Immunopathogenesis of Primary Cicatricial Alopecia

A thesis submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Medical and Human Sciences

2013

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Figure 3.16 Q-PCR of E-Cadherin gene expression in the bulge epithelial of the lesional hair follicle
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<tr>
<td>α- MSH</td>
<td>Alpha-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>AKN</td>
<td>Acne keloidalis nuchae</td>
</tr>
<tr>
<td>AM</td>
<td>Alopecia mucinosa</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>APM</td>
<td>Arrector pili muscle</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>BMZ</td>
<td>Basement membrane zone</td>
</tr>
<tr>
<td>CCCA</td>
<td>Central centrifugal cicatricial alopecia</td>
</tr>
<tr>
<td>CCLE</td>
<td>Chronic cutaneous lupus erythematosus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous leukocyte antigen</td>
</tr>
<tr>
<td>CTS</td>
<td>Connective tissue sheath</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diamobenzidine</td>
</tr>
<tr>
<td>DCS</td>
<td>Dissecting cellulitis of the scalp</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double strand DNA</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eHFSC</td>
<td>Epithelial hair follicle stem cell</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transformation</td>
</tr>
<tr>
<td>EPDS</td>
<td>Erosive pustular dermatosis of the scalp</td>
</tr>
<tr>
<td>Fas</td>
<td>Factor of apoptosis stimulus</td>
</tr>
<tr>
<td>FasL</td>
<td>Factor of apoptosis stimulus ligand</td>
</tr>
<tr>
<td>FB</td>
<td>Foreign body</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FD</td>
<td>Folliculitis decalvans</td>
</tr>
<tr>
<td>FFA</td>
<td>Frontal fibrosing alopecia</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>FM</td>
<td>Follicular mucinosis</td>
</tr>
<tr>
<td>GLS</td>
<td>Graham little syndrome</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HF</td>
<td>Hair follicle</td>
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<td>HIER</td>
<td>Heat induced epitope retrieval</td>
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<td>HIS</td>
<td>Hair immune system</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HPF</td>
<td>High power field</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3deoxygenase</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
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<tr>
<td>INF (γ)</td>
<td>Interferon gamma</td>
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<tr>
<td>IP</td>
<td>Immune privilege</td>
</tr>
<tr>
<td>IR</td>
<td>Immune reactivity</td>
</tr>
<tr>
<td>IRS</td>
<td>Inner root sheath</td>
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<tr>
<td>KFSD</td>
<td>Keratosis follicularis spinulosa decalvans</td>
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<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
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<tr>
<td>LFT</td>
<td>Liver function test</td>
</tr>
<tr>
<td>LPP</td>
<td>Lichen planopilaris</td>
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<tr>
<td>LRC</td>
<td>Label retaining cells</td>
</tr>
<tr>
<td>MAC</td>
<td>Macrophages</td>
</tr>
<tr>
<td><strong>MC</strong></td>
<td>Mast cells</td>
</tr>
<tr>
<td><strong>MHC</strong></td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td><strong>MIF</strong></td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td><strong>n.s.</strong></td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>NAHRS</strong></td>
<td>North American hair research society</td>
</tr>
<tr>
<td><strong>NK cells</strong></td>
<td>Natural killer cells</td>
</tr>
<tr>
<td><strong>OCT</strong></td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td><strong>ORS</strong></td>
<td>Outer root sheath</td>
</tr>
<tr>
<td><strong>PB</strong></td>
<td>Pseudoplade of Brocq</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td><strong>PCA</strong></td>
<td>Primary cicatricial alopecia</td>
</tr>
<tr>
<td><strong>PICC</strong></td>
<td>Peri-follicular infiltration cell culture</td>
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<tr>
<td><strong>POD</strong></td>
<td>Programmed organ deletion</td>
</tr>
<tr>
<td><strong>PPAR-γ</strong></td>
<td>Peroxisome-proliferator-activated receptor (gamma)</td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td><strong>RT (q) PCR</strong></td>
<td>Real time (quantitative) polymerase chain reaction</td>
</tr>
<tr>
<td><strong>SCC</strong></td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>Standard error of mean</td>
</tr>
<tr>
<td><strong>TBS</strong></td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td><strong>TGF (β)</strong></td>
<td>Transforming growth factor (beta)</td>
</tr>
<tr>
<td><strong>TNF (α)</strong></td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td><strong>Tregs</strong></td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td><strong>U&amp;E</strong></td>
<td>Urea and electrolyte</td>
</tr>
<tr>
<td><strong>UK</strong></td>
<td>United Kingdom</td>
</tr>
<tr>
<td><strong>UV</strong></td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td><strong>VVG</strong></td>
<td>Verhoeff-van Gieson (elastin) stain</td>
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Abstract

The University of Manchester – School of Medicine – MPhil Abstract - Omima Abojela Eruk

Immunopathogenesis of Primary Cicatricial Alopecia

The current thesis project contributes to examining the immunopathogenesis of lichen planopilaris (LPP), one of the most frequent and the best-studied forms of scarring hair loss (primary cicatricial alopecia [PCA]) and an excellent model for the lymphocytic, inflammatory type of PCA. Current LPP pathobiology concepts propose that scarring hair loss in LPP results from an inflammatory attack that is associated with a collapse of the relative immune privilege of the hair follicle (HF), which irreversibly damages the epithelial stem cells in the bulge region of the HF. This is thought to limit the HF’s capacity to cyclically regenerate itself, thus causing permanent hair loss.

The current thesis project aimed to establish a basic understanding of human HF anatomy, function, and immunology and to acquire fundamental methodological skills for investigating human hair follicle pathology by employing appropriate histochemical and immunohistological markers.

An array of histochemical, immunohistological techniques for examining the inflammatory infiltrates of lesional versus non-lesional HFs in scalp skin biopsies from patients with LPP and HFs from healthy scalp skin was established. This included H&E, mast cell histochemistry (toluidine blue) and immunohistochemistry (tryptase), and immunohistological markers for macrophages (CD163), natural killer (NK) cells (CD161, V alpha 24), regulatory T cells (FOXP3), and EMT markers (E-cadherin, vimentin).

Perifollicular mast cells and macrophages were also evaluated. This revealed an increased number of degranulated mast cells in the perifollicular mesenchyme of LPP HFs compared to controls. It could also be shown that the number of resident (CD163+) macrophages in the HF’s connective tissue sheath, unexpectedly, is significantly reduced around LPP HFs compared to controls.
Quantitative immunohistomorphometry was employed to gauge whether there are any phenomenological indications that HF epithelial stem cells are undergoing a transition to mesenchymal cells (EMT). The epithelial cell-specific adhesion molecule, E-cadherin, was significantly reduced in the bulge area of lesional LPP HFs, while the mesenchyme-specific marker, vimentin, was up-regulated here. These immunohistomorphometry results were supplemented by quantitative RT-PCR, which showed a trend towards a reduction of E-cadherin and towards an up-regulation of vimentin expression in the bulge region of lesional HFs from LPP patients, though significance was not reached.

Taken together, the current thesis project has made an instructive panel of antigens and new immunostaining protocols available for systematic quantitative analysis in future LPP research. Most importantly, the project has generated the first exciting pointers in support of the hypothesis that the extensive scarring in PCAs may indeed result from pathological EMT in the epithelial stem cell zone of the HF, the bulge.
Declaration

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Chapter 1: INTRODUCTION
1.1 Project Overview

Besides hair follicle-derived tumours, irreversible hair loss in the form of scarring alopecia (primary cicatricial alopecia [PCA]) constitutes the most severe form of human hair follicle pathology. PCA can cause lasting disfigurement and major secondary morbidity, and thus can greatly reduce the quality of life of affected patients. Unfortunately, no causal therapy is available for any form of PCA, and PCA therapy is often highly unsatisfactory. Moreover, knowledge of the immunopathogenesis of PCA remains very incomplete. Therefore, advances in understanding the aetiology and pathobiology of PCA needs to be made in order to greatly improve the management of these chronic, permanent hair loss disorders.

Lichen planopilaris (LPP) is one of the most frequent and the best-studied forms of PCA, and provides an excellent model for lymphocytic, inflammatory PCA. Current LPP pathobiology concepts propose that scarring hair loss in LPP results from an inflammatory attack that is associated with a collapse of the relative immune privilege of the hair follicle (HF), which irreversibly damages the epithelial stem cells in the bulge region of the HF. This is thought to limit the HF’s capacity to cyclically regenerate itself, thus causing permanent hair loss.

With this in mind, using LPP as a model disease, the current thesis project aimed to establish a basic understanding of human HF anatomy, function, and immunology and to acquire fundamental methodological skills for investigating human hair follicle pathology by employing appropriate histochemical and immunohistological markers. On the basis of this, optimal markers for investigating hair follicle immunopathology in LPP were to be selected and immunostaining protocols to be established, specifically for studying the role of mast cells and macrophages in LPP pathobiology. Finally, the hypothesis was to be explored that the excessive scarring associated with LPP might result from pathological epithelial-mesenchymal transition (EMT) events in the epithelial stem cell area of the HF.

To this end, the relevant background literature was synthesized, and a systematic literature search strategy defined, which was then used to assist in selecting appropriate markers. For these markers, all necessary histochemical and immunohistological staining techniques
were established, along with appropriate positive and negative controls and quantitative (immuno-) histochemistry techniques.

The obtained results are reported and documented below. Discussing these results in the light of the relevant literature, the thesis concludes with an outline of some clinically relevant perspectives that arise from the (preliminary) findings generated in the current research project.

1.2 Human hair follicle anatomy and cycling

1.2.1 Functional anatomy

Although HFs show significant variation in size and shape according to their distribution over the body, they have a similar anatomical structure. The HF is generally classified into three main types: (1) lanugo hair follicles, produced during foetal development; (2) non-pigmented, thin vellus hair that appears in childhood; and (3) pigmented terminal hair which is present in adulthood.

The mature HF consists of a distal part that is classified as the compartment closest to the epidermis (Müller-Röver et al 2001). This is the so-called permanent portion of the HF, as it does not undergo major cyclical transformation changes. This region of the HF is further subdivided into two parts: the first is the infundibulum, which runs from the opening of the hair canal through the layers of the skin to the insertion of the sebaceous gland; the second is the isthmus, marking the area between the entry of the sebaceous gland and the attachment of the arrector pili muscle (Schneider et al 2009). The arrector pili muscle unit is located in the area where the infundibulum and isthmus join together.

The bulge region is located within this area, i.e. in the permanent and non-cycling proximal part of the HF’s basal layer of the outer root sheath (ORS) (Figure 1.2). This region contains the HF epithelium (keratinocytes), Langerhans cells, (dendritic antigen presenting cells), Merkel cells (specialised neurosecretory cells), and melanocyte hair follicle stem cells, all of which play an important role in wound healing after epidermal injury, as well as in protecting the hair follicle from exterior pathogens and from activation of the immune system (Ito et al. 2005, Schneider et al. 2009, Chou et al. 2013; Harries et al 2013).
The proximal part of the hair follicle which is located near to panniculus carnosus in mice (Müller-Röver et al 2001) and embedded into the subcutis in human skin, regenerates continuously, and is composed of two parts: the bulb region that contains both the matrix keratinocytes and the mesenchymal dermal papilla, and the hair shaft unit (Figure 1.2). The anagen bulb also contains the pigmentary unit of the HF, in addition to the matrix keratinocytes, which give rise to the hair shaft and its surrounding inner root sheath (Marlonet et al, 2009). The inner root sheath bundles the upwards-moving hair matrix cells and gives shape to both the HF as a whole and to the hair shaft. The ORS also harbours antigen presenting cells and a melanocytic melanocytes (Blume et al. 2011) and may be the major energy reservoir and energy production compartment of the HF (Vidali et al. 2013). The anatomical terms used to define each compartment of the hair follicle have been summarised in the Table 1.1.
**Figure 1.1 Histomorphology of the hair follicle.**

(A) Sagittal section through a human scalp hair follicle (anagen VI) showing the permanent (infundibulum, isthmus) and anagen associate (suprabulbar and bulbar area) components of the hair follicle. (B) High magnification image of the isthmus. The dashed square indicates the approximate location of the bulge. (C) High magnification image of the bulb. (D) Schematic drawing illustrating the concentric layers of the outer root sheath (ORS), inner root sheath (IRS) and shaft in the bulb. (BM: basal membrane; APM: arrector pili muscle; CTS: connective tissue sheath; DP: dermal papilla; M: matrix; HS: hair shaft, IRS: inner root sheath; ORS: outer root sheath; SG: sebaceous gland) (Schneider et al 2009)
Table 1.1 Definition of the corresponding anatomical terms in the hair follicle: adapted from Schneider et al (2009)

<table>
<thead>
<tr>
<th>Arrector pili muscle</th>
<th>Definition</th>
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<tbody>
<tr>
<td><strong>Bulb</strong></td>
<td>Thickening of the proximal end of the hair follicle. Contains rapidly proliferating, rather undifferentiated matrix cells (transit amplifying cells), melanocytes and outer root sheath cells.</td>
</tr>
<tr>
<td><strong>Bulge</strong></td>
<td>Convex protrusion of the outer root sheath in the most distal permanent portion of the hair follicle, just below the sebaceous gland and at the insertion site of the muscle arrector pili. Contains the hair follicle stem cells.</td>
</tr>
<tr>
<td><strong>Club hair</strong></td>
<td>Fully keratinized, dead hair (telogen follicle) formed during catagen and telogen.</td>
</tr>
<tr>
<td><strong>Dermal papilla</strong></td>
<td>Mesodermal signalling centre of the hair follicle consisting of closely packed specialized mesenchymal fibroblasts. Framed by the enlarged bulb matrix in anagen.</td>
</tr>
<tr>
<td><strong>Hair canal</strong></td>
<td>Tubular connection between the epidermal surface and the most distal part of the inner root sheath. Contains the hair shaft.</td>
</tr>
<tr>
<td><strong>Hair germ</strong></td>
<td>Also called ‘hair placode’ depending on the developmental stage. Bud-like thickening in the fetal epidermis consisting of elongated keratinocytes, which at the distal end are in contact with numerous aggregated specialized dermal fibroblasts, the dermal condensate.</td>
</tr>
<tr>
<td><strong>Hair peg</strong></td>
<td>Column of keratinocytes growing into the dermis during embryonic hair follicle development (developmental stages 3-5). The concave proximal end starts to encase the dermal condensate, the future dermal papilla.</td>
</tr>
<tr>
<td><strong>Hair shaft</strong></td>
<td>The hair per se, composed of trichocytes, which are terminally differentiated hair follicle keratinocytes. It is composed of the medulla, the central part with loosely connected keratinized cells and large air spaces, and the cortex, which is the bulk of the hair shaft, consisting of keratinized cells, keratin filaments, and melanin granules in pigmented hairs.</td>
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<td><strong>Infundibulum</strong></td>
<td>Most proximal part of the hair follicle relative to the epidermis, extending from the sebaceous duct to the epidermal surface. Includes the hair canal and the distal outer root sheath.</td>
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<tr>
<td><strong>Inner root sheath</strong></td>
<td>A multi-layered rigid tube composed of terminally differentiated hair follicle keratinocytes, surrounded by the outer root sheath.</td>
</tr>
<tr>
<td><strong>Isthmus</strong></td>
<td>Middle part of the hair follicle extending from the sebaceous duct to the bulge.</td>
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Outer root sheath | The outermost layer of the hair follicle. Merges proximally with the basal layer of the interfollicular epidermis and distally with the hair bulb.
---|---
Sebaceous gland | Acinar gland composed of lipid-filled sebocytes, located close to the insertion of the arrector pili muscle. Secretes sebum to the epidermal surface via a holocrine mechanism. Sebum helps to make hair and skin waterproof. Together with the hair follicle and the arrector pili muscle, it forms the pilosebaceous unit.

