2013

The histopathology, direct immunofluorescence and immunoperoxidase staining in the distinction between lichen plano-pilaris and central centrifugal cicatricial alopecia

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Dissertation

THE HISTOPATHOLOGY, DIRECT IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE STAINING IN THE DISTINCTION BETWEEN LICHEN PLANO-PILARIS AND CENTRAL CENTRIFUGAL CICATRICIAL ALOPECIA

by

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Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Science

2013

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DEDICATION

To

my parents,

Wirat and Veeraya Rattanasirivilai,

who made all of this possible

through their endless encouragement and patience.

And also to

Dr. Amal Kurban,

who inspired dermatologists to be their very best.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help of many people in so many ways. I would like to take this opportunity to acknowledge the numerous people who have helped and supported me along the way. I would like to thank Dr. Thomas Ruenger, my program director, the second reader and also thesis committee member, who always encouraged and supported me through these four years. I would like to thank Dr. Lynne Goldberg, an amazing advisor, who has been tremendously inspiring and has guided me through a graceful learning experience in the clinic and at the microscope. Her dedication to her work, her patients and her students is truly beyond description. I would like to thank Dr. Jag Bhawan, the third reader and Director of Dermatopathology. I am highly grateful for his advice and support. I would like to thank Dr. Shi Yang, who kindly agreed to be the thesis committee chair. Your kind and sincere words in each meeting were significantly helpful as my research progressed.

I would like to acknowledge Timothy Heeren, professor in biostatistics. His very special ability to teach me biostatistics has been tremendously helpful in my research. I would like to sincerely thank my fellow international graduate trainees in dermatology for your support and encouragement. Lastly, I would like to acknowledge Dr. Somphote Angkhanawiriyarak, without whose support I would not be where I am today.

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ABSTRACT

Lichen planopilaris (LPP) and central centrifugal cicatricial alopecia (CCCA) are lymphocytic scarring alopecias. They share overlapping clinical and histopathologic findings. The goal of this dissertation has been to find reliably distinguishing features between these two conditions. Toward this goal, three different studies were conducted.

Study 1 was a retrospective cross-sectional data analytic review of histologic features from patients identified by diagnosis of LPP or CCCA. Horizontal sections at level of the infundibulum, isthmus and inferior from scalp biopsies of 24 patients (19 CCCA and 5 LPP) were analyzed. The findings of unaffected follicular units, retained sebaceous glands and mild perifollicular inflammation were found to favor the diagnosis of CCCA. Dilated eccrine glands and heavy perifollicular inflammation were found to favor the diagnosis of LPP.

Study 2 was a prospective cross-sectional data analysis study designed to identify and compare direct immunohistochemical findings in patients with LPP or CCCA. Vertical frozen sections of scalp biopsies from eleven patients (4 CCCA and 7 LPP) were stained with IgG, IgA, IgM, C3 and fibrinogen. No DIF finding that reliably distinguishes LPP from CCCA was found. The presence of a positive DIF was significantly correlated with the amount of inflammation.

Study 3 investigated and compared T lymphocyte subsets, including T helper cells, cytotoxic T cells, Th 17 lymphocytes and regulatory T cells between LPP and CCCA cases. Subjects in this study were identical to study 2. There were no significantly distinctive T lymphocyte populations that differentiate between CCCA and LPP. There were higher numbers and percentages of CD8 positive cells in LPP compared to CCCA. The CD4:CD8 ratios were decreased in LPP and increased with duration of disease approaching proportions found in the normal hair follicle and CCCA. There were higher CD1a:CD3 ratios in LPP compared to CCCA. The CD1a:CD3 ratios in LPP decrease over time, approaching those found in CCCA. Results of this study confirm role of Langerhans cells as antigen presenting cells and role of cytotoxic lymphocytes in pathogenesis of LPP in early disease. Th17 lymphocytes and Tregs may have a role in both CCCA and LPP.

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Abbreviations	Meaning
BMZ	basement membrane zone
BUMC	Boston University Medical Center
CCCA	central centrifugal cicatricial alopecia
DAB	diaminobenzidine
DIF	direct immunofluorescence
DLE	discoid lupus erythematosus
FAPD	fibrosing alopecia in a pattern distribution
FDS	follicular degeneration syndrome
FFA	frontal fibrosing alopecia
FoxP3	Forkhead family transcription factor 3
INKers	Individual necrotic keratinocytes
IRB	Institutional Review Board
IRS	inner root sheath
LP	lichen planus
LPP	lichen planopilaris
NAHRS	North America Hair Research Society
n.s.	not significant (difference not statistically significant)
NZB/KN	New Zealand black mouse model
ORS	outer root sheath
PDIRS	premature desquamation of the inner root sheath
PPARγ	peroxisome proliferator-activated receptor gamma

INTRODUCTION

Biology of the hair follicle

Hair is found only in mammals, where its functional properties include thermoregulation, collecting sensory information, protection against environmental trauma, social communication, and mimicry.[1] Human skin contains approximately 5 million hair follicles, 100,000 of which are on the scalp. During one's lifetime, the average hair density on the scalp is the most when we are born (1,135/cm^2). This decreases to 615/cm^2 in adolescence and 435/cm^2 in elderly adults.[2] Hair density and volume are different among ethnicities. Caucasians have significantly higher hair density and volume compared to Asians and African-Americans, respectively.[3,4]

In utero, hair follicle formation begins on the head, and then moves downward to the remainder of the body. It is first visible on the eyebrow, upper lip and chin regions. Morphologically, hair follicle development has been divided into eight consecutive stages[1,5] as shown in Figure 1. Each stage is characterized by unique expression patterns of growth factors and their receptors, growth factor antagonists, adhesion molecules, and intracellular signal transduction components. [1,5,6]

Stage 1 of hair follicle development first appears at 10 weeks gestation when collections of undifferentiated epithelial cells form epithelial placodes. In stage 2, epithelial placodes develop and expand vertically to form the primary hair germ. Dermal cells beneath the hair germ form a cluster, which later develops into a dermal papilla. In stage 3-4, the hair germ elongates into a cord of epithelial cells called a peg. The mesenchymal cells at the sides of the peg will develop into the fibrous sheath of the hair

follicle. In stage 5, the deepest portion of the follicle peg forms a bulbous structure, which surrounds the underlying mesenchymal cells destined to become the dermal papilla. Follicular keratinocytes begin to form the inner root sheath. At the same time, the follicular pigmentary unit generates melanin. [1,5,6]

In stage 6, the outer root sheath forms two bulges on the side of the hair follicle forming an obtuse angle with the surface of the skin. The superficial bulge will develop into the sebaceous gland. The deeper bulge serves as the future site of epithelial stem cells that generate the new lower follicle during hair follicle cycling. The arrector pili muscle attaches in the bulge area. In the axillae, anogenital region, areolae, periumbilical region, eyelids, and external ear canals, a third bulge develops superficial to the sebaceous gland bud and gives rise to the apocrine gland. In stage 7, the necrosis and cornification of epithelial cells in the infundibulum form a central lumen, where the hair shaft will emerge in stage 8. [1,5,6]

There are three main types of hair follicles: lanugo, vellus and terminal, which share the same basic anatomy (Figure 2). However, their growth, size, shape, pigmentation, and other characteristics differ widely, based on body location and variation among individuals. Terminal hairs are typically greater than 60 μm in diameter, possess a central medulla, and can grow to well over 100 cm in length, depending on ethnicity. In contrast, vellus hairs are typically less than 30 μm in diameter, do not possess a medulla, and are less than 2 cm in length. The hair bulbs of vellus hairs in anagen are located in the reticular dermis, whereas hair bulbs of terminal hairs are in the subcutis. Terminal hairs are found on the scalp, eyebrows, and eyelashes at birth. Vellus

hairs are found elsewhere, and, at puberty, vellus hair follicles in the genitalia, axillae, trunk, and beard area in men transform into terminal hair follicles under the influence of sex hormones.[6]

The first hairs formed in the embryo are lanugo hairs, which are non-pigmented, soft, and fine. Lanugo hair is typically shed between the 32nd and 36th weeks of gestation, although approximately one-third of newborns still retain their lanugo hair for up to several weeks after birth. After formation of lanugo hairs, terminal hairs and vellus hairs are formed.[1]

The hair follicle is divided into three major portions along its length, which are, from superficial to deep, the infundibulum, isthmus and inferior follicle. These portions are divided by the opening of sebaceous gland (Figure 3, upper arrow) and the insertion of arrector pili muscle, which is also called the bulge region (Figure 3, lower arrow). The length of each portion in the vellus and terminal hair follicle is shown in Figure 2. The infundibulum and isthmus portions are permanent, whereas the inferior portion changes with the hair cycle, regressing then regenerating via the activity of multipotent stem cells residing in the bulge region.[7] These multipotent stem cells can repopulate the epidermis, hair follicles and sebaceous glands. Inflammatory alopecias that destroy the bulge region may lead to scarring alopecia or permanent loss of hair, due to loss of these stem cells.[6]

The lowermost part of the inferior follicle forms a bulbous structure known as the hair bulb. The bulb epithelium surrounds a mesenchymal structure called the dermal papilla. The dermal papilla is comprised of fibroblasts, collagen bundles, a mucopolysaccharide-rich stroma and capillary loops. It has the ability to induce hair

follicle formation, and determines the size of the hair shaft. The volume of the dermal papilla correlates with the number of matrix cells, and thus the size of the hair shaft.[8]

The hair follicle is also divided circumferentially into layers, or compartments. From outermost to innermost these are the connective tissue sheath, the outer root sheath (ORS), the inner root sheath (IRS), the hair shaft cortex, and the hair shaft medulla (Figure 4). Each epithelial compartment expresses distinct hair follicle-specific keratins.[9] The connective tissue sheath is composed of type III collagen. It envelops the epithelial components of the hair follicle and is also continuous with the interfollicular basement membrane.

The ORS is continuous with the epidermis at the infundibulum, continues to form the bulge at the base of isthmus and extends to the bulb. In the infundibulum, the ORS resembles the epidermis and forms a granular layer during keratinization. In the isthmus, the ORS keratinizes without a granular layer, so called trichilemmal keratinization. In the inferior follicle, the ORS cells are larger and paler due to abundant glycogen (Figure 5). The functions of ORS include serving as a stem cell reservoir, a support and guidance structure for the IRS and hair shaft and as a transit route of nutrients and oxygen to the hair shaft via the IRS. The ORS also produces hair cycle-modulatory factors, such as fibroblast growth factor 5, neurotrophins and prolactin.[10]

The IRS arises from hair matrix keratinocytes in the bulb. It consists of three separate layers, which from outer to inner are called Henle's layer, Huxley's layer and the IRS cuticle. The IRS, specifically Huxley's layer, dictates the shape of the hair by funneling the hair shaft cells as they are produced from the bulb.[6] The IRS cuticle

attaches firmly to the hair shaft cuticle and desquamates at the mid isthmus (as shown in Figure 5B). The loss of the IRS below this point has been described as premature desquamation of the IRS, which is a feature of scarring alopecia.[11]

The hair shaft arises from hair matrix keratinocytes, which reside at the apex of dermal papilla (shown in Figure 6). It is composed of two layers, the hair cortex, which is the main structure of the hair shaft, and the hair medulla. The hair cortex consists of densely packed, spindle-shaped, fully cornified trichocytes. They are oriented longitudinally, parallel with the hair axis. The hair cortex is covered by the cells of the cuticle, which protect the shaft from weathering, fracture, breakage, and splitting.[6]

During postnatal life, individual hair follicles perpetually cycle through three phases[12-14]: (i) intensive growth and hair shaft production (anagen), (ii) apoptosis-driven regression (catagen) and (iii) relative resting (telogen) as shown in Figure 1. Approximately 85-90% of scalp follicles are in anagen, 10-15% in telogen and less than 1% in catagen. The length of anagen determines the final length of the hair shaft. Terminal scalp hair spends 2-6 years in the anagen phase, 2-3 weeks in the catagen phase and 3 months in the telogen phase. The anagen duration of vellus scalp hair is 6-12 weeks. There are substantial variations in anagen duration by part of the body. The anagen duration of moustache hair is 4-14 weeks, arms 6-12 weeks and legs 19-26 weeks.[12]

The formation of a new lower follicle in anagen phase begins with growth of the dermal papilla and increased mitotic activity in the overlying epithelium. This process is followed by formation of the hair bulb, and bulb matrix differentiation to follicular structures including the hair shaft. Once a telogen hair is dislodged, a new hair shaft

emerges up through skin surface and grows in a stable rate depending on the location of the hair follicle. The growth rate of terminal scalp hair is approximately 0.35 mm/day.[6]

The catagen phase is a regression phase. It is characterized by apoptotic activity in lower portion of hair follicle, which is associated with a decrease in mitotic activity of matrix cells. The lower portion of hair follicle retracts upward, while the perifollicular sheath collapses and forms a fibrous streamer. The dermal papilla also moves upward and situates below the bulge at the lower portion of the isthmus. Pigmentation by melanocytes ceases, and leads to a non-pigmented end of the hair shaft. Once the catagen phase is complete, the lower portion of hair follicle becomes club shaped in the next phase, called telogen. The telogen phase is a so called resting phase, as mitotic activity of hair shaft is at its lowest and the hair shaft is ready for expulsion from the scalp. There is sometimes a lag period after shedding of the telogen hair and the formation of the next anagen hair, particularly in patients with androgenetic alopecia.[15]

Other than anagen, catagen and telogen, a distinct phase of the hair follicle called exogen was described in 2002. The exogen phase is a unique shedding phase that differs from the shedding of the hair club found in telogen. The shedding activity of exogen is associated with increasing proteolytic enzymes, and it occurs in mid anagen phase. It is a highly controlled and timed event, which explains seasonal shedding in mammals and shedding of human hair following starting of medications such as minoxidil.[16]

Alopecia

Loss of hair (alopecia) can lead to significant psychological and emotional distress, and it supports a multibillion-dollar pharmaceutical and cosmetic effort aimed at restoring hair. There are many causes of alopecia, and an accurate diagnosis is crucial to the prognosis and the determination of a treatment plan. The diagnosis is made by correlation of history, clinical findings, and histopathologic findings. Alopecia is categorized clinically as either focal or diffuse, and histopathologically by the presence or absence of scarring.

Non-scarring alopecia refers to hair loss without permanent destruction of the hair follicle. Examples include female and male pattern hair loss, telogen effluvium, alopecia areata, anagen effluvium, inherited disorders of the hair shaft, and hair breakage related to external factors.

Scarring (cicatricial) alopecia refers to hair loss associated with inflammation, fibrosis and ultimately replacement of the hair follicle by scar tissue. Hair loss in scarring alopecia is permanent and the hair follicles may become completely destroyed. Examples include lichen planopilaris (LPP), central centrifugal cicatricial alopecia (CCCA), discoid lupus erythematosus, acne keloidalis, folliculitis decalvans, and dissecting cellulitis.

Some alopecias demonstrate a biphasic pattern in which non-scarring alopecia is observed early and permanent hair loss appears later. These diseases include patterned hair loss, alopecia areata and traction alopecia. These forms of alopecia are classified as non-scarring, but after a long duration of active disease, permanent follicular drop-out occurs.[17]

Cicatricial alopecia

Cicatricial alopecia is divided into primary and secondary forms. In primary cicatricial alopecia, the destruction of the hair follicle is caused by a targeted inflammatory process, which leads to permanent hair loss.[11,18] These types of alopecia are categorized by the type of inflammatory cells present in the biopsy; those with neutrophils are called neutrophilic scarring alopecias, those with lymphocytes are called lymphocytic scarring alopecias. There is also a mixed group, as well as an end stage, non-specific group. Each of these groups is further subdivided based on a classification scheme devised at a special workshop of The North America Hair Research Society[17] (NAHRS) as shown in Table 1.

In secondary cicatricial alopecia hair follicles are destroyed nonspecifically as innocent bystanders, due to other diseases affecting the dermis. Example of diseases that can cause secondary scarring alopecia include cutaneous sarcoidosis, morphea, necrobiosis lipoidica and lupus vulgaris.[19]

While various forms of cicatricial alopecia have distinctive histopathological findings, an accurate diagnosis of cicatricial alopecia almost always requires good correlation of clinical and histopathological findings. This is because histopathological findings of some entities overlap and distinction between these entities can be exceedingly difficult.

The diseases being studied in this dissertation are lichen planopilaris (LPP) and central centrifugal cicatricial alopecia (CCCA). They are in the category of lymphocytic scarring alopecia. There is currently no gold standard to distinguish these entities, and

although LPP and CCCA overlap histologically, treatments for these disorders differ. It is thus essential to be able to distinguish them, not only for proper patient management, but also to further our knowledge on their pathophysiology.

Lichen planopilaris

Lichen planopilaris (LPP), a type of primary lymphocytic scarring alopecia, is also known as lichen follicularis or follicular lichen planus of the scalp.[20, 21] Initially described in 1895 by Pringle, it is believed to be a follicular variant of lichen planus (LP)_, an extremely itchy eruption composed of purple, polygonal papules that have a predilection for the extremities and mucosal areas.[20, 21]

The 2003 NAHRS working classification of primary cicatricial alopecias[17] categorized LPP into three main clinical variants: classic LPP, frontal fibrosing alopecia (FFA) and Lassueur Graham-Little Piccardi syndrome. Others have proposed adding fibrosing alopecia in a pattern distribution (FAPD) as a presentation of LPP.[22]

Classic LPP commonly involves the vertex, although any region of the scalp can be affected. The classic early findings are perifollicular violaceous erythema and perifollicular scales or keratotic plugs. After inflammation and hair shedding, scarring alopecic patches replace previously inflamed lesions. These are smooth surfaced, skin colored patches that may be patchy in a reticular pattern or become large, confluent areas of scarring alopecia.[21] Careful inspection often reveals active perifollicular inflammation at the periphery.

The typical age of onset of classic LPP is around the fifth decade.[23] Women are more affected than men, and account for 60 to 90% of cases.[24, 25] Caucasians are more affected than dark skinned individuals. Extra-cranial lichen planus involving nails, mucous membrane and glabrous skin, may occur. The reported rate of extra-cranial involvement was variable, ranging from 17 to 50% of patients. [20, 26]

FFA is a variant of LPP seen mostly in postmenopausal women, and rarely in men.[27] It presents as a progressive symmetrical band of scarring alopecia affecting the frontal hairline, the preauricular scalp and the retroauricular scalp. Eyebrows are often affected before the scalp, and inflammation in this area is generally absent or subtle. Facial papules may be observed.[28]

Lassueur Graham-Little Piccardi syndrome consists of scarring patchy alopecia of the scalp, non-scarring axillary and pubic hair loss, and a lichenoid follicular eruption on the body, scalp, or both. Scalp alopecia often precedes follicular papules.[29, 30] FAPD presents with perifollicular erythema and scales as is commonly seen in classic LPP, but it involves an area on the crown of the scalp that is seen in female pattern hair loss.[22, 31, 32] FAPD is often underdiagnosed early in the disease, and requires histopathologic confirmation.

Pathophysiology of LPP

The etiology of lichen planopilaris is unknown, but it is presumed to be a cell-mediated immune reaction, similar to lichen planus. In active lesions of LPP, there is an inflammatory process mediated by T lymphocytes (CD4 and CD8 positive), supporting

the notion that cell-mediated immunity plays a major role in the triggering the clinical expression of the disease.[33, 34] These lymphocytes attack and destroy particular antigens on keratinocytes in follicular epithelium. As a result of follicular epithelial destruction, the hair cycle is disrupted causing alopecia. The inflammation is mostly located around the bulge area where multipotential stem cells are present; their destruction leads to permanent loss of the follicles.[34, 35]

The nature of targeted follicular antigens is unknown. The antigen may be an autoreactive peptide, thus classifying LPP as an autoimmune disease. Alternatively, it may represent an exogenous antigen such as a medication, a contact allergen, an infectious agent, or an unidentified immunogenic target. Medications that induce LPP include etanercept and infliximab, which are both anti-tumor necrosis factors used for treatment of psoriasis.[36, 37] A series of patients with LPP occurring years after hair transplantation for male pattern hair loss have been reported. The possible explanations for this phenomenon include koebnerization, whereby a condition develops or spreads due to trauma, and immune privilege collapse, whereby the hair follicle, previously unrecognized by the body's immune system, becomes subject to attack.[38, 39]

The possible etiologies of LPP other than cell-mediated immunity include altered follicular integrin expression in the hair follicle[40] and decreased expression of peroxisome proliferator-activated receptor gamma[41] (PPARγ). Integrin is a transmembrane receptor found on all animal cells. It mediates the attachment between a cell and its surroundings and also transduces signals between cell and extracellular matrix. Altered integrin distribution at the basement membrane of follicular epithelium has been shown in active

LPP lesions, but not in normal follicles.[40] This may cause the affected areas to be more susceptible to antigen presentation, and the resultant immune response could explain the phenomenon of easy epilation of anagen hairs with an abnormal gelatinous root sheath that is seen in active scarring alopecia.

