, and MC1R antibodies, respectively. All healthy control sera were negative, indicating a high disease-related specificity for the antibodies. The frequencies of the novel antibodies were relatively high compared to antibody frequencies reported for other pigmentation-related proteins, including tyrosinase (9%), tyrosinase-related protein-1 (6%), tyrosinase-related protein-2 (6%) and melanocyte-specific protein PMEL (6%) (Kemp et al., 1998a). Both GPNMB and OCA2 antibodies were similar in prevalence to tyrosine hydroxylase (19%) and transcription coactivator p75 antibodies (20%) (Okamoto et al., 2004, Kemp et al., 2011b). Antibodies against MC1R were closer in frequency to those against retinol-binding protein-4 (67%) (Ahn et al., 2011), and the hair follicle antigens trichohyalin (100%) and keratin-16 (80%) (Leung et al., 2010).

Antibody titrations indicated that the majority of patients (7/10 = 70%) with GPNMB antibodies had titres of between 1:500 and 1:1000. Most patients with MC1R antibodies (16/21 = 76.2%) had titres of 1:1000 or above. OCA2 antibodies were found at a range of titres, but 3/8 (37.5%) were only at 1:200. For antibody specificity, absorption experiments showed that GPNMB, OCA2, and MC1R antibodies reacted specifically with their target antigen with no cross-reactivity to the other melanocyte proteins melanin-concentrating hormone receptor 1 and tyrosinase. This is as expected since they amino acid sequence and structural sequence similarities.

Comparisons of GPNMB, OCA2, and MC1R antibody-positive and antibody-negative patients demonstrated that the antibodies were not associated with additional autoimmune disease, including vitiligo, the severity of alopecia areata, or the presence of nail changes. This was also the case for atopy with the exception of OCA2 antibodies that were more prevalent in

patients without atopic disease. In addition, there were no apparent correlations between the presence of GPNMB, OCA2, and MC1R antibodies and immunoreactivity against either tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, melanocyte-specific protein PMEL, or tyrosine hydroxylase. Furthermore, there was no significant association between GPNMB, OCA2, and MC1R antibodies and either patient sex or disease duration. For GPNMB antibodies, there was no association with age or alopecia areata onset age. For MC1R antibodies, there was no association with age, but antibody-positive patients had a significantly lower age of alopecia areata onset. Finally, for OCA2 antibodies, antibody-positive patients had a significantly increased age and age of alopecia areata onset.

Previous studies have also largely reported no associations of antibodies in alopecia areata patients with any specific clinical or demographic feature of the disease (Okamoto et al., 2004, Leung et al., 2010, Kemp et al., 2011b). An association between tyrosine hydroxylase antibodies and a disease duration of < 10 years was, however, noted (Kemp et al., 2011b).

5.4.2 Potential for a pathogenic role of GPNMB, OCA2 and MC1R antibodies in alopecia areata

The stimulus for the production of GPNMB, OCA2, and MC1R antibodies remains to be elucidated. They might arise from a genetic predisposition to autoimmunity or emerge as a consequence of injury to hair follicle cells. Such antibodies may exacerbate alopecia areata or play no part in its pathogenesis. However, they might indicate the existence of autoreactive T lymphocytes that are capable of destroying hair follicles. Indeed, T cells against peptide epitopes the MC1R have been detected in alopecia areata patients (Wang et al., 2016).

Primarily, both GPNMB and OCA2 localise intracellularly, although GPNMB has been observed occasionally to be expressed on the melanocyte surface (Tomihari et al., 2009a), and a mutant OCA2 has been reported to mislocalise to the melanocyte membrane (Sitaram et al., 2009). However, their predominant intracellular location suggests that antibodies against GPNMB and OCA2 are most likely to arise following antigen exposure due to the destruction of melanocytes by oxidative stress or cytotoxic T cells. Several hypotheses including neo-antigen formation, exposure of cryptic epitopes, and protein modification during apoptosis have been suggested to account for antibody reactivity to cytoplasm-located melanocyte antigens (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011a). Following processing by

dendritic cells, proteins can be then be presented to either autoreactive T cells 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., 2011a). Subsequently, antibodies could be produced if autoreactive B lymphocytes are triggered by activated CD4+ T cells (Namazi, 2007). Due to restricted contact with their intracellular target, GPNMB and OCA2 antibodies are not likely to adversely affect the function of their target, so they may just be indicators of cellular destruction and T lymphocyte responses in alopecia areata patients.

With regard to MC1R, it is possible that the function of the receptor could be affected adversely by antibody interaction, as in the case of the thyrotropin receptor in Graves' disease (Weetman and McGregor, 1994), the melanin-concentrating hormone receptor-1 in vitiligo (Gottumukkala et al., 2006), and the acetylcholine receptor in myasthenia gravis (Hoedemaekers et al., 1997).

5.5 Conclusions

In conclusion, GPNMB, OCA2, and MC1R have been identified as a novel antibody targets in alopecia areata. This further supports the involvement of autoimmune responses in the aetiology of alopecia areata. The finding that a high frequency of patients (43.8%) had MC1R antibodies warranted further investigations regarding their usefulness as serum markers for alopecia areata as well as their role in the pathogenesis of hair-loss.

Chapter 6

Characterisation of Autoantibodies against the Melanocortin 1 Receptor in Alopecia Areata

6 Characterisation of Autoantibodies against the Melanocortin 1 Receptor in Alopecia Areata

6.1 Introduction

6.1.1 The melanocortin 1 receptor

The melanocortin 1 receptor (MC1R) is a seven-transmembrane G protein-coupled receptor with an intracellular carboxy-terminus and an extracellular amino-terminus (Figure 6.1) (Donatien et al., 1992, Wolf Horrell et al., 2016). It was originally cloned and identified by two independent groups (Mountjoy et al., 1992, Chhajlani and Wikberg, 1992). The receptor is composed of 317 amino acids and maps to chromosome 16q24.3 (Gantz et al., 1994, García-Borrón et al., 2005). It is primarily found in skin and hair follicle melanocytes (Cai and Hruby, 2016), but can also be expressed in keratinocytes, antigen-presenting cells, endothelial cells, and fibroblasts (Luger et al., 1999). Normally, the expression of MC1R is low, with an estimated 700 copies per melanocyte (Donatien et al., 1992, Roberts et al., 2006).

Cell signalling via the MC1R is coupled to the cellular cAMP pathway which is responsible for most of, if not all, the melanogenic actions of its ligand α -MSH (Figure 6.2). Activation of MC1R by α -MSH results in the synthesis of black/brown eumelanin (Figure 6.3) and enhances melanosome transfer to keratinocytes (Buscà and Ballotti, 2000). Antagonism of α -MSH by the direct action of agouti signalling protein (ASIP) blunts α -MSH-induced signalling and results in the production of yellow/red pheomelanin (Figure 6.3) (Virador et al., 2002, Gantz and Fong, 2003, García-Borrón et al., 2005, Wolf Horrell et al., 2016).

The MC1R is highly polymorphic and, in humans, loss-of-function variants are associated with red coloured hair (Smith et al., 1998, Abdel-Malek et al., 2014). Individuals with a dysfunctional MC1R are also fair-skinned and have an increased sensitivity to UV radiation due to decreased eumelanin synthesis (Palmer et al., 2000, Landi et al., 2005).

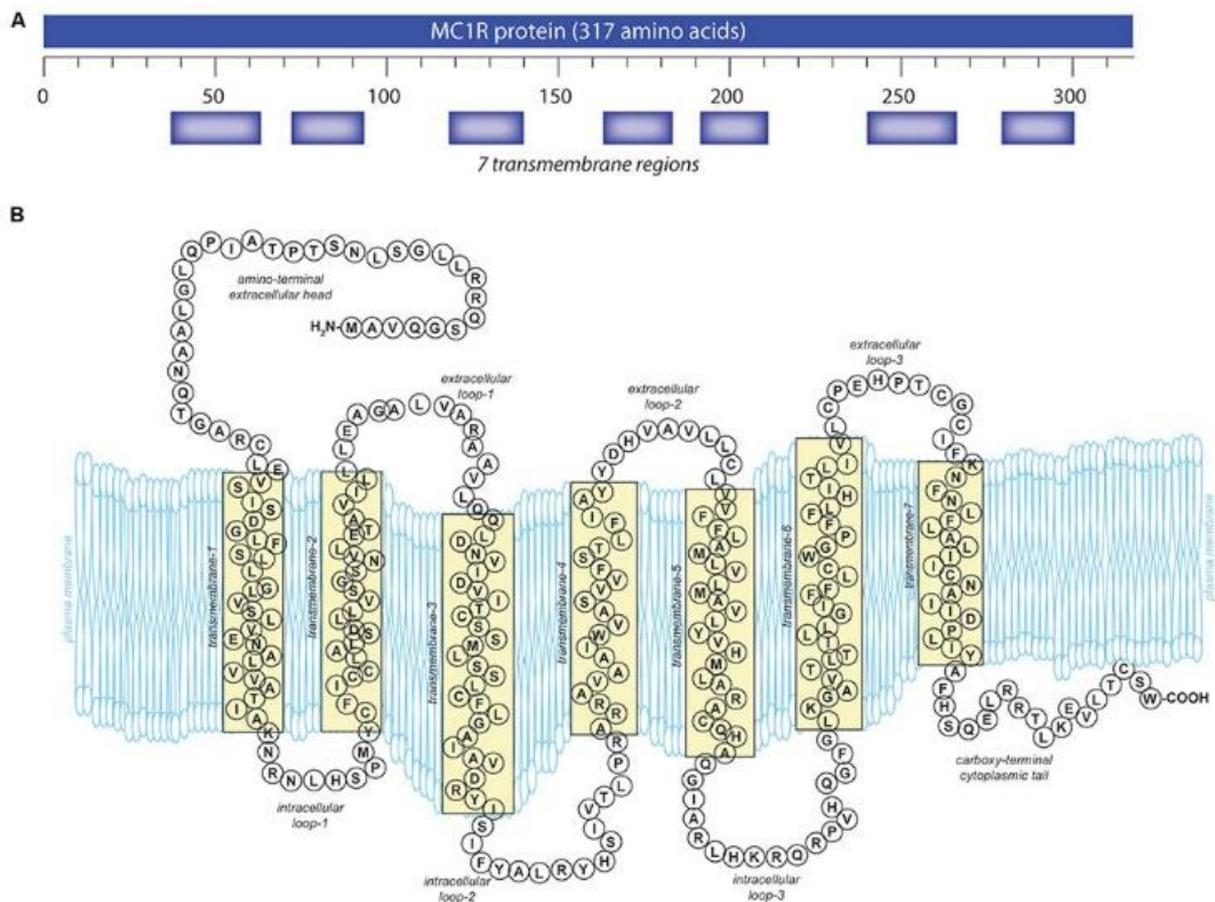


Figure 6.1: Melanocortin 1 receptor gene structure and protein structure.

(A) The human melanocortin 1 receptor (MC1R) locus is at cytogenetic location:16q24.3. **(B)** The mature MC1R is a G-protein coupled receptor that spans the cell membrane seven times. The amino-terminal extracellular domain and transmembrane domains interact with MC1R ligands such as α -melanin-stimulating hormone (α -MSH). The carboxy-terminus intracellular domain and the transmembrane domains regulate adenylyl cyclase interactions and cyclic adenosine monophosphate (cAMP) signalling. The figure, taken from a paper by Wolf Horrell et al, 2016, is used with kind permission from Holtzbrinck Publishing Group.

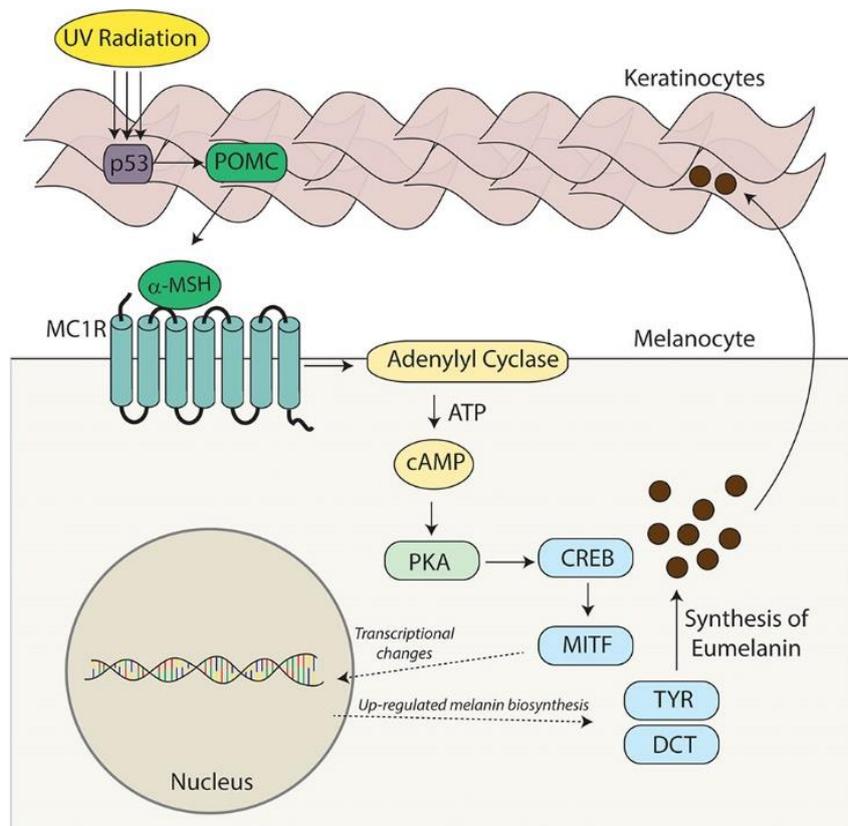


Figure 6.2: The melanocortin 1 receptor signalling pathway.

α -Melanin-stimulating hormone (α -MSH) is produced basally by the pituitary and induced in the skin after ultra-violet (UV) radiation injury. Binding to the melanocortin 1 receptor (MC1R) promotes physiologic changes in melanocytes to protect the skin from UV radiation damage. The MC1R activates adenylyl cyclase and stimulates cyclic adenosine monophosphate (cAMP) production. In turn, several downstream effector pathways are activated including those of the cAMP response element-binding protein (CREB) and the microphthalmia-associated transcription factor (MITF). The activity of protein kinase A (PKA) is increased. The expression several of melanogenic enzymes including tyrosinase (TYR) and dopachrome tautomerase (DCT) is increased and melanin synthesis is up-regulated. Melanin produced in organelles termed melanosomes, is transferred to neighbouring keratinocytes such that a UV-protective layer of pigment established in the skin's epidermis. The melanocyte genomic stability is also enhanced through improved DNA repair. In the absence of functional melanocortin signalling, the control pathways are blunted so that the skin doesn't contain sufficient melanin. As a result, melanocytes accumulate more UV-induced mutations. In this way, individuals with inherited defects in MC1R signalling are at an increased melanoma risk. ATP, adenosine triphosphate; POMC, proopiomelanocortin; p53, tumour protein p53. The figure, taken from a paper by Wolf Horrell et al, 2016, is used with kind permission from Holtzbrinck Publishing Group.

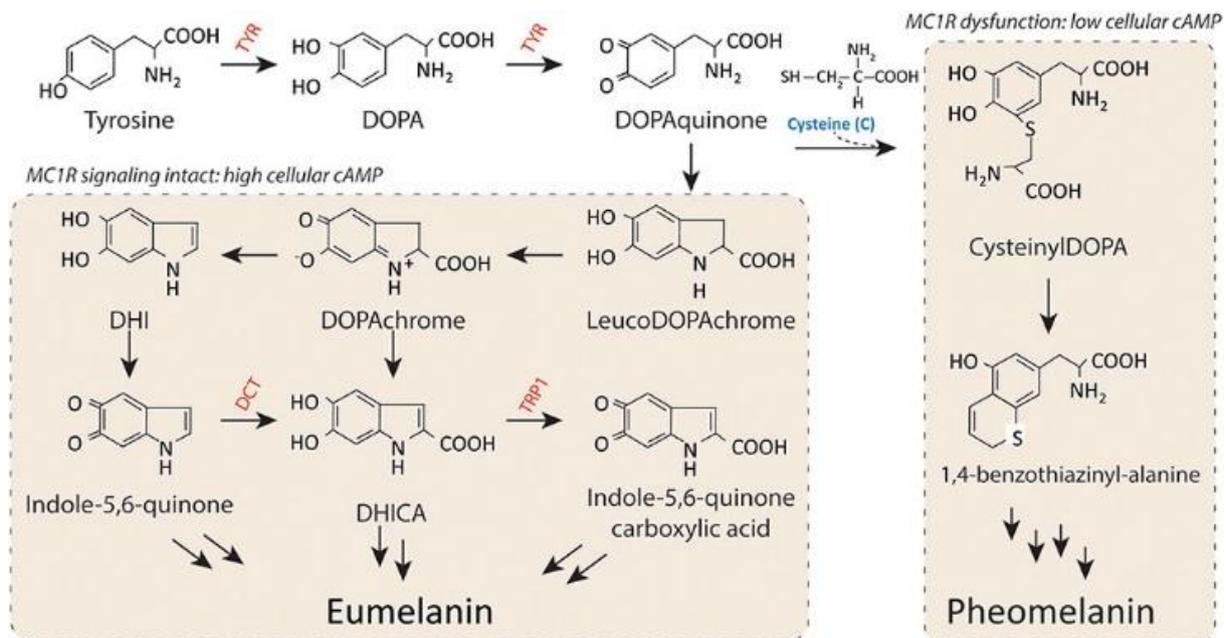


Figure 6.3: Melanin synthesis.

There are two main types of melanin pigment: the dark brown/black eumelanin and the red/yellow pheomelanin. Each is derived from progressive cyclisation and oxidation of tyrosine. Tyrosinase (TYR), the rate-limiting enzyme for melanogenesis, catalyses the first two stages of melanin biosynthesis. When the melanocortin 1 receptor (MC1R) is functional and cyclic adenosine monophosphate (cAMP) levels are high, melanocytes produce eumelanin. When MC1R is dysfunctional and cAMP levels are low, cysteine is incorporated and pheomelanin is produced. Important pigment enzymes which cause hypopigmentary phenotypes when defective include tyrosinase (TYR), dopachrome tautomerase (DCT), and tyrosinase related protein-1 (TRP1). DHI, dihydroxyindole; DHICA, dihydroxyindole-2-carboxylic acid; DOPA, dihydroxyphenylalanine. The figure, taken from a paper by Wolf Horrell et al, 2016, is used with kind permission from Holtzbrinck Publishing Group.

6.1.2 Antibody properties

The following antibody properties are described and discussed as they will be analysed in relation to the antibodies against the MC1R.

6.1.2.1 Antibody epitopes

The term epitope describes the precise amino acids on an antigen that are recognised by an antibody (Westwood and Hay, 2001). Epitopes differ in amino acid sequence and length as well as in secondary structure, and they can involve post-translational modifications of the antigen (Westwood and Hay, 2001). Usually, epitopes are defined as conformational or linear (Roitt and Delves, 1997). Characterised by amino acid sequence, linear epitopes range in size from 5-6 up to 15-22 amino acid residues (von Mikecz et al., 1995). Conformational epitopes depend upon the secondary or tertiary shape of the antigen (Roitt and Delves, 1997). Often, they are part of the antigen surface and are hydrophilic in nature (Westwood and Hay, 2001).

The approaches also be identified by testing antibody reactivity directly against antigen peptide fragments or representative synthetic peptides (Pettersson, 1992). Both of the latter techniques mainly allow linear epitopes to be identified, although they have been used to characterise conformational antibody binding sites on chimeric antigens (Hu et al., 2007). Phage-display systems have been employed to identify both available for mapping antibody epitopes include X-ray crystallography and nuclear magnetic resonance (Amit et al., 1986, Morris, 2001, Hore, 2015). Both techniques are especially feasible for identifying conformational epitopes. A second method uses the testing of the immunoreactivity of an antigen whose primary sequence has been altered by deletion or substitution (Pettersson, 1992, Westwood and Hay, 2001). Any variation in the pattern of immunoreactivity can then be used to identify amino acid residues that are essential for antibody binding. Epitopes can conformational and linear antibody binding sites (Williams et al., 2001).

Information on antibody binding sites is helpful in several ways including how antibodies might adversely affect the function of a receptor (Kifor et al., 2004, Kemp et al., 2009), the development specific antibody assays, providing insights into autoimmune disease initiation such as molecular mimicry (Wucherpfennig, 2001), antibody light chain usage (McIntosh et

al., 1997), and the engineering of functional antibodies that can be used for disease treatment.

6.1.2.2 IgG antibody subclasses

The total immunoglobulin in humans consists of 75% IgG, which is the major effector of the antibody response, and 25% IgE, IgD, IgM, and IgA (Spiegelberg, 1974). The activity of IgG antibodies mainly occurs during a secondary antibody response following recurring interaction with an antigen. This antibody class persists in the circulation for long time periods. IgG antibodies have relatively high affinities compared with other antibody classes.

IgGs are classed as IgG1, IgG2, IgG3 or IgG4 (Gergely et al., 1967). Their properties are compared in Table 6.1. In particular, interactions with Fc receptors (FcγR) differ between the IgG subclasses such that they have differing effects on effector cell functions including antigen presentation, phagocytosis, and antibody-mediated cellular cytotoxicity (van de Winkel and Capel, 1993, Flesch and Neppert, 2000). Notably, the IgG4 subclass is unable to cause complement system activation (Flanagan and Rabbitts, 1982, van Loghem, 1986).

IgG subclass restriction has been observed in the case of autoantibodies. In Addison's disease and premature ovarian failure, autoantibodies against 21-hydroxylase and 17 α -hydroxylase are of the IgG1 subtype (Bøe et al., 2004, Brozzetti et al., 2010). Likewise, in type 1 diabetes, IgG1 autoantibodies are the predominant subclass against glutamic acid decarboxylase 65 and protein tyrosine phosphatase IA-2 and glutamic acid decarboxylase (Bonifacio et al., 1999, Hawa et al., 2000). In autoimmune hypothyroidism, thyroid peroxidase autoantibodies are mainly of the IgG1 and IgG4 subtypes (Kohno et al., 1993, Silva et al., 2003, Xie et al., 2008), while in Graves' disease, IgG2 thyroglobulin autoantibodies have been reported to be the predominant subtype (Caturegli et al., 1994). In autoimmune hypocalciuric hypercalcemia, autoantibodies against the calcium-sensing receptor were only of the IgG4 subclass (Pallais et al., 2004).

Table 6.1: Properties of the different IgG subclasses

| Property of the IgG subclass | IgG1 | IgG2 | IgG3 | IgG4 |
|--|-------|-------|---------|----------|
| Mean serum concentration in g/l | 6.98 | 3.8 | 0.51 | 0.56 |
| Percentage of total IgG | 43-75 | 16-48 | 1.7-7.5 | 0.8-11.7 |
| Anti-protein activity | ++ | +/- | ++ | +/- |
| Anti-polysaccharide activity | + | ++ | - | - |
| Anti-allergen activity | + | - | - | ++ |
| Complement C1q binding | ++ | + | +++ | - |
| FcγRI receptor (CD64:monocytes, macrophages, neutrophils, dendritic cells) | ++ | - | +++ | + |
| FcγRII receptor (CD32):monocytes, macrophages, neutrophils, eosinophils, platelets, B cells, dendritic cells, endothelial cells) | ++ | - | +++ | - |
| FcγRIIa-H131 receptor | ++ | +++ | +++ | - |
| FcγRIIa-R131 receptor | ++ | - | ++ | - |
| FcγRIII receptor (CD16:neutrophils, eosinophils, macrophages, NK cells, subsets of T cells) | ++ | - | ++ | - |
| FcγRIIIb-NA1 receptor | +++ | - | +++ | - |
| FcγRIIIb-NA2 receptor | ++ | - | ++ | - |

+++, very strongly positive; ++, strongly positive; +, positive; +/-, weakly positive; -, negative. The table information is adapted from Roitt and Delves, 1997.

6.1.2.3 Antibody functional affinity (avidity)

Antibody affinity is defined as the strength of binding between an antibody and its epitope (Roitt and Delves, 1997). Practically, functional affinity (or avidity) is used to determine the overall binding strength between multivalent antigens and antibodies (Roitt and Delves, 1997). There have only been a few examples of autoantibodies that have been shown to have increased functional affinity (Charavi and Reiber, 1996). These are autoantibodies against ribosomal P protein and double-stranded DNA in systemic lupus erythematosus, anti- β 2-glycoprotein I autoantibodies in antiphospholipid syndrome (Takeda et al., 2001, Cucnik et al., 2004, Cucnik et al., 2011).

6.1.2.4 Antibody functional effects

Cell surface receptors are the targets of autoantibodies in several different autoimmune diseases. These include the thyroid-stimulating hormone receptor in Graves' disease where stimulating autoantibodies cause overproduction of thyroid hormones (Morgenthaler et al., 2007). The acetylcholine receptor is blocked by autoantibodies in myasthenia gravis leading to errors in transmission of nerve impulses to the muscles (Jacob et al., 2012). In addition, the calcium-sensing receptor can be stimulated by autoantibodies that cause a lack of PTH secretion resulting in hypoparathyroidism (Kifor et al., 2004, Kemp et al., 2009).

6.2 Aims

The aims of this part of the project were to further characterise alopecia areata patient autoantibodies against the MC1R by:

- Identifying MC1R epitopes.
- Determining the IgG subclass of MC1R antibodies.
- Investigating functional effects of MC1R antibodies.

6.3 Materials and methods

6.3.1 Patients

Details of the 21 MC1R antibody-positive alopecia areata patients used in this part of the study are in Table 6.2.

6.3.2 MC1R peptide ELISAs

Patient antibodies against peptides of the MC1R were detected using ELISAs as described before (Kemp et al., 2010). The MC1R peptides (Table 6.3) were obtained in lyophilised form. They were and stored in accordance with the supplier's directions (Cambridge Peptides Ltd., Birmingham, UK). When required for ELISAs, peptides were diluted in PBS to a concentration of 200 ng/ml. Aliquots of 100 μ l were used to coat the wells of 96-well microtitre plates, which were then stored at 4°C overnight.

Excess peptide was removed from the wells, which were blocked with blocking buffer (PBS containing 0.1% Tween-20 and 3% bovine serum albumin) at 37°C for 1 h. Wells were washed six times with washing buffer (PBS containing 0.1% Tween-20). Samples of 100 μ l of serum diluted 1:100 in blocking buffer were added to the wells. Where available, a positive MC1R antibody (Table 6.3) was used in the relevant ELISA at a 1:1000 dilution. Samples of PBS were included on each ELISA plate to detect secondary antibody non-specific binding. ELISA plates were incubated for 1 h at room temperature.

ELISA plate wells were washed six times. A 100- μ l sample of 1:2000 diluted goat anti-human antibody-alkaline phosphatase conjugate (Sigma-Aldrich) was added to wells for 1 h at room temperature. In the case of positive MC1R antibodies (Table 6.3), goat anti-rabbit antibody-alkaline phosphatase conjugate (Sigma-Aldrich) was used as the secondary antibody at a 1:2000 dilution. For IgG subclass determination, anti-human IgG1, IgG2, IgG3 or IgG4 antibody conjugated to alkaline phosphatase (SouthernBiotech, Birmingham, AL, USA) was applied as the secondary antibody at a 1:2000 dilution. Plates were washed six times. A 100- μ l aliquot of Sigma Fast p-nitrophenyl phosphate (Sigma-Aldrich) was applied to each well and the plates incubated at room temperature. After colour development for 15 min, absorption readings at 405 nm were taken using a Microtitre Plate Reader. Sera were tested in duplicate and the average OD₄₀₅ value was calculated.

The antibody reactivity of sera was expressed as an antibody index as: mean OD₄₀₅ of tested serum/mean OD₄₀₅ of 30 control sera. Each serum was tested in at three experiments. The upper limit of normal for each ELISA was: the mean antibody index + 3SD of 30 control sera. Any patient serum sample with an antibody index higher than the upper limit of normal was designated as positive for MC1R antibodies.

ELISA precision and reproducibility were expressed as the intra- and inter-assay percentage coefficient of variation, respectively. The ELISA precision was assessed by the intra-assay coefficient of variation: different serum samples that were measured within the same ELISA. ELISA reproducibility was assessed by the inter-assay coefficient of variation: different serum samples that were measured in several consecutive ELISAs.

Table 6.2: Details of MC1R antibody-positive alopecia areata patients

| Patient | Sex | Age at sample (years) | Age at onset (years) | Disease duration (years) | Alopecia areata severity | Atopy | Autoimmune disease |
|----------------|------------|------------------------------|-----------------------------|---------------------------------|---------------------------------|--------------|---|
| AA02 | Female | 24 | 16 | 8 | Patchy | Yes | Autoimmune hypothyroidism; lichen sclerosus |
| AA03 | Female | 60 | 50 | 10 | Totalis | No | None |
| AA04 | Female | 66 | 53 | 13 | Totalis | Yes | None |
| AA06 | Male | 38 | 18 | 20 | Patchy | Yes | None |
| AA09 | Female | 26 | 25 | 1 | Patchy | Yes | None |
| AA10 | Female | 33 | 28 | 5 | Universalis | No | None |
| AA13 | Female | 61 | 53 | 8 | Patchy | No | Autoimmune hypothyroidism |
| AA14 | Female | 37 | 20 | 17 | Patchy | No | None |
| AA16 | Male | 42 | 35 | 7 | Patchy | Yes | None |
| AA18 | Male | 34 | 25 | 9 | Patchy | Yes | None |
| AA20 | Male | 35 | 23 | 12 | Patchy | No | None |
| AA25 | Female | 52 | 25 | 27 | Universalis | Yes | None |
| AA27 | Female | 54 | 33 | 21 | Totalis | Yes | None |
| AA29 | Female | 51 | 50 | 1 | Totalis | Yes | None |
| AA32 | Female | 49 | 32 | 17 | Totalis | Yes | None |
| AA34 | Male | 53 | 49 | 4 | Patchy | Yes | None |
| AA39 | Male | 45 | 7 | 38 | Patchy | Yes | Vitiligo |
| AA41 | Female | 31 | 31 | 1 | Patchy | Yes | Vitiligo |
| AA43 | Female | 46 | 8 | 38 | Patchy | Yes | Autoimmune hypothyroidism; Vitiligo |
| AA46 | Female | 34 | 32 | 2 | Patchy | Yes | Vitiligo |
| AA48 | Female | 36 | 32 | 4 | Patchy | Yes | Vitiligo |

Table 6.3: MC1R peptides and positive control MC1R antibodies used in ELISAs

| MC1R peptide | Domain | Peptide sequence | MC1R antibody used as a positive control |
|---------------------|------------------------------|--|---|
| 1-36 | Extracellular amino terminal | MAVQGSQRRLLGSLNSTPTA IPQLGLAANQTGARCL | Rabbit polyclonal antibody against amino acids 1-37 (PA5-97961) (Thermo Fisher Scientific, (Waltham, MA, USA) |
| 101-113 | Extracellular loop 1 | LEAGALVARAAVL | Not available |
| 182-191 | Extracellular loop 2 | YDHVAVLLCL | Not available |
| 266-277 | Extracellular loop 3 | LCPEHPTCGCIF | Rabbit polyclonal antibody against amino acids 253-317 (PA5-21911) (Thermo Fisher Scientific) |
| 219-236 | Intracellular loop 3 | QGIARLHKRQRPVHQGFG | Rabbit polyclonal antibody against amino acids 217-232 (PA5-77487) (Thermo Fisher Scientific) |

6.3.3 Cell culture

Human embryonic kidney cells expressing the MC1R (HEK293-MC1R) were a gift from Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK). HEK293 cells were from European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in T75 tissue culture flasks (Nalge Nunc International, Rochester, NY, USA) in Dulbecco's DMEM medium containing 4.5 g/L glucose, 110 mg/ml sodium pyruvate, 10% fetal calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate, 2 mM L-glutamine (All from Invitrogen, Paisley, UK), in a humidified atmosphere of 5% CO₂ at 37°C. After the cells reached 80% confluence, the medium was removed and the cells washed twice with phosphate-buffered saline (pH 7.4). The cells were then incubated for 1-2 min in Gibco® Cell Dissociation Buffer (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. The buffer was removed and the cells were resuspended in medium and then counted using a haemocytometer. Cells were passaged 2-3 times before use in experiments.

6.3.4 α-MSH-stimulation experiments

The response of the HEK293-MC1R cell line to stimulation with α-MSH (Sigma-Aldrich) was assessed by measuring intracellular adenosine 3',5'-cyclic monophosphate (cAMP) synthesis (Figure 6.2) (Newton et al., 2005). Initially, HEK293-MC1R or HEK293 cells were seeded in 96-well cell culture plates (Nalge Nunc International) at 1×10^3 cells per well in 100 µl of medium.

After 48 h at 37°C, the medium was removed, replaced with 100 µl of serum-free medium and the cells incubated for 30 min at 37°C. A 100-µl sample of medium alone or medium containing 400 µM 3-isobutyl-1-methylxanthine (IBMX) (non-specific inhibitor of cyclic nucleotide phosphodiesterases used to enhance intracellular cAMP levels) (Sigma-Aldrich) with 200 nM α-MSH or 20 µM forskolin (an adenylate cyclase activator used to increase cAMP levels) (Sigma-Aldrich) was then added to the cells for 20 min at 37°C.

To measure the effects of 21 alopecia areata patient IgG and 10 control IgG (Section 2.3) on α-MSH-stimulation of HEK293-MC1R cells, the cells, after 48 h at 37°C, were pre-incubated with 100 µl serum-free medium alone or serum-free medium containing IgG at a 1:100 dilution and 400 µM IBMX for 30 min at 37°C. A 100-µl sample of medium alone or medium containing 200 nM α-MSH or 20 µM forskolin was then added to the cells for 20 min at 37°C.

After this time, excess culture medium was decanted and 200 μ l of lysis reagent 1B from a cAMP enzyme immunoassay (GE Healthcare Life Sciences, Little Chalfont, UK) were added to each well. After adding the lysis reagent, culture plates were shaken on a microtitre plate shaker for 10 min. The concentration of cAMP in 100- μ l duplicate samples of the cell extracts was measured immediately according to a cAMP enzyme immunoassay. If required, samples were diluted to bring the cAMP concentrations to within the range of the standard curve of the assay of 25–6400 fmol/well.

6.3.5 cAMP assay

Intracellular cAMP measurement was carried out according to a cAMP enzyme immunoassay, which is a competitive enzyme immunoassay. The assay is based on competition between unlabelled cAMP, present in a standard or in an experimental sample, and horse-radish peroxidase-labelled cAMP (cAMP-HRP) for binding sites on a cAMP-specific rabbit antibody (Figure 6.4). The cAMP-specific rabbit antibody interacts with a donkey anti-rabbit IgG that is bound to the wells of a microtitre plate. The greater the amount of unlabelled cAMP, the less binding of the cAMP-HRP occurs. The resulting reduction in HRP activity is detected by a decrease in optical density at 450 nm due to a reduced amount of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) that changes colour.

All reagents were equilibrated to room temperature before use. Assay buffer, wash buffer, lysis reagents, cAMP standards, cAMP-HRP conjugate, and cAMP-specific rabbit antibody were then prepared according to the protocol. Assay buffer was 0.05 M acetate (pH 5.8) containing 0.02% bovine serum albumin. Wash buffer was 0.01 M phosphate buffer (pH 7.5) containing 0.05% Tween 20. Lysis reagent 1B was assay buffer containing 0.25% of dodecyltrimethylammonium. cAMP standards ranging from 25–6400 fmol were made by serial dilution of the stock standard (64 pmol/ml) in lysis reagent 1B. cAMP-specific rabbit antibody was made up in lysis reagent 2B. The cAMP-HRP conjugate was made up in assay buffer.

To set up the assay plate, duplicate 100- μ l aliquots of experimental samples, and of cAMP standards (0-6400 fmol) were added to the appropriate wells of the microtitre plate. 100 μ l of lysis reagent 1B and 100 μ l of lysis reagent 2B were placed in wells to measure non-specific binding (NSB). Two wells were designated as blank wells. Samples of 100- μ l of cAMP-specific

rabbit antibody was placed into all wells except the blank and NSB wells. The plate was covered and incubated at 4°C for 2 h with gentle mixing. A 50- μ l aliquot of the cAMP-HRP conjugate was added into all wells except the blank. The plate was incubated at 4°C for 1 h with gentle mixing. The wells were washed four times with 400 μ l of wash buffer and 150 μ l of TMB added into all wells. The plate was incubated for 1 h at room temperature with shaking. On the development of a blue colour in the wells, the reaction was stopped by adding 100 μ l of 1.0 M sulphuric acid into each well. The optical density of the wells was measured in a plate reader at 450 nm within 30 min of the addition of the acid.

The mean optical density (OD) for each set of replicate wells was calculated. The percentage cAMP-HRP bound for each standard and sample was calculated using the following: $\%B/B_0 = (\text{standard or sample OD} - \text{NSB OD}) / (\text{zero standard OD} - \text{NSB OD}) \times 100$. A standard curve was then generated by plotting the $\%B/B_0$ as a function of the \log_{10} of the cAMP concentration of the standards. A typical standard curve is shown in Figure 6.5. The cAMP concentration of the samples was then calculated from the standard curve.

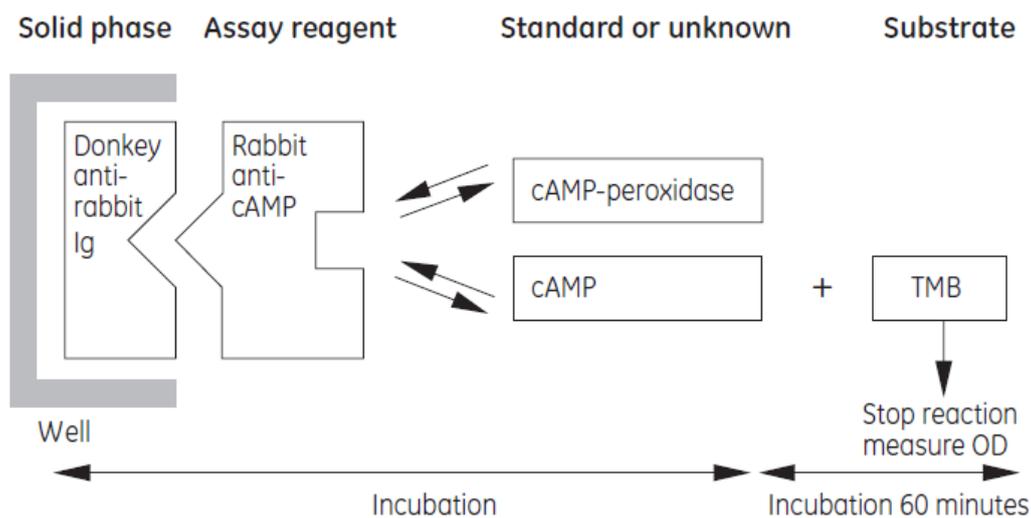


Figure 6.4: Enzyme immunoassay for cAMP measurement.

Intracellular cAMP measurement was carried out according to a cAMP enzyme immunoassay, which is a competitive enzyme immunoassay. The assay is based on competition between unlabelled cAMP, present in a standard or in an experimental sample, and horse-radish peroxidase-labelled cAMP (cAMP-HRP) for binding sites on a cAMP-specific rabbit antibody. The cAMP-specific rabbit antibody interacts with a donkey anti-rabbit IgG that is bound to the wells of a microtitre plate. The greater the amount of unlabelled cAMP, the less binding of the cAMP-HRP occurs. The resulting reduction in HRP activity is detected by a decrease in optical density at 450 nm due to a reduced amount of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) that changes colour.

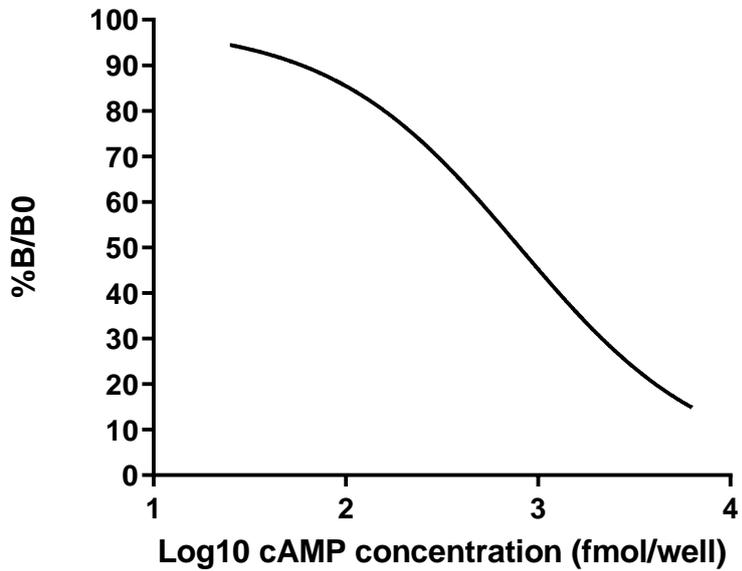


Figure 6.5: Example standard curve for cAMP measurement in the enzyme immunoassay.

From the cAMP enzyme immunoassay, the mean optical density (OD) for each set of replicate standards was calculated. The percentage cAMP-HRP bound for each standard was calculated using the following: $\%B/B0 = (\text{standard or sample OD} - \text{non-specific binding OD}) / (\text{zero standard OD} - \text{non-specific binding OD}) \times 100$. The standard curve was then generated by plotting the %B/B0 as a function of the \log_{10} of the cAMP concentration of the standards.

6.4 Results

6.4.1 Binding of alopecia areata patient MC1R antibodies in peptide ELISAs

Peptide ELISAs were used to investigate the potential epitopes of MC1R antibodies in alopecia areata patients. Putative epitope domains of the extracellular amino terminal, and extracellular loops 1-3 were initially identified by their cell surface exposure (Figure 6.1). In addition, intracellular loop 3 was included as an ELISA substrate as it was initially assumed an internal peptide would not be an epitope domain and would represent a negative control ELISA. Serum samples from 21 alopecia areata patients with MC1R antibodies and 20 healthy controls were analysed in an MC1R peptide (Table 6.3) ELISAs, as in Section 6.1.2.

In Figure 6.6, the results of the MC1R peptide ELISAs are shown with the antibody indices of the alopecia areata patient and healthy control sera. Table 6.4 summarises the upper limit of normal of each MC1R peptide ELISA as well as the mean antibody indices of the patient and control groups the positive control MC1R antibodies, and the intra- and inter-assay coefficients of variation.

Antibodies against the different MC1R peptides were not detected in healthy control (Table 6.4). The frequency of antibody-positivity against MC1R peptides in the alopecia areata patients is summarised in Table 6.4. No antibodies against MC1R peptides 101-113, 266-277 and 219-236 were detected in any of the alopecia areata patients. Antibodies against MC1R peptides 1-36 and 182-191 were detected in 21/21 (100%) and 2/21 (9.5%) patients, respectively. Only in the case of antibodies against the MC1R peptide 1-36 was their frequency significantly higher in the alopecia areata patients than in the controls: $P = < 0.0001$ (Fisher's exact test for 2 x 2 contingency tables).

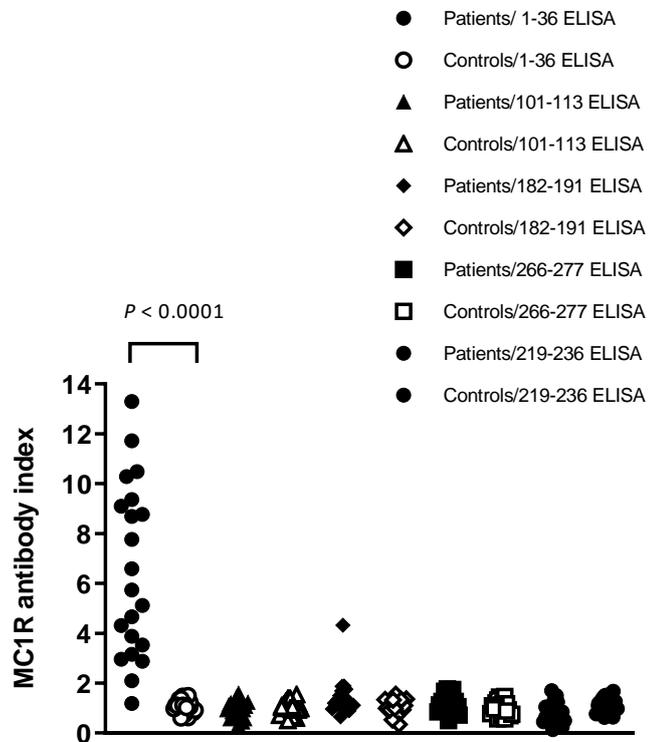


Figure 6.6: Results of MC1R peptide ELISAs.

Sera from 21 alopecia areata patients and 20 healthy controls were tested in ELISAs for antibodies against MC1R peptides 1-36, 101-113, 182-191, 266-277, and 219-236. The graph shows the mean antibody index for each serum sample determined from three separate ELISAs. *P* values were calculated in Fisher's exact test for comparing MC1R antibody-positive frequency in patients and controls. *P* values < 0.05 were considered significant. Only in the case of antibodies against MC1R peptide 1-36 was there a significant difference in their frequency between patients and controls.

Table 6.4: MC1R peptide ELISA data

| MC1R peptide | Mean (\pm SD) antibody index of patients ($n = 21$) | Mean (\pm SD) antibody index of controls ($n = 20$) | Antibody index - upper limit of normal | Antibody index of positive MC1R antibody | Intra-assay coefficient of variation | Inter-assay coefficient of variation | MC1R-antibody-positive patients (%) | MC1R antibody-positive controls (%) | <i>P</i> value ¹ |
|--------------|--|--|--|--|--------------------------------------|--------------------------------------|-------------------------------------|-------------------------------------|-----------------------------|
| 1-36 | 6.45 \pm 3.47 | 1.08 \pm 0.26 | 1.85 | 17.5 | 5.5% | 9.5% | 21/21 (100) | 0/20 (0) | < 0.0001 |
| 101-113 | 0.94 \pm 0.33 | 1.02 \pm 0.27 | 1.83 | Not available | 4.3% | 9.6% | 0/21 (0) | 0/20 (0) | NA |
| 182-191 | 2.08 \pm 3.08 | 1.02 \pm 0.30 | 1.93 | Not available | 3.8% | 10.5% | 2/21 (9.5) | 0/20 (0) | 0.49 |
| 266-277 | 1.14 \pm 0.34 | 0.99 \pm 0.27 | 1.79 | 20.3 | 4.5% | 7.4% | 0/21 (0) | 0/20 (0) | NA |
| 219-236 | 0.79 \pm 0.42 | 1.10 \pm 0.30 | 2.00 | 15.6 | 2.9% | 8.6% | 0/21 (0) | 0/20 (0) | NA |

¹NA, not applicable; *P* values were calculated in Fisher's exact test for comparing MC1R antibody-positive frequency in patients and controls. *P* values < 0.05 was considered significant.

Table 6.5: A summary of MC1R epitopes recognised by alopecia areata patients

| MC1R peptide | Alopecia areata patient |
|---------------------|--|
| 1-36 | AA02, AA03, AA04, AA06, AA09, AA10, AA13, AA14, AA16, AA18, AA20, AA25, AA27, AA29, AA32, AA34, AA39, AA41, AA43, AA46, AA48 |
| 101-113 | None detected |
| 182-191 | AA18, AA25 |
| 266-277 | None detected |
| 219-236 | None detected |

6.4.2 Determination of alopecia areata patient MC1R antibody IgG subclass

The antibody subclass of MC1R antibodies in alopecia areata patient was investigated using MC1R peptide ELISAs with peptides 1-36 and 182-191, and IgG1-4 subclass-specific secondary antibodies, as in Section 6.1.2. Serum samples from 21 alopecia areata patients with MC1R antibodies and 20 healthy controls were analysed.

The results of the MC1R peptide ELISAs are shown in Figure 6.7. Table 6.6 summarises the upper limit of normal of each MC1R peptide ELISA as well as the mean antibody indices of the patient and control groups, and the intra- and inter-assay coefficients of variation.

No control sera were positive for antibody binding (Table 6.6). In all alopecia areata patients, antibodies against the MC1R epitope 1-36 were of the IgG1 subclass. The antibody response, in the two patients with antibodies against the MC1R epitope 182-191, were of the IgG3 subclass.

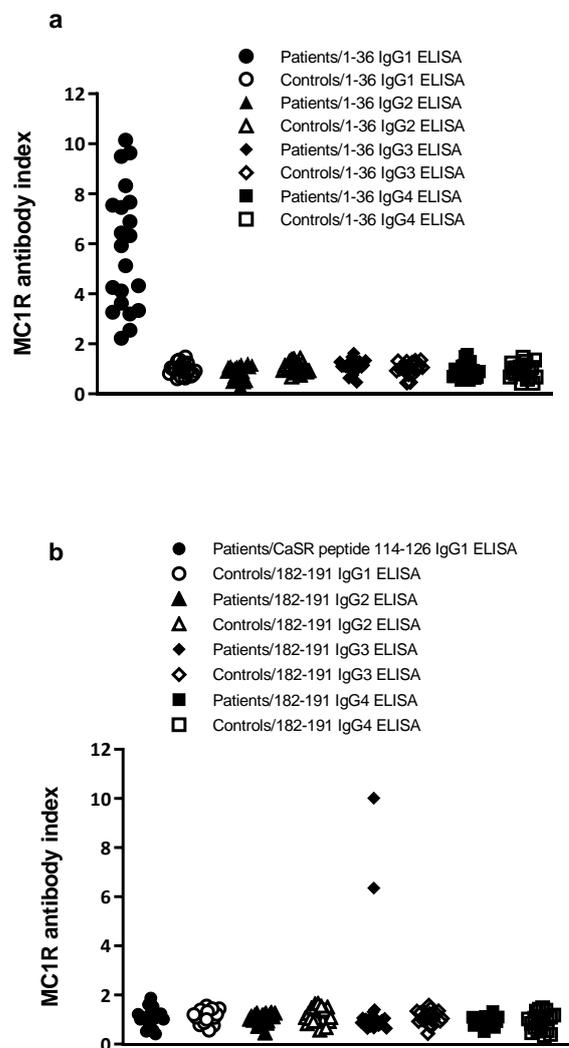


Figure 6.7: IgG subclass of MC1R antibodies from alopecia areata patients.

The sera from the 21 MC1R antibody-positive alopecia areata patients and 20 controls were analysed in MC1R peptide ELISAs with IgG1-4 subclass secondary antibodies. The antibody index shown is the mean from three experiments. The results are shown for **(a)** MC1R peptide ELISAs using peptide 1-36 with IgG1-4 subclass secondary antibodies; and **(b)** MC1R peptide ELISAs using peptide 182-191 with IgG1-4 subclass secondary antibodies.

Table 6.6: IgG subclass ELISA data

| MC1R peptide/subclass of secondary antibody | Mean (\pm SD) antibody index of patients ($n = 21$) | Mean (\pm SD) antibody index of controls ($n = 20$) | Antibody index - upper limit of normal | Intra-assay coefficient of variation | Inter-assay coefficient of variation | MC1R-antibody-positive patients (%) | MC1R antibody-positive controls (%) |
|--|--|--|---|---|---|--|--|
| 1-36/IgG1 | 5.80 \pm 2.45 | 0.98 \pm 0.25 | 1.73 | 6.5% | 10.9% | 21/21 (100) | 0/20 (0) |
| 1-36/IgG2 | 0.84 \pm 0.30 | 1.06 \pm 0.22 | 1.71 | 7.1% | 11.7% | 0/21 (0) | 0/20 (0) |
| 1-36/IgG3 | 1.10 \pm 0.27 | 1.02 \pm 0.26 | 1.81 | 4.2% | 9.7% | 0/21 (0) | 0/20 (0) |
| 1-36/IgG4 | 0.94 \pm 0.30 | 0.92 \pm 0.30 | 1.81 | 6.9% | 14.3% | 0/21 (0) | 0/20 (0) |
| 182-191/IgG1 | 1.08 \pm 0.38 | 1.12 \pm 0.26 | 1.90 | 8.4% | 12.7% | 0/21 (0) | 0/20 (0) |
| 182-191/IgG2 | 1.02 \pm 0.22 | 1.15 \pm 0.31 | 2.09 | 10.6% | 9.8% | 0/21 (0) | 0/20 (0) |
| 182-191/IgG3 | 1.62 \pm 2.26 | 1.07 \pm 0.27 | 1.88 | 5.9% | 13.2% | 2/21 (9.5%) | 0/20 (0) |
| 182-191/IgG4 | 0.96 \pm 0.17 | 0.93 \pm 0.36 | 2.00 | 9.1% | 11.2% | 0/21 (0) | 0/20 (0) |

Table 6.7: A summary of MC1R antibody IgG subclasses

| Alopecia areata patient | Antibody reactivity against MC1R peptide 1-36¹ | IgG subclass | Antibody reactivity against peptide 182-191¹ | IgG subclass |
|--------------------------------|--|---------------------|--|---------------------|
| AA02 | + | IgG1 | - | - |
| AA03 | + | IgG1 | - | - |
| AA04 | + | IgG1 | - | - |
| AA06 | + | IgG1 | - | - |
| AA09 | + | IgG1 | - | - |
| AA10 | + | IgG1 | - | - |
| AA13 | + | IgG1 | - | - |
| AA14 | + | IgG1 | - | - |
| AA16 | + | IgG1 | - | - |
| AA18 | + | IgG1 | + | IgG3 |
| AA20 | + | IgG1 | - | - |
| AA25 | + | IgG1 | + | IgG3 |
| AA27 | + | IgG1 | - | - |
| AA29 | + | IgG1 | - | - |
| AA32 | + | IgG1 | - | - |
| AA34 | + | IgG1 | - | - |
| AA39 | + | IgG1 | - | - |
| AA41 | + | IgG1 | - | - |
| AA43 | + | IgG1 | - | - |
| AA46 | + | IgG1 | - | - |
| AA48 | + | IgG1 | - | - |

¹+, MC1R antibody-positive; -, MC1R-antibody-negative.

6.4.3 α -MSH-stimulation of HEK293-MC1R and HEK293 cells

The results of α -MSH-stimulation on HEK293-MC1R and HEK293 cells are illustrated in Figure 6.8. A response to α -MSH-stimulation was only evident in the HEK293-MC1R cells which showed cAMP accumulation when treated with the peptide hormone.

6.4.4 Effect of alopecia areata patient IgG on stimulation of the MC1R by α -MSH

To ascertain the effects of IgG from 21 MC1R antibody-positive alopecia areata patients and 10 healthy controls upon the function of the MC1R, HEK293-MC1R cells were incubated with IgG before being treated with 200 nM α -MSH or 20 μ M forskolin. Experiments included HEK293-MC1R cells that were not pre-incubated with any IgG prior to treatment. Subsequently, cAMP accumulation was assessed in a cAMP enzyme immunoassay. The results of pre-incubation are in Figure 6.9.

The accumulation of intracellular cAMP was compared between α -MSH-stimulated and unstimulated cells using Student's paired t tests. None of the 10 control IgG samples tested had a statistically significant effect upon cAMP accumulation in HEK293-MC1R cells when compared with stimulation with α -MSH-stimulation alone; *P* values were > 0.05 . Equally, 19/21 alopecia areata patient IgGs had no statistically significant effect on cAMP accumulation; *P* values were two-tailed were > 0.05 .

Pre-incubation with IgG from two alopecia areata patients, AA18 and AA25, resulted in a statistically significant decrease in cAMP accumulation in response to α -MSH stimulation in HEK293-MC1R cells compared with α -MSH stimulation alone; *P* values were < 0.0001 . The results indicated the presence of MC1R-blocking activity in these two alopecia areata patient IgG samples.

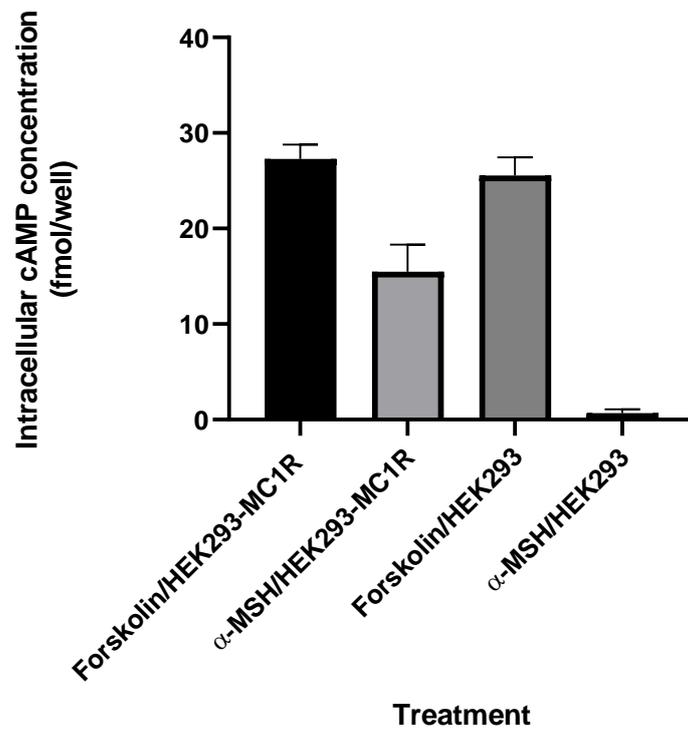


Figure 6.8: Measuring the effect of the stimulation of HEK293-MC1R cells with α -MSH.

HEK293-MC1R and HEK293 cells were treated with 3-isobutyl-1-methylxanthine (IBMX) and either α -MSH or forskolin. Intracellular cAMP was measured in a cAMP Enzyme Immunoassay. The results show the mean (\pm SD) intracellular cAMP concentration from four experiments.

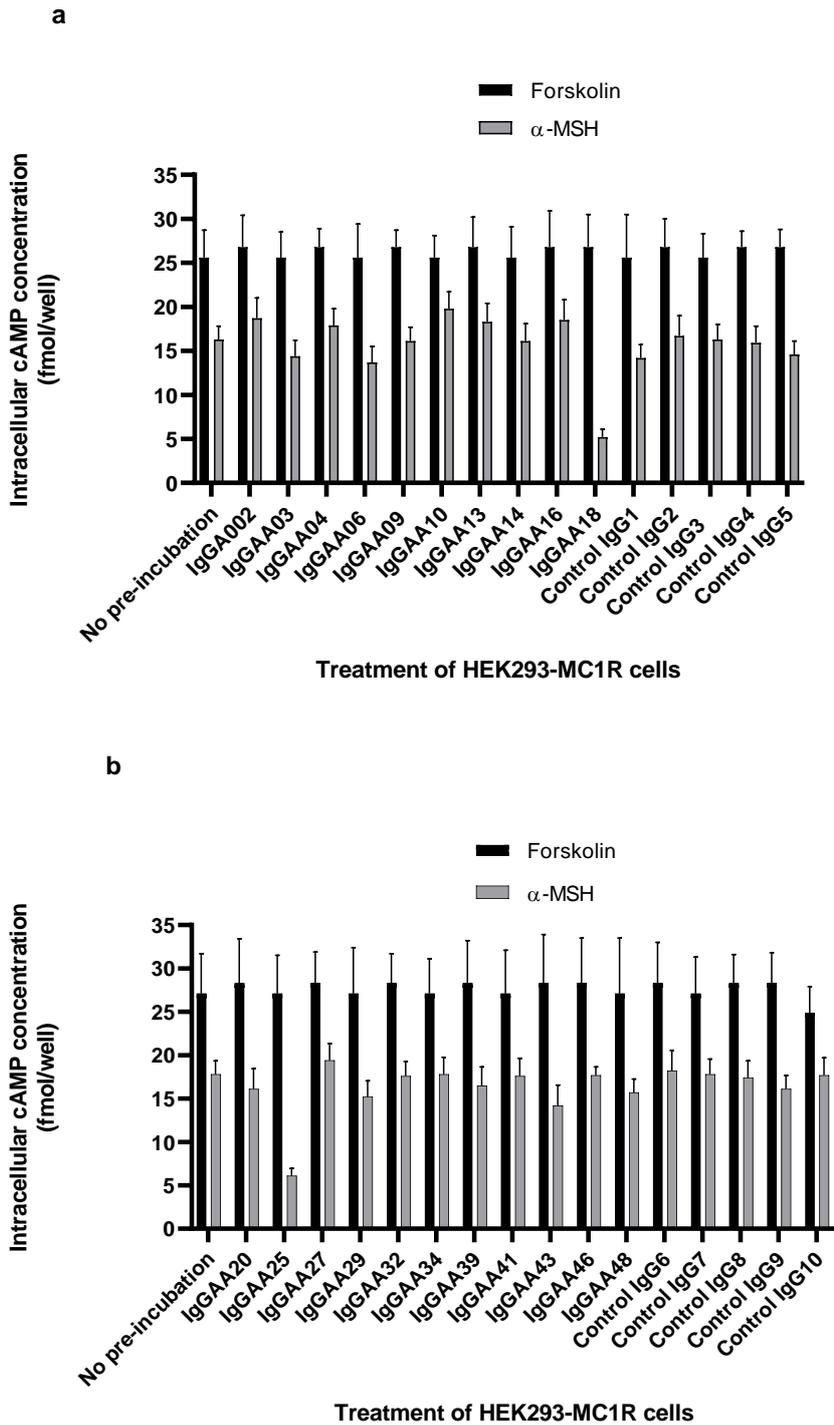


Figure 6.9: Measuring the effect of alopecia areata patient IgG on MC1R activity.

The results show the mean (\pm SD) cAMP accumulation from four experiments. Pre-incubation with IgG from two alopecia areata patients, AA18 and AA25, resulted in a statistically significant decrease in cAMP accumulation in response to α -MSH stimulation in HEK293-MC1R cells compared with α -MSH stimulation alone; P values were < 0.0001 .

6.5 Discussion

6.5.1 Epitope identification

The MC1R antibodies detected in 21 alopecia areata patients recognised epitope 1-36, at the extracellular terminal of the receptor (Figure 6.1). In addition, two patients also had MC1R antibodies that recognised epitope 182-191 at extracellular loop 2 of the receptor (Figure 6.1). None of the patients had MC1R antibodies against extracellular loops 1 or 3 or against intracellular loop 3 (Figure 6.1). Since all patient sera reacted against epitope 1-36, this could represent an immune-dominant MC1R antibody binding site. The peptide might be used in a simple assay to test a larger panel of sera for MC1R antibodies.

The characterised MC1R antibody binding sites are linear in nature as recognition of them by patient MC1R antibodies was determined using ELISAs with synthetic peptides. However, linear epitopes can contribute to discontinuous/conformational antibody binding sites on the receptor. Generally, it is assumed that antibodies affecting receptor function and lead to disease bind to conformational epitopes. For example, antibodies against the thyroid-stimulating hormone receptor, which cause hyperthyroidism in Graves' disease, recognise several conformational epitopes on the receptor (Morgenthaler et al., 1999). An important future goal would be to identify MC1R conformation-dependent antibody binding sites.

Immuno-reactivity to more than one epitope was only detected in two patients. In other autoimmune diseases such as type 1 diabetes and autoimmune thyroid disease, antibodies have been reported to react against several epitopes on the respective antigens tyrosine phosphatase-like IA2 and thyroid peroxidase (Zanelli et al., 1992, Lampasona et al., 1996). This phenomenon has been referred to as intramolecular spreading of the antibody response from one to multiple epitopes as the disease progresses. In type 1 diabetes, a temporal spreading of the antibody responses has been described. Antibodies against immune-dominant epitopes on tyrosine phosphatase-like IA2 have been reported in the early pre-clinical phase of type 1 diabetes. Antibodies to lesser immunogenic epitopes manifest in later stages of the disease (Naserke et al., 1998). However, no evidence of such a process of epitope spreading could be provided in this study as longitudinal patient sera were not available.

Using sera to map autoantibody epitopes can be problematic because they can contain multiple antibody species even against specific autoepitopes and the immune response can also become more diverse as diseases progress. To characterise the variety of antibodies against a particular autoantigen would require the isolation of monoclonal antibodies. An example of this has been is in the characterisation of glutamic acid decarboxylase antibodies in type 1 diabetes (Syren et al., 1996). This might be a useful approach to further the study of MC1R antibodies from alopecia areata patients allowing a greater detailed characterisation of them.

6.5.2 IgG subclasses

Like previous studies that have investigated IgG subclass in autoimmune disease (Boe et al., 2004, Brozzetti et al., 2010), the antibodies against the receptor were mainly of the IgG1 subclass. A minority of patients had antibodies to epitope 182-191 that were of subtype IgG3. Antibodies of the IgG1 and IgG3 subclasses have the ability to cause cell-mediated effector mechanisms and are able to activate the complement system (Flanagan and Rabbitts, 1982, van Loghem, 1986). These potential pathogenic mechanisms need to be investigated with respect to MC1R antibodies.

The profile of IgG subclasses is influenced by the T helper cell Th1/Th2 balance with respect to the immune response (Finkelman et al., 1990, Snapper and Paul, 1987). The Th1 cell subset promotes macrophage and cytotoxic T lymphocyte activation and the IgG subclass switching to IgG1 and IgG3 (Romagnani, 1995, Mosmann and Sad, 1996). Indeed, alopecia areata is considered a Th1 cell-mediated autoimmune disease characterised by the production of IFN- γ , IL-2, and TNF- β (Gilhar and Kalish, 2006, Ma et al., 2017), and in this study, IgG1 and IgG3 subclass antibodies predominated with respect to the MC1R antibody response.

It has been shown that fractionating into different IgG subclasses can identify the presence of all IgG subtype antibodies. This has been done for thyroid peroxidase and thyroglobulin antibodies in Hashimoto's thyroiditis where IgG4 antibodies were also identified in addition to IgG1 and IgG2 (Weetman et al., 1989).

6.5.3 MC1R antibody effects on the receptor

Only two patients had MC1R antibodies that appeared to block the activity of the receptor when stimulated with α -MSH. This was determined in HEK293 cells expressing the MC1R. However, further work is needed to determine if the same effects would be evident in melanocytes and the work needs to be extended to investigate how this could be related to hair-loss in alopecia areata. Both patients had antibodies against epitopes 1-36 and 182-191. However, at present, it is not known which of these antibody types could be responsible for the blocking activity or if indeed the effects are due to a patient MC1R antibody that has yet to be identified.

Chapter 7

General Discussion

7 General Discussion

7.1 Summary discussion of the results

Alopecia areata is the most common chronic inflammatory disease of the anagen hair follicle that results in non-scarring hair loss. It affects almost 2% of the general population at some point during their lifetime (Messenger et al., 2012a, Pratt et al., 2017). Until now, the exact aetiology and pathogenesis of alopecia areata are still not completely understood and need to be fully resolved. Various studies and hypotheses have been suggested environmental trigger factors, genetic susceptibility, immune factors as factors leading to autoimmunity and breakdown of hair follicle immune privilege and these are discussed in Sections 1.3.3, 1.3.4, 1.3.5, 1.3.8, 1.3.11, and 1.4.2.

Identification of autoantigens and characterising the autoimmune responses against hair follicles may help reveal the aetiological mechanisms involved in the disease. In addition, identifying new immune targets could be useful for developing new therapeutic and diagnostic tools. Therefore, the work in this thesis aimed to describe the Sheffield alopecia areata patient cohort regarding demographic and clinical profiles, and to identify novel disease autoantigens in alopecia areata.

7.1.1 The alopecia areata patient cohort clinical details and demographic

Alopecia areata is clinically classified as patchy or severe, the latter including totalis, universalis, and ophiasiform. Many epidemiological studies have reported that patchy type alopecia areata is the most common form occurring in 75-90% of total cases. Severe and rare forms of alopecia areata have been documented to represent 10–25% of total cases (Nanda et al., 2002, Alkhalifah et al., 2010a, Finner, 2011). In the current study, patchy alopecia areata was predominant with 68.8% of patients diagnosed with this type. Severe and rare forms of alopecia areata represented 31.3% of patients. These results were of a comparable frequency to previous studies.

Former epidemiological studies are conflicted as to whether men and women are affected by alopecia areata at the same frequency or whether there is a preponderance of females (Darwin et al., 2018). In conformity with previous studies (Tan et al., 2002b, Guzman-Sanchez et al., 2007, Lundin et al., 2014), female patients were more affected than men amongst our

analysed patient data with a ratio of affected males to females of 1:2.7. This may be due to a higher female cosmetic concern regarding hair loss and their more readiness to consult a dermatologist for further treatment. Some studies have reported a male predominance with a male to female ratio ranging from 2:1 to 1.1:1 (Sharma et al., 1996a, Jain and Marfatia, 2003, Yang et al., 2004, Kavak et al., 2008). Other studies have reported an equal frequency between male and female (Villasante Fricke and Miteva, 2015, Sobolewska-Wlodarczyk et al., 2016, Strazzulla et al., 2018).

In alopecia areata all age groups are at risk of developing alopecia areata, but the mean age at disease onset is 25-36 years (Tan et al., 2002b, Yang et al., 2004, Finner, 2011, Mirzoyev et al., 2014, Villasante Fricke and Miteva, 2015). In the present study, the mean age of onset was 36 years, which is compatible with previous studies. Most patients (81.3%) in the current study developed alopecia areata before the age of 50 years, which is closely compatible with several earlier studies (Kavak et al., 2008, Kyriakis et al., 2009, Trueb and Dias, 2018, Lyakhovitsky et al., 2019). In this study, 4/48 (8%) cases started in childhood, differing from some studies that state 20-50% of cases of alopecia areata develop between 1-16 years of age (Muller and Winkelmann, 1963, Sharma et al., 1996a).

Nail changes are a common feature of alopecia areata and one prognostic factor in the disease with a reported incidence ranging from 19-64% (Tosti et al., 1994, Sharma et al., 1996a, Gandhi et al., 2003, Kasumagic-Halilovic and Prohic, 2009, Roest et al., 2018). Nail pitting and trachyonychia are the most common manifestations in alopecia areata (Tosti et al., 1994, Chelidze and Lipner, 2018). The results of the current study reported an incidence of 35.4% in agreement with previous studies. In addition, 70.6% of the patients with severe forms of alopecia areata had nail involvement, which is compatible with several studies that have reported nail changes being an important prognostic indicator of alopecia areata severity (Sharma et al., 1996a, Gandhi et al., 2003).

Our findings in this study support and confirm previous evidence of correlations between alopecia areata with atopy (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Lyakhovitsky et al., 2015, Villasante Fricke and Miteva, 2015, Laitinen et al., 2020). There are frequent observations of a high prevalence of atopic diseases of between 39% and 61% amongst patients with alopecia areata (Tan et al., 2002b, Goh et al., 2006, Barahmani et al.,

2009, Chu et al., 2011). Our findings in the current study report that 75% of the alopecia areata patients had atopy which is like previous studies. There was no significant association of atopy with alopecia areata type; atopy was present in 81.8% of patients with patchy alopecia areata, and 60% of patients with severe forms. This is compatible with the results reported by Tan and colleagues who found no significant association between atopy and the severity of alopecia areata (Tan et al., 2002b). However, another study reported atopic disease, specifically atopic dermatitis, was significantly associated with alopecia areata, totalis, and universalis (Goh et al., 2006, Barahmani et al., 2009).

Alopecia areata has been associated with an increased risk of one or more other autoimmune diseases. Such comorbid conditions include systemic and dermatological autoimmune disorders (Tan et al., 2002b, Goh et al., 2006, Barahmani et al., 2009, Lyakhovitsky et al., 2015, Villasante Fricke and Miteva, 2015, Laitinen et al., 2020). Vitiligo is an autoimmune skin disease that has been reported widely to accompany alopecia areata (Goh et al., 2006, Barahmani et al., 2009, Kos and Conlon, 2009, Alkhalifah et al., 2010a). Only a single clinical study in India revealed no relationship between the two diseases (Thomas and Kadyan, 2008). The finding in current study was that the frequency of vitiligo in alopecia areata patients was 25%, an approximate four-fold increase when compared with the frequency of 1.8-7% found in other studies (Tan et al., 2002b, Chu et al., 2011, Huang et al., 2013) . Our findings confirm previous evidence (Muller and Winkelmann, 1963, Tan et al., 2002b, Kasumagic-Halilovic, 2008, Chu et al., 2011, Huang et al., 2013, Diaz-Angulo et al., 2015, Lyakhovitsky et al., 2015, Han et al., 2018, Lee et al., 2019b) of correlations between alopecia areata and autoimmune hypothyroidism and rheumatoid arthritis with frequencies at 16.7% and 4.2%, respectively.

Overall, in the present study, the clinical profiles and demographic of the alopecia areata patients were generally in agreement with results from previous reports.

7.1.2 Use of phage-display technology to identify autoantigens

One aim of this thesis was to identify and characterise novel antibody targets in alopecia areata using the pJuFo phage-display approach (Cramer and Suter, 1993, Cramer et al., 1994). This method was previously used for the successful enrichment of several different autoantigens in vitiligo and other autoimmune diseases (Cramer and Blaser, 1996, Cramer

et al., 1996, Lindborg et al., 1999, Kemp et al., 2002b, Waterman et al., 2010). In the current study, a panel of 14 different autoantigens was reported.

7.1.2.1 Previously identified autoantigens

Several were major melanocyte-specific proteins that had already been identified as autoantigens in alopecia areata in other studies (Kemp et al., 2011a). These included tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and melanocyte-specific protein PMEL (Kemp et al., 2011a). In the present study, the frequency of tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and melanocyte-specific protein PMEL antibodies ranged from 8-23%, a slightly increased frequency compared with 6-9% in an earlier report (Kemp et al., 2011b).

Tyrosine hydroxylase, the enzyme responsible for initiating the synthesis of melanin by converting tyrosine to L-dopa (Barbas, 1993), was identified as a B cell autoantigen in patients with autoimmune polyendocrine syndrome type 1, where immunoreactivity against tyrosine hydroxylase strongly associated with the presence of alopecia areata (Hedstrand et al., 2000). In addition, antibodies against tyrosine hydroxylase have been detected in patients with vitiligo and alopecia areata (Kemp et al., 2011a, Kemp et al., 2011b, Rahoma et al., 2012). Antibodies against tyrosine hydroxylase were detected in the current alopecia areata patient cohort at a frequency of 27.1%,

Some of the identified autoantigens have been reported in other autoimmune disorders, for example, HSP90 is an autoantigen in systemic lupus erythematosus (Conroy et al., 1994) and in vitiligo (Waterman et al., 2010). Therefore, HSP90 antibodies are potentially general markers of autoimmunity. The protein stabilises proteins against heat stress and assists in keeping proteins in activation-competent conformation (Tukaj and Węgrzyn, 2016, Zuehlke et al., 2018). It also has a role in immune responses including antigen presentation, the activation of lymphocytes and macrophages, the maturation and activation of dendritic cells, and in the induction of inflammation (Srivastava, 2002). Recent studies reported that HSP90 inhibitors have been shown to improve rheumatoid arthritis, systemic lupus erythematosus, autoimmune encephalomyelitis, and dermatological disorders like epidermolysis bullosa acquisita, bullous pemphigoid and mucous membrane pemphigoid (Tukaj et al., 2013, Kasperkiewicz et al., 2015, Tukaj and Węgrzyn, 2016).

Lamin A is a protein located in the nucleus of the cell and has been proposed to play an important role in the morphogenesis of the dermis and subcutaneous tissue involved in the maintenance of cellular morphology. It can be expressed on the surface of melanocytes so may be accessible to the immune system (Cui et al., 1995, Zhu et al., 2015, Thanomkitti et al., 2018). Antibodies against laminar proteins have been detected in patients with other autoimmunities such as autoimmune liver disease (Wesierska-Gadek et al., 1988), systemic lupus erythematosus (Senecal et al., 1999, Zhang et al., 2016), and vitiligo (Cui et al., 1995, Li et al., 2011). Recently, Thanomkitti and colleagues reported for that HSP90 and Lamin A/C physically interact with each other and play an essential role in hair follicle generation, growth, migration, and self-aggregation of dermal papilla cells and may be linked to the disease mechanism of alopecia areata via dermal papilla cells dysfunction (Thanomkitti et al., 2018).

Several novel autoantigens were also identified from the phage-display experiments including MC1R, OCA2 P protein, and glycoprotein non-metastatic melanoma protein b. These are discussed in the next section as they were investigated and validated in further experiments.

7.1.2.2 Newly identified autoantigens

The melanocortin 1 receptor (MC1R) is a seven-transmembrane G protein-coupled receptor with an intracellular carboxy-terminus and an extracellular amino-terminus. It regulates ultraviolet radiation responses by increasing skin pigmentation, through its agonist α -MSH (McRobie et al., 2014, Wolf Horrell et al., 2016, Salinas-Santander et al., 2018). Interestingly, black-coloured hair regrowth in a red-haired alopecia areata patient after cyclophosphamide administration, raises some interesting questions regarding the significance of pigmentation and the MC1R in alopecia areata pathogenesis (Ramot et al., 2012). In the current work, antibody reactivity to the MC1R was found at a frequency of 43.8%, much higher than that of other antibodies against tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, GPNMB, OCA2 protein, melanocyte-specific protein PMEL, tyrosine hydroxylase, and transcription coactivator p75 (Okamoto et al., 2004, Kemp et al., 2011b).

The frequency of antibodies against MC1R was closer to that of antibodies against retinol-binding protein-4 (67%) (Ahn et al., 2011), and the hair follicle antigens trichohyalin (100%) and keratin-16 (80%) (Leung et al., 2010). There were no associations between MC1R

antibody-positivity and the presence of autoimmune disorders, nail changes, atopy, and alopecia type.

The glycoprotein non-metastatic melanoma protein b (GPNMB) is a 560 amino acid protein encoded by the GPNMB gene localised to chromosome 7q15 (Kuan et al., 2006). It is expressed in different cell types including melanocytes and retinal pigment epithelial cells as well as antigen-presenting cells and macrophages (Owen et al., 2003, Kumagai et al., 2015). In pigment cells, the protein is localised in late-stage melanosomal organelles and enables keratinocyte-melanocyte adhesion, and so is thought to play a role in the melanosomes maturation and then the transporting of melanosomes to keratinocytes (Tomihari et al., 2009b, Hoashi et al., 2010, Maric et al., 2013). Expression of the protein on the surface of melanocytes is upregulated by both IFN- γ and TNF- α (Tomihari et al., 2009b). To our knowledge, GPNMB has not previously been reported as an antigen in alopecia areata. In the current study, antibody reactivity to GPNMB was found at frequency of 20.8%, similar to the prevalence of tyrosine hydroxylase (19%) and transcription coactivator p75 antibodies (20%) antibodies (Okamoto et al., 2004, Kemp et al., 2011b). GPNMB antibody-positivity was not associated with the presence of alopecia type, autoimmune disorders, nail changes, atopy, or patient demography.

The OCA2 protein was another novel autoantigen detected in this study. The function of this protein is not clear, although some studies suggest it participates in the transportation of tyrosinase to melanosomes as well as in maintaining the pH of melanosomes (Kemp et al., 1997a, Kemp et al., 1997b, Kemp et al., 1998a, Kemp et al., 1998b, Waterman et al., 2010, Kemp et al., 2011a, Li et al., 2011). OCA2 protein antibodies were detected at a frequency of 16.7% in the alopecia areata patient group, similar in prevalence to tyrosine hydroxylase (19%) and transcription coactivator p75 antibodies (20%) in patients with alopecia areata (Okamoto et al., 2004, Kemp et al., 2011b). There were no associations between OCA2 antibody-positivity and the presence of autoimmune disorders, nail changes, and alopecia type. There appeared to be associations between the absence of atopy, an increased patient age, and increased age of alopecia areata onset.

7.1.3 Properties of MC1R antibodies

The MC1R antibodies detected in 21 alopecia areata patients recognised epitope 1-36, at the extracellular terminal of the receptor. In addition, two patients also had MC1R antibodies that recognised epitope 182-191 at extracellular loop 2 of the receptor. None of the patients had MC1R antibodies against extracellular loops 1 or 3 or against intracellular loop 3. The antibodies against the receptor were mainly of the IgG1 subclass. A minority of patients had antibodies to epitope 182-191 that were of subtype IgG3. Only two patients had MC1R antibodies that appeared to block the activity of the receptor when stimulated with α -MSH. Further work is needed to determine if the same effects would be evident in melanocytes and the work needs to be extended to investigate how this could be related to hair-loss in alopecia areata. Both patients had antibodies against epitopes 1-36 and 182-191. However, at present, it is not known which of these antibody types could be responsible for the blocking activity or if indeed the effects are due to a patient MC1R antibody that has yet to be identified.

7.2 Future work and implications

7.2.1 Increasing case numbers

Although this study only included a small number of participants, this work could be progressed by the analysis of more alopecia areata patient serum samples for the newly identified antibody responses. Furthermore, in order to extend the preliminary findings, additional cases with patchy alopecia and severe type of alopecia, with and without nail changes, alopecia alone or associated with other inflammatory and autoimmune diseases should be tested for the occurrence of antibodies against the novel autoantigens. However, it is unlikely that appropriate numbers of patients within these categories will be available in the short-term.

7.2.2 Investigating the distribution of identified antibodies

All the antibodies identified in the present work were found due to the examination of patient serum samples. In further studies, it would be of interest to ascertain if any antibody specificities are present at the site of active alopecia areata patches, as well as in the circulation. This could be done using double indirect immunofluorescence.

7.2.3 Origin of autoantibodies in alopecia areata

Many of the autoantigens that were identified in the phage-display experiments were cytoplasmic antigens and it is not clear how antibodies against these targets could originate. Several theories have been postulated and these are discussed below.

Early studies reported that intracellular antigens in cells undergoing apoptosis can be included in apoptotic blisters or microbodies (Herrmann et al., 1998, Casciola-Rosen et al., 1999). In different autoimmune diseases, a failure to remove such apoptotic cell material has been reported. It has been suggested that this leads to the accumulation of cytoplasmic proteins that can become a source for activating dendritic cells and then triggering of a specific immune response (Muñoz et al., 2010, Mahajan et al., 2016).

Furthermore, the development of autoreactive B cells and the induction of antibodies reactive to intracellular antigens could result from changes in epigenetic modification including histone acetylation and DNA methylation (Renaudineau et al., 2010, Mahajan et al., 2016). For example, some medications such as procainamide or hydralazine prevent DNA methylation and lead to the development of a systemic lupus erythematosus-like syndrome (Dubroff and Reid, 1980, Mahajan et al., 2016).

Other theories put forward to explain antibody responses against intracellular antigens include the formation of neo-antigens, the exposure of cryptic epitopes, and the modification of proteins during apoptosis (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011a). If these altered antigens are exposed to and processed by dendritic cells and presented to autoreactive T cells that were not clonally deleted or to naïve T cells that were not tolerised against cryptic epitopes, then they could initiate antibody production from B cells activated by autoreactive CD4+ T lymphocytes (Namazi, 2007, Westerhof and d'Ischia, 2007, Kemp et al., 2011a).

Currently, it is not known which of these or other mechanisms allow the production of antibodies against the intracellular antigens such as tyrosinase, OCA2 P protein, and GPNMB that were identified in the present study. However, this could be a subject of future work.

7.2.4 Pathogenic effects of autoantibodies in alopecia areata

The existence of antibodies to melanocytes and keratin in alopecia areata patients poses the question of how are they implicated in disease pathogenesis. It is presently unknown if antibody abnormalities detected in alopecia areata patients are a primary or secondary cause of the disease. Indeed, they may only serve as markers for the disease or for the presence of autoreactive T lymphocytes that are capable of destroying hair follicles.

Autoantibodies may take part in antibody-dependent cellular cytotoxic or complement activity. In vitiligo, antibodies against melanocytes have been demonstrated to exert complement-mediated damage and antibody-dependent cellular cytotoxicity against pigment cells (Norris et al., 1988). Additionally, the injection of IgG samples from patients with vitiligo can destroy melanocytes in human skin that had been grafted onto nude mice (Gilhar et al., 1995). Such experiments could be repeated using antibodies isolated from patients with alopecia areata. Interestingly and in relation to intracellular antigens, in systemic lupus erythematosus and Sjögren's syndrome, it has been shown that antibodies against the SSA autoantigen can exert their cytopathic effects inside cells (Mallery et al., 2010, Racanelli et al., 2011).

Some antibodies can have an effect upon receptor activity, as shown for MC1R in the current project. Other examples of pathogenic antibodies include the thyrotropin receptor in Graves' disease (Weetman and McGregor, 1994), the acetylcholine receptor in myasthenia gravis (Hoedemaekers et al., 1997), and the calcium-sensing receptor in hypoparathyroidism (Kemp et al., 2009).

Alopecia areata is believed to be a cell-mediated autoimmune disease of hair loss, so it is likely that antibodies detected in patients are markers of T lymphocyte activity. Indeed, alopecia areata-affected subjects present with an increased frequency of cytotoxic T cells that are responsive to epitopes originating from keratinocytes and melanocytes (Ito et al., 2013a). Such T cell autoantigens include trichohyalin, tyrosinase, tyrosinase-related protein-2, and melanocyte-associated antigen-3 (Ito et al., 2013a), all of which have also been identified as targets of the humoral immune response (Leung et al., 2010, Kemp et al., 2011b).

7.3 Overall conclusion

The exact relationship between previously reported and novel antibodies identified in this project and alopecia areata pathogenesis remains to be fully determined. Further study of them may help to resolve the questions as to whether the antibodies can cause or exacerbate the disease, or if they are just markers that indicate the presence of a cellular immune response that may itself be responsible for hair follicle damage in alopecia areata. The phage-display method has been useful in identifying novel autoantigens that may ultimately be the targets of cytotoxic T cells in alopecia areata.

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