1.2.2 Hair cycle

Hair is formed by rapid proliferation of matrix keratinocytes in the bulb at the base of an active growing (anagen) HF, with growth potentially continuing for several years (in human scalp skin). This proliferation stage is accomplished by the dermal papilla secreting numerous important factors (“papilla morphogens”) (Stenn and Paus 2001, Schneider et al. 2009), which function via interacting with their respective receptors in/on hair matrix cells, such as insulin-growth factor-1 (IGF-1) and fibroblast growth factor-7 (FGF-7). Previous studies have shown that mice lacking IGF-1 and its receptors are unlikely to produce a new HF, whereas FGF-7 deficient mice maintain quite normal hair growth. However, abnormality of the FGF-7 receptor, which is also a receptor for FGF-2, leads to a significant reduction of HF development. Towards the end of the anagen phase, the HFs express FGF-5, which terminates this stage of the cycle and induces HF regression (catagen). Mice lacking FGF-5 demonstrate a longer anagen phase - double that of the normal HF (Danilenko et al 1996; Stenn et al 1996; Rosenquist et al 1996; Hebert et al 1994; Paus et al 2012).

During catagen, hair matrix keratinocytes stop proliferating and the lower follicle regresses by switching on apoptosis. This usually occurs over a short and temporary period of a few weeks. Furthermore, follicular melanogenesis also stops during catagen (Tobin 2011). Prior to the end of catagen, the dermal papilla moves upward to a position adjacent to the HF bulge and the so-called secondary hair germ (Cotsarelis and Millar 2001). Should the dermal papilla be unable to make this transition, the HF will remain in catagen and cease cycling.

Subsequent to the catagen regression phase, the follicle enters telogen, which is generally believed to be a phase of relative quiescence that may last for months. However, it now understood that even telogen is not a “resting” period, as the HF shows intense and telogen-specific activities (Geyfman et al. 2012). The percentage of telogen HFs in any given
skin area may differ considerably depending on body region. If the percentage of telogen scalp HF is high, hair loss may ensue ("telogen effluvium"), thus it is interesting to pursue the reduction of telogen HFs as a novel treatment in hair loss. Upon activation of the DP, proliferation of matrix cells is resumed and new hair bulb is produced. Thus anagen resumes and the hair cycle is once again initiated (Figure 1.3) (Paus and Cotsarelis 1999; Stenn and Paus 2001; Schneider et al. 2009; Harries et al. 2013).

![Figure 1.2 Normal hair cycles](image)

*Figure 1.2 Normal hair cycles:* The hair follicle enters a continuous cyclical process of organ regression (catagen), a relative ‘resting’ phase (telogen) and a growth phase (anagen) in the hair cycle. Dermal papilla (DP), Outer root sheath (ORS), Hair shaft (HS), inner root sheath (IRS), Sebaceous gland (SG). (Schneider et al. 2009)
1.3 The bulge region

The bulge region is located in a specific area of the ORS, found at the lower end of the “permanent” portion of the hair follicle, as described above (Figure 1.2 and Table 1.1). It has been identified by different sources, in relation to previous observations. In 1876, Unna described the bulge region as the “haarbett” which means ‘hair bed’. Following this, in 1904, Stohr named this area a “wulst”, to describe the swelling or bulge in the rodent hair follicle (Unna 1876; Stohr 1904).

The identification of the bulge region has been established through recognition of two anatomical markers in the HF. The first is the presence of small protrusions from the bulge region, termed the “follicular trochanter” (Tiede et al 2007). The second is the APM, considered a fundamental anatomical landmark for distinguishing the bulge region in the hair follicle (Tiede et al. 2007; Harries et al 2013). The bulge region has been identified as the seat of eHFSC by the observation that it contains the most slow cycling cells (“label retaining cells” [LRC]) of the entire pilosebaceous unit (Cotsarelis et al, 1990). The bulge region also contains melanocyte stem cells that undergo proliferation during early anagen, and remain quiescent throughout the rest of the hair cycle (Nishimura al 2002; Cotsarelis, 2006). However, these cells can be recruited to the epidermis under conditions of wounding, at least in mice (Chou et al. 2013, Paus et al 2013).

1.4 Epithelial hair follicle stem cell biology

Cotsarelis 2006 defines somatic stem cells as “The cells of origin for terminally differentiated cells in adult tissues” (Cotsarelis, 2006). These stem cells are found in many different tissues and are characterised by their ability to self-renew, as well as having multipotent features. In skin, they are not restricted to the hair follicle, but also exist in the sebaceous gland and the epidermis (Plikus et al 2012). Indeed, it was previously assumed that stem cells were present in the basal layer of the epidermis, which was produced as a consequence of the proliferation that occurs in the basal layers. This was described in 1974 by Potten, who named it “Epidermal proliferative unite” (EPU). The stem cells, which reside in the EPU, control the continuous renewal processes in the epidermis (Allen et al 1974; Ito et al 2005; Cotsarelis 2006). In contrast, eHFSC appear to go through cyclical bursts of regenerative...
activity. Recent studies have identified additional stem cell populations in the HF epithelium or in the mesenchyme adjacent to it, several of which may be fundamental to wound healing, regeneration of the HF mesenchyme and HF-adipose tissue interactions (Tiede et al 2007a, Plikus et al. 2012, Schmidt and Horsley 2012; Goldstein and Horsley 2012). Basic features of eHFSCs are summarized in Table 1.2.

Table 1.2 Characteristics of epithelial hair follicle stem cells (eHFSCs).

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<tr>
<td>1)</td>
<td>Relative quiescence (Morris et al 1999; Lyle et al 1998)</td>
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<tr>
<td>2)</td>
<td>Slow cycling cells (“Label retaining cells). (Wright and Alison 1984; Cotsarelis et al 1990)</td>
</tr>
<tr>
<td>3)</td>
<td>Undifferentiated cells.</td>
</tr>
<tr>
<td>4)</td>
<td>Limited plasticity (differentiation into epidermal or HF keratinocytes, or into sebocytes)</td>
</tr>
<tr>
<td>5)</td>
<td>A high proliferative potential, which is represented in two categories: The first is the holoclone phenotype (a holoclone is a colony-forming stem cell which has a high reproduction rate and a low rate of differentiation (Barrandon and Green 1987; Blanpain, Lowry et al 2004, Cotsarelis 2006). The second is high colony forming efficiency, presented as each cell that is plated having a colony (Jones et al 1993; Lowry et al 2004, Cotsarelis 2006).</td>
</tr>
<tr>
<td>6)</td>
<td>Stem cells control regeneration of the tissue including the entire HF, epidermis and sebaceous gland it is believed that bulge stem cells proliferate at the anagen phase and have a role in maintaining the lower hair follicle. (Weinberg, Goodman et al. 1993; Oshima, Rochat et al. 2001; Blanpain, Lowry et al. 2004; Claudinot, Nicolas et al. 2005)</td>
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<td>7)</td>
<td>Extended life-span compared to their differentiated/committed progeny (Castilho et al. 2009)</td>
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<tr>
<td>8)</td>
<td>Residence within a specialized “stem cell niche” (Goldstein and Horsley 2012)</td>
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There has been some debate in previous studies as to how convincing evidence is that the hair matrix keratinocytes in the bulb are the progeny of bulge-derived stem cells. Indeed, no study has yet provided data for the number of bulge-derived stem cells that are contributing to the formation of the new anagen hair follicle (Jun Kamimura et al 1997; Cotsarelis et al. 2006). It is believed that eHFSCs participate in anagen induction, moving downwards to proliferate in the ORS and to form the next bulb (Oshima et al 2001; Cotsarelis 2006; Schneider et al. 2009; Hsu et al 2011).

Given the vital role in HF growth and cycling, protection of the bulge region stem cells from both cytotoxic damage and immune destruction is vital. Indeed, it has been noted that infiltration of inflammatory cells into the eHFSCs in the bulge region is a mechanism by which permanent hair follicle loss can occur, whereas inflammation affecting only the bulbar region produces reversible damage (alopecia areata), confirming the notion that the bulge, while remaining intact, is able to produce a new lower anagen hair. This therefore defines a specific biological advantage for follicular stem cells to be resident in the permanent portion of the HF (bulge region) rather than to be located in the cyclical bulb region, as described in further detail in section 1.5 (Cotsarelis 1990; Mobini et al 2005; Cotsarelis 2006). This is consistent with the suggestion that the bulge region and lower hair follicle are relatively protected from immunological insult a concept known as “immune privilege” (Meyer et al. 2008). The loss of this protection, including damage of the bulge area of the hair follicle, is the hypothesis for PCA pathology.

1.5 The bulge region as a niche for the hair follicle stem cells

As discussed above, there are a number of biological advantages to the follicular stem cells residing in the bulge region. Firstly, despite the fact that the bulge area is located in the non-cycling permanent portion of the hair follicle, the stem cells are appropriately located to meet the upcoming dermal papilla cells in the late stage of telogen or early anagen in order to regenerate a new follicular in the early stage of anagen (Cotsarelis et al 1990). Secondly, protection of the stem cells from accidental damage due to plucking is achieved by their being situated in the outer root sheath, away from the hair shaft (Bassukas et al 1989, Cotsarelis et al 1990). Thirdly, the bulge region area is well vascularised (Ellis and Moretti et al 1959; Montagna, et al 1952, Cotsarelis et al 1990).
1.6 Epithelial hair follicle stem cells biomarkers

There are numerous publications describing the identification of the epithelial hair follicle stem cells (eHFSCs) in the bulge region, as defined by positive and negative bulge stem cell markers. Negative markers in human HF s include 1) missing expression of CD34 by bulge stem cells (Kloepper et al., 2008; Trempus et al., 2003); 2) a lack of the gap junction protein, connexin 43, from the basal layer of the ORS bulge (Matic et al 1997; Matic et al 2002; Kloepper et al 2008), and 3) of the intermediate filament protein, nestin, a neural stem cell marker (Lardon et al 2002; Schultz et al 2006; Kloepper at al 2008; Lau et al 2009).

Meanwhile, the best-known positive markers of human eHFSCs are: Cytokeratin 15 (C8/144B monoclonal antibody), whose immunoreactivity pattern is strikingly up-regulated in follicular keratinocytes of the bulge region in both mice (Lyle et al 1998) and humans (Lyle et al 1999; Kloepper et al 2008); Cytokeratin 19 expression has also been demonstrated in in the bulge of mice and humans (Michel 1996; Kloepper et al 2008); CD200 expression (Kloepper et al., 2008; Ohyama et al., 2006). Cytokeratin 15, cytokeratin 19 and CD200 have all been found to be up regulated in the bulge region and are therefore considered being positive markers for the bulge stem cells (Lyle et al 1998; Trempus et al 2003; Lyle et al 2004; Cotsarelis 2006). It must however be noted that none of these markers are entirely specific to eHFSCs alone.

1.7 Hair follicle immunology

The cellular composition of the immune system in defined HF compartments has been demonstrated by immunohistochemical localisation of various immune cells, as summarized in (Figure 1.4) (Christoph et al. 2000; Ito et al 2005a). The immunoreactivity pattern of specific cellular biomarkers was utilised to confirm the immune cellular composition in perifollicular and interfollicular tissue around the bulge region. These cell types and their expression patterns are described in more detail in the following sections.

1.7.1 Mast cells.

Previous studies have shown that mast cells play a central role in multiple different aspects of skin biology (Maurer and Metz 2005), and their contribution to skin immune response can be classified into three essential features. Firstly, they are essential in inflammation and it has been shown that they play a considerable role in allergic encephalomyelitis (Hatfield et
of mast cells. Secondly, they have a role in wound healing and angiogenesis, and thirdly they are involved in innate and acquired immunity through T cell activation and regulation (Ito et al. 2010; Maurer et al. 1998; Weber et al. 2003; Theoharides and Cochrane, 2004; Theoharides et al. 2004a; Theoharides et al. 2004b; Maurer and Metz, 2005; Schroeder et al. 2006; Weller et al., 2006; Metz and Maurer, 2007; Metz et al., 2008; Stelekati et al. 2009).

Researchers have also identified that mast cells make a significant contribution to IP damage as well as neurogenic skin inflammation, through observations of degranulated mast cells around the bulge region in mice in response to psychoemotional stress (Arck, Handjiski et al. 2003; Peters, Kuhlmei et al. 2005; Arck, Slominski et al. 2006). Interestingly, no intraepithelial mast cells are found in the normal anagen HF, whereas they are richly present in the hair follicle cutaneous tissue sheath (CTS) (Christoph et al. 2000, Sugawara et al. 2012)(Figure 1.4).

In lymphocytic PCA, namely in LPP, their number is also greatly increased and they show signs of increased activation (Harries et al. 2013). Further, a high density of immature mast cells was observed around the bulge region of LPP patients, which suggests that maturation of immature mast cells present in the CTS of human scalp HFs (Ito et al. 2010, Sugawara et al. 2012) is induced. This study (Harries et al. 2013) also presented evidence for mast cell-T cell interactions in lesional LPP samples, consistent with other previously published work in mice (Stelekati et al. 2009).

1.7.2 Macrophages
Monocytes are believed to be attracted to damaged tissue by different microenvironmental stimuli, where they differentiate into macrophages (Lehtonen et al. 2007) Macrophages are customarily classified into two broad phenotypes according to their activation. The first is the M1 phenotype (“proinflammatory”), which displays the IL-12 high, IL-13 high, IL-10 low phenotype and is activated by interferon γ (IFNγ), a range of microbial stimuli and/or Th1 lymphocyte-related responses. Upon activation these cells release specific inflammatory cytokines such as TNF-α, IL-6, IL-1β, and transforming growth factor (TGF-β). The second macrophages population, the M2 phenotype (“reparative”), is activated by Th2 lymphocyte-driven responses, IL-13 and IL-4 (Clark et al. 2006; Higashi-Kuwata 2010; Juniantito et al. 2012).
These two macrophage phenotypes are considered to be versatile cells and fulfil distinct functions. They play a role in various processes including; 1) the removal and ingestion of cellular debris in late stage inflammation, 2) the regulation of the immune response through scavenging debris and dead cells in order to present antigens to T helper cells, 3) functioning in vascularisation and wound healing, and 4) playing a crucial role in fibrotic diseases as they are a significant source of TGF-β.

It has been suggested that M1 macrophages express CD68, whereas M2 macrophages express high levels of CD163 (Higashi-Kuwata et al., 2010; Juniantito et al., 2012). In addition to the M1 and M2 classifications, macrophages are categorised into three subtypes; oxidative, resident, and antigen presenting cells. These subtypes are recognised immunohistochemically in the rat via expression of CD68 (ED1) for oxidative macrophages (Damoiseaux et al 1994; Juniantito et al 2012), CD163 for resident macrophages (ED2)(Polfliet et al 2006; Juniantito et al 2012), and MHC II molecules for antigen presenting macrophages (OX6)(Picard et al 2008;Juniantito et al., 2012). However the relationship between the functions of these macrophages and their immunophenotype needs to be investigated in more detail. (Hume et al 2008; Juniantito et al 2012).

Recent human HF research has detected the highest number of macrophages in the perifollicular mesenchyme (CTS), with increased numbers around the hair bulb, whereas macrophages are absent in the epithelial part of the human HF (Christoph et al 2000). Furthermore, macrophage numbers are believed to increase in the catagen phase of the hair cycle (Westgate et al 1991), with evidence suggesting that macrophages may exert some control over hair-cycle regulation (Parakkal 1969; Parakkal 1969; Paus et al 1999, Stenn and Paus 2001).

The importance of macrophages in HF physiology and pathology is demonstrated by reports indicating that removal of damaged HF by physiological mechanisms (termed “programmed organ deletion”) is mediated by activated macrophages (Eichmuller et al 1998). Indeed, the distribution of macrophages is altered in PCA: By analysing CD68 immunohistochemistry, lesional LPP hair follicles were found to show a very high density of macrophages in the CTS (compared to non-lesional and healthy control HFs) and were found to invade the epithelial HF in advanced stage LPP (Harries et al. 2013) – just as has been described for the process of “programmed organ deletion” of HFs in mice (Eichmüller et al. 1998).
1.7.3 T Lymphocytes

There are different subtypes of T cells with distinct functions, which are generally classified into six subtypes; 1) helper, 2) cytotoxic, 3) memory, 4) regulatory, 5) natural killer, 6) gamma-delta T cells. T-cells are further subdivided into those positive or negative for CD4+ and CD8+ (Akbar et al 1988; Watanabe et al 2005; Gutcher et al 2007; Jiang et al 2004; Hanabuchi, et al 2010).