PPARγ is a member of the nuclear receptor super-gene family, which regulates the expression of genes involved in inflammation and lipid homeostasis. It was recently shown to have a role in the pathogenesis of LPP.[41,42] A significantly decreased expression of multiple genes required for fatty acid oxidation, cholesterol biosynthesis, and peroxisome biogenesis, including PPARγ, were identified in LPP scalp tissue.[41-43] Moreover, targeted knockout of PPARγ in the stem cells of the bulge causes scarring alopecia.[41,43] Thiazolidinedione, a PPARγ agonist, has been shown to improve clinical symptoms, decrease perifollicular erythema, halt the spread of old patches and decrease inflammatory infiltration in LPP patients.[43,44]

Clinical presentation of LPP

The clinical course and pattern of hair loss in LPP may be insidious or fulminant, and the pattern of hair loss on the scalp is highly variable. Most commonly there are several scattered foci of partial hair loss on the crown. Disease activity is best appreciated at the periphery of active lesions, which exhibit characteristic perifollicular erythema and follicular hyperkeratosis.[20] The inflammation affects the anchoring of anagen follicles, and they can be pulled out easily in active lesions. Eventually scattered foci of complete hair loss form, and sometimes merge to form large areas of hair loss. Late lesions lack of

erythema and scale, and close inspection reveals loss of follicular orifices on the scalp surface. The sites of predilection in classic LPP are the frontocentral scalp and crown. Associated cutaneous, nail, and mucous membrane lichen planus may be present in one third to half of cases.[21, 24, 25, 45] LPP may be asymptomatic, but scalp pruritus and dysesthesia (pain, discomfort and burning) are often present.

Histopathologic findings of LPP

Histopathologic findings vary according to the stage of the disease. Early on there is a lichenoid lymphocytic infiltrate affecting the infundibulum and isthmus, sparing the lower portion of hair follicle.[20, 21, 25] Interface dermatitis occurs between the follicular epithelium and adjacent dermis with loss of the basement membrane zone. Sebaceous glands are lost in early lesions and the root sheaths of the hair follicles are destroyed. There is no increase in dermal mucin and abnormal changes in the structure of the vascular plexus as in discoid lupus erythematosus. Later on, extensive perifollicular lamellar fibrosis develops, especially around the isthmus. In end stage disease, hair follicles are completely replaced by fibrosis and it is not possible to distinguish LPP from other primary cicatricial alopecias. [20, 21, 25] Several studied have been published on the use of elastic fiber abnormalities in LPP,[46, 47] including wedge shape loss of elastin in the upper dermis.

Central centrifugal cicatricial alopecia (CCCA)

CCCA is a progressive primary lymphocytic scarring alopecia of the central scalp that occurs primarily in African American women.[48,49] It was first described in 1968 by Lopresti et al[50], as "hot comb alopecia" based on the hypothesis that heat from hot combing was responsible for the alopecia. The term "follicular degeneration syndrome" was later proposed in 1996, as no etiological relationship between use of a hot comb and the scarring alopecia was found.[51] Other terms including, scarring alopecia in African Americans and chemically induced cosmetic alopecia were also proposed.[52] Finally, the term "central centrifugal cicatricial alopecia" was proposed by Sperling[19] in 2000 and adopted by the North American Hair Research Society in 2001.[17]

CCCA is the most common type of primary scarring alopecia in African American women. Women are more affected than men with a ratio of 4:1 or more.[53-55] The mean age of presentation is 36 years in females and 31 years in males. The prevalence is estimated as high as 5.6%-17% of the African American population. [53-57]

Pathophysiology of CCCA

The etiology of CCCA is unknown. It is hypothesized that it is multifactorial, incorporating a genetic predisposition with follicular damaging hairstyles.[57] Genetic predisposition may leave the hair follicle predisposed to external injury, and the use of hair relaxers, traction, or other chemicals might create irritation, inflammation and resultant scarring alopecia. However, reports of familial cases without any evidence of

mechanical or chemical damage to the hair question the contribution of styling practices, and strengthen the role that genetics plays in CCCA.[56, 58]

Follicular damaging hairstyles are strongly associated with CCCA.[59, 60] These include the use of hair weaves, cornrows, braided hairstyles and artificial hair extensions. These hairstyles may cause chronic chemical irritation or mechanical trauma to the structure of the hair follicle and the follicular stem cells, and result in permanent destruction of hair follicles.[59]

Altered retinoid metabolism has been recently proposed as an etiology for CCCA.[61] Retinoic acid and retinoid metabolism have critical roles in the development and maintenance of multiple epithelial tissues, including skin, hair, and sebaceous glands.[56, 62] These include maintenance of hair growth and hair cycling.[56, 63] A recent study has shown an association of altered retinoid metabolism with cicatricial alopecia similar to CCCA in a mouse model.[56] In humans, many retinoid metabolic proteins are altered in mild CCCA.[56] Furthermore, retinoid supplementation in patients with low serum retinol levels has been shown to improve CCCA.[61]

Clinical presentation of CCCA

CCCA presents as a roughly circular alopecic patch at the crown and/or vertex of the scalp, which develops over time and gradually progresses centrifugally.[49, 55, 64] Similar to other scarring alopecias, the affected scalp ultimately becomes smooth and shiny, with loss of follicular openings. A few short brittle hairs may remain. The most active disease is at the periphery. Eventually the disease becomes quiescent.[49] Dysesthesia (mild

pruritus, pain, or tenderness) may occur in involved areas but is usually not severe, and symptoms may be trivial or absent.

Histopathologic findings of CCCA

An early histologic change of CCA is lymphocytic infiltration of the upper follicle, from the lower follicular infundibulum to the upper isthmus.[49-52] Concentric lamellar fibroplasia occurs around the mid to upper follicle. Sebaceous glands are lost, as in other types of scarring alopecia. A mild perivascular lymphocytic infiltrate may be present. The number of terminal hair follicles is decreased and there is an increase in the number of fibrous tracts, reflecting loss of hair follicles. Interface alteration at the dermo-epidermal junction and around the hair follicle, dyskeratotic cells, mucin and perieccrine inflammation are usually absent. [49]

In the normal hair follicle, the IRS attaches firmly to the hair shaft as it arises from the hair matrix then desquamates at the level of sebaceous gland opening or isthmus.[1] The loss of the IRS below this point has been described as premature desquamation of the IRS (PDIRS). It is found in association with perifollicular inflammation in some cases of primary scarring alopecias, as result the result of cell-mediated injury or trauma.[65, 66] Although it is nonspecific, it is hypothesized that premature desquamation of inner root sheath occurs early in CCCA before marked inflammation or degenerative follicular changes, and its finding early is distinctive to the diagnosis of CCCA.[66]

Perifollicular inflammation in CCCA may cause changes in follicular unit morphology, forcing the follicular canal into an eccentric position and causing follicular

asymmetry. Progressive thinning of the external root sheath leads to follicular distention, rupture, hair granulomas and dense fibrosis.[49] The end stage changes, however, are indistinguishable from other primary scarring alopecias. Verhoeff-Van Gieson staining for elastin in CCCA has shown broad fibrous tracts with intact elastic sheath and thick elastic fibers throughout the dermis.[47]

The distinction of LPP and CCCA

LPP and CCCA are two of the most common lymphocytic scarring alopecias. They have distinctive clinical features and occur in different groups of populations, LPP in Caucasian and Hispanic and CCCA in African American. CCCA usually starts at the vertex or crown then progress centrifugally, whereas LPP is typically patchier in distribution, although it can become confluent on the crown. In patients in whom the clinical presentation is not characteristic, a biopsy is required. While a biopsy can distinguish LPP from other lymphocytic scarring alopecias such as discoid lupus erythematosus (DLE), Mirmirani et al. have suggested that LPP is histologically indistinguishable from CCCA.[18] Thus, clinico-pathological correlation is always required in making these diagnoses. Recent study by Elston et al. [47] suggested the use of EVG to differentiate LPP and CCCA, wedge shape scars in LPP and broad fibrous tracts with intact elastic sheath in CCCA.[47]

Direct immunofluorescence in LPP and CCCA

Immunofluorescence staining is an immunohistochemical technique using fluorescent dye labeled antibodies to identify antigens, such as antigens on the surface of bacteria, or antigens in cells in histologic sections or in other specimens. In cutaneous disease, the antigen in question is usually an antibody itself, and fluorescent-labeled antibodies are used to detect them either in the blood (indirect immunofluorescence) or in tissue sections (direct immunofluorescence). In indirect immunofluorescence, a control tissue is chosen as a substrate, and a fluorescent-labeled antibody is used to detect circulating antibodies in the patient's serum after it is applied to the substrate. In direct immunofluorescence (DIF), the fluorescent labeled antibodies are applied directly to the patient's tissue, to detect antibodies deposited there. Once the fluorescent-labeled antibody interacts with antigen of interest, it can be visualized using a special microscope that emits ultraviolet light.

DIF is routinely performed in many dermatologic disorders to identify immunoglobulins deposited in different layers of affected skin. In immunobullous diseases such as pemphigus vulgaris and bullous pemphigoid, DIF is used diagnostically to determine whether autoantibodies and complement are deposited within the epidermis (pemphigus) or at the dermoepidermal junction (pemphigoid). DIF is also used in collagen vascular diseases such as lupus erythematosus, where multiple immunoglobulins and complement are found at the dermoepidermal junction. It is occasionally used in inflammatory skin conditions such as lichen planus. However, there are only a handful studies on the use of DIF in the diagnosis of LPP[21, 25, 26, 67] and none on CCCA.

In LPP and CCCA, the possible locations where immunoglobulins can be deposited are the basement membrane zone (BMZ) of the hair follicle, around the vasculature, and on apoptotic keratinocytes, also called colloid bodies, at the DEJ. Typically, fluorescent tagged antibodies against IgG, IgM, IGA, C3 and fibrinogen are used. The pattern can be linear, granular or speckled.

Immunoperoxidase staining

Immunoperoxidase staining is an immunohistochemical technique that uses a peroxidase-catalyzed reaction to visualize antibodies that bind with antigens of interest. It is used to determine the presence, intensity and quantity of a particular antigen. The first step is the binding of the specific (primary) antibody to the cell or tissue sample, usually prepared and fixed on a slide. The detection of the primary antibody can be then accomplished directly with a secondary antibody labeled with a peroxidase enzyme, which is used to catalyze a chemical reaction to generate a colored product. An example of the peroxidase staining is diaminobenzidine (DAB) method, which was used in our study. It visualizes the presence of enzyme-antibody-antigen complex fixed to tissue as a stable brown product.

In clinical diagnostics, immunoperoxidase staining can be used on a tissue biopsy for a more detailed histopathologic study, including sub-classifying tumors and lineage of lymphocytes. In dermatopathology, immunoperoxidase staining is used to differentiate carcinoma, melanoma, sarcoma, neural neoplasms and lymphoma. A panel of antibodies is generally recommended rather than a single stain.

Immunoperoxidase staining is not used in the diagnosis of alopecia, but it has been useful as a research tool, to identify subtypes of inflammatory cells and to better understand disease pathogenesis. In alopecia areata, a non-scarring alopecia exhibiting lymphocytes at the bulb area, CD8 positive T-cells and Th1 cytokines were found to play a key role in the autoimmune attack that occurs in this disorder.[68] In primary scarring alopecia, loss of stem cell markers (CK15, CD34 and nestin) is related to permanent follicular damage.[69] In DLE, CD8 positive T-cells were increased in patients with scarring disease.[70] In LPP, immunoperoxidase staining identified both CD4 and CD8 positive T-cells, which were activated by Langerhans cells that are increased in the dermis and epidermis.[25] This finding suggested a cell-mediated autoimmune process to a specific antigen in LPP. CCCA is known as a lymphocytic scarring alopecia, however, the role of T-cells and Langerhans cells has not been elucidated.

PURPOSE OF THE STUDY

It has been shown that scarring alopecia affects the quality of life of its sufferers, regardless of age or extent. A recent symposium brainstormed on the future direction of cicatricial alopecia research and identified a better classification system as one of its goals. LPP and CCCA are diseases with overlapping clinical and histopathologic findings. A study to better define the histopathologic characteristics of LPP and CCCA was initiated. Direct immunofluorescent features and subtypes of inflammatory cell populations using immunoperoxidase staining in both diseases in hopes of furthering our understanding of these conditions were studied. It is our hope that a better delineation of

these entities under the microscope will lead to further insight into their pathogenesis and hopefully increased therapeutic options.

Several histologic features that might distinguish LPP from CCCA under the microscope were evaluated. The features of the hair follicles were evaluated, including the number of follicles, both terminal and vellus, and the presence and number of follicular units, which are groups of sebaceous glands and hair follicles at the level of the isthmus. In alopecia, hair follicles are reduced in number, sebaceous glands are destroyed, there is thinning of follicular epithelium and follicular asymmetry, and there can be premature desquamation of the inner root sheath. The inner root sheath normally presents and desquamates at the isthmus level, just below the entry of the sebaceous duct. Premature desquamation of the inner root sheath (PDIRS) is said to occur when the inner root sheath desquamates at a lower level

The pattern and degree of perifollicular and perivascular inflammation were evaluated. The types of inflammatory cell were noted, as was the location, anatomic level, pattern and density of the inflammation. Associated findings such as individually necrotic keratinocytes and lymphocytes within the follicular epithelium were recorded. Individual necrotic keratinocytes (INKers) are a feature of interface dermatitis, and their presence in follicular epithelium has been described in lymphocytic scarring alopecia.[11] Subsets of T-lymphocytes and Langerhans cells were quantified using immunoperoxidase staining.

The last histologic feature evaluated was the presence and patterns of fibrosis and scarring. Destruction of the hair follicle leaves a scar where the hair follicle once stood,

which is also called a fibrous or fibrotic tract. It is the replacement of the follicle by amorphous connective tissue, which signifies follicular dropout.[71, 72] Naked hair shafts may be present in fibrotic tracts, which are hair shafts without any epithelium, sometimes surrounded by multinucleated giant cells forming a foreign body granuloma.[71] These were searched for and quantitated at different levels of the hair follicle. The presence or absence of perifollicular mucin was noted, which was hypothesized to be related to the chronicity of the perifollicular fibrosis.[73] Of note, fibrous scars found in cicatricial alopecia are different from follicular streamers found in non-scarring alopecia. These streamers, also known as follicular stelae, are elastin rich angiofibrotic whorls of connective tissue, remnants of a follicle that has cycled from anagen to catagen/telogen. They are a crucial element in hair cycling, as they serve as the mesenchymal column leading to new follicular papilla formation. This serves as the pathway for downward regrowth of a new anagen follicle.

Lastly, we used DIF and immunoperoxidase staining to see if there are features that distinguish LPP from CCCA, as there are few studies on the former, and no reported studies on the latter.

HYPOTHESES AND SPECIFIC AIMS OF THE STUDY

The study was based on three hypotheses: one, that there are histologic features that distinguish LPP from CCCA; two, that there are DIF features that distinguish LPP and CCCA, and three, that there are different populations of inflammatory cells that

distinguish LPP from CCCA. The specific aim of the study was to identify distinguishing findings between LPP and CCCA.

RESEARCH DESIGN

Two research protocols were designed. The first protocol was designed to answer the first hypothesis. It was a retrospective, cross-sectional data analytic review of histologic features from patients diagnosed with either LPP or CCCA from a data repository of alopecia patients (Hair Data Repository, IRB# H-27150, PI Dr. Goldberg) and the records of the Skin Pathology Laboratory at Boston University between January 1, 2000 and December 31, 2011.

The second protocol was designed to answer the second and the third hypotheses. It was a prospective, cross-sectional data analytic study, designed to identify immunofluorescent and immunohistochemical features that distinguish between LPP and CCCA.

STUDY 1. The histopathologic distinction between lichen planopilaris and central centrifugal cicatricial alopecia

Study 1. Materials and Methods

A retrospective, cross-sectional data analytic review was conducted. The study was approved by the Institutional Review Board (IRB) at the Boston University Medical Center (BUMC) under protocol number H-31124. Dr. Goldberg's Hair Data Repository and records of the Skin Pathology Laboratory at Boston University were used to select

patients diagnosed with either LPP or CCCA between January 1, 2000 and December 31, 2011. All selected patients underwent chart review, and those that were clinically evaluated and biopsied by Dr. Goldberg were included in the study. Patients with exclusion criteria (see below) were excluded.

Nineteen cases of CCCA and five cases of LPP were studied. Investigators were blinded from the diagnosis at time of slide review. The form used to collect data can be found in the Appendix 1. In each case, we reviewed all available consecutive horizontal sections to find the levels of interest for data collection, which included the isthmus and the inferior follicle. The isthmus level is the level where sebaceous glands are largest in cross section, and hair follicles are normally grouped with sebaceous glands in follicular units. The inferior follicle level chosen was the dermal subcutaneous junction, where there is an equal amount of dermal collagen and subcutaneous tissue.

Data on select histopathologic features that we thought might contribute to distinguishing CCCA from LPP at both the level of the isthmus/infundibulum and the inferior follicle was collected. At the isthmus/infundibulum, these included the presence of epidermal involvement, the number of hair follicles, the number of fused follicles, and the number of normal follicular units. Fused follicles are conjoined follicles with a shared follicular epithelium. The numbers of fibrous tracts were categorized as absent, 1-5, 6-10 and >10.

To grade the degree of follicular asymmetry, the thickest part of the outer root sheath was evaluated and compared it to what estimated normal thickness would be for that follicle. A follicle where the thickest part of the outer root sheath was over twice

normal was considered severely asymmetric, over 1.5 times normal was considered moderately asymmetric, and between 1 and 1.5 times normal was considered mildly asymmetric. To grade the degree of perifollicular fibrosis, the diameter of the fibrosis with the diameter of the outer root sheath was compared. If the fibrosis was thicker than the thickness of the outer root sheath, it was considered severe, if it was over half the thickness of the outer root sheath it was considered moderate, and if it was less than half the thickness of the outer root sheath it was considered mild.

Mucin, naked hair shafts, INKers and intrafollicular lymphocytes, and plasma cells were recorded as present or absent. Perifollicular inflammation was considered present if it involved at least half the circumference of the hair follicle. It was considered marked if it was thicker than the thickness of the outer root sheath, moderate if it was over half the thickness of the inner root sheath, and mild if it was less than half the thickness of the outer root sheath. Perivascular inflammation was graded similarly. It was considered either mild or moderate, depending on its approximate thickness in comparison to the outer root sheath. Moderate inflammation was thicker in diameter than the outer root sheath, and mild inflammation was thinner in diameter than the thickness of the outer root sheath.

Similar data was collected from the inferior follicle at the dermal-subcutaneous junction, adding the presence or absence of naked hair shafts. During data collection dilation of eccrine ducts was noted, and this finding was added to the list of histologic features being collected.

Once data collection was complete, the medical records of the patients were reviewed for relevant information including patient age, sex, ethnicity, age at the time of biopsy and duration of disease prior to biopsy. Once unblinded, the data was separated into the two groups of CCCA and LPP. The diagnosis was made on the basis of the clinical presentation. Then statistical analysis was done to identify distinguishing features of the two diseases.

Study 1. Inclusion and Exclusion Criteria

Patients who have been evaluated and biopsied by Dr. Goldberg and found to have a diagnosis of either LPP or CCCA from January 1, 2000 to December 31, 2011 were included. Exclusion criteria included patients whose biopsy slides were not available, those whose slides were cut in vertical sections, those whose slides had inadequate tissue (lacking epidermis or the dermal subcutaneous junction), those that were coded as scarring incorrectly, and those whose scarring alopecia was end-stage (less than three remaining hair follicles in one horizontal section of 4 mm. punch biopsy).

Study 1. Sample Size

The study sample was obtained from two sources. Patients with either LPP or CCCA were identified in the Hair Data Repository and from the records of the Skin Pathology Laboratory at Boston University. The Hair Data Repository identified 9 patient biopsies of LPP and 27 of CCCA. The Skin Pathology Records revealed additional 9 biopsies of LPP and 18 of CCCA, for a total of 18 LPP biopsies and 45 of CCCA. Many

biopsies met exclusion criteria (Fig. 1), leaving 5 available biopsies of LPP and 19 of CCCA. The most common exclusion was that the slides were cut vertically rather than horizontally, followed by insufficient tissue (chosen levels not available) and inadequate numbers of follicles due to end-stage disease. Three patients were excluded because their slide review revealed non-scarring alopecia.

Study 1. Data Analysis

Statistical analysis was performed using R, Statistical Software, version 2.14.1 (2011-12-22) and AnalystSoft Inc., StatPlus: Mac, version 2009. Quantitative variables were calculated in mean, minimum and maximum. T-test was used for intergroup mean comparison. Qualitative variables were calculated in percentages and Fisher exact test was used for intergroup comparison. A p-value of less than 0.05 was considered statistically significant (Figure 7).

Study 1. Results

The demographic data of both groups is shown in Table 2. Most patients were female, with the exception of one male patient with CCCA. All CCCA patients were African American, while LPP patients were Caucasian or Hispanic. About 80% of patients in both groups were between 30-60 year old, with 2 cases of CCCA who were under 30 years old and one case of CCCA over 60 years of age. The mean age of the LPP group was higher than the CCCA group, 49.4 and 44.3 year old, respectively. The mean duration of disease prior to the biopsy in the CCCA group was highly variable from 1 to

20 years with a mean of 7.4 years, whereas in the LPP group, the duration of disease prior to biopsy ranged from 1 to 10 years with a mean of 3.8 years.

The numbers of follicles at the isthmus and inferior level are shown in Table 3. The mean number of follicles in the LPP group was higher than in the CCCA group at the isthmus and inferior level. Most follicles in the LPP group were terminal, with a ratio of terminal to vellus follicles of 11:1. The ratio of terminal to vellus follicles in CCCA group was 7:3. The ratio of terminal to vellus hair in LPP is higher than in the CCCA group. However, there was no significant difference in the number of follicles between the two groups. The numbers of fibrous tracts at the isthmus and inferior level can be found in in Table 4. There was no difference in the mean number of fibrous tracts between CCCA and LPP at either level.

Selected histologic findings in the two diseases can be found in Table 5. There was no epidermal hyperplasia, interface dermatitis or hypergranulosis in any patient in either group. We found the absence of normal follicular units in all LPP cases and in some cases of CCCA (42%), which was statistically significant ($p<0.05$). There was complete loss of sebaceous glands in 60% of the LPP group but only in 11% of the CCCA group. The difference was statistically significant ($p<0.05$).

