Thesis submitted for the degree of Master of Science in the Faculty of Health Science, Department of Medicine at the University of Cape Town.

Title:

Assessment of progressive pigmentary changes in interface dermatosis using longitudinal histopathology, immunohistochemistry, and electron microscopy at three-time intervals in Fitzpatrick skin types III – VI.

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Year: 2023

University of Cape Town

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Date: 08/06/2023

Dedication:

I dedicate this thesis to my family for supporting me through this journey.

Acknowledgements:

I would like to thank:

My supervisors, Dr. Thuraya Isaacs and A/Prof. Rannakoe Lehloenya, of the Division of Dermatology, Department of Medicine at Groote Schuur Hospital.

My co-supervisor, Prof. Komala Pillay, of the Anatomical Pathology Department at UCT.

The laboratories and personnel who played a vital role in helping me produce accurate results namely, Red Cross Hospital, Histology Laboratory (NHLS) and Groote Schuur Hospital, Histology Laboratory (NHLS).

Dr. Solal Noubuadinan at Groote Schuur Hospital, Division of Dermatology Department for doing the biopsies, making sure patients give consent and filling in the Case Report Forms.

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Abstract

Interface dermatoses (ID) are characterized by lymphocyte infiltration of the dermo-epidermal junction. The spectrum of ID includes lichen planus, lupus erythematosus, fixed drug eruption and Stevens-Johnson syndrome. A major sequela of ID in pigmented skin is post-inflammatory hyperpigmentation (PIHP). The frequency and severity of PIHP varies amongst different ID for unclear reasons. PIHP can be disfiguring following many dermatoses and some cosmetic and medical interventions, especially in dark-skinned individuals. There is limited understanding of PIHP pathomechanisms and little progress has been made in prevention and treatment of PIHP. ID provide a platform to understanding PIHP, a condition that predominantly affects darker skin. In this study we aimed to determine the progression of PIHP by longitudinal examination of the histopathological features of interface dermatitis at three-time intervals (acute stage, during resolution and upon complete healing) using electron microscopy and light microscopy.

This was a prospective study undertaken at the Groote Schuur dermatology clinic over a 16-month period (March 2021 – June 2022). The study population included all patients with interface dermatitis that were diagnosed by the attending dermatologist and confirmed on biopsy during March to December 2021 and subsequently followed up for 6 months. Biopsies were taken at three-time intervals and compared morphologically by using transmission electron microscopy and light microscopy.

In this study, it was noted that the degree of melanophages present in the majority of the participants correlate with the degree of chronic inflammatory cell (CIC) infiltration. The only 2 factors that are common amongst all the patients across all the periods were the presence of CIC infiltrate and melanophages. FDE and BFDE were assessed, and the key difference was the presence of full thickness epidermal necrosis due to the necrotic keratinocytes in the epidermal layer of BFDE.

Upon assessing HMB45 and Melan-A staining, it was found that in participants with atrophic epidermal features, that the HMB45 and Melan-A staining count was decreased or absent. This was also observed in participants with a necrotic epidermal layer and participants with DLE, however with regeneration improvement in the HMB45 and Melan-A staining results were observed. No correlation was found between the quantity of melanophages and extracellular melanin when assessing CD68 and MF stain results. Chronic inflammatory cell infiltrate and melanophages persisted in all participants from initial to final biopsy. All data showed no statistical significance, a larger population size could be used to further asses any significance in correlation seen.

Abbreviations:

- ACTH – Adrenocorticotropic Hormone
- α-MSH - α-Melanocyte-stimulating hormone
- ASP – Agouti Signalling Protein
- BFDE – Bullous Fixed Drug Eruption
- cAMP – Cyclic Adenosine Monophosphate
- DNA - Deoxyribonucleic Acid
- DOPA - L-3,4-dihydroxyphenylalanine
- EM – Electron Microscopy
- FDE – Fixed Drug Eruption
- FSPC – the Fitzpatrick Skin Phototype Classification
- H&E – Haematoxylin and Eosin
- HIV – Human Immunodeficiency Virus
- Hsp70-1A – Heat Shock Protein 70 – 1A
- IHC - Immunohistochemistry
- LP – Lichen Planus
- MALDI-TOF – Matrix Assisted Laser Desorption Ionization- Time of Flight
- MC1-R – Melanocortin 1 Receptor
- PI – Pigment Incontinence
- PIHP – Post Inflammatory Hyperpigmentation
- SJS – Stevens-Johnson Syndrome
- TEM – Transmission Electron Microscopy
- TEN – Toxic Epidermal Necrolysis
- TYR – Tyrosine-Related Protein
- TYRP1 – Tyrosinase-Related Protein 1
- TYRP2 – Tyrosinase-Related Protein 2
- UV - Ultraviolet

Classification of terms:

- Action spectrum- the wavelength of ultraviolet radiation leading to carcinogenicity
- Bullous - the blister formation of the skin
- Fitzpatrick skin classification - numeric classification of skin type according to the constitutive skin colour and reaction to sun exposure
- Histochemistry - histological technique to identify chemical constituents of tissue using stains
- Immunohistochemistry - histological technique to identify antigens in tissue using antibodies and chromogen
- Interface dermatosis -a group of disorders characterised by an inflammatory infiltrate of the dermo-epidermal junction resulting in morphological changes of the basal keratinocytes, the dermo-epidermal junction, and the papillary dermis
- Keratinocytes - specialised epithelial cells found in the epidermis that produce keratin
- Melanin - natural pigment which is produced by melanocytes that gives human skin and hair their colour
- Melanocyte- specialised melanin-producing, neural crest-derived cells located in the basal layers of the skin and hair follicles
- Melanogenesis - it is the process of melanin production in melanosomes
- Melanosomes - organelles found within melanocytes that are responsible for synthesis, storage, and transport of melanin
- Pigment incontinence - a histopathological term used to describe the deposition of melanin in the papillary dermis
- Ultrastructure - cell architecture only visible using an electron microscope

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Chapter 1

1.1 Introduction and Significance of Research

South Africa has a highly diverse population made up of people with different ethnic origins, mixed ethnicities, and skin colors. Pigmentation of the skin plays important roles in human lives including biological functions and social identity. The type and quality of normal skin pigmentation impacts on pigmentary disorders. In this dissertation we will be looking at pathological characteristics and evolution of post-inflammatory hyperpigmentation (PIHP) in patients with Interface dermatoses seen by the Dermatology Unit at Groote Schuur Hospital in Cape Town, South Africa. The patients were clinically diagnosed with either lichen planus (LP); discoid lupus erythematosus (DLE); bullous fixed drug eruption (BFDE); fixed drug eruption (FDE) or lichenoid drug reaction (LDR). A better understanding of the pathogenesis of PIHP could aid in the development of preventative and therapeutic strategies of this non-life threatening but debilitating condition. Abnormal skin pigmentation may lead to social embarrassment or even self-isolation. LP; BFDE; FDE; DLE and LDR with its various clinical severities that seem contrasting with the degree of PIHP, provide a solid basis to study evolution of PIHP at different intervals.

Classification of Skin Pigmentation

A scale was developed by Thomas B. Fitzpatrick to predict patients' sensitivity to phototoxic reactions during phototherapy. This classification is known as the Fitzpatrick Skin Phototype Classification (FSPC) (Magin and Pond, 2012; D'Orazio et al, 2013). The classification is demonstrated in table 1 and in more detail in Figure 12.

TABLE 1: FITZPATRICK CLASSIFICATION (MAGIN AND POND, 2012)

Type I	Always burns, never tans
Type II	Always burns, sometimes tans
Type III	Sometimes burns, always tans
Type IV	Never burns, always tans
Type V	Brown skin of Asian descent
Type VI	Black skin

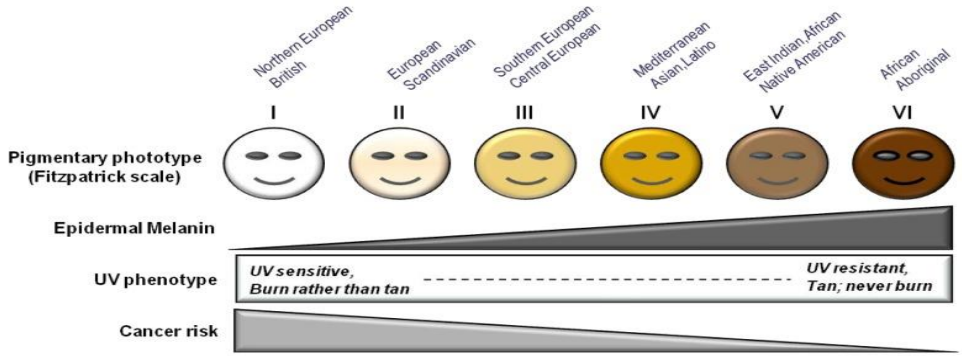

FIGURE 1: DEMONSTRATION OF THE FITZPATRICK CLASSIFICATION RELATED TO SKIN CANCER PREVALANCE. (D'ORAZIO ET AL, 2013)

Classification and characteristics of PIHP

PIHP is defined as a reactive process resulting from increased melanin or abnormal distribution of melanin secondary to inflammatory skin conditions, dermatologic therapies, and external stimuli (Shenoy and Madan, 2020). PIHP is broadly classified as either epidermal or dermal. In epidermal PIHP there is an increase in epidermal pigmentation and melanogenic activity by melanocytes. In dermal PIHP there is an increase in dermal pigmentation, decrease in epidermal pigmentation with an increased melanogenic activity. In both types, the number of melanocytes were found to be the same, however increased melanogenic activity was observed in both types (Lehloenya et al 2022; Park et al, 2017).

Mechanism of Dermal pigmentation:

Dermal hyperpigmentation:

Dermal PIHP occurs due to the destruction of basal keratinocytes, these keratinocytes contain large amounts of melanin as melanosomes are transferred to basal keratinocytes upon maturation. The degenerated basal keratinocyte loses its melanin and is phagocytosed by surrounding macrophages in turn becoming melanophages, which enter the dermis. The mechanism of dermal PIHP has been associated with basal layer vacuolar degeneration, weakening or loosening of the basal layer causing a disruption of melanin pigment being transferred to keratinocytes and extensive perivascular lymphocytic infiltrate as well as higher numbers of mast cells and macrophages (Park et al., 2017; Gru and Salavaggione, 2017). Melanophages tend to accumulate at the site of injury, which is characterised by marked increased dermal pigmentation and decreased epidermal pigmentation. Dark-gray or blue-gray patches are present on the skin in dermal PIHP, and without treatment these patches may take years to heal or become permanent (Callender et al. 2011; Park et al., 2017).

Another theory suggests that PIHP occurs due to the oxidation of arachidonic acid by peroxides, cyclo-oxygenase and 5-lipoxygenase which results in the formation of intermediates that form prostaglandins, leukotrienes and thromboxanes. Epidermal melanocytes become hypertrophic due to the stimulation of

prostaglandins, leukotrienes and thromboxanes, which leads to the increase in melanin synthesis. This leads to the transfer of melanin to keratinocytes and dermal macrophages, resulting in hyperpigmented lesions (Cardinali et al, 2012).

Epidermal hyperpigmentation

Epidermal PIHP occurs due to the overstimulation of melanocytes leading to an increase of melanin synthesis, which is exerted by the presence of cytokines, inflammatory mediators, and reactive oxygen species. Thus, there is an increase in number (hyperplasia), size (hypertrophy) and melanogenic activity. Tan to dark brown patches are present in epidermal PIHP, and without treatment may take months to years to resolve (Callender et al. 2011).

- melanocytes become donors of dermal melanosomes through gaps in the basal lamina
- free melanosome uptake by macrophages, which migrate to the dermis
- Macrophage uptake of abnormal/damaged keratinocytes together with their melanosomes, the macrophages end up in the dermis
- Cytokine stimulation by damaged keratinocytes maintains macrophages

Causes and triggers of PIHP

Extrinsic factors include UV radiation and certain chemical compounds such as drugs like tenofovir, an antiretroviral drug commonly used to treat HIV, and chemical peels and microneedling. Intrinsic factors include molecules secreted by keratinocytes, fibroblasts, inflammation, and conditions such as pregnancy. It has been proposed that the response to stimulatory conditions such as pregnancy and diabetes is mediated by the cutaneous neuroendocrine system (D'Mello et al, 2016; Gupta et al, 2015; Eluwa et al 2012).

Risk Factors

Various studies regarding PIHP indicates that it's more frequently associated with Fitzpatrick's phototypes IV to VI (Kaufman et al, 2018).

In a study done by Kang et. al. it was concluded that velvety skin with smaller pores is more susceptible to PIHP, when using a QS 532 NY laser. This laser treatment is frequently used to treat solar lentigines and pigmented lesions (Kang et al., 2017 and Chandrashekar et al., 2018). However, Fitzpatrick's phototype was not found significant relating to the incidence of PIHP amongst patients according to the study, rather the presence of erythematous lentigines was more likely to result in PIHP (Kang et al., 2017). Hyperpigmentation is most frequent complication after chemical peeling with trichloroacetic acid, with skin types III-VI. Fitzpatrick's phototype I and II are high risk when these individuals have early sunlight exposure without the use of sunscreen. Individuals taking oestrogen containing medication are also considered high risk. (Nikalji et al., 2012). This was also seen in individuals who had microneedling done, whereby Fitzpatrick's phototype III-VI experienced PIHP (Cary et al, 2019)

1.2 Aim:

To determine the progression of PIHP in patients with interface dermatitis at three-time intervals (acute stage, during resolution and upon complete healing).

1.3 Objectives:

1. Identify and correlate the characteristics of interface dermatitis at three-time intervals using Haematoxylin and Eosin (H&E) staining.
2. Use the special stain, Masson-Fontana to quantify extracellular melanin at three-time intervals in patients with interface dermatitis.
3. Quantify melanophages at three-time intervals in patients with interface dermatitis using CD68 immunohistochemical staining.
4. Quantify melanocytes at three-time intervals in patients with interface dermatitis using Melan-A immunohistochemical staining.
5. Quantify the immunoreactivity of melanocytes at three-time intervals in patients with interface dermatitis using HMB45 immunohistochemical staining.
6. Use electron microscopy (EM) to characterise keratinocytes, melanocytes, and melanosomes ultrastructurally.

Chapter 2: Background and Literature review

2.1 Background:

Interface dermatoses (ID) are characterized by lymphocyte infiltration of the dermo-epidermal junction. The spectrum of ID includes lichen planus and its variants, lupus erythematosus, fixed drug eruption. A major sequela of ID in pigmented skin is post-inflammatory hyperpigmentation (PIHP). The frequency and severity of PIHP varies amongst different ID for unclear reasons. PIHP can be disfiguring following many dermatoses and patients with darker skin are predisposed to PIHP following certain cosmetic and medical interventions such as laser therapy. There is limited understanding of PIHP pathomechanisms and little progress has been made in prevention and treatment of PIHP. ID provide a platform to understanding PIHP, a condition that predominantly affects darker skin.

2.2 Literature Review

2.2.1 Skin

Functions of the skin

The human skin plays a crucial role in the body by serving as a protective barrier against a wide range of external factors such as physical trauma, harmful chemicals, pathogens, immunogenic substances, ultraviolet (UV) radiation, and free radicals. In addition to its protective function, the skin also acts as a sensory organ, endocrine organ, regulator of body temperature and blood pressure, excretory organ, and immune organ (McGrath, 2020; James et al., 2019).

Skin as a physical barrier:

Keratinocytes are specialised cells which produce keratin and filaggrin, which serves as the physical barrier of the skin (Proksch et al, 2008).

This is achieved by terminal differentiation of keratinocytes, which leads to the formation of cornified cell envelopes, which are highly insoluble. The cornified cell envelope forms in the stratum corneum as keratinocytes differentiate. The cornified cell envelope increases in rigidity and thickens by the sequential cross-linking of several structural components, such as involucrin, loricrin, SPRRs, elafin, cystatin A, S100 family proteins, and certain desmosomal proteins. These components are catalysed by transglutaminases (Ishida-Yamamoto and Ilzuka, 1998).

The stratum corneum is composed of an intercellular lipid bilayer matrix and hygroscopic molecules, which also play a role in retaining hydration (Verdier-Sévrain and Bonté, 2007).

Skin as an immunological organ:

Keratinocytes also serves as a chemical barrier maintained by the expression of S100A7, which is an antimicrobial substance capable of killing *Escherichia coli* effectively. Skin contains antimicrobial peptides which are effective against a broad spectrum of microorganisms (McGrath, 2020). Keratinocyte-derived endogenous β-defensins and cathelicidins also provide an innate immune defence against bacteria, viruses, and fungi (Braff et al., 2005). Langerhans cells found in the epidermis determine the adaptive immune response required by interpreting the microenvironment and presenting as antigen presenting cells (Clayton et al., 2017).

Protection against UV radiation:

Melanin provides protection against deoxyribonucleic acid (DNA) damage in epithelial cells by absorbing or scattering UV radiation (James et al., 2019).

As an endocrine and thermoregulatory organ:

Skin also plays a vital part in vitamin D_3 synthesis and thermo-regulation. Thermo-regulation is achieved by vasodilation or vasoconstriction of blood vessels regulating heat loss. Eccrine sweat glands are responsible for sweat secretion to keep the body's temperature constant in hot conditions (James et al., 2019).

As a sensory organ:

Skin contains multiple nerve endings and Merkel cell-neurite complexes which allows for the sensation of temperature, touch, pressure, and vibration (James et al., 2019).

Scaffolding, insulation, and shock absorption roles:

Subcutaneous fat plays a role in cushioning against trauma and provides insulation protecting the body from heat and cold, and mechanical shock (James et al., 2019).

Blood pressure regulation:

The skin is able regulate blood pressure by regulating sodium by the skin interstitium (Martin, 2023).

Secretory function including drugs:

The detection of drugs, or lipophilic substances reach the skin surface via the sebum, whereas hydrophilic substances utilize the sweat for delivery to the skin surface (Bittremieux et al, 2022).

The structure of the skin and its appendages

The skin is a large organ and made up of three layers: the epidermis, dermis, and subcutaneous fat. The epidermis and dermis are separated by a complex structure called the basement membrane. This membrane is also known as the dermal-epidermal junction or the interface. Keratinocytes and melanocytes invaginate from the epidermis, extending into the dermis to form hair follicles. The skin consists primarily of three cell types: keratinocytes, melanocytes, and fibroblasts. The dermal layer also contains macrophages, lymphocytes, and mast cells (Hirobe, 2014; Nguyen and Soulika, 2019)(Figure 1).

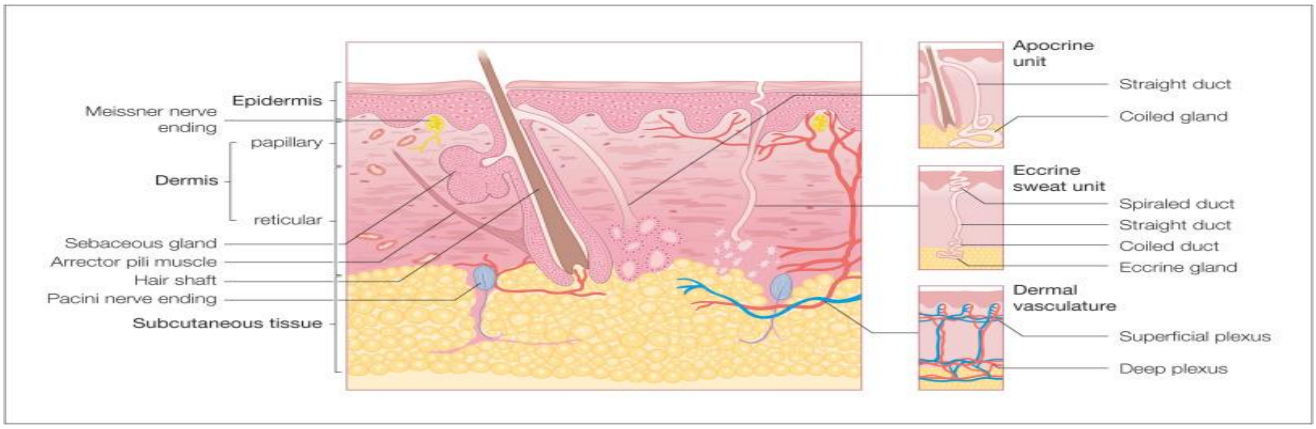

FIGURE 2: DIAGRAMMATIC CROSS SECTION OF SKIN (JAMES ET AL, 2019)

Epidermis

The epidermis can be divided into 4 layers:

Basal cell layer (stratum basale):

The basal cell layer is characterised by cuboidal or columnar cells with large nuclei containing nucleolus. The clear cells observed with haematoxylin and eosin (H&E) staining represents the melanocytes (McGrath, 2020). The characteristic mitotic activity of the keratinocytes in the stratum basale can also be observed with H&E staining (Junqueira and Carneiro, 1980).

Spinous cell layer (stratum spinosum):

The spinous cell layer is characterised by polygonal prickle cells, which contains eosinophilic cytoplasm and oval vesicular nuclei with nucleoli. Langerhans cells are present throughout the epidermis; however, they are most easily recognised within this layer, and form part of the immune system sentinels. The keratinocytes have visible intercellular bonds presented like spiny projections; thus, it is referred to as the spinous cell layer. In this layer the keratinocytes start synthesizing fibrillar proteins called cytokeratins. Cytokeratins build up within the cells, aggregating together to form tonofibrils. The tonofibrils go on to form desmosomes. Desmosomes are the major intercellular adhesion complexes in the epidermis, which are responsible for the strong bonding between adjacent keratinocytes. The desmosomes can be observed by using electron microscopy (EM) as seen in Figure 4 (James et al, 2019). This is due to

desmosomes containing electron-dense plaque which are present along the cytoplasm parallel the junctional region (McGrath, 2020).

Granular cell layer (stratum granulosum):

The granular cell layer is characterised by the presence of basophilic and electron-dense ovoid granules called keratohyalin granules, which are proteins situated in the cytoplasm of keratinocytes. The keratinocytes form 3-5 tightly packed layers and becomes flattened and takes on a tetrakaidecahedron shape with central nuclei (McGrath, 2020; Junqueira and Carneiro, 1980).

Keratohyalin granules can be identified with H&E staining and distinguished from the other epidermal layers due to its basophilic nature. EM identification can also be done to identify keratohyalin granules due to its electron density (McGrath, 2020; Young et al. 2014). Keratohyalin granules are responsible for the keratinisation, whereby keratinocytes in the granular layer are differentiated into corneocytes. During the process of keratinisation, the cross-linking and aggregation of keratin precursors form bundles. Keratohyalin granules contain filaggrin and trichohyalin intermediate filament associated proteins, which are modified and subsequently released into fully mature keratinocytes where they function in the role of aggregating keratin filament bundles (Manabe and O'Guin, 1992). Lamellar granules initially formed in the spinous layer and accumulate at the granular layer provide structural protein, proteases and protease inhibitors required for cell-cell adhesion structures and timely desquamation. Desmosomes play a major role in intercellular adhesion in the basal layer to the granular layer. In the granular layer, corneodesmosomes are released from the lamellar granules and are incorporated into the cornified cell envelope. Transglutaminase 1 is the main enzyme involved in catalysing cornified cell envelope cross-links (Ishida-Yamamoto et al, 2018).

Cornified layer (stratum corneum)

The cornified layer is characterised by flattened keratinocytes that lose their nuclei. The cytoplasm comprises of dense compact keratin, embedded in a dense amorphous matrix. Keratin is a birefringent filamentous scleroprotein. Cells are continuously shed from the surface as keratinization progresses (McGrath, 2020; Junqueira and Carneiro, 1980). Between the cornified cells, intercellular lipids and lipid processing enzymes derived from lamellar granules are responsible for water permeability barrier function (Ishida-Yamamoto et al, 2018).

Stratum lucidum

In certain areas of the body such as the soles and palms; and where skin is chronically scratched or rubbed, an additional acellular layer known as stratum lucidum is seen. The stratum lucidum consists of 2 to 3 flattened, diamond-shaped cell layer containing eleidin. Eleidin is a clear intracellular protein and transformation product of keratohyalin (Yousef et al, 2021). The layer may appear clear to pink on H&E staining due eleidin and located between the granular and corneocyte layers (James et al, 2019; McGrath et al, 2020; Nguyen and Soulika, 2019). Refer to Figures 2 and 3.

Anatomical variation of the epidermis

Glabrous skin (non-hair bearing skin) such as the palms and soles consist of a thicker and more compact stratum corneum compared to hair-bearing skin. Glabrous skin does not contain hair follicles or sebaceous glands; however, it contains encapsulated sense organs within the dermis. The stratum spinosum is thicker with more abundant tonofibrils in these areas of the body (Junqueira and Carneiro, 1980). In response to friction in these areas, keratin production is enhanced. The spinous cell layer forms more layers, and the granular layer becomes thicker and more prominent. Keratin in the corneocyte layer becomes thicker with more density (James et al, 2019).

Hair bearing skin does not contain encapsulated sense organs; however, it contains hair follicles and sebaceous glands. The size, structure and density of hair follicles vary in different anatomical sites. For example, the scalp has a larger hair follicle than the forehead. In older individuals, epidermal thinning and dermal atrophy can be observed (McGrath, 2020).

In a study conducted by Yamaguchi et al, it was reported that the melanocyte density in palms and soles of human skin (palmoplantar skin) is five times lower than non-palmoplantar skin. This is due to the palmoplantar fibroblasts suppressing the growth and pigmentation of melanocytes (Yamaguchi et al, 2004). The number of melanocytes is higher in areas of the body that is more exposed to UV light (Brenner and Hearing, 2008). Dickkopf 1 expression by fibroblasts are responsible for inhibiting the function and proliferation of melanocytes by suppressing beta-catenin and microphthalmia- associated transcription factor. The expression of Dickkopf 1 is significantly higher in palmoplantar skin than non-palmoplantar skin, this explains why palmoplantar epidermis is paler than non-palmoplantar epidermis (Yamaguchi, et al. 2007)

A study conducted by Thibodeau and D'Ambrosio, reflectance spectrophotometry was used to measure the melanin and haemoglobin pigmentation in lips and skin in individuals. It was found that significant differences exist in melanin pigment in various anatomical sites and haemoglobin levels were not significantly different in various anatomical sites, however a difference was seen amongst various skin types. The lower and upper lips had higher levels of melanin and haemogloblin compared to the other anatomical locations examined (medial aspect of lower right arm, the left cheek below the level of zygoma) independent of individual skin type (Thibodeau and D'Ambrosio, 1997). Age also plays a role in the distribution of skin colour consistency in various anatomical sites. Melanin and erythema indexes were found to be distributed more evenly in younger individuals compared to older ones, various anatomical sites measured also had a variation in melanin measurement using a spectrophotometer (Yun et al, 2010).

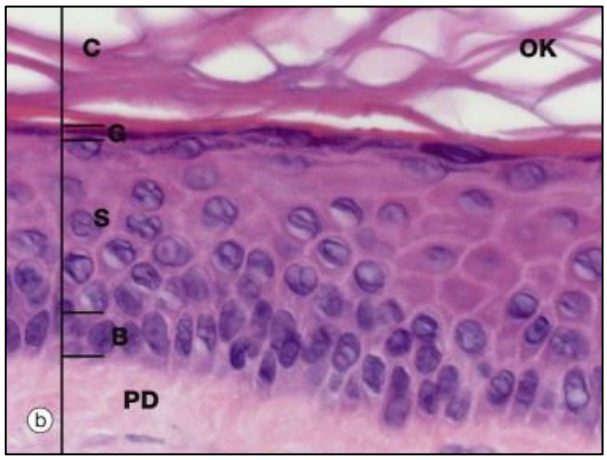

FIGURE 3: TRANSVERSE EPIDERMIS (H&E STAIN AT HIGH MAGNIFICATION POWER) (YOUNG ET AL, 2014)

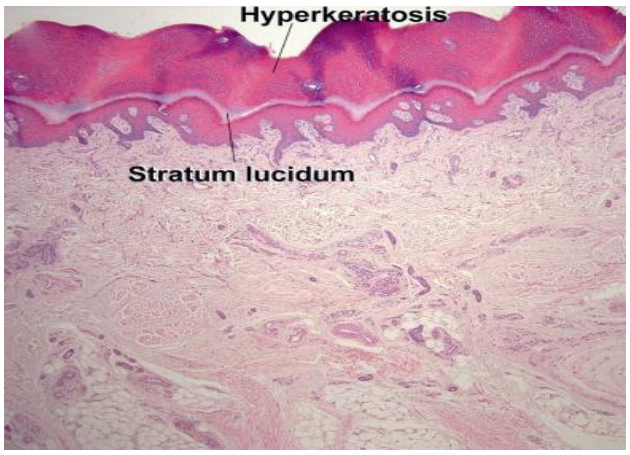

FIGURE 4: VOLAR SKIN DEMONSTRATING STRATUM LUCIDUM (H&E STAIN X100) (JAMES ET AL, 2019)

PD- papillary dermis; B- stratum basale; S- stratum spinosum; G- stratum granulosum; C- stratum corneum; OK- orthokeratosis

2.2.2 Cells of the epidermis:

The epidermis consists primarily of two cell types, keratinocytes and melanocytes; and a host of resident and migrating immune cells.

Keratinocytes:

The keratinocytes proliferate and migrate from the basal cell layer to the corneocyte layer in a process called keratinization. During this process the keratinocytes in the stratum basale undergo mitotic division readily replacing cells in the stratum spinosum by upward migration with associated maturation. In the stratum spinosum the keratinocytes synthesize cytokeratins, which accumulate in aggregates to form tonofibrils. These tonofibrils then bind to desmosomes to form strong contacts with neighbouring keratinocytes. Keratinocytes then progress to the stratum granulosum where they acquire dense basophilic keratohyalin granules. These granules interact with the tonofibrils to produce keratin. Further maturation to the stratum corneum leads to the loss of their nuclei and cytoplasm to become keratin squames. This programmed process is also known as terminal differentiation (James et al,2019; Young et al, 2014). Keratinocytes can be observed by using H&E staining as seen in Figure 2. The use of electron microscopy allows for the observation of keratinocytes in more detail, making it possible to observe the tonofibrils, keratohyalin granules and desmosomes (James et al, 2019). Refer to Figure 5 and 6.

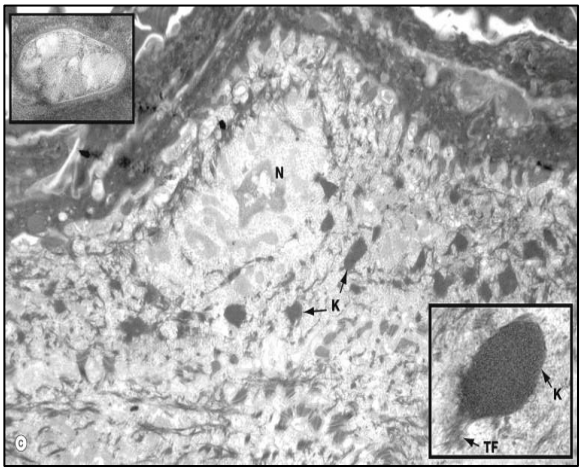

FIGURE 5: KERATINOCYTE IN THE GRANULAR LAYER (EM X12 000) (JAMES ET AL, 2019)

N – nucleus of keratinocyte, K – keratohyalin granules

The inset demonstrates the desmosome and tonofibril linkage between keratinocytes.

