MONASH University

Lymphatic Patterns in Primary Cutaneous Squamous Cell Carcinomas of the Head and Neck

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the degree of Master of Surgery

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Contents

List of Tables

List of Figures

Abstract

Introduction

In primary cutaneous squamous cell carcinoma (cSCC), the risk of metastases increases in the presence of clinical immunosuppression and also depends upon the histological grading of the cSCC (i.e., whether a cSCC is graded as well, moderately or poorly differentiated). Less well-differentiated cSCCs and more infiltrative growth patterns have been associated with an increased risk of recurrence and metastases. A well-differentiated cSCC is usually considered as low risk for local, regional and distant metastases. However, a recent study found that 12 of 78 patients (i.e., 15 per cent) with well-differentiated primary cSCCs of the head and neck developed metastatic cSCCs. In addition, perineural and lymphovascular invasion was an absolute risk factor for the progression of metastatic disease. Thus, the hypotheses that tumour cells invade the lymphatic system by passive mechanisms (e.g., as advancing tumour fronts that erode the walls of any vessels in their path and via metastases that occur by passive drainage) may not be correct. Indeed, recent evidence suggests that a complex interaction occurs between tumour cells and the lymphatic endothelium. This study aimed to identify the changes in lymphatic behaviour within the intra and peritumoral region of different cSCCs grading. The difference in lymphatic numbers, size and density are compared. This was done by performing a retrospective microscopic examination of head and neck cSCCs comparing lymphatic vessels between the various differentiation groups.

Methodology

Paraffin blocks of cutaneous squamous cell carcinoma specimens of head and neck region were recalled from the Peninsula Health tissue storage facility. The tissues were then deparaffinised, prepared and stained with podoplanin (D2-40) to identify lymphatic vessels. Each specimen was then examined under the microscope and images were taken using the microscope camera. These images were then reconstructed and image analysis software (i.e., ImageJ™) was used to assess and analyse the lymphatic vessels.

Results

In a total of 74 specimens, 21 well, 34 moderately and 19 poorly differentiated cSCC specimens were examined. Compared to the lymphatic vessel specimens of the other two groups,

specimens in the less well-differentiated cSCC group had intratumoural and peritumoural lymphatic vessels (p-value = 0.0001) that were larger in size, greater in number (p-value = 0.0003) and higher in density (p-value = 0.0002). There was no difference in cancer size (p-value = 0.3918) or depth of invasion (DOI; p-value = 0.5874) across the three groups.

Conclusion

Less well-differentiated cSCCs of the head and neck contained a higher number of lymphatic vessels. These lymphatic vessels were also larger in diameter and higher in density. This may have contributed to a higher incidence of lymphatic metastasis in these groups as reported in literatures. It is hypothesised that there is an interaction between cSCC differentiation and lymphangiogenesis. Thus, including lymphatic patterns as a prognostic indicator may improve the accuracy of cSCC prognoses and treatments.

Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

The design, data collection, computer image analysis data analysis and interpretation of results were performed by myself under the guidance of my supervisors. Assistance and resources for performing immunohistochemistry and specimen review were done in conjuction with Pathology Department, Frankston Hospital and Dorevitch Pathology, Heidelberg.

I consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Abbreviations

AJCC	American Joint Committee on Cancer
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
BCC	Basal cell carcinoma
BEC	Blood endothelia cell
cSCC	Cutaneous squamous cell carcinoma
D2-40	Podoplanin antibodies
DOI	Depth of invasion
DTH	Delayed-type hypersensitivity
EC	Endothelial cell
ECM	Extracellular matrix
FOXC2	Forkhead box transcription factor
H&E	Hematoxylin and eosin
HA	Hyaluronan
IHD	Ischaemic heart disease
LCM	Laser capture microdissection
LEC	Lymphatic endothelial cell
LVD	Lymphatic vessel density
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor 1

PDG	Platelet-derived growth factor
PROX1	Prospero related homeobox-1
RT	Reverse transcription
SCC	Squamous cell carcinoma
SLN	Sentinel lymph node
SYK	Spleen tyrosine kinase
TME	Tumour microenvironment
TNM	Tumour, nodes and metastases
UICC	International Union Against Cancer
UV	Ultraviolet
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
ZO-1	Zonula Occludens-1

Chapter 1: Literature Review

1.1 Introduction

This chapter considers the potential value of developing a new parameter for determining the poor prognostic factors associated with cutaneous squamous cell carcinoma (cSCC). It identifies and outlines the current classification system, the lymphatic endothelial markers, the lymphatic networks of normal tissues and the lymphatic changes that occur in the presence of tumour tissues. It also considers the potential effects of lymphatic pattern recognition in cSCC. Additionally, it discusses the histopathology of primary cSCC and the current classification system. Specifically, it considers how current classifications are being performed and interpreted and how this affects patients' prognoses and thus their management.

This chapter begins by introducing the current concept of the lymphatic vascular system in relation to normal tissue. It then discusses the changes that occur via lymphangiogenesis that are secondary to growth factor secretion by tumour cells. Following this, the biological cascades between lymphatic endothelial receptors are considered in relation to the growth, character and survival of the lymphatic vessels. Next, the use of lymphatic endothelial antibodies to visualise and identify lymphatic vessels under microscopic examinations is discussed. Finally, the potential implications related to measuring lymphatic vessels in primary cSCCs to determine prognoses and manage these patients are considered.

cSCC is one of the most common forms of skin cancer in Caucasians, second only to basal cell carcinoma (BCC) [1]. It is believed that all cSCC lesions begin following a repeated and uncontrolled division of cancer stem cells of epithelial lineage. An accumulation of such cancer cells causes a microscopic focus of abnormal cells that are, at least initially, locally confined within the specific tissue in which the primary tumour cell resides. This condition is called squamous cell carcinoma (SCC) *in situ* and is diagnosed as such when the tumour has not yet penetrated the basement membrane or another delimiting structure to invade the adjacent tissues. Once a lesion grows and progresses to the point that it has breached, penetrated and infiltrated adjacent structures, it is referred to as 'invasive' cSCC. An invasive carcinoma can potentially spread to other organs. This process is referred to as 'metastasis' and can result in a secondary tumour forming some distance from the primary site. Once metastasis has occurred, its process is systemic. Indeed, it is difficult to achieve good outcomes for patients

once the process has begun. Aggressive treatments, including surgical excision, chemotherapy, radiotherapy, immunotherapy or a combination of all four [2], are often required. However, once a tumour has advanced to this stage, the outcome can be fatal for patients [2–5].

1.1.1 Incidence

cSCC epidemiology often combines the data of BCC and other non-melanocytic skin tumours. Such studies have yielded widely variable results. In Australia, the Department of Health reported that, as of 2008, the lifetime risk for SCC was 9 to 14 per cent among men and 4 to 9 per cent among women. It has been estimated that there are 138,000 new cases of cSCC per year [4]. This number accounts for about 30% of non-melanoma skin cancer [Cancer Council Australia]. However, it should be noted that in Australia, non-melanoma skin cancer (unlike other types of cancer, such as melanomas) is not reportable to cancer registries. Thus, the true incidence of cSCC is difficult to capture accurately. It is more likely that the true incidence of these conditions is much higher than what is stated.