In the human HF, CD4+ and CD8+ T-cells are found primarily in the distal ORS and the CTS, whereas they are found only very rare in the isthmus and virtually absent in the hair bulb. High densities of CD4+ cells are found, in comparison with the number and distribution of CD8+ cells (Christoph et al 2000)(Figure 1.4). In contrast, in lesional LPP hair follicles, CD4+ and CD8+ cells are both detected as highly expressed and accumulate around the distal HF as well as infiltrating the epithelium of the infundibulum and bulge region. These findings suggest a contribution for CD4+ and CD8+ T-cells in IP damage in LPP (Harries et al. 2013).

Regulatory T cells (Tregs) are commonly named suppressor T cells; they have a crucial role in the control of immune response, including in certain immunoprivileged tissue (Waldmann 2006). Tregs can be categorised into natural (constitutive) Tregs, which are produced in the thymus and transferred into peripheral tissue, where they interact with effectors cells, and adaptive regulatory Tregs, with various subsets that include TGF-β-, IL-10, FOX P3+ inducible T cells (Lourenco et al 2012).

It has been noted that the number of Tregs decreases in autoimmune diseases such as systemic lupus erythematosus (Miyara et al 2005; Lee et al 2006; Mellor-Pita et al 2006; Lyssuk et al 2007; Valencia et al. 2007; La cava2008; Zhang et al 2008; Kuhn et al 2009), morphea and multiple sclerosis (Viglietta et al 2004; Matarese et al 2005; Paust et al 2005; Antiga et al. 2010; Lourenco2012). Conversely, Tregs numbers are up regulated in other inflammatory disorders, for instance, psoriasis and lichen planus (Franz et al 2007). Recently Tregs have been found to be increased in the lesional hair follicles of LPP patients (Harries et al 2013).
1.7.4 Gamma-delta T cells

Normal murine epidermis displays a dendritic phenotype of intra-epithelial T cells (DETC), which display invariant gamma-delta chains on the T-cell receptor (e.g., Vc3/Jc1-Cc1 and Vd1/Dd2/Jd2-Cd chains in the skin of mice [Shekhar et al 2012]). DETCs are best appreciated to execute three distinct functions: 1) directing skin inflammatory response, e.g. as part of antigen-independent anti-infection defences or as “danger alert” cells, 2) a role in wound healing, 3) recognition and elimination of malignant cells. They achieve these functions by the production of a distinct array of chemokines, cytotoxic molecules and growth factors that can recruit additional immune cells to the skin (Goldstein et al 2012; Fan et al 2011; Macleod et al 2011) Most recently, it has been found in mice that gamma-delta T cells are also involved in hair cycle control (Harries et al. 2013)(Figure 1.4), and in wound healing-associated HF neogenesis in adult murine skin (Plikus et al. 2013).

1.7.5 Natural killer and natural killer T cells:

Natural killer cells are only infrequently seen around healthy human anagen HF (Christoph et al 2000)(Figure 1.4), while they are greatly increased around HFs in alopecia areata (Ito et al. 2008). NK cells display an array of different receptors, including the heterodimer CD94/MCG2A and NKG2D (an NK-activating receptor) (Long & Rajagopalan1999; Bynoe et al 2005; T Ito et al 2008), and killer cell Ig-like receptors (KIRs). Until now, only relatively little research has been performed on the role of NK and NKT cells in HF health and disease. However, recent studies which suggest a key role for NKG2D+ NK and/or NKT cells and NKG2D-activating ligands of the MICA family (Ito et al 2008, Petukhova et al. 2010; Schafer et al 2010; Gilhar et al. 2012,) have placed these cells into the centre of HF immunology research, namely in inflammatory HF diseases.

The role of macrophage migrating inhibitory factor (MIF) in immune privilege maintenance has been confirmed by studies noting a reduction in NK cell stimulation in the anterior eye chamber (Apte et al 1998). MIF may play a similar role in the IP of human HFs, MIF immunoreactivity is up regulated in the entire epithelium of the normal anagen scalp HF, mainly in the IRS and ORS of the proximal HF, whereas it is reduced in the lesional HF in
alopecia areata and PCA (Ito et al 2008), suggesting that tissue sites of IP collapse are unable to suppress excessive NK cell activity.

NK cells are activated by IFN-γ and can also secrete this IP collapse-inducing cytokine; this secretory activity plays major role in host defence against foreign antigens and malignant tumours and is implicated in many autoimmune diseases (Novelli et al 1991; Bryceson et al 2006; T Ito et al 2008; Gilhar et al. 2012). Intriguingly, IFN-γ was found to be a key cytokine in bringing about HF IP collapse and in promoting the development of alopecia areata (Rückert et al 1998; Paus et al 2003; Ito et al 2004; Freyschmidt-Paul et al 2006; Gilhar et al 2007 + 2012; Ito et al 2008).

### 1.7.6 Langerhans cells

Langerhans cells (LC) are a subtype of dendritic cells of the epidermis and professional, MHC class II + antigen-presenting cells (Romani et al 003; Novak et al 2004.). LCs can be found in considerable numbers in the distal ORS and extremely rarely in the hair bulb, where they do not express MHC class II and thus cannot present antigens to CD4+ T cells (Christoph et al. 2000, Ito et al. 2004)(Figure 1.4). Recently, it has been documented that there was no substantial difference in the expression of LC markers (CD1a-CD209) between lesional and non-lesional LPP HFs, thus questioning whether LCs are critically involved in the immunopathogenesis of LPP (Harries et al 2013).
**Figure 1.3 Schematic representation of terminal hair follicle** (anagen VI phase), displaying the distribution of the immunoreactivity pattern of specific immune cells markers in the hair follicle: 1) Langerhans cells (CD1a & MHCII) are highly expressed in the basal layer epidermis (Bos 1997) and the distal HF outer root sheath (ORS) with a sharp decline in the isthmus ORS (Christophe et al 2000). 2) T cells (CD+4 helper/inducer T cells & CD+8 Cytotoxic / suppressor T cells) are detected to be up-regulated in the perifollicular epidermis (Ledbetter et al 1981) and the distal ORS with remarkable accumulation in the connective tissue sheath (CTS) around the distal HF; the isthmus is characterised by a sharp decline in the number of CD+4 & CD+8 T cells. (The proportion of CD+4 to CD+8 is increased in the normal HF (Christoph et al 2000). 3) Macrophages are predominantly distributed in the perifollicular CTS of the entire HF (Bos 1997) with some macrophages detected in CTS of the hair bulb, and a few melanophages entering dermal papilla (DP) (Christorph et al 2000). 4) Mast cells also are identified in high density in the perifollicular CTS from the distal to the proximal HF, with virtual absence throughout the hair follicle epithelium. (Christorph et al 2000). 5) B cells are significantly rarely seen and are only located in the hair ORS at the level of the sebaceous gland (Christorph et al 2000). 6) Natural Killer (NK cells) are identified in small numbers in the perifollicular CTS, with no intraepithelial expression in the HF (Christoph et al 2000; Ito et al. 2005). 7) γδT cells are rarely detected in the distal ORS of human HFs, with no expression in the entire HF epithelium; generally γδT cells are resident in the human epidermis (Christoph et al 2000; Macleod et al 2011). Schematic representation of the HF: Omima Eruk
1.8 Hair follicle immune privilege

1.8.1 General features of immunoprivileged tissue sites
Immune privilege (IP) is restricted to vital compartments within the body, including the anterior chamber of the eye, cortex of the adrenal gland, ovary, testis, foetotrophoblast and parts of the central nervous system (Medawar 1948; Niederkorn et al 2003; Paus et al 2005; Cobbold et al 2006; Meyer et al 2008). Within the skin appendages, IP is restricted to the HF and possibly exists in the human nail apparatus as well (Ito et al 2005a). It is important to note that IP damage has a crucial role in autoimmune diseases such as mumps, orchitis, autoimmune uveitis, hepatitis, (Mellor and Munn 2000; Streilein 2003; Monney et al 2002).

1.8.2 Immune privilege in the human hair follicle
That the HF also has established a relative IP (Paus et al. 2005) was discovered in a famous transplantation study by Billingham (Billingham, and Silvers 1971). In this study, the epidermis of black guinea pigs was grafted onto a genetically distinct strain of white guinea pigs. The pigmentation of the transplanted epidermal graft was initially lost due to rejection of the epidermal melanocytes; however a few months later, black hair was seen to grow in the area of the transplanted and rejected epidermis. This indicated that migrating donor melanocytes had escaped destruction by the host immune system by seeking shelter in the anagen hair bulbs of host HFs. The concept of HF IP has been extended and confirmed by several subsequent studies (see Ito et al. 2004; Ito et al. 2005, Gilhar et al. 2012).

1.8.2.1 Bulb immune privilege: Long after Billingham had proposed that the anagen hair bulb is immunologically privileged (Billingham and Silver 1971), it was postulated that alopecia areata primarily results from a collapse of this bulb IP and that alopecia areata can serves as a unique model disease to elucidate the consequence of the IP collapse in the human HF (Paus et al. 1993 + 2005). IP collapse is thought to be triggered by several predisposing factors (e.g. infection, trauma, and other psychoemotional stressors); consequently, INF-γ and or the stress-associated neuropeptide, substance P, is secreted which up-regulate MHC class I, β2 Microglobulin and is associated with induced down-regulation of the “IP guardians” which maintain IP in the HF, such as TGF-β1, α-MSH and IGF-1, as well as MIF (macrophages migrating inhibitory
factor). The immunologically compromised HF is then constantly subjected to auto reactive cytotoxic CD8+ T-cell attack, with CD8+, CD4+ and NK/NK T cells massively accumulating around the HF bulb (Paus et al 2005; Paus et al 2007; Peters et al. 2007; Ito et al 2008; Gilhar et al. 2012).

1.8.2.2 Bulge immune privilege:
Recent studies have disproved the previous belief that IP is limited to the anagen hair bulb by confirming constitutive gene and protein expression patterns that are in line with a relative IP being established also in the bulge in line areas of the HF (Morris et al 2004a; Ohyama et al 2006; Meyer et al 2008; Tiede et al. 2009). Most recently, this has also been confirmed for the bulge of murine vibrissae HFs (Bertolini et al. 2013).

Reduced histocompatibility expression of genes such as: H2-D2, H2L, H2-Q2 and β2 Microglobulin in the bulge label –retaining cells has been identified in the mouse and human bulge (Morris et al 2004a, Ohyama et al. 2006; Cotsarelis 2006; Meyer et al. 2008; Tiede et al. 2009). Bulge cells also show an up-regulation of (immunosuppressive) TGF-β2 mRNA. Furthermore, this evidence is supported by highly sensitive Immunohistochemical studies and quantitative immunohistomorphometry for various markers, which clearly show a substantial reduction of MHC class Ia, and MHC class II. Independently, the experiments revealed up-regulation of IDO (indoleamine 2,3’ dioxygenase), increased immunoreactivity of local immunosuppressant components (α-MSH, TGF-β2) as well as the bulge cells showing up-regulation of MIF (Morris et al 2004a, Ohyama et al. 2006; Cotsarelis 2006; Meyer et al. 2008; Tiede et al. 2009).

Feature of bulge IP include: 1) the up-regulation of cell surface glycoprotein CD200, which functions as an immunosuppressant to the immune system and is known as “no danger” (Rosenblum et al 2004; Meyer et al 2008). The CD200-CD200R complex has been confirmed to produce immune inhibitory signals to inhibit proinflammatory cytokines, APC activity and influence IDO production. 2) A specific feature of bulge IP is the up-regulation of non-classical MHC I b (i.e. HLA-E); both act as immunomodulators against immune attack and are likely to be expressed throughout the HF cycle, not only restricted to the anagen phase. 3) IP collapse in the bulge is preferentially induced by higher doses of proinflammatory stimulus.
(INF-γ), more than 10 times than that required to induce collapse in the HF bulb. (Meyer et al 2008). (Figure 1.5). 4) There is very suggestive evidence of bulge IP collapse in lesional LPP HFs (Harries et al. 2013).

**Figure 1.4 Immune privilege of hair follicle bulge:** Normal human anagen hair follicle bulge represents the expression of the immunoreactivity of the IP markers and locally generated immunosuppressant. (Meyer et al 2008)
# 1.9 Immunopathology of the hair follicle: Primary cicatricial alopecia (PCA)

Cicatricial alopecia (scarring, permanent alopecia) represents a group of disorders, the end result of which is permanent hair loss due to complete destruction of the hair follicle unit and replacement with fibrous tissue (Dawber and Fenton 1997).

Cicatricial alopecia is generally classified into two types. The first is primary cicatricial alopecia (PCA), in which the main target for the inflammatory destruction is the hair follicle. The other is secondary cicatricial alopecia, where the hair follicle (by-stander) is damaged as a result of destructive processes to the scalp skin, such as infiltrative processes (e.g. carcinoma, sarcadiosis), or trauma (e.g. burns, infection). Loss of follicular ostia in the scalp skin of primary scarring alopecia patients is considered to be a distinctive clinical feature. A notable histopathological feature is the formation of scar tissue, resulting from the replacement of the hair follicle with fibrous material (Figure1.1)(Ross et al, 2005; Trueb et al, 2009; Harries et al, 2009).

Recent developments in understanding the pathogenesis of primary cicatricial alopecia reveal that the epithelial hair follicle stem cells, which are located in the bulge region of the hair follicle, are lost. This is a primary cause of the irreversible hair damage (Harries et al 2009; Harries et al 2010a; Harries and Paus 2010iMcElwee 2008).

The concept of the bulge region destruction and loss of eHFSCs as a result of bulge IP collapse (Harries et al. 2013) is an important advance in the understanding of PCA. These findings may also encourage clinicians and stem cell biologists to focus on PCA as a model system to explain how inflammatory disorders and disruption of the immune system may impact on stem cells. This may well allow researchers to obtain essential pointers to the future prevention of stem cell depletion and to generate new management strategies for PCA. Therefore, it is important to further characterize the steps in the immunopathology of model PCAs, like LPP, that lead to bulge IP collapse and to dissect the specific contributions of other, as yet insufficiently examined immunocyte populations in LPP pathogenesis, such as mast cells, macrophages, and gamma-delta T cells.
Figure 1.5 Diagnostic signs of alopecia: (a) Visible follicular ostia in alopecia areata (b) Histological features of the non-scarring alopecia (c) Loss of follicular Ostia in cicatricial alopecia (d) Histology of primary cicatricial alopecia (PCA). Clinical images photographed by Omima Eruk (Dermatology Hospital, Libya, 2005). Histological images reproduced from Harries & Paus (2010).
1.9.1 Clinical and histological classification of PCA

The clinical classification of the primary cicatricial alopecia, as proposed by the North American Hair Research society, depends on the predominant type of inflammatory cells: lymphocytic, neutrophilic or mixed, with subsequent sub-classification according to clinical features (Table 1.3 & Table 1.4). All have in common the loss of the follicular ostia or orifices in areas where alopecia is occurring and usually come with manifestations of cutaneous inflammation, for instance perifollicular scales, erythema, telange, which result in epidermal atrophy (Hordinsky et al 2008).