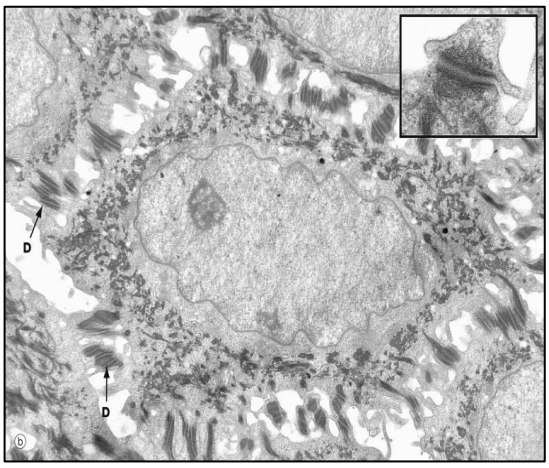

FIGURE 6: KERATINOCYTE IN THE STRATUM SPINOSUM (EM X15 000) (JAMES ET AL, 2019)

D – desmosomes

The inset demonstrates the desmosome and tonofibril linkage between keratinocytes.

Melanocytes:

Melanocytes are derived from neural crest cells and are classified as dendritic cells. They are found in the stratum basale of the epidermis. Melanocytes do not keratinise like keratinocytes. The dendrites expand within the epidermis and reach multiple keratinocytes to form an epidermal melanin unit. On H&E-stained tissue sections, melanocytes are characterised by their vacuolated cytoplasm, which is an artefact caused by fixation (Hirobe, 2014; Riley, 1997; McGrath, 2020). This is due to the melanocytes not forming desmosomal attachments with keratinocytes, due to their lack of tonofibrils (James et al, 2019). Refer to Figure 7 and 8. Ultrastructurally keratinocytes are differentiated from melanocytes by the cell-cell junctions between keratinocytes. Melanocytes are recognised by the presence of melanosomes and pale cytoplasm. They also demonstrate the lack of tonofilaments, hemidesmosomes and desmosomes (James et al, 2019). Refer to Figure 7 and Figure 8. By using a melanocyte antigen such as Melan-A, mature melanocyte staining is specific (Blessing et al, 1998).

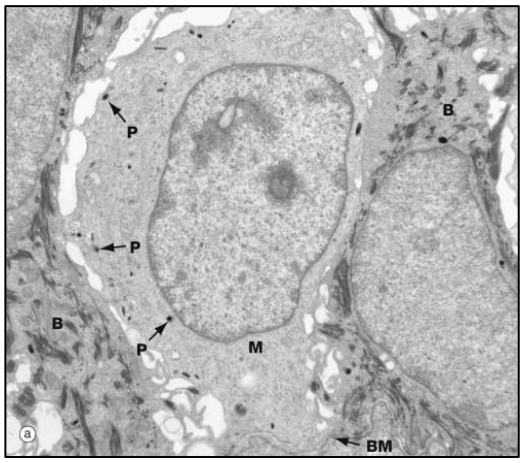

FIGURE 7: MELANOCYTE (EM X1500) (YOUNG ET AL, 2014)

P- melanosomes, M- melanocyte, B- basal keratinocyte, BM – basement membrane

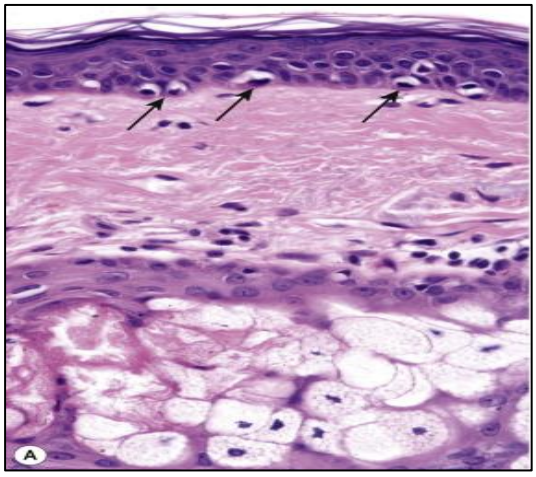

FIGURE 8: TRANSVERSE SKIN (H&E) (MCGRATH, 2020)

Melanocytes denoted by arrows.

2.2.3 Melanosomes

Melanosomes are cytoplasmic organelles found in melanocytes and derived from the smooth endoplasmic reticulum of the skin, hair, and retinal epithelial cells (McGrath, 2020). Melanosomes originate from early endosomes, and its biogenesis occurs in melanocytes. Melanosomes are classified as lysosome-related organelles and share similar characteristics with other lysosomes which include biogenesis, maturation, trafficking, recycling, and migration. Mature melanosomes are transported to dendrites from which it is transferred to connecting keratinocytes and accumulate in the cytosol to protect against DNA damage. The role of melanosomes is melanogenesis, storage and transportation of melanin (Hirobe, 2014; James et al, 2019; Wiriyasermkul, 2020). Melanosome maturation has four developmental stages, in which melanin is formed. This takes place in the Golgi zone of the granular endoplasmic reticulum of the melanocyte. Stages 1 and 2 melanosomes are unmelanised immature premelanosomes and stages 3 and 4 are melanised melanosomes (Hirobe, 2014; Junqueira and Carneiro, 1980).

Melanosome transportation:

The transportation of melanosomes to keratinocytes has been extensively researched; however, a defining conclusion is yet to be made as various research produced different results (Wu and Hammer, 2014; Delevoye, 2014). Wu, X. and Hammer, J. (2014) explains the four (4) possible mechanisms for transportation as either by:

- Cytophagocytosis
- Membrane fusion
- Shedding-phagocytosis
- Exocytosis-endocytosis

Cytophagocytosis - This mechanism of transport is achieved by phagocytosis of the dendrite tip of the melanocyte by the keratinocyte. This is initiated by the dendrite making contact with surrounding keratinocyte. The keratinocyte then reacts by engulfing the dendrite tip with villus-like cytoplasmic projections. It is then pinched off resulting in the formation of a cytoplasmic pouch filled with melanosomes. The melanocyte membrane surrounding the melanosomes is then degraded, as well as the cytoplasmic constituents by phagolysosomes. The phagolysosomes disintegrate into smaller vesicles containing either single or multiple melanin granules, which is dispersed in the keratinocyte cytoplasm (Van Den Bossche et al, 2006).

According to Van Den Bossche et al, various authors could not achieve the same results as that of the original study. In the initial study time-lapse cinematography was done using light microscopy. Transmission electron microscopy and scanning electron microscopy was also used for observation (Okazaki et al, 1976). The cross-section images obtained by using these techniques was interpreted as a melanocyte dendrite being surrounded by keratinocyte cytoplasm, however this could also represent an enfolded dendrite that is still attached to the melanocyte (Van Den Bossche et al, 2006).

Membrane fusion – Membrane fusion occurs when the melanocyte plasma membrane fuses with the keratinocyte plasma membrane, which results in the formation of a pore or a channel connecting the cytoplasms allowing the transportation of the melanosomes. It was suggested that the pore or channel formation is a filopodia or tunnelling nanotubes (Van Den Bossche et al, 2006).

The optical properties of melanocyte and keratinocyte membranes are similar under light microscopy making it difficult to observe the fusion. However, thin projections can be seen connecting melanocyte and keratinocyte cytoplasm via electron microscopy. It is suggested that more research is required to determine the function of the melanocytic filopodia, to determine if they serve as conduits for melanin transfer or melanocyte-keratinocyte adhesion (Van Den Bossche et al, 2006).

Shedding-phagocytosis - The shedding–phagocytosis mechanism proposed by (Ando et al, 2012) and (Wu et al, 2012) involves the release of melanosomes in a micro-vesicle from the dendrites of melanocytes and the capture by the micro-villi of the keratinocytes. A micro-vesicle is a single membrane vesicle which transports a variety of substances. Micro-vesicles may interact specifically with recognition signals on target cells. The membrane of the micro-vesicle is then degraded resulting in the melanosomes being spread into the cytosol (Wu and Hammer, 2014; Ando et al, 2012; Wu et al, 2012).

Exocytosis-endocytosis - The exocytosis-endocytosis study by (Tarafder et al, 2014) was conducted by using electron microscopic analysis. They noted the presence of melanin granules without membranes in the extracellular space between the melanocytes and the keratinocytes. Following keratinocyte invagination, individual melanin granules were surrounded by a single membrane. It was also noted that TYRP1, a marker for melanosome membrane was absent in the keratinocytes suggesting that the melanocyte-derived melanosomes membrane is absent (Tarafder et al, 2014).

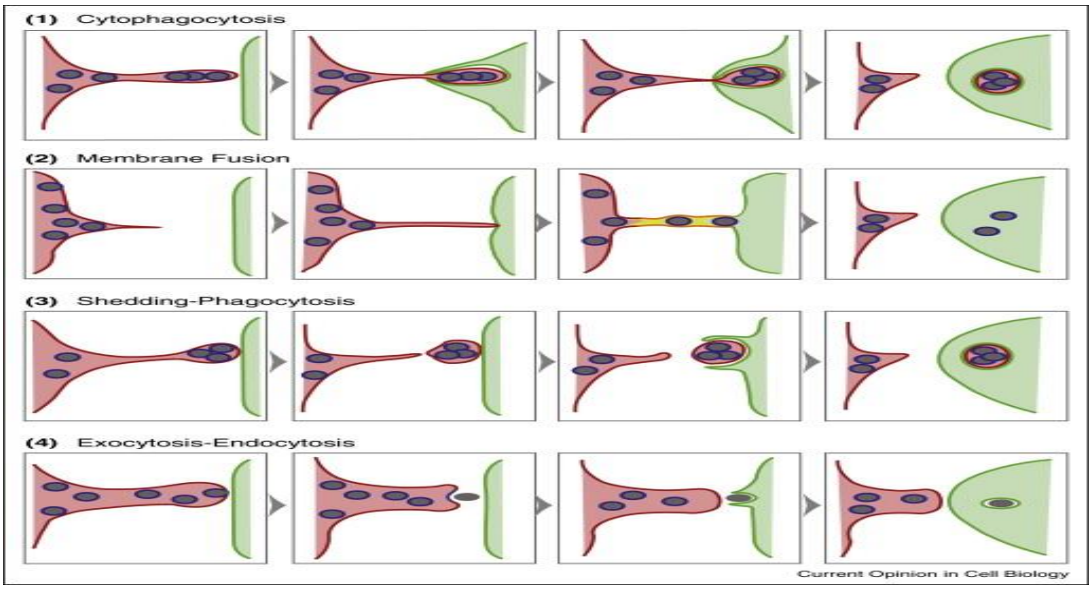

FIGURE 9: PURPOSED MELANOSOME TRANSFER MECHANISMS (WU AND HAMMER, 2014)

Light skinned individuals have fewer and smaller melanosomes compared to dark skinned individuals. The melanosomes are also packaged within membrane-bound complexes, whereas in dark skinned individuals it is singly dispersed (James et al, 2019). Melanosomes are produced in various shapes, sizes, numbers, and densities. Melanosomes containing eumelanin are elliptical with longitudinal depositions of pigments in the intraluminal fibrils. Melanosomes containing pheomelanin are spherical with granular depositions of pigments within multivesicular bodies (Hirobe, 2014).

2.4 Melanin

Melanin refers to the natural pigment synthesised by melanocytes located in the stratum basale, within melanosomes during melanosome development (Young et al, 2014), refer to Figure 10. In Figure 11 the Masson-Fontana (MF) stain demonstrates the presence of melanin in melanocytes and keratinocytes as expected in dark skinned individuals (McGrath, 2020). Melanin in human skin is sub-divided into eumelanin and pheomelanin. Eumelanin is the black-brown insoluble pigment and pheomelanin refers to the yellow to red brown alkali-soluble pigment (Ito and Wakamatsu, 2008). The type of melanin produced is dependent on the function of the melanogenic enzymes and availability of substrates during melanogenesis (d'Ischia et al, 2015; Wiriyasermkul et al, 2020).

Eumelanin is the major factor contributing to skin colour, as eumelanin is predominately produced in darker skin and pheomelanin is predominately in lighter skin. Thus, the ratio of eumelanin to pheomelanin determines skin pigmentation (Lin and Fisher, 2007; McGrath, 2020; Wiriyasermkul et al, 2020). Eumelanin and pheomelanin have different responses to UV radiation. It is generally accepted that eumelanin is a photoprotective antioxidant and pheomelanin is a phototoxic pro-oxidant. Moreover, the antioxidant properties of melanin are related to the ratio of eumelanin to pheomelanin; the greater the ratio of pheomelanin to eumelanin; the greater the chance of phototoxicity (Ito and Wakamatsu, 2008).

Melanin quantity and quality is determined by the differentiated state and number of melanocytes present, the degree of melanogenesis, blood supply, dendricity and environmental factors. It is also determined

by genetics and epigenetic factors (Hirobe, 2014). It has been determined that white skin is approximately 70 times more likely to develop skin cancer than black skin due to differences in constitutive pigmentation (d'Ischia et al, 2015). This is due to pheomelanin being less protective against UV radiation and reactive oxygen species than eumelanin, because of their different chemical structures and optical properties (Wiriyasermkul et al, 2015).

Phenotypically these two melanin sub-types can be identified by looking at the human anatomy. The phenotype of hair colour correlates with the ratio of more eumelanin to pheomelanin content, however red hair contains four times the amount of pheomelanin compared to the eumelanin (Ito and Wakamatsu, 2003). It was concluded in a study using high performance liquid chromatography, that eumelanin and pheomelanin levels were ten-times higher in deeply pigmented skin compared to light pigmented skin. The study also concluded that there was no difference in the eumelanin/pheomelanin ratio between the two groups (Hunt et al., 1995).

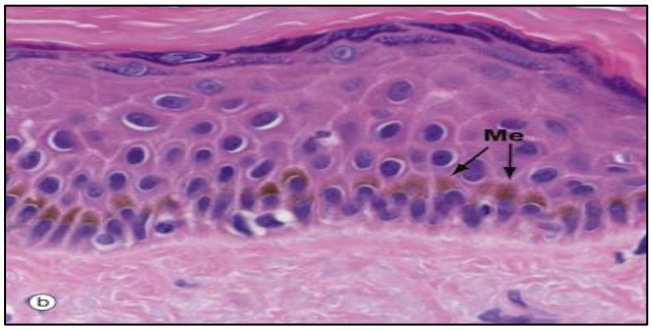

FIGURE 10: MELANIN DISTRIBUTION (H&E STAINED) (YOUNG ET AL, 2014)

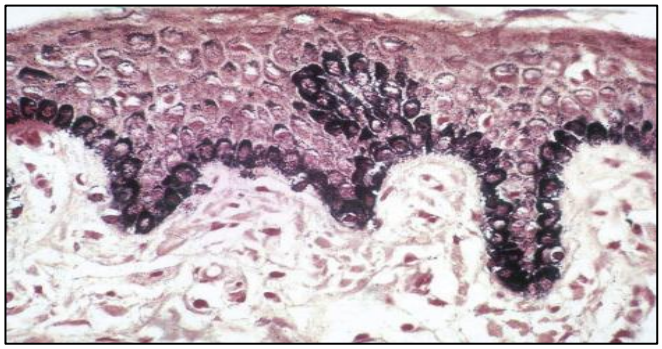

FIGURE 11: MELANIN DISTRIBUTION IN DARK SKIN (MASSON – FONTANA STAINED) (MCGRATH, 2020)

2.2.5 Immune cells of the skin:

Myeloid and lymphoid cells are found in the skin in steady state, however some of these resident immune cells migrate to lymph nodes in immune response or tolerance to tissue self-antigens. Each layer of the skin is occupied by different immune cell population and can be identified by their differential expression of markers (Yanez et al, 2017).

Macrophages/Melanophages

Macrophages are skin-resident immune cells in the dermal layer, which are part of the myeloid lineage. Under inflammatory conditions they are able to migrate to the lymph nodes (Nestle et al, 2009). It was found that dermal macrophages derive from two sources: those that develop in the yolk sac from erythro-myeloid progenitors and persist in adult tissue as resident cells, and those that form in adult tissue from bone marrow-derived circulating monocytes. Several sub-sets of macrophages exist, of which some contribute to homeostasis and other facilitate tissue repair, and others eliminate disease (Rodrigues and Gurtner, 2017). They maintain homeostasis by promoting or suppressing inflammation, and are able to do this as they can change their genetic profile (Yanez et al, 2017)

In the event of pigment incontinence, macrophages engulf the melanin by phagocytosis when it enters the dermis to form melanophages (Ortonne and Bissett, 2008). Melanophages can be distinguished from melanocytes by their aggregation around papillary dermal capillaries and their larger cell size, with the presence of bright and often coarser cytoplasmic granules (Busam et al, 2001).

Langerhans cells

Langerhans cells are present throughout the epidermis; however, they are most easily recognised within the stratum spinosum layer, and forms part of the immune system sentinels. They are derived from macrophage precursors and enters the epidermis at about 12 weeks after birth. They can be identified by H&E staining by their clear cytoplasm and irregular lobulated nuclei (McGrath, 2020; Young et al. 2014). During an immune response, which is generated by signals from keratinocytes they migrate into the dermis and enter the lymphatic vessels and drain the lymph nodes, initiating a T-cell mediated adaptive immune response or promote homeostasis. While in the steady state Langerhans cells sample antigens and upon activation they are able to extend their processes towards the stratum corneum or stratum basale (Heath and Carbone, 2013). Langerhans cells found in the epidermis determine the adaptive immune response required by interpreting the microenvironment and presenting as antigen presenting cells (Clayton et al., 2017).

Dermal immune cells

Dermal dendritic cells are situated in the dermis, and also migrate to the lymph nodes inducing T-cell proliferation (Nestle et al, 2009). They are efficient at priming adaptive immune responses. Dermal dendritic cells play a role in maintaining homeostatic interactions between host and skin-resident commensal bacteria. Plasmacytoid dendritic cells are also found in the skin during inflammatory conditions and are essential for viral defence. Mast cells enters the skin from bone marrow as progenitors and mature locally in response to environmental signals. Resident skin mast cells express tryptase and chymase, whereas in other anatomical sites only tryptase is expressed. Mast cells are also known to be involved in allergic reactions as they produce histamine. Eosinophils are skin-resident cells and are known

to promote host defence against parasitic infections (Nguyen and Soulika, 2019; Heath and Carbone, 2013)

Lymphoid cells

A variety of lymphoid cells are present in the skin and are important in both the steady state and pathophysiology. This includes T- lymphocytes and B – lymphocytes (Nguyen and Soulika, 2019). In addition to Langerhans cells in the epidermis, CD8+ T-cells can also be found in the stratum basale and stratum spinosum. The dermal layer also contains, CD4+ T-helper cells, γδ T-cells and natural killer T-cells (Nestle et al, 2009).

Inflammatory cells persist and localise within the inflamed region of the tissue. This is due to the ability of fibroblasts to govern the persistence of inflammation and establishing immunological memory. It is suggested that inflammatory cells should not be considered in isolation and that non-immune cells provide a crucial role in the formation and persistence of infiltration (Buckley, 2011). It was noted that effector T-cells are recruited to the skin during cutaneous inflammation, and some do not return into circulation post inflammation. These non-circulating T-cells are tissue resident memory T-cells, and the longevity of these cells differ between residing tissue (Honda et al, 2019).

2.2.6 Hair follicles

Hair follicles house the skin stem cells, which are able to maintain, repair and regenerate itself. These cells are based in the bulge area of the hair follicle, the base layer of the interfollicular epidermis and the base of the sebaceous glands. The stem cells only function in pilosebaceous unit homeostasis and regenerates interfollicular skin when skin is wounded (McGrath, 2020). They are also reservoirs for Langerhans cells and melanocytes. Hair follicles have a sensory apparatus for shaft movement detection and regulate body temperature (Yanez et al, 2017).

2.2.7 Melanogenesis

Melanin synthesis takes place in melanosomes via the Raper-Mason biochemical pathway and is regulated by multiple enzymes and melanin-specific genes. Eumelanin and pheomelanin are derived from the common precursor dopaquinone (Joly-Tonetti et al, 2016). Melanogenesis can be influenced by genetic, extrinsic, and intrinsic factors. Extrinsic factors include UV radiation and certain chemical compounds such as Tenofovir, which is found in HIV treatment. Intrinsic factors include molecules secreted by keratinocytes, fibroblasts, inflammation, and conditions such as pregnancy. It has been proposed that the response to stimulatory conditions such as pregnancy and diabetes is mediated by the cutaneous neuroendocrine system (D'Mello et al, 2016; Gupta et al, 2015; Eluwa et al 2012). It is hypothesised that skin colour is an evolutionary adaptation to counter UV-induced skin damage. In individuals whose ancestors recently resided in lower latitude areas tend to have darker skin compared to those from higher latitude areas (Murase et al, 2016).

Pigment production

Melanosome synthesis and melanogenesis is induced by L-tyrosine. Eumelanin and pheomelanin synthesis are initiated by the oxidation of L-tyrosine to the common precursor dopaquinone by tyrosinase (d'Ischia et al, 2015).

Eumelanin synthesis is initiated in the absence of cysteine, which is a sulfhydryl compound. The enzyme, tyrosine-related protein (TYR) catalyses the rate-limiting synthetic reaction of hydroxylating L-tyrosine to L-3,4-dihydroxyphenylalanine (DOPA). Mutations causing a disruption in TYR functionality results in albinism, which is an inherited pigmentary disorder (Yamaguchi et al, 2007). DOPA upregulates melanin synthesis (D'Mello et al, 2016). Cyclodopa is produced by intramolecular cyclization of dopaquinone. The redox exchange with dopaquinone produces DOPA and DOPAchrome, an orange-red intermediate. DOPAchrome rearranges to form 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by tyrosinase-related protein 2 (TYRP2) also known as dopachrome tautomerase. The DOPAchrome rearrangement also leads to the production of 5,6-dihydroxyindole (DHI). DHI and DHICA is oxidised to form eumelanin. DHICA makes use of tyrosinase-related protein 1 (TYRP1) also known as tyrosinase in catalysing the production of eumelanin (Wiriyaermkul et al, 2020; Yamaguchi, 2007).

Pheomelanin is produced in the presence of L-cysteine, which stoichiometrically reacts with dopaquinone to form thiol adducts of DOPA (cysteinylDOPAs) which is a condensation product. CysteinylDOPA is then oxidised to form 1, 4-benzothiazine intermediates, which converts to pheomelanin (d'Ischia et al, 2015; Wiriyasermkul et al, 2020; D'Mello et al, 2016).

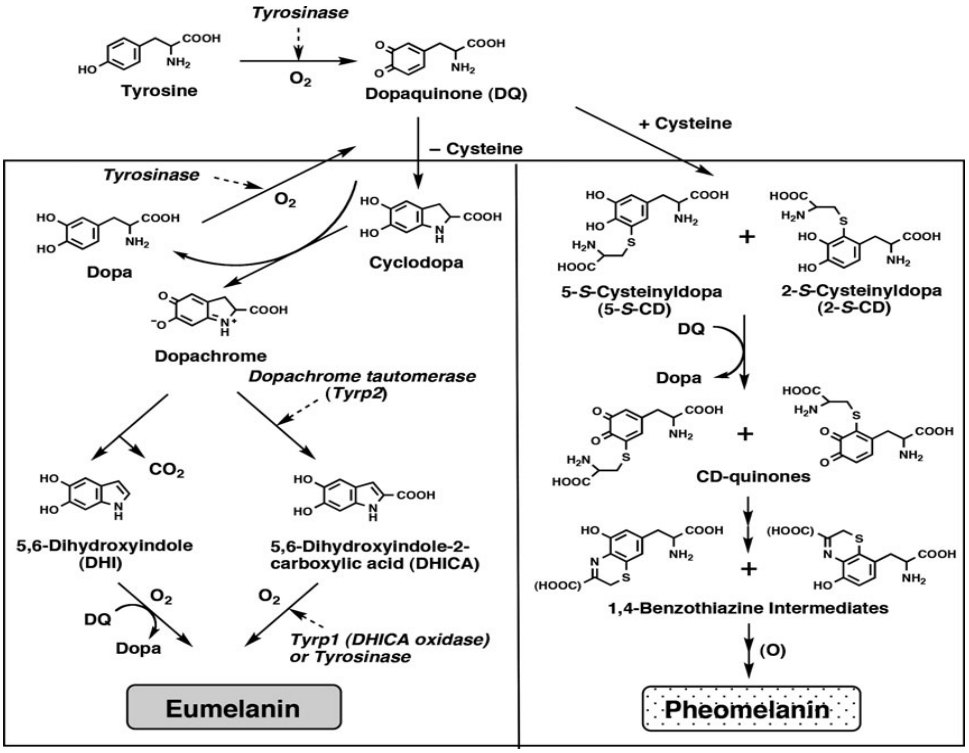

FIGURE 12: MELANIN SYNTHESIS (D'ISCHIA ET AL, 2015)

Molecular pigment regulators

Melanocortin 1 receptor (MC1-R) is a member of a subgroup of G-protein coupled receptors. MC1-R regulates the quantity and quality of melanin produced, and controls which melanin is produced between eumelanin and pheomelanin. α-melanocyte-stimulating hormone (α-MSH) binds to MC1-R which activates adenylate cyclase leading to elevated levels of intracellular cyclic adenosine monophosphate (cAMP). Tyrosinase is activated by α-MSH stimulation via cAMP pathway. The proportion of pheomelanin and eumelanin is regulated by α-MSH via the MC1-R. Eumelanin synthesis is stimulated via the MC1-R agonist α-MSH and adrenocorticotropic hormone (ACTH). Pheomelanin synthesis is stimulated by agouti signalling protein (ASP) (d'Ischia et al, 2015; Wiriyasermkul et al, 2020). Various genes have been identified which play a role in determining skin colour, an example is heat shock protein 70-1A (Hsp70-1A). Hsp70-1A expression is correlated with the level of tyrosinase and is higher in darker skin (Murase, 2016).

2.2.8 Melanocyte and melanosome stimulation

The number of melanocytes present in dark and light skinned individuals is not significantly different. The number of melanocytes differs in areas of the body that is more exposed to UV light. This is due to the UV – induced DNA damage and/or its repair, which initiates an increase in melanogenesis after UV irradiation. This can be observed after skin tanning, which is acquired pigmentation. Larger melanosomes are also produced after chronic sun exposure, mimicking the distribution pattern seen within keratinocytes of dark-skinned individuals. The action spectrum involved in skin tanning is almost identical to the spectrum that induces erythema (James et al, 2019; Brenner and Hearing, 2008).

Inflammation due to endogenous or exogenous stimuli results in the release of a variety of inflammatory mediators, which have an influence of melanogenesis stimulation (Fu et al, 2020). This is discussed in more detail under Pigmentary disorders.

The characteristics of melanosomes differ among Fitzpatrick skin types. In light skin, melanosomes accumulate more in clusters than as isolated melanosomes, whereas in dark skin they are present more as isolated melanosomes than in clusters. The melanosomes in Fitzpatrick skin types I to III are smaller than that of Fitzpatrick skin types IV to VI. In Fitzpatrick skin types I to III the poorly pigmented melanosomes cluster above the nucleus and in Fitzpatrick skin types IV to VI the melanosomes are distributed individually in keratinocytes. The number of melanosomes is up to five times greater in highly pigmented skin (Wiriyaserumkul et al, 2020; Ando et al, 2012; Yamaguchi et al, 2007; Hurbain et al, 2018). TYR is also quantitatively more active in melanogenesis in Fitzpatrick skin types IV to VI than I to III (Murase et al, 2016).

Melanosome:	**Fitzpatrick I to III**	**Fitzpatrick IV to VI**
Distribution	Clusters	Isolated
Size	Smaller	Larger
Number	Less	More

TABLE 2: CHARACTERISTICS OF MELANOSOMES IN DIFFERENT FITZPATRICK SKIN PHOTOTYPES

2.2.9 Melanosome degradation

The distribution of melanosomes differs in the epidermis based on pigmentation level. This occurrence has been suggested to be due to melanosome degradation. Literature suggests that melanosomes are degraded based on an autophagy-based pathway and is supported due to the lysosomal nature of melanosomes. However, no formal evidence has been presented to support this theory (Joly-Tonetti et al, 2018). Most of the melanosomes are situated in keratinocytes in the stratum basale and becomes less abundant towards the stratum corneum. In light skin melanosomes are completely degraded in the corneocytes and in dark skin some melanosomes remain intact in the corneocytes (Ebanks et al, 2011). Refer to Figure 9. This can also be seen in hyperproliferative skin diseases involving keratinocytes such as psoriasis (Joly-Tonetti et al, 2018; Hurbain et al, 2018).

The release and the process involved of degrading melanosomes or other cellular organelles are not fully established (Ebanks et al, 2013). It has been observed that melanin was degraded more rapidly in Fitzpatrick skin types I and II than Fitzpatrick skin types III to VI due to the up-regulated presence of several acid hydrolases in the suprabasal layers of the skin. The enzyme cathepsin L2, a member of the class of papain-like cystein proteinases, is overexpressed in light skin compared to dark skin. It has been reported that cysteine cathepsins participate in proteolytic cascades, which leads to cleavage of proteinaceous substrates. A recent study has also concluded that cathepsin V, a lysosomal protease is involved in melanosome degradation. It is reduced in the basal layer compared to the stratum corneum of hyperpigmented skin. Cathepsin V is expressed across the entire epidermis in normal skin and upregulated and more active in light skin compared to dark skin (Homma et al, 2018). Another study concluded that melanin granules are redistributed to the non-differentiating daughter cells in progenitor keratinocytes post-mitosis. This is done via asymmetric organelle inheritance, thus the second daughter cell destined for differentiation lacks significant amount of melanin. During stressful conditions the melanin granules are inherited symmetrically, thus both daughter cells inherit melanin granules in response to environmental conditions such as UV radiation (Joly-Tonetti et al, 2018).

2.2.10 Pigmentary disorders

Melanin production is dependent on the following: structural proteins of melanosomes; enzymes required for melanin synthesis; and proteins required for melanosomes transport. Any disturbances in these factors lead to pigmentary disorders. The pigmentary disorders are classified as hyperpigmentation, hypopigmentation, or mixed hyper-/hypopigmentation. The disruption of melanin production can occur either congenitally or be acquired (Yamaguchi and Hearing, 2014).

Hypopigmentation refers to the loss or reduction of pigmentation and is sub-categorised as either complete or incomplete depigmentation. This may occur due to the loss of melanocytes or the failure to transport melanosomes or inability of melanogenesis. As mentioned before, mutations causing a disruption in TYR functionality results in albinism, which leads to impaired pigmentation of skin, hair, and eyes. This is an inherited pigmentary disorder (Yamaguchi et al, 2007). The gene mutations identified involving hypopigmentation are linked to the intracellular transportation of protein to lysosome-related organelles and keratinocytes, as well as transportation of organelles to the peripheries of the cell. Most acquired hypopigmentation disorders are inflammatory induced, and studies have shown that increased tumour necrosis factor-α (TNF-α) and interleukin-17 levels suppress pigment-related signalling and melanogenesis partly via MC1R (Yamaguchi and Hearing, 2014).

Vitiligo, an acquired hypopigmentation disorder presents with an increased level of cytokines, which includes TNF-α and interferon-γ (Wang et al, 2013) and progressive depigmentation due to the loss of functioning melanocytes. Moreover, it was reported that $CD8^+$ T-cells are present in hypopigmentation disorders suggesting that the pathogenesis is T-cell mediated (Grimes, 2005).