Over the past 20 years, the incidence of cSCC has increased in the United States and other countries [2, 3, 5–7]. ALam et al reported the incidence of 100 to 150 per 100,000 persons per year, and among persons over the age of 75 years is approximately 10 times that rate [8]. This increase is secondary to lifestyle changes during the past 50 years including voluntary exposure of higher levels of ultraviolet (UVB) resulting in p53 mutation [8] (e.g., from sunlight and the use of solarium tanning beds), an ageing population and smoking [2, 4]. It is also likely to be secondary to increases in community awareness and diagnosis of the condition. Immunosuppression both secondary to diseases process (HIV, chronic burn wound) or treatment (post organ transplant medication or chronic steroid use) are also well known risk factors for the development of cSCC. These factors contributed to an increase incidence of cSCC in the developed world especially in Caucasians population [8]. In contrast, cSCC in the under develop countries are more likely to be associated with carcinogens such as Arsenic and polycyclic aromatic hydrocarbons exposure both are product of from the combustion and distillation of carbon compounds such as coal tar, cutting oils, and pitch. Other factors include lupus vulgaris infection and inheritance syndromes (eg. Xeroderma pigmentosum and albinism) have been reported in the sub-continence [9]. Joseph et al reported a metastatic rate of cSCC amongst Australian population as high as 4.9% with an overall mortality rate of 70.6% [10]. In 2015, Cancer Council Australia reported that 642 people died from non-melanoma skin cancer and a majority of those from cSCC. cSCC contributes substantially to morbidity and

mortality among the elderly; however, its true incidence and its associated mortality rate cannot be determined due to multiple confounding factors. Fransen et al. reported a significant increase in the total number of both surgical and non-surgical treatment for non-melanoma skin cancers (i.e., an increase from 412,493 in 1997 to 938,991 in 2015) [11]. These data were based on the Medicare Benefit Schedule claims. The number of such services has more than doubled over the last two decades. Thus, it is not surprising that there has also been a significant increase in health sector costs. In 2015, the total cost for treating non-melanoma skin cancer was estimated to be A$703 million [11].

1.1.2 Histopathology

cSCC refers to a group of non-melanoma skin cancers that are characterised by nests of squamous epithelial cells that arise in the epidermis and extend for variable distances into the dermis. The cells have abundant eosinophilic cytoplasm that are large and often with vesicular nuclei. Central keratinisations and horn pearl formations vary, depending on the differentiation of the tumour. Several studies have identified the risk factors associated with local recurrences and metastases [1,2,5,6,12]. Local recurrences have been associated with infiltrative features, perineural invasion, inadequate excision margins and desmoplastic lesions. [2]. Metastatic risk factors include tumour size, depth of invasion (DOI), an infiltrative growth pattern, mitosis, a spindle cell pattern, acantholysis (i.e., a loss of cellular adhesion) and poor differentiation [2].

Descriptions of the degree of differentiation are classified into three categories: well, moderately and poorly differentiated. A well differentiated cSCC has a histological appearance of malignant cells (as described above), but maintains a normal architecture that is similar to that of a normal epidermis. A poorly differentiated cSCC has a distorted architecture that is unrecognisable without the presence of keratin markers and in which, the products of differentiation (e.g., keratin, desmosomal attachments and glandular differentiation) are poorly expressed. Descriptions of moderately differentiated cSCCs are controversial and unclear; this type of cSCC is often described subjectively and may vary among pathologists. A full review of the histological features of cSCC is provided further below (see Section 1.3).

1.1.3 Metastasis

An invasive cSCC could potentially recur and metastasise. In the literature, the local 5 year recurrence rate of a primary cSCC ranges from 5 to 8 per cent while the 5 year rate of lymph node metastasis (regional or distant) ranges from 0.5 to 5 per cent [8]. The incidence of lymph node metastasis from cSCC may be considerably higher in certain situations (e.g., if the cSCC is associated with immune-suppression, if radiotherapy has been used previously and if the cSCC arises in chronically inflamed or irritated lesions, such as Marjolin ulcers.) Tumour thickness is another factor which contributed to increase incidence of metastasis. Brantsch et al reported Metastases occurred in 12 (4%) of 318 tumours between 2·1 mm and 6·0 mm in thickness, and in 14 (16%) of 90 tumours with a thickness greater than 6·0 mm [13]. Further, several studies have shown an increase in the correlation between the degree of differentiation and the metastasis rate in cSCC. Poorly differentiated neoplasms have twice the local recurrence rate and three times the metastatic rate than those of well-differentiated neoplasms [14]. Rowe et al. identified a significant relationship between mortality rates and tumour grades. Specifically, they found the five-year curative rate after treatment was 61.5 per cent for poorly differentiated cSCCs and 94.6 per cent for patients with well-differentiated cSCCs [14]. Individual risk factors for metastatic spread are well recognised (as evidenced by the guidelines of the National Comprehensive Cancer Network, the Royal College of Pathologists and those provided in Motley's study) [2, 8, 15, 16]. Such factors include DOI, tumour diameter, perineural invasion, lymphovascular invasion and the degree of differentiation.

Most patients with primary cSCC have an excellent prognosis. However, the presence of metastatic disease has been shown to portend extremely poor outcomes for patients. In many such cases, a palliative approach is considered. In United States, a systemic review by Karia PS et al reported the average assumption estimates indicate that 186,157 persons were given a diagnosis of cSCC, 5604 developed nodal metastases (3.0%), and 3932 (2.1%) died from cSCC [17]. The prognosis for these advance stage diseases may be improved with the use of an innovative treat immunomodilator medication such as human anti-programmed death (PD-1) monocolonal antibody (Cetuximab and Panitumumab) or checkpoint inhibitors (nivolumab) which has shown promising result in stage 4 melanoma skin cancer. Its use in cSCCs is still in clinical trial, thus the long term outcome is yet to be seen. However, Furerst et al, has reported a 69% overall survival with use of panitumumab at 7 months [19]. A systematic metastasis has been shown to involve regional lymph nodes in approximately 85 per cent of cases and in

approximately 15 per cent of cases involve secondary deposits at distant sites, including the lungs, the liver, the brain, cutaneous deposits and bones [2,13]. Thus, there appears to be a connection between solid organ metastasis and the lymphatic system. Peat et al. conducted a study to develop a system of risk stratification and identified the clinical and histological factors related to metastasis from cSCC in the head and neck region. They found that 15 per cent of patients in the known metastatic cohort originated from what was once thought to be a good prognostic group (i.e., the well-differentiated cSCC group). Consistent with previous research, they also found that 54 per cent of patients in their study originated from the poorly differentiated cohort [12].

1.2 Cutaneous Squamous Cell Carcinoma Classification

A histological grading classification is an important prognostic tool, as it is used to predict the clinical and biological behaviours of cancer. This grading forms part of a prognostic indicator, which in turn determines patients' treatment options. An ability to identify the high-risk prognostic features of cSCC helps to stratify patients and to assess tumour-specific risks. Several proposed methods have been reported in the literature for categorising cSCC. Classification systems include Broder's classification system, the American Joint Committee on Cancer's (AJCC) staging of cutaneous non-melanoma skin cancer and histological grading by differentiation.