Table 1.3 The North American Hair Research Society (NAHRS) classification of primary cicatricial alopecia (PCA)(Olsen et al 2003)

<table>
<thead>
<tr>
<th>Primary scarring alopecia according to the proposed working classification of primary cicatricial alopecia</th>
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<tbody>
<tr>
<td>1. Lymphocyte- associated primary cicatricial alopecia:</td>
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<tr>
<td>1.1 Lichen planopilaris (Fig 3)</td>
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<tr>
<td>• Classic planopilaris</td>
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<tr>
<td>• Frontal fibrosing alopecia</td>
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<td>• Graham Little syndrome</td>
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<td>1.2 Chronic cutaneous lupus erythematosus (Fig 5)</td>
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<tr>
<td>1.3 Pseudoplaide of Brocq (PB)</td>
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<tr>
<td>1.4 Central centrifugal cicatricial alopecia (CCCLE)</td>
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<td>1.5 Keratosis follicularis spinulosa decalvans (KFSD) (Fig4)</td>
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<td>1.6 Alopecia mucinosa (AM)</td>
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<td>2. Neutrophil- associated primary cicatricial alopecia</td>
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<tr>
<td>2.1 Folliculitis decalvens (FD)</td>
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<td>2.2 Dissecting cellulitis of the scalp (DCS)</td>
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<tr>
<td>3. Mixed inflammatory primary cicatricial alopecia</td>
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<tr>
<td>3.1 Acne keloidalis nuchae (AKN)</td>
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<td>3.2 Erosive pustular dermatosis</td>
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<tr>
<td>3.3 Acne necrotica varioliform (ANV)</td>
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<td>4. Nonspecific primary cicatricial alopecia</td>
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</table>
Table 1.4 Clinical and histological features of PCA:

Clinical and histopathological features of each PCA subtype (defined using the NAHRS classification system) are summarised in table 1.4

Table from Harries; (Harries et al 2009), adapted from Sellheyer and Bergfeld (Sellheyer and Bergfeld 2006).

Omima Eruk took all clinical photos at Dermatology Hospital-Libya, and Dr M Harries has taken Histological photos.

<table>
<thead>
<tr>
<th>Lymphocytic group</th>
<th>LPP</th>
<th>CCLE</th>
<th>PB</th>
<th>CCCA</th>
<th>KFSD</th>
<th>AM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical features</strong></td>
<td>Central scalp lesion: hyperkeratotic follicularis; papules surrounded by erythematic margin; characterised by multifocal pattern. LPP may exist with KFP in eyebrow, and/or axilla, and extend to the trunk (Ross et al 2005; Hordinsky 2008; Al-refu et al 2009).</td>
<td>Women &gt;Men Central scalp discoid lesion: chronic erythema; scaly papules; follicular plugging; scar formation; loss of follicular ostia; depigmentation (Ross et al 2005; Hordinsky 2008; Al-Refue et al 2009).</td>
<td>Women&gt;Men Recognised by non-inflammatory signs and by slow progressive destruction of the HF in early stages; small patches of scarring alopecia called “footprints in the snow” in advanced stages (Moretti et al 2004; Sellheyer et al 2006).</td>
<td>Common in African-American women: starting centrally over the vertex and progressing outwards centrifugally; deficiency of PPAR-Ƴ recently suggested (Mirmirani et al 2009; Summer et al 2011).</td>
<td>Onset in childhood: X-linked inherited disorder characterised by follicular keratotic papules starting in face, eyelashes, eyebrow and scalp. KFSD may exist with corneal dystrophy, blepharitis, photophobia and palmo-plantopilaris (Baden et al 1994).</td>
<td>Affecting men &amp; women equally: erythematous patches and boggy plaques (Whiting 2001).</td>
</tr>
<tr>
<td><strong>Histological features</strong></td>
<td>Lymphocytic infiltration in infundibulum and isthmus; interface dermatitis (lichened reaction) - this accompanied by destruction of SG and replaced with fibrous tissue (Whiting et al 2001).</td>
<td>Lymphocyte cells infiltrate: infundibulum and isthmus; interface dermatitis: vacuolar immunofluorescence: granular deposition of IgG and C3 at the dermoepidermal junction (Whiting 2001).</td>
<td>Significant lymphocytic infiltration around infundibulum and mid-portion of the HF; scar formation in the late stage of CA(Whiting 2001).</td>
<td>Lymphocytes predominate infiltration; no specific interface changes; early loss of SG (Sperling et al 2009).</td>
<td>Lymphocytic infiltration are recognised as being replaced later by neutrophilic cells around infundibulum, and isthmus associated with hypergranulosis (Baden et al 1994; Klaus et al 2006).</td>
<td>Diagnostic sign; deposit of mucin in ORS; perifollicular &amp; intra-follicular lymphocytic infiltration (Whiting 2001; Sellheyer et al 2006).</td>
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<tr>
<td>Histopathological appearance</td>
<td>Clinical appearance</td>
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<td><img src="image1" alt="Histopathological Image 1" /></td>
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<td><img src="image5" alt="Histopathological Image 3" /></td>
<td><img src="image6" alt="Clinical Image 3" /></td>
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<td><img src="image11" alt="Histopathological Image 6" /></td>
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<tr>
<td>Neutrophilic and mixed group</td>
<td>FD</td>
<td>DC</td>
<td>AKN</td>
<td>EPDS</td>
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<tr>
<td><strong>Clinical features</strong></td>
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<tr>
<td>Adults</td>
<td>Adults</td>
<td>Black male</td>
<td>African-American men</td>
<td>Rare form of cicatricial alopecia</td>
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<tr>
<td>Irregular patches of alopecia surrounded by crops of painful pustules, thickened hypertrophic scarring and hair tufting (Whiting et al 2001).</td>
<td>DC is one of 3 diseases making up the follicular occlusion triad, along with acne conglobate and hydradenitis suppurative. Characterised by linear plaques of abscess, sinus tract and spontaneous drainage occurs resulting in keloid formation (Wollina et al 2012).</td>
<td>Usually starts in occipital scalp and may extend to the neck; characterised by papules and follicular pustules that change later to fibrous tissue to form keloid (Whiting et al 2001; Khumalo et al 2011).</td>
<td>Distinguished by sterile pustules and crusted lesion on different parts of the scalp. Predisposing factors: sun damage, trauma and skin grafting (Mastroianni et al 2005; Harries et al 2009). Strong association with increased risk of secondary carcinoma (Lovell et al 1980).</td>
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</table>
### Histological features

| | Perifollicular and intrafollicular neutrophilic, infiltration lymphohistocytic lymphohistocytic and plasma cells also identified in later stages. Abscess formed in the dermis; no sinus tracts; variable dermal fibrosis and follicular plugging (Powel et al 1999; Mihaljevic et al 2012). | Follicular ostium is blocked by follicular hyperkeratosis; associated with perifollicular neutrophilic, lymphocytic and histocytes infiltration (Sperling et al 2001). | Mixed inflammatory infiltration; lymphocytic and neutrophilic cells are located in perifollicular dermis and within follicular; infundibulum and isthmus, leads to weakening of the follicular wall that induces rupture and abscess formation in the dermis; Hypertrophic scar formation is the diagnostic sign (Sperling et al 2001; Sperling et al 2000). | Lymphocytic and neutrophilic infiltration with plasma cells is seen in scalp biopsy of perifollicular dermis and within follicle, associated with loss of pilosebaesous unite which is replaced by fibrous tissue (Mastroianni et al 2005) |

### Treatment

**1.9.2 Lichen planopilaris**

Lichen planopilaris (LPP) is a relatively rare inflammatory hair loss disorder of unknown cause, in which the inflammatory cells infiltrate the scalp hair follicle, which then evolves into irreversible hair damage with fibrous tissue replacement, eventually developing cicatricial alopecia (Figure 1.6). While it may sometimes be triggered by trauma (Cho et al 2010; Griffin et al 2012; Harries 2013), convincing trigger factors are generally not evident. LPP has histopathological and immunohistopathological features that are very similar to lichen planus, sharing the same pattern of lichenoid inflammation (Harries et al. 2009; Harries and Paus 2010). LPP symptoms are a consequence of lymphocytic inflammatory infiltration of the diseased hair follicle. (Smith et al 2000; McElwee et al 2008; Harries et al 2013).

LPP is divided into three subtypes according to the clinical presentation (Table 1.4), these being: classic lichen planopilaris, Piccardi-Lassueur-Graham syndrome and frontal fibrosing alopecia, with all three displaying similar histopathological features. The clinical and histological features of classic lichen planopilaris are similar to those of discoid lupus erythematosus, clinically described as showing hyperkeratotic follicularis papules surrounded by the erythematic margin (Sellheyer et al 2006).

*Figure 1.6 Primary Cicatricial alopecia presented by lichen planopilaris (LPP).*
Photographed by Omima Eruk (Dermatology Hospital-Libya 2005)
Graham Little syndrome, also called Picardi-Lassueur-Graham syndrome, or Graham Little-Feldman syndrome, commonly affects the scalp causing scarring alopecia, but also affects the eyebrows, axilla, pubic hair and extends to the trunk and extremities, causing keratosis follicularis papules. Whose nature seems to be scarring (Whiting et al 2001; Ross et al 2005). Frontal fibrosing alopecia commonly affects women, starting in the frontal line and progressing backwards. This pattern of cicatricial alopecia may overlap with discoid lupus erythematosus.

**Histopathology**: LPP presents with lymphocytic inflammatory infiltration; lichenoid dermatitis is usually found in the dermo-epidermal junction, which prominently targets the infundibulum and isthmus (Whiting et al 2001). This is accompanied by complete loss of the sebaceous epithelium in the early stages, and no mucin deposition in the dermis and epidermis; later the destruction of HF's has been identified, which replaced by a fibrous tract instead (Sellheyer et al 2006).

**Differential diagnosis**: Early onset LPP is hardly distinguishable from the early stages of Lupus erythematosus, Pseudoplate of Brocq, folliculitis decalvans, keratosis follicularis spinulosa de calvar and alopecia mucinosa. (Whiting et al 2001; Ross et al 2005)

**Treatment**: Topical and oral steroids, intralesional corticosteroids such as a triamcinolone acetonide injection employed in active lesions; hydroxychloroquine and cyclosporine are also known to be effective (Whiting et al 2001).

### 1.10 Pathogenesis of PCA, with emphasis on LPP

#### 1.10.1 General principles of inflammatory PCA pathobiology

Despite a great deal of research being done to investigate the underlying mechanisms of primary cicatricial alopecia, the primary cause is still not clear. However, several pathogenic factors are considered to be new insights into the etiopathogenesis of PCA (McELwee et al 2009, Harries and Paus 2010, Ohyama 2012 (Figure 1.7).

The first question to pose is whether the damage to the bulge region is the primary factor in the development of cicatricial alopecia. Cicatricial alopecia is distinguished from non-cicatricial alopecia by examining the location of inflammatory infiltration. It is hypothesized that inflammatory cells infiltrate the peri-and interfollicular bulb in alopecia areata, in
contrast to scarring alopecia, where the inflammatory cells are predominately found in the distal, non-cycling region of the hair follicle, where the epithelial stem cells are resident. Consequently, damage of the stem cells is associated with permanent hair loss and scar formation (McEwlee et al 2008).

Histological studies have suggested the mechanisms for damage to the bulge region to take place in the early active stage, the hair follicle, particularly in the bulge region, is affected by lymphocytic infiltration (Mobini et al 2005; Harries et al. 2013), whereas the bulb, (dermal papilla), is not affected by inflammatory infiltration. In later (fibrotic) stages, both the HF and sebaceous glands are replaced by fibrous tissue, resulting in the destruction of the pilosebaceous unit and damaging of the bulge stem cells which are considered to be essential cells in HF regeneration (Harries et al. 2009, 2013).

1.10.1.1 Inflammatory damage to hair follicle epithelium stem cells in LPP

Inflammatory infiltrates in LPP are composed predominantly of CD8+ T lymphocytes, resulting in loss of Ki-67 positive transit amplifying cells (TACs) (Mobini et al. 2005). These cells are generated from eHFSCs, so the disappearance of [TACs] is indicative of stem cell damage and CD8-rich inflammatory infiltration, and support the concept that CD8+ cytotoxic T-cell mediated destruction of the bulge region in the pathogenesis of LPP (Pozdnyakova et al 2008; Harries et al. 2013). That the CK15 gene and protein expression in lesional LPP bulges is also reduced (Harries et al. 2013), further supports the concept that the high infiltration of CD8+ lymphocytes in association with bulge IP collapse destroys eHFSCs and their progeny in LPP (Al-Refu et al 2008, Harries 2013).

1.10.1.2 Programmed organ deletion

Programmed organ deletion (POD) is a basic physiological process responsible for tissue remodelling through the removal and destruction of malfunctioning HFs. Programmed organ deletion is commonly seen during frog metamorphosis and also during embryogenesis in mammals (Eichmüller et al. 1998). Regular perifollicular inflammatory cluster cells (PICC), found around the bulge region of the normal back skin of adolescent mice, were present in 1-2% of all HF examined (Eichmüller et al. 1998). The predominant cells of the PICC, activated macrophages, along with a few CD4+ T-cells, aggregate around the bulge, thus
inducing degenerative phenomena such as ectopic MHC class II expression, basement membrane thickening, and ORS keratinocyte apoptosis ORS; all of these eventually leads to HF destruction and replacement by fibrotic tissue, which was detected in 10% of PICC+ HFs. Therefore, POD may be a physiological pathway for the targeted removal of malfunctioning HFs. It has been proposed that physiological POD turns into a pathological, PCA-promoting event when it becomes excessive (Harries and Paus 2010).

1.10.2 Precipitating and predisposing factors in LPP
Neutrophilic types of PCA, such as folliculitis decalvans, present with crops of pustules. The presence of staphylococcus aurous cultured from alopecic lesions has been noted (Bogg 1963; Powell et al 1999; Powell and Dawber 2001), which secretes staphylococcus enterotoxin, a bacterial superantigen. This results in massive activation and cytokine release from T-cells, including interleukin 2 (IL-2). As a consequence, abnormal complexes of superantigen and major histocompatibility (MHC II) receptors may stimulate T-cells (Marrack and Kappler 1990), and promote inflammatory responses (Brook and Griffiths 2001).

Traumatic trigger factors may underlie the development of another form of PCA, central centrifugal cicatricial alopecia, which includes three variants: 1) hot comb alopecia 2) follicular degeneration syndrome and 3) central elliptical pseudoplate in white women. CCCA is slowly progressive scarring hair loss, usually beginning at the crown and advancing to the surrounding areas (Olsen et al 2008; Summers et al 2011), where the hair is replaced by fibrous tissue. Moreover, erosive pustular dermatitis of the scalp is involved in the pathogenesis of lesional PCA, and often develops following trauma or surgery (Summers et al 2011).

Several genetic disorders directly cause PCA, one of which is keratosis pilaris atropicans. This includes many forms of keratosis pilaris, with cicatrical alopecia types including keratosis pilaris atropicans faciei, atrophoderma vermiculatum, keratosis follicularis spinulosa decalvans and ichthyosis follicularis (Arnold et al 2006).
1.10.3 Potential role of the sebaceous gland in LPP
Mice displaying spontaneous autosomal mutation, asebia (Scd1ab) have been proposed to offer an excellent model for PCA as display scarring alopecia phenotype (Zheng et al 1999; Stenn et al 2001) since these animals show scanty or absent hair, with fibrous tissue replacing the hair follicle in the area of scarring alopecia, and absence of sebaceous glands. One of the main enzymes of the sebaceous gland is steroyl COA desaturase, which is important in lipogenesis in both the sebaceous glands and adipose tissue. Defects in steroyl COA desaturase results in sebaceous gland hypoplasia, regression of the hair shaft and enhanced inflammatory infiltration. In addition, hair shafts adhere to the inner root sheath and eventually damage to the sebaceous gland leads to complete destruction of the hair follicle (Stenn et al. 2001; Al-Zaid et al 2011). This have invited the hypothesis that sebaceous gland damage is the primary defect in PCA, followed subsequently by inflammatory infiltration as a secondary event, which eventually leads to scarring alopecia via destruction of the bulge (for discussion, see Harries and Paus 2010).