Hyperpigmentation refers to the increase of pigmentation, which can be acquired or due to congenital disease. Hyperpigmentation can be caused by different factors such as exposure to UV radiation or FDE, as well as certain congenital conditions like generalized lentiginosis. (Yamaguchi and Hearing, 2014).

Melanin synthesis can also be stimulated during the inflammation of the epidermis. This occurs due to the release of various cytokines, prostaglandins, and leukotrienes, which stimulates the melanocytes to produce melanin resulting in PIHP, particularly in Fitzpatrick phototypes IV to VI (Ortonne and Bissett, 2008). It has been reported that endothelin-1 and stem cell factor involved in the development of senile lentigo (Ünver, N et al, 2006).

The presence of variable melanin incontinence within the dermis is a result of the disruption in the transfer of melanin pigment to keratinocytes. This interruption leads to the pigment becoming free lying in the dermis due to vacuolar degeneration (Gru and Salavaggione, 2017). The macrophages engulf the melanin produced by phagocytosis when it enters the dermis to form melanophages (Ortonne and Bissett, 2008). Another theory suggests that PIHP occurs due to the oxidation of arachidonic acid by peroxides, cyclooxygenase and 5-lipoxygenase which results in the formation of intermediates that form prostaglandins, leukotrienes and thromboxanes. Epidermal melanocytes become hypertrophic due to the stimulation of prostaglandins, leukotrienes and thromboxanes, which leads to the increase in melanin synthesis. This leads to the transfer of melanin to keratinocytes and dermal macrophages, resulting in hyperpigmented lesions (Cardinali et al, 2012).

Another theory suggests that macrophages migrate to the epidermis from the dermis and phagocytise the dyskeratotic cells containing tonofibrils, nuclear chromatin and melanosomes. The melanophages containing the material then migrate back to the dermis and digest the material phagocytosed. However, it is found that melanosomes are more resistant to the digestion and become phagosomes. This theory was proposed in a study where hyperpigmentation occurred due to fixed drug eruption (Masu and Seiji, 1983).

2.2.11 Interface dermatitis

The epidermis is bound to the dermis by the dermo-epidermal junction. Damage caused to the dermo-epidermal junction can lead to its separation. In earlier literature cell damage at the basal cell layer by apoptosis or necrosis was referred to as lichenoid tissue reaction (Panja and Banerjee, 1989). The terms lichenoid tissue reaction and interface dermatitis are often used interchangeably to refer to the inflammatory response and damage to the basal epidermal layer, which includes keratinocytes and melanocytes (Sontheimer, 2009).

The term interface dermatitis refers to the inflammatory infiltrate of the dermo-epidermal junction, which includes the damage of the basal keratinocytes of the epidermis. The cellular damage seen microscopically is associated with the infiltration of T-cells in the upper dermis due to an auto-immune attack, this is also seen as the primary pathological event of interface dermatitis (Shiohara and Mizukawa, 2005). Lichenoid interface dermatitis is characterised by epidermal basal cell injury and cell-rich lymphohistiocytic infiltration of the superficial dermis and epidermis (Shiohara and Mizukawa, 2005). Lymphocytes are the most common inflammatory cells present during interface reactions, other cells such as plasma cells, eosinophils, neutrophils, mast cells and histiocytes may also be present (Joshi, 2013).

Lichen planus represents the cell-rich interface dermatitis prototype (Shiohara and Mizukawa, 2005). Cell-poor interface dermatitis occurs due to cellular cytotoxicity caused by autoantibodies targeting

components of the basement membrane (Altilli, 2019; Gru and Salavaggione, 2017; Crowson et al, 2008). Erythema multiforme and lupus erythematosus are examples of cell-poor interface dermatitis and represent the cell-poor interface dermatitis prototype (Crowson et al, 2008; Shiohara and Mizukawa, 2005)

Basal cell keratinocyte damage, changes to the basement membrane and the effects of the inflammatory cell infiltrate are the primary morphological changes. Secondary changes to follow may affect the epidermis, the papillary dermis, or both. Secondary changes vary morphologically, which characterises each interface dermatitis (Joshi, 2013). The disruption caused to the epidermal melanin unit is a major sequela leading to pigment incontinence (PI). PI refers to the melanin granules being present in the upper dermis or within macrophages due to compensatory accumulation. PI causes post inflammatory hyperpigmentation (PIHP) (Crowson et al, 2008; Hedge and Urmila, 2014). The severity and presence of PI differ with each type of interface dermatitis (Sehgal et al, 2011). In an experiment using mice that were induced with different concentrations of $CD4^+$ autoreactive T-cell clones, it was found that the epidermal damage is proportional to the dosage of the T-cell clone injected. When a suboptimal dosage was induced cell-poor interface dermatitis was observed. Cell-rich interface dermatitis was mimicked when optimal doses were administrated and at high doses full-thickness epidermal necrosis and detachment indistinguishable from toxic epidermal necrolysis (TEN) was observed (Shiohara and Mizukawa, 2005).

2.2.12 PIHP conditions

2.2.12.1 Lichen Planus

Lichen planus (LP) is an inflammatory disease of the stratified squamous epithelia and affects oral mucosa as well as mucosa of the skin, it may also be encountered on the scalp, hair, and nails (Gru and Salavaggione, 2017).

Clinical Features

The classic lesions of cutaneous LP are characterised by its pruritic, violet, smooth, shiny, flat-topped polygonal papules, and plaques (Gru and Salavaggione, 2017; Calonje et al, 2020). In a study of 444 patients with LP, pruritus was the most prominent feature associated with cutaneous LP (Schwager et al, 2019). Classical LP and its variants can serve as a prototype for interface dermatitis (Gru and Salavaggione, 2017; Calonje et al, 2020).Refer to Figure 13.

Epidemiology

Studies have shown that LP is more common in female adults compared to male adults, with the average age of the patients diagnosed with LP being between 40 to 45 years old (Diop et al, 2019; Irvine et al, 1991). LP has been described in children, but at a much lower prevalence compared to adults. The prevalence of LP was found to be higher in African American children, with female children accounting for a higher portion (Walton et al, 2010).

Aetiology

LP has been with various disorders including auto-immune disease, malignancy, immunodeficiency, metabolic disease, and drug use. However, it is classified as being caused by an unknown aetiology as the evidence supporting these associations is mainly circumstantial and weak.

LP is hypothesized to be a delayed hypersensitivity reaction, caused by a combination of an external antigen coupled with an internal antigen in the epidermis. An example is Human Immunodeficiency Virus (HIV) combined with the use of drug treatment (Calonje et al, 2020).

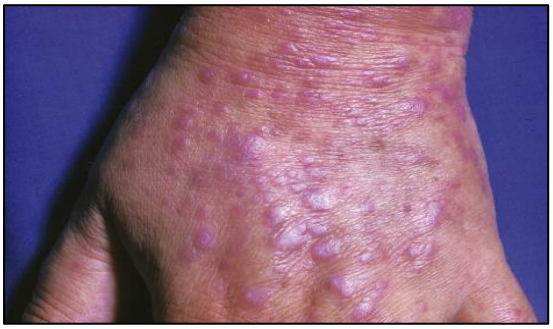

FIGURE 13: VIOLET PAPULES ON DORSUM OF THE HAND. (CALONJE, 2020)

Pathophysiology:

Lymphocytic infiltrate is composed of mostly of cytotoxic T-cells, and suggests that cytotoxic T-cells, which are activated CD8+ lymphocytes trigger the apoptosis of keratinocytes in LP lesions (Sugerman et al, 2000). Cytotoxic T-cells program their target cells expressing foreign peptides bound to MHC class 1 molecules and the Fas ligand to undergo cell death by apoptosis (Janeway et al, 2001). Granzyme B-positive CD8+ T-cells induce keratinocyte apoptosis (Shimizu et al, 1997).

A study comparing the cytotoxicity of dermal T-cell lines of normal skin and lesional skin from LP patients concluded that T-cell lines from lesional skin had a higher cytotoxicity (Sugerman et al, 2000). This study showed variations in the levels of cytotoxicity between the different morphological types of LP.

Microscopic features:

Microscopically LP is characterised by the presence of damaged basal layer keratinocytes manifesting as, vacuolar degeneration and, apoptotic cells as well as, a lichenoid band of lymphohistiocytic cells obscuring the dermo-epidermal junction, and eosinophilic colloid bodies in the dermis. The irregular rete ridges pattern forms an elongated ‘sawtooth’ configuration (Weedon, 1980; Gru and Salavaggione, 2017). The cause of cell damage is due to apoptosis, which is known as programmed cell death. Apoptosis of basal cells leads to the formation of colloid bodies. The fragments of these apoptotic fragments then migrate to the dermis to form colloid bodies (Weedon, 1980). The various theories regarding the mechanism of melanin incontinence has been described above. Refer to Figure 14 and 15. The presence of variable melanin incontinence within the dermis is a result of the interruption in the transfer of melanin pigment to keratinocytes.) Gru and Salavaggione explain that these changes cause variable melanin

incontinence in the dermis due the disruption of melanin pigment being transferred to keratinocytes and becomes free-lying due to vacuolar degeneration. The macrophages engulf the melanin produced by phagocytosis when it enters the dermis to form melanophages (Ortonne and Bissett, 2008).

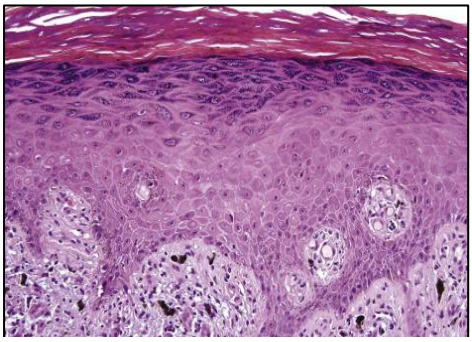

FIGURE 14: LICHEN PLANUS (H&E) IRREGULAR ACANTHOSIS, PIGMENT INCONTINENCE AND HYPERKERATOSIS ARE NOTED. (CALONJE, 2020)

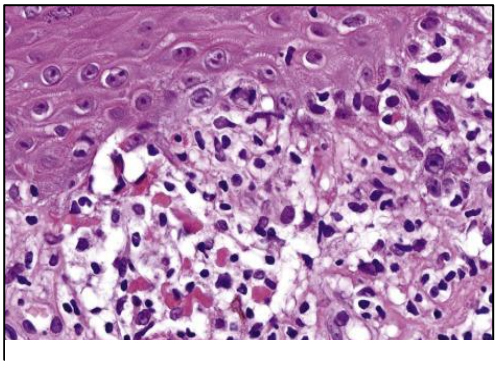

FIGURE 15: VACUOLAR DEGENERATION (H&E) EOSINOPHILIC COLLOID BODIES AND BASAL CELL VACUOLAR DEGENERATION IS NOTED. (CALONJE, 2020)

Lichen planus variants

LP has a number of variants apart from the classic form of LP, which will be used for the study that are worth briefly mentioning.

- Atrophic LP is seen upon resolution of the active phase of classic LP. In atrophic LP, the epidermis becomes thin, and the normal rete pattern is lost (Gru and Salavaggione, 2017; Calonje et al, 2020).
- In hypertrophic LP, the lesions are typically manifest as highly pigmented warty plaques and occur in long-standing lesions of LP. These lesions are very persistent and extremely itchy. Microscopically it is characterised by epidermal hyperplasia with marked hyperkeratosis (Gru and Salavaggione, 2017; Calonje et al, 2020).
- Ulcerative LP, which is a chronic variant has a predilection for feet and genital sites. Ulcerated LP also has a likelihood of developing squamous cell carcinoma (Gru and Salavaggione, 2017; Calonje et al, 2020).
- Lichen planopilaris, is the most common form of scarring alopecia. Clinically it manifests as patches of hair loss with perifollicular erythema, scaling, and the absence of follicular ostia. Lichen planopilaris is not limited to the scalp as frontal fibrosing alopecia, a variant of Lichen planopilaris is more limited to the frontal and temporal hairline and associated with the loss of eyebrows (Gru and Salavaggione, 2017).
- LP pigmentosus (erythema dyschromicum perstans), the lesions are manifested clinically as a progressive, slow growing eruption and appears ‘ashy’ or as brown macular hyperpigmentation. In this variant of LP, pigmentary incontinence is present in the dermis, frequently in the absence of interface changes. In its active phase, a lichenoid reaction that is milder than classic LP with a deeper dermal extension (Gru and Salavaggione, 2017).

- Nail involvement in LP, is encountered in up to 10% of patients with LP. It generally associated with mucocutaneous LP involving other anatomical sites, particularly oral LP. The development of dorsal pterygium is encountered in 4% of nail LP, which is an irreversible destruction of the nail matrix with a ‘V’ shaped extension of the proximal nailfold (Grover and Baran, 2022). It also has a predilection for fingernails over toenails. Dorsal pterygium may lead to permanent disfigurement, which has functional and psychological effect on the individual (Wechsuruk et al, 2021).

2.2.12.2 Lichenoid drug reaction / Lichenoid drug eruption

Lichenoid drug reaction (LDR) refers to a poorly characterized group of delayed hypersensitivity reactions affecting the skin, hair and mucosa that resemble lichen planus. LDE manifests as pruritic, violaceus, symmetrical, scaly, eczematous or psoriasiform patches, papules or plaques that are primarily located on the trunk, extremities, and mucosa. On the skin, the lesions are often photo distributed or photo accentuated. Prolonged exposure to the offending drug can result in PIHP and depigmentation, both of which may be reversible. LDR is less likely to involve flexures, mucosa, and genitalia. LDR can be caused by numerous drugs and identifying the causative drug may be difficult as onset sometimes occurs several months to years after initiation of the medication. (Boch et al, 2021; Cheraghlou and Levy, 2020).

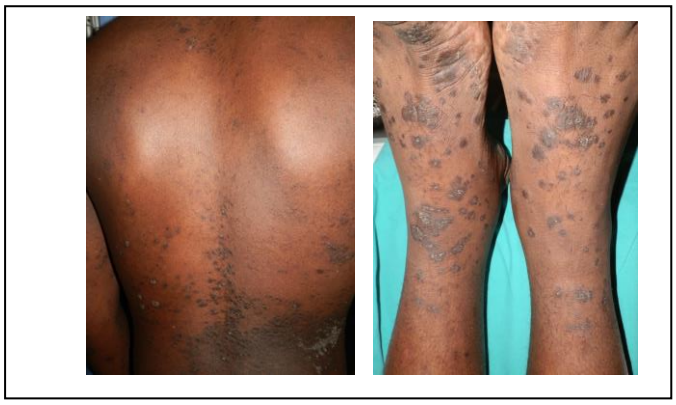

FIGURE 16: LICHENOID DRUG REACTION (LEHLOENYA ET AL, 2020)

LDR is often associated with thiazides, angiotensin-converting enzyme inhibitors, antituberculosis drugs, beta blockers, gold, penicillamine and antimalarials (Byun et al, 2014; Kakande, 2015). As mentioned previously the onset of LDR takes months to years after initiation of the causative drug, however the lesions resolve weeks after cessation of the offending drug which leads to the formation of PIHP (Cheraghlou & Levy, 2020). It has been reported that a patient has spontaneously become tolerant to terbinafine, which is an anti-fungal agent which caused the onset of LDR (Teraki & Shiohara, 2004). The pathogenesis of LDR is not well established, however idiopathic LP may provide some context as to how the LDR occurs. It is thought that LDR is mediated by the medication that is acting as haptens to incite injury (Cheraghlou & Levy, 2020).

Microscopic features

LDR has the similar histologic features as idiopathic LP, however LDR include a high-level of cytoid bodies and eosinophils within the dermal infiltrate (Calonje et al, 2020). However, a study done using direct immunofluorescence has indicated that, the presence of focal parakeratosis, focal interruption of the granular layer is more indicative of LDR (Cheraghlou & Levy, 2020). The presence of necrotic keratinocytes forming clusters in all layers of the epidermis may also be noted (Weyers and Metze, 2011). Refer to Figure 17.

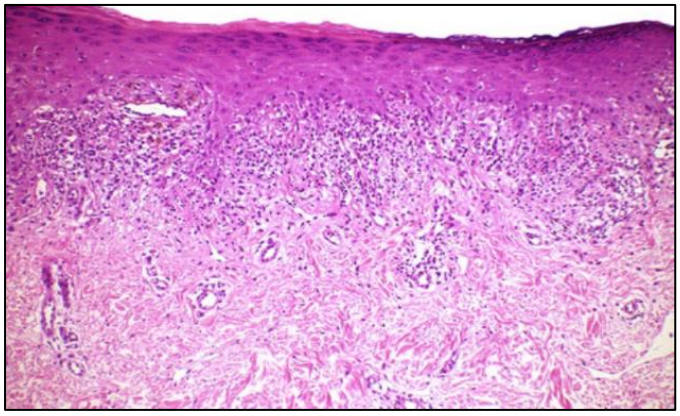

FIGURE 17: LDR RESEMBLES LP WITH THE PRESENCE OF ACANTHOSIS. (H&E STAINING) (WEYERS AND METZE, 2011)

2.2.12.3 Fixed drug eruption:

The term adverse drug reaction is described by the World Health Organization as “a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function” (World Health Organization Technical Report Series no. 498, 1972). It is advised that whenever a patient develops a new medical problem after or during drug treatment, an adverse drug reaction should be included in the differential diagnoses (Hausmann et al, 2012).

The skin is the most frequently affected by adverse drug reactions. Adverse cutaneous drug reactions are mostly benign in nature and occur mostly as maculopapular eruptions or urticarial. Only up to 2 % of all adverse cutaneous drug reactions are severe and life-threatening. The most common hypersensitivity reaction of the skin is drug-induced exanthematous, also known as morbiliform drug eruption makes up approximately 2 % of hospitalised patients. Cutaneous exanthematous drug reaction most frequently appears as a maculopapular rash or can present in an eczematoid-, psoriasis form-, or lichenoid-like pattern. Furthermore, the exanthematous lesions appear on the ventral and dorsal trunk before expanding to the proximal extremities (Hoetzenecker et al., 2016)

Fixed drug eruptions (FDE) can develop within hours of taking the drug, and present as solitary or multiple lesions, which then forms hyperpigmented macules upon resolution. Fixed drug eruptions rarely become bullous in nature. These changes reoccur at the same site when taking the same causative agent (Gru and Salavaggione, 2017). FDE is a localised form of drug induced exanthems; however, a generalised form may occur which can mimic Stevens - Johnson syndrome (SJS) / TEN. Clinically the patient will show solitary or few well-circumscribed, round and/or oval erythematous macules and plaques with dusky centres on the skin. During the early stages the skin becomes dusky red and oedematous in appearance (Joshi, 2013). Refer to Figure 16. Furthermore, itching or a burning sensation may accompany the lesions of FDE.

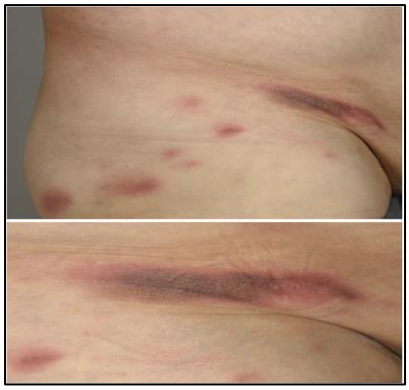

FIGURE 18: FIXED DRUG ERUPTION MACULE (HOETZENECKER ET AL., 2015)

Microscopic features

Skin biopsies taken from blistered or necrotic areas will show similar features to TEN and SJS, these features include vacuolar interface dermatitis, necrotic keratinocytes, and pigment incontinence. A subepidermal blister can be a result of full thickness necrosis in rapidly evolving lesions (Anderson and Lee, 2021). Recurrent FDE shows changes similar to erythema multiforme with the presence of eosinophils, neutrophils, and abundance of melanophages in the dermis (Gru and Salavaggione, 2017). Refer to Figure 18.

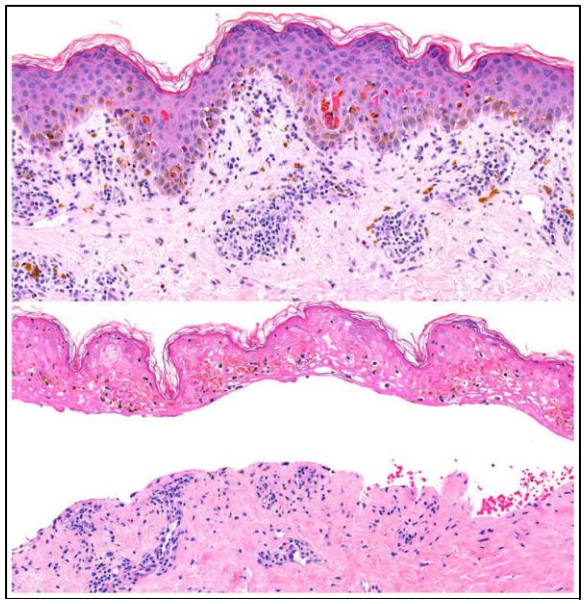

FIGURE 19: CHARACTERISTIC FDE WITH H&E STAINING (ANDERSON AND LEE, 2021).

2.2.12.4 Bullous fixed drug eruption

Bullous fixed drug eruption (BFDE) is a form of FDE that is associated with localised or generalised cutaneous blister formation and erosions (Zaouak et al., 2019). Clinically the patients tend to present with widespread macules or plaques with overlying bullae (Cheraghlou. & Levy, 2020). A case has been reported of an immunocompromised patient developing BFDE three days after the quadrivalent influenza vaccine, she presented with proximal distribution of lesions of 2.5 to 4 cm distributed over a background of diffuse PIHP, and rim of erythema. This is supportive of BFDE (Byrd et al., 2018). It has been reported that BFDE has a mortality rate of 22% in among elderly patients in a study involving 58 patients diagnosed with BFDE (Lipowicz et al., 2013). Individuals with recurrent FDE have an increased risk of developing BFDE. (Patel et al., 2020). BDFE generally resolves within 2 weeks after cessation of offending drug (Cheraghlou. & Levy, 2020).

Microscopic features

Microscopic features of BFDE include inflammation with eosinophils, necrotic keratinocytes and dermal melanophages. Compared to SJS/TEN, the inflammation with eosinophils and the presence of dermal melanophages are more severe and fewer necrotic keratinocytes are present in BFDE. Furthermore, BFDE may develop full-thickness epidermal necrosis which is also seen in SJS/TEN (Patel et al., 2020). Refer to Figure 19

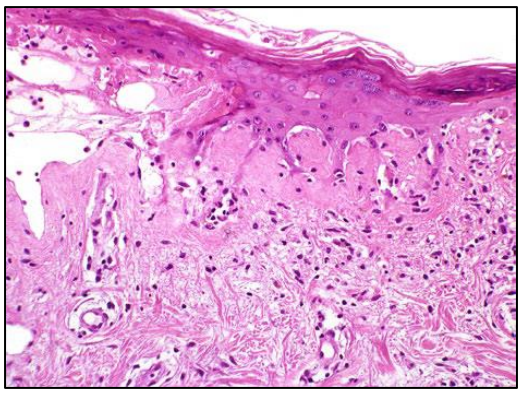

FIGURE 20: BFDE (H&E STAIN) (WEYERS AND METZE, 2011)

2.2.12.5 Discoid Lupus erythematosus

Lupus erythematosus is a multisystem auto-immune disease with associated inflammation affecting the connective tissue, which mostly affects the skin and can occur in all age groups (Gilliam and Sontheimer, 1981).

There are several subtypes of cutaneous lupus erythematosus, however the most frequent subtypes are acute cutaneous lupus, subcutaneous lupus, and discoid lupus erythematosus (DLE). DLE is categorised as a chronic cutaneous lupus erythematosus according to the classification suggested by Gilliam (Gilliam and Sontheimer, 1981). This classification is most commonly used to categorise cutaneous lesions in lupus erythematosus and is based on whether interface dermatitis is seen or not microscopically. Furthermore, the categories were acute cutaneous lupus erythematous, subcutaneous lupus erythematous and chronic lupus erythematosus. Systemic lupus erythematosus is associated with systemic involvement which includes the heart, lungs, brain, kidneys, and other organs (Panjwani, 2009).

The clinical features of DLE are defined by its characteristic skin lesions, which appear as erythematous papules or plaques, with hyperpigmentation at the periphery and hypopigmentation with atrophy, scarring and telangiectasia at the centre of the lesion. (Panjwani, 2009). Refer to Figure 20.

FIGURE 21: CUTANEOUS DLE (PNJWANI, 2009)

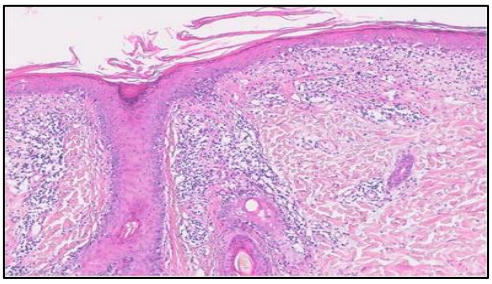

FIGURE 22: CUTANEOUS LUPUS ERYTHEMATOSUS (H&E) (RIBERO ET AL, 2017)

Microscopic features

The microscopic features of DLE include interface dermatitis with associated basement membrane thickening, vacuolar degeneration of the basal cells, lymphohistiocytic infiltration in the reticular dermis. In mature lesions hyperkeratosis is more frequent and follicular plugging may be seen (Panjwani, 2009). The CD68 antibody stain is up-regulated in DLE lesional skin compared to normal skin, indicating that the presence of macrophages are more than normal in DLE lesional skin (Thorpe et al, 2014). It has been postulated that UV irradiation is responsible for inducing keratinocyte to produce pro-inflammatory cytokines and apoptosis, which results in the recruitment of lymphocytes perpetuating the cutaneous immune response resulting in the development of DLE (Zhou et al, 2021).

Microscopic features seen in H&E staining:

TABLE 3: MICROSCOPIC FEATURES OF SELECTED ID

Disease	Microscopic feature
LP	• Damaged basal layer keratinocytes, vacuolar degeneration of keratinocytes, apoptotic cells, a lichenoid band of lymphohistiocytic cells obscuring the dermo-epidermal junction, and eosinophilic colloid bodies in the dermis. • The irregular rete ridges pattern forms an elongated 'sawtooth' configuration cause of cell damage is due to apoptosis. • Colloid bodies • Melanin incontinence in the dermis.
LDR	• LDR has the similar histologic features as idiopathic LP, however LDR include a high-level of cytoid bodies and eosinophils within the dermal infiltrate. • Focal parakeratosis and focal interruption of the granular layer. • Necrotic keratinocytes forming clusters in all layers of the epidermis may be noted
FDE	• Focal parakeratosis • Numerous apoptotic keratinocytes and basal cell vacuolation. • The upper dermis will have sparse infiltrate of lymphocytes and eosinophils • Recurrent FDE present with eosinophils, neutrophils, and abundance of melanin incontinence in the dermis
BFDE	• Inflammation with eosinophils, necrotic keratinocytes, and dermal melanin incontinence • May develop full-thickness epidermal necrosis
DLE	• Interface dermatitis with associated basement membrane thickening • Vacuolar degeneration of the basal cells, lymphohistiocytic infiltration in the reticular dermis. • In mature lesions hyperkeratosis is more frequent and follicular plugging may be seen

2.13 Post Inflammatory Hyperpigmentation

PIHP can be classified histologically by the location of the excess melanin pigment, as dermal or epidermal PIHP. Hyperpigmentation can also occur in both the epidermis and in the dermis. In the dermis, melanin is located between bundles of collagen and within melanophages (Nieuweboer-Krobotova, 2013). Interface dermatitis is associated with dermal PIHP as damaged basal keratinocytes releases large amounts of melanin, which is then phagocytosed by macrophages (Ortonne and Bissett, 2008). Based on current literature, the epidermal PIHP and dermal PIHP have an increase in melanogenic activity by melanocytes however, the number of melanocytes remains normal (Markiewicz and Idowu, 2020). This was confirmed by analysing NKI/beteb levels in patients with PIHP. NKI/beteb is a marker of melanogenic activity. Glycoproteins of pmel-17 are recognised by pmel-17, which located in premelanosome vesicles (Park et al, 2017).

Dermal PIHP is also characterised by perivascular lymphocytic infiltrate and vacuolar alteration seen on H&E staining (Park et al, 2017). These characteristics can be seen in LP and its variants. Dermal melanophages are common in several inflammatory conditions and may also be seen in normal skin. In dermal hyperpigmentation, melanophages are more abundant than epidermal pigmentation, hypopigmentation, and normal skin (Joshi, 2018; Ortonne and Bissett, 2008).

Epidermal PIHP clinically presents as tan to dark brown patches and in dermal PIHP is displayed as a dark grey to blue-grey patches. PIHP presents itself as macules and patches, in the area where the inflammation or trauma took place (Park et al, 2017; Davis and Callender, 2010). PIHP is common, persistent, and more severe in Fitzpatrick skin types III to VI in comparison to Fitzpatrick skin types I and II (Nieuweboer-Krobotova, 2013; Davis and Callender, 2010).

TABLE 4: EPIDERMAL PIHP VS DERMAL PIHP

	Epidermal PIHP	**Dermal PIHP**
Clinical features	tan to dark brown patches	dark grey to blue-grey patches
Epidermal pigmentation	increased	decreased
Melanophages	Less than dermal PIHP	more than epidermal PIHP
Melanogenesis	increased	Increased
Resolution	Months to years	Permanent or resolve over a protracted period of time

Epidemiology:

PIHP is common in darker skin. Zulu et al investigated PIHP in acne, the most common skin disorder in black adults from Durban, South Africa. They found that over 80 percent of patients developed PIHP (Zulu et al, 2017). In a comparative study of 1412 patients, it was found that dyschromia was more commonly seen in black patients than white patients, although the number of patients between the two races had similar frequencies related to acne and eczema (Alex, A.F. et al, 2007). A study conducted in KwaZulu-Natal, South Africa reported that PIHP was the second most common encountered dyschromia in a public

hospital setting, whereby Africans made up most of the population (Dlova et al, 2018). The incidence of PIHP was also found to be common in patients of Asian descent with darker skin types (Abad-Casintahan, F. et al, 2016). This relates to the observation that the degree of skin pigmentation influences the frequency and severity of PIHP, rather than ethnicity (Kaufman et al, 2018). Thus, it was decided to include participants that have Fitzpatrick skin types III to VI in this study. This study will be fitting for the location where it will be conducted, as the black African population constitutes approximately 81% of South Africa's population in the year 2019 (Stats SA, 2019).

Prognosis of PIHP

Epidermal hyperpigmentation may take months to years to resolve, and dermal hyperpigmentation may either be permanent or resolve over a protracted period, if left untreated. PIHP can also worsen with ultraviolet irradiation and/or recurrent inflammation (Gru and Salavaggione, 2017; Davis and Callender, 2010).

Management and response to therapy

Epidermal pigmentation can be treated with depigmentation cream such as hydroquinone, but it has many disadvantages. Dermal pigmentation is however less responsive to laser therapy and local therapy such as depigmentation cream (Nieuweboer-Krobotova, 2013). There is no strategy that has been developed to prevent or lessen the clinical manifestation of PIHP in recent studies.

2.14 Histopathological Techniques

2.14.1 Fixation:

To achieve optimal tissue morphology and antigen preservation for the various histological techniques to be performed, the tissue must be preserved in their respective fixatives as soon as possible. The tissue is preserved by chemical means, whereby the chemical structures of the tissue are altered for preservation of the tissue to appear in a "life-like" state (Layton and Suvarna, 2013). In this study 10% Neutral Buffered Formalin and 3% glutaraldehyde with 0.1 M phosphate buffer was used.