1.2.1 Broder's Classification System

In the earlier years of histological examination, cSCC was classified according to Broder's classification [20]. In 1927, Broder suggested that a system based on histological subtype be used to grade various tumours, including cSCCs. He used various staining mediums to examine histological specimens and quantify the amount of differentiation within tumour cells. Under Broder's system, a lower portion of well-differentiated features within a tumour indicates a higher grade. A Grade 1 classification is given to a tumour in which 75 per cent or more of the lesion is well differentiated, a Grade 2 classification is given to a tumour in which 50 per cent to 75 per cent of the lesion is well differentiated, a Grade 3 classification is given to a tumour in which 25 per cent to 50 per cent of the lesion is well differentiated and a Grade 4 classification is given to a tumour in which less than 25 per cent of the lesion is well differentiated (see Table 1). Broder described the features of a well-differentiated cSCC as having its cell architecture resemble that of normal tissue, but with a large amount of keratin. Broder contended that a well differentiation cSCC is less likely to metastasise than that of a higher grade of differentiation (ie. poorly differentiated). Table 2 shows the different features of low-grade and high-grade cSCCs [20,21].

Table 1. 1: Broder's Classification System

Grade 1	75 per cent or more of the lesion is well differentiated
Grade 2	50 per cent to 75 per cent of the lesion is well differentiated
Grade 3	25 per cent to 50 per cent of the lesion is well differentiated
Grade 4	Less than 25 per cent of the lesion is well differentiated

Table 1. 2: Histological Differences between Low-Grade and High-Grade Cutaneous Squamous Cell Carcinomas

Low-Grade cSCC	Well to moderately differentiated: intercellular bridges and keratin pearls Tumour cells arranged in solid or sheet-like patterns. Associations with solar damage and precursor actinic keratosis Diameter less than 2 cm Depth less than 2 mm
High-Grade cSCC	Poorly differentiated: clear-cells, sarcomatoid or single cell features The presence of infiltrating individual tumour cells Arising *de novo* or in site of prior injury (ulcer, burn scar or osteomyelitis) Perineural and/or perivascular invasion Diameter greater than 2 cm Depth greater than 2 mm

Broder's classification system was used for many years in cSCC. Several classifications were then developed that modified Broder's classification system and applied to oropharynx SCC, including systems by Jakobbson et al., Crissman et al., Lund et al. and Anneroth et al. [21-24]. In addition to including the morphologic parameters, these systems also require the analysis of 'structure', the 'tendency to keratinization', 'nuclear aberrations' and the 'number of mitoses'. Such systems were developed following an increased understanding of tumour cell proliferation and cell receptors, as well as advancements in immunohistochemistry. These systems also evaluated tumour-specific interaction parameters with the host, including the 'regularity of the tumour border', the 'stage of invasion', 'vascular invasion' and the 'degree of lymphoplasmocytic infiltration' (see Table 3). Broder's and subsequent classification systems have been found to have a low correlation between the degree of differentiation and the prognosis. This is largely because cSCCs usually exhibit heterogeneous cell populations that differ in the degree of their differentiation [24]. Thus, a multifactorial malignancy grading system was developed to obtain more precise morphological evaluations of the growth potential, malignancy risks and prognoses of cSCCs [25].

Table 1. 3: Jakobbson et al. (1973) Histologic Grading System

Histologic grading of malignancy (based on tumour cell population)				
Tumour cell population	**1**	**2**	**3**	**4**
Structure	Papillary and solid	Strands	Small cords and groups of cells	Marked cellular dissociation
Differentiation	Highly (keratinisation)	Moderately (some keratinisation)	Poorly (minimum keratinisation)	Poorly (no keratinisation)
Nuclear polymorphism	Few enlarged nuclei	Moderate number of enlarged nuclei	Numerous	Anaplastic immature enlarged nuclei
Mitoses	Single	Moderate number	Great number	Numerous
Histologic grading of malignancy (based on tumour-host relationship)				
	1	**2**	**3**	**4**
Mode of invasion	Well-defined borderline	Cords, less marked borderline	Groups of cells, no distinct borderline	Diffuse growth
Stage of invasion	Possibly	Microcarcinoma (few cords)	Nodular, into connective tissue	Massive
Vascular invasion	None	Possibly	Few	Numerous
Cellular response (plasmalymphocytic infiltration)	Marked	Moderate	Slight	None

1.2.2 The Seventh Edition American Joint Committee on Cancer Staging System for Cutaneous Non-Melanoma Skin Cancer

The American Joint Committee on Cancer (AJCC) and the International Union Against Cancer's (UICC) tumour, nodes and metastases (TNM) staging system for SCC of the skin has also been developed. Under the AJCC/UICC staging system, the designation 'T' refers to a primary tumour that has not been previously treated, the symbol 'P' refers to a pathological classification of the TNM (as opposed to a clinical classification and is based on gross and microscopic examination of tumour tissue). pT entails a resection of the primary tumour or a biopsy that is adequate to evaluate the highest pT category. pN entails an adequate removal of nodes to validate lymph node metastasis. pM refers to a microscopic examination of distant lesions. The clinical classification of TMM is usually carried out by a referring physician before treatment and during an initial evaluation of a patient or when pathologic classification is not possible. Pathologic staging is usually performed after the surgical resection of the primary tumour and depends on a pathologic documentation of the anatomic extent of disease (whether or not the primary tumour has been completely removed). If a biopsied tumour cannot be resected for any reason and if the highest T and node (N) categories or the M1 category of the tumour can be confirmed microscopically, the criteria for pathologic classification and staging can be satisfied without the total removal of the primary cancer [25]. However, it should be noted that the guidelines still contain a number of significant limitations. In particular, several recommendations are largely impractical. Specifically, it is neither economically feasible nor medically justifiable to apply the full clinic-pathologic staging criteria to the verrucous variant of cSCC and the small superficial SCCs that could be cured with primary biopsies.

1.2.3 Histological Differentiation Grading

The most widely accepted method for grading cSCC is histological differentiation grading. This system is used around the world in many pathology departments. It assists clinicians to make decisions about treatment options based on prognosis and clinical behaviours. 'Differentiation' is a term used to describe the appearance of malignant tumours. It refers to the extent to which a tumour resembles its tissue of origin. Well-differentiated tumours closely resemble their tissue of origin. Conversely, poorly differentiated tumours barely resemble their tissue of origin. Descriptions and illustrations of the different histologic grades are given below [26].

Under the haematoxylin and eosin stain, the normal head and neck skin is composed of different layers including the thin keratin, epidermis (stained dark purple), thin layer of dermoepidermal junction and dermis which contain blood vessels.