1.10.4 PPAR gamma defect in LPP
Another potential mechanism for the etiopathogenesis of PCA arises from evidence of the dysfunction of lipid biogenesis and peroxisome metabolism, which is essential for HF maintenance (Karnik et al. 2009b, Harries and Paus 2009a). According to recent studies who analysed gene expression in LPP patients, a defect in peroxisome proliferative activated receptor gamma (PPARγ) function was recognized, which is expected to result in defects in lipid metabolism and peroxisome biosynthesis, thus causing pilosebaceous unit destruction (Karnik et al. 2009; Mirmirani and Karnik 2009). This is thought to induce a proinflammatory signalling milieu of lipid-mediated programmed cell death (Lipoapoptosis). Histological observation of LPP sections revealed that lesional skin exhibits hyperkeratosis and defects of the sebaceous gland, perfollicular lymphocytic infiltration, and perfollicular fibrosis accompanied by lipid accumulation around the follicle, which lead to HF damage (Karnik et al. 2009). A recent study treated one therapy-resistant LPP patient with an oral PPARγ agonist (pioglitazone hydrochloride) over the course of several months, resulting in a notable decrease in inflammation and rapid improvement of clinical symptoms (Mirmirani and Karnik 2009).
Figure 1.7 several pathogenic factors that may be involved in hypotheses for the development of primary cicatricial alopecia. (Ohyama 2012, Harries and Paus 2010)
1.11 Morphological and molecular characteristics of epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a distinct phenotypic pattern, which includes up-regulation of mesenchymal markers such as vimentin, fibronectin and N-Cadherin in epithelial cells (Shook and Keller 2003; Thiery et al 2009). It has also been observed that the expression of extracellular matrix remodelling enzymes (i.e. matrix metalloproteinase) is altered. EMT is instigated by molecular and cellular mechanisms described in Figure 1.8). In addition, a down-regulation of epithelial markers such as E-cadherin (CDH1), which plays a key role in epithelial homeostasis and cell-cell adhesion, is seen, accompanied by a loss of apical-basal cell polarity. CDH1 loss may also reduce the expression of other epithelial markers such as tight junctions and desmosomal proteins (Thiery et al 2009; Kalluri and Weinberg 2009; Moreno-Bueno et al 2009; Nakamura and Tokura 2011).

Various transcription factors (e.g; SNAIL1, SNAIL2, TWIST1) have been identified as (EMT) inducers, which in turn are induced by several types of factors such as TGF-β, EGF, Wnt-β, and Catenin. This leads to loss of an essential epithelial marker (E-cadherin, CDH1) and the acquisition of a functionally and morphologically mesenchymal phenotype, thus executing the epithelial-mesenchymal transition process (Kalluri and Neilson 2003; Vega et al 2004; Liu et al 2010; Lee et al 2011; Lee et al 2012).
Epithelial mesenchymal transition (EMT)

Figure 1.8 Epithelial mesenchymal transition mechanism: the transcription factors (SNAIL 1, SNAIL2, and TWIST) are expressed by generating signals from EMT inducers such as TGF-β, EGF, Wnt-β and β-catenin in the epithelial cells, which will lead to loss of E-cadherin as an epithelial marker and increased expression of N-cadherin, vimentin and fibronectin as mesenchymal markers, to acquire the function and morphology of the mesenchymal phenotypic pattern. (Schematic summary: Omima Eruk).

It is yet to be ascertained whether EMT is associated with immune privilege collapse in lesional LPP hair follicles. However, it is known that EMT plays a role in a number of different processes, including: 1) wound healing, 2) skin morphogenesis, 3) skin malignancy, 4) and cutaneous fibrosis (Figure 1.9) (Nakamura and Tokura 2011).
Figure 1.9 Model to describe the biological subtypes of EMT.

EMT has been demonstrated in different fibrotic disorders in the human body such as pulmonary fibrosis, renal fibrosis, and recently it has been demonstrated in patients with postmenopausal frontal fibrosing alopecia (FFA). The expression of SNAIL1, a transcriptional factor in the interfollicular dermis, is increased, suggesting a role for EMT in the FFA pathogenesis (Nakamura and Tokura 2010; Nakamura and Tokura 2011). Given that FFA may represent a variant of LPP (Harries et al. 2009), it is conceivable that EMT also plays a role in LPP-associated scarring.
1.12 Aims
This MPhil thesis project aimed to

1. Develop full understanding of human HF anatomy and physiology

2. Obtain training and acquire fundamental skills in human skin and HF histology and immunohistology. Establish an extended range of histochemical and immunohistological markers for analysing the immunopathogenesis of LPP in paraffin and cryosections

3. Learn how to quantitatively assess the difference in immunoreactivity in defined HF reference areas (quantitative immunohistomorphometry) for selected antigens

4. Obtain pilot data on mast cells and macrophage-related abnormalities in lesional LPP hair follicles versus normal scalp hair follicles.

5. Obtain pilot data on whether or not EMT of epithelial progenitor cells occurs in the bulge region of lesional LPP hair follicles.

The methodology chosen to meet these aims is delineated in the next chapter.
Chapter 2: METHODS AND MATERIALS
2.1 Literature search and analysis strategy
A major initial challenge of the current project was to choose suitable markers. The immune cell and EMT markers selected for the study had to be carefully selected, since for each of these markers a well-working immunostaining protocol had to be established, along with appropriate positive and negative controls. Extensive literature analysis suggested to opt for the markers explained below.

2.1.1 Selection strategy of optimal hair follicle immune cell markers

CD163: (also known as M130, MM130) This is a protein, which serves as a member of the scavenger receptor cystein-rich super family (SRCR) and is almost always expressed on monocytes/macrophages. It plays a role as an acute phase-regulated receptor, contributing to the clearance and endocytosis of haemoglobin/haptoglobin complexes and the restriction of oxidative damage which may occur in free haemoglobin; it is also involved in the uptake and recycling of iron through the endocytosis of haemoglobin-haptoglobin with its consequent debris of haem (Law et al 1993; Møller et al 2012). It is localised predominantly to the plasma membrane of macrophages, although weak to moderate cytoplasmic staining has been noted.

CD161: (known as killer cell lectin-like receptor super family member 1, KLRB). KLRB is a protein expressed on natural killer cells, and is involved in regulating NK cell cytotoxicity. Its activation is enhanced by T cell proliferation induced by CD3, and it is also involved in IFN\(\gamma\) production. Expression is localised to the cell membrane. (Litwin et al 1994; Cameron2002; Germain et al 2011).

V\(\alpha\)24: Natural killer T cells as an distinct subset of T lymphocyte that can express NK cell markers such as CD94, CD 161 and T cell receptor (TCR) with their both segments; Variable (V)\(\alpha\) and Junctional (J)\(\alpha\) segment. NK cells differentiate from NKT cell by lack of TCR expression (Balato et al 2009). NKT cells are restricted and activated by CD1d, which is expressed by antigen-presenting cells such as dendritic cells, instead TCR on NKT cells does not recognise the classical major histocompatibility complex type I and II that present peptide antigen. This results in the production of cytokines including IL-4, IL-13, IFNy and TNF\(\alpha\). However, the iNKT cells have cytolytic activity similar to NK cells; the prominent role of iNKT cells is considered to be as regulators in the immune system. Human V\(\alpha\)24 cells can
be classified phenotypically and functionally into two subtypes: CD4+ and CD4−, and their ratio plays a role in the immunoregulation of iNKT cells (Dellabona et al 1994; Godfrey et al 2000; Sandberg et al 2004).

**FOX P3**: (Known as Scurfin) is a protein involved in the regulation of the immune system, it has been shown that the function of FOX P3 is a master regulator in the proliferation and function of regulatory T cells; in addition it down-regulates the immune system (Cobbold et al 2006; Waldmann et al 2006) by employing the function of the control and development of other natural regulatory proteins such as CD4 and CD25 (Jang et al 2008). Expression is localised to the nucleus.

**Mast cell tryptase**: Mast cells have been identified as playing a major role in allergy and anaphylaxis; in this mechanism, the antigen interacts with the IgE on the Fc receptor on the mast cell in order to enhance the release of antimicrobial cytotoxic molecules from granules. This process is called ‘degranulation’, and preformed mediators include: histamine, proteoglycans and a distinct group of enzymes called serine proteases. A member of this family, known as mast cell tryptase (MCT) is used to identify mature and immature mast cells (Yamasaki S et al 2005; Prussin C et al 2003; Sugawara et al. 2012).

### 2.1.2 Selection strategy for EMT markers

**E-cadherin**: Epithelial cadherin is also known as CD324. The encoded protein CDH1 is a calcium-dependent cell-cell adhesion molecule and is involved in the regulation of such adhesion as well as the mobility and proliferation of epithelial cells. It also has a function as an invasive suppressor in the epithelial tissue. It is localised to the cell junctions associated with the cell membrane (Moreno-Bueno et al 2009).

**Vimentin**: a class-III intermediate filament (IF), it is preferentially expressed in non-epithelial cells, particularly the mesenchymal cells in the connective tissue. Previous studies have demonstrated the specific functions of the vimentin and its role in different processes such as organogenesis (Guarino, 1995; Hay, 1995; Gilles et al 1999), wound healing (SundarRaj et al., 1992; Gilles et al 1999) and tumour invasion (Ramaekers et al., 1983; Savagner et al 1994; Guarino, 1995; Gilles and Thompson, 1996; Gilles et al 1996a; Gilles et al 1996b; Gilles et al 1999). Vimentin also is very prominently expressed in fibroblasts (Iwano et al., 2002; Moreno-Bueno et al 2009; Nakamura and Tokura 2011).
2.2 Buffers and Reagents

Buffers

TBS
Tris...........6g
NaCl........43.8g
DH2O........5L
pH 7.5

EDTA buffer
EDTA.........0.37g
DH2O.........1L
Tween20......0.5ml
pH 8.0.

Citrate buffer
Citrate.......2.94g
DH2O.........1L
Tween 20......0.5ml
PH 6.0.

1. Blocks

1% Block
Bovine Serum Albumin (BSA) ......50mg
TBS ..........5ml

3% block
Gout Serum ......150μl
BSA ..............150mg
TBS .............5ml

0.6% hydrogen peroxidase

30% H2O2........180μl
Methanol........10ml

1% Hydrogen Peroxidase
30 % H2O2......1ml

D H2O..............29ml

0.1% Triton-X 100 in PBS
PBS (10X)...100μl

D H2O..900μl

Triton –X 100...1ml

Trypsin Stock Solution (0.5% in Distilled Water)
Trypsin...............50 mg
Distilled water.... 10 ml
Mix to dissolve and Store at -20 ºC.

Calcium Chloride Stock Solution (1%):
Calcium chloride ...0.1 g
Distilled water..... 10 ml
Mix well and store at 4 ºC.

Trypsin Working Solution (0.05%):
Trypsin stock solution (0.5%) ......1 ml
Calcium chloride stock solution 1%.....1 ml
Distilled Water ......8 ml
PH to 7.
Store at 4 ºC for one month or -20 ºC for long term storage
Reagents

Vectastain® ABC reagent

1 drop (50mcl) Solution A
1 drop (50mcl) Solution B
Add one drop of each to 5ml of the buffer
Stand for 30 minutes prior to use

Dako 3, 3-diaminobenzidine (DAB)

1 drop of the dilution buffer
1-drop stock (DAB)
2.3 Human tissue sources
Dr Harries, in 2008 collected all samples from LPP patients, after informed patient consent and ethics approval. The consultation, assessment and diagnosis were taking place at Salford Royal Foundation Trust, Pontefract General Hospital or Macclesfield District General Hospital. The clinical diagnosis of LPP was confirmed histologically (see details, see Harries et al. 2013)

All samples were stored and filed in the Manchester Biobank, Inst. of Inflammation and Repair, and processed in the Stopford Building, The University of Manchester.

2.4 Sample Processing:
All lesional hair follicle samples were collected from volunteers with scarring alopecia, in accordance with the NAHRS classification system for PCA (Olsen et al 2003)(see above). Therefore, all samples were obtained from lichen planopilaris (LPP), as they were the commonest type of PCA. Following identification of the patients, clinical and histopathological assessments were performed.

Biopsies were taken from active hair bearing lesional scalp skin; further biopsies were taken from clinically uninvolved (non-lesional) scalp skin. Some of these biopsies were placed in “optimal cutting temperature” (OCT) compound (Tissue-Tek™, Ted Pella Inc, CA, USA), snap frozen in liquid nitrogen then stored at -80°C. The rest of the biopsies were placed in 10% natural buffered formalin for paraffin embedding. Samples from healthy scalp skin have been used in this study; theses samples were taken from Manchester Biobank, The University of Manchester.

2.4.1 Tissue sectioning: Formalin fixed paraffin embedded (FFPE) tissue blocks were sectioned by inserting the block into the microtome chucks, placing a fresh blade on the microtome and setting the dial to cut 4 to 5um sections, picking up the section with a fine brush or a forceps to float the section on the surface of warm water (37°C); the floating section was picked up by new IHC slides. The slides were dried on a warm surface oven overnight at 65°C, checking the sections on a light microscope to ensure that the sections had taken properly before storing them at room temperature.
2.4.2 Deparaffinisation: A fundamental process, in which the paraffin sections were dewaxed before proceeding with IHC staining. The staining process was begun by loading the sections in the rack (slide holder); they were then deparaffinised in Xylene twice for 5 minutes each time, and dehydrated in a graded series of industrial methylated spirit (IMS): IMS 100% twice for 4 minutes, IMS 90% 2 minutes, IMS 70% for 2 minutes, and finally IMS 50% for 2 minutes, before finish washed in distilled water for 2 minutes.

2.4.3 Antigen retrieval: The crucial stage in IHC staining of formalin fixed paraffin embedded sections is antigen retrieval. The concept of antigen retrieval is to break the methylene bridges, which are formed during fixation, which cross-links proteins, thus masking antigenic sites of the tissue (Leong et al 1993; Henwood 1982; Henwood 2012).

There are two different techniques employed in order to expose the protein and allow the antibody to bind to the antigen of interest, enabling the antigen –antibody complex to form. These are heat induced epitope retrieval (HIER), and proteolytic induced epitope retrieval (PIER).

2.4.3.1 Heat induced epitope retrieval (HIER):

A. Microwave: A bath was filled with the recommended buffer as listed in the IHC staining protocol, i.e. EDTA (ethylenediaminetetraacetic acid) or Citrate acid (Sodium citrate): see appendix. The solution was then heated in a microwave at full power for 20 minutes in a non-sealed container. The slides were immersed in the boiling solution and left covered for a further 20 minutes to allow them to cool down before continuing with the IHC staining protocol.

B. Hot Plate: The appropriate antigen retrieval solution was brought to the boil using a hot plate inside a fume cupboard. Slides were then immersed in the boiling buffer (using forceps), boiled for 15 to 20 minutes, as recommended by the protocol, and were transferred to cold running tap water for 10 minutes, before continuing with the recommended IHC staining protocol.(NB. A third method, not used in the present project would be antigen retrieval by pressure cooker, which uses the same technique as
for hot plate retrieval. However, the pressure cooker needs to be sealed with a lid and removed at the end of the process.)

2.4.3.2 Proteolytic induced epitope retrieval (PIER)

There are numerous enzymes that may help to unmask the antigen, namely trypsin, proteinase K and pepsin:

A. Trypsin: Preparation of the working trypsin solution requires two stock solutions:

Trypsin stock solution: Trypsin was diluted in distilled water and stored at -20°C.

Calcium chloride stock solution (1%): Calcium chloride was added to distilled water, the solution can stay for longer at 4°C

Trypsin working solution (0.05%): equal amounts of the trypsin stock solution (0.5%) and the calcium chloride stock solution 1% are added to distilled water and the pH is adjusted to 7.8 (see appendix).

Procedure: The slides were covered with the stock solution and placed in the incubator for 15 minutes to warm up. The slides were then placed under cold water, and the IHC staining protocol was followed.

B. Proteinase K: Proteinase K 20mg/ml was diluted with PBS 1:1000; the solution was added to the tissue sections in sufficient amounts (around 70-100ul per section) and the slides were placed in a humidified chamber for 15 minutes at room temperature. The solution was removed by washing in the preferred buffer (TBS or PBS) and the immunostaining was performed.