If the tissue is not preserved promptly and adequately it will experience agonal changes. These agonal changes are due to catabolic enzymes causing a breakdown of protein and eventual liquefaction of the cell, this is known as autolysis. These enzymes are released by the lysosomes within the cells. Swelling of cytoplasm and the inability to retain stains adequately is also a feature of autolysis due to the formation of a granular homogenous mass. This will affect the significance of staining results. Putrefaction may also occur whereby the tissue experiences a bacterial attack state (Layton and Suvarna, 2013; Jenkins and Burg, 2003).

10% Neutral buffered formalin is an aldehyde fixative and the most widely used fixative for light microscopy. It reacts with amino groups to form methylene bridges. Gluteraldehyde is also an aldehyde fixative, however it is a bifunctional aldehyde with an aldehyde group on both end of the molecules. By having more than one aldehyde group attached to the molecule it allows for better cross-linkage with the protein however, unreacted aldehyde groups may interact with antibodies. Thus, makes it unsuitable for IHC and making it the most widely used fixative for electron microscopy, due to its preservation

characteristics of ultrastructure in the tissue as it forms cross-links with protein faster than formalin as it is a bifunctional aldehyde (Layton and Suvarna, 2013).

2.14.2 Processing and sectioning:

The aim of tissue processing is to stabilize the tissue adequately for support when it is to be sectioned for microscope examination. In order to cut thin sections of the tissues, it should have suitable hardness and consistency when presented to the knife-edge. In order to achieve the suitable support for tissue that require IHC, H&E and MF staining the tissue should be impregnated with paraffin wax, this enables the tissue to be sectioned at 2-3µm with a microtome (Feldman and Wolfe, 2014).

Specimens that require EM are impregnated with epoxy resin. Epoxy resin is used as it is resistant to the force of the electron beam and hard enough to permit sections as thin as 80 nm to be cut using an ultratome. The same supporting medium used for infiltration is used to embed the tissue to create a tissue "block". Both procedures require the tissue to undergo fixation, dehydration, clearing, infiltration and embedding (Woods and Stirling, 2019).

2.14.3 H&E and MF staining:

The H&E staining technique is used routinely to stain tissue sections in histopathology. The haematoxylin dye stains cell nuclei blue – black and demonstrates good intranuclear detail, and eosin dye stains cell cytoplasm and extracellular matrix various shades of pink. This stain ultimately allows for the clear demonstration of different tissue structures of paraffin-embedded tissue. Melanin can also be observed with H&E staining however it is non-specific (Orchard, 2013). This is demonstrated in Figures 9 and 10.

Histochemical stains such as MF staining method for melanin and modified Warthin-Starry (WS) stain (pH 3.2) is more specific for demonstrating melanin (Joly-Tonetti et al, 2018; Orchard, 2013; Joly-Tonetti et al, 2016). WS stain highlights stage 3 melanosomes (Joly-Tonetti et al, 2016). In this study the MF staining method will be used. The MF staining method is based on the ability of melanin to reduce ammoniacal silver to from a metallic silver without the use of an external reducer, this known as the argentaffin reaction. The melanin will appear black with a red background stain is the expected end result (Orchard, 2013).

2.14.4 IHC staining

Immunohistochemical (IHC) staining with the use of HMB-45, Melan-A and/or S100 antibodies may also be used to assess the PIHP and PI. A comparison study has shown that S100 antibody is the most sensitive marker for melanocytic differentiation, compared to Melan-A antibody and HMB-45 antibody. HMB-45 antibody was declared the least sensitive. However, Melan – A antibody and HMB-45 antibody is more specific melanocytic markers, of which Melan-A is more sensitive and more specific for melanocytic lesions (Blessing et al, 1998).

Kikuchi, A. et al explains that HMB-45 is a monoclonal antibody, raised against Pmel 17, which demonstrates immunoreactivity in premature developing melanocytes and immature melanosomes

during melanogenesis. HMB45 is absent in normal adult melanocytes (Kikuchi et al, 1996). HMB-45 antigens are also expressed when melanocytes are reactive due to inflammation (Bacchi et al, 1996). The Pmel17 expressed, occur during melanogenesis, whereby it serves as a fibrillary matrix on which melanin gets deposited (Hoashi et al, 2006).

Melan-A recognises a 20-22 kDa doublet protein in Melan-A mRNA positive melanoma and melanocytic differentiated cells in normal skin (Blessing et al, 1998).

CD68 is a transmembrane glycoprotein and expressed in macrophages and other mononuclear phagocytes (Chistiakov et al, 2017). IHC staining with CD68 antibody allows for the identification of macrophages, as well as melanophages (Mäkitie et al, 2001). The use of red chromogen was found to eliminate melanin pigmentation as a visual perplexing factor in heavily pigmented skin (Jiang et al, 2014). However, alkaline phosphatase with red chromagens is sensitive to light and heat. Peroxidase chromagens are also susceptible to loss after preparation, but at a slower rate (Sanderson et al, 2019).

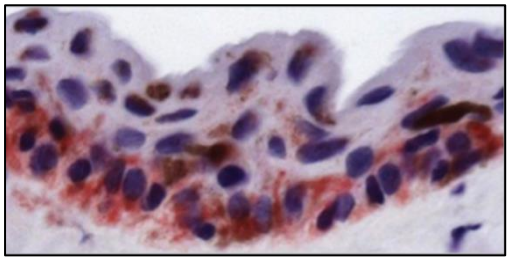

FIGURE 23: HMB45 WITH RED CHROMOGEN SUBSTRATE (JIANG ET AL, 2014)

It was identified that IHC staining with Melan-A as a single marker can lead to a diagnostic pitfall, in what has been described as “pseudomelanocytic nests”. These “pseudonests” have been identified in lesions caused by discoid lupus erythematosus, lichen planus and fixed drug eruption. These “nests” as it is described, stain with melanocytic markers, however it does not correlate with the clinical picture of the patients and further investigation is required. This occurs due to Melan-A’s cytoplasmic antigen in melanosomes being transferred between cells during inflammation (McClanahan et al, 2019).

2.14.5 EM

Electron microscopy (EM) is used to examine the ultrastructure of individual cells. This is possible due to its high resolution; this is approximately 1000 times higher magnification than light microscopy. To obtain specimens suitable for ultrastructure examination a series of histology techniques have to be performed. These techniques vary from that used to obtain suitable specimens for routine and IHC staining. Transmission electron microscopy (TEM) is a form of EM, which makes use of electron beams through the specimen to produce the image (Mainini et al, 2014). EM has been used in previous studies to obtain data related to melanin and the distribution thereof and for diagnostic purposes of interface dermatitis (Orchard, 2013; Joly-Tonetti et al, 2016; Jaunzems and Woods, 1997). Melanin granules are electron-dense which allows for it to be detected and analysed using EM (Joly-Tonetti et al, 2016). Melanosome differentiation can also be observed using EM. For example, immature melanosomes have been

observed in keratinocytes in patients with seborrheic keratosis (Noh et al, 2019). EM Figures can be observed in Figures 5, 6 and 7.

Chapter 3: Methods and materials

3. Method:

3.1 Research design:

3.1.1 Study population:

Participants for this study include 12 adult patients who have been diagnosed by the dermatology service at Groote Schuur Hospital with either active lesions of fixed drug eruption; bullous FDE; discoid lupus erythematosus; lichen planus or lichenoid drug reactions.

3.1.2 Data Collection:

The data collected from the patients consist of clinical data, demographic data, and photographs of the site of the biopsy before and after every biopsy. This information was obtained by the consulting dermatologist and recorded on the case report form. A copy of the case report form can be found in Appendix II.

3.1.3 Criteria:

Inclusion criteria:

- Skin lesion diagnosed clinically by at least two dermatologists as bullous FDE, FDE, DLE, lichen planus, or lichenoid drug reaction.
- Adults above the age of 18 able to give informed consent.
- Patients able to conform to follow-up visits.

Exclusion criteria:

- Children under the age of 18.
- Patients unwilling or unable to give informed consent.
- Patients not willing to adhere to follow-up visits.

3.2 Ethics

The study was approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee (HREC REF 713/2020).

3.3 Specimen collection:

1. The participants were followed from baseline, 6 weeks after baseline and 6 months after the initial visit to the dermatology clinic.
2. Clinical examination, detailed medication history and clinical photographs were taken at each visit. At the follow-up visits, skin biopsies were repeated at the same anatomical location at the least and as close to the original as possible, avoiding exposed parts of the body like the face and chest if possible. Photographs will be taken at the site of the biopsy. An example of the case report form is found in Appendix I

- At each visit, an appointed qualified dermatologist performed one 4mm punch biopsy and one 2mm punch biopsy under local anaesthesia of active lesions which represents biopsy 1 and biopsy 2 respectively:
    - Biopsy 1 was placed in a 10% neutral buffered formalin fixative immediately after collection, these biopsies were used to perform H & E staining, MF staining, immunohistochemical staining (CD68; Melan-A and HMB-45), and MALDI-TOF.
    - Biopsy 2 was placed in a 3% glutaraldehyde buffered fixative with 0.1 M phosphate buffer immediately after collection, EM was performed on these biopsies.

These biopsies were processed and stained by a qualified Medical Technologist at the National Health Laboratory Service (NHLS), according to the laboratory's standard operating procedure. All safety procedures were followed.

3.4 Processing and cutting for H&E staining, MF staining and Immunohistochemistry:

The tissue was fixed in a 10% neutral buffered formalin fixative for 60 minutes at 37^{o}C. It was then dehydrated in increasing concentrations of alcohol (70%, 90%, 90% and 100%) for 60 minutes at 37^{o}C in each container of alcohol concentration. This was done to remove water and formalin from the tissue. Xylene was used as the clearing agent for 60 minutes at 37^{o}C using two containers of xylene. This was to remove alcohol from the tissue. The tissue was then impregnated with paraffin wax for 90 min at 62^{o}C. The tissue was then embedded in paraffin wax, to create a formalin-fixed paraffin embed (FFPE) block. This processing schedule is used for routine specimens and set up by the laboratory. The tissue processor used was a Leica TP1020.

The FFPE block was sectioned at 3 microns using a semi-automated rotary microtome. These sections were then placed in a thermostatically controlled floatation bath at 48^{o}C, it was then attached to a glass slide to be stained. The slides used for H&E staining were plain microscope glass slides. HistoBond® microscope slides were used for MF staining and immunohistochemistry staining. The difference in the

slides is that HistoBond® microscope slides are positively charged. Before staining commenced the slides with the sections attached were incubated at 65^{o}C to allow the sections to dry.

3.5 Staining:

Tissue controls were stained to verify quality control of the staining by a qualified and registered Medical Technologist. The H&E -, MF - and IHC - stained slides were reviewed using a light microscope by Professor K. Pillay. A subjective and qualitative analysis was performed regarding the presence of melanophages and chronic inflammatory cell infiltrate.

3.5.1 H&E stain

H&E stain was done using an automated stainer (Tissue-Tek® DRS™ 2000, Sakura)

The tissue was then rehydrated before commencing staining protocol. Rehydration was achieved by immersing the slides in xylene then in descending concentrations of alcohol (100% alcohol, 95% alcohol then 70% alcohol) before rinsing it in distilled water.

H & E staining procedure according to the laboratory's operating procedure:

1. Immerse slides in Haematoxylin (Gadson's Haematoxylin) for 5 minutes
2. Rinse in distilled water
3. 2 dips in 1% Acid alcohol
4. Place sections in running tap water for 5 minutes until sections are blue.
5. Counterstain in Eosin 2:1 Phloxine for 4 minutes
6. Rinse in distilled water
7. Dehydrate using ascending concentrations of alcohol (70% alcohol, 95% alcohol then 100% alcohol)
8. Clear in Xylene
9. Coverslip with a glass coverslip using Entellan® mounting media.

TABLE 5: PRODUCTS AND SUPPLIERS FOR H&E STAINING

Product	Supplier
Haematoxylin powder	Sigma-Aldrich®, USA
Eosin powder	Merck, Germany
Phloxine B (C.I. 45410) powder	Merck, Germany
Entellan®	Merck, Germany
Microscope slides	Lasec® Diagnostics, South Africa

MF staining and immunohistochemistry was performed after assessing the tissue sections microscopically to see if the skin morphology is adequate (containing epidermis and dermis) on the slide and the staining was satisfactory according to Bancroft and Layton, whereby the nuclei stain blue-black, showing intracellular detail and the cell cytoplasm and most connective tissue fibres stain various shades of pink, orange and red (Bancroft and Layton, 2019). The characteristics of the tissue sample was determined by the H&E stain.

3.5.2 MF staining procedure

The tissue was then rehydrated before commencing staining protocol. Rehydration is achieved by immersing the slides in xylene then in descending concentrations of alcohol (100% alcohol, 95% alcohol then 70% alcohol) before rinsing it in distilled water.

Ammoniacal silver solution preparation:

1. 20ml of 10% aqueous silver nitrate solution was placed in a glass flask.
2. Concentrated ammonia was added dropwise while agitating the flask
3. This was done until the formed precipitate *almost* dissolved and a faint opalescence was observed.
4. Distilled water was then added to make up 50ml. The solution was then filtered into the coplin jar.

The staining then commenced as follows:

1. Immerse slides into a coplin jar of ammoniacal silver solution overnight in a black box at room temperature at a slant.
2. Wash well in distilled water
3. Immerse slides into a coplin jar of 1% Gold chloride for 1 minute
4. Rinse in distilled water
5. Treat sections with 5% aqueous sodium thiosulphate for 5 minutes

6. Rinse in distilled water
7. Counterstain with 1% aqueous Neutral Red for 2 minutes, by using a filter paper to prevent deposits.
8. Dehydrate using increasing concentrations of alcohol (70% alcohol, 95% alcohol then 100% alcohol)
9. Clear in Xylene
10. Coverslip with a glass coverslip using Entellan®

TABLE 6: PRODUCTS AND SUPPLIERS FOR MF STAINING

Product	**Supplier**
Silver Nitrate powder	Sigma-Aldrich®, USA
Gold Chloride powder	Sigma-Aldrich®, USA
Sodium Thiosulphate powder	Merck, Germany
Neutral Red powder	Merck, Germany

MF staining was deemed satisfactory if melanin and melanophages were staining adequate, without overbearing silver and 1% Neutral Red dye deposits. The amount of extracellular melanin was determined from the MF stain and categorised as being mild <10, moderate 10-20, severe >/= 21. This count was done using a light microscope at x40 magnification over 3 fields.

3.5.3 Immunohistochemistry (IHC):

IHC was performed using Ventana BenchMark ULTRA system, an automated slide staining instrument. This was done at the IHC laboratory at the National Health Laboratory Service, Groote Schuur Hospital. All the reagents used, besides the antibodies are produced by Roche.

The test sections were accompanied by a known positive control to confirm the staining result. The antibodies used for the relevant tests were CD68, Melan-A and HMB-45. All the antibodies were stained using the ultraView Universal Alkaline Phosphatase Red Detection Kit, which is a red detection alkaline phosphatase kit. All slide and section preparations were the same as for H&E staining and special stains, as mentioned above. Before staining commenced the slides with the sections attached were incubated at 65^{o}C to allow the sections to dry on HistoBond® microscope slides.

Procedure:

The procedure using the Ventana Benchmark Ultra for each antibody stain has its own protocol that is pre-loaded by a qualified Medical Technologist and quality control procedures are followed according to laboratory protocol.

After each step described below SSC wash buffer is used to rinse the slides.

1. Deparaffinization of the tissue was achieved using EZ prep solution.
2. Antigen retrieval:
    - Heat induced epitope retrieval:

CD68 antibody staining required heat induced epitope retrieval, this was performed by using Ultra Cell Conditioning solution (CC1) 64 min at 32 °C.

Melan – A antibody staining required heat induced epitope retrieval, this was performed by using CC1 solution for 64 min at 32 °C.

- Enzyme induced epitope retrieval

HMB-45 antibody staining required enzyme induced epitope retrieval this was performed by using Protease 1 enzyme for 12 min at 32 °C.

Epitope retrieval is performed to aid immuno-recognition as fixation involving formaldehyde. Cross-linking of proteins during tissue preservation by formaldehyde fixation masks the epitopes, making antigen binding sites unavailable (Sanderson et al, 2019)

3. The application of Primary Antibody is done manually using a micropipette.

TABLE 7: ANTIBODY CLONE AND MANUFACTURER

Antibody	Clone	Manufacturer
CD68	Monoclonal mouse antibody	Dako
Melan-A	Monoclonal mouse antibody	Leica
HMB-45	Monoclonal mouse antibody	Dako

4. Application of the detection system used to label the primary antibodies, using ultraView Universal Alkaline Phosphatase Red Detection Kit.

TABLE 8: DILUTION OF REAGENTS FOR IHC STAINING

Reagent	Dilution
EZ prep solution	1:10
CC1	Neat
Protease 1 enzyme	Neat
SSC wash buffer	1:10
Melan-A antibody	1:20
HMB-45 antibody	1:50
CD68 antibody	1:1000

The amount of HMB45 and Melan-A positive staining was determined from the HMB45 immunohistochemical stain. This count was done using a light microscope at x40 magnification over 3 fields. The CD 68 immunohistochemical stain was devised for the study to determine the quantification of macrophages that have engulfed free-lying melanin. The amount of melanophages was categorised as being mild <30, moderate 30-39, severe >/= 40.

3.6 Processing for EM staining

Biopsy 2 was preserved and fixed in 30% glutaraldehyde buffered with 0.1 M phosphate buffer (3% buffered glutaraldehyde) immediately after collection and stored in a refrigerator at 5°C.

The tissue was trimmed with a razor blade to less than 0.25 mm, longitudinally to include the epidermis and dermis. The selected tissue was placed in a filter paper and placed in a specimen basket then placed in the automated processor (Leica EM TP).

The tissue was first placed in 3% buffered gluteraldehyde for further fixation. It was then placed in cacodylate buffer to rinse the tissue of gluteraldehyde as it is a reducing agent. It was then placed in osmium tetroxide which is the secondary fixative, which fixes the lipids and phospholipids and can be retained during the dehydration process. Osmium tetroxide is an oxidising agent, thus the tissue had to be rinsed to prevent a redox reaction between the two fixatives (Woods and Stirling, 2019).

The tissue was then transferred to 2% aqueous uranyl acetate; this is an en bloc staining procedure which adds to the contrast to the final sections in addition to improvement to preservation (Woods and Stirling, 2019) The tissue was transferred to increasing concentrations of alcohol (70%, 90%, 90% and 100%).

The tissue was then impregnated with epoxy resin. Before was placed in pure epoxy resin it was placed in a 50:50 mix of resin and absolute alcohol. Epoxy resin is used as it is resistant to the force of the electron beam and hard enough to permit sections as thin as 30-40 nm to be cut using an ultratome.

Embedding:

The tissue was embedded in epoxy resin. The tissue was placed in a BEEM® capsule with the participant number, which were then filled with epoxy resin. It was then placed in the incubator at 60 °C to polymerise.

Sectioning and staining

Glass knives were used, which were obtained from glass strips. These strips were broken into angulated glass knives with a cutting edge. A float out boat (trough) was made, which was attached to the knife, located below the cutting edge. The ultratome is attached to a stereomicroscope, which facilitates the viewing of the sections.

The tissue blocks were trimmed with an old glass knife at 1 – 3 µm to remove excess resin and expose the whole tissue. Semi-thin sections were then cut at 0.5 µm which is viewed under the light microscope, to ensure that the morphology required is present (epidermis and dermis). These sections were stained using 1 % Toluidine blue in 1% Borax. This ensures the correct orientation of the tissue and sufficient components for analysis.

Ultrathin sections were cut at 60 – 120 nm and picked up using a water-permeable copper grid from the trough. The sections were then stained with lead citrate and uranyl acetate. The samples were viewed with a transmission electron microscope.

Images of the keratinocytes, melanocytes and melanosomes were analysed and captured, using the JOEL electron microscope imaging system.

Chapter 4: Results

The patient data and results were logged on Microsoft Excel spreadsheet.

4.1 Patient data:

TABLE 9: PATIENT CLINICAL DIAGNOSIS AND DEMOGRAPHICS (N=12)

Participant number	Clinical Diagnosis	Age	Gender	Fitzpatrick phenotype
01	Lichenoid drug reaction	24	F	V
02	Lichen planus	34	F	IV
03	Lichen planus	33	F	IV
04	Fixed drug eruption	39	F	IV
05	Fixed drug eruption	25	F	IV
06	Lichen planus	30	F	IV
07	Discoid lupus erythematosus	37	F	V
08	Discoid lupus erythematosus	36	F	V
09	Bullous fixed drug eruption	41	F	IV
10	Bullous fixed drug eruption	51	M	VI
11	Lichenoid drug reaction	44	M	V
12	Bullous fixed drug eruption	49	F	VI

4.1.1 Study demographics:

The study population included 12 individuals who attended the GSH dermatology clinic in Cape Town, with PIHP for the period of February 2021 to June 2022, 8 out of the 12 individuals completed all 3 visits to the dermatology clinic in order to complete the study data collection. The 3 visits consist of the initial visit, first follow-up (6 weeks after initial visit) and second follow-up (6 months after initial visit). There were 11 participants who had more than 1 visit. The patient demographics for the study showed a 5:1 female to male ratio in participants. The average age of these patients was 39 years old (range: 24 to 51 years of age). The Fitzpatrick phenotype count is IV = 6, V =4 and VI =2 (Table 7).

Unfortunately, not all 12 participants were able or willing to complete the study by attending the clinic for follow up biopsies due to COVID-19 and biopsy dates that were due in December to January 2021. Whilst 1 patient with BFDE only attended the clinic for the initial biopsy and did not return as he relocated to another province.

TABLE 10:DATES OF PARTICIPANT VISITATION

Participant number	Duration of onset before biopsy	Initial visit of biopsy	6 week follow up	6 month follow up
01	2 months	24/03/2021	13/06/2021	09/11/2021
02	4 months	08/04/2021	27/05/2021	07/10/2021
03	4 months	24/05/2021	Missed	12/11/2021
04	4 months	27/05/2021	29/06/2021	missed
05	4 days	25/06/2021	10/08/2021	missed
06	1 year	08/07/2021	26/08/2021	21/01/2022
07	Unknown amount of years	08/07/2021	Missed	12/11/2021
08	1 year	12/08/2021	19/11/2021	11/02/2022
09	1 day	29/09/2021	Missed	21/01/2022
10	1 week	08/10/2021	Missed	missed
11	1 month	10/11/2021	21/01/2022	missed
12	1 week	10/11/2021	Missed	04/02/2022

4.2H & E staining results:

TABLE 10: : H&E STAINING RESULTS AFTER INITIAL VISIT (INFLAMMATORY FEATURES)

Participant	Spongiosis	Necrotic Keratinocytes	Chronic Inflammatory Cell (CIC) Infiltrate*	Focal Interface Dermatitis	Eosinophils	Melanophages*
01	0	1 (Focal)	Mild (no band-like infiltrate)	1	0	Mild
02	0	0	Mild with a few plasma cells (no band-like infiltrate)	1	0	Mild
03	0	0	Mild with a few plasma cells (no band-like infiltrate)	1	0	Mild
04	0	1 (Scattered)	Moderate	1	0	Moderate
05	1	1 (Numerous)	Mild with focal plasma cells	1	1 (focal)	Mild
06	1	1 (Focal)	Moderate (no band-like infiltrate)	0	0	Mild
07	0	0	Mild (dermal fibrosis)	0	0	Moderate
08	0	0	Moderate (few mast cells)	1	0	Severe
09	0	1 (Necrotic epidermal layer)	Mild	1	1 (focal)	Very Mild
10	1	1 (Necrotic epidermal layer)	Mild	1	0	Mild
11	0	0	Mild	1	0	Mild
12	1	1 (Necrotic epidermal layer)	Mild	1	0	Mild

Key: 0: absent; 1: present

*A qualitative analysis was done regarding the presence of melanophages and chronic inflammatory cell (CIC) infiltrate

TABLE 11: H&E STAINING RESULTS AFTER INITIAL VISIT (EPIDERMAL FEATURES)

Participant	Orthokeratosis	Hyperkeratosis	Parakeratosis	Acanthosis	Atrophy
01	0	1	1	Mild	0
02	1	0	0	0	0
03	0	1	0	0	0
04	0	1 (with hypergranulosis)	0	1	0
05	0	0	1	0	0
06	0	1 (with hypergranulosis)	1	1 (psoriasiform)	0
07	0	1	0	0	1
08	0	1	0	0	0
09	0	0	0	0	0
10	0	1 (with hypergranulosis)	1	0 (mild regenerative)	0
11	0	1	0	0	0
12	0	1	0	0	0

Key: 0: absent; 1: present

TABLE 12: H&E STAINING RESULTS AFTER 6 WEEK FOLLOW-UP (INFLAMMATORY FEATURES)

Participant	Spongiosis	Necrotic Keratinocytes	CIC Infiltrate*	Focal Interface Dermatitis	Eosinophils	Melanophages*
01	0	1	Mild	1	0	Marked
02	0	0	Mild	1	0	Mild
03	N/A	N/A	N/A	N/A	0	N/A
04	0	0	Mild	0	0	moderate
05	mild	1	Mild	0	0	Mild
06	0	1	Mild	0	0	mild
07	N/A	N/A	N/A	N/A	N/A	N/A
08	mild	0	Moderate	0	0	marked
09	0	0	Mild	1	0	mild
10	N/A	N/A	N/A	N/A	N/A	N/A
11	0	0	Mild	0	0	mild
12	0	0	Mild	0	0	mild

Key: 0: absent; 1: present; N/A: Not applicable as no biopsy performed

*A qualitative analysis was done regarding the presence of melanophages and CIC infiltrate

TABLE 13: H&E STAINING RESULTS AFTER 6-WEEK FOLLOW-UP (EPIDERMAL FEATURES)

Participant	Orthokeratosis	Hyperkeratosis	Parakeratosis	Acanthosis	Atrophy
01	0	1	0	Mild	0
02	1	0	0	0	1
03	N/A	N/A	N/A	N/A	N/A
04	1	0	0	mild	0
05	0	1	0	mild	0
06	0	1	0	0	1
07	N/A	N/A	N/A	N/A	N/A
08	0	1	0	0	1
09	0	1	0	0	0
10	N/A	N/A	N/A	N/A	N/A
11	1	0	0	0	1
12	0	1	0	0	1

Key: 0: absent; 1: present; N/A: Not applicable as no biopsy performed

TABLE 14: H&E STAINING RESULTS AFTER 6-MONTH FOLLOW UP (INFLAMMATORY FEATURES)

Participant	Spongiosis	Necrotic Keratinocytes	CIC Infiltrate*	Focal Interface Dermatitis	Eosinophils	Melanophages*
01	0	0	mild	0	0	mild
02	0	0	mild	0	0	mild
03	0	0	Moderate (scattered mast cells)	0	0	mild
04	N/A	N/A	N/A	N/A	N/A	N/A
05	N/A	N/A	N/A	N/A	N/A	N/A
06	0	0	mild	0	0	mild
07	0	0	mild	1	0	mild
08	1	0	moderate	1	0	marked
09	N/A	N/A	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A	N/A	N/A
11	N/A	N/A	N/A	N/A	N/A	N/A
12	0	0	mild	0	0	mild

Key: 0: absent; 1: present; N/A: Not applicable as no biopsy performed

*A qualitative analysis was done regarding the presence of melanophages and inflammatory cell infiltrate

TABLE 15: H&E STAINING RESULTS AFTER 6-MONTH FOLLOW UP (EPIDERMAL FEATURES)

Participant	Orthokeratosis	Hyperkeratosis	Parakeratosis	Acanthosis	Atrophy
01	0	1	0	0	0
02	0	1	0	0	1
03	0	1	0	1	0
04	N/A	N/A	N/A	N/A	N/A
05	N/A	N/A	N/A	N/A	N/A
06	0	1	0	0	0
07	0	1	0	1	0
08	0	1	0	0	1
09	N/A	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A	N/A
11	N/A	N/A	N/A	N/A	N/A
12	0	1	0	0	1

Key: 0: absent; 1: present; N/A: Not applicable as no biopsy performed

4.3 IHC and MF results:

TABLE 16: IHC AND MF STAINING RESULTS AFTER INITIAL VISIT

	Immunohistochemistry			Special Stain
Participant	**HMB45***	**Melan-A***	**CD68 #**	**Masson's Fontana+**
01	28	37	3	2
02	5	35	2	1
03	5	16	1	1
04	39	37	3	2
05	20	6	3	2
06	34	7	2	1
07	8	0	2	3
08	8	0	2	3
09	0	2	2 (intra-epidermal macrophages present)	3
10	7	24	1	1
11	5	18	1	2
12	0	0	3	3

Key: *: Quantity per 3 high power fields (40X); #: Severity of infiltrate - mild <30 (1), moderate 30 – 39 (2), severe >/= 40 (3); +: Severity of infiltrate - mild <10 (1), moderate 10 – 20 (2), severe >/= 21 (3)

TABLE 17: IHC AND MF STAINING RESULTS AFTER 6-WEEK FOLLOW-UP

	Immunohistochemistry			Special Stain
Participant	**HMB45***	**Melan-A***	**CD68 #**	**Masson's Fontana+**
01	21	31	1	3
02	7	62	1	2
03	N/A	N/A	N/A	N/A
04	10	11	2	1
05	30	9	2	1
06	0	0	1	1
07	N/A	N/A	N/A	N/A
08	0	0	3	3
09	7	38	1	1
10	N/A	N/A	N/A	N/A
11	6	34	2	2
12	10	32	1	2

Key: *: Quantity per 3 high power fields (40X); #: Severity of infiltrate - mild <30 (1), moderate 30 – 39 (2), severe >/= 40 (3); +: Severity of infiltrate - mild <10 (1), moderate 10 – 20 (2), severe >/= 21 (3); N/A: Not applicable as no biopsy performed

TABLE 18: IHC AND MF STAINING RESULTS AFTER 6-MONTH FOLLOW UP

	Immunohistochemistry			Special Stain
Participant	**HMB45***	**Melan-A***	**CD68 #**	**Masson's Fontana+**
01	17	17	1	3
02	0	4	1	1
03	5	11	2	3
04	N/A	N/A	N/A	N/A
05	N/A	N/A	N/A	N/A
06	7	16	1	2
07	19	32	1	2
08	0	0	3	3
09	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A
11	N/A	N/A	N/A	N/A
12	0	0	1	2

Key: *: Quantity per 3 high power fields (40X); #: Severity of infiltrate - mild <30 (1), moderate 30 – 39 (2), severe >/= 40 (3); +: Severity of infiltrate - mild <10 (1), moderate 10 – 20 (2), severe >/= 21 (3); N/A: Not applicable as no biopsy performed

4.4 Lichen planus:

This diagnostic group comprised a total of 3 female participants (Participant 2, 3 and 6) each with Fitzpatrick phenotype IV and an average age of 33 years. Participants 2 and 6 completed all 3 visits, whilst Participant 3 attended the initial visit and returned after 6 months for the last biopsy. All 3 patients had no previous topical steroid treatment and had pruritus upon their initial biopsy.

Participant 6 presented at the clinic 1 year after the onset of the lesions as opposed to Participants 2 and 3, who presented within 4 months of onset.