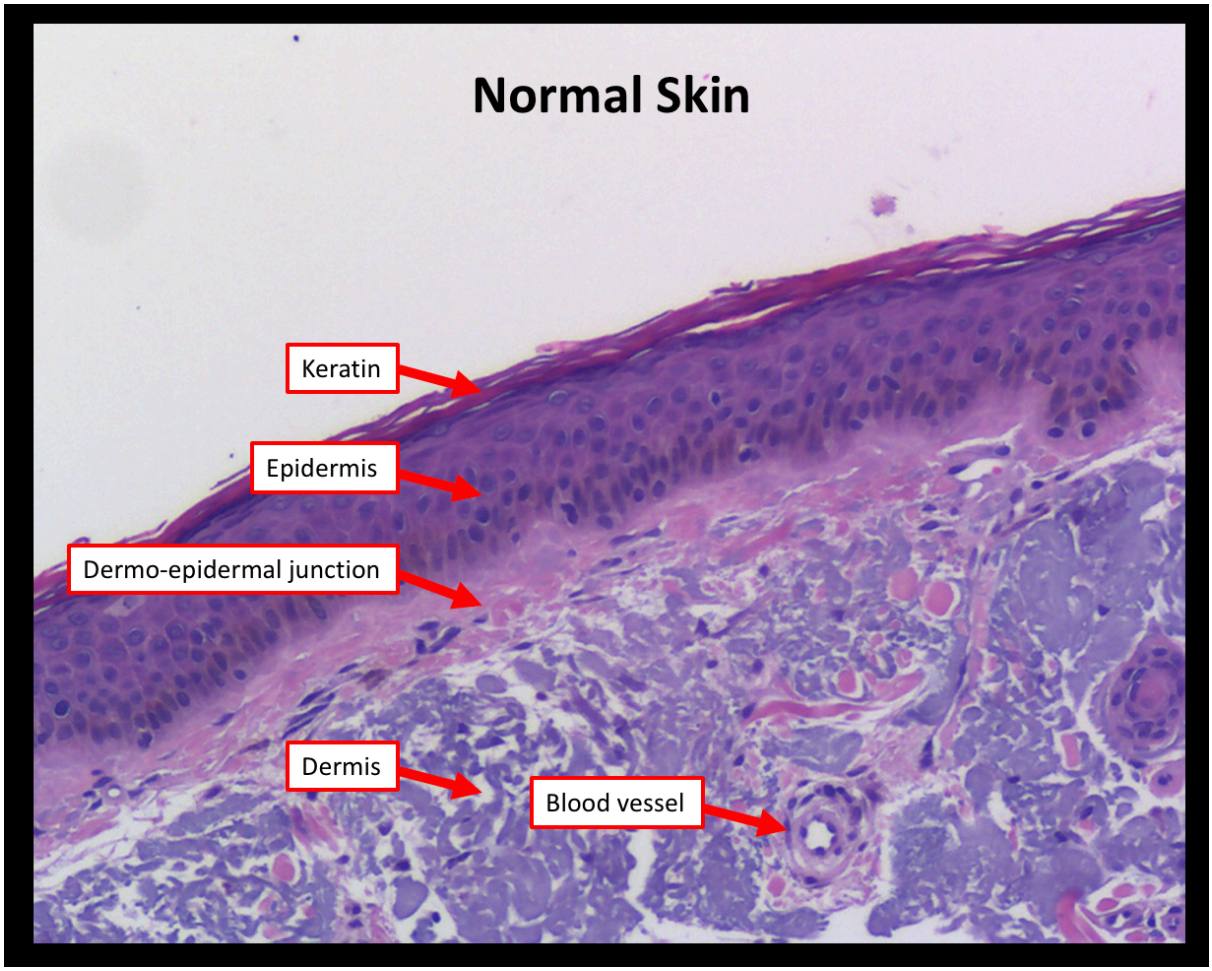

Figure 1. 1: Normal skin.

Grade 1: Well-differentiated tumours are characterised by a squamous epithelium that frequently shows easily recognisable and often abundant keratinisation. Intercellular bridges are readily apparent. Pleomorphism is minimal and mitotic figures are mainly located basally.

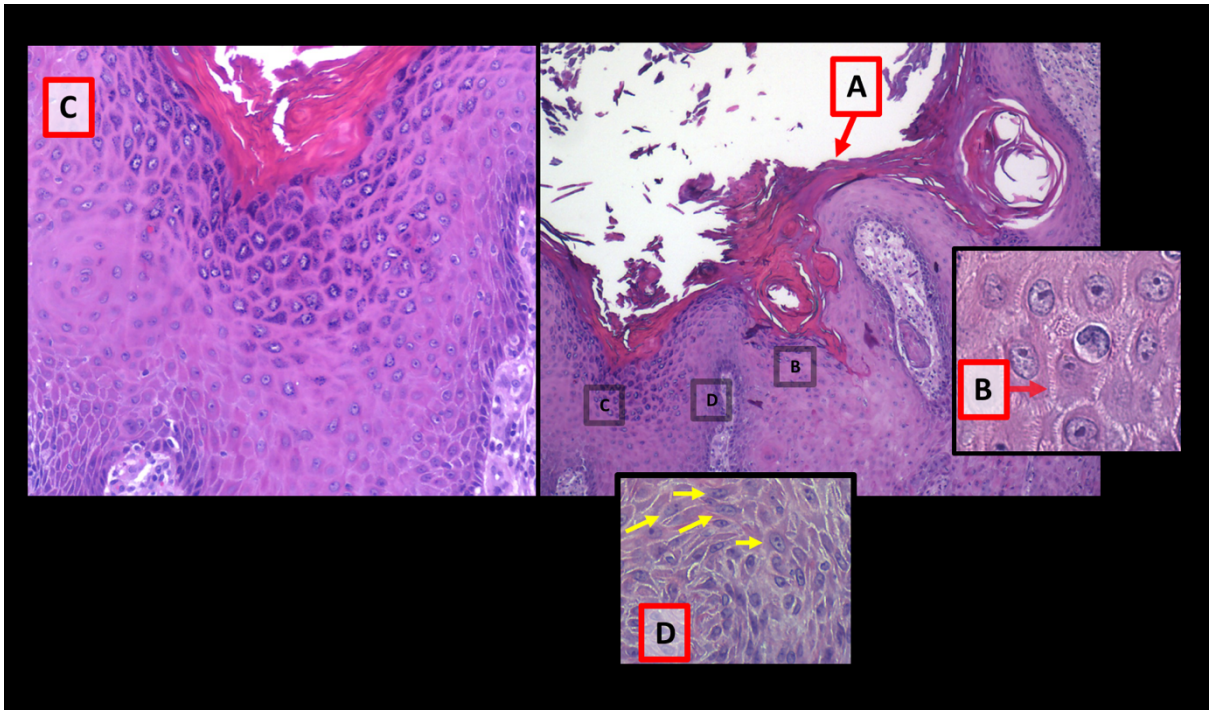

Figure 1. 2: Well-Differentiated Cutaneous Squamous Cell Carcinoma (Haematoxylin and Eosin Stained) – A) Hyperkeratinisation; B) abundant Intercellular bridges; C) minimal Pleomorphism (similar variability in the size, shape and staining of cells and/or their nuclei); D) minimal mitotic rate (cells undergoing mitosis is indicated with yellow arrows).

Grade 2: Moderately differentiated tumours show more structural disorganisation and squamous epithelial derivation is less obvious. Nuclear and cytoplasmic pleomorphisms are more pronounced and mitotic figures may be numerous. Keratin formation is typically limited to keratin pearls, horn cysts and scattered individually keratinised cells.