Fused follicles were found more at the isthmus level compared to the inferior level. At the level of isthmus, fused follicles were found in some cases with CCCA and in almost all LPP cases, 52% and 80% respectively. At the inferior level, a fused follicle was found in only one case, which was LPP. There was no difference in the presence of fused follicles between CCCA and LPP at either the isthmus or inferior level.

Premature desquamation of the inner root sheath was found in most cases of CCCA and LPP, 74% and 60% respectively, with no statistical significance. Dilated eccrine glands were found in 10% of the CCCA group compared to 60% of the LPP group ($p<0.05$).

More follicular INKERs at the inferior level compared to the isthmus level were observed in both groups. At the level of isthmus, follicular INKERs were seen in 31.6% of the CCCA group and 40% of the LPP group. At the inferior level follicular INKERs were found in 52% of the CCCA group and 100% of the LPP group (n.s.).

More naked hair shafts were noted at the inferior level compared to the isthmus level. The presence of naked hair shafts was found more often in LPP cases compared to CCCA cases. These were present in 60% of LPP patients and 16% of CCCA patients at the level of isthmus, and 60% and 37%, respectively, at the inferior level (n.s.).

More intrafollicular lymphocytes at the isthmus level were found compared to the inferior level. At the isthmus level, intrafollicular lymphocytes were found in 60% of LPP cases and 37% of CCCA cases. At the inferior level, no intrafollicular lymphocytes were seen in any cases (n.s.).

The number of plasma cells was more at the isthmus level when compared to the inferior level. Plasma cells were found more often in CCCA cases compared to LPP, 68% and 20% at the isthmus level and 24% and 0% at the inferior level (n.s.). Mucin was found more often at the level of isthmus compared to the inferior level. Mucin was observed in 68% of CCCA cases and 20% of LPP cases at the level of the isthmus, in

24% of CCCA cases and in none of the LPP cases at the inferior level. However, the difference was not statistically significant.

Data on follicular asymmetry can be found in Table 6, and examples of follicles with mild asymmetry and marked asymmetry can be found in Figure 8. Most cases of CCCA and LPP had mild follicular asymmetry, 79% and 60% respectively at the isthmus level and 53% and 60% at the inferior level. There was no statistically significant difference in follicular asymmetry between the two groups.

Data on perifollicular fibrosis can be found in Table 7. At the isthmus level, most cases of CCCA had mild and moderate perifollicular fibrosis, whereas most cases of LPP had moderate perifollicular fibrosis. At the inferior level, most cases of CCCA and all cases of LPP had mild perifollicular fibrosis. Results from both isthmus and inferior levels were not significantly different.

Data on the amount of perifollicular inflammation can be found in Table 8. At the isthmus level, we found that most CCCA cases had mild or absent perifollicular inflammation (89.4%), whereas most (60%) LPP cases had moderate or severe perifollicular inflammation. This difference was statistically significant ($p=0.04$). At the level of the inferior follicle, mild or absent perifollicular inflammation was noted in most cases of both CCCA and LPP (89.4% and 80%) with no significant difference.

The degree of perivascular inflammation can be found in Table 9. At the isthmus level, we found that most CCCA cases (68%) had mild perivascular inflammation, whereas LPP cases had variable perivascular inflammation, with 20% of cases having none, 40% mild, 40% moderate. There was no significant difference between the degree

of inflammation in CCCA and LPP at the isthmus level. At the inferior level, most cases of both CCCA and LPP had no perivascular inflammation, although some cases had mild perivascular inflammation, with no significant difference.

Study 1. Discussion

In 1968, Lopresti et al.[50] described the histologic findings of CCCA, called "hot comb alopecia" at that time, as superficial perifollicular lymphocytic infiltrate with degeneration of ORS, epidermal atrophy with elongated rete ridges, with fibrotic replacement of the follicular unit sparing the arrector pili muscle. In 1992, Sperling and Sau[51] proposed the term "follicular degeneration syndrome" (FDS) and described additional, unique findings that they felt were not found in other scarring alopecias, including premature desquamation of the IRS and migration of the hair shaft through the ORS. Other histologic findings found in, but not limited to, CCCA include a mononuclear cell infiltrate and lamellar fibroplasia at the level of the isthmus, disintegration of the follicular epithelium, "naked" hair shafts with surrounding foreign body giant cell reaction, and replacement of the entire follicle by thick fibrous tracts.[51] In 1993, Nicholas et al.[52] described histologic findings similar to those previously described in hot comb alopecia and FDS, with perifollicular chronic inflammatory cells, perifollicular fibrosis in a lamellar pattern, naked hair shafts within the dermis, and eventual total replacement of hair follicles by fibrosis. They described these findings under the term "chemically induced cosmetic alopecia".

Similar to what was previously reported in CCCA cases, a decrease in density of terminal and vellus follicles, a decreased or absent number of normal follicular units, perifollicular lymphocytic inflammation with perifollicular mucinous fibrosis, PDIRS, loss of sebaceous glands, a decreased number of normal follicular units, follicular epithelial thinning, follicular asymmetry, naked hair shafts, plasma cells, increase fibrotic tracts and fusion of follicular epithelium of several follicles. In contrast to Lopresti, epidermal atrophy or elongated rete ridges was not seen. INKERs and lymphocytes within the follicular epithelium were found in some cases.

In 1996, Headington[57] described PDIRS as a non-specific finding present in a variety of scarring alopecias. Headington also described histologic findings similar to those described by Sperling and Sau, including primary follicular scarring with decreased follicular density, loss of sebaceous epithelium, follicular fusion and slight perivascular lymphocytic inflammation. Late-stage lesions were characterized by destruction of pilosebaceous units, dermal scarring, and dermal lymphocytic and plasma cell infiltration. The concept that PDIRS was not a specific finding in CCCA was supported by Horenstein and Simon in 2007.[65] However, Sperling proposed in 2007 that PDIRS as an early finding, without significant inflammation or fibrosis, is distinctive of CCCA.[66]

PDIRS was observed in 74% of patients with CCCA cases and 60% of patients with LPP, consistent with Headington's and Horenstein and Simon's assertion that this finding is non-specific for any particular cicatricial alopecia. This is probably the result of cell-mediated injury to the follicular epithelium.[65] PDIRS was not found in LPP cases with duration of disease less than 2 years or with no perifollicular inflammation.

However, PDIRS was seen in many cases of CCCA with duration of disease less than 2 years, even with absent perifollicular inflammation. Our findings support Sperling's assertion that the presence of PDIRS in early lesions without significant inflammation is distinctive of CCCA.

The histopathologic findings of LPP were described by Ackerman et al. in 1988 as compact orthokeratosis, zones of hypergranulosis around hair follicles, and a band-like infiltration of lymphocytes.[74] In 1992, Mehregan et al.[21] studied 45 cases of LPP and described perifollicular lymphocytic inflammation at the infundibulum and isthmus, which, in most cases, did not involve every hair follicle. Loss of elastic fibers, follicular plugging, vacuolar degeneration of the ORS, cytoid bodies and fibrosis were found in over 50% of cases. Interfollicular epidermal involvement was found in less than 10% of cases. In 2003, Chieregato et al.[25] reported findings from 30 cases of LPP, described as a band-like lymphocytic infiltrate with destruction of the follicular basement membrane and the ORS between the infundibulum and isthmus. They also described hypergranulosis, hyperkeratosis, acanthosis, degeneration of basal keratinocytes and destruction of epidermal basement membrane in the epidermis. In chronic lesions, they found a basophilic fibrous stroma with reduction of sebaceous glands and arrector pili muscles and the presence of colloid bodies.

In 2008, Tandon et al.[20] reviewed 27 LPP cases and reported lymphocytic perifollicular and perivascular inflammation, marked reduction of hair density with loss of vellus hairs, reduction or complete absence of arrector pili muscles, and complete absence of sebaceous glands of involved follicles (in most cases). Contrary to other

reports, they found PDIRS in only one case, follicular plugging in a few cases and no significant interfollicular epidermal change. They also described mucinous perifollicular fibroplasia in one third of cases.

In this study, LPP cases were found to have moderate to severe perifollicular and perivascular lymphocytic inflammation with perifollicular mucinous fibrosis, follicular INKers, naked hair shafts, follicular asymmetry and PDIRS. There was loss of sebaceous glands in most of our cases and absent normal follicular units in all. In contrast to the literature, no vacuolization of the ORS, or epidermal involvement was noted. There was no parakeratosis, hyperkeratosis, hypergranulosis or follicular plugging. Dilation of eccrine ducts in 60% of LPP cases was observed, which has not been reported. In contrast to intrafollicular lymphocytes in 60% of our patients with LPP, the presence of intrafollicular lymphocytes has not been reported in LPP.

Histopathologic findings of CCCA and LPP are compared in Table 10. A previous study in 2005 suggested that lymphocytic alopecias are not distinguishable by histopathologic findings.[18] An attempt was made to see if distinctive findings can be identified which maybe specific for either CCCA or LPP. Only patients who were evaluated and had a biopsy by a single clinician, Dr. Goldberg, in order to ensure the clinical diagnoses of CCCA and LPP were included. The site of biopsy in all cases was chosen from the most active area of the affected scalp.

Nineteen cases of CCCA and 5 cases of LPP were evaluated. The limited number of subjects in this study was due to the fact that many cases presented with late or end stage disease, and that early in the study period slides were routinely *cut vertically* rather

than horizontally (as required for quantification). Among all the histopathologic features collected, there were four that showed a statistically significant difference between the LPP and CCCA groups (Table 11). These were the presence of normal follicular units, complete loss of sebaceous glands, dilation of eccrine ducts and perifollicular inflammation. The presence of normal follicular units unaffected by the scarring process suggested the diagnosis of CCCA. Normal follicular units in 58% of CCCA cases and in none of LPP cases. The morphologically affected follicular units exhibited diminished or absent sebaceous glands and a decreased number of follicles per unit.

Similarly, CCCA exhibited less loss of sebaceous glands (complete loss of sebaceous glands was found in 10% of CCCA cases vs. 60% of LPP cases). The remaining cases of CCCA had only partial or no loss of sebaceous glands. The finding that in CCCA some follicular units are spared and sebaceous glands are often retained suggests that pathogenic pathways that lead to scarring via loss of sebaceous glands, such as the PPAR gamma pathway, may not be as important in CCCA as in LPP. While sparing of some hair follicles has also been reported in LPP,[21] the authors did not comment on the presence or absence of sebaceous glands.

This study is the first to describe dilation of eccrine ducts in LPP. Dilation of eccrine ducts was present in the reticular dermis at the level of the inferior hair follicle in 60% of LPP cases, without significant association of the presence of dilation of eccrine ducts and degree of inflammation or duration of disease. The finding of dilated eccrine ducts has been previously reported in CCCA, although it appeared to play no role in the etiology of the hair loss.[75, 76] In 2012, Miteva and Tosti[76] found dilated syringoma-like

eccrine ducts in over half of their CCCA cases and reported it to be a clue to the diagnosis. In contrast, the dilation of eccrine ducts was found in 60% of LPP cases and 10% of CCCA cases in this study.

The role of dilation of eccrine ducts in the pathogenesis of CCCA is unknown. It may represent a reactive process, similar to that which has been reported in a variety of benign neoplasms, malignant neoplasms, and non-scarring alopecia, including patterned hair loss[77] and alopecia areata.[78] In patterned alopecia and alopecia areata, eccrine ductal proliferation was found as a reactive process in the reticular dermis along with miniaturized follicles and telogen follicles. These ductal structures varied in size and degree of cystic dilation.[77,78]

Perifollicular inflammation at the level between the infundibulum and isthmus, with sparing of the lower follicle, has been described in both LPP and CCCA. However, the degree of inflammation in both conditions has not been compared. In this study, perifollicular inflammation was found to be more in intensity in LPP in comparison to CCCA. Moderate to severe perifollicular inflammation was found in 60% of LPP cases, whereas 90% of CCCA cases showed either mild or no perifollicular inflammation. The difference of degree of perifollicular inflammation may be the result of the difference in duration of disease between both groups, 3.8 years in LPP cases and 7.4 years in CCCA cases. In the author's opinion, this difference in perifollicular inflammation reflects the clinical presentation of both diseases; evident perifollicular erythema in active lesions of LPP and subtler perifollicular erythema in CCCA. The perifollicular inflammation in lymphocytic scarring alopecias is a cell-mediated immune reaction to an unknown but

specific follicular antigen.[35] The antigens that cause both diseases may be different, explaining the difference in perifollicular inflammation.

In addition to the abovementioned features, there were histologic findings that differed between CCCA and LPP but did not reach statistical significance. These findings included the absence of vellus follicles, the presence of naked hair shafts, presence of follicular INKers and intrafollicular lymphocytes, which favored the diagnosis of LPP, while the presence of plasma cells and mucin, which favored the diagnosis of CCCA. In CCCA, there is a marked reduction of terminal hair follicles with some persistence of vellus hair follicles.[49,71,79] In LPP, Tandon et al.[20] described a reduction of terminal hair follicles and disappearance of vellus follicles. However, in this study marked reduction of terminal and vellus follicles was observed in both CCCA and LPP. However, the absence of vellus follicles was found more often in LPP supporting the findings reported by Tandon et al. This suggests that in LPP the putative antigen that induces the inflammatory process leading to permanent follicular destruction is present not only in the terminal follicle but in the vellus follicle as well.

Naked hair shafts are remnants of destroyed hair follicles that are often found in fibrotic streamers, usually surrounded by giant cells forming foreign body granulomas. Naked hair shafts have been described in many types of scarring alopecia including CCCA[48,80] and LPP[57]. Miteva and Tosti described them in most cases of CCCA.[76] At the isthmus level, naked hair shafts were seen in 60% of LPP cases and 16% of CCCA cases in this study (n.s.). The percentage of CCCA cases with naked hair shafts in this series was lower than that reported in the literature, suggesting that this finding might not be

specific to CCCA. The absence of naked hair shafts in our CCCA cases might be due to the late stage of disease, as naked hair shafts may eventually degenerate and be replaced by fibrotic scars. However, cases with less than three follicles were excluded in this study.

Follicular INKers have been described in LPP in association with lichenoid lymphocytic infiltration at the level of the follicular infundibulum and isthmus.[20,79,81] In CCCA, in contrast, they were not described as a diagnostic finding.[49] Follicular INKers were found in all LPP cases and in 50% of CCCA cases at both the isthmus and the level of the inferior follicle. There was no statistical significance between this finding in CCCA and LPP. Furthermore, the presence of follicular INKers correlated with the degree of perifollicular inflammation; follicular INKers were found more in cases with moderate to severe perifollicular erythema compared to cases with absent or mild perifollicular erythema. Thus, follicular INKers appear to reflect the degree of perifollicular inflammation and may not be independent distinguishing feature between LPP and CCCA.

Intrafollicular lymphocytes were not specifically described as findings in either CCCA or LPP.[20,49,73] The finding of intrafollicular lymphocytes in 60% of LPP cases and 37% of CCCA cases in this study represents another histologic finding in lymphocytic scarring alopecia. Surprisingly, the presence of intrafollicular lymphocytes did not parallel the degree of follicular inflammation. Intrafollicular lymphocytes were found in 41% of all CCCA and LPP cases with absent or mild perifollicular inflammation, and in 43% of all CCCA and LPP cases with moderate to marked perifollicular inflammation. It

may be that any amount of perifollicular inflammation is sufficient to enter the epidermis, or alternatively that the amount of perifollicular inflammation is dynamic; cases with mild inflammation may have had heavier inflammation at some point in time. This remains to be elucidated.

Plasma cells have been described as a histologic feature in CCCA,[11, 20, 57] folliculitis decalvans,[11] alopecia syphilitica[82] and erosive pustular dermatosis of the scalp,[83] but not as often in LPP.[84] In CCCA, perifollicular plasma cells were found along with lymphocytes at levels between the infundibulum and isthmus.[11, 48, 57] Plasma cells represent a chronic inflammatory process, as they are found in later stages of CCCA and folliculitis decalvans.[11] In this study plasma cells were found at the isthmus level in 68% of CCCA cases and 20% of LPP cases. This finding may reflect the longer duration of disease in CCCA cases compared to LPP.

Mucinous perifollicular fibrosis has been described in the upper dermis in LPP, but not in CCCA.[20, 25] In LPP, perifollicular mucin was found in the upper dermis in up to 1/3 of the cases but was absent in the interfollicular dermis.[20] It was described in chronic lesions together with perifollicular fibrosis.[20, 25] Perifollicular mucin was observed in 20% of LPP cases and 68% of CCCA cases at the level of the isthmus. Mucin was absent from the interfollicular dermis in all cases. The percentage of cases with perifollicular mucin in our LPP cases was not different from those reported in the abovementioned publication.[20] In addition, the finding of perifollicular mucin in many of our CCCA cases suggests that this is not a distinguishing feature between the two diseases.

Many of our histologic findings were previously described in both LPP and CCCA and did not distinguish the two diseases. These include the presence of fibrous tracts at the isthmus and inferior levels, follicular asymmetry, the presence of fused follicles and perifollicular fibrosis.

Fibrotic tracts lacking elastic fibers were described in scarring alopecia.[72] Variable numbers of fibrotic tracts were found in our cases, with no difference between LPP and CCCA. The fibrotic tracts were the result of permanent follicular damage destroying follicular stem cells at the bulge region. There was no linear association of the number of fibrotic tracts and the number of remaining hair follicles. This random number of the fibrotic tracts raise the hypothesis that not every destroyed hair follicle results in a tract, and that after a certain period of time it is possible that fibrotic tracts are no longer evident with H&E staining.

Follicular asymmetry has been described in scarring alopecia, non-scarring alopecia, and in normal scalp of African American patients.[76] In CCCA, the hair shaft occupies an asymmetric position within both terminal and vellus follicles with an asymmetric ORS.[76] The hair shaft may then migrate toward one side and ultimately perforate through the ORS.[51, 80] Two or three follicular infundibula often fuse and form compound structures.[76] In LPP, Headington described eccentric hair shaft placement within the follicle and fusion of damaged infundibula creating a compound follicle.[57, 85] Follicular asymmetry along with compound follicles was observed in both CCCA and LPP. There was no difference in the level or character of compound follicles among the two diagnoses. These results support the notion that follicular asymmetry and follicular

fusion are histologic findings in lymphocytic scarring alopecia in general, and are not the distinguishing findings between LPP and CCCA.

Perifollicular fibrosis is a feature of scarring alopecia. In CCCA, concentric perifollicular lamellar fibrosis has been found at the level of the isthmus.[48,51,57,85] In LPP, concentric lamellar fibrosis was also described along the follicle at the level of infundibulum and isthmus.[20,57] Neither study commented on its degree. Perifollicular fibrosis was noted in the majority of our cases – all LPP cases and 90% of CCCA cases, at both the isthmus and the level of the inferior follicle (n.s.). There was no correlation between the degree of perifollicular fibrosis and the degree of perifollicular inflammation. The presence of perifollicular fibrosis was a diagnostic finding of scarring alopecia, but the presence and degree of fibrosis did not distinguish between CCCA and LPP.

No epidermal involvement was found in any cases of LPP or CCCA. These findings differed from those of Tandon et al.[20] and Annessi et al.,[81] who described interfollicular lichenoid infiltration along with hypergranulosis, vacuolar changes and INKers in some LPP cases. No epidermal thinning was observed in CCCA cases, which is different from the epithelial atrophy described by Lopresti et al.[50] Although fewer cases were available to compare, epidermal involvement was not a helpful distinguishing feature between LPP and CCCA. This may be due to the lower number of cases, as compared to Tandon et al or Annessi et al.

The main limitation of this study was the small sample size. Cicatricial alopecias are rare diseases, and scalp biopsies need to be cut horizontally for adequate

quantification of findings. This limited number of cases hindered the finding of statistical significance for many of the different histologic features of CCCA and LPP. Another limitation of this study was the fact that it was retrospective in nature. Some data in the medical record were missing or briefly described. A prospective study that provides systematic and standardized data collection is needed to confirm our results.

Study 1. Conclusion

CCCA and LPP are lymphocytic scarring alopecia those share many overlapping findings histologically. Clinico-pathological correlation is always required for the definite diagnosis. This study found that the findings of unaffected follicular units, retained sebaceous glands and mild perifollicular inflammation favors a diagnosis of CCCA, and dilated eccrine glands and heavy perifollicular inflammation favor LPP.

STUDY 2. Direct immunofluorescent staining in the distinction between lichen planopilaris and central centrifugal cicatricial alopecia

Study 2. Materials and Methods

A prospective, cross-sectional data analytic study to identify immunofluorescent and immunohistochemical features that distinguish between LPP and CCCA was designed. The study was approved by the Institutional Review Board (IRB) at the Boston University Medical Center under protocol number H-31220.

Patients being evaluated by Dr. Goldberg who had suspected lymphocytic scarring alopecia and required a biopsy were invited to participate. After informed consent was obtained, the patients underwent two biopsies, one for standard diagnostic testing and one for research purposes. Patients being evaluated by Dr. Goldberg who already had a biopsy for standard diagnostic testing were invited to participate by undergoing a biopsy for research purposes (Figure 9). Each research biopsy was transferred to the Skin Pathology Laboratory in Michel's transport medium and was vertically cut in half, with half frozen for immunofluorescent staining for Study 2, and half put in formalin for immunoperoxidase staining for Study 3 (Figure 10).