The H&E stain of the initial biopsy of Participant 2 demonstrated orthokeratosis, mild CIC infiltrate with a few plasma cells, mild melanophages, focal interface dermatitis and no necrotic keratinocytes. Participants 3 demonstrated hyperkeratosis, mild CIC with a few plasma cells and mast cells; mild melanophages and the presence of focal interface dermatitis with no necrotic keratinocytes. Participant 6 showed hypergranulation with hyperkeratosis; parakeratosis; spongiosis; focal necrotic keratinocytes; moderate CIC infiltrate; mild melanophages and features of psoriasiform epidermal hyperplasia. All 3 participants did not present a lichenoid inflammatory cell band-like infiltrate and no eosinophils. Participants 2 and 3 did not present with elongated rete-ridges and spongiosis.

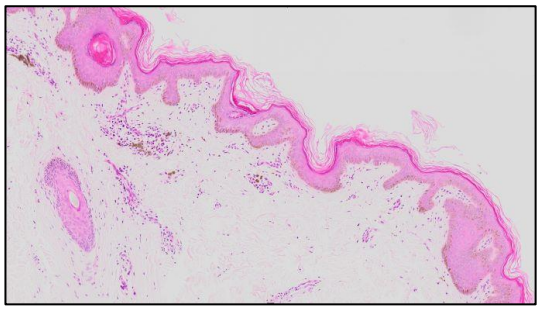

FIGURE 24: PARTICIPANT 3 INITIAL BIOPSY (H&E, X100)

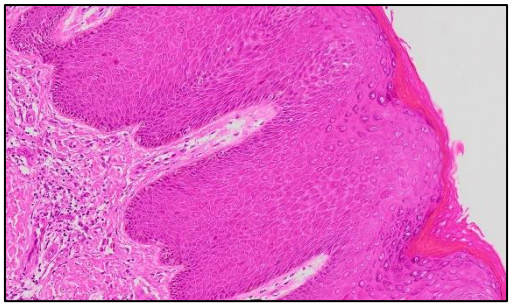

FIGURE 25: PARTICIPANT 6 INITIAL BIOPSY (H&E, X100)

The H&E staining of the first follow up biopsy of Participant 2 presented with orthokeratosis; atrophic epidermal layer; mild CIC infiltrate; mild melanophages and focal interface dermatitis. The H&E staining of the first follow up biopsy of Participant 6 presented with hyperkeratosis; spongiosis; necrotic keratinocytes; mild CIC infiltrate; mild melanophages and epidermal atrophy. Both participants did not present with a lichenoid inflammatory cell band-like infiltrate.

After 6 months Participant 2 presented with hyperkeratosis; mild CIC infiltrate; mild melanophages and mild atrophy. Participant 3 presented with hyperkeratosis; atrophic epidermal layer; moderate CIC infiltrate with scattered mast cells and mild melanophages. Participant 6 presented with hyperkeratosis; mild CIC infiltrate; mild melanophages. All 3 participants did not present lichenoid inflammatory cell band-like infiltrate.

TABLE 19: LICHEN PLANUS H&E STAINING RESULTS AFTER INITIAL VISIT

	Participant 2	**Participant 3**	**Participant 6**
Orthokeratosis	1	0	0
Hyperkeratosis	0	1	1 (with hypergranulosis)
Parakeratosis	0	0	1
Spongiosis	0	0	1
Necrotic Keratinocytes	0	0	1 (focal)
Chronic Inflammatory Infiltrate	Mild (no band-like infiltrate)	Mild (no band-like infiltrate)	Moderate (no band-like infiltrate)
Focal interface Dermatitis	1	1	0
Melanophages	mild	mild	mild
Eosinophils	0	0	0
Acanthosis	0	0	1 (psoriasiform)

Key: 0: absent; 1: present

TABLE 20: LICHEN PLANUS H&E STAINING RESULTS AFTER 6 WEEKS

	Participant 2	Participant 6
Orthokeratosis	1	0
Hyperkeratosis	0	1
Necrotic Keratinocytes	0	1
Chronic Inflammatory Infiltrate	1	1
Focal interface Dermatitis	1	0
Melanophages	1	1
Acanthosis	0	0

Key: 0: absent; 1: present

TABLE 21: LICHEN PLANUS H&E STAINING RESULTS AFTER 6 MONTHS

	Participant 2	Participant 3	Participant 6
Hyperkeratosis	1	1	1
Chronic Inflammatory Infiltrate	1	1	1
Melanophages	1	1	1
Acanthosis	0	1	0

Key: 0: absent; 1: present

The Masson-Fontana stain was performed to quantify extracellular melanin deposits; the stain indicated that there was a mild deposition in all 3 participants at the time of initial biopsy. Participant 2 had a moderate deposition (Figure 26) and Participant 6 remained mild at the time of the second biopsy (Figure 25). The final biopsy indicated that Participant 2 had less extracellular melanin deposits present as it returned to a mild deposition of melanin. Participant 6 had a moderate case of extracellular melanin deposits and Participant 3 had a severe deposition at the final biopsy.

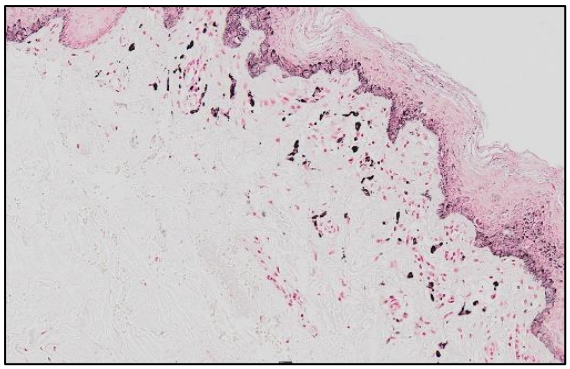

FIGURE 26: PARTICIPANT 2 FIRST FOLLOW UP BIOPSY (MF, X350)

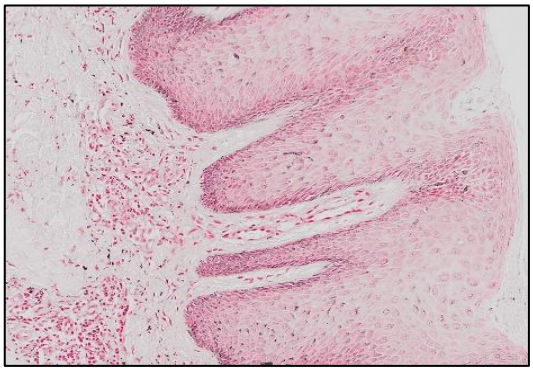

FIGURE 27: PARTICIPANT 6 INITIAL BIOPSY (MF, X350)

The immunohistochemistry stain with Human Melanoma Black (HMB45) was performed of which Participant 2 had a count of 5/3hpf upon initial visit. There was an increase of 7/3hpf in the second biopsy (Figure 28), and a major decrease of 0/3hpf in the third biopsy. Participant 3 had a count of 5/3hpf upon initial biopsy and last biopsy. Participant 6 had a count of 34/3hpf staining upon initial visit (Figure 27). The second biopsy had a count of 0/3hpf and 7/3hpf was counted in the last biopsy.

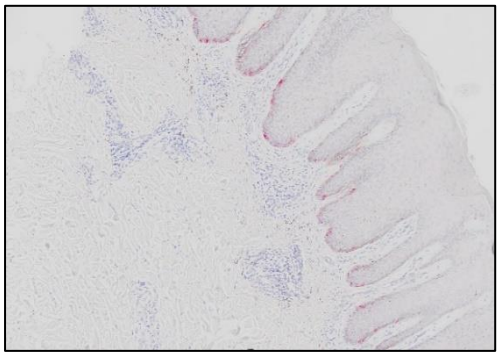

FIGURE 29: PARTICIPANT 6 INITIAL BIOPSY (HMB45, X200)

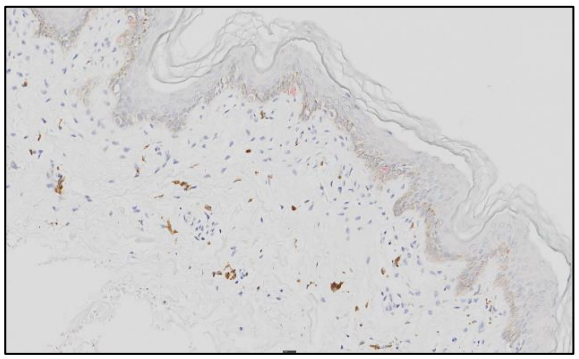

FIGURE 28: PARTICIPANT 2 FIRST FOLLOW UP BIOPSY (HMB45, X350)

The (melanoma antigen) Melan-A staining of Participant 2 had a count of 35/3hpf upon initial visit and an increase of 62/3hpf (Figure 30), and a major decrease of 4/3hpf. Participant 3 had a count of 16/3hpf Melan-A staining upon initial visit and a count of 11/3hpf in the last biopsy. Participant 6 had a count of 7/3hpf melanocytes upon initial visit (Figure 29). There was 0/3hpf count in the second biopsy and 16/3hpf in the third biopsy of Participant 6.

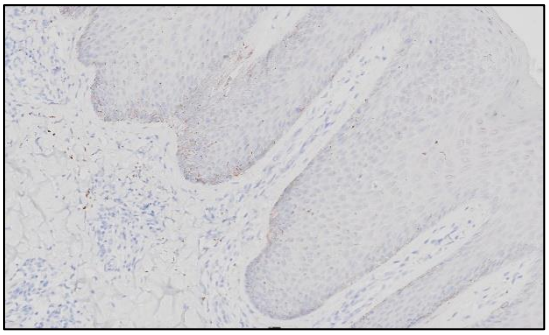

FIGURE 30: PARTICIPANT 6 INITIAL BIOPSY (MELAN-A, X100)

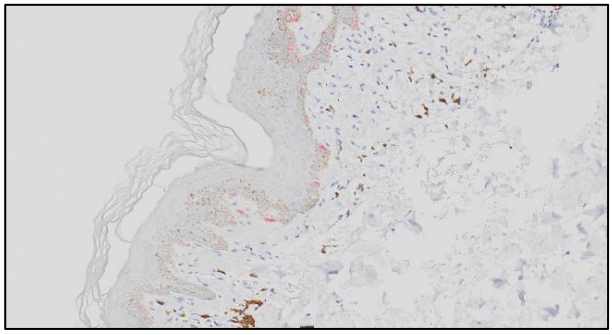

FIGURE 31: PARTICIPANT 2 FIRST FOLLOW UP BIOPSY (HMB45, X100)

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Participant 2 was classified as having a moderate melanophage infiltrate upon initial visit. A decrease of melanophage infiltrate in the second (Figure 32) and third biopsy of Participant 2 was noted. Participant 3 had a mild melanophage infiltrate upon initial visit and moderate infiltrate at the last biopsy. Participant 6 had a moderate melanophage infiltrate upon initial visit (Figure 31). A decrease of melanophage infiltrate in the second and third biopsy of Participant 6 was seen. A decrease in the presence of melanophages was noted after the initial biopsy of Participants 2 and 6, however Participant 3 experienced an increase in CD68 positive staining.

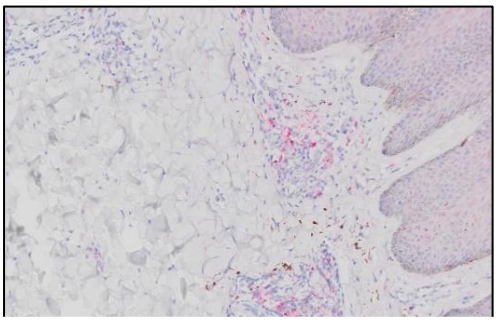

FIGURE 32: PARTICIPANT 6 INITIAL BIOPSY (CD68, X100)

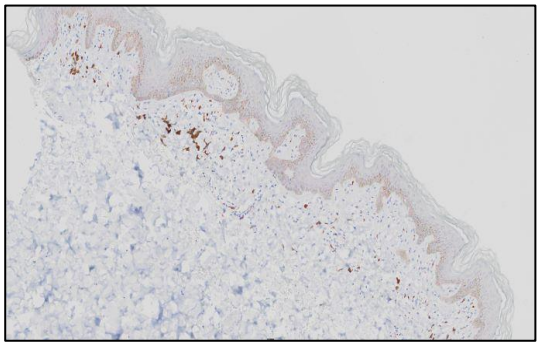

FIGURE 33: PARTICIPANT 2 FIRST FOLLOW UP BIOPSY (HMB45, X100)

4.5 Fixed Drug Eruption

In the second diagnostic group with a diagnosis of FDE there were two female participants (Participant 4 and 5), both with Fitzpatrick phenotype IV at the average age of 32 years old. Both participants are infected with human immunodeficiency virus (HIV) and follows a fixed dose of medication, which includes tenofovir disoproxil, lamivudine, dolutegravir (TLD) drug regime to treat HIV/AIDS. Anti-retroviral medication is known offenders to cause adverse cutaneous drug reactions (Carlson et al, 2002). Both participants have returned for only their first follow up biopsies. The date of onset of Participant 4 when arriving at the clinic was within 4 months, compared to Participants 5, which was within 4 days.

The H&E staining of the initial biopsy of Participant 4 presented with hyperkeratosis together with hypergranulosis, moderate CIC infiltration, moderate melanophages, no eosinophils, acanthosis and scattered necrotic keratinocytes, focal interface dermatitis upon initial visit. Participant 5 presented with parakeratosis; spongiosis; numerous necrotic keratinocytes; mild CIC infiltration with focal plasma cells; focal interface dermatitis; mild melanopahges as well as focal eosinophils upon initial visit. Refer to Figures 34 and 35.

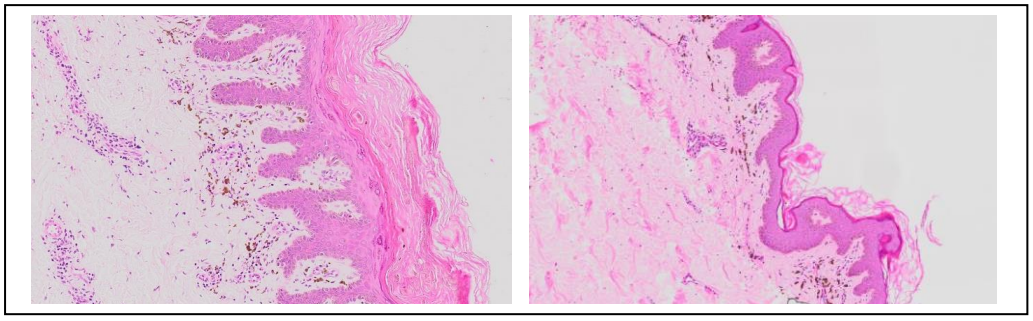

FIGURE 34: PARTICIPANT 4 (H&E, X350) FROM LEFT TO RIGHT: INITIAL VISIT AND FINAL FOLLOW UP BIOPSY

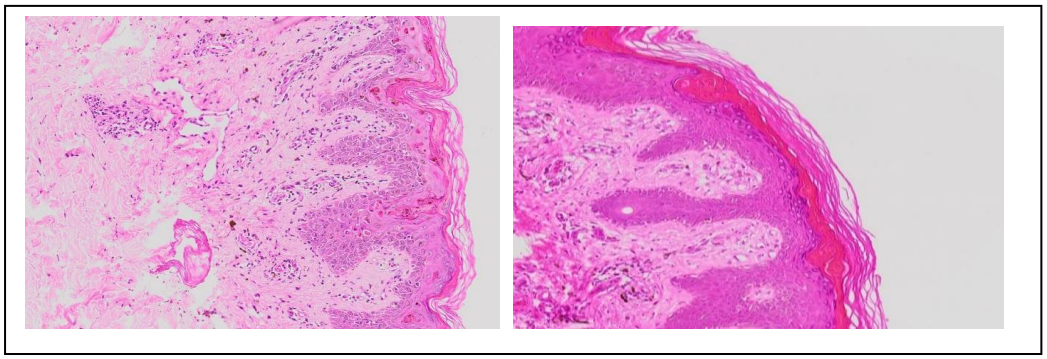

FIGURE 35: PARTICIPANT 5 (H&E, X350) FROM LEFT TO RIGHT: INITIAL VISIT AND FINAL FOLLOW UP BIOPSY

The second biopsy of Participant 4 presented with hyperkeratosis; the presence of mild CIC infiltration and moderate melanophages as well as mild acanthosis. (Figure 34). The second biopsy of Participant 5 presented with hyperkeratosis; spongiosis, necrotic keratinocytes, mild CIC infiltration; mild melanophages and epidermal layer atrophy (Figure 35).

TABLE 22: FIXED DRUG ERUPTION H&E STAINING RESULTS AFTER INITIAL VISIT

	Participant 4	**Participant 5**
Hyperkeratosis	1 (with hypergranulation)	0
Parakeratosis	0	1
Spongiosis	0	1
Necrotic Keratinocytes	1 (scattered)	1 (numerous)
Chronic Inflammatory Infiltrate	1 (moderate)	1 (mild + focal plasma cells)
Melanophages	1 (moderate)	1 (mild)
Eosinophils	0	1 (focal)
Acanthosis	1	0

Key: 0: absent; 1: present

TABLE 23: FIXED DRUG ERUPTION H&E STAINING RESULTS AFTER 6 WEEK VISIT

	Participant 4	Participant 5
Orthokeratosis	1	0
Hyperkeratosis	0	1
Spongiosis	0	1
Necrotic Keratinocytes	0	1
Chronic Inflammatory Infiltrate	1	1
Melanophages	1	1

Key: 0: absent; 1: present

Participants 4 and 5 had a moderate infiltrate of extracellular melanin in their initial biopsy. Both Participants 4 and 5 had a mild infiltrate of free-lying melanin at the time of the second biopsy. Which indicates that both patients had less extracellular melanin in their second biopsy. Refer to Figures 36 and 37.

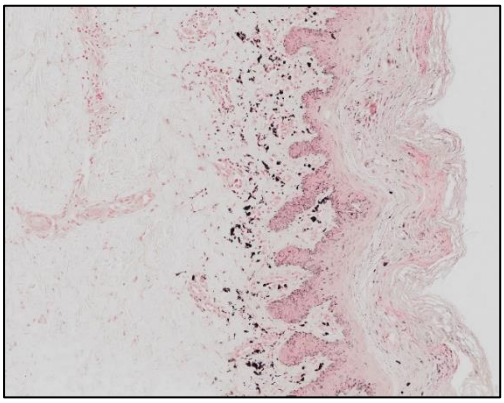

FIGURE 36: PARTICIPANT 4 INITIAL BIOPSY (MF, X 350)

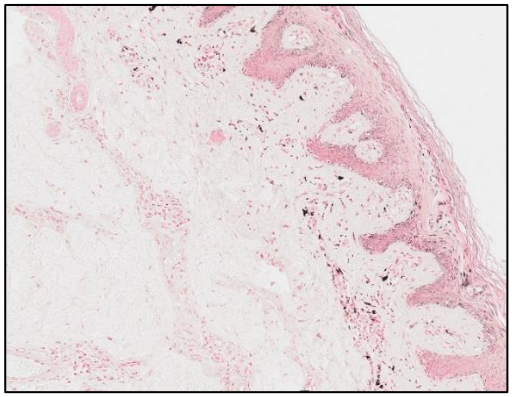

FIGURE 37: PARTICIPANT 5 FINAL FOLLOW-UP BIOPSY (MF, X 350)

The HMB45 staining of Participant 4 had a count of 39/3hpf upon initial visit. The second biopsy had a decrease to 10/3hpf. Participant 5 had a count of 20/3hpf upon initial visit, however there was an increase of HMB45 staining identified of 30/3hpf. The HMB45 staining had variable results between the two participants as Participant 4 had a decrease in HMB45 staining, whilst Participant 5 had an increase. Refer to Figure 38 and Figure 39.

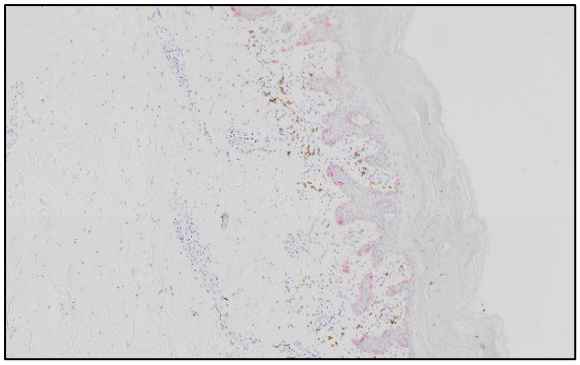

FIGURE 38: PARTICIPANT 4 INITIAL BIOPSY (HMB45, X 350)

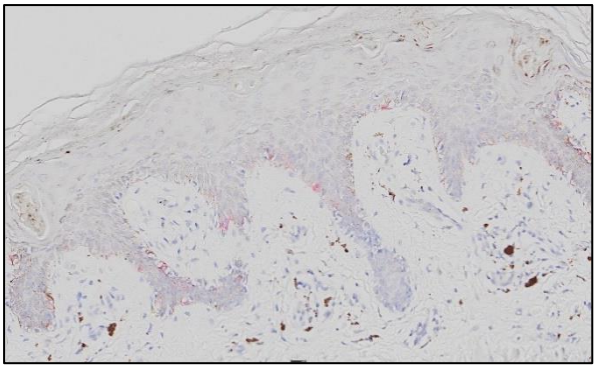

FIGURE 39: PARTICIPANT 5 FINAL FOLLOW-UP BIOPSY (HMB45, X350)

Participant 4 had a count of 27/3hpf Melan-A staining upon initial visit. There was a decrease of Melan-A stain identified of 11/3hpf in the second biopsy. Participant 5 had a count of 6/3hpf melanocytes upon initial visit, however there was an increase of Melan-A staining of 9/3hpf. Refer to Figures 40 and 41.The participants are not following the same trend regarding the positive staining of HMB45 and Melan –A staining.

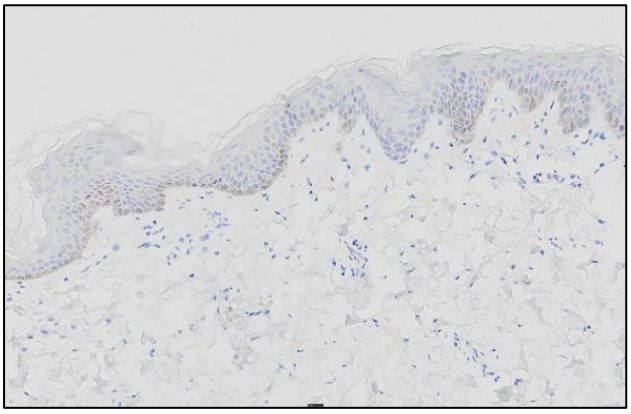

FIGURE 41: PARTICIPANT 5 FINAL FOLLOW-UP BIOPSY (MELAN-A, X 350)

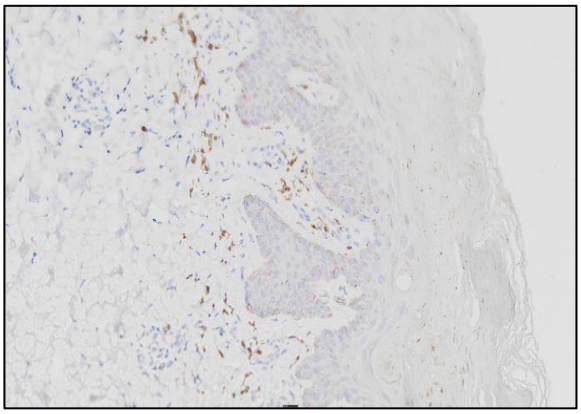

FIGURE 40: PARTICIPANT 4 INITIAL BIOPSY (MELAN-A, X 200)

The initial biopsy done on Patient 4 and 5 indicated that there was a severe infiltrate of melanophages, which were identified with CD68 immunohistochemical staining. Refer to Figures 42 and 43. The second biopsy of both patients indicated a moderate infiltration.

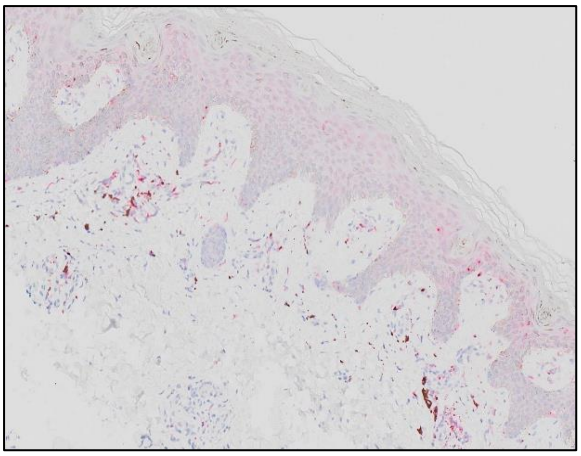

FIGURE 42: PARTICIPANT 5 FINAL FOLLOW-UP BIOPSY (CD 68, X350)

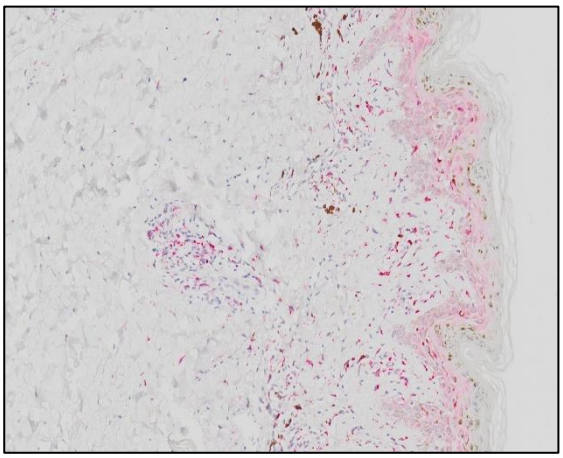

FIGURE 43: PARTICIPANT 4 INITIAL BIOPSY (CD68, X 350)

4.6 Bullous Fixed Drug Eruption

In diagnostic group 3 there were 3 participants (Participant 9, 10 and 12) with the diagnosis of BFDE. The 3 participants consisted of 2 female participants and 1 male participant. The 2 females presented with Fitzpatrick phenotype IV and VI respectively and the male with VI. The average age was 47 years old. All 3 participants are infected with human immunodeficiency virus (HIV) and follows a fixed dose of medication, which includes tenofovir disoproxil, lamivudine, dolutegravir (TLD) drug regime to treat HIV/AIDS. Anti-retroviral medication is known offenders to cause adverse cutaneous drug reactions (Carlson et al, 2002). All three participants also presented with epidermal necrolysis upon initial visit. Participant 9 presented at the clinic 1 day after the onset of the lesions as opposed to Participants 10 and 12, who presented within 1 week of onset.

The H&E staining of the initial biopsy of Participant 9 presented with mild CIC infiltration, focal interface dermatitis, very mild melanophages and focal eosinophils upon initial visit. Participant 10 presented with hyperkeratosis together with hypergranulation, parakeratosis, mild spongiosis, mild inflammatory cell infiltration, focal interface dermatitis, and mild melanopahges upon initial visit. Participant 12 presented with hyperkeratosis, spongiosis, mild CIC infiltration, focal interface dermatitis and mild melanophages upon initial visit. No eosinophils were noted in Participants 10 and 12. All 3 participants presented with epidermal necrosis and a portion of intact epidermal regenerative tissue intact.

The second biopsy of Participant 9 presented with hyperkeratosis, mild CIC infiltration; focal interface dermatitis and mild melanophages. The second biopsy of Participant 12 presented with hyperkeratosis; mild melanophages and mild CIC infiltration (Figure 46). The final biopsy of Participant 12 presented with an atrophic epidermis; mild CIC infiltrate and mild melanophages.

TABLE 24: BULLOUS FIXED DRUG ERUPTION H&E STAINING RESULTS AFTER INITIAL BIOPSY

	Participant 9	Participant 10	Participant 12
Hyperkeratosis	0	1(mild hypergranulation)	1
Parakeratosis	0	1	0
Spongiosis	0	1	1
Necrotic Keratinocytes	1 (necrotic epidermal layer)	1(necrotic epidermal layer)	1(necrotic epidermal layer)
Chronic Inflammatory Infiltrate	1 (mild)	1 (mild)	1 (mild)
Focal interface Dermatitis	1	1	1
Melanophages	1 (very mild)	1 (mild)	1 (mild)
Eosinophils	1 (focal)	0	0
Acanthosis	0	1(mild regenerative)	0

Key: 0: absent; 1: present

TABLE 25: BULLOUS FIXED DRUG ERUPTION H&E STAINING RESULTS AFTER 6 WEEK FOLLOW UP

	Participant 9	Participant 12
Hyperkeratosis	1	1
Chronic Inflammatory Infiltrate	1	1
Focal interface Dermatitis	1	0
Melanophages	1	1

Key: 0: absent; 1: present

TABLE 26: BULLOUS FIXED DRUG ERUPTION H&E STAINING RESULTS AFTER 6 MONTH FOLLOW UP

	Participant 12
Hyperkeratosis	1
Acanthosis	1

Key: 0: absent; 1: present

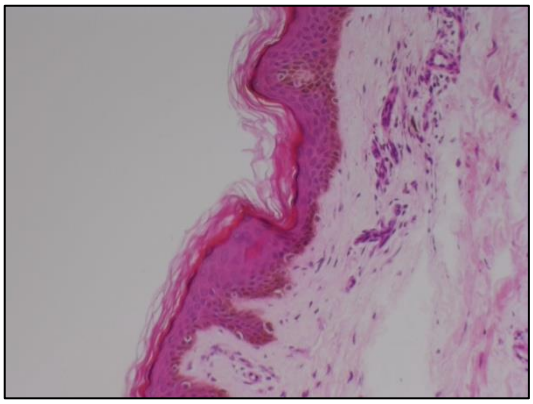

FIGURE 44: PARTICIPANT 9 FIRST FOLLOW UP BIOPSY (H&E, X200)

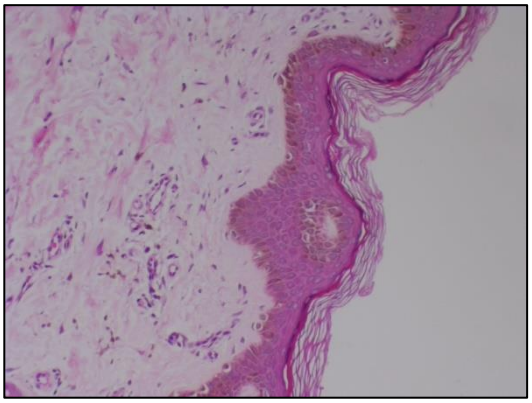

FIGURE 45: PARTICIPANT 12 FIRST FOLLOW UP BIOPSY (H&E, X200)

Participants 9 had a severe deposition of extracellular melanin in the epidermal layer in the initial biopsy (Figure 47) and mild deposition in the follow up biopsy. Participant 10 had a mild infiltrate of extracellular melanin in the initial biopsy. Participant 12 had a severe deposition upon his initial biopsy and a moderate deposition in the first follow up biopsy (Figure 46) and mild deposition in the final biopsy.