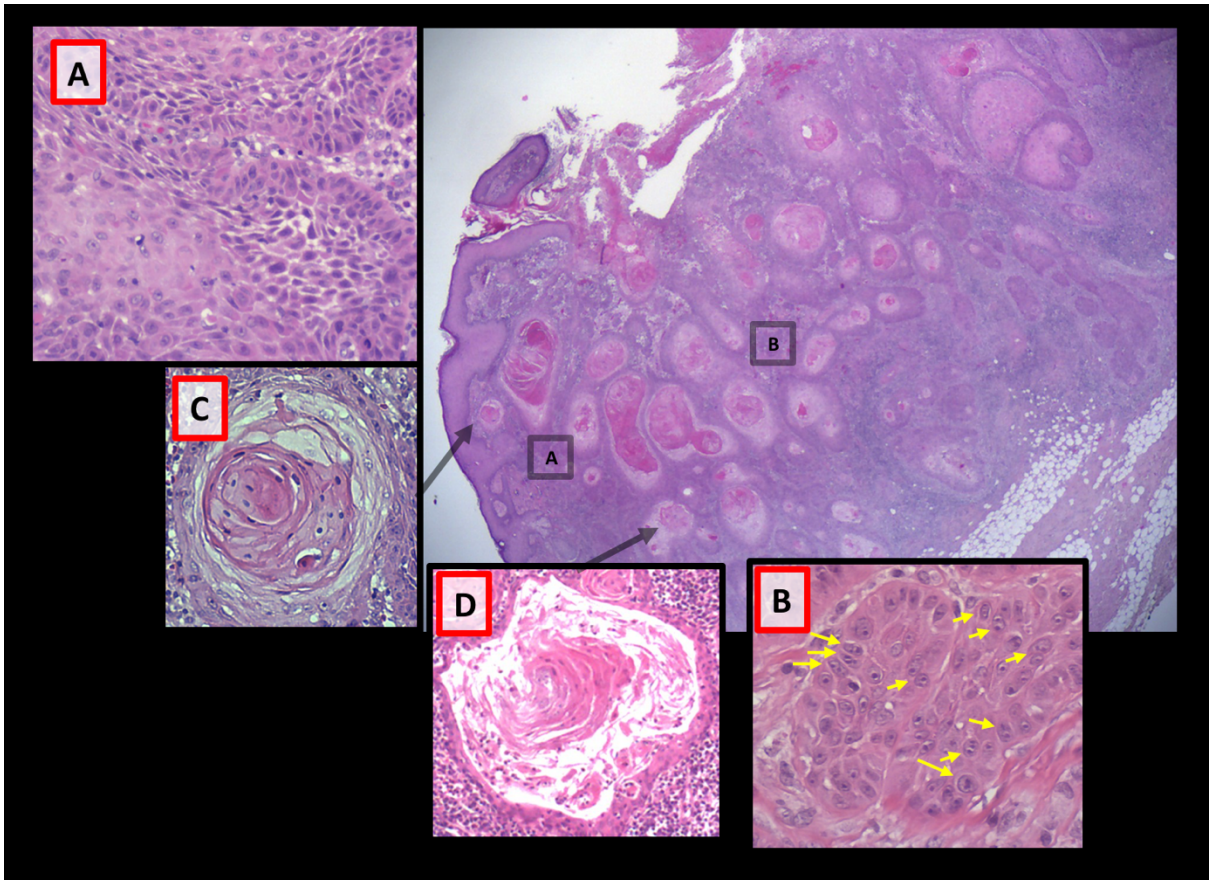

Figure 1. 3: Moderately Differentiated Cutaneous Squamous Cell Carcinoma (Haematoxylin and Eosin Stained) – A) moderate pleomorphisms and B) mitotic rate; Keratin formation with keratin pearls (C) and horn cysts (D).

Grade 3: In poorly differentiated tumours, it may be difficult to establish squamous differentiation; however, it can usually be identified by highly distorted cellular architecture, high mitotic rate and rare intercellular bridges or small foci of keratinisation.

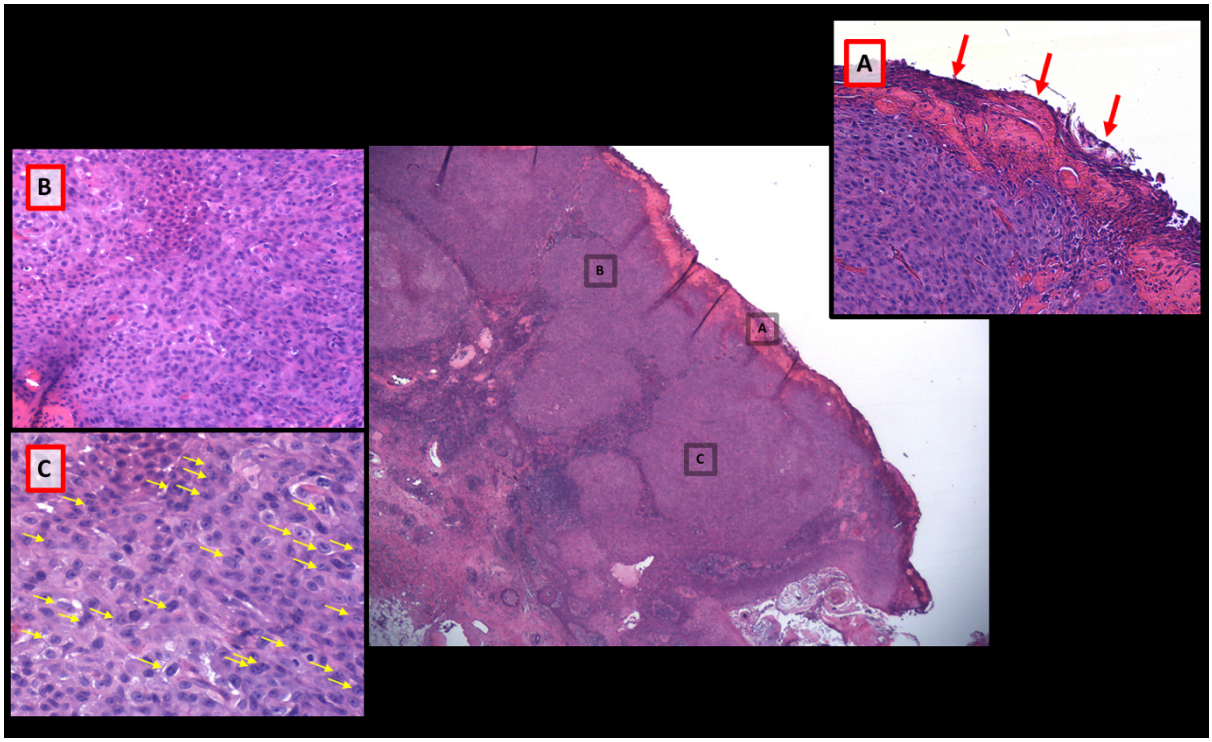

Figure 1. 4: Poorly Differentiated Cutaneous Squamous Cell Carcinoma (Haematoxylin and Eosin Stained) – A) small foci of keratinisation, B) highly distorted cellular architecture, C) high mitotic rate and rare intercellular bridges.