Each half specimen for Study 2 was embedded in cryo-embedding media and stored at -80 °C until ready for sectioning. They were later cut in four micron cryostat sections and rinsed in PBS. Two hundred ul of immunofluorescent antibodies to IgG, IgM, IgA, C3, and fibrinogen were applied, one per slide, for 30 minutes, rinsed with buffer, mounted with fluorescent mounting medium, coverslipped and stored at 2-8 °C in the dark. One section was stained with H&E for anatomical comparison. All antibodies

were run with positive controls, provided by the Skin Pathology Laboratory.

All direct immunofluorescent stains were interpreted by both Dr. Rattanasirivilai and Dr. Goldberg. The immunofluorescent findings were reported as either positive or negative. Positive findings were subdivided as to the pattern of the staining. The data collection form can be found in the Appendix 2.

Study 2. Inclusion and Exclusion Criteria

Patients who were being evaluated by Dr. Goldberg for standard clinical care who had suspected lymphocytic scarring alopecia were invited to participate. Both patients who required a biopsy for diagnosis and those who already had a biopsy interpreted as lymphocytic scarring alopecia were eligible to participate. Exclusion criteria included those patients whose biopsies did not show scarring alopecia.

Study 2. Sample Size

Two patients were informed consent and agreed to undergo a research biopsy in addition to their diagnostic biopsy. Nine patients, who had already been biopsied for diagnostic purposes, were informed consent and agreed to undergo a research biopsy. We did not exclude any cases from the study. Total of eleven patients participated in this study.

Study 2. Data analysis

Statistical analysis was performed using R, Statistical Software, version 2.14.1 (2011-12-22) and AnalystSoft Inc., StatPlus:Mac, version 2009. Qualitative variables were calculated in percentages and Fisher exact test was used for intergroup comparison. A p-value of less than 0.05 was considered statistically significant.

Study 2. Results

Four patients with CCCA and seven with LPP consented to participate in the study. Once the data was collected, medical records were reviewed. Relevant demographic data is shown in Table 12. Most patients were female, with the exception of two male patients with LPP. All CCCA patients were African American, while all LPP patients were Caucasian. The mean age of patients in the LPP group was higher than in the CCCA group, 54.3 and 40.3 years old, respectively. The mean duration of disease prior to the biopsy in the CCCA group was highly variable from 2 to 20 years, with a mean duration of 7 years. In the LPP group, the duration of disease prior to biopsy ranged from 1 to 7 years, with a mean duration of 3.2 years.

Positive DIF findings were found in 3 of 11 cases, two in LPP (cases LGP-004 and LGP-008), and one in CCCA (LGP-007). The positive reactants were IgG in two cases, C3 in two cases and fibrinogen in one case. The patterns of deposition were varied, including linear, shaggy and granular. The location of deposition varied as well, from the isthmus to the deep perifollicular level (Table 13). Result of DIF along with clinical information including diagnosis, duration of disease, degree of perifollicular erythema, degree of perifollicular inflammation and previous treatments is shown in Table 14.

There was no appreciable difference in duration of disease between the positive and negative cases, although the positive cases tended to have more inflammation and were not previously treated with either intralesional steroid injection or systemic treatment.

Of the seven patients with LPP, two had a positive DIF, cases LGP-004 and LGP-008 (Table 15). In case LGP-004, linear, shaggy deposition of IgG at the perifollicular basement membrane at the inferior portion of hair follicle were noted. There was no positive staining of cytoid bodies or of the interfollicular dermis. All the other stains were negative, as shown in Figure 11.

Shaggy/granular deposition of IgG and C3 at the perifollicular basement membrane of the hair follicle at the isthmus level was found in case LGP-008, as shown in Figure 12. There was no positive staining of cytoid bodies or of the interfollicular dermis, and other stains were negative.

Of the four patients with CCCA, one (LGP-007) had a positive DIF (Table 16). In case LGP-007, shaggy deposition of C3 at the perifollicular basement membrane of the hair follicle at the infundibulum and isthmus levels was observed, as shown in Figure 13. There was no positive staining of cytoid bodies or of the interfollicular dermis. Other stains were negative.

Study 2. Discussion

Previous studies on DIF findings in LPP reported highly variable results. In 1992, Ioannides and Bystryn[67] found immunoglobulins at the DEJ of the follicular epithelium without interfollicular deposits or deposition on cytoid bodies in all 7 LPP cases studied.

These immunoglobulins consisted solely of IgG or IgA, or a combination of IgG and IgA. Linear deposition of a combination of IgG, IgM and fibrin at the BMZ were sometimes present (Table 17). The details of where along the hair follicle the immunoreactants were located were not reported. Their DIF results were different from those reported in lichen planus (Table 18). While LPP and LP are commonly thought to share the same etiology, this difference raised questions about the relationship between LPP and LP.

During the same year, Mehregan et al.[21] described deposits of immunoglobulin at the DEJ of follicular epithelium in some cases of LPP, and interfollicular deposits and cytoid body deposition in many, similar to the findings in LP (Table 18). Further details of the anatomical location of the positive findings in follicular epithelium were not given. From their findings, they supported the idea of LPP and LP sharing the same etiology but manifest on different areas of the body. In 2003, Chieregato et al.[25] studied DIF findings in 30 cases of LPP and found linear fibrinogen deposition and positive cytoid bodies with IgM deposition up to 43% of cases at dermoepidermal junction, and non-specific deposition in the rest (Table 17) [86-88]. There were no details provided on the non-specific cases. Their results were similar to Mehregan et al. [21] None of these studies discussed contributing factors to positive or negative DIF findings, or which antigens might be being targeted.

The sample size of this study was limited by the number of patients who consented to participate. A total of 11 patients consented to participate, 4 who were found to have CCCA, and 7 LPP. Our DIF results were different from those previously reported. Only a few cases with strongly positive DIF were observed in both the LPP and

the CCCA groups. Only two of seven cases of LPP had positive DIF. Both had IgG staining of the perifollicular BMZ in combination with a second reactant (fibrinogen in LGP-004 and C3 in LGP-008). However, the pattern and location of deposition of IgG in the cases was different; one was linear/shaggy at the inferior portion of the follicle, whereas the other had shaggy/granular deposition at the isthmus portion of the follicle. Only one of four CCCA cases had positive DIF. Unlike in LPP there was no IgG staining. Positive C3 staining in a shaggy/granular pattern at the isthmus level were observed. None of the positive cases of both diseases exhibited positive staining of cytoid bodies or of the interfollicular basement membrane. There was no consistency in the antibody deposited or the pattern of the deposition. These findings in LPP were similar to those of Ioannides and Bystryn, with IgG deposits at the follicular epithelium without IgG interfollicular or colloid body staining. These cases did not exhibit a specific anatomic follicular location of immune reactant deposition.

Positive DIF was found to be associated with moderate perifollicular inflammation, both from a clinical standpoint and what was found on H&E staining. The association of a positive DIF and moderate perifollicular inflammation is statistically significant, $p=0.006$. This association has not been previously described. While the cause is unknown, the patients with moderate inflammation may represent those with active disease; hence immunoglobulin deposition was found. In addition, all of the positive cases had not received treatment with intralesional steroid injection or systemic agents. These treatments might reduce the degree of inflammation and thus deposition of

immunoglobulin. Of note, the association of previous treatment and positive DIF was not statistically significant.

The duration of the disease in the positive cases was 2, 2.5 and 3 years. There was no appreciable difference in duration of disease between the positive and negative cases. None of the cases with duration of disease longer than 3 years had positive DIF staining, although some cases with duration of disease 1 or 2 years with mild inflammation also had a negative DIF. This shows that duration of disease may not be related to disease activity, and that duration of disease activity differs from case to case. Those cases with negative findings could be due to low disease activity from partial treatment, long standing disease prior to the biopsy, or inflammation that has subsided due to the natural course of the disease. With the limited number of positive cases, no pattern of association between the type of the immunoglobulin deposit and duration of disease was observed.

Study 2. Conclusion

There are no DIF findings that can reliably distinguish LPP from CCCA. The presence of a positive DIF is correlated with the amount of inflammation ($p=0.006$). It is possible that long duration of disease, disease activity, and treatment of disease affect the sensitivity of DIF. Future studies should be limited to patients with new, untreated and active disease.

STUDY 3. The immunohistochemical distinction between lichen planopilaris and central centrifugal cicatricial alopecia

Study 3. Materials and Methods

Immunohistochemical staining was used to compare T-cell subsets and Langerhans cells in LPP and CCCA. A prospective, cross-sectional data analytic study was designed to identify both immunohistochemical features that distinguish between LPP and CCCA. The details of patient selection can be found in the Material and Methods of Study 2.

After the biopsy was obtained, the tissue was transferred to the Skin Pathology Laboratory in Michel's transport medium and was vertically cut in half, with half frozen for immunofluorescent staining for Study 2, and half put in formalin for immunoperoxidase staining for Study 3. Each half of vertically cut specimen for Study 3 was fixed in formalin, embedded in paraffin, cut vertically, and stained for the following: CD3, CD4, CD8, CD1a, CCR6, FoxP3 and H&E. Details can be found in the Appendix 3. All immunohistochemical stains were run with a positive control (tonsil), provided by the Skin Pathology Laboratory. Details of the antibodies used can be found in the Appendix 4.

Data interpretation was done by both Dr. Rattanasirivilai and Dr. Goldberg, who were blinded from diagnosis at the time of data collection. For each case, the area with the greatest amount of inflammation on the H&E slide were chosen and photographed at 200x (Olympus DP26 Digital Camera). This same area was identified for each antibody and photographed. The number of positive cells in the photographs were counted

manually for each antibody. A positive cell was considered when greater than or equal to half of the cell was present in the section.

Results were reported as the number of each antibody positive cells per photographed 200x field, the percentage of CD4, CD8, CCR6 and FoxP3 positive cells compared to CD3 positive cells, the ratio of CD4: CD8 positive cells, and the ratio of CD1a:CD3 positive cells. Cases were then unblinded and grouped by diagnosis into LPP and CCCA. The LPP group was also further subdivided by duration of disease. Medical records were reviewed for relevant demographic and clinical data, including age, sex, duration of disease and previous treatment.

For each group, the mean number of positive cells for each antibody, the mean percentages of CD4, CD8, CCR6 and FoxP3 (which mark T-helper cells, cytotoxic T-cells, Th17 cells and regulatory T cells, respectively), and the mean ratios of CD4:CD8 and CD1a:CD3 were calculated. In order to determine if there was a difference in the degree of inflammation in different stages of disease, the LPP group was divided by duration into greater or less than 1 year.

Study 3. Inclusion and Exclusion Criteria

Patients who were being evaluated by Dr. Goldberg for standard clinical care and who suspected to have lymphocytic scarring alopecia were invited to participate. Both patients who required a biopsy for diagnosis and those who already had a biopsy interpreted as lymphocytic scarring alopecia were eligible to participate. Exclusion criteria included those patients whose biopsies did not show scarring alopecia.

Study 3. Sample Size

Two patients agreed to undergo a research biopsy in addition to their diagnostic biopsy. Nine patients who had already been biopsied, and had a confirmed diagnosis of LPP or CCCA, agreed to undergo a research biopsy. One 4-mm punch biopsy was obtained from each case.

Study 3. Data analysis

Statistical analysis was performed using R, Statistical Software, version 2.14.1 (2011-12-22) and AnalystSoft Inc., StatPlus: Mac, version 2009. Quantitative variables were calculated in means and range of minimum to maximum, and T-test was used for intergroup comparison of means, percentages and ratios. Qualitative variables were calculated in percentages and Fisher exact test was used for intergroup comparison. A p-value of less than 0.05 was considered statistically significant.

Study 3. Results

Patients recruited in this study were identical to the patients in Study 2 (see page 47). Examples of the chosen area with the most inflammation and the photographs of the immunohistochemical stains are shown in Figure 14. The chosen area for each case was either perifollicular or perivascular, superficially at the level of the isthmus. The number of positive cells for all antibodies is shown in Table 21.

The number of CD3 positive cells was highly variable, ranging from 9-340 cells/200x field. There were three cases, LGP-002, LGP-005 and LGP-010, with less than 50 CD3 positive cells/200x field. These were cases that either had longer duration of disease (LGP-005, 7 years and LGP-010, 20 years) or had less perifollicular erythema clinically (LGP-002). The number of CD3 positive cells was higher than the other antibodies studied. There were 5 cases with a higher number of CD4 positive cells compared to CD8, 5 cases with a higher number of CD8 positive cells compared to CD4, and one case with equal numbers of CD4 and CD8 positive cells. The number of CCR6 positive cells was less than the number of both CD4 and CD8 cells in 6 cases, greater than the number of CD4 positive cells in 3, and greater than the number of CD5 positive cells in 2. The presence of FoxP3 positive cells was very limited, with a mean of 2 cells. CD1a positive cells were counted from the chosen area in the dermis, and not from the follicular epithelium or epidermis. The number of CD1a positive cells ranged from 1-27 cells/200x field with mean of 7 cells.

Tables 22 and 23 show the number of positive cells grouped by diagnosis. The comparison of the mean number of CD3, CD4, CD8, CCR6, FoxP3 and CD1a positive cells in LPP and CCCA is shown in Figure 15. The LPP group had slightly higher means of CD3, CD4, CD8 and CCR6 positive cells, although this was not statistically significant. The mean numbers of FoxP3 and CD1a positive cells in both groups was similar.

The percentages of the different T-cell subsets compared to the total number of CD3 cells and the ratios of CD4 positive to CD8 positive cells and CD1a positive to CD3

positive cells for LPP is shown in Table 24. CD4 positive cells outnumbered CD8 positive cells in 3 cases, and CD8 outnumbered CD4 in 4. One case exhibited equal numbers of CD3 positive and CD8 positive cells. In all but three cases, there were less CCR6 positive cells than CD4 or CD8. FoxP3 positive cells were scarce except for in one case. The CD4:CD8 ratios were highly variable. The ratio of CD1a to CD3 cells was low in all cases. There was no statistical significance in these differences.

The percentages of the different T-cell subsets compared to the total number of CD3 cells and the ratios of CD4 positive to CD8 positive cells and CD1a positive to CD3 positive cells for CCCA is shown in Table 25. CD4 positive cells outnumbered CD8 positive cells in 2 cases, and CD8 outnumbered CD4 in 2. There were less CCR6 positive cells than CD4 or CD8 in all 4 cases. FoxP3 positive cells were scarce in all cases. The CD4:CD8 ratios were highly variable. The ratio of CD1a to CD3 cells was low in all cases. There was no statistical significance in these differences.

The means ratios of CD4 to CD8 positive cells and CD1a to CD3 positive cells in LPP and CCCA are shown in Figure 16. The mean CD4 to CD8 ratio in the CCCA group was almost twice that of the LPP group, shown in Figure 17. The mean CD1a to CD3 ratio was about the same in both groups.

The number of positive T-cell subsets and CD1a positive cells for LPP with duration of disease less than 1 year and greater than 1 year are shown in Tables 26 and 26, respectively.

Percentages of CD4, CD8, CCR6 and FoxP3 positive cells compared to the number of CD3 positive cells and the ratios of CD4 to CD8 positive cells and CD1a to

CD3 positive cells in LPP with duration of disease less than 1 year and greater than 1 year are shown in Tables 27 and 28, respectively, and compared in Figure 12. In the two cases of LPP with duration of disease less than 1 year, the percentage of CD8 positive cells compared to CD3 positive cells was higher than the CD4 percentage.

The percentages of CD4, CD8, CCR6 and FoxP3 positive cells compared to the number of CD3 positive cells in both LPP with duration of disease less than 1 year, greater than one year, and CCCA, are shown in Figure 18. The percentage of CD4 in LPP with duration of disease less than 1 year is lower than other groups. The mean percentage of CD8 in both groups of LPP was higher in comparison to CCCA. However, this difference was not statistically significant.

The mean CD4:CD8 ratios in each group are shown in Figure 19. The mean CD4:CD8 ratios in LPP with duration of disease less than 1 year is lower than in the other groups, and it increased in LPP with duration of disease over 1 year, approaching the CCCA group and what is found in the normal hair follicle.

The mean CD1a:CD3 ratio in each group is shown in Figure 20. The CD1a:CD3 in LPP with duration of less than 1 year is almost twice that of LPP with duration over 1 year and CCCA. There was no statistical significance.

Study 3. Discussion

LPP and CCCA are scarring alopecias that manifest with lymphocytic perifollicular infiltration. The immunopathogenesis of the inflammatory cells in these diseases has not been well studied. It is possible that these lymphocytes are the primary

mediators of follicular destruction via a cell-mediated immune reaction to an endogenous or exogenous follicular antigen.[89-91]

In cell-mediated immune reactions in general, Langerhans cells play a role as antigen-presenting cells that present an as yet unknown antigen to CD4 positive T-lymphocytes. Following antigen recognition, CD8 positive cells are activated and undergo clonal expansion. These activated lymphocytes release cytokines and chemokines, such as interleukin (IL)-2, IL-4, IL-10, interferon-gamma, tumor necrosis factor alpha and transforming growth factor beta. These cytokines attract and regulate the trafficking of more lymphocytes to the affected area. As a result, the inflammatory process induces keratinocyte apoptosis. If this cell-mediated immune process at the hair follicle affects the bulge area where multipotent stem cells reside, permanent follicular destruction and scarring alopecia may be the consequence.[89-91]

Previous studies[34, 92] evaluating the cellular components of the inflammation in LPP have supported the theory of a cell-mediated immune process. One study by Mobini in 2005 showed an increase in CD8 T lymphocytes compared to CD4.[34] They found a reverse in the CD4:CD8 ratio of 1:1-2, whereas in the normal hair follicle it is 2:1.[93] They also found a decrease in the number of Langerhans cells in the bulge region in affected, as compared to normal follicles.[34]

The work of Hutchens et al.[92] also supported a cytotoxic inflammatory process in LPP by comparing Langerhans cell concentrations in LPP and traction alopecia. There was a significantly higher ratio of Langerhans cells to T lymphocytes in LPP compared to what was found in traction alopecia (1.3 vs 0.6). This demonstrated the usefulness of the

ratio of CD1a positive cells to CD3 positives cells and suggested separate inflammatory pathways for LPP and traction alopecia, both of which result in scarring alopecia. In LPP, Langerhans cell triggered a cytotoxic inflammatory process and destroyed the hair follicle including the bulge region, home to multipotent stem cells, resulting in permanent hair loss. In traction alopecia, less evident inflammatory cells despite the presence of Langerhans cells suggested a different pathogenesis. It has been suggested that the helical shape of the hair shaft in African Americans is more vulnerable to traumatic injury, and likely to be the cause of chronic follicular damage in traction alopecia leading to permanent hair loss.[76]

A New Zealand black mouse model (NZB/KN) established for the study of chronic rheumatoid arthritis and systemic lupus erythematosus was found to also have autoimmune-induced scarring alopecia.[94] These mice had normal hair morphogenesis and hair cycling in early life and developed progressive alopecia as they grew. The study of inflammatory cells around the hair follicles revealed increased number of CD3, CD4 and CD8 positive cells around the bulge of hair follicles and blood vessels.[94] This study supported the notion that perifollicular lymphocytic inflammation involving the hair bulge, and not an exogenous triggering antigen, contributes to lymphocytic scarring alopecia.

In this study, variable numbers of CD3, CD4 and CD8 positive cells in inflammatory areas were found in both conditions. When comparing between the CCCA group and the LPP group, the number and percentage of CD8 positive cells in the LPP

group was higher than in the CCCA group. There is no previously published data with which to compare these findings.

In this study, the CD4:CD8 ratio in CCCA (2.15:1) was similar to what is found in the normal hair follicle (~2:1).[93] On the contrary, the CD4:CD8 in LPP in this study was 1.25:1, suggesting that there may be a cytotoxic inflammatory process in this condition. This finding is consistent with previous data by Mobini.[34] However, the difference of these ratios was not statistically significant.

LPP cases were stratified by duration of disease and found a trend in the altered CD4:CD8 ratio. In early disease (duration <1 year), the CD4:CD8 was as low as 0.5, and it increased to 1.55 in cases with duration >1 year. This finding has not been described in the literature and suggests a role of a cytotoxic inflammatory process in early disease. The inflammatory process remodels as the disease progresses, and the role of cytotoxic inflammation in later lesions was not as significant as in early lesions. This was suggested by the change in CD4:CD8, which evolved toward what is found in normal hair follicles.

The number of CD1a positive cells in the CCCA and LPP groups were not different, but the ratio of CD1a:CD3 in the LPP group was slightly higher. The CD1a:CD3 represents the role of Langerhans cell in lymphocytic scarring alopecia. This ratio in LPP was different from traction alopecia.[92] In this study, the difference of CD1a:CD3 between LPP and CCCA was not statistically significant.

When stratified by duration of disease, we found that the CD1a:CD3 in LPP with duration <1 year (0.14) was almost twice that of LPP with duration >1 year (0.08) and

CCCA (0.07). These findings were consistent with data published by Hutchens et al.[92] in 2011 comparing CD1a:CD3 in LPP and traction alopecia, which suggested role of CD1a as antigen-presenting cells in LPP, especially in early disease. These findings are consistent with previous data by Pozdnyakova and Mahalingam showing the presence of CD1a cells in early stages of scarring alopecia.[95] Of note, this latter study compared the findings among stages of disease but did not distinguish between different types of scarring alopecia. The number of cases was insufficient to stratify CCCA by duration of disease in this study.

Recently, subtypes of CD4 positive cells were found to play a key role in T lymphocyte mediated inflammatory processes such as psoriasis. These cells include Th17 positive lymphocytes and regulatory T lymphocytes (Tregs).[96-98] Th17 lymphocytes differentiate from naïve CD4 positive cells and respond to certain extracellular pathogens and fungi. Th17 lymphocytes express CCR6, IL-23R, CXCR4, CD161 and multiple CD49 integrins and produce IL-17 and IL-17F.[99] Other dermatoses with increased Th 17 positive lymphocytes include atopic dermatitis, drug-induced hypersensitivity syndrome and acute generalized exanthematous pustulosis.[100, 101] Increased Th17 lymphocytes and its cytokines are associated with alopecia areata and alopecia universalis.[100 102]

CCR6, a marker of Th17 lymphocytes, is a transmembrane protein regulating migration and recruitment of T cells during inflammation and immunologic responses. We found CCR6 positive lymphocytes in both CCCA and LPP. This finding suggests a role of Th17 lymphocytes in lymphocytic scarring alopecia. The Th17 lymphocytes may regulate the migration and recruitment of other T cells in the follicular inflammatory

process, which subsequently results in follicular destruction and permanent hair loss. This finding is important, as the finding of Th17 lymphocytes in other dermatoses, such as psoriasis, has had therapeutic implications. Ustekinumab, a monoclonal antibody targeting Th17 lymphocyte activators, IL-12 and IL-23, blocks Th17 lymphocyte differentiation and amplification. Ustekinumab has been shown to significantly improve psoriasis.[103] The finding of CCR6 in cicatricial alopecia is preliminary, and further studies are needed to confirm their presence and perhaps identify elevated levels of Th17 cytokines. If confirmed, targeting of Th17 lymphocytes may be a promising strategy for a novel treatment of LPP and CCCA.

Treg cells are a distinct, mature subpopulation of T lymphocytes derived from naïve CD4 positive T lymphocytes. They account for 5-10% of the total CD4 positive T cell population. They are characterized by expression of the Forkhead family transcription factor 3 (FoxP3), which is a transcription factor crucial in Treg cells development and function.[104] Tregs serve to regulate the cutaneous immune response, maintain immune tolerance and control autoreactive T cells. Dysfunction of Tregs could significantly contribute to autoimmune disease susceptibility.[105] An increased number of Tregs were found in psoriasis, but they had impaired function.[106] Other dermatoses with increased numbers of impaired Tregs include atopic dermatitis and systemic lupus erythematosus.[107] Tregs were found to be diminished in cutaneous lesions of thymoma-associated autoimmune disorder.[108]

In alopecia areata, Tregs play a significant role in immunopathogenesis as pro-inflammatory cells.[105] Prior studies found a decreased number of, and defective

function[102] of, Tregs in lesional skin of alopecia areata.[109] Decreased numbers of Tregs and defective Treg function may contribute to a decreased threshold of autoreactive perifollicular inflammation and subsequent alteration of the follicular cycle. The function of Tregs in peripheral blood of alopecia areata patients has also found to be defective.[102, 110] There have been no previous studies on the role of Tregs in scarring alopecia.

The main purpose of this study was to establish any difference in Tregs between LPP and CCCA. The FoxP3 positive cells were similarly rare in patients with LPP and CCCA, and there was no significant difference between the groups. There was no difference of FoxP3:CD3 among the LPP and the CCCA groups. The limited number of Treg may decrease protection against an autoreactive inflammatory process such as lymphocytic scarring alopecia. This hypothesis remains to be elucidated. To establish the pathogenic role of Tregs in scarring alopecia, future studies should compare the number of Tregs, and the ratio of Tregs and Treg secretory cytokines such as IL-12, IFN-γ and TNF-α in actively involved hair follicles vs. normal hair follicles.

The major limitation of this study was the limited number of patients who consented to participate. This curtailed our ability to establish statistical significance. Moreover, some of our cases had limited inflammation, presumably either due to prior treatment or to the fact that they were biopsied at a late stage of disease. In addition, the inflammatory cells were counted from chosen 200x fields. These may not representative the disease process as a whole. Although biopsies were obtained from perceived active areas, one could argue that the site of the biopsy on the scalp poses a similar representation issue, as inflammation in scarring alopecia can be focal and is subject to

sampling error. Future studies on inflammation in cicatricial alopecia may be limited to untreated patients with new onset and active disease.

Study 3. Conclusion

No significantly distinctive T lymphocyte populations that could differentiate between CCCA and LPP were found. However, several differences between the two diseases were noted. LPP had higher numbers and percentages of CD8 positive cells compared to CCCA. The CD4:CD8 ratio increased with duration of disease, approaching proportions similar to those found in the normal hair follicle and CCCA. A higher CD1a:CD3 in LPP compared to CCCA and a trend of decreased CD1a:CD3 over time, approaching the ratio found in CCCA was observed. Our data confirmed the presence of CD8 positive lymphocytes and Langerhans cell as antigen presenting cells in early LPP, suggesting a cytotoxic inflammatory process at this stage of the disease. The study of Th17 lymphocytes and Tregs in scarring alopecia was pioneered. Th17 lymphocytes may have a role in pathogenesis of both CCCA and LPP. Insufficient function of Tregs may also be contributing.

In conclusion, there are histopathologic findings that help in the distinction between LPP and CCCA. The findings of unaffected follicular units, retained sebaceous glands and mild perifollicular inflammation favor a diagnosis of CCCA, and dilated eccrine glands and heavy perifollicular inflammation favor a diagnosis of LPP. There are no DIF findings that reliably distinguish LPP from CCCA, although presence of a

positive DIF has found to correlate with the amount of inflammation. Future studies should be limited to patients with new, untreated, active disease. There are no specific T lymphocyte populations that differentiate between CCCA and LPP. However, this study confirmed the presence of CD8 positive lymphocytes and Langerhans cell as antigen presenting cells in early LPP, suggesting a cytotoxic inflammatory process at this stage of the disease. This study also suggested possible role of Th17 lymphocytes and Tregs in both CCCA and LPP. Our findings set the stage for larger studies of patients with early, active disease to further elucidate the pathogenesis of LPP and CCCA and the histologic features that set them apart. The possible role of Th 17 lymphocytes raises the possibility of use of an anti-Th 17 medication such as Ustekinumab as a novel treatment for LPP and CCCA.

FIGURES

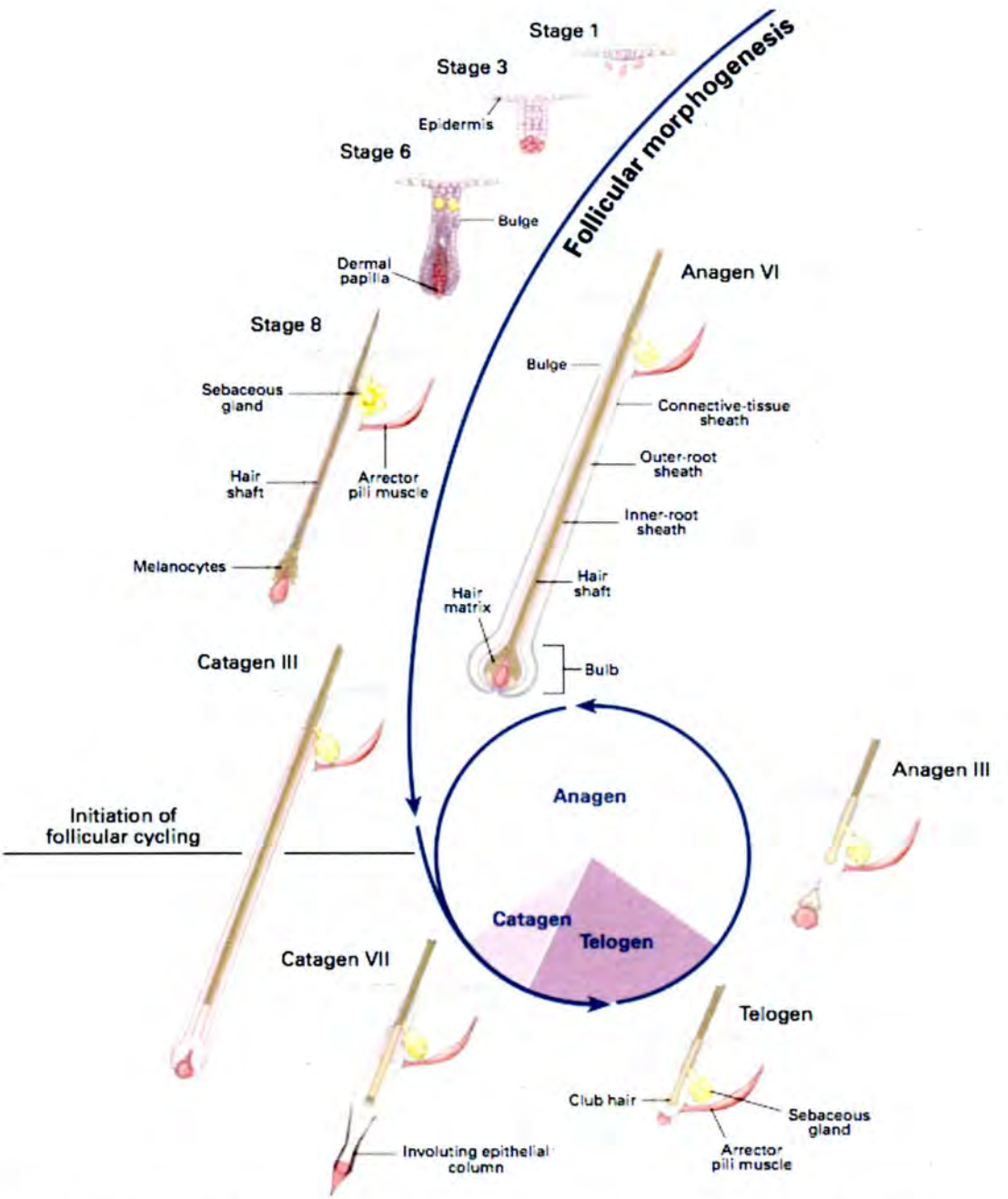

Figure 1. Schematic representation of follicle development and hair cycle. Selected stages from 8 stages of the morphogenesis of hair follicles and follicular cycling (anagen, catagen and telogen) are shown, adapted from Paus and Cotsarelis[6].

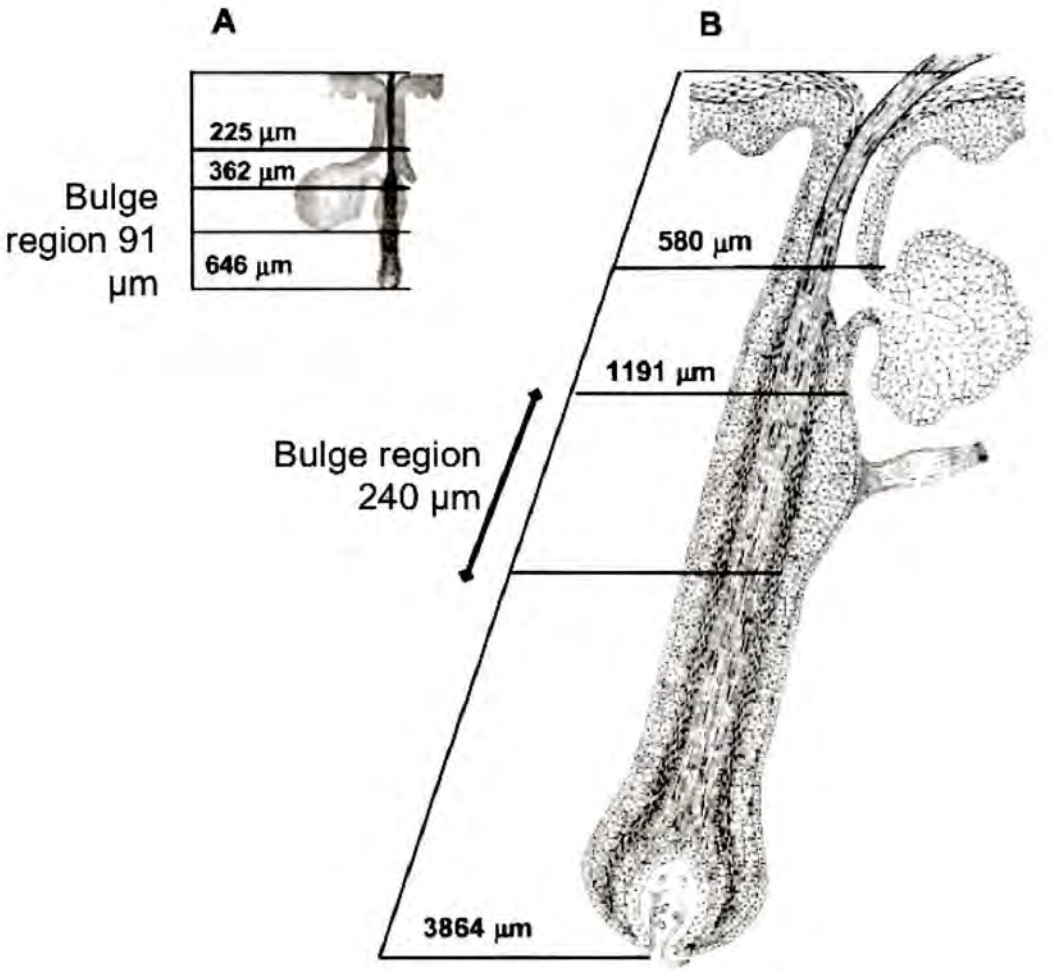

Figure 2. Vellus follicle (A) and terminal follicle (B) share similar basic anatomy but with different size. Terminal hair is about 5 times larger than vellus hair, adapted from Knorr et al.[111]

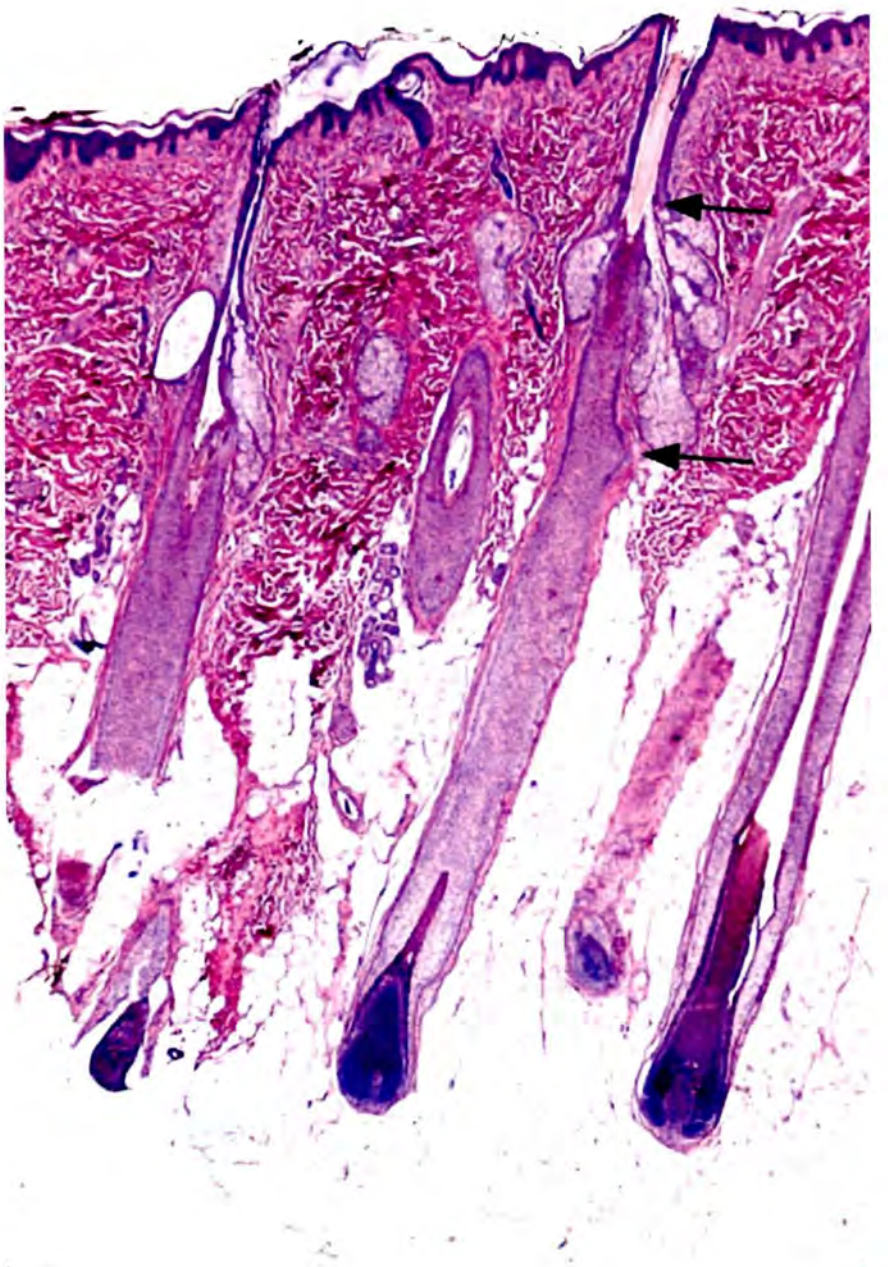

Figure 3. Anagen hair anatomic reference sites. The entry of the sebaceous duct (upper arrow) divides the infundibulum above from the isthmus below, and the insertion of the erector pili muscle, also called bulge region (lower arrow) divides the isthmus above from the inferior follicle below (from Dermatopathology Interactive Atlas, 2001, by Bhawan, Sau and Byers, with permission from Dr. Jag Bhawan).[112]

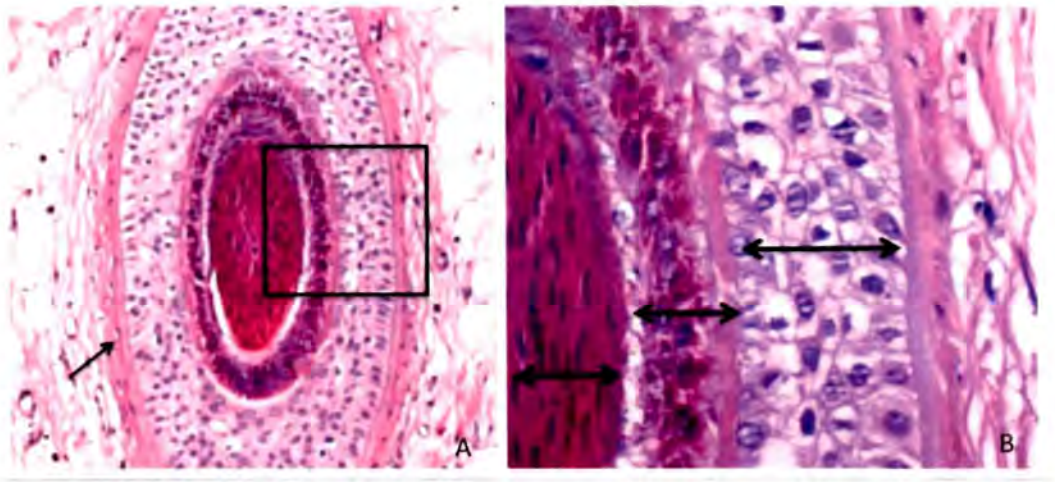

Figure 4. (A) Cross section of an anagen hair follicle at an inferior level, showing concentric layers of hair follicle. Arrow shows the connective tissue sheath. Square frames area in (B). **(B) Different layers of hair follicle from innermost to periphery depicted by arrows.** The lowest arrow shows the hair matrix which will form the hair shaft. The middle arrow shows the inner root sheath (IRS), which consists of the IRS cuticle, Huxley's layer and Henley's layer. The highest arrow shows the outer root sheath (from Dermatopathology Interactive Atlas, 2001, by Bhawan, Sau, Byers, with permission from Dr. Jag Bhawan).[112]

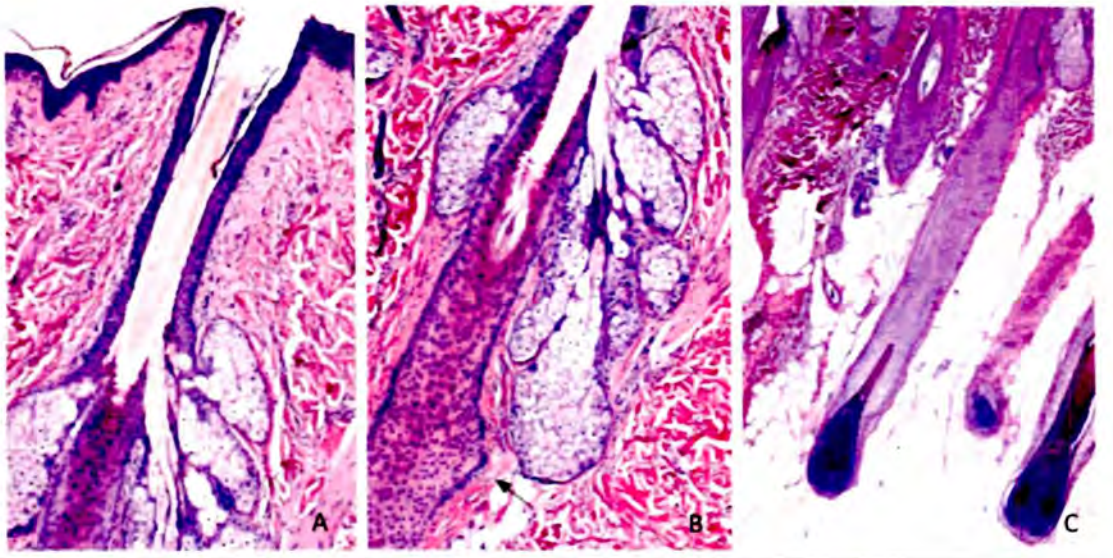

Figure 5. Outer root sheath (ORS) at different portions. (A) Infundibulum portion. Notice the continuity of ORS and epidermis. (B) Isthmus portion. Notice trichilemmal keratinization (epithelium with no granular layer). Arrow shows the hair bulge, which contains multipotent stem cells. (C) Inferior portion. ORS continues downward to the hair bulb where it becomes paler and larger (from Dermatopathology Interactive Atlas, 2001, by Bhawan, Sau and Byers, with permission from Dr. Jag Bhawan). [112]

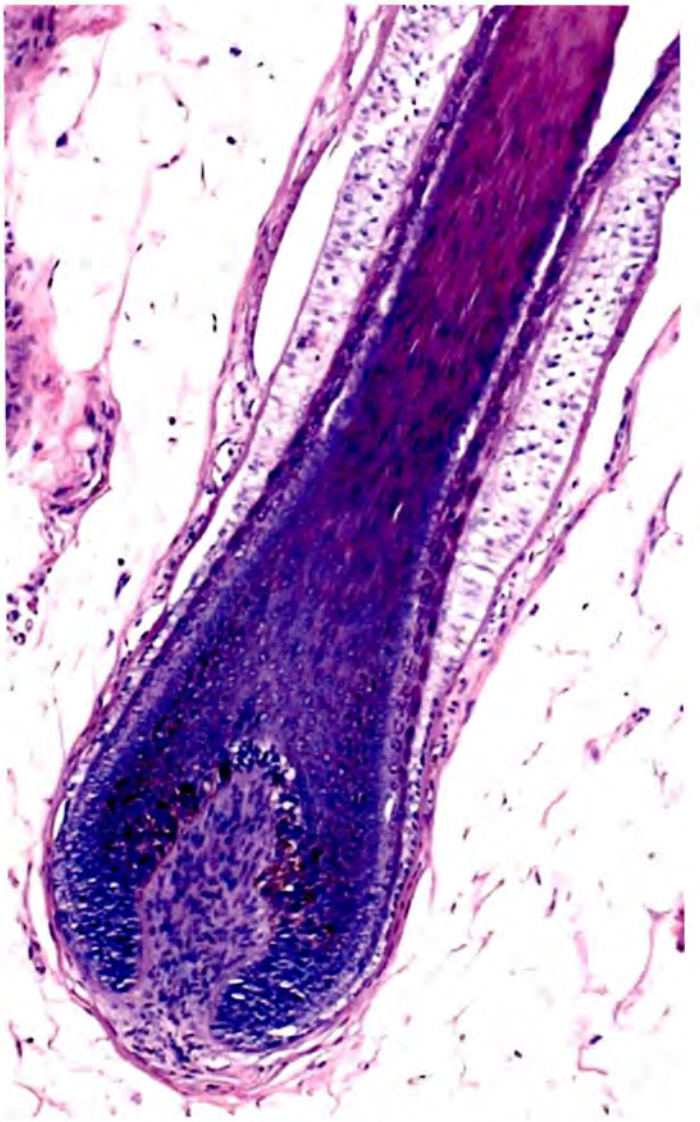

Figure 6. Vertical section of anagen hair bulb showing hair matrix. Notice the hair matrix is continuous with the newly formed hair shaft. The hair matrix is surrounding the dermal papilla in "ball and claw" configuration (from Dermatopathology Interactive Atlas, 2001, by Bhawan, Sau and Byers, with permission from Dr. Jag Bhawan). [112]

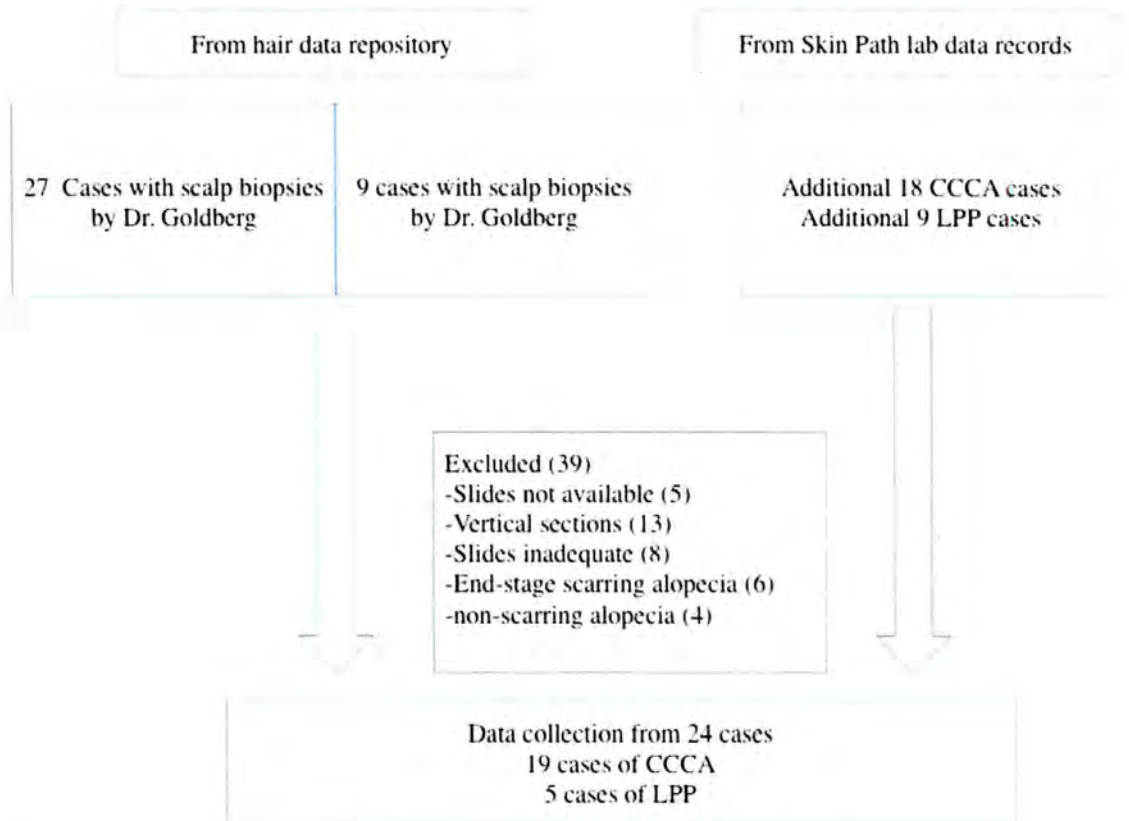

Figure 7. Study 1 Sample size

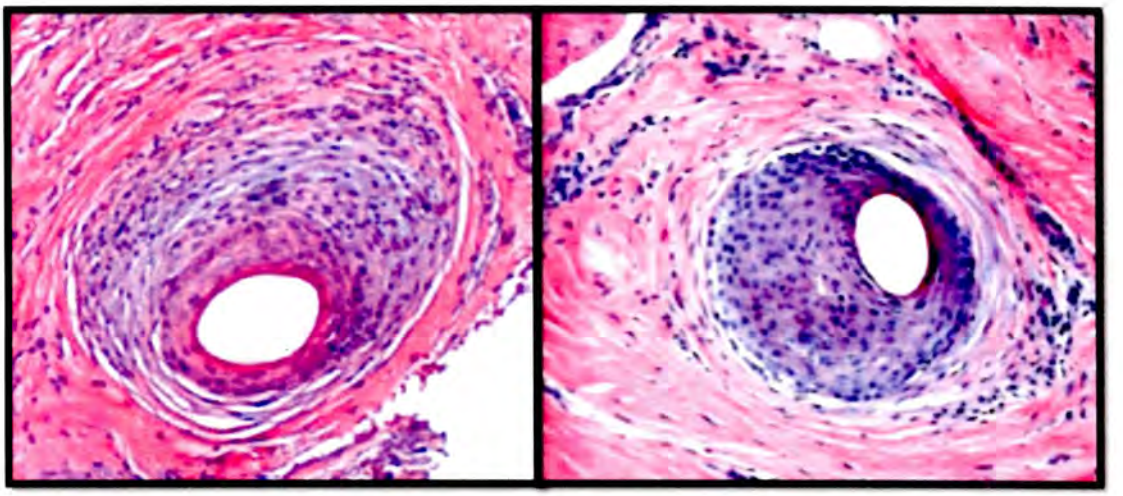

Figure 8. Examples of follicles with mild asymmetry and marked asymmetry (from Dermatopathology Interactive Atlas, 2001, by Bhawan, Sau and Byers, with permission from Dr. Jag Bhawan).[112]

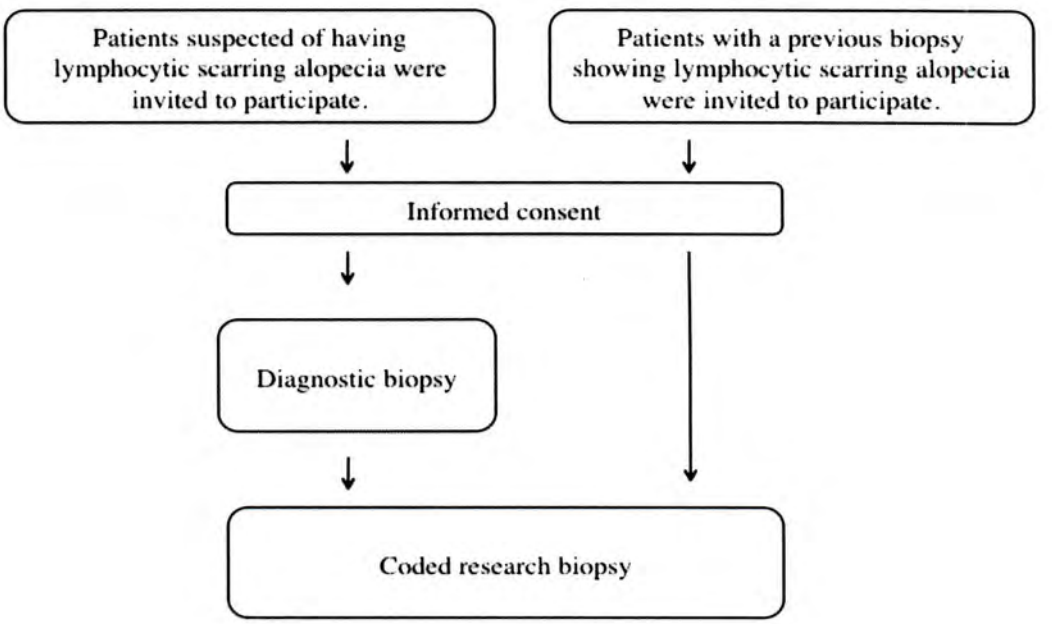

Figure 9. Subject recruitment for Studies 2 and 3

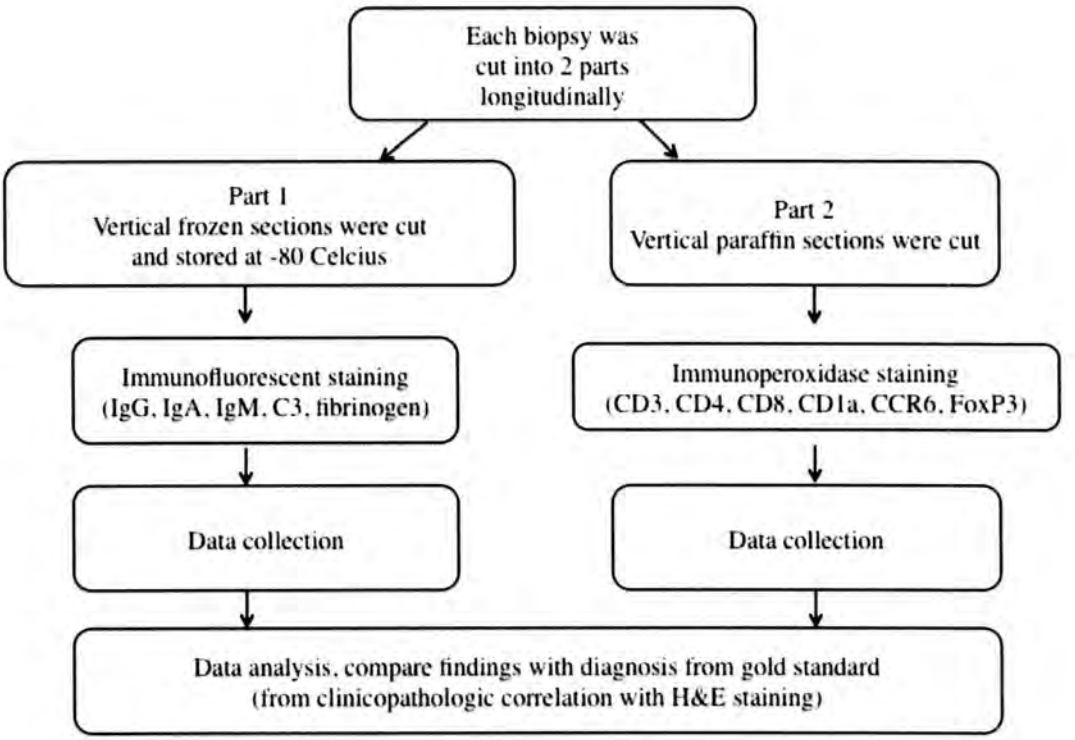

Figure 10. Tissue processing for Studies 2 and 3

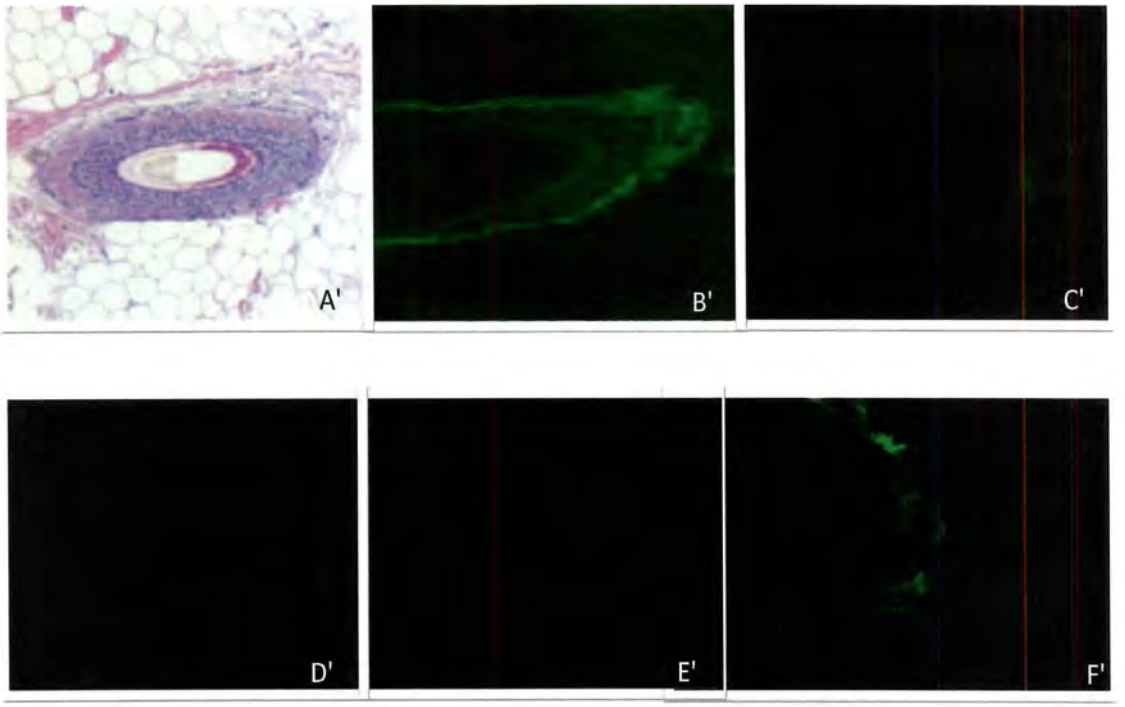

Figure 11. Positive DIF findings in case LGP-004. (A) Vertical section of an anagen hair follicle at the inferior portion, H&E stain, 200x; (B) Linear/shaggy IgG deposition at the perifollicular BMZ; (C) Negative IgM staining; (D) Negative IgA staining; (E) Negative C3 staining; (F) Shaggy fibrinogen deposition at the perifollicular BMZ

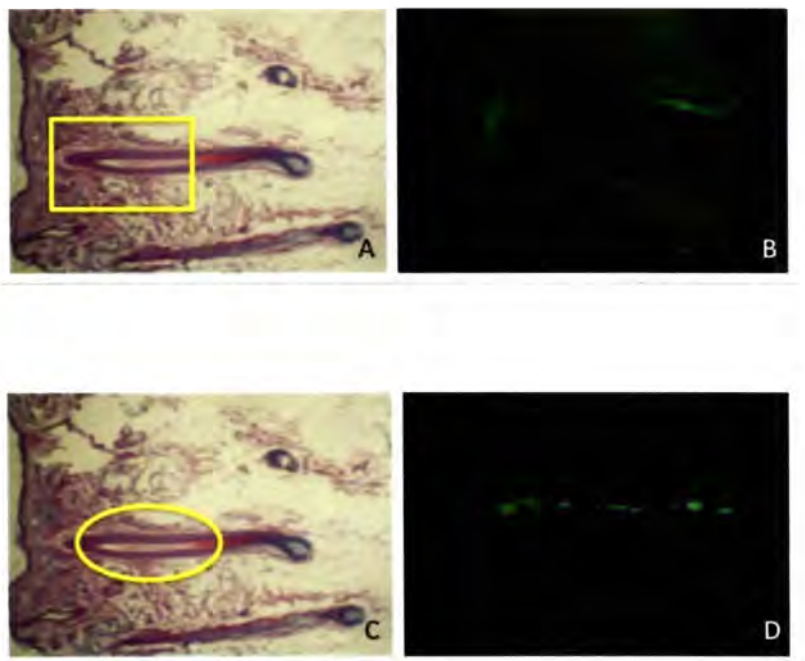

Figure 12. Positive DIF findings in case LGP-008. (A) Vertical section of an anagen hair follicle. The rectangle frames the area of positive IgG deposition, H&E stain, 200x; (B) Shaggy/granular IgG deposition at the perifollicular BMZ at the isthmus level; (C) Vertical section of an anagen hair follicle. The oval frames the area of positive C3 deposition; (D) Shaggy/granular C3 deposition at the perifollicular BMZ at the isthmus level, 400x.

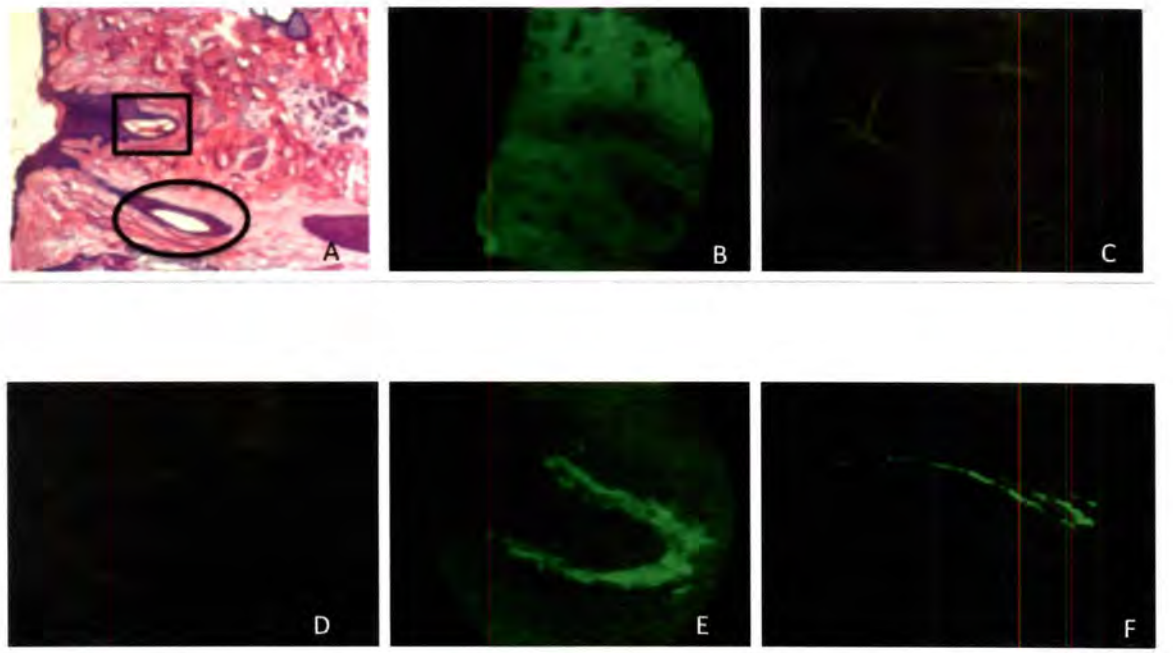

Figure 13. Positive DIF findings in case LGP-007. (A) Infundibulum/isthmus level. The rectangle and oval frame areas of C3 deposition. H&E stain, 200x; (B) Negative IgG staining; (C) Negative IgM staining; (D) Negative IgA staining; (E) Shaggy C3 deposition at the perifollicular BMZ at the infundibulum level; (F) Shaggy C3 deposition at the perifollicular BMZ at the isthmus level.

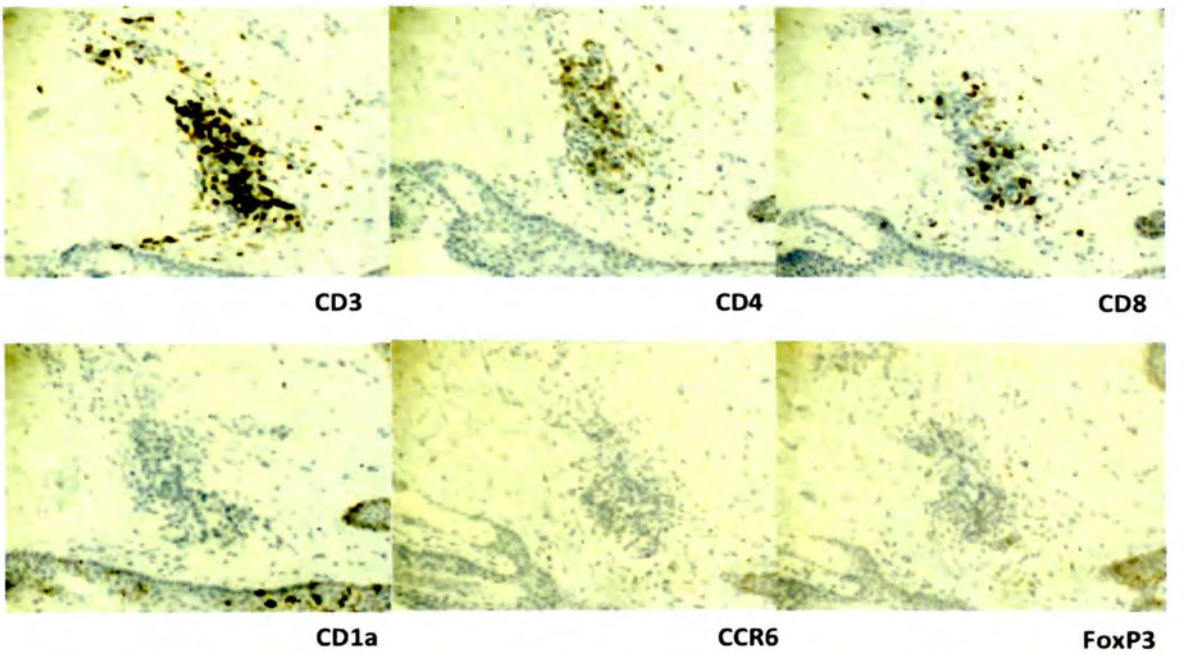

Figure 14. Case LGP-007 immunoperoxidase photographs

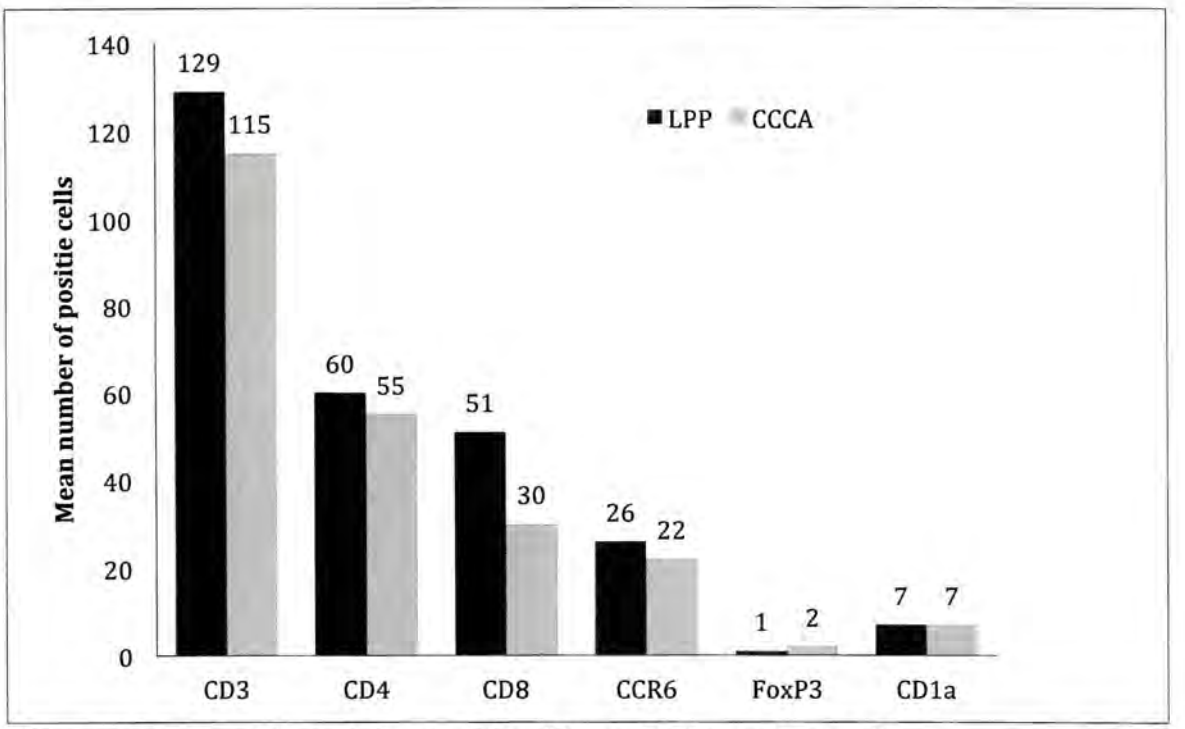

Figure 15. Comparison of means of positive cells from photos of immunohistochemical stains. None of the differences between LPP and CCCA were statistically significant.

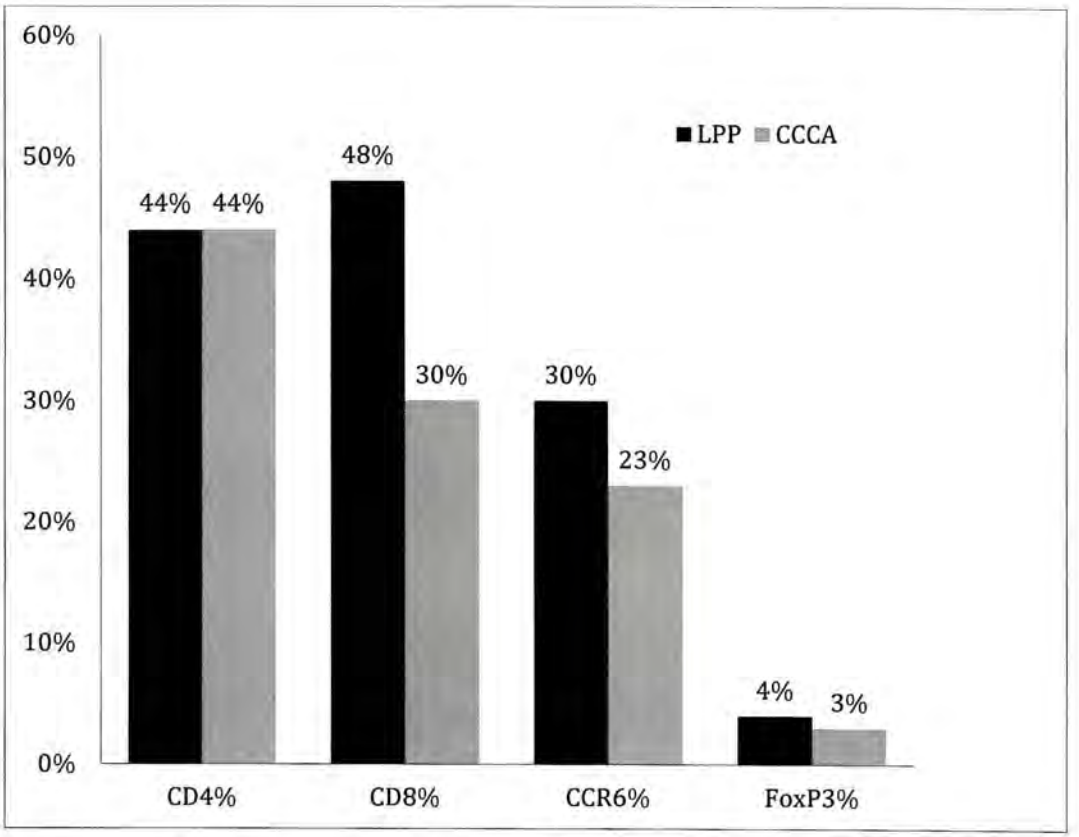

Figure 16. Comparison of mean percentages of positive cells from photos of immunohistochemical stains of LPP and CCCA. None of the differences between LPP and CCCA were statistically significant.

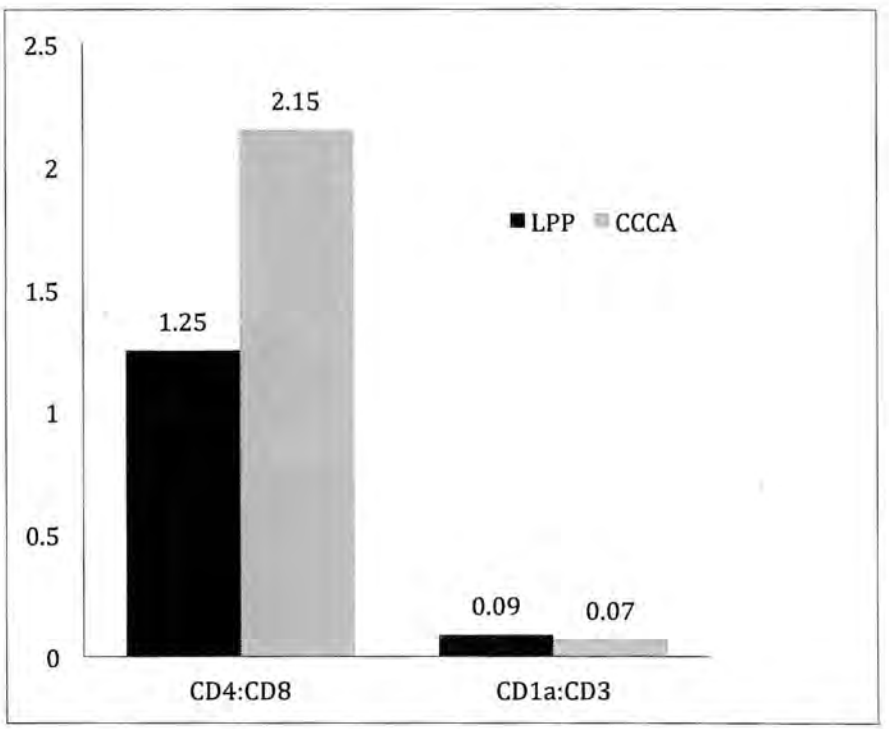

Figure 17. Comparison of ratios of positive cells from photos of immunohistochemical stains of LPP and CCCA. None of the differences between LPP and CCCA were statistically significant.

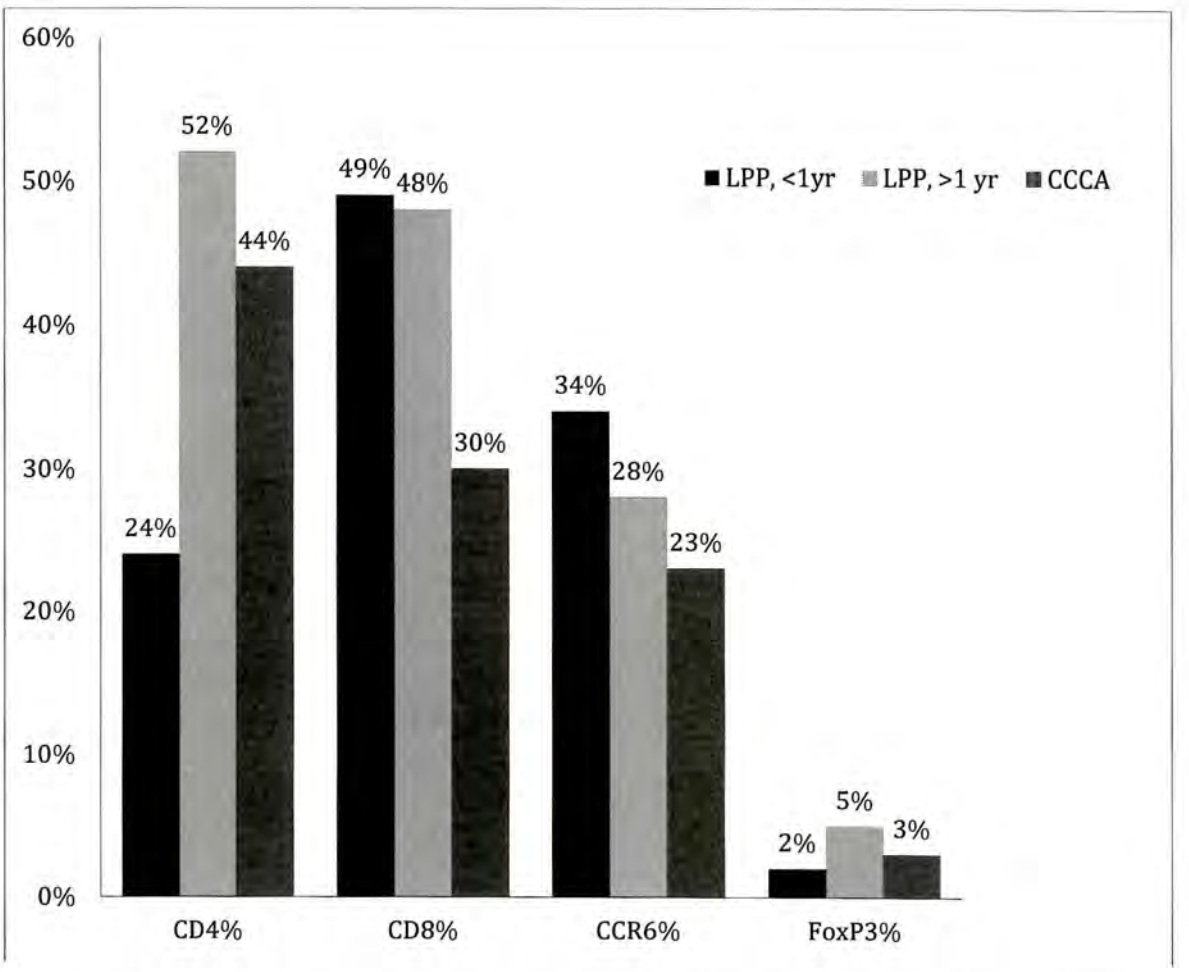

Figure 18. Comparison of means of percentage of positive cells from photos of immunohistochemical stains of LPP with duration less than 1 year, more than 1 year, and CCCA. None of the differences between LPP and CCCA were statistically significant.

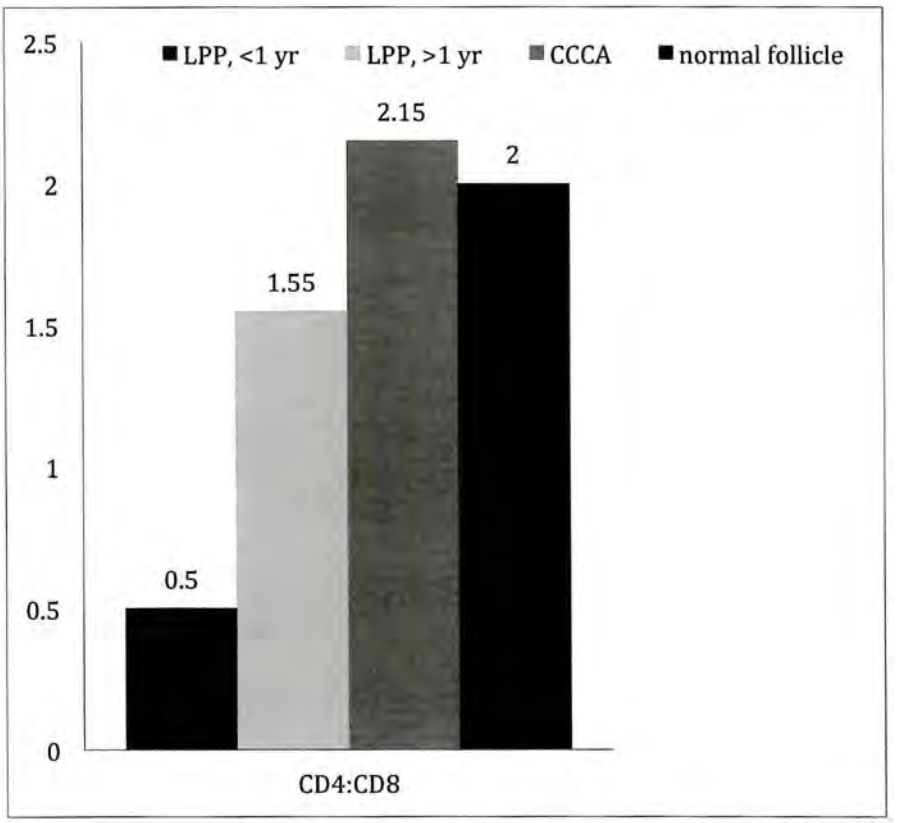

Figure 19. Comparison of the CD4:CD8 of photos of immunohistochemical stains in LPP with duration less than 1 year, more than 1 year, CCCA, and normal follicles. The difference between LPP and CCCA was statistically significant.

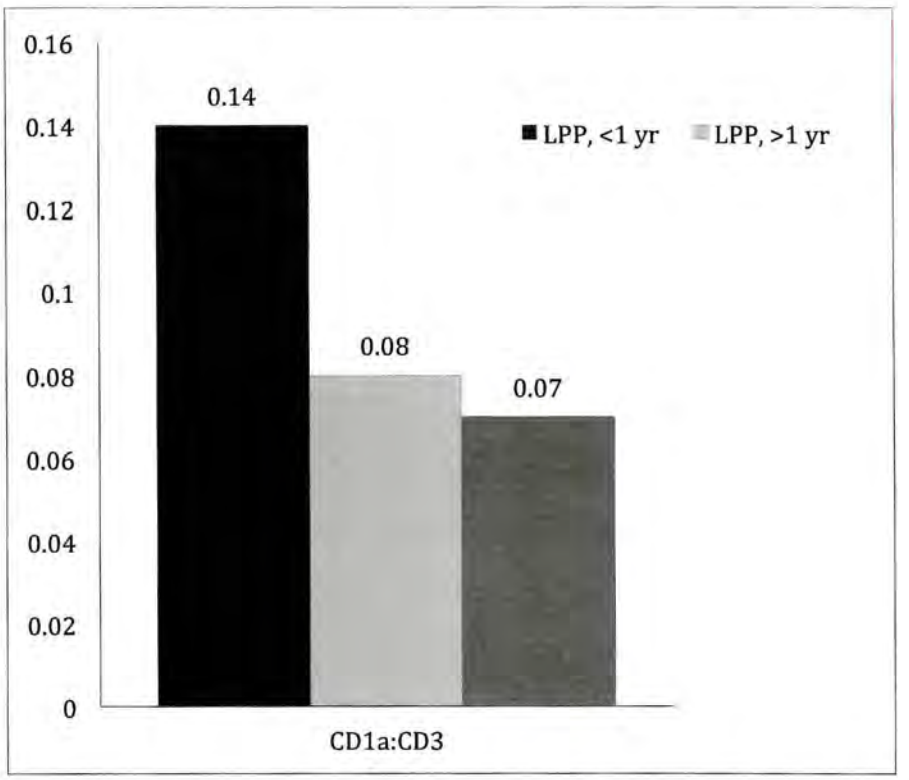

Figure 20. Comparison of the CD1a:CD3 in photos of immunoperoxidase stains of LPP with duration less than 1 year, more than 1 year, and CCCA. The difference between LPP and CCCA was statistically significant.

TABLES

Table 1. Classification of primary cicatricial alopecia, modified from the summary of North American Hair Research Society (NAHRS)-sponsored Workshop on Cicatricial Alopecia [17].