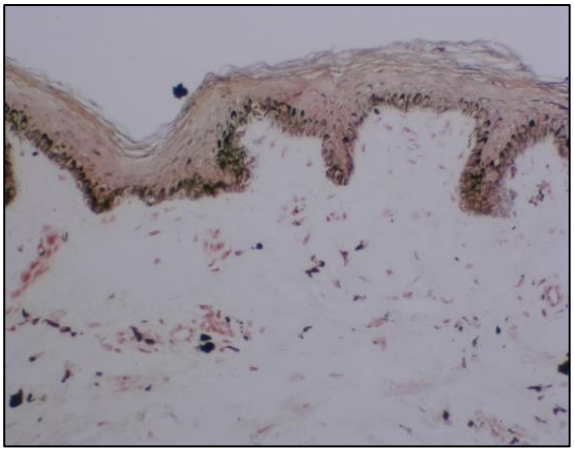

FIGURE 46: PARTICIPANT 12 FIRST FOLLOW-UP BIOPSY (MF)

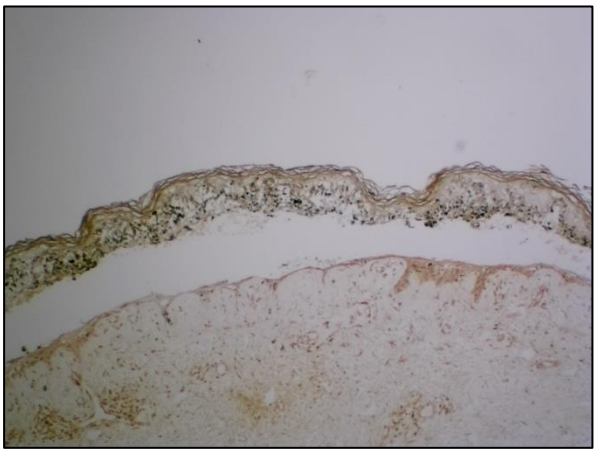

FIGURE 47: PARTICIPANT 9 FIRST FOLLOW-UP BIOPSY (MF)

The HMB45 staining of Participant 9 had a count 0/3hpf count upon initial biopsy and 38/3hpf upon second visit. Patient 10 had a count of 7/3hpf melanocyte immunoreactivity upon initial visit. Participant 12 a count 0/3hpf count upon initial biopsy and 10/3hpf upon second visit. The Melan-A staining of Participant 9 had a count of 2/3hpf positive staining upon initial visit. There was an increase to 38/3hpf positive staining at second visit. Patient 10 had a count of 24/3hpf melanocytes upon initial visit. Participant 12 a count 0/3hpf count upon initial biopsy and 32/3hpf upon second visit. A trend was noted in this case whereby the number of HMB45 staining correlated with the presence of Melan-A positive staining.

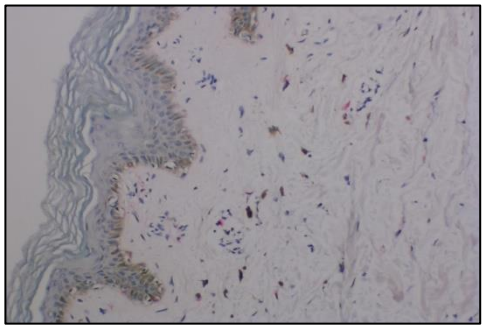

FIGURE 49: PARTICIPANT 9 FIRST FOLLOW-UP BIOPSY (HMB45 X 200)

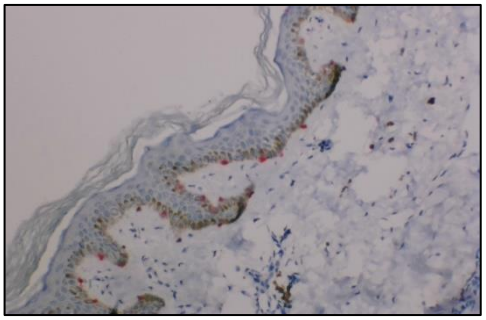

FIGURE 48: PARTICIPANT 12 FIRST FOLLOW-UP BIOPSY (MELAN-A, X 200)

The initial biopsy done on Participant 9 indicated that there was a moderate infiltrate of melanophages. The second biopsy showed a decrease of melanophages of a mild infiltrate. The initial biopsy done on Participant 10 indicated that there was a mild infiltrate of melanophages. Participant 12 had a severe infiltrate of melanophages upon initial biopsy; a decrease in infiltration was noted in the second biopsy of which was moderate, and the third biopsy was mild.

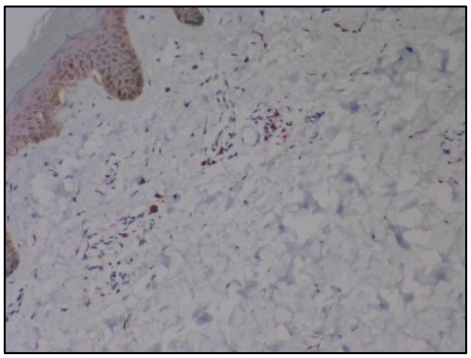

FIGURE 50: PARTICIPANT 12 FIRST FOLLOW-UP BIOPSY (CD68)

4.7 Lichenoid Drug Reaction

In diagnostic group 4 there were 2 participants (Participant 1 and 11) with the diagnosis of LDR. The 2 participants consisted of a female participant and 1 male participant, both presenting with Fitzpatrick phenotype V. The age of the participants were 24 and 44 years old. Only Participant 1 has completed all 3 visits, whilst Participant 11 only attended the initial visit and the first follow up. Participant 1 presented at the clinic 2 months after the onset of the lesions as opposed to Participants 11, who presented within 1 month of onset.

Participant 1 presented with hyperkeratosis, parakeratosis, necrotic keratinocytes, mild CIC infiltrate, focal interface dermatitis and mild melanophages upon initial biopsy (Figure 53). The second biopsy of Participant 1 persisted with hyperkeratosis; necrotic keratinocytes; mild CIC infiltrate and focal interface dermatitis, however marked melanophages were noted, as well as mild acanthosis. The final biopsy presented with orthokeratosis; mild CIC infiltrate and the presence of mild melanophages. Participant 11 presented with hyperkeratosis, mild CIC infiltrate, focal interface dermatitis and mild melanophages upon initial biopsy. The second biopsy presented with orthokeratosis; atrophic epidermal layer; mild CIC infiltrate and mild melanophages. Both participants did not present eosinophils in both biopsies taken.

TABLE 27: LICHENOID DRUG REACTION H&E STAINING RESULTS AFTER INITIAL VISIT

	Participant 1	**Participant 11**
Hyperkeratosis	1	1
Parakeratosis	1	0
Necrotic Keratinocytes	1 (focal)	0
Chronic Inflammatory Infiltrate	1 (mild, no band-like infiltrate)	1 (mild no band-like infiltrate)
Focal interface Dermatitis	1	1
Melanophages	1 (mild)	1 (mild)

Key: 0: absent; 1: present

TABLE 28: LICHENOID DRUG REACTION H&E STAINING RESULTS AFTER 6 WEEK FOLLOW UP

	Participant 1	Participant 11
Orthokeratosis	0	1
Hyperkeratosis	1	0
Necrotic Keratinocytes	1	0
Chronic Inflammatory Infiltrate	1	1
Focal interface Dermatitis	1	0
Melanophages	1	1

Key: 0: absent; 1: present

TABLE 29: LICHENOID DRUG REACTION H&E STAINING RESULTS AFTER 6 MONTH FOLLOW UP

	Participant 1
Orthokeratosis	0
Hyperkeratosis	1
Parakeratosis	0
Spongiosis	0
Necrotic Keratinocytes	0
Melanophages	1

Key: 0: absent; 1: present

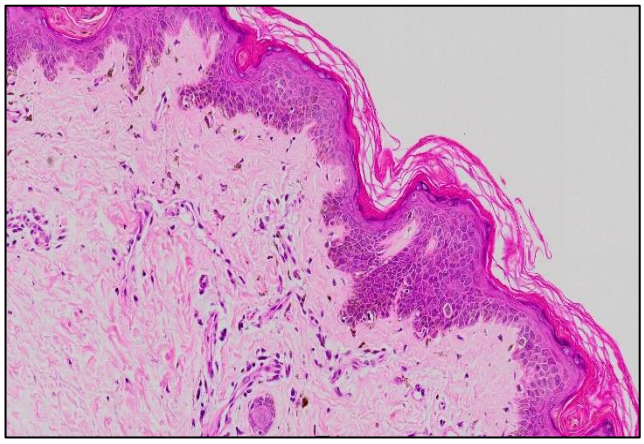

FIGURE 52: PARTICIPANT 1 INITIAL BIOPSY (H&E X 350)

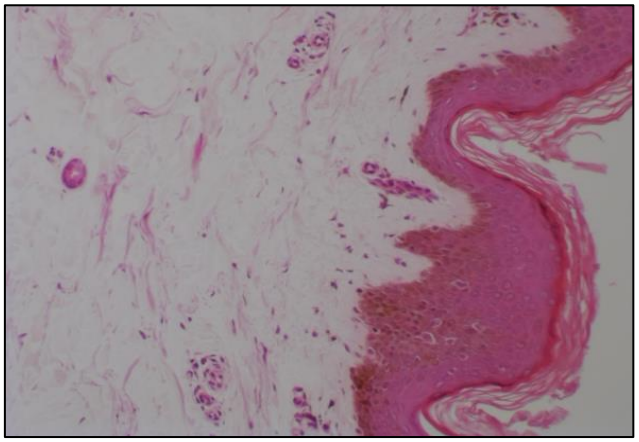

FIGURE 51: PARTICIPANT 11 INITIAL BIOPSY (H&E)

Participants 1 had moderate deposition of extracellular melanin in the initial biopsies, however a severe deposition was noted in both follow up biopsies. Participant 1 presented with a moderate extracellular melanin deposition upon the initial biopsy which became a severe deposition at the time of the second biopsy.

Participant 1 had a HMB45 IHC staining count of 28/3hpf upon initial visit, 21/3hpf on the second biopsy and 17/3hpf on the final biopsy. Participant 11 had a count of 5/3hpf upon initial biopsy and the second biopsy of Participant 1 had a count of 6/3hpf.

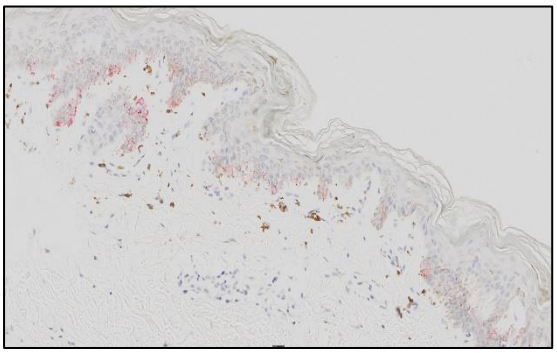

FIGURE 54: PARTICIPANT 1 INITIAL BIOPSY (HMB45 X 200)

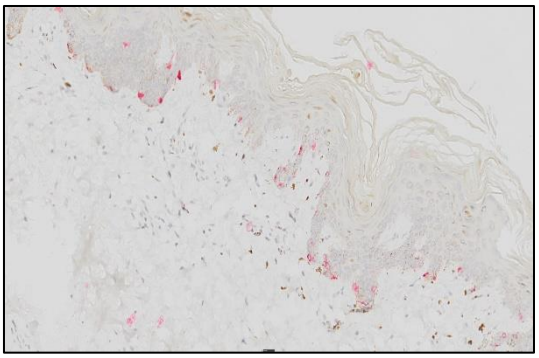

FIGURE 53: PARTICIPANT 1 INITIAL BIOPSY (MELAN-A X 200)

The Melan-A positive staining count for Participant 1 was 37/3hpf upon initial visit, 31/3hpf on the second biopsy and 17/3hpf on the third. Participant 11 had a count of 11/3hpf positive staining upon initial visit and an increase to 34/3hpf upon second biopsy.

The initial biopsy done on Participant 1 indicated that there was a severe infiltrate of melanophages, however a significant amount of non-specific staining was noted (Figure 57). The second and third biopsies showed a decrease of melanophages of a mild infiltrate. The initial biopsy done on Patient 11 indicated that there was a mild infiltrate of melanophages, with an increase to moderate infiltration of melanophages.

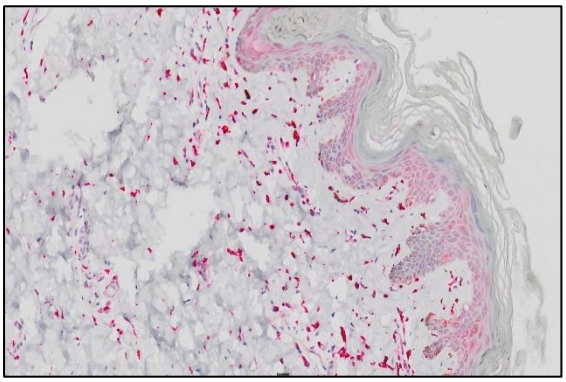

FIGURE 55: PARTICIPANT 1 INITIAL BIOPSY (CD68 X 200)

4.8 Discoid Lupus Erythematosus

In diagnostic group 5 there were 2 participants (Participants 7 and 8) with the diagnosis of DLE. The participants were both female and presented with Fitzpatrick phenotype V. The age of the participants was 36 and 37 years old. Only Participant 8 has completed all 3 visits, whilst Participant 7 only attended the initial visit and the second follow up. Participant 7 presented at the clinic after an unknown number of years after the onset of the lesions as opposed to Participants 8, who presented within 1 year of onset.

Participant 7 and 8 both presented with hyperkeratosis, mild CIC infiltration with dermal fibrosis, the presence of moderate melanophages, focal interface dermatitis and the absence of eosinophils upon the initial biopsy. Atrophy was also noted in all the biopsies of Participants 7 and 8, however regeneration of the epidermis was noted in the second biopsy of Participant 7.

TABLE 30: DISCOID LUPUS ERYTHEMATOUS H&E STAINING RESULTS AFTER INITIAL VISIT

	Participant 7	**Participant 8**
Hyperkeratosis	1	1
Chronic Inflammatory Infiltrate	1 (mild + dermal fibrosis	1 (moderate + mast cells)
Focal interface Dermatitis	0	1
Melanophages	1 (moderate)	1 (marked)
Acanthosis	1 (atrophic)	0

Key: 0: absent; 1: present

TABLE 31: DISCOID LUPUS ERYTHEMATOUS H&E STAINING RESULTS AFTER 6 WEEK FOLLOW UP

	Participant 8
Hyperkeratosis	1
Spongiosis	1
Chronic Inflammatory Infiltrate	1
Melanophages	1

Key: 0: absent; 1: present

TABLE 32: DISCOID LUPUS ERYTHEMATOUS H&E STAINING RESULTS AFTER 6 MONTH FOLLOW UP

	Participant 7	Participant 8
Hyperkeratosis	**1**	**1**
Spongiosis	**0**	**1**
Chronic Inflammatory Infiltrate	**1**	**1**
Focal interface Dermatitis	**1**	**1**
Melanophages	**1**	**1**

Key: 0: absent; 1: present

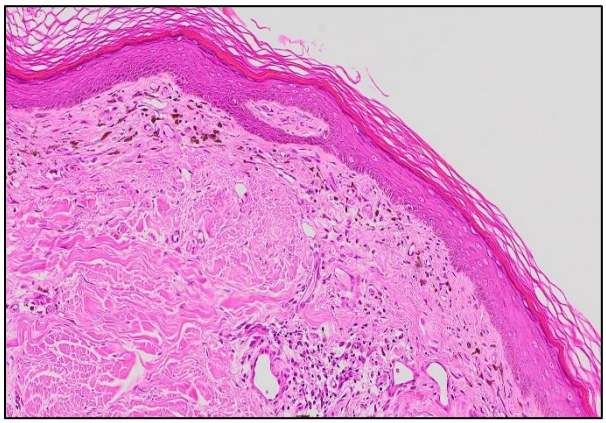

FIGURE 57: PARTICIPANT 7 INITIAL BIOPSY (H&E, X350)

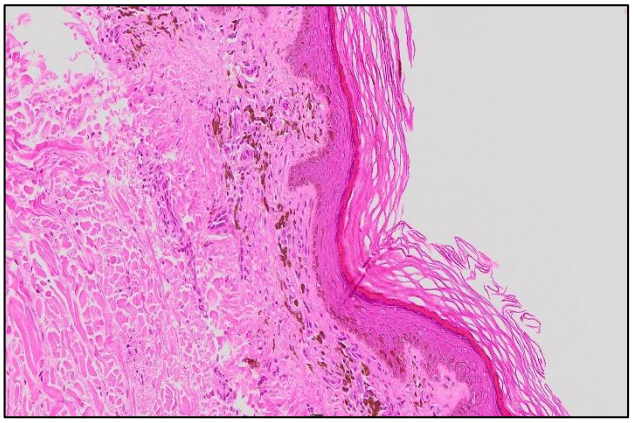

FIGURE 56: PARTICIPANT 8 INITIAL BIOPSY (H&E, X350)

Participant 7 and 8 both presented with severe extra-cellular melanin. The second biopsy of Participant 7 presented with moderate extracellular melanin. The second and third biopsy of Participant 8 remained severe. There was, however, a marked decrease in melanin within the keratinocytes in Participant 8 from the initial biopsy to the last biopsy, whilst Participant 7 did not experience these changes. Participant 8 experienced hypopigmentation as the disease progressed, whilst Participant 7 experienced hyperpigmentation. Refer to Figures 53 and 54.

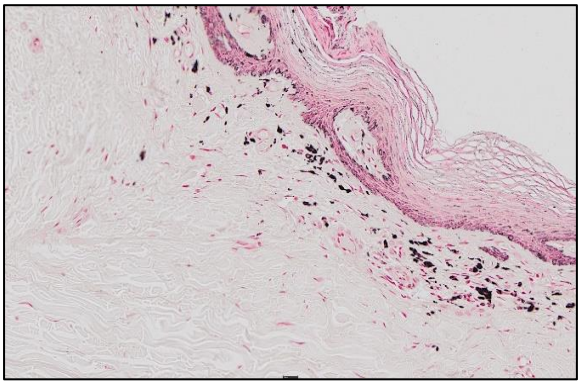

FIGURE 58: PARTICIPANT 7 INITIAL BIOPSY (MF, X 350)

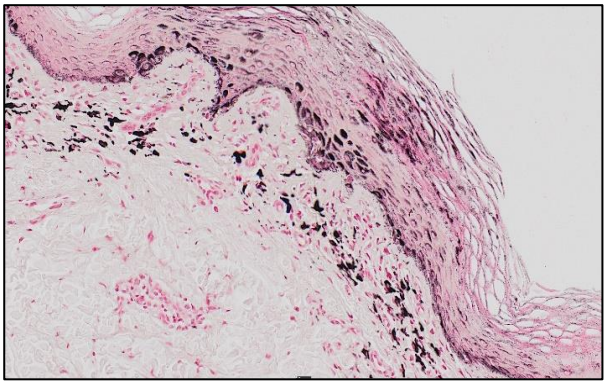

FIGURE 59: PARTICIPANT 8 INITIAL BIOPSY (MF, X 350)

Participants 7 and 8 had a count of 8/3hpf for HMB45 positivity staining upon initial visit. Participant 8 had 0/3hpf positive staining for HMB45 at the second and last visit. The HMB45 positive staining was seen in areas that were not pigmented in the epidermis of Participant 8 (Figure 62) and in the final biopsy positive staining was seen in the dermal area, which is we classified as non-specific staining (Figure 63). However, Participant 7 had a count of 17/3hpf at the last biopsy, which is an increase from the first biopsy.

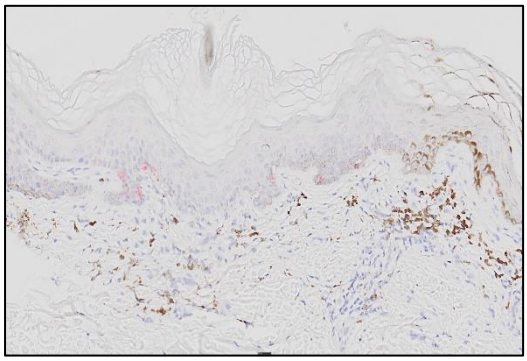

FIGURE 60: PARTICIPANT 8 INITIAL BIOPSY (HMB45, X 350)

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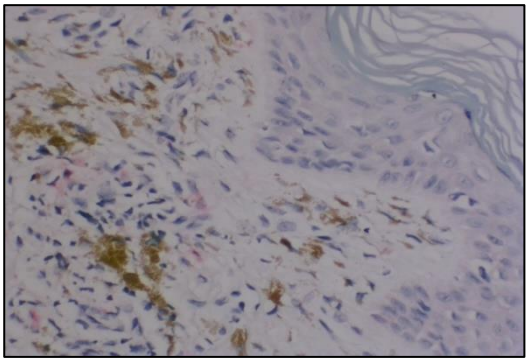

FIGURE 61: PARTICIPANT 8 FINAL BIOPSY (HMB45)

It was noted that atrophy and interface dermatitis was present in the biopsies of Participants 7 (initial biopsy) and 8 (all three biopsies), which correlates with the loss of melanocytes and the 0/3hpf melanocyte count with Melan-A IHC staining. Refer to Figures 64 and 65.

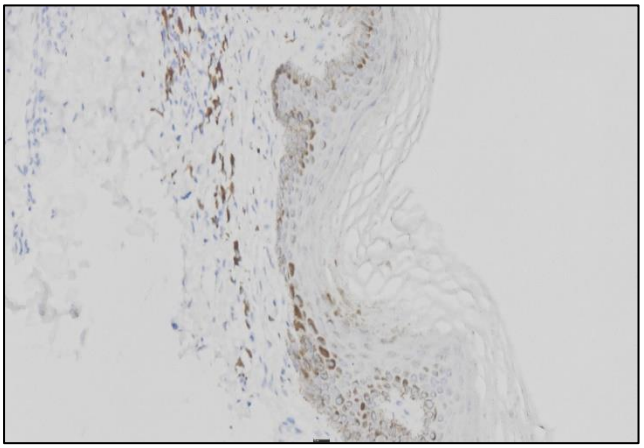

FIGURE 62: PARTICIPANT 8 INITIAL BIOPSY (MELAN-A, X350)

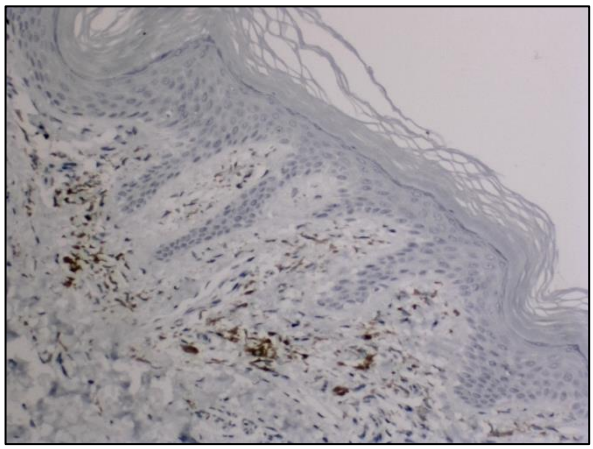

FIGURE 63: PARTICIPANT 8 FINAL BIOPSY (MELAN-A)

The initial biopsy done on Participant 7 and 8 indicated that there was a moderate infiltrate of melanophages. In Participant 8, there was a significant increase of melanophages as the melanophage infiltrate remained severe from the second biopsy to the last biopsy, however Participant 7 had a decrease in melanophage infiltration in the last biopsy.

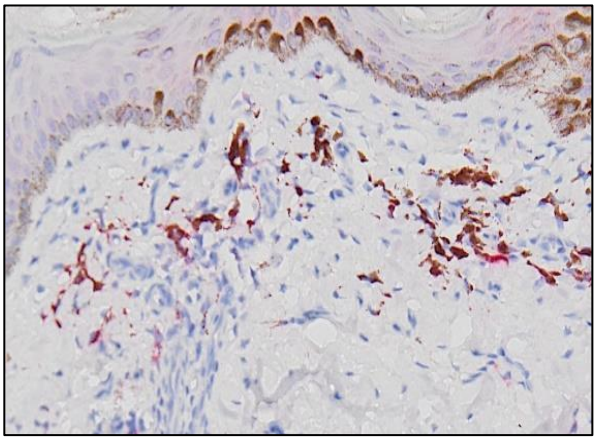

FIGURE64: PARTICIPANT 8 INITIAL BIOPSY (CD68, X400)

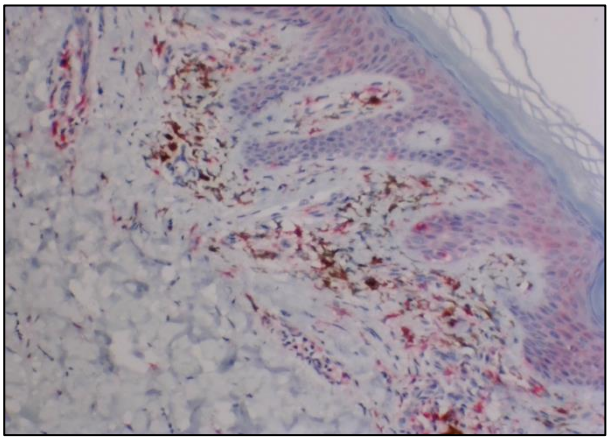

FIGURE 65: PARTICIPANT 8 FINAL BIOPSY (CD68)

4.9 Electron Microscopy:

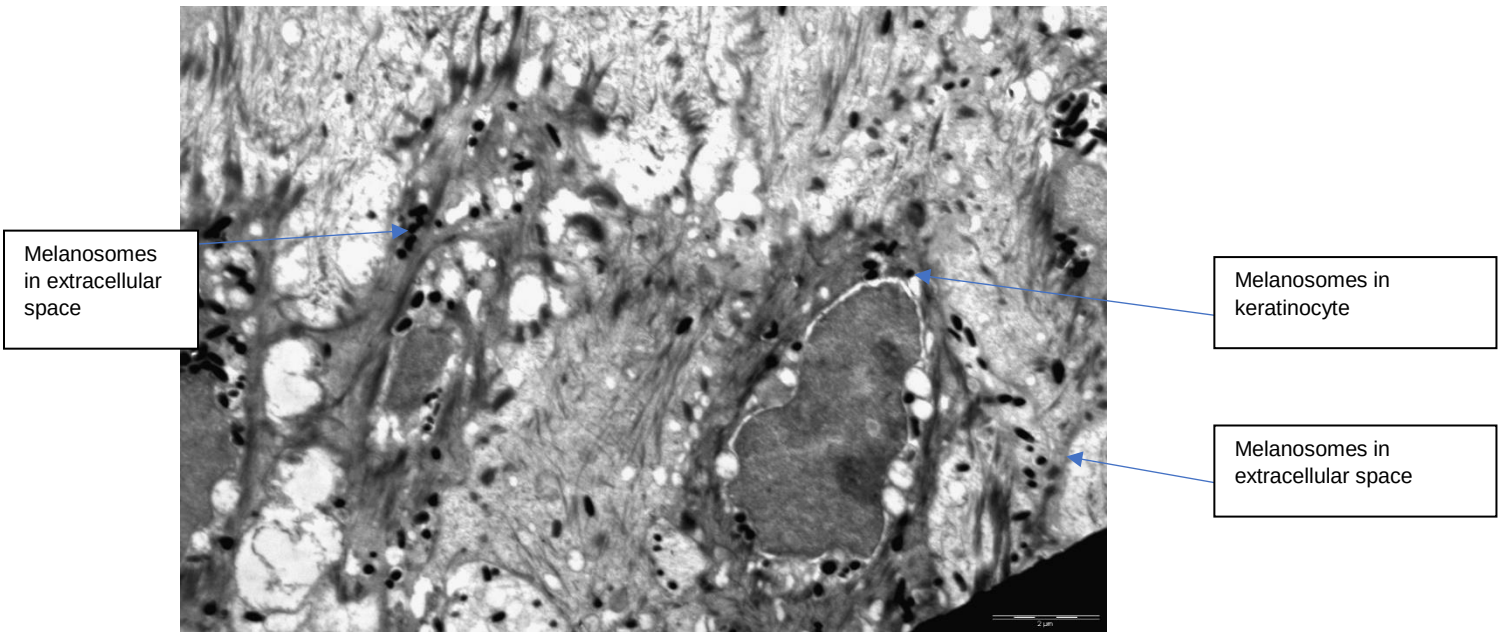

FIGURE 66: ALL MELANIN WITHIN KERATINOCYTES AND EXTRACELLULAR SPACE (ARROW DENOTE MELANOSOMES)

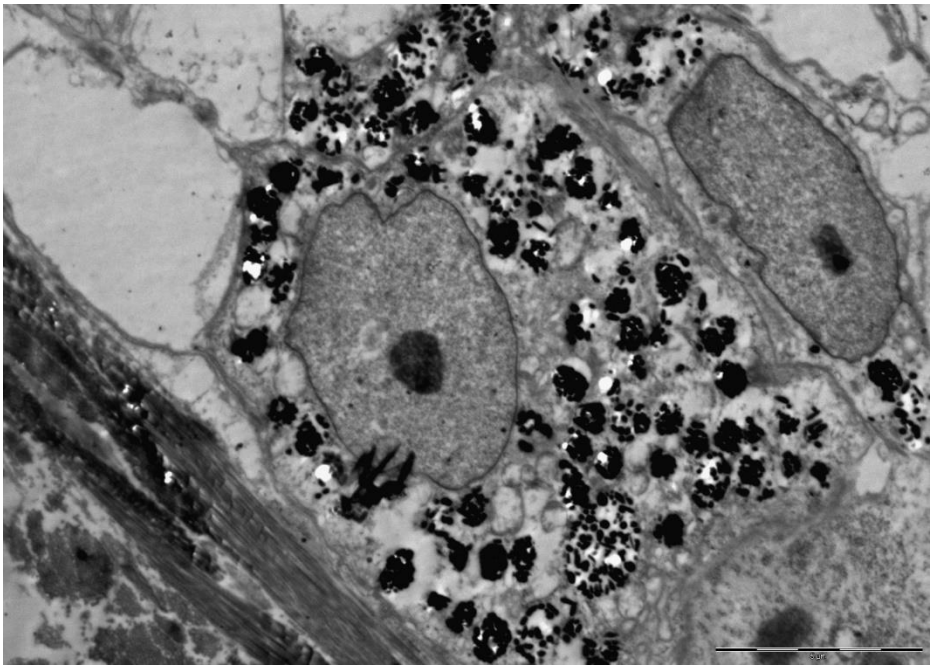

FIGURE 67: MELANOSOMES CONTAINED WITHIN A MELANOPHAGE

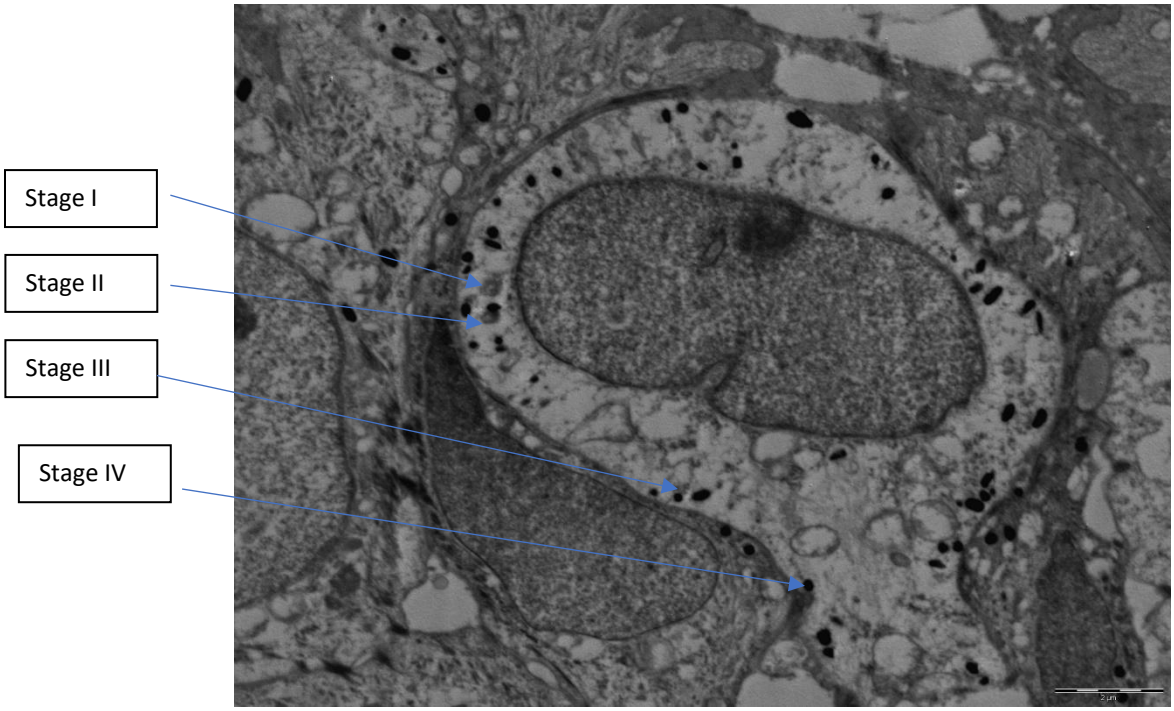

FIGURE 68: MELANOCYTE WITH VARYING DEGREE OF MELANOGENESIS

4.10 Inflammatory markers vs Melanophages

TABLE 33: MELANOPHAGE RESULTS

	Initial visit	6 week follow up	6 month follow up
Participant 1	Mild	Severe	Mild
Participant 2	Mild	Mild	Mild
Participant 3	Mild	N/A	Mild
Participant 4	Moderate	Moderate	N/A
Participant 5	Mild	Mild	N/A
Participant 6	Mild	Mild	Mild
Participant 7	Moderate	N/A	Mild
Participant 8	Severe	Severe	Severe
Participant 9	Mild	Mild	N/A
Participant 10	Mild	N/A	N/A
Participant 11	Mild	Mild	N/A
Participant 12	Mild	Mild	Mild