Table 1. 4: Cutaneous Squamous Cell Carcinoma Differentiation Histological Features

	Well Differentiated	**Moderately Differentiated**	**Poorly Differentiated**
Keratin pearls	Present	Present	Reduced number of Keratin pearls
Pleomorphism	Absent	Moderate degree of nuclei atypia with frequent mitosis	High degree of nuclei atypia with frequent mitosis
Intercellular bridges	Present	Present	Present
Degree of architecture distortion of tumour	Low	Moderate	High
Degree of keratinisation	Low	Moderate	High

1.3 The Lymphatic Network

The lymphatic network comprises several components, including the lymphatic vessels that carry lymph, lymphocytes, lymph nodes and lymphatic organs. It has an important function in maintaining health and wellbeing. Lymph is a thin watery fluid that is similar in composition to blood plasma. It is filtered out through the blood capillary wall and forms the interstitial fluid that bathes the tissues. The transport of fluid between the blood capillary wall and the interstitial space is dictated by hydrostatic and colloid osmotic pressure. The hydrostatic pressure in the capillary's forces fluid and its soluble substances through the capillary pores into the interstitial space where the fluid moves down a pressure gradient. Conversely, colloid osmotic pressure, caused by changes in the concentration of plasma proteins, causes fluid to move from the interstitial spaces into the blood. Normally, 2 to 3 litres of lymph are returned each day [27]. The lymph returned to circulation is crucial to maintain normal homeostasis, as substances of high molecular weight (e.g., proteins) cannot be absorbed from the tissues in any other way. The high molecular weight structure can enter the lymphatic capillaries with very little impediment due to the special valve that is created by the attachment of the anchoring filaments to the lymphatic endothelial cell (LEC) wall, which in turn attaches itself to the surrounding connective tissue. At the junctions of adjacent endothelial cells (ECs), the edge of one EC overlaps with the edge of the adjacent cell due to the formation structure. The result is a minute valve that opens to the interior of the lymphatic capillary, as the overlapping edges flap freely inwards. Thus, the interstitial fluid and its suspended particles can push the valve open and flow directly into the lymphatic capillary. The valve itself is one-way, preventing any backflow of fluid due to the closure of the flap [27, 28].

The process of lymph fluid transport between cells and interstitial space is similar to the processes of the blood capillary wall and interstitial space. In the lymphatic system, when fluid enters the terminal lymphatic capillaries, the lymph vessel walls automatically contract for a few seconds to pump the fluid into the blood circulation. This process creates an overall slightly negative pressure that has been measured for fluid in the interstitial spaces. Almost all of the body's tissues have special lymph channels that drain excess fluid directly from the interstitial spaces. Exceptions include the bones, the endomysium of the muscles, the central nervous system and the superficial portions of the skin. These tissues have minute interstitial channels called pre-lymphatics through which interstitial fluid can flow and empty into the lymphatic vessels. An intricate network of thin-walled vessels, which constitutes the lymphatic

vasculature, commences within the superficial dermis of the skin, a highly permeable blind-ending sac. These vessels are referred to as 'lymphatic capillaries' or 'initial lymphatics'. These superficial or initial lymphatics of the dermis communicate with the deeper layer within the dermis that acts as a pre-collecting lymphatic vessel. The pre-collection systems then drain the lymphatic fluid into the cutaneous lymphatic system. They have segments that contain valves and are surrounded by basement membranes, smooth muscles, cells and intraluminal valves [28, 29]. This complex system regulates the flow of lymphatic fluid before it enters into a series of lymph nodes or other secondary lymphoid organs.

It is then emptied into the regional collecting systems prior to returning to the heart. Lymph from the left side of the head and neck enters the thoracic duct before being emptied into the left internal jugular vein and left subclavian vein. Lymph from the right side of the head and neck enters the right lymph duct (which is much smaller than the thoracic duct) and is then emptied into the juncture of the right internal jugular and right subclavian vein.

1.4 Lymphatic Endothelium Markers

1.4.1 Podoplanin Marker (D2-40)

Previously, when no effective lymphatic marker of paraffin sections existed, conventional haematoxylin and eosin (H&E) staining was used to identify lymphatic invasion. The presence of tumour invasion could be observed as tumour emboli within the single layer EC lining. It is difficult to obtain a visualisation of the single cell lymphatic vessel wall using the H&E technique. However, when this technique is used, the tumour emboli that obliterate the lymphatic lumen can be observed as a simple extension of the tumour with no lymphatic structure [30,31].

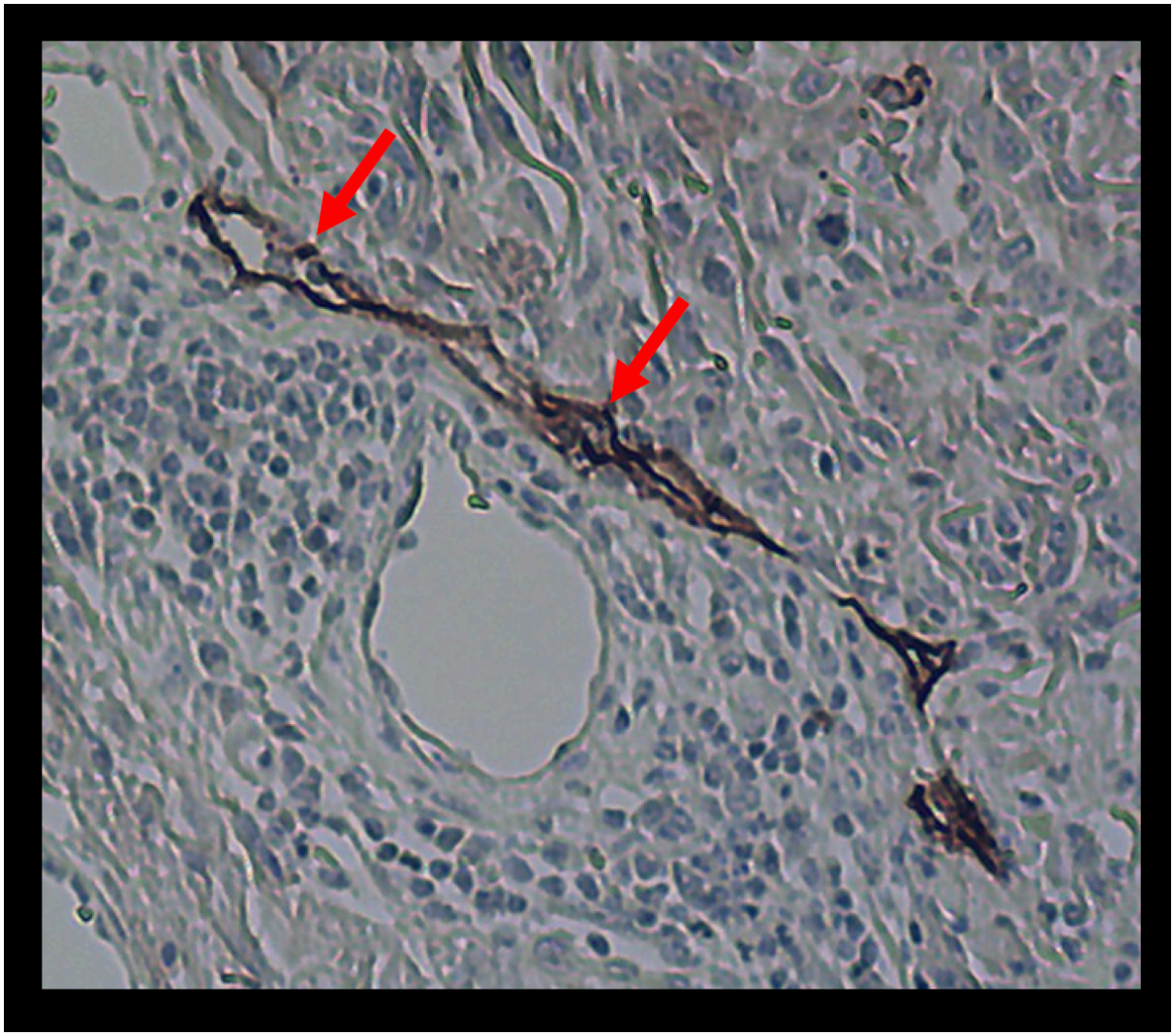

Figure 1. 5: D2-40 Stain showing Lymphatic Invasion (Tumour cells stained blue found within the lymphatic vessel stained brown)

Podoplanin (D2-40) is a novel monoclonal antibody that acts against the mucin-type transmembrane glycoprotein podoplanin that is specifically expressed by lymphatic ECs. D2-40 was originally used against M2A antigens, a surface sialoglycoprotein that was first associated with germ cell neoplasia and foetal testicular gonocytes [32]. However, it was later found D2-40 could also detect lymphatic specific ECs. Consequently, the use of D2-40 has expanded and it is now used to analyse lymphatic vessel in other solid organs. The major advantage of D2-40 is that it is a selective marker of lymphatic endothelium. It does not react to vascular endothelium and thus can be used to identify lymphatic invasions of primary tumours shown to have tumour cells within its lumen shown in figure 5 [30,31]. D2-40 binds with lymphatic endothelia leaving dark brown stains that easily differentiate between lymphatic vessels and other cell structures shown in figure 6.