Inflammatory cells	Cicatricial alopecia
Lymphocytes	Lichen planopilaris (LPP) Frontal fibrosing alopecia Graham Little syndrome Central centrifugal cicatricial alopecia (CCCA) Alopecia mucinosa Keratosis follicularis spinulosa decalvans
Neutrophils	Folliculitis decalvans Dissecting cellulitis/folliculitis (Perifolliculitis abscedens et suffodiens)
Mixed	Folliculitis (acne) keloidalis Folliculitis (acne) necrotica Erosive pustular dermatosis
Nonspecific or end stage	Pseudopelade of Brocq

Table 2. Demographic data of subjects in study 1

Characteristics	CCCA, n=19	LPP, n=5
Sex F M	18 (94.7%) 1 (5.3%)	5 (100%) 0
Ethnicity Arican-American Causasian Hispanic	19 (100%) 0	2 (40%) 3 (60%)
Age at time of biopsy<30 years 30-60 years >60 years	2(10.5%) 16 (84.2%) 1 (5.3%)	0 4 (80%) 1 (20%)
Mean age, range	44.3 years (26-68)	49.4 years (33-64)
Mean duration of disease, (range)	7.4 years (1-20)	3.8 years (1-10)

Table 3. The number of follicles at the isthmus and the inferior follicle

	CCCA, n=19 Mean (range)	LPP, n=5 Mean (range)	p-value
Isthmus			
Total number of follicles	10.8 (4-17)	12.4 (7-19)	0.49916
Number of terminal follicles	7.4 (3-16)	11.6 (7-19)	0.06129
Number of vellus follicles,	3.15 (1-14)	1 (0-2)	0.1389
Inferior follicle			
Total number of follicles	7.35 (3-16)	11.4 (3-19)	0.0592

Table 4. The number of fibrous tracts at the isthmus and the inferior follicle

	CCCA, n=19 Mean	LPP, n=5 Mean	p-value
Isthmus None 1-5 6-10 >10	 1 (5.3%) 6 (31.6%) 7 (36.8%) 5 (26.3%)	 0 3 (60%) 1 (20%) 1 (20%)	0.75
Inferior follicle None 1-5 6-10 >10	 0 4 (21%) 5 (26.3%) 10 (52.6%)	 0 2 (40%) 1 (20%) 2 (40%)	0.81

Table 5. Select histopathologic findings in CCCA and LPP

Findings	CCCA, n=19	LPP, n=5	p-value
Epidermal involvement Present Absent	 0 19 (100%)	 0 5 (100%)	0.9
Normal follicular units Present Absent	 11 (57.9%) 8 (42.1%)	 0 5 (100%)	<0.05*
Loss of sebaceous glands None or partial Complete	 17 (89%) 2 (11%)	 2 (40%) 3 (60%)	<0.05*
Fused follicles, isthmus Present Absent	 10 (52.6%) 9 (47.4%)	 4 (80%) 1 (20%)	0.36
Fused follicles, inferior Present Absent	 0 19 (100%)	 1 (20%) 4 (80%)	0.36
PDIRS Present Absent	 14 (74%) 5 (26%)	 3 (60%) 2 (40%)	0.61
Dilation of eccrine glands Present Absent	 2 (10.5%) 17 (89.5%)	 3 (60%) 2 (40%)	<0.05*

FINKERs, isthmus Present Absent	6 (31.6%) 13 (68.4%)	2 (40%) 3 (60%)	0.9
FINKERs, inferior follicle Present Absent	10 (52.6%) 9 (47.4%)	5 (100%) 0 (0%)	0.12
Naked hair shafts, isthmus Present Absent	3 (15.8%) 16 (84.2%)	3 (60%) 2 (40%)	0.08
Naked hair shafts, inferior Present Absent	7 (36.8%) 12 (63.1%)	3 (60%) 2 (40%)	0.61
Intrafollicular lymphocytes, isthmus Present Absent	7 (36.8%) 12 (63.1%)	3 (60%) 2 (40%)	0.61
Intrafollicular lymphocytes, inferior Present Absent	0 (0%) 19 (100%)	0 (0%) 5 (100%)	0.99
Plasma cells, isthmus Present Absent	13 (68.4%) 6 (31.6%)	1 (20%) 4 (80%)	0.12
Plasma cells, inferior Present Absent	4 (24%) 15 (76%)	0 (0%) 5 (100%)	0.54
Mucin, isthmus Present Absent	13 (68.4%) 6 (31.6%)	1 (20%) 4 (80%)	0.54
Mucin, inferior Present Absent	4 (24%) 15 (76%)	0 (0%) 5 (100%)	0.54

Table 6. Grade of follicular asymmetry at the isthmus and the inferior follicle

Grade of follicular asymmetry	CCCA, n=19	LPP, n=5	p-value
Isthmus			
No asymmetry Mild Moderate Marked	1 (5.3%) 15 (78.9%) 1 (5.3%) 2 (10.5%)	0 3 (60%) 1 (20%) 1 (20%)	0.44
Inferior follicle			
No asymmetry Mild Moderate Marked	3 (15.9%) 10 (53%) 5 (26.5%) 1 (5.3%)	0 3 (60%) 1 (20%) 1 (20%)	0.59

Table 7. Grade of perifollicular fibrosis at the isthmus and inferior follicle

Grade of perifollicular fibrosis	CCCA, n=19	LPP, n=5	p-value
Isthmus			
None Mild Moderate Severe	2 (10.5%) 7 (36.8%) 7 (36.8%) 3 (15.7%)	0 0 3 (60%) 2 (40%)	0.75
Inferior follicle			
None Mild Moderate Severe	2 (10.5%) 15 (79%) 0 2 (10.5%)	0 5 (100%) 0 0	0.9

Table 8. Grade of perifollicular inflammation at the isthmus and the inferior follicle

Grade of perifollicular inflammation	CCCA, n=19	LPP, n=5	p-value
Isthmus			
Mild or absent Moderate or severe	17 (89.4%) 2 (10.5%)	2 (40%) 3 (60%)	0.04
Inferior follicle			
Mild or absent Moderate or severe	17 (89.4%) 2 (10.5%)	4 (80%) 1 (20%)	0.52

Table 9. Grade of perivascular inflammation at the isthmus and the inferior follicle

Grade of perivascular inflammation	CCCA, n=19	LPP, n=5	p-value
Isthmus			
None Mild Moderate	2 (10.5%) 13 (68.4%) 4 (21.0%)	1 (20%) 2 (40%) 2 (40%)	0.57
Inferior follicle			
None Mild Moderate	12 (63.2%) 7 (36.8%) 0	4 (80%) 1 (20%) 0	0.52

Table 10. Histopathologic findings in LPP versus CCCA

Characters	CCCA[11, 19, 48-50, 71]	LPP[20, 21, 25, 34, 57, 79, 81]
Number of hair follicles	Markedly reduced of terminal follicle, some vellus hair persist	Marked reduced of both terminal and vellus
Epidermal keratin	Absent	Parakeratosis, hyperkeratosis, hypergranulosis and follicular plugging
Interfollicular epidermal involvement	Absent Or epidermal atrophy	Absent in most cases, some cases with lichenoid infiltration, hypergranulosis, vacuolar changes and INKers
Sebaceous glands	Absent	Absent
Arrector pili muscle	Retained	Diminished
Perifollicular inflammation	Variable dense lymphocytic perifollicular infiltration	Lichenoid lymphocytic infiltration at infundibulum and isthmus
Follicular INKers	Absent	Present
Perivascular inflammation	Lymphocytic infiltrate	Lymphocytic infiltrate
Premature desquamation of inner root sheath (PDIRS)	Present in early stage, along with thinning of follicular epithelium	Present together with inflammatory damage
Follicular asymmetry and polytrichia	Present	Present
Naked hair shaft	Present	Present

Concentric lamellar perifollicular fibrosis	Present	Present
Clefting between stroma and follicular epithelium	Absent	May present
Perifollicular mucin	May present	May present
Elastin change	Thicken dermal elastic fiber in hyalinized dermis	Loss of elastin in papillary dermis

Table 11. Summary of distinctive histologic findings of CCCA and LPP

Histologic findings	CCCA	LPP	p-value
Presence of normal follicular units	58%	0%	0.04
Complete loss of sebaceous glands	10%	60%	0.04
Moderate to severe perifollicular inflammation	10%	60%	0.04
Dilatation of eccrine ducts	10%	60%	0.04

Table 12. Demographic data of patients with CCCA and LPP in study 2

Characteristics	CCCA, N=4	LPP, N=7
Sex Female Male	 4 (100%) 0	 5 (71%) 2(29%)
Ethnicity African American Caucasian	 4 (100%) 0	 0 7 (100%)
Mean age (range)	40.3 years (28-59)	54.3 years (37-72)
Mean duration of disease	7 years (2-20)	3.2 years (1-7)

Table 13. Immunofluorescence results

Case	IgG	IgM	IgA	C3	fibrinogen
LGP-001	Negative	Negative	Negative	Negative	Negative
LGP-002	Negative	Negative	Negative	Negative	Negative
LGP-003	Negative	Negative	Negative	Negative	Negative
LGP-004	Linear/ shaggy deep PF deposits	Negative	Negative	Negative	Shaggy deep PF deposits BMZ/PFCT
LGP-005	Negative	Negative	Negative	Negative	Negative
LGP-006	Negative	Negative	Negative	Negative	Negative
LGP-007	Negative	Negative	Negative	Shaggy PF deposits at infundibulum/isthmus	Negative
LGP-008	Shaggy/ granular PF deposits at isthmus	Negative	Negative	Shaggy/ granular PF deposits at isthmus	Negative
LGP-009	Negative	Negative	Negative	Negative	Negative
LGP-010	Negative	Negative	Negative	Negative	Negative
LGP-011	Negative	Negative	Negative	Negative	Negative

PF = perifollicular, BMZ=basement membrane zone, CT = connective tissue

Table 14. DIF results and clinical history

Case	DIF result	Diagnosis	Duration of disease, year(s)	Perifollicular inflammation /erythema	Previous treatment
LGP-001	Negative	LPP	2	Mild	ILK, doxycycline
LGP-002	Negative	LPP	1	Mild	Topical steroid
LGP-003	Negative	LPP	1	Mild	ILK
LGP-004	IgG & fibrinogen positive	LPP	2	Moderate	Minoxidil
LGP-005	Negative	LPP	7	Mild	Topical steroid
LGP-006	Negative	CCCA	2	Mild	ILK, topical steroid
LGP-007	C3 positive	CCCA	3	Moderate	Topical steroid
LGP-008	IgG & C3 positive	LPP	2.5	Moderate	Topical steroid
LGP-009	Negative	LPP	7	Mild	Plaquenil, topical steroid
LGP-010	Negative	CCCA	20	Mild	None
LGP-011	Negative	CCCA	3	Mild	Topical steroid

Table 15. Immunofluorescence results in the LPP group

Case	IgG	IgM	IgA	C3	fibrinogen
LGP-001	Negative	Negative	Negative	Negative	Negative
LGP-002	Negative	Negative	Negative	Negative	Negative
LGP-003	Negative	Negative	Negative	Negative	Negative
LGP-004	Linear/ shaggy deep PF deposits	Negative	Negative	Negative	Shaggy deep PF deposits
LGP-005	Negative	Negative	Negative	Negative	Negative
LGP-008	Shaggy/ granular PF deposits at isthmus	Negative	Negative	Shaggy/ granular PF deposits at isthmus	Negative
LGP-009	Negative	Negative	Negative	Negative	Negative

Table 16. Immunofluorescence results in the CCCA group

Case	IgG	IgM	IgA	C3	fibrinogen
LGP-006	Negative	Negative	Negative	Negative	Negative
LGP-007	Negative	Negative	Negative	Shaggy PF deposits at infundibulum/isthmus	Negative
LGP-010	Negative	Negative	Negative	Negative	Negative
LGP-011	Negative	Negative	Negative	Negative	Negative

Table 17. DIF findings in LPP from previous studies

Author, year	Number of cases	Deposition at DEJ of follicular epithelium, number of positive cases	Deposition at DEJ of interfollicular epithelium, number of positive cases	Presence of cytoid body
Ioannides[67], 1992	7	IgG 3/7, IgA 2/7, IgG+IgA 1/7, IgG+IgM+fibrinogen1/7 (linear pattern)	None	Negative
Mehregan[21], 1992	28	Fibrinogen 9/28, IgM 2/28, C3 1/28 (Patchy pattern)	IgG 7/28	IgM+IgA, rare C3 18/28
Chieregato[25], 2003	30	Non-specific, 17/30	Fibrinogen, 13/30	IgM 13/30

Table 18. Direct Immunofluorescence findings in lichen planus and LPP, as reported by Ioannides and Bystryn [67]

Lichen planus	Lichen planopilaris (LPP)
- Fibrinogen deposits in fibrillar pattern at basement membrane zone (BMZ), more than 90% of cases - Ig and/or complement deposits at cytoid body at BMZ, more than 90% of cases	- Linear IgG at BMZ of follicles 70% with IgG or IGA 30% with IgG +others - 14% present with linear fibrin at BMZ - Negative cytoid body

Table 19. Demographic data of LPP patients in Study 3

Case number	Ethnicity	Age, year	Sex	Duration of disease
LGP-001	Caucasian	64	Male	2 years
LGP-002	Caucasian	47	Female	1 year
LGP-003	Caucasian	54	Female	1 year
LGP-004	Caucasian	38	Female	2 years
LGP-005	Caucasian	72	Female	7 years
LGP-008	Caucasian	68	Female	2.5 years
LGP-009	Caucasian	37	Male	7 years

Table 20. Demographic data of CCCA patients in Study 3

Case number	Ethnicity	Age, year	Sex	Duration of disease
LGP-006	African American	28	Female	2 years
LGP-007	African American	36	Female	3 years
LGP-010	African American	38	Female	20 years
LGP-011	Caucasian	59	Female	3 years

Table 21. Number of positive cells from photos of immunohistochemical stain

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
LGP-001	134	78	45	43	0	1
LGP-002	39	14	19	18	0	4
LGP-003	62	8	31	13	2	11
LGP-004	110	44	14	6	2	27
LGP-005	9	5	9	6	2	1
LGP-006	122	37	45	9	1	13
LGP-007	86	30	34	15	2	1
LGP-008	340	132	132	55	1	2
LGP-009	206	140	108	44	2	2
LGP-010	48	23	15	22	4	6
LGP-011	203	129	24	42	1	7
Mean	124 (9-340)	58 (5-140)	43 (9-132)	25 (6-55)	2 (0-4)	7 (1-27)

Table 22. LPP. Number of positive cells from photos of immunohistochemical stains

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
LGP-001	134	78	45	43	0	1
LGP-002	39	14	19	18	0	4
LGP-003	62	8	31	13	2	11
LGP-004	110	44	14	6	2	27
LGP-005	9	5	9	6	2	1
LGP-008	340	132	132	55	1	2
LGP-009	206	140	108	44	2	2
Mean	129 (9-340)	60 (5-140)	51 (9-132)	26 (6-55)	1 (0-2)	7 (1-27)

Table 23. CCCA. Number positive cells from photos of immunohistochemical stains

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
LGP-006	122	37	45	9	1	13
LGP-007	86	30	34	15	2	1
LGP-010	48	23	15	22	4	6
LGP-011	203	129	24	42	1	7
Mean	115 (48-203)	55 (23-129)	30 (15-45)	22 (9-42)	2 (1-4)	7 (1-13)

Table 24. LPP. Percentages and ratios of positive cells from photos of immunohistochemical stains

Case	CD4%	CD8%	CCR6%	FoxP3%	CD4:CD8	CD1a:CD3
LGP-001	58%	34%	32%	0%	1.73	0.01
LGP-002	36%	49%	46%	0%	0.74	0.10
LGP-003	13%	50%	21%	3%	0.26	0.18
LGP-004	40%	13%	5%	2%	3.14	0.25
LGP-005	56%	100%	67%	22%	0.56	0.11
LGP-008	39%	39%	16%	0%	1.00	0.01
LGP-009	68%	52%	21%	1%	1.30	0.01
Mean	44% (13-68)	48% (13-100)	30% (5-67)	4% (0-22)	1.25 (0.26-3.14)	0.09 (0.01-0.25)

Table 25. CCCA. Percentages and ratios of positive cells from photos of immunohistochemical stains

Case	CD4%	CD8%	CCR6%	FoxP3%	CD4:CD8	CD1a:CD3
LGP-006	30%	37%	7%	1%	0.82	0.11
LGP-007	35%	40%	17%	2%	0.88	0.01
LGP-010	48%	31%	46%	8%	1.53	0.13
LGP-011	64%	12%	21%	0%	5.38	0.03
Mean	44% (30-64)	30% (12-40)	23% (7-46%)	3% (0-8%)	2.15 (0.82-5.38)	0.07 (0.01-0.13)

Table 26. LPP duration less than 1 year. Number of positive cells from photos of immunohistochemical stains

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
LGP-002	39	14	19	18	0	4
LGP-003	62	8	31	13	2	11
Mean	50.5 (39-62)	11 (8-14)	25 (19-31)	15.5 (13-18)	1 (0-2)	7.5 (4-11)

Table 27. LPP duration greater than 1 year. Number of positive cells from photos of immunohistochemical stains

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
LGP-001	134	78	45	43	0	1
LGP-004	110	44	14	6	2	27
LGP-005	9	5	9	6	2	1
LGP-008	340	132	132	55	1	2
LGP-009	206	140	108	44	2	2
Mean	159.8 (9-340)	79.8 (5-140)	61.6 (9-132)	30.8 (6-55)	1.4 (1-2)	6.6 (1-27)

Table 28. LPP duration less than 1 year. Percentages of T-cell subsets and ratios of positive cells from photos of immunohistochemical stains

Case	CD4%	CD8%	CD4:CD8	CCR6%	FoxP3%	CD1a:CD3
LGP-002	36%	49%	0.74	46%	0%	0.10
LGP-003	13%	50%	0.26	21%	3%	0.18
Mean	24% (13-36)	49% (49-50%)	0.50 (0.26-0.74)	34% (21-46)	2% (0-2)	0.14 (0.1-0.18)

Table 29. LPP duration over 1 year. Percentages of T-cell subsets and ratios of positive cells from photos of immunohistochemical stains

Case	CD4%	CD8%	CD4:CD8	CCR6%	FoxP3%	CD1a:CD3
LGP-001	58%	34%	1.73	32%	0%	0.01
LGP-004	40%	13%	3.14	5%	2%	0.25
LGP-005	56%	100%	0.56	67%	22%	0.11
LGP-008	39%	39%	1.00	16%	0%	0.01
LGP-009	68%	52%	1.30	21%	1%	0.01
Mean	52% (39-68)	48% (13-100)	1.55 (0.56-3.14)	28% (5-67)	5% (0-22)	0.08 (0.07-0.25)

APPENDIX

Appendix 1

Histopathologic data collection form-Isthmus & Infundibulum level

Epidermal Involvement	( ) Present ( ) Absent
The number of terminal follicles The number of vellus follicles The number of fused follicles The number of follicular units	
The number of fibrous tracts	( ) Absent ( ) 1-5 ( ) 6-10 ( ) >10
Follicular asymmetry	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Perifollicular fibrosis	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+

Mucin	( ) Present ( ) Absent
Perifollicular inflammation	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Naked hair shaft	( ) Present ( ) Absent
Follicular individual necrotic keratinocytes (INKers)	( ) Present ( ) Absent Number of involved follicles_____
Intrafollicular lymphocytes	( ) Present ( ) Absent Number of involved follicles_____
Perivascular inflammation	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Plasma cells	( ) Present ( ) Absent
Dilatation of eccrine ducts	( ) Present

	( ) Absent

Inferior level (at dermal subcutaneous junction)

The number of follicles	
The number of fibrous tracts	( ) Absent ( ) 1-5 ( ) 6-10 ( ) >10
Follicular asymmetry	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Perifollicular fibrosis/mucin	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Perifollicular inflammation	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
PDIRS	( ) Present ( ) Absent

Follicular INKers	( ) Present ( ) Absent Number of involved follicles_____
Intrafollicular lymphocytes	( ) Present ( ) Absent Number of involved follicles_____
Perivascular inflammation	( ) Absent ( ) 1+ ( ) 2+ ( ) 3+
Plasma cells	( ) Present ( ) Absent

Appendix 2

Direct immunofluorescent data collection

Case	IgG	IgM	IgA	C3	Fibrinogen
Patient #1					
Patient #2					
Patient #3					

Appendix 3

Details of tissue processing and immunohistochemical staining

The dehydration process was accomplished by passing the each specimen through a series of increasing alcohol concentrations from 30% to and 100% for about two hours each. The specimen was then placed in a second 100% ethanol solution to ensure that all water was removed. After dehydration, the tissue blocks were transferred to pure paraffin in the oven for 1 hour and then into melted paraffin for an additional 2-3 hours. During this time, the tissue block was completely infiltrated with melted paraffin. The tissue was then placed into an embedding mold and melted paraffin was poured into the mold to form a block. The blocks were allowed to cool, were cut into three-micrometer sections using a microtome, and layered on a glass slide. These were stored in a labeled box in Skin Pathology Laboratory until the time of staining. Just prior to staining, sections mounted on glass slides were deparaffinized and rehydrated through a series of graded alcohol to water.

For H&E staining, the slide was immersed in hematoxylin for 4 minutes, rinsed with water, immersed in 0.3% acid alcohol, rinsed with water, immersed in eosin for 2 minutes. Once staining was complete they were run through alcohols and clearing solvents, dried, and coverslipped.

For CD3, CD4, CD8 and CD1a staining, after deparaffinization and rehydration the specimen was incubated with Peroxidase Block Reagent for 5 minutes at room temperature, rinsed with Phosphate-buffered saline (PBS), then incubated with the primary antibody (CD3, CD4, CD8 and CD1a, all at a concentration of 1:50) for 20

minutes and rinsed. The slide was then incubated with horseradish peroxidase (HRP) solution for 20 minutes, rinsed and incubated with diaminobenzidine (DAB) peroxidase substrate solution for 10 minutes, rinsed and incubated with hematoxylin for 5 minutes and rinsed. Then, they were run through alcohols and clearing solvents, dried, and coverslipped.

For CCR6, after deparaffinization and rehydration the specimen was incubated with peroxidase block solution for 10 minutes at room temperature, rinsed with PBS, then incubated with the primary antibody (concentration of 1:50) for 40 minutes and rinsed. Then the slide was then incubated with HRP solution for 20 minutes, rinsed and incubated with DAB peroxidase substrate solution for 7 minutes, rinsed, incubated with hematoxylin for 5 minutes, and rinsed. Once staining was complete they were run through alcohols and clearing solvents, dried, and coverslipped.

For FoxP3, after deparaffinization and rehydration the specimen was incubated with DEEB for 10 minutes at room temperature, rinsed with PBS followed by incubation in the primary antibody (concentration of 1:500) for 30 minutes, and rinsed. Then the slides were incubated with goat probe solution for 20 minutes, rinsed twice with PBS, incubated with goat polymer solution for 20 minutes, rinsed twice with PBS, incubated with DAB peroxidase substrate solution for 7 minutes, rinsed, incubated with hematoxylin for 5 minutes, and rinsed. Once staining was complete, they were run through alcohols and clearing solvents, dried, and coverslipped.

Appendix 4

Immunohistochemical staining antibodies in Study 3

Antibody	Clone	Manufacturer	Dilution	Incubation time (minutes)
CD3	F7.2.38	Dako	1:50	30
CD4	4B12	Dako	1:50	30
CD8	C8/144B	Dako	1:50	30
CD1a	010	Dako	1:750	30
CCR6	MAB195	R&D Systems	1:50	40
FoxP3	AF3240	R&D Systems	1:500	30

Appendix 5

Immunoperoxidase data collection

Case	CD3	CD4	CD8	CCR6	FoxP3	CD1a
Patient #1						
Patient #2						
Patient #3						

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Curriculum Vitae

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