TABLE 34: CHRONIC INFLAMMATORY CELL INFILTRATE RESULTS

	Initial visit	6 week follow up	6 month follow up
Participant 1	Mild	Mild	Mild
Participant 2	Mild	Mild	Mild
Participant 3	Mild	N/A	Moderate
Participant 4	Moderate	Mild	N/A
Participant 5	Mild	Mild	N/A
Participant 6	Moderate	Mild	Mild
Participant 7	Mild	N/A	Mild
Participant 8	Moderate	Moderate	Moderate
Participant 9	Mild	Mild	N/A
Participant 10	Mild	N/A	N/A
Participant 11	Mild	Mild	N/A
Participant 12	Mild	Mild	Mild

TABLE 35: HMB45 RESULTS

	Initial visit	6 week follow up	6 month follow up
Participant 1	28	21	17
Participant 2	5	7	0
Participant 3	5	N/A	5
Participant 4	39	10	N/A
Participant 5	20	30	N/A
Participant 6	34	0	7
Participant 7	8	N/A	19
Participant 8	8	0	0
Participant 9	0	7	N/A
Participant 10	7	N/A	N/A
Participant 11	5	6	N/A
Participant 12	0	10	0

TABLE 36: NECROTIC KERATINOCYTES

	Initial visit	6 week follow up	6 month follow up
Participant 1	1	1	0
Participant 2	0	0	0
Participant 3	0	N/A	0
Participant 4	2	0	N/A
Participant 5	3	1	N/A
Participant 6	1	1	0
Participant 7	0	N/A	0
Participant 8	0	0	0
Participant 9	4	0	N/A
Participant 10	4	N/A	N/A
Participant 11	0	0	N/A
Participant 12	4	0	0

TABLE 37: INTERFACE DERMATITIS

	Initial visit	6 week follow up	6 month follow up
Participant 1	1	1	0
Participant 2	1	1	0
Participant 3	1	N/A	0
Participant 4	1	0	N/A
Participant 5	1	0	N/A
Participant 6	0	0	0
Participant 7	0	N/A	1
Participant 8	1	0	1
Participant 9	1	1	N/A
Participant 10	1	N/A	N/A
Participant 11	1	0	N/A
Participant 12	1	0	0

TABLE 38: MELAN-A RESULTS

	Initial visit	6 week follow up	6 month follow up
Participant 1	37	31	17
Participant 2	35	62	4
Participant 3	16	N/A	11
Participant 4	37	11	N/A
Participant 5	6	9	N/A
Participant 6	7	0	16
Participant 7	0	N/A	32
Participant 8	0	0	0
Participant 9	2	38	N/A
Participant 10	24	N/A	N/A
Participant 11	18	34	N/A
Participant 12	0	32	0

Inflammatory markers as well as Melan-A and HMB45 are tabulated above to confirm if there are any correlation between the degree of inflammation and the presence of melanophages among all the participants. The use of Melan-A was to determine if varying degree of inflammation is related to the number of melanocytes. The use of HMB45 was used to determine if the production of melanosomes had an impact in determining the degree of free-lying melanin and melanophages. Inflammation due to endogenous or exogenous stimuli results in the release of a variety of inflammatory mediators, which have an influence of melanogenesis stimulation (Fu et al, 2020).

The results above indicate that upon initial visit 8/12 participants had mild melanophages infiltrate, as well as mild CIC infiltrate, and 1/12 participants had moderate CIC infiltrate as well moderate melanophages infiltrate. Thus, 9/12 participants had the same degree of CIC infiltrate and melanophages presence.

After 6 weeks 6/9 participants had a mild degree of CIC infiltrate and melanophages, and 2/9 participants had severe melanophages infiltrate with 1 having moderate CIC infiltrate and the other having mild CIC infiltrate. One participant had moderate melanophage infiltrate with a mild CIC infiltrate.

After 6 months, 5/7 participants had a mild melanophage infiltrate, as well as mild CIC infiltrate, 1/7 participants had a mild melanophages infiltrate and moderate CIC infiltrate. One participant had severe melanophages infiltrate and moderate CIC infiltrate.

Chapter 5: Discussion:

5.1 Study objective:

South Africa has a highly diverse population made up of people with different ethnic origins, mixed ethnicities, and skin colours. Pigmentation of the skin plays important roles in human lives including biological functions and social identity. The type and quality of normal skin pigmentation impacts on pigmentary disorders. The frequency and severity of PIHP varies amongst different ID for unclear reasons. PIHP can be disfiguring following many dermatoses and some cosmetic and medical interventions. There is limited understanding of PIHP pathomechanisms and little progress has been made in prevention and treatment of PIHP. ID are strongly associated with PIHP, thus provide a platform to understand PIHP, a condition that predominantly affects darker skin. A better understanding of the mechanisms that drive the pathogenesis of PIHP could aid in the development of preventative and therapeutic strategies of this non-life threatening but socially debilitating condition.

In this dissertation our aim was to describe light microscopy, immunohistochemistry and electron microscopy characteristics and evolution of a spectrum of interface dermatoses and the resultant post-inflammatory hyperpigmentation (PIHP) in patients seen by the Dermatology Unit at Groote Schuur Hospital in Cape Town, South Africa. The interface dermatoses that were studied participants were: lichen planus; discoid lupus erythematosus; bullous fixed drug eruption; fixed drug eruption or lichenoid drug reaction.

5.2 Key findings

5.2.1 The degree of inflammation vs the degree of pigmentation

The disruption caused to the epidermal melanin unit is a major sequela leading to pigment incontinence (PI). PI refers to the melanin granules being present in the upper dermis or within macrophages due to compensatory accumulation. PI causes post inflammatory hyperpigmentation (PIHP) (Crowson et al, 2008; Hedge and Urmila, 2014). The severity and presence of PI differ with each type of interface dermatitis (Sehgal et al, 2011). In an experiment using mice that were induced with different concentrations of $CD4^+$ autoreactive T-cell clones, it was found that the epidermal damage is proportional to the dosage of the T-cell clone injected. When a suboptimal dosage was induced cell-poor interface dermatitis was observed. Cell-rich interface dermatitis was mimicked when optimal doses were administrated and at high doses full-thickness epidermal necrosis and detachment indistinguishable from toxic epidermal necrolysis was observed (Shiohara and Mizukawa, 2005). In another study using mice, it was observed with the inducing of chronic contact dermatitis using a hapten, it led to the infiltration of inflammatory cells and resulted in features PIHP (Nakano et al, 2021).

To determine the relationship between the degree of inflammation and PIHP, the following characteristics were compared: Chronic inflammatory cell (CIC) infiltrate, necrotic keratinocytes, interface dermatitis and HMB45 vs the presence melanophages. It was noted that the degree of melanophages present in the majority of the participants correlate with the degree of CIC infiltration. The other factors used did not correlate with degree of melanophages as well as the persistence thereof. The only 2 factors that are common amongst all the patients across all the periods were the presence of CIC infiltrate and melanophages. Thus, the degree of PIHP can be determined by the amount of melanophages present. Due to HMB45 being an indicator of melanogenesis and inflammation, it was expected that HMB45 would correlate with the presence of free-lying melanin and CIC infiltrate. However, they did not correlate as expected.

5.2.2 Fixed Drug Eruption (FDE) vs Bullous Fixed Drug Eruption BFDE

BFDE is due to recurrent FDE, which is associated with severe inflammation and blister formation. The most notable difference observed in our study between FDE and BFDE was the full-thickness epidermal necrosis due to necrotic keratinocytes in the epidermis of BFDE participants. This is also seen in toxic epidermal necrolysis (TEN) (Patel et al., 2020).

Epidermal necrosis was absent in the follow up biopsies of both participants 9 and 12. We hypothesise that due to the cleft being detached from the dermal layer, macrophages could not attain the free-lying melanin, thus the degree of pigmentation is less than in FDE as demonstrated below. The results obtained indicates that macrophages were present with the use of CD68 stain in both FDE and BFDE.

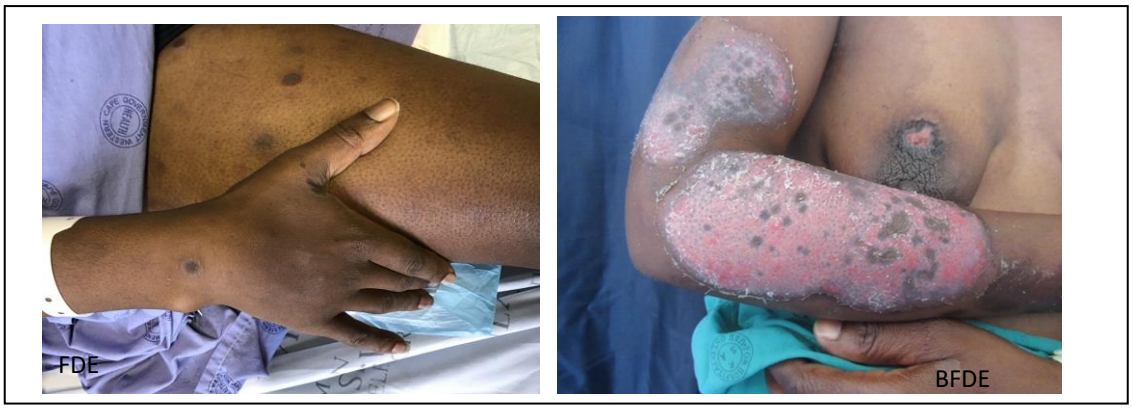

FIGURE 69: FROM LEFT TO RIGHT: PARTICIPANT 5 AND PARTICIPANT 12: INITIAL CLINICAL FEATURES FDE VS BFDE

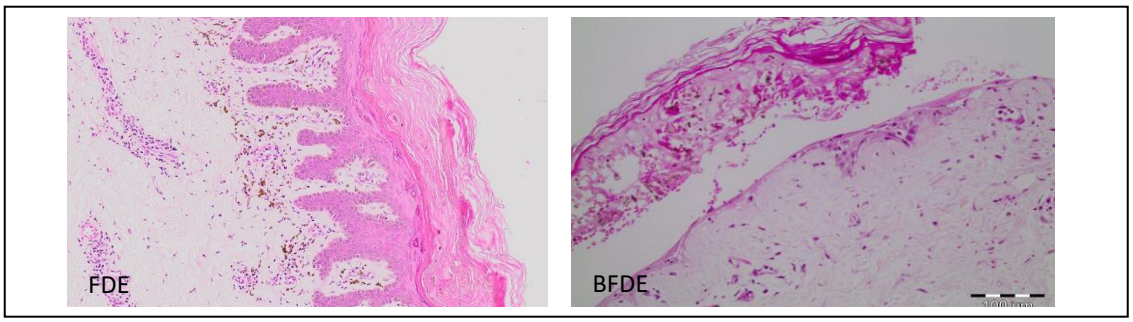

FIGURE 70: FROM LEFT TO RIGHT: PARTICIPANT 5 (H&E) AND PARTICIPANT 9 (H&E): INITIAL BIOPSIES FDE VS BFDE

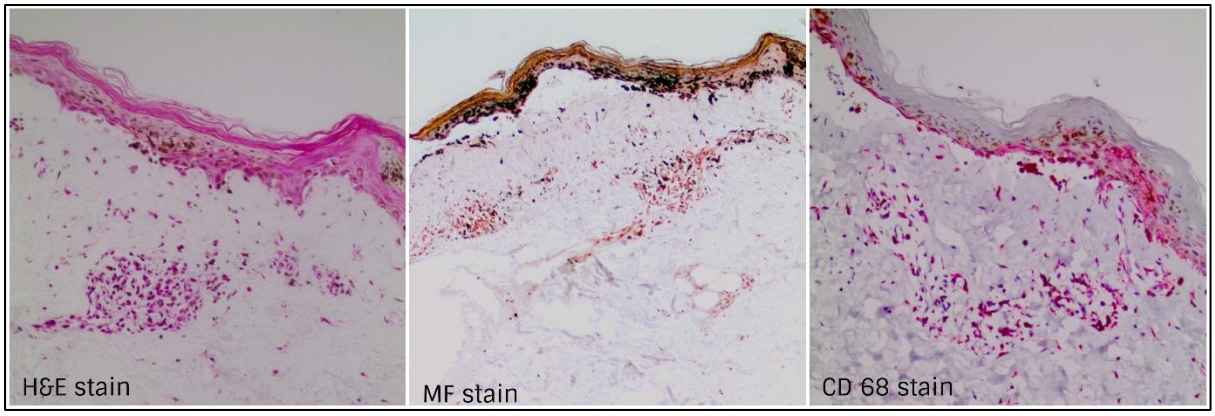

FIGURE 71: PATIENT 12 INITIAL BIOPSY (DEMONSTRATION OF CLEFT)

5.2.3 Absence of Immunohistochemistry (IHC) markers

The immunochemistry markers assessed were HMB-45, Melan A and CD68. HMB45 was initially developed for melanoma detection, however it was later found that it had the ability to detect PMEL17 which is linked to the early production of melanosomes, thus being linked to early melanogenesis (Hoashi et al, 2006). Diagnostic pitfalls have been discovered when Melan-A immunohistochemistry staining is used in isolation, due to Melan-A's cytoplasmic antigen in melanosomes being transferred between cells during inflammation leading to "pseudomelanocytic nests (McClanahan et al, 2019). The expression of HMB45 alongside Melan-A, can serve as an indication that melanogenesis was active with the presence of functional melanocytes.

In our study it was found that if the participant had atrophic epidermal features, that the HMB45 and Melan-A staining count was decreased or absent. This was also observed in participants with a necrotic epidermal layer and participants with DLE. The loss of melanocytes has been described to be due to the destruction of melanocytes (França, A. F. & De Souza, E. M. 2010). In the study conducted by França. & De Souza, the absence of Melan-A and HMB45 was also observed and hypothesised that melanogenesis is still active as the presence of melanin granules were present despite the loss of melanocytes. In our study Participant 7 had the presence of HMB45 stain, which is an indicator of melanogenesis and loss of Melan-A staining. In the follow up biopsy, these markers are both present, which is an indication that melanocyte regeneration is in process. In Participant 8, only HMB45 is present and a complete loss of HMB45 and Melan-A is observed.

The Melan-A count of Participant 10 who had a necrotic epidermal layer was similar to the participants who did not have atrophy as epidermal regeneration was observed. Epidermal shedding was noted with partial regeneration of the epidermis layer. The initial biopsies of participants 9 and 12 with BFDE had the majority of the melanin in the epidermal layer, which was necrotic, thus the HMB45 and Melan-A staining was sparse to absent.

The HMB45 staining count was increased in participants who had a longer onset before treatment, in the sub-categories LP; LDR and FDE.

5.2.4 Patient Demographics

Twelve participants were enrolled into the study, LP (n=3), BFDE (n=3), FDE (n=2), DLE (n=2) and LDR (n=2). There was a 5:1 female to male ratio. Their median age was 39 years old (range: 24 to 51 years). 6/12, 4/12 and 2/12 had Fitzpatrick skin phototypes: IV, V and VI respectively. The predominance of females in ID was previously noted in studies by Dhar *et al* and Hegde *et al* who reported that females accounted for (59%) and (58%) respectively. However, Batchu *et al*, and Sarin *et al* reported males outnumbering females, 67% and 57% respectively in their series of LP patients. The higher proportion of females in this study may not reflect the true population prevalence as this was a convenience sample and a very small population. The heterogeneity of the conditions studied also may not reflect gender distribution in the individual ID in the general population. Some previous studies have shown the average age of patients with LP at diagnosis to be between 40 and 45 years old (Diop et al, 2019; Irvine et al, 1991). A large study by Sarin *et al*, found the age of presentation to be between 8 – 50 years of

age. Similar arguments relating to female gender bias mentioned above apply to age in our study population.

5.2.5 Lichen Planus (LP)

All three participants with LP reported pruritus as a major feature at presentation and the histopathological features were classic LP (Gorouhi et al, 2014). Prutitus is a major feature of LP, and this finding was not unexpected (Schwager et al, 2019). All three participants had not been treated with topical steroids at presentation. Chronicity and severity of clinical LP was reflected on light microscopy, with the initial biopsy from participant 6 who had untreated LP for a year, showing psoriasiform changes and more pronounced parakeratosis; spongiosis and necrotic keratinocytes compared to participants 2 and 3 who had untreated LP for four months. The histological features of LP that persisted in all three participants for up to six months, despite treatment with topical steroids, were the chronic inflammatory infiltrate, melanophages and focal interface dermatitis. In LP, the interface reaction results in the damage of both the basal keratinocytes and melanocytes and subsequent melanin incontinence. Dermal macrophages engulf this extracellular melanin, seen as melanophages microscopically. (Ortonne and Bissett, 2008).

As reported in Saharkhiz *et al*, blunt rete ridge and saw tooth rete ridges can be found in cutaneous LP as well as oral LP. The formation of the rete-ridges is dependent on the sub-type of cutaneous LP (Gorouhi et al, 2014). This was identified in Participant 6 as the rete-ridges are elongated and rounder, however Participants 2 and 3 did not demonstrate prominent feature of elongated rete-ridges. There was significant thinning of the epidermis after the second visit of Participant 6 and regeneration at the third visit. The epidermal thickness of Participants 2 and 3 did not have significant thickening or thinning at any of the follow up visits, the significance of the degree of epidermal thickness was not investigated in detail in this study, however the observation was noted.

The CIC infiltrate was identified qualitatively with H & E staining in all 3 participants. It was identified as mild in participants 2 and 3, and moderate in Participant 6 in their initial biopsies, however no marked band-like lymphohistiocytic infiltrate at the dermal-epidermal junction was noted in any of the biopsies as expected. Prominent features expected to be identified in cutaneous LP such as extended “saw-tooth” rete-ridges and a marked lichenoid band of lymphohistiocytic cells obscuring the dermo-epidermal junction were not identified in any of the biopsies as described by Gru and Salavaggione, 2017. The persistence of chronic inflammatory cells seen after treatment is observed due to the presence of the resident memory T cells. The duration of its longevity is unknown (Honda, 2019).

Clinically, the papules features Participant 6 with a longer onset was more raised compared to the other participants. Below are the initial photographs of Participant 6 compared to Participant 3.

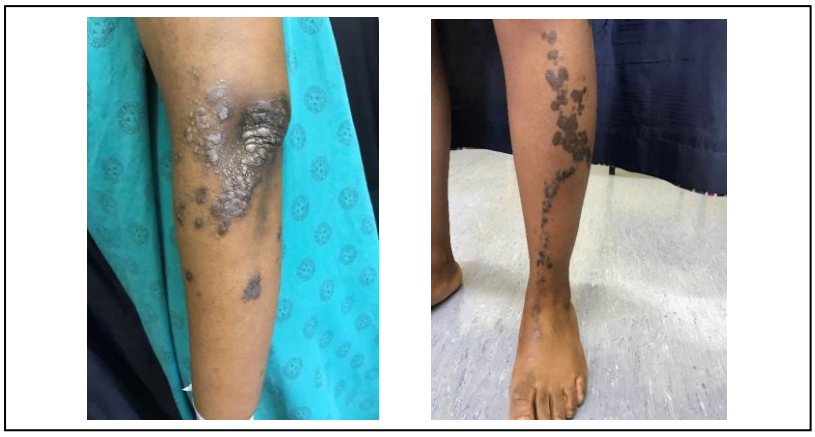

FIGURE 72: INITIAL CLINICAL FEATURES OF PARTICIPANT 6 (LEFT) AND PARTICIPANT 3 (RIGHT)

5.2.6 Lichenoid Drug reaction (LDR):

LDR has the similar histologic features as idiopathic LP, however LDR can be favoured diagnostically by the presence of a high-level of cytoid bodies and eosinophils within the dermal infiltrate (Calonje et al, 2020). A high level of cytoid bodies is also found in idiopathic LP by using direct immunofluorescence, however it was not compared to LDR in the study conducted (Chularojanamontri et al, 2010). A comparative study has indicated that the number of necrotic keratinocytes (grouped in clusters), plasma cells and eosinophils present in the infiltrate has significant value in LDR diagnosis over idiopathic LP (Lage et al, 2012; Weyers and Metze, 2011). The presence of focal parakeratosis, focal interruption of the granular layer is also more indicative of LDR (Cheraghlou & Levy, 2020).

By comparing Participant 1 and 11, more features of inflammation were identified in Participant 1 than Participant 11. The epidermal features in the initial biopsy of Participant 1 demonstrated parakeratosis; mild acanthosis and focal necrotic keratinocytes which did not feature in the initial biopsy of Participant 11.

These features observed in Participant 1 are key features in the diagnosis of LDR as mentioned above, however the duration of onset in Participant 1 was 1 month longer than that of Participant 11. After 6 weeks Participant 1 persisted with mild acanthosis and hyperkeratosis, whilst Participant 11 had orthokeratosis and atrophy of the epidermal layer. The features seen in the initial biopsies of the participants were similar to that of LP, thus the differential diagnosis can be challenging, and the clinical history and clinical differences should be considered (Weyers and Metze, 2011). Distinguishing features were noted in Participant 1, which included parakeratosis and focal necrotic keratinocytes, instead of abundant necrotic keratinocytes as mentioned in Weyers and Metze. Another distinguishing feature mentioned was the presence of slight spongiosis, which was not observed in Participant 1 nor 11. Participant 6, with LP displayed features of slight spongiosis, focal necrotic keratinocytes and

parakeratosis. This indicates that the clinical history and features are very important in aiding distinguishing the two diseases.

The CIC infiltrate and melanophages observed with H&E staining was both subjectively categorised as mild in both in the initial biopsy and the biopsy taken after 6 weeks in Participants 1 and 11. The CD68 staining however demonstrated that Participant 1 had a severe infiltrate of melanophages compared to Participant 11 who had a mild infiltrate. In the first follow up biopsy Participant 1 had mild CD68 staining, and Participant 11 had moderate staining. Macrophages were also identified with CD68 staining within the dermis of these patients. We could not identify if these melanophages have phagocytosed necrotic keratinocytes with melanin.

No eosinophils were seen in any of the biopsies; thus, it does not correlate with the literature mentioned in Calonje *et al.* The presence of eosinophils varies in cases of LDR as a study by Weyers and Metze, indicated that not all patients demonstrate eosinophils in LDR and those who demonstrate eosinophils vary in severity.

5.2.7 Fixed Drug Eruption (FDE):

The 5 participants (2 FDE and 3 BFDE) were treated for HIV using fixed dose combination ART, which includes tenofovir, disoproxil, lamivudine, dolutegravir (TLD) drug regime to treat HIV/AIDS. Anti-retroviral medication is known offenders to cause adverse cutaneous drug reactions (Carlson et al, 2002).

The initial biopsy of Participant 4 demonstrated features of hyperkeratosis with hypergranulosis, scattered necrotic keratinocytes with acanthosis. Participant 5 presented with parakeratosis, moderate spongiosis and numerous necrotic keratinocytes, with focal eosinophils. Both participants demonstrated focal interface dermatitis. These features are commonly seen in FDE and other drug eruptions (Perron et al, 2021 ; Weyers and Metze, 2011). Participant 4 had a longer onset than Participant 5, however Participant 5 had spongiosis and more necrotic keratinocytes. Spongiosis and dermal vacuolization was found to be the most common feature of FDE in a study by Weinborn *et al*.

Upon the second visit, Participant 4 had a decrease in the length of the rete ridges, however Participant 5 had no significant changes. Moreover, it was also noted that the keratin layer of Participant 4 transformed from hyperkeratosis to orthokeratosis and Participant 5's keratin layer transformed from parakeratosis to hyperkeratosis (refer to Figure 34 and 35). The presence of mild CIC infiltration and mild melanophages with mild acanthosis persisted. (Figure 34). The second biopsy of Participant 5 presented with mild spongiosis, scattered necrotic keratinocytes, mild CIC and mild melanophages with mild acanthosis (Figure 35).

5.2.8 Discoid Lupus Erythematosus (DLE)

Lesional DLE are characterised by the presence of interface dermatitis, lymphocytic infiltration, and pigmentary incontinence. The epidermal features include hyperkeratosis with keratotic follicular plugging, featuring variable acanthosis and atrophy (Crowson and Margo, 2001). The presence of T-cells, B- cells and macrophages were expected to be higher in DLE lesional skin compared to normal skin due to the inflammatory process (Thorpe et al, 2014).

The initial biopsy done on Participant 7 and 8 indicated that there was a moderate infiltrate of melanophages. Participant 7 and 8 both presented with hyperkeratosis, variable chronic inflammatory infiltration, and the presence of melanophages upon the initial biopsy. Interface dermatitis was only demonstrated in Participant 8, in the initial biopsy and after 6 months. However, in Participant 7 it was detected only after 6 months. The initial biopsy of Participant 7 demonstrated atrophy at the initial biopsy and acanthosis after 6 months. In Participant 8 atrophy was noted in both follow-up biopsies. These changes are consistent with the literature, which indicates that these microscopic features are a manifestation of DLE (Rothfield et al. 2006; Crowson and Margo, 2001).

It was noted that both participants with DLE had the highest degree of free-lying melanin in the study, particularly Participant 8, who had severe free-lying melanin. The free-lying melanin was confined to the dermis and did not reach the hypodermis. This was noted across all participants in the study, which could be the result of persistent hyperpigmentation.

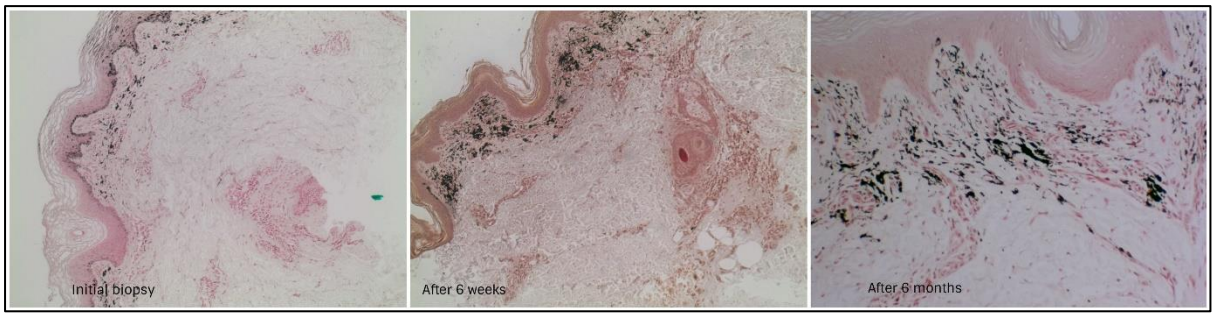

FIGURE 73: MF STAINING OF PARTICIPANT 8 FROM INITIAL BIOPSY TO FINAL BIOPSY

5.2.8 Immunohistochemistry:

In a study by Carlson *et* al, it was noted that HMB45 positive staining was found to be higher in cutaneous LP than in normal skin and in individuals with hypopigmented lesions (Carlson et al, 2002). A similar observation was noted in participant 6 with LP, with initial biopsy values of 34 and decreasing to absent by the second biopsy. By the third biopsy, decreased atrophy is seen with increased presence of regeneration. This pattern was also seen in participant 2 with LP. Thus, we hypothesise that HMB45 is indeed an indicator of melanogenesis.

Participants with LDR, FDE and DLE also showed a similar HMB45 pattern, however, it is not a conclusive finding for the entire study population as not all participants were monitored over the entire 6-month period. This was a limitation of the study. It would be interesting to note the presence of pre-

melanosomes within the melanophages, unfortunately with the high degree of mature melanosomes within the melanophages we were not able to investigate HMB45 staining within melanophages. It was noted that presence of Melan-A coincided with the presence of HMB45.

The CD68 stain was used to quantify melanophages and the Masson's Fontana stain was used to quantify the extracellular melanin. There was no correlation seen between the amount of melanophages compared to extracellular melanin, which did not follow a significant trend according to our observation. As mentioned above the free-lying melanin were restricted to the dermis, however CD68 staining was observed in the hypodermis, well below the free-lying melanin. All participants, besides Participant 8 had a decrease in free-lying melanin, which is attributed to the presence of macrophages performing phagocytosis and causing degradation of the melanin. The enzymatic activity involved in degradation is due to the rich acid phosphatase presence in melanophages, which is also seen in the stratum granulosum (Mishima, 1966).

There was a decrease of CD68 staining was observed in 7/9 participants who had their second biopsy done at 6 weeks, and an increase in 2/9 participants. A decrease of extracellular melanin was seen in 4/9 participants who had their second biopsy done at 6 weeks. An increase was seen in 2/9 participants, while 3/9 participants remained the same. Of the 12 participants, 7 participants returned for the 6-month follow up biopsy. We observed a decrease in melanophages in 5/7 participants, and an increase in 2/7 participants. The extracellular melanin was decreased in 2/7 participants and increase in 3/7 participants whilst 2/7 participants had the same amount.

5.2.7 Electron microscopy:

The electron microscopy images obtained correlated with the features seen in the H&E staining of the study and the literature regarding post-inflammatory hyperpigmentation. The images did not contribute to the study, however further investigation can be done regarding the ultrastructure in future studies.