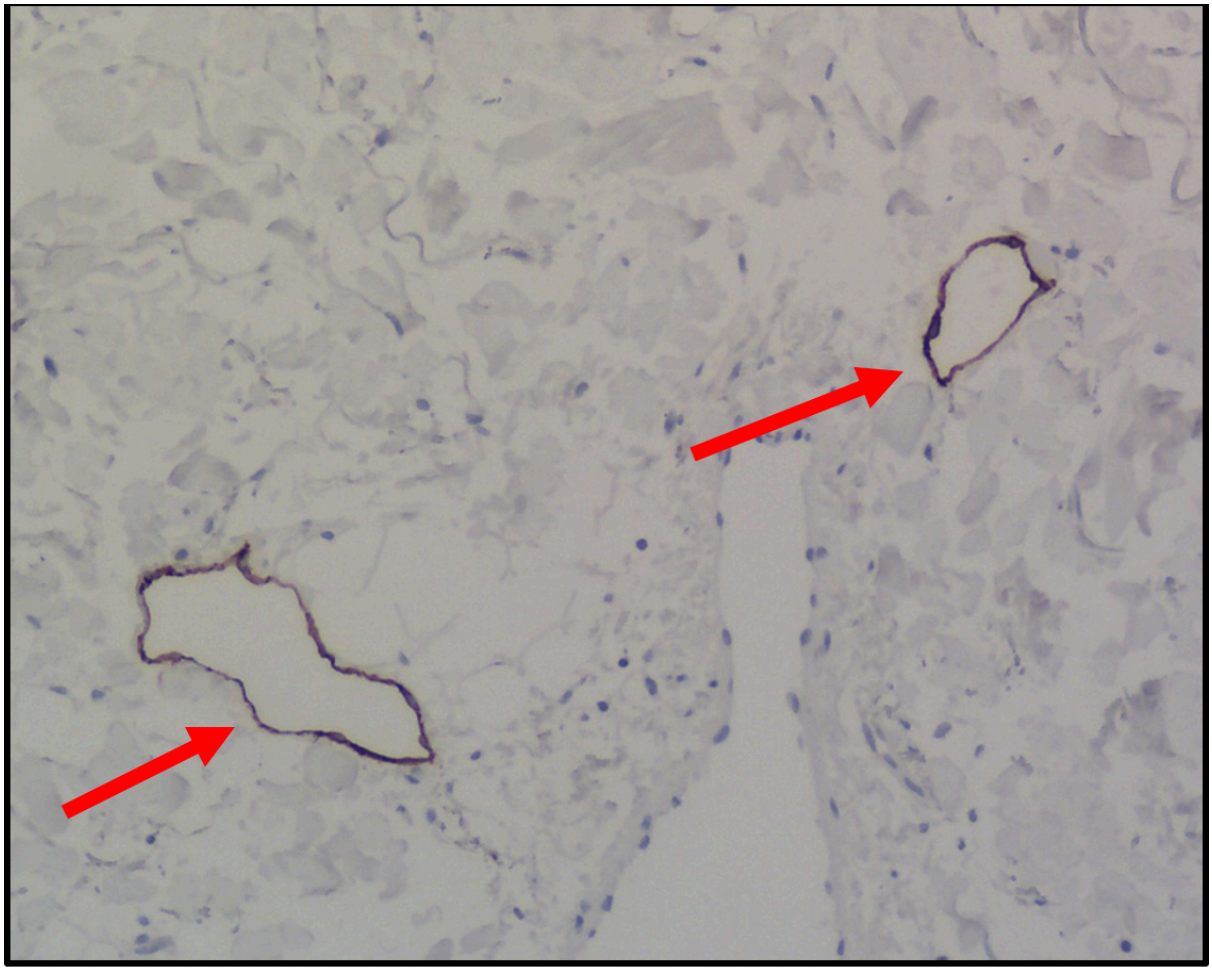

Figure 1. 6: D2-40 Stain showing a Lymphatic Vessel Highlighted in Black/Dark Brown

A previous study that compared D2-40 stains and H&E stains showed that D2-40 stains were significantly better at detecting lymphatic invasion. A pilot study by Kahn et al. examined 50 cases of breast cancer and observed that D2-40 stains detected 88 per cent of lymphatic invasion in lymph node positive cases, but that H&E stains only detected 72 per cent of lymphatic invasion. Consequently, it was concluded that H&E staining has a false negative rate of 18 per cent [31]. Similar results have been found in respect of other tumours, such as vascular, mesothelioma, oesophagus, lung, colon, gastric, uterine, malignant melanoma, adrenal and renal tumours. Many studies of non-small cell lung cancers have shown a correlation between lymphatic vessel density (LVD), lymph node metastasis and poor prognoses using D2-40 [32–36]. Minardi et al. also reported a strong correlation between the grade of cell differentiation and D2-40 immunoreactivity in penile SCC samples. Strong cellular D2-40 immunoreactivity has also been identified as a negative prognostic factor [37].

In relation to cSCC, very few papers have described the role of D2-40 in identifying lymphatic vessels. A study by Toll et al. in 2012 looked at the intensity of D2-40 expression in metastatic

and non-metastatic cSCCs. Specifically, Toll et al. observed the intensity of D2-40 staining at different magnifications. The lower the magnification required to detect staining is considered a higher expression/intensity (higher upregulation). Toll et al found that metastatic cSCCs have higher expressions and areas of D2-40 upregulation than non-metastatic cSCCs. They also observed that they were able to identify more lymphatic invasion using D2-40 stains than standard H&E stains. Further, D2-40 staining enabled them to differentiate vascular invasion from lymphatic invasion in four of the positive H&E stained specimens [38].

The existence of peri-tumoural lymphatics is well recognised. However, the presence of intra-tumoural lymphatics in solid tumours, particularly in breast cancer, continues to be a hotly debated issue. Initial studies reported that breast cancers did not have intra-tumoural lymphatics, owing to the increased interstitial pressure created by the proliferating cancer cells. Vleugel et al and Williams et al. failed to detect lymphangiogenesis in breast cancer using the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) as a marker of lymphatic vessels [39,40]. However, recent studies have shown that intra-tumoural lymphatics are detectable when new specific markers of lymphatic vessels, such as D2-40 and podoplanin, are used [41,42]. Further, such markers allow researchers and physicians to analyse the effects of tumours on lymphatic vessels in much greater detail.

1.5 Lymphatic and Vascular Structure

The lymphatic vasculature forms a unidirectional network of blind-ended capillaries (i.e., terminal lymphatics) that collect protein-rich fluid from blood vessels. It is then drained through a conduit system of collecting vessels, lymph nodes, lymphatic trunks and ducts so that it can enter the venous circulation as shown in figure 7. In mammalians, the lymphatic system has a share origination with embryonic veins, its morphogenesis involves the identification, acquisition and separation of lymphatic endothelia cells (LECs) from the blood vessel network. Thus, the ECs of blood vessels or blood endothelial cells (BECs) and LECs share many features, such as their flat shape, strong apical-basal polarity and expression of certain endothelial markers. The functional roles of the two vascular networks are distinct and specialised. The main difference between BECs and LECs is a lack of a continuous basement membrane. Further, the contact between LECs is not tightly sealed by junctional complexes due to the anchoring filaments that link LECs to the interstitial extracellular matrix (ECM) as shown in figure 8. Thus, fluid accumulation in the tissue opens up inter cellular gaps and

enhances the uptake of interstitial fluid. The distinct architecture and function of lymphatic vessels also require some specific gene products that are not shared with the BECs [43,44].

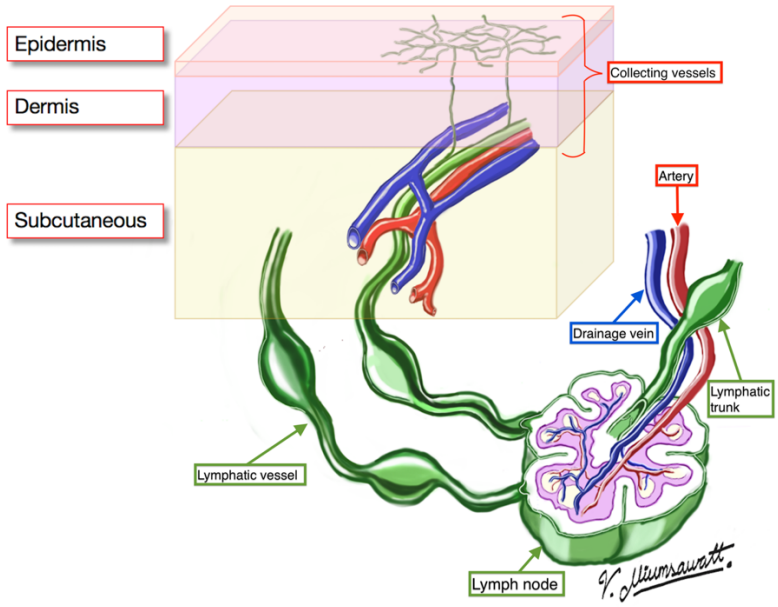

Figure 1. 7: Lymphatic system

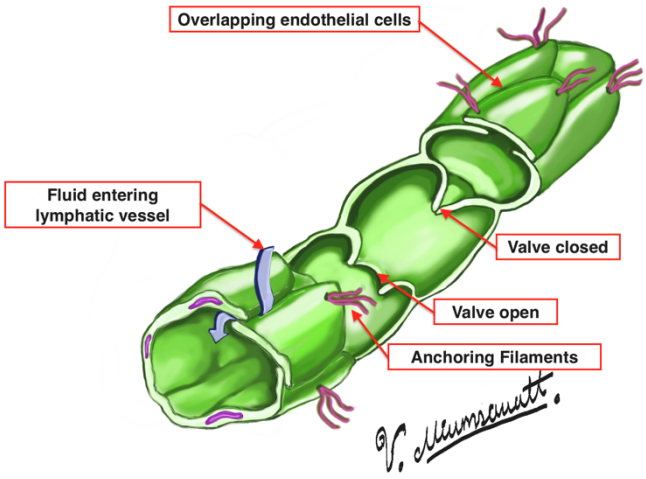

Figure 1. 8: Lymphatic vessel

1.6 Lymphangiogenesis: Growth Factors

In mouse embryos, a distinct cluster of endothelial cells (EC) on the dorsal side of the jugular vein express the Prospero related homeobox-1 (PROX1) transcription factor. These ECs differentiate into the first LECs. It is still unknown which transcription factor induces the activity. However, the role of PROX1 in the upregulation of many lymphatic specific genes and the suppression of certain blood vessel markers has been confirmed by gene expression profiling studies of cultured cells [44]. An inactivation of PROX1 has been shown to result in a defective extension of ECs from the cardinal vein, cause a loss of lymphatic marker expression and effect the lymphatic vasculature [45,46]. It has been shown that PROX1 heterozygous mutated mice exhibit viable mispatterned, dysfunctional and ruptured lymphatic vessels. The importance of separating blood vessels and lymphatic vessels has also been demonstrated in studies of mice that lack the cytoplasmic spleen tyrosine kinase (SYK) or the adaptor protein SLP76 [47,48]. Mutations of the two growth factors will result in abnormal connections between BECs and LECs during embryogenesis. The role of SYK and SLP76 in this process remains unclear; however, both molecules are involved in integrin signalling and could help to direct circulating precursor cells to specific vascular beds (e.g., by modulating their adhesion or migration) [47].

1.6.1 Lymphatic Endothelial Cells: Proliferation and Extension

In relation to the growth of blood vessels, vascular endothelial growth factor C (VEGF-C) functions as an important regulator of lymphangiogenesis. Specifically, it promotes the extension of the first LECs from the jugular vein and induces LEC proliferation [49]. VEGF-C knockout mice are unable to form primary lymph sacs, lack all types of lymphatic vessels, develop severe oedema and die before full-term gestation. The loss of a single VEFF-C allele in heterozygous mutants results in lymphatic vessel hypoplasia and lymphoedema in the skin [49]. VEGF-D also affects lymphangiogenic activity; however, it is not crucial to the development of the lymphatic vasculature. VEGF-C and VEGF-D signal via VEGFR-2 and VEGFR-3. The expression of VEGFR-3 becomes increasingly confined to the lymphatic vasculature. Consequently, any disruptions to VEGF-C–VEGFR-3 signalling will selectively compromise lymphangiogenesis. In tumours and wounds, inhibitions of angiogenesis have also been observed to correlate with the re-expression of VEFGR-3 in blood vessels.

NRP-2 assists in the development of lymphatic vessel and in differentiation. NRP-2 is expressed in lymphatic vessels and can interact with VEGFR-3 and bind to VEGF-C and VEGF-D. NRP2-knockout mice show reduced LEC proliferation and fail to develop small-diameter lymphatic vessels and capillaries [43,49].

Other growth factors and chemokines that have been suggested to effect lymphangiogenic activity include fibroblast growth factors, hepatocyte growth factors, platelet-derived growth factors (PDGFs), the insulin-like growth factor-1 and the insulin-like growth factor-2. However, some of these molecules may stimulate lymphatic growth indirectly or only in pathological settings [43].

1.6.2 Lymphatic Vessel Differentiation

VEGF-C is essential until developmental lymphangiogenesis has been completed postnatally. After which, angiopoietin-2 (Ang-2) becomes essential to patterning the lymphatic network and smoothing muscle cell recruitment to the collecting lymphatics. Angiopoietin-1 (Ang-1) has been found to promote lymphangiogenesis, trigger LEC proliferation and rescue the lymphatic defects of Ang-2 knockout mice. Ephrin-B2 is involved in the angiogenic growth of both blood vessels and lymphatic vessels, as shown by