2.5 Histological staining:

2.5.1 Haematoxylin and eosin staining (H&E):

The formalin fixed paraffin embedded tissue sections were routinely stained with haematoxylin and eosin stain. The sections were deparaffinised in Xylene twice for 5 minutes, then rehydrated by immersing the slides in a series of graded industrial methylated
spirits (IMS) sequentially: 100% IMS twice for 2 minutes, 90% IMS once for 2 minutes, 70% IMS once for 2 minutes, 50% IMS twice for 2 minutes and finally in running tap water for 1 minute. The slides were then stained in Meyer’s haematoxylin stain for 6 minutes and rinsed under running tap water for 2 minutes. The slides were differentiated in 0.2% acid/water for 20 seconds followed by running water for 1 minute. The slides were then “blued” in 37mM ammonia for 2 minutes followed by rinsing under running water for 1 minute. The sections were rinsed in 70% IMS for 1 minute, then counterstained in eosin for 1 minute. The sections were dehydrated in 70% and 100% IMS for 3 minutes and 20 seconds respectively, and cleared in 2 changes of xylene for 1 minute each. Finally the slides were permanently mounted in Pertex R mounting medium.

2.5.2 Toluidine blue staining protocol to identify mast cells:

The toluidine blue staining technique has been widely used to detect mast cells. The main components of mast cell granules are mucopolysaccharides and glycoaminoglycans. The toluidine blue stains the background blue (orthochromatic staining), and mast cells red – purple (metachromatic staining).

Paraffin embedded sections were dewaxed in Xylene twice for 5 minutes each change, followed immediately by rehydration in a series of graded industrial methylated spirits (IMS) for 2 minutes in each grade. The sections were stained in Toluidine blue working solution for 2-3 minutes. The stained sections were then washed with distilled water 3 times. The slides were dipped in 95% IMS and each dipped 10 times in 2 changes of 100% IMS until the stain faded. They were then cleared by immersion in 2 changes of Xylene for 3 minutes in each. Finally, the slides were mounted in Pertex R mounting medium and covered with a glass cover slip.
2.6 Immunohistochemical staining:

2.6.1 Formalin-fixed samples

2.6.1.1 Envision method – Horseradish peroxidase on formalin fixed paraffin embedded skin tissue FFPE (Dako- Hamburg, Germany):

The antigens of interest were localised on paraffin embedded skin tissue sections by using monoclonal mouse anti-human antibodies: CD163, FOX P3, mast cell tryptase and E-Cadherin. Following dewaxing and heating of the slides in the preferred buffer, the endogenous peroxide activity was blocked with the peroxidase block, (provided in the Envision kit) for 5 minutes; the primary antibody concentrations, dilutions and the durations are listed in table 2.1.

Sections were incubated for 30 minutes with the secondary antibody (labelled polymer conjugated to goat anti-mouse antibody HRP) (Envision kit, Dako), the stain was visualised by 3.3’ diaminobenzidine substrate and counterstained with Meyer’s haematoxylin.

N.B the CD163 stained sections had been washed with 0.2% Tween 20 in TBS and incubated with 3% hydrogen peroxidise in TBS before the antigen retrieval step.
**Table 2.1** Antibodies used on formalin fixed paraffin embedded tissue, employing the Envision kit HRP protocol (Dako-Hamburg, Germany)

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen retrieval buffer</th>
<th>Permeabilisation</th>
<th>Pre-incubation</th>
<th>Primary antibody titre/dilution</th>
<th>Secondary antibody</th>
<th>Dilution and duration</th>
<th>Chromogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD163</td>
<td>Citrate buffer Ph 6.0</td>
<td>No</td>
<td>3%H₂O₂ in TBS for 10 mins at RT</td>
<td>CD163 incubated for 1hr in closed chamber</td>
<td>Labelled Polymer anti-mouse conjugated HRP (Envision Kit)</td>
<td>One drop per section applied for 1hr at RT</td>
<td>DAB substrate for 10 min at RT</td>
</tr>
<tr>
<td>FOX P3</td>
<td>EDTA Ph9.0</td>
<td>No</td>
<td>Peroxidase block (0.3% H₂O₂ containing sodium azide (Dako))</td>
<td>1:100 Incubated for 30 mins at RT</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse Ig (Dako)</td>
<td>One drop per section for 30 mins at RT</td>
<td>DAB chromogen for 6 mins at RT</td>
</tr>
<tr>
<td>Protein</td>
<td>Buffer</td>
<td>Blocking</td>
<td>Antibody</td>
<td>Incubation</td>
<td>Labeling</td>
<td>DAB Time</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>----------</td>
<td>----------------</td>
<td>------------</td>
<td>---------------------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Mast cell tryptase</td>
<td>Citrate Ph6.0</td>
<td>No</td>
<td>0.3%H₂O₂ in PBS for 10 mins</td>
<td>1:100 diluted in 1% BSA, incubated for 1hr at RT</td>
<td>Labelled Polymer anti-mouse conjugated HRP</td>
<td>One drop per section for 30 mins at RT</td>
<td>DAB chromogen for 10 mins</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Citrate Ph6.0</td>
<td>No</td>
<td>Peroxidase block (0.3% H₂O₂ containing sodium azide (Dako))</td>
<td>1:25, incubated for 1hr at RT</td>
<td>Labelled polymer anti-rabbit conjugated HRP (Envision Kit)</td>
<td>One drop per section for 30 mins at RT</td>
<td>DAB chromogen for 4 mins</td>
</tr>
</tbody>
</table>
2.6.1.2 Vectastain-ABC peroxidase method

Immunohistochemical staining was carried out on formalin fixed paraffin embedded sections; normal human skin was used as a positive control for vimentin. Subsequent to the appropriate antigen retrieval the non specific binding was reduced by 1% bovine serum albumin for 25 minutes, then the endogenous peroxide activity was inhibited by 1% H2O2 in distilled water for 10 minutes. The sections were incubated with the primary antibody, (which was applied as shown in table 2.2), for 30 minutes in the incubator at 37°C. Next the secondary antibody, the HRP labelled polymer conjugated goat anti mouse antibody, was applied and the sections were incubated for 30 minutes at 37°C They were then washed with PBS 0.1% triton X-100 three times for 5 minutes. Avidin-Biotin peroxidase block was prepared in advance (30 minutes before application) and applied for 30 minutes. The bound antibody was visualised by DAB chromogen and the sections were counterstained with Meyer’s haematoxylin.
Table 2.2 Antibodies used on formalin fixed paraffin embedded tissue employing the Envision kit HRP protocol and the Vectastain ABC-Peroxidase kit (Vector Laboratories- Burlingame, Germany)

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Buffer/antigen retrieval</th>
<th>Permeabilisation</th>
<th>Pre-incubation</th>
<th>Block</th>
<th>Primary antibody titre and duration</th>
<th>Antibody diluents</th>
<th>Secondary antibody</th>
<th>Signal amplification</th>
<th>Chromogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>Citrate Buffer Ph6.0</td>
<td>0.1% triton-X100 in PBS</td>
<td>1% H$_2$O$_2$ in distilled water for 25 mins</td>
<td>1% BSA in PBS for 10 mins</td>
<td>1:500 Incubated for 30 mins at 37C</td>
<td>Dako Diluent ready to use</td>
<td>Labelled polymer anti mouse conjugated HRP (Envision Kit)</td>
<td>Peroxidase conjugated avidin0Biotin complex (Vectastain-Peroxidase Kit)</td>
<td>DAB chromogen for 4 mins at RT</td>
</tr>
</tbody>
</table>
2.6.2 Frozen samples:

Immunohistochemical staining was performed on frozen skin tissue as a positive control. Two techniques were employed for two different monoclonal antibodies: CD161 and Vα24.

2.6.2.1 ABC immunostaining method on frozen normal skin tissue (Vector Laboratories- Burlingame, Germany):

For CD161, following fixation in chilled acetone for 10 minutes, the sections were pre-incubated with 0.6% hydrogen peroxide in methanol for 30 minutes in a humidified chamber to block the endogenous peroxidase. The non-specific binding was reduced by applying normal horse serum, which was provided in the kit, for 30 minutes. Sections were incubated with primary and secondary antibodies as described in table 2.3. Avidin-Biotin peroxidase reagent was applied to the sections for 30 minutes and DAB chromogen was applied for 10 minutes.

2.6.2.2 Envision method – Horseradish peroxidase on frozen normal skin tissue (Dako- Hamburg, Germany):

For Vα24, subsequent to fixation in chilled acetone, endogenous peroxidase was blocked by 0.3% hydrogen peroxidise containing sodium azide, (Envision Kit, Dako), for 5 minutes. The sections were immunostained using Vα24 diluted with 1% BSA and applied for 30 minutes. This was followed by incubation with HRP labelled polymer conjugated goat anti-mouse antibody (Envision Kit) as a secondary antibody, which was also applied for 30 minutes. The staining was visualised by DAB chromogen for 3 minutes. The antibody concentrations, dilutions and the durations are listed in table 2.3 below:
### Table 2.3 Antibodies used on frozen samples with two different kits; ABC-peroxidase and Envision HRP kit.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Fixation</th>
<th>Pre-incubation</th>
<th>Block</th>
<th>Primary antibody titre and duration</th>
<th>Antibody diluent</th>
<th>Secondary antibody</th>
<th>Dilution and duration</th>
<th>Signal amplification</th>
<th>Chromogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD161 IHC</td>
<td>Acetone for 10 mins</td>
<td>0.6%H₂O₂ in methanol for 30 mins in humidified chamber</td>
<td>Normal horse serum Incubated for 30 mins (Vectastain-ABC kit) for 30 mins</td>
<td>1:40 incubated for 30 mins at RT</td>
<td>1% BSA</td>
<td>Goat antimouse secondary antibody 1:200 (Vectastain-ABC kit)</td>
<td>1:200 1%BSA incubated for 30 mins at RT</td>
<td>ABC-peroxidase (Avidin-Biotin) reagents for 30 mins</td>
<td>DAB chromogen applied for 10 mins at RT</td>
</tr>
<tr>
<td>Va24</td>
<td>Acetone for 10 mins</td>
<td>Peroxidase block 0.3%H₂O₂ containing sodium azide (Dako)</td>
<td>No</td>
<td>1:100 Applied for 30 mins at RT</td>
<td>1% BSA</td>
<td>Labelled polymer anti-mouse conjugated HRP (Envision Kit)</td>
<td>One drop per section incubated for 30 mins at RT</td>
<td>HRP Polymer</td>
<td>DAB Chromogen for 5 mins</td>
</tr>
</tbody>
</table>
2.7 Real time – quantitative polymerase chain reaction (qPCR)

RNA was extracted from tissue sections by laser capture microdissection. The amplification system, which was used to amplify the very small quantities of RNA obtained, was the Ovation™ Pico WTA amplification system (NuGEN™ Technologies Inc., San Carlos, Ca), which is used to amplify RNA to microgram amounts of complementary DNA (cDNA).

The next step was purification of the amplified cDNA. This step was achieved by using a QIAquick® PCR purification kit (Qiagen Ltd). In addition, photospectometry analysis was used to verify the purity and quantity of cDNA, a technique adopted from Harries 2011.

Dr Matt J Harries had performed all the RNA extraction, amplification and purification of complementary DNA (for details, see Harries et al. 2013).

q-PCR analysis was performed by using Human TaqMan® gene expression assays and the StepOne Plus™ Real-Time PCR system (Applied Biosystems, Warrington, UK).

The relative qualification was to compare un-lesional with lesional hair follicles. q-PCR reaction was performed as described previously (Harries et al. 2013). The total volume of the reaction was 20ul, containing 2ul cDNA (5ng μl-1 cDNA), 7ul RNase-free water, 10ul of TaqMan universal fast PCR (2X) and 1μl (20X) TaqMan® gene expression assay. The samples were run in triplicate reactions to detect the expression relative to standard volumes obtained from the housekeeping gene peptidyl prolyl isomerase (PPIA) TaqMan® Human Endogenous Control Hs99999904_m1, RefSeq: NM_021130.3, amplicon length 98 bp). The q-PCR protocol was performed as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles of 15 minutes at 95°C s followed by 1 minute at 60°C.

The analysis was carried out using inventoried gene expression assays validation (Applied Biosystems, Warrington, UK), and by using the ΔΔCT method, as shown below:

\[
\Delta CT = Ct \text{ gene of interest} - Ct \text{ reference gene}
\]

\[
\Delta\Delta CT = (Ct \text{ gene of interest} - Ct \text{ reference gene}) \text{ target} - (Ct \text{ gene of interest} - Ct \text{ reference gene}) \text{ calibrator}
\]
Relative quantification = $2^{-\Delta\Delta CT}$

[Reference gene = PPIA; target = lesional samples; calibrator = non-lesional samples]

### 2.8 Hair follicle compartment and bulge identification

Identification of the hair follicle compartments has already been accomplished in previous work using quantitative immunohistomorphometry, as described below (Christoph et al 2000). The immunoreactivity pattern was based on quantitating positively stained cells per section of the HF. This identification approach is precisely designed to calculate the mean score of each individual region of intact HFs obtained from different patients.

To generate accurate results, sufficiently reliable areas of the sections are placed precisely in the centre of the microscope and are vertically oriented to provide a good section for counting (figure 2.1). For estimation of the location in the defined reference region of the HF, the standardised high power field- HPF (500μm vertical plane; Keyence BZ8000 microscope; x20 lens; x13 optical zoom) has been used to determine and measure the compartments of the hair follicle.

The ultimate target of this approach is the analysis of the immunoreactivity pattern of positively stained cells by the provision of a clear criterion obtained by using HPF (the Keyence BZ8000 microscope). The primary regions of the hair follicle analysed were: 1) the epithelial hair follicle compartment, and 2) the perifollicular mesenchyme, which includes the connective tissue sheath (CTS).

#### 2.8.1 Epithelial hair follicle compartment:

The evaluation of defined hair follicle compartments has previously been described by Harries (2011), who provides guidelines for precisely determining the location of each of these compartments, according to their particular placement within the microscope field: (see table 2.4).
Table 2.4 Microscopic field placements for analysis: using the Keyence microscope for analysing the defined reference region in the hair follicle.

<table>
<thead>
<tr>
<th>Hair follicle epithelial compartment</th>
<th>Position of high power field (HPF) during analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td>Field centred over edge of follicular ostia. Epidermis was counted on each side of the follicular ostia.</td>
</tr>
<tr>
<td>Infundibulum</td>
<td>Inferior edge of field aligned level with the sebaceous gland duct opening.</td>
</tr>
<tr>
<td>Bulge</td>
<td>Field centred over arrector pili muscle (APM) insertion and inferior pole of the sebaceous gland.</td>
</tr>
<tr>
<td>Proximal Hair follicle</td>
<td>Size of proximal follicle very variable due to angle of sectioning, etc. Therefore, fields were randomly and (where possible) multiply positioned over the proximal follicle with values averaged. Care was taken not to encroach on the bulge region (distally) or bulb (proximally).</td>
</tr>
<tr>
<td>Bulb</td>
<td>Field centred over the hair bulb matrix.</td>
</tr>
</tbody>
</table>

The number of intact HF s per section varied from patient to patient, generally only one or two sections per patient were found to include an intact hair follicle. The sections were derived from different patients. Only identifiable intact hair follicles in the section were counted and included in analysis.
**Fig 2.1 Hair follicle epithelial compartments.** Defined hair follicle epithelial compartments are displayed in the boxes (Epidermis, Infundibulum, Bulge region, Proximal follicle and Bulb). Identified areas used for immune cell counts and immunohistomorphometric analysis. (X20 lens; x13 optical zoom, vimentin+ cells).
2.8.2 Perifollicular mesenchyme compartment (connective tissue sheath):

The identification of positive immunoreactivity was performed blindly. Within the CTS, positive staining was quantified at two different distances: 30um and 100um. Both distances were measured from the epithelial basement membrane outwards to the CTS (Harries 2011). The size of the normal hair follicle CTS is 30 um from the basement membrane, hence it was chosen as a standard distance measurement. As peri-follicular fibrosis often occurs in lesional hair follicles, the distance of CTS from basement membrane increases, an additional measurement of 100um was included to account for these changes.

![Perifollicular mesenchyme analysis](image)

**Figure 2.2 Perifollicular mesenchyme analysis.** Both a 30µm (green) and 100µm (red) peri-follicular zone measured on both sides of the follicle to identify the hair follicle connective tissue sheath (CTS; 30 µm) and peri-follicular mesenchyme in each defined compartment. [APM = arrector pili muscle; IRS=Inner root sheath; ORS = outer root sheath]
2.8.3 Identifying the bulge region in the hair follicle

Identification of the bulge region in the hair follicle has been demonstrated by using two distinct landmark criteria: morphological landmarks and immunohistological landmarks, (shown in the table 2.5 and figure 2.3). Morphologically, the bulge region has been identified where the APM inserts into the ORS of the HF. More recently, the follicular trochanter has also been indicated as being an additional morphological landmark (Tiede et al 2007). The fact that the immunoreactivity pattern of K15 and CD200 act as an immunohistologically positive landmark criteria has also been firmly established. (by the same studies)

Table 2.5 Morphological and Immunohistochemical landmark criteria for identifying the bulge in human HF

<table>
<thead>
<tr>
<th>Morphological landmark</th>
<th>Immunohistochemical landmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrector pili muscle (APM): is inserted into the middle of</td>
<td>CD200 immunoreactivity pattern: is used as an epithelial hair follicle stem cell marker</td>
</tr>
<tr>
<td>the distal ORS of the human HF (Cotsarelis 2006; Cotsarelis</td>
<td>(Ohyama et al 2006)</td>
</tr>
<tr>
<td>2006a)</td>
<td></td>
</tr>
<tr>
<td>Follicular trochanter: is a protrusion which comes out from</td>
<td>Cytokeratin 15 Immunoreactivity pattern: K15 is predominantly expressed in and out of the</td>
</tr>
<tr>
<td>ORS of the human HF (Tiede et al 2007)</td>
<td>ORS, which is used as a marker for the bulge region (Lyle et al 1998; Cotsarelis 2006; Cotsare</td>
</tr>
<tr>
<td></td>
<td>lis 2006a; Ohyama et al 2006)</td>
</tr>
</tbody>
</table>
Figure 2.3 Hair follicle bulge region identification: anatomical landmarks: (a) follicular trochanter (FT); (b) arrector pili muscle (APM) sebaceous gland (SG), hair follicle (HF), scale bar=30um).

2.8.4 Identifying the bulge region in a lesional HF

The above landmark criteria are restricted to the normal hair follicle, as certain structures of the HF (APM, SG, FT etc.), which normally serve as morphological markers to indicate the bulge, are lost during the disease process. In addition, the expressions of the IR of CD200 and/or K15, which are normally employed as distinctive bulge stem cell markers, are reduced in the early stages of inflammatory disease of the HF (LPP), which are normally employed as distinctive bulge stem cell markers.

2.8.5 Quantitative Immunohistomorphometry:

Semi-quantitative immunohistomorphometry was used in this project to evaluate the immunostaining intensity in the defined bulge area of the HF by using Image J software (National institute of health-NIH). To estimate the intensity, the representative digital image was uploaded onto the Image J software and by using the options provided in the Image J platform; the area that needed to be evaluated was highlighted as illustrated in (Figure 2.4) Preceding the calculation of the intensity by image J, the digital image was converted to a negative image and all of the values were saved to a separate spreadsheet (Harries 2011).
Figure 2.4 “Image J” semi-quantitative immunohistomorphometry: (a) image of bulge region up-loaded into “Image J” software for analysis; and (b) area highlighted using software drawing tool, followed by the image being inverted (shown as blue) before analysis is performed. (MHC I IHC staining) This photo from (Harries 2011)
Chapter 3: RESULTS
The first main aim of the current project was to select and establish a panel of histochemical, immunohistological read-out parameters for use in either FFPE or OCT-embedded cryopreserved tissue (for methodological details, see Chapter 2 and Tables 2.1, 2.2, 2.3).

3 Selection of optimally suited markers for studying hair follicle immunopathology, bulge immune privilege, and EMT

3.1 Regulatory T + cells
Tonsil, Spleen and other lymphoid tissue were immunolabeled as a positive control to identify Tregs +cells by using FOX P3 immunostaining (Carreras et al 2006). Unfortunately, the current project did not examine the Treg+ cells on lymphoid tissue, as they were not available on paraffin tissue. Based on the Tregs +cells distribution in the normal human skin tissue, FOX P3 has been previously used to investigate Tregs+ cells (DeBoer et al 2007), the present study determine the immunoreactivity of the FOX P3 positive Tregs cells on normal human skin tissue.

First, an immunostaining protocol for FOXP3+ Trges was established on paraffin section. The expression pattern of Tregs is identified by FOX P3 positive immunoreactivity, and was examined first by establishing IHC staining on healthy human skin sections (FFPE). Clear nuclear staining in the dermis of normal skin tissue was observed (Figure 3.1).
Figure 3.1 FOX P3+ cells in the normal human (healthy) skin tissue (FFPE): (a) no specific staining in negative section; FOX P3+ cells are localised in the dermis (b); (c) high power view of the dermis (Scale bar=50um)

3.2 Natural killer and Natural killer T cells
The distribution of NK and NKT cells in and around the human HF has as yet been incompletely characterized (Christoph et al. 2000, Ito et al. 2008, Petukhova et al. 2010). Previous literature has reported that NKT cells are very rarely seen around the HF, and where a few do occur, they would only be in MHC I+ cells on the distal ORS and CTS (Christoph et al 2000; Ito et al. 2008). CD56 has previously been used to demonstrate NK cells in normal and diseased human scalp skin. Very few CD65+ cells are mainly found in the perifollicular mesenchyme compartments, and there was no difference between lesional and control HFs (Christoph et al 2000; Ito et al. 2008; Harries et al. 2013). The present study has focused on different antigenic markers for NK & NKT cells that have not yet been examined. CD161 was selected as a marker for NK cells for the reasons outlined above (page 60). The immunostaining protocol established is only applicable to frozen tissue sections. As there was only access to FFPE LPP scalp skin sections, CD161 IR could not be tested in lesional and non-lesional LPP samples. In healthy scalp skin cryosections, CD161 expression was observed in the membrane of dermal cells, as shown in Figure 3.2.
Figure 3.2 CD161+ cells in FFPE sections of normal (healthy) human skin: (a) negative control; (b) it shows that (a) a few CD161+ cells are localised in the dermis; (c) high power view of the dermis to show the CD161+ cells (Scale bar= 50um).

NKT+ cells have not previously been mapped in the human HF. The Vα24 antibody is considered to be a specific marker for NKT cells (Dellabona et al 1994; Godfrey et al 2000; Sandberg et al 2004). Vα24+ cells were observed in one lesional LPP HF, but not in the normal (non-lesional) HFs. The IR pattern displayed Vα24 IR in the ORS and CTS of a lesional LPP HF (Figure 3.3). The monoclonal antibody tested was only effective on when cryosections were used.
Figure 3.3 V$\alpha$24+ cells expression in human hair follicle epithelium in (LPP) using immunohistochemistry on frozen samples: Increased number of V$\alpha$24+ cells in the ORS of a lesional LPP HF (b & c) compared with an uninvolved HF from the same patient (a). (Scale bar =30um, ORS= Outer root sheath, IRS-inner root sheath, HF= hair follicle, CTS= Connective tissue sheath)
3.3 Mast cell abnormalities in LPP

Previous research has demonstrated that both the total number and the degranulation of mast cells were increased around lesional HFs (LPP). Mast cells were clustered at high density in the perifollicular mesenchyme adjacent to the bulge and infundibulum (Harries et al. 2013). The mast cell immunoreactivity pattern for tryptase was examined in normal human skin (FFPE), with intact and degranulated mast cells being located in the dermis of human skin (Figure 3.4).

To double-check these findings, mature mast cell expression on FFPE lesional HF from LPP patients and HF from healthy scalp skin was examined in the present project, using mast cell histochemistry (toluidine blue). As expected, no TB+ mast cells were found in the HF epithelial (lesional, non-lesional, or healthy skin HFs). Instead, the number and degranulation of TB+ mast cells were increased around the bulge and in the CTS of lesional LPP HFs compared to HF from healthy scalp skin (Figure 3.5, Figure 3.6 and Figure 3.7).
Figure 3.4 Mast cell tryptase + cells in normal human skin tissue: (a) Negative section: MCT +cells are expressed in the dermis of the human normal skin tissue 20X. (b), and (c) show positive MCT cells in high power magnification 40X (MCT=mast cell tryptase).
Figure 3.5 Histological staining of LPP hair follicle using Toluidine blue: increased expression of MCT+ cells, both whole and degranulated, in the perifollicular mesenchymal tissue adjacent to the bulge region in LPP (c & d) compared with the HF from healthy scalp skin (a & b). Total mast cells indicated with solid arrow and degranulated highlighted with open arrow. (CTS=connective tissue sheath, ORS=outer root sheet, SG=sebaceous gland, MCT=Mast cell tryptase)
Figure 3.6 Mast cells tryptase +cells in the 30μm perifollicular mesenchyme compartments of LPP. Increased number of total mast cells in the (30μm) CTS adjacent to the infundibulum and bulge in LPP compared with control HF from healthy scalp skin (n.s= non significant +/- SD)
Mast cells in defined 100μm perifollicular mesenchyme compartments.

Figure 3.7 Mast cell tryptase +cells (MCT) in the 100μm perifollicular mesenchyme compartments of LPP. Increased number of total and degranulated mast cells in the (100μm) CTS adjacent to the infundibulum and bulge in LPP, compared with the HF from healthy scalp skin (P* = 0.0354, t-test +/- SD, n.s= non significant)

These data independently confirm the analyses of Harries et al. 2013 and demonstrate that a greater number of (activated) mast cells is found in the close vicinity of the HF’s stem cell zone (i.e. in the peri isthmus HF and skin mesenchyme). This raises the possibility that mast cells may participate in LPP pathogenesis.
3.4 Macrophage abnormalities in LPP
Macrophages with different immunophenotypes have been identified around the normal human HF. CD68 labels oxidative macrophage (Damoiseaux et al 1994; Juniantito et al 2012), MHC II has been found to be expressed on antigen presenting cells, some of which are macrophages (Picard et al 2008; Juniantito et al., 2011) CD163 is expressed on resident macrophages (Polfliet et al 2006; Juniantito et al 2012). The majority of macrophages are located in the perifollicular mesenchyme of the normal HF, from proximal to distal, but are completely absent in the HF epithelium. It has been shown that the number of CD68+ cells increases in the lesional HF, in contrast with the normal HF, where was no positive cells are expressed (Christoph et al 2000; Harries 2011)

This study attempts to identify an additional marker for macrophages. As such an anti-CD163 antibody (ED2) was used to identify CD163 cells. Interestingly, the occurrence of CD163+ cells was much less frequent in the CTS adjacent to the infundibulum and bulge of LPP HF compared to HF from healthy scalp skin (Figure 3.8-figure 3.10). This significant reduction in the number of resident CTS macrophages in LPP suggests that the majority of CTS macrophages got activated and switch into oxidative phenotype, which may explain the drop in the number of CD163+ CTS cells. Therefore, future work should dissect whether this putative switch in macrophage phenotype is important for LPP pathogenesis and whether it deserves to be targeted therapeutically.
Figure 3.8 CD163 + cells expression in the bulge epithelium of LPP hair follicle: Reduced expression of CD163 in both epithelium and perifollicular mesenchyme tissue of lesional HF (a) compared with HF from healthy scalp skin (b). (APM= arrector pili muscle, CTS= connective tissue sheet, ORS=outer root sheet, IRS=inner root sheath, scale bar=30um).
Figure 3.9 CD163 immune reactivity in defined 30 microns perifollicular mesenchymal compartments in LPP HF compared with HF from healthy scalp skin: down-regulation of CD163+ cell expression in the infundibulum and bulge perifollicular tissue (30μm), with virtual absence of positive cells in the epithelial hair of LPP, compared with HF from healthy scalp skin. (P*=0.038 (infundibulum mesenchyme) t-test +/- SD, n.s=non significant in bulge mesenchyme)
Figure 3.10 CD163+ve cells in defined 100 microns perifollicular mesenchymal compartments in LPP HF compared with HF from healthy scalp skin: down-regulation of CD163+ cell expression in the infundibulum (100μm) hair of LPP compared with HF from healthy scalp skin, with considerable increase in expression of CD163+cells in the bulge, proximal follicle and bulb. (n.s=non significant in bulge and infundibulum mesenchyme; t-test +/-SD)
3.5 Epithelial-mesenchymal transition (EMT) is a component of LPP pathobiology: Preliminary evidence

To assess the dramatic morphological and functional changes in the epithelial cells needed to acquire the fibrosing phenotype in PCA, the expression of transcription factors related to the process of epithelial-mesenchymal transition (EMT) had previously been investigated in skin biopsies from FFA patients. (Nakamura et al 2010, 2011) demonstrated that both SNAIL 1 & SNAIL2 expression is altered in these patients, whose form of PCA may be a variant of LPP (Harries et al. 2009, Racz et al. 2013, Dlova et al. 2013), suggesting that the EMT process has a potential role in the scarring process associated with PCAs, namely with LPP. However, some experts have recently disputed it whether FFA really represents a variant of LPP, rather than a distinct PCA entity (E. Poblet/F. Jimenez, personal communication). Therefore, the current project focused on evaluating evidence for the occurrence of EMT in LPP by examining the epithelial marker (E-cadherin) and the mesenchymal marker (vimentin) in LPP lesional HF s compared to HF from healthy scalp skin, at both the protein and gene levels.

This showed that vimentin+ cell numbers are increased in the lesional bulge epithelium and in the 30um reference area that was examined in the perifollicular mesenchyme compared with the HF from healthy scalp skin. While significance for the up-regulation of vimentin in the HF bulge epithelium was not reached, any expression of vimentin in this area is to be considered abnormal. Furthermore, in 3 patients out of 5, vimentin IR was noticed as a single ectopic infiltration in the bulge epithelium (Figures 3.11 and figure 3.12).

Moreover, attempts were made to examine the expression of E-cadherin in LPP HF s. Intense immunoreactivity for CDH1 was observed in healthy epidermal sections used as positive control tissue. Repeated staining of healthy scalp and LPP sections displayed much weaker immunoreactivity, which may indicate a technical issue with the protocol. Importantly, a significant difference in staining intensity was still observed between healthy scalp follicles and LPP follicles (Figure 3.13 and figure 3.14).
The IHC data strongly support the concept that the bulge and possibly other areas of the HF epithelium undergo EMT during the course of LPP development.

**Figure 3.11 Vimentin+ cells in the bulge epithelium and perifollicular mesenchyme adjacent to the bulge of LPP HF (30um):** increased expression of vimentin in the epithelium and CTS of the bulge in lesional LPP HFs compared with the HF from healthy scalp skin (APM=arrector pili muscle, ORS=Outer root sheath, scale bar=30um).
Figure 3.12 Vimentin+ cells in the bulge epithelium and perifollicular mesenchyme tissue adjacent to the bulge: total numbers of Vimentin+ cells are increased in the bulge epithelium and CTS of LPP hair compared with the HF from healthy scalp skin with a significant increase occurring in the CTS of LPP hair (Number per HPF)(P*< 0.05 t-test; +/-SD).
**Figure 3.13 E-Cadherin+ cells in the bulge epithelium of LPP HF:** increased expression of E-Cadherin +cells in the epithelium of the normal HF (a&b) and reduced CDH +cells expression in the epidermis(c) with absolute absence of CDH +cell in the HF (d).
Figure 3.14 E-cadherin immuneactivity (IR) in hair follicle bulge epithelium in LPP:

Significant Increased expression of E-Cadherin IR in hair follicle bulge epithelium in normal hair follicle comparing with LPP (P* < 0.001 paired t-test; +/- SD)
3.6 Q-PCR results

In order to compare the protein expression in situ data obtained by IHC with transcriptional evidence, quantitative real time PCR (q-RT-PCR) was carried out using a probe for the reference housekeeping gene peptidylprolyl isomerise A (PPIA) and using probesets for vimentin and E-cadherin. For this, mRNA was examined that had been extracted in the context of a previous study in the lab from the bulge region of lesional versus non-lesional HFs, using laser capture microdissection (see Harries et al. 2013 for details).

As shown in Fig. 3.15, quantitative RT-PCR demonstrated a trend towards a reduction of E-cadherin transcription in the bulge of lesional LPP HFs. Instead, there was a trend towards an up-regulation of vimentin mRNA expression in the bulge region of lesional HFs from LPP patients (Figure 3.14). However, likely due to the small sample size, significance was not reached in either case.

Nevertheless, combined, the current IHC and qRT-PCR results suggest that vimentin +cells are up-regulated in and around the bulge epithelium, at both protein and gene levels, accompanied by a relative decline in E-cadherin+ epithelial cells in the bulge. Taken together, this is very suggestive of pathological EMT actually occurring during LPP.
Figure 3.15 Q-PCR of Vimentin gene expression in the bulge epithelial of the lesional hair follicle: real time PCR in the bulge of the lesional HF is up regulated compared to the HF from healthy scalp skin [P > 0.05= non-significant]
Figure 3.16 Q-PCR of E-Cadherin gene expression in the bulge epithelial of the lesional hair follicle: real time PCR in the bulge of the lesional HF is up regulated compared to the HF from healthy scalp skin [P> 0.05=non significant]
Chapter 4: DISCUSSION
The current thesis project contributes to a better understanding of the as yet unclear immunopathogenesis of LPP in several respects:

- It has established an extended array of LPP immunopathology markers that have not yet been systematically applied to LPP research and that are now available for future related studies in the supervisor’s lab;
- it suggests that perifollicular mast cells and macrophages may play a more important and more complex role in the disease than previously appreciated;
- and it provides the first phenomenological evidence in support of the hypothesis that the extensive scarring in LPP may indeed result from pathological EMT in the epithelial stem cell zone of the HF, the bulge.

Moreover, from a personal growth perspective, this thesis has provided an excellent opportunity to establish a basic understanding of human HF anatomy, function, and immunology and to acquire fundamental methodological skills for investigating human hair follicle pathology. These skills were acquired and exercised by employing, photodocumenting, and critically evaluating immunostaining results with the help of appropriate positive and negative controls, and was complemented by quantitative immunohistomorphometry and statistical data analysis. These newly acquired techniques will serve as an invaluable asset for the candidate’s future activities in clinical research.

The systematic establishment of histochemical and immunohistological techniques for examining the inflammatory infiltrates of lesional versus non-lesional HFs in scalp skin biopsies from patients with LPP and HF from healthy scalp skin, served as an excellent testing ground for the challenges and problems one typically encounters when performing antigen detection on tissue sections, and for developing professional strategies how to overcome any obstacles in this respect (e.g. selecting optimally suited primary and secondary antibodies, dealing with non-specific background immunoreactivity and insufficiently strong immunoreactivity signals, selecting instructive positive and negative controls).
The evaluation of perifollicular mast cells and macrophages performed in the current project revealed an increased number of degranulated mast cells in the perifollicular mesenchyme of LPP HFs compared to controls. Instead, quite to the contrary of what had been expected, the number of resident (CD163+) macrophages in the HF’s connective tissue sheath is significantly reduced around LPP HFs compared to controls. Quantitative immunohistomorphometry also revealed that the epithelial cell-specific adhesion molecule, E-cadherin, is significantly reduced in the bulge area of lesional LPP HFs, while the mesenchyme-specific marker, vimentin, is up-regulated. We shall discuss the implications of this finding, which suggests that epithelial HF stem cells are being pushed into a fibroblastoid transformation during the course of LPP, further below.

Thus, the current project has further contributed to dissecting the immunopathogenesis and etiopathogenesis of LPP, as an essential basis for the development of novel, more effective therapeutic strategies for managing this permanently disfiguring form of PCA.

The experimental design of the present study was based on previous work in the supervisor’s lab, which the immune cellular composition was analysed in the normal human HF, with special reference to infiltrating inflammatory cells around normal and LPP-afflicted human scalp HFs (Christoph et al 2000; Meyer et al 2008; Harries 2010a). The main emphasis of previous work in the lab had been on the physiological immune privilege of the human HF’s stem cell zone, the bulge (Meyer et al. 2008), and its collapse in PCA (Harries et al. 2010a), namely in LPP and in the contribution that CD8+ T cells and interferon-gamma secretion may play in this bulge immune privilege collapse (Harries et al. 2013). Previously, work in the supervisor’s lab has focused on the role of immune privilege collapse in the bulge area of lesional LPP HFs. The interesting distribution pattern of FOXP3+ regulatory T cells, along with the reduced number of resident macrophages and the increased number of activated (degranulating) mast cells around lesional HFs that the current project has revealed now shifts the interest to these key players of innate immunity.

With a view to detecting the infiltration of Treg + Cells in the hair follicle compartments, particularly in the bulge region where the immune privilege is localised (Meyer et al. 2008, Harries et al. 2013), FOX P3+ cells were examined on FFPE normal skin tissue as a positive control, by using IHC staining (figure 3.1). Clear nuclear staining was identified in the dermis...
of the normal skin tissue. An increased expression of FOX P3 + cells in inflammatory skin disorders such as lichen planus and psoriasis has previously been demonstrated (Franz et al 2007). In addition, recently FOXP3+ cells have been observed in the bulge epithelium and perifollicular mesenchyme of LPP HFs, in contrast to non-lesional HFs, where, in striking contrast, FOX P3 was seen to be reduced in autoimmune diseases including: systemic lupus erythematosus (Miyara et al 2005; Lee et al 2006; Mellor-Pita et al 2006; Lyssuk et al 2007; Valencia et al. 2007; La cava 2008; Zhang et al 2008; Kuhn et al 2009), morphea and systemic sclerosis (Viglietta et al 2004; Matarese et al 2005; Paust et al 2005; Antiga et al. 2010; Lourenco 2012). Further research now needs to determine the physiological functions of Tregs in HF immune privilege (e.g., are their activities required for immune privilege maintenance?), and their role in the collapse of bulge immune privilege collapse, e.g. in LPP (Harries et al. 2013). NK cells are rarely seen in or around the normal human hair follicle, with only a few NK cells seen around the ORS of the distal HF (Christoph et al. 2000; Ito et al. 2008). Previously, the NK number and distribution pattern in association with normal human scalp HFs had been assessed by studying two immune markers: neural cell adhesion molecule (CD56) and the human natural killer cell marker (CD57). Interestingly these authors did not find any CD57 in the HF, along with only very few CD56+ cells (Christoph et al. 2000; Ito et al. 2008) These findings suggest that either there are no NK cells in/around the normal HF or that these antigens are not expressed on perifollicular NK cells.

Previous work has proposed the association between NK cells and the damage of the immune privilege by autoimmune disorders. (Ito et al 2008) conducted their experiments on alopecia areata as a perfect model. They established that, despite the down-regulation of MHC I by the HF epithelium, which should expose it to attack by NK cells, the HF normally escapes NK cell attack by down-regulating ligands that activate NK cell receptors and by up-regulating factors that suppress NK cell function, such as macrophage migration inhibitory factor (MIF) (Ito et al. 2008). Failure of this system may play an important role in bringing about the most common autoimmune hair loss disorder, alopecia areata (Ito et al. 2008; Petukhova L et al. 2010; Gilhar et al 2012; Gilhar et al 2013).

A number of studies have demonstrated the expression of NKT cells in various types of autoimmune diseases such as type I diabetes mellitus, autoimmune encephalomyelitis, rheumatoid arthritis, myasthenia gravis, psoriasis and allergic contact dermatitis. In the last
two conditions, cellular infiltration occurred mainly in the dermis and in the mature diseased epidermis (Cameron et al., 2002; Vissers et al., 2004; Bovenschen et al., 2005; Liao et al., 2006; Ottaviani et al., 2006).

Instead, the role of NK cells and NKT cells in PCA, namely in LPP, remains relatively unstudied. Therefore, the present project attempted to investigate the role of NK and NKT cells with regard to IP collapse and the immunopathogenesis of LPP, using CD161 and Vα24 as markers, respectively. CD161+ cells were examined on the frozen normal skin section, revealing the membranous expression on NK cells in the dermis (figure 3.2), but could not be detected in PFF HF sections. Vα24+ cells were absent in normal HF tissue sections, while Vα24 positivity greatly increased in lesional LPP. This represents the first immunohistochemical mapping of Vα24 in lesional LPP and normal HFs. These two antigens could not be detected in FFPE skin tissue, suggesting that investigating NK and NKT cells with these primary antibodies is best done on cryopreserved tissue (Figure 3.2-3.3).

Mast cells are recognised as having a major function in anaphylactic and allergic reactions, as well as for their participation in innate and acquired immunity (Sayed and Brown 2007; Walker, Hatfield et al. 2011; Theoharides et al. 2012). Recent evidence has demonstrated that mast cells occur in inflammatory diseases, which are stimulated by non-allergic factors such as cytokines and neuropeptides (Dillon et al. 2004; Bischoff, S. C. 2007; Theoharides et al. 2007). Mast cells are increasingly appreciated to play a crucial role in the immunopathogenesis of a large number of inflammatory diseases and are associated with controlling and arranging the autoimmune responses of T-cells, such as atopic dermatitis, psoriasis, and multiple sclerosis (Ito et al. 2010; Maurer et al. 1998; Weber et al. 2003; Theoharides and Cochrane, 2004; Theoharides et al. 2004; Maurer and Metz, 2005; Paus et al., 2006; Weller et al., 2006; Metz and Maurer, 2007; Metz et al., 2008; Stelekati E et al. 2009). (Harries et al. 2013) Furthermore, the same study identified that the number of immature mast cell progenitors are higher than mature mast cells which suggests that immature mast cells can grow and actively become mature mast cells in the lesional LPP; this suggests a role of mast cells in the collapse of bulge IP and the subsequent damage of the HF. Moreover, mast cells have been shown to contribute to the damage of the bulge region in mice and to the occurrence of IP collapse and neurogenic skin inflammation (Arck et al. 2003; Peters et al. 2005; Arck et al. 2006). In the present study, no intraepithelial
toluidine blue+ mast cells were identified in the LPP hair follicle, whereas by using this histological staining (Figure 3.4-3.5), mast cell up-regulation was observed in the perifollicular mesenchyme close to the bulge and infundibulum of the LPP, in contrast with the normal HF. This observation, which is in agreement with results of previous research (Christoph et al 2000; Harries et al 2013). Together, this data suggests that mast cells play a significant role in the immunopathogenesis of LPP by participating in IP collapse and irreversible hair loss. Therefore there may be the potential for mast cells to be targeted in future LPP therapy, using drugs that inhibit mast cell degranulation and or that antagonize key mast cell products.

Macrophages are prominent in the perifollicular mesenchyme tissue, but never present in the epithelial of the normal human hair follicle (Christoph et al 2000), with the possible exception of catagen (Paus et al 1998). A previous study in mice suggests that activated macrophages are critically involved in “programmed organ deletion” of the HF, which is suggested as a process for the removal of the damaged HF (Eichmuller, Paus et al. 1998) and which may occur in greatly exaggerated and uncontrolled form in PCA (Harries and Paus 2010). Indeed, using CD68 as a pan-macrophage marker, the number of macrophages is greatly increased in both the CTS and the epithelium of lesional LPP HFs in the late stages of the disease (Harries et al. 2013).

It is therefore of interest that the present study detected the number of resident macrophages, identified by CD163 expression, to be significantly reduced in the perifollicular mesenchyme tissue, compared with the normal HF (figure 3.8). Macrophage subtypes (Proinflammatory (M1) and reparative (M2)) may participate in HF damage during LPP by means of their properties such as producing fibrogenic factors, phagocytosis and the scavenging of dead cells, is still unresolved, as is whether they have a potential role in the pathogenesis of LPP. The current study encourages one to, next, systematically characterize different macrophage sub-populations (resting versus activated ones), e.g. by double-immunostaining of CD68/CD163 comparing lesional and non-lesional LPP HFs as well as healthy human scalp HFs.
Epithelial-mesenchymal transformation (EMT) is a process characterized by the loss of the epithelial phenotypic property of cells and tissues and the acquisition of a mesenchymal phenotype. This mechanism selectively involves the downregulation of the epithelial phenotype, E-cadherin, and the upregulation mesenchymal markers such as vimentin (Nakamura and Tokura 2011). In fact, it is now recognized that EMT takes prominently place during the development of organ fibrosis such as pulmonary fibrosis, renal fibrosis and the lymphocytic type of PCA; postmenopausal frontal fibrosing alopecia, which may represent a variant of LPP (Nakamura and Tokura 2011).

Therefore, it is particularly important and clinically relevant that this thesis project provides the first intriguing pointers in support of the hypothesis that the extensive scarring in LPP may indeed result from pathological EMT in the epithelial stem cell zone of the bulge of HF. In fact, this has most recently helped the laboratory to win a new pilot grant from the Cicatricial Alopecia Research Foundation (C.A.R.F, USA) that will allow to systematically pursuing the EMT leads generated by the current MPhil project.

If confirmed in the planned follow-up studies and if backed-up by additional evidence that EMT occurs during LPP pathogenesis (e.g. up-regulation of SNAIL1 and/or SNAIL 2 [see Nakamura and Tokura 2011]), this could have major clinical implications: The suppression of EMT in the bulge could then become a very important new therapeutic strategy in the future clinical management of LPP and other forms of PCA so as to prevent both, the cosmetically disfiguring scarring in PCAs, which also greatly impairs the overall function of scalp skin, and the irreversible transformation of epithelial stem cells to fibroblastoid cells, which may greatly contribute to destroying the HFs capacity for regeneration and growth. Since clinically available drugs that may also inhibit pathological EMT (e.g. simvastatin (Yang et al 2013) and the development of novel EMT inhibitors (e.g. Shi Z et al. Cancer Res 2013), are a current focus of investigation in both oncology and fibrotic diseases, this line of research promises the development of important novel forms of LPP therapy.

Future work that will follow-up the current MPhil project will have to take into account several important methodological issues that need to be addressed. Most notably the number of individuals examined should be increased as much as is possible so as to enhance the chance to observe significant differences between test and control groups (the relatively
small number of samples that was available for study, often precluded a meaningful quantitative analysis or rendered it difficult to obtain statistically significant data. Resolutions of this issue may also be achieved by expansion of the size of the samples. In addition, it is very important and instructive to compare both main types of PCA, i.e. neutrophilic and lymphocytic subtypes (Harries and Paus 2010), with respect to the read-out parameters established in the present study.

Taken together, the current thesis project has made an instructive panel of antigens and new immunostaining protocols available for systematic quantitative analysis in future LPP research in the lab. This serves greatly facilitates all subsequent LPP studies planned in 2013/2014 (in fact, a new post-doc, who will join the lab from Japan this autumn, will utilize the immunostaining protocols and immunohistomorphometry techniques established in the current thesis project to further investigate the immunopathogenesis of LPP). As a result of the current thesis project, one emphasis in these future LPP studies in the lab will be placed on clarifying the as yet obscure role of perifollicular mast cells and macrophages in LPP pathogenesis, to which the present study has called attention. The other emphasis will be on the role of EMT in LPP, and how to suppress this – ideally with widely approved drugs already used in daily clinical medicine that can be repositioned for this purpose.
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Dedication

This thesis is dedicated to my father, **Professor Abojela Eruk**, who was not here to witness the achievement of my postgraduate education. You are loved and missed. You were always supportive of my dreams in medical sciences and I hope to have made you proud.

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