Chapter 6: Conclusion:

It was noted that the degree of melanophages present in the majority of the participants correlate with the degree of CIC infiltration. The only 2 factors that are common amongst all the patients across all the periods were the presence of CIC infiltrate and melanophages.

FDE and BFDE were assessed, and the key difference was the presence of full thickness epidermal necrosis, creating complete detachment/cleft from the underlying tissue in BFDE. We hypothesise that due to the cleft being detached from the dermal layer, macrophages could not attain the free-lying melanin and migrate into the dermis, and thus the degree of pigmentation is less than in FDE. Similarly, the pigment drop-off from the keratinocytes and melanocytes as a result of basement membrane disruption is reduced. This is illustrated by the supra-cleft localization of both the keratinocytes and melanocytes in Figures 70 and 71. Epidermal regeneration was noted also below the sub-epidermal cleft.

In our study it was found that participants with atrophic epidermal features, that the HMB45 and Melan-A staining count was decreased or absent. This was also observed in participants with a necrotic epidermal layer and participants with DLE, however with regeneration improvement in the HMB45 and Melan-A staining results were observed.

No correlation was found between the quantity of melanophages and extracellular melanin when assessing CD68 and MF stain results. Chronic inflammatory cell infiltrate and melanophages persisted in all participants from initial to final biopsy. All data showed no statistical significance, a larger population size could be used to further asses any significance in correlation seen.

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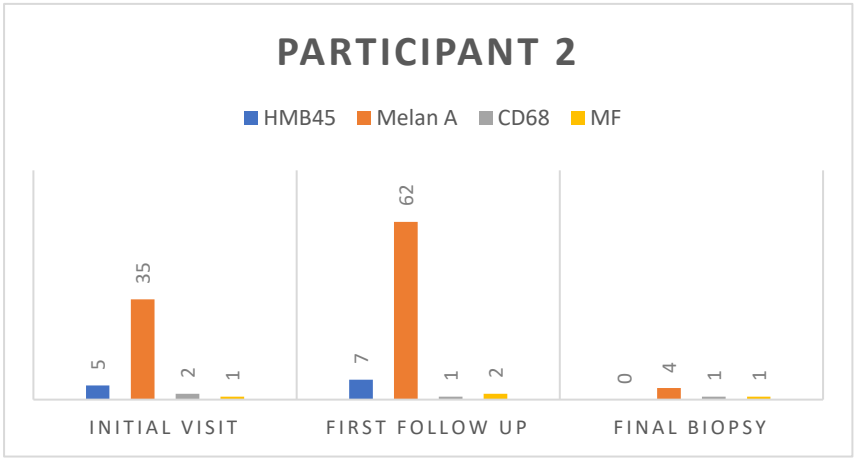
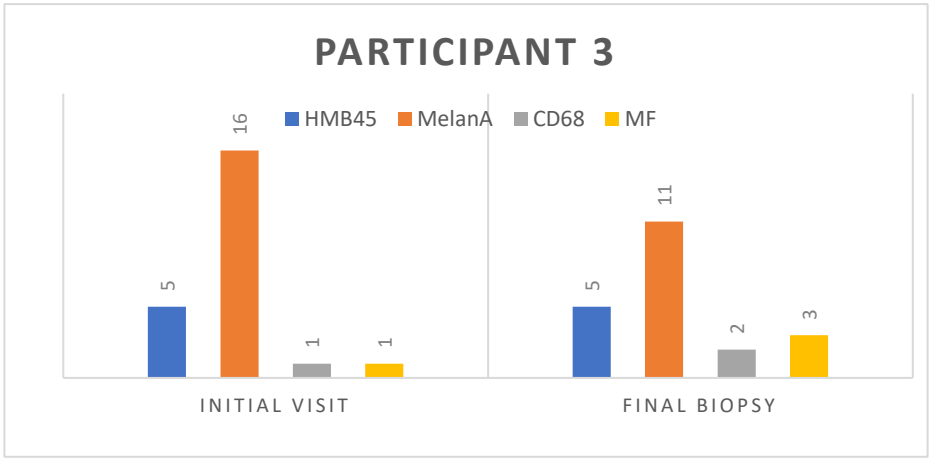
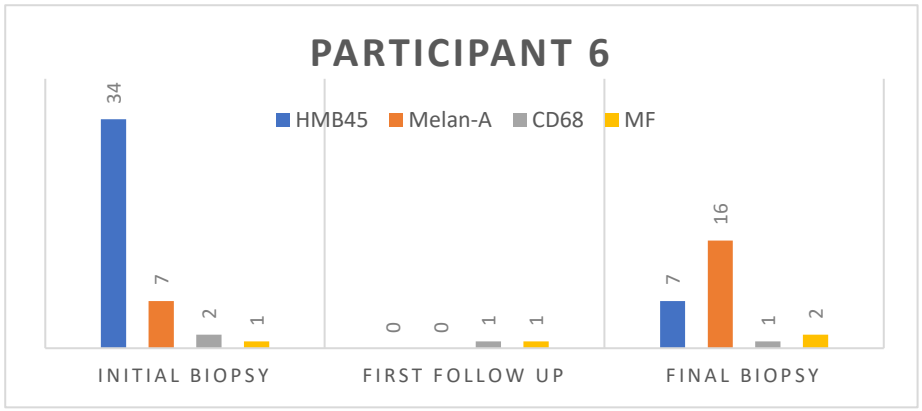
Appendix A:

Bar graph comparison:

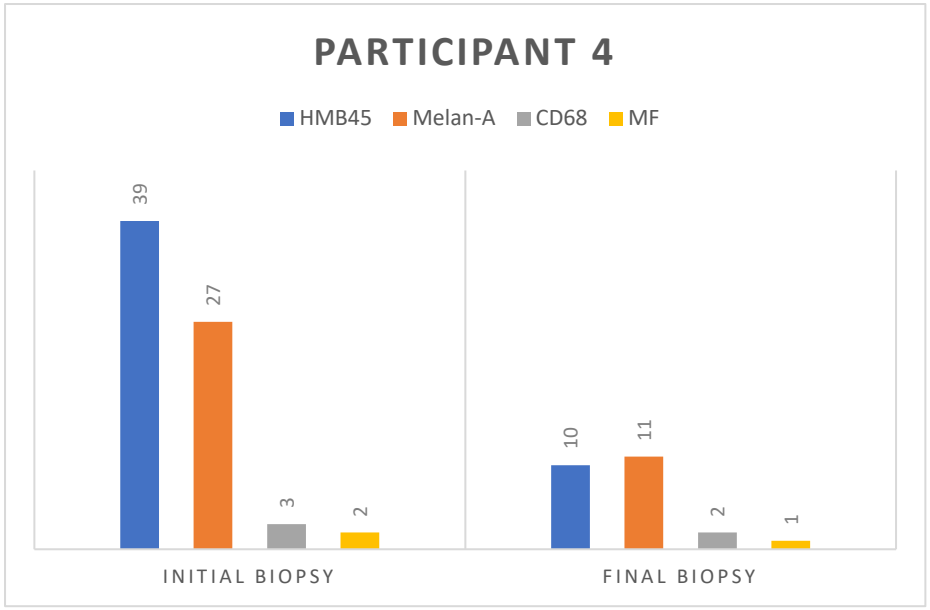
A. Lichen planus

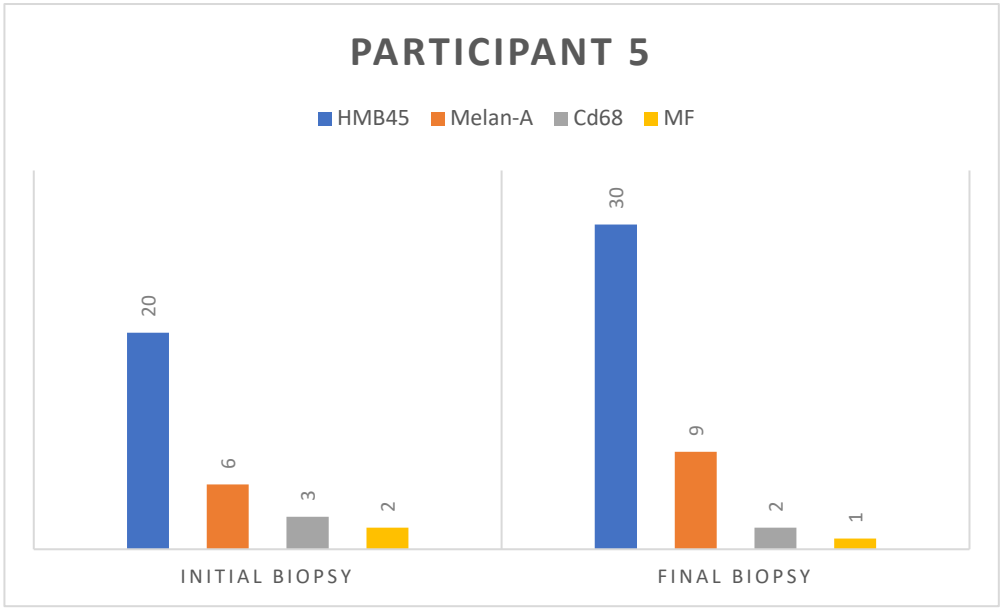




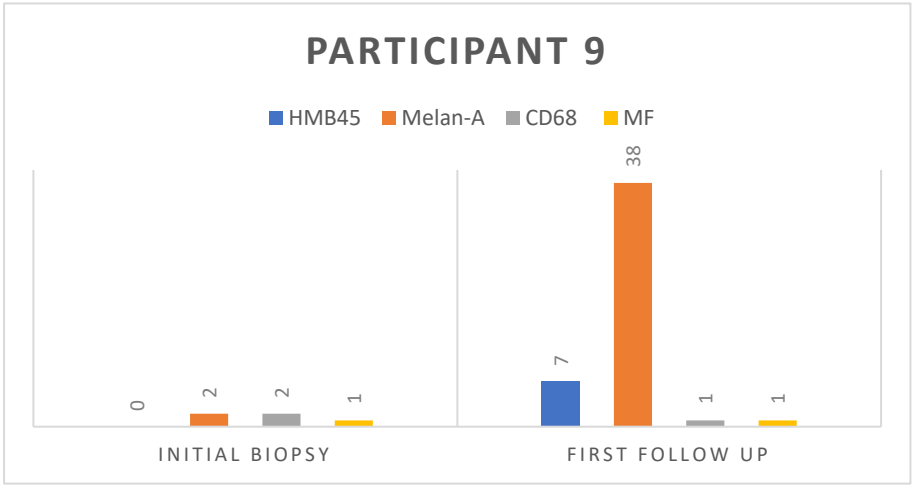


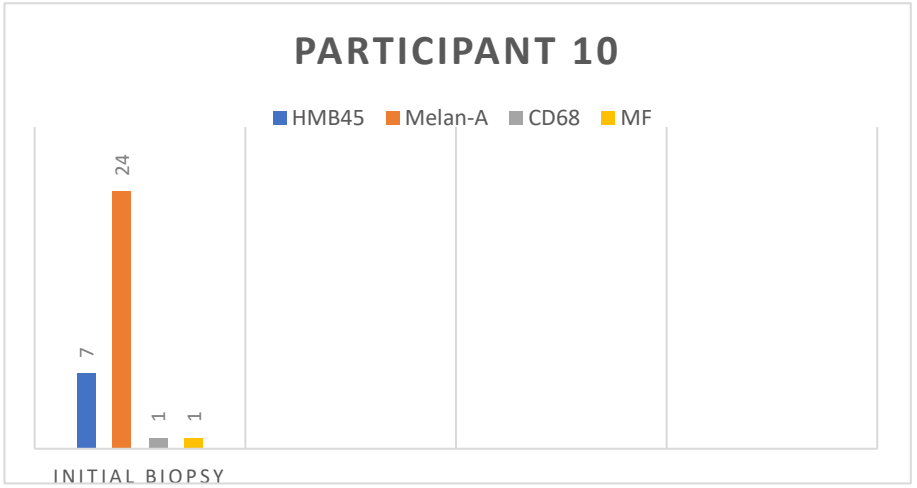
B. Fixed Drug Eruption

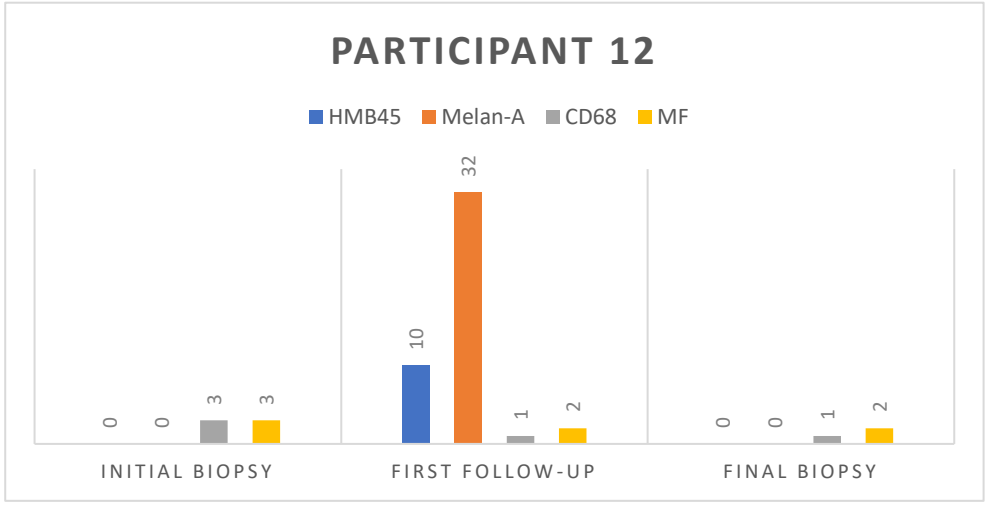




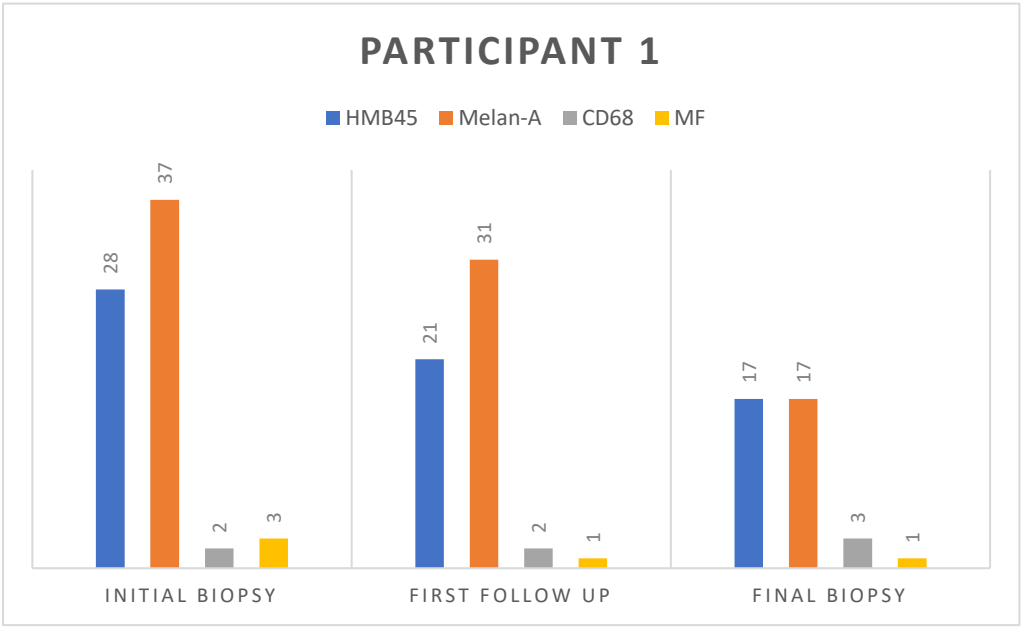
C. Bullous Fixed Drug Eruption

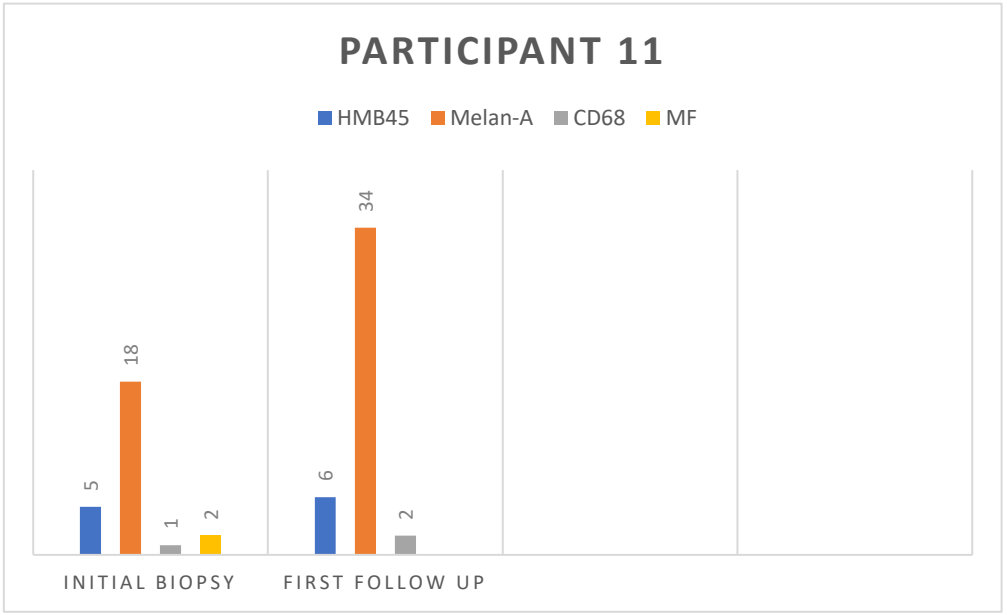




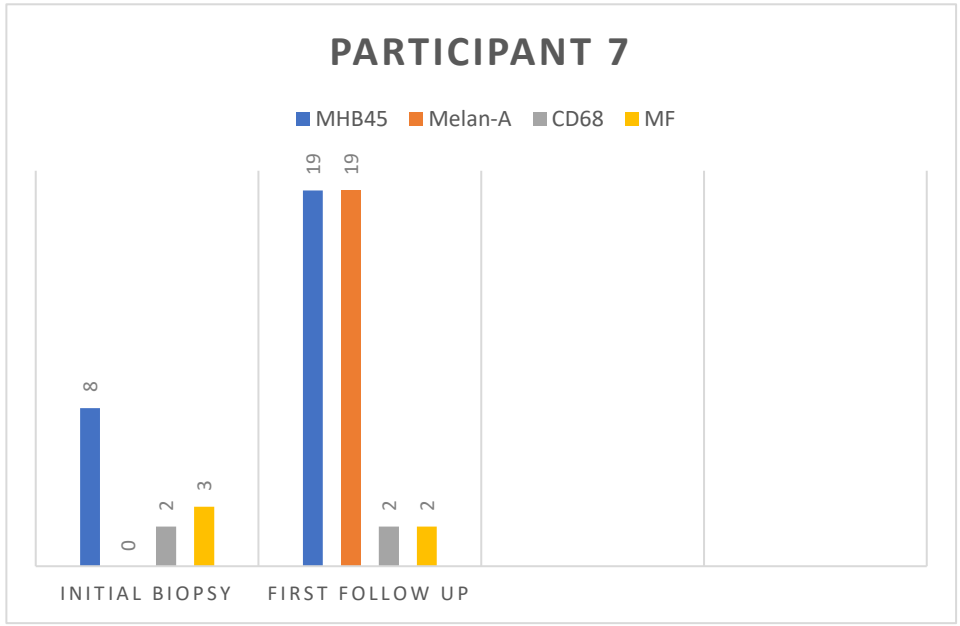


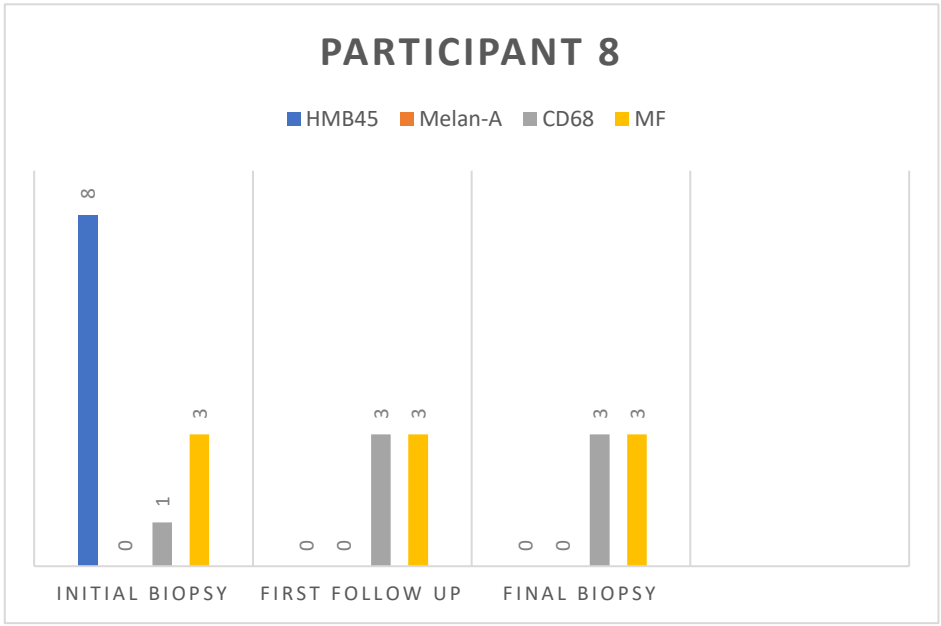
D. Lichenoid drug reaction





E. Discoid lupus erythematosus





Appendix B:

Consent Form: Information sheet

Title:

Longitudinal study of dyspigmentation associated with interface dermatosis using histopathology techniques and MALDI-TOF imaging in Fitzpatrick skin types IV – VI.

Principal Investigator:
Dr. Thuraya Isaacs, Division of Dermatology, Department of Medicine, University of Cape Town.
thuraya.isaacs@uct.ac.za Tel: 021 404 5265

Participant Information Sheet and Consent Form: IM-ADRs Cases

INTRODUCTION

We are approaching you because you have developed a skin condition that falls under a group called interface dermatoses. These conditions are variable in their severity although they may be similar. One of their main problems is that even after healing they often leave dark marks on your skin that take a long time to resolve, if at all. We are collecting information and skin samples from people like you who have one of these conditions. Please read through this document carefully, or listen to whoever is reading this information sheet to you, and talk about it to your family if you want. Please ask if there is anything that you don't understand or if there is something that you would like to know.

WHY ARE WE COLLECTING YOUR INFORMATION AND SAMPLES?

We are collecting information and skin samples because we want to learn more about how people like yourself with lichenoid dermatoses develop dark marks and how we can prevent others in the future from getting these marks. This may not benefit you directly but we hope it will benefit others in the future with the same disease as well as others diseases that leave marks on the skin. We are asking you for permission to collect your clinical information, photographs of the affected skin and skin biopsies to analyse in the laboratory.

WHAT INFORMATION WILL BE COLLECTED?

We will collect routine clinical information about your background, medical and medication history such as other conditions you may have. We will also collect results of your laboratory tests, how your skin is being treated. This information will be collected from your paper based and electronic hospital records.

WHAT SAMPLES WILL BE COLLECTED?

We will collect two small pieces of skin from the affected and unaffected skin at different points. These will be when the lesions are new, when they are healing and when they have completely resolved. We will also like take photographs of your lesions at each time point. Photographs will be stored in our password protected database and access will be restricted to the clinical team. For confirmation of your diagnosis a small group of experts will use these photographs. When being used, they will be de-identified and only your clinical information will be known. We would like to follow-up your response for six months

PROCEDURES AND ASSOCIATED RISKS OF SKIN BIOPSIES

If your doctors decide that it is indicated to perform a skin biopsy to aid in the diagnosis of your reaction, we will ask for collection of additional one 3mm size punch biopsy and one 2mm size punch biopsy. The biopsies will all be performed by experienced doctors, trained in how to perform these procedures with minimal discomfort. The procedures will be explained to you fully by the treating doctor, and you will be asked to sign the hospital consent form for the procedure. The form will list all the risks associated with the procedure. The rare complications associated with these procedures include reaction to the local anaesthetic, bleeding and wound infection.

DO I HAVE TO TAKE PART?

It is your choice whether you would like your information and specimens to be collected and stored or not. If you do not want to, you do not have to give a reason and you can change your mind at any time. If you decide that you don't want your information and samples to be stored now or later, you will continue to receive the same care.

PARTICIPATION REQUIREMENTS

To be included in the study, you will be required to travel to the Dermatology Clinic at Groote Schuur Hospital twice after the initial biopsies. The follow up biopsies will be done 6 weeks after initial biopsy and 6 months after the initial biopsy.

WHERE WILL MY INFORMATION AND SAMPLES BE STORED?

Your samples will be stored at the Hair and Skin Research Laboratory at the University of Cape Town until processed. Your identity will be removed from all samples; stored samples will be labelled with a database number. All information collected will be stored securely in a UCT-based database with password protection. Research study results generated from your samples will be published in scientific journals. These will not include any information which identifies you personally.

All viable samples and sample information will be kept at UCT in a biobank for future scientific advancements and innovation.

WILL I BE PAID TO TAKE PART?

You will not be paid for participation in the study or for specimen collection. However, we will cover the cost of your transport each time you return to the clinic for study-related follow-up. You will be paid R150 for each visit to cover your travel costs.

WHAT IF SOMETHING GOES WRONG?

The University of Cape Town (UCT) undertakes that in the event of you suffering any significant deterioration in health or well-being, or bodily injury because you are taking part in the study, that is caused by your participation in the study, it will provide immediate medical care. UCT has appropriate insurance cover to provide prompt payment of compensation for any trial-related injury according to the guidelines outlined by the Association of the British Pharmaceutical Industry, ABPI 1991. Broadly-speaking, the ABPI guidelines recommend that the insured company (UCT), without legal commitment, should compensate you without you having to prove that UCT is at fault. An injury is considered trial-related if, and to the extent that, it is caused by study activities. You must notify the study doctor immediately of any side effects and/or injuries during the trial, whether they are research-related or other related complications.

UCT reserves the right not to provide compensation if, and to the extent that, your injury came about because you chose not to follow the instructions that you were given while you were taking part in the study. Your right in law to claim compensation for injury where you prove negligence is not affected. Copies of these guidelines are available on request.

If you are harmed and the insurer pays for the necessary medical costs, usually you will be asked to accept that insurance payment as full settlement of the claim for medical costs. However, accepting this offer of insurance cover does not mean you give up your right to make a separate claim for other losses based on negligence, in a South African court. It is important to follow the study doctor's instructions and to report straightaway if you have a side effect from the study medicine.

WHAT DO I DO IF I HAVE ANY FURTHER QUESTIONS?

You are encouraged to ask the investigators should you have any questions or concerns; you can call Dr. Thuraya Isaacs on 021 404 5265.

UNIVERSITY OF CAPE TOWN HUMAN RESEARCH ETHICS COMMITTEE

If you have any queries related to the ethical conduct and approval of this study or would like to report any concerns about study conduct, you can directly contact the Chairperson of the Human Research Ethics Committee at the University of Cape Town Professor Blockman on 021 406 6492.

Appendix C

INFORMED CONSENT FORM

I.....give the investigator permission to:

	Photographs
	Collect one 3mm skin biopsy of skin
	Collect one 2mm skin biopsy of skin
	Collect and store my clinical information for this study and future studies

I am aware of all the procedures and associated risks. I understand that I may withdraw at any time without giving a reason and I will continue to receive the same care.

The information sheet and this form have been explained to me. The investigator has answered all my questions. I fully understand what will happen with my information and samples and I consent.

Participant Signature: **Date:**

Witness Signature: **Date:**

Statement of Investigators Responsibility

I have explained the nature, purpose, procedures, benefits, risks and alternatives to this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Researcher's Signature: **Date:**

Appendix D

Case report form

Title:

Longitudinal study of dyspigmentation associated with interface dermatosis using histopathology techniques and MALDI-TOF imaging in Fitzpatrick skin types IV – VI.

Principal Investigator:
Dr. Thuraya Isaacs, Division of Dermatology, Department of Medicine, University of Cape Town.
thuraya.isaacs@uct.ac.za Tel: 021 404 5265

Baseline Data

Section	Item type	Response Type	Response Options
Identification	Participant Number		1 to 12
	Gender		Anonymous , Female, Male
	Hospital number		
	Fitzpatrick skin type		IV, V, VI
Baseline	Did the patient give informed consent?		
	Date of consent		
	Date of visit		
	Presenting History (signs and symptoms)		
HIV	HIV status		[0] refused to take test, [1] positive, [2] negative
	Current ART regimen start date?		Date, Not stated
	Treatment		ABC, AZT, D4T, TDF, other, not stated
TB	TB status		[1] positive, [2] negative
	Start of current TB treatment		Date, Not stated
	Treatment		Rifampin, PZA, Ethambutol, Levofloxacin, other

Co-morbidities			
Drugs	Drug 1		
	Drug 2		
	Drug 3		
	Drug 4		
ID	Provisional phenotype of ID		FDE, BFDE, DLE, LP
	Body surface area percentage documented		Percentage, Not stated, Description
	Localization		
	Date of onset		
	Treatment		
	Signs and symptoms after hospital administration		

Laboratory Confirmation

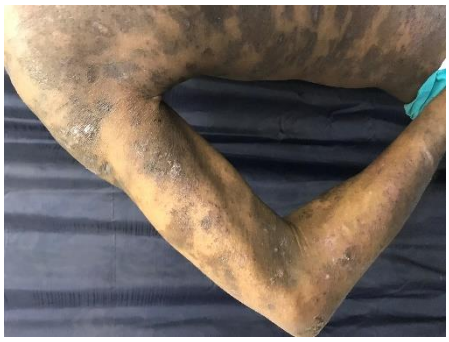
Section	Item type	Response Type	Response Options
	Date biopsies taken		
	Are the biopsies adequate for histology processing?		Yes or no
	Lab results date		
	Is the patient's clinical and laboratory phenotype consistent with ID		none selected, yes, no

Study Investigations and visits

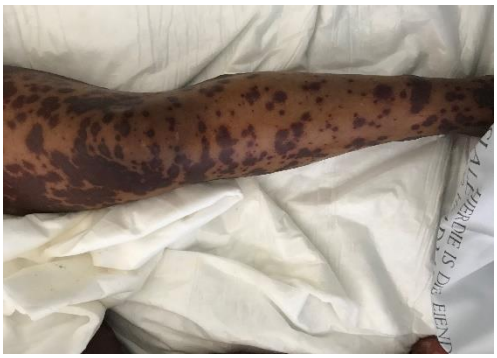
Section	Item type	Response Type	Response Options
Visits	Visit		1,2,3, other (specify)
	Date of Visit		
	Biopsies taken		Yes or no
	Anatomical Location of biopsy		
	Photographs		Yes or no
Results	EM		
	MALDI		
	H and E		
	Special stain (Mel Bleach)		
	Immunohistochemistry		
	Scans of original reports		Yes or no
	Which skin problems are still present?		hypopigmentation, hyperpigmentation, dyspigmentation, depigmentation, milia, xerosis, chronic eczema, other (specify)

Appendix E:

Photographs of some of the Participants (Consent was given).



Participant 7 initial visit



Participant 9 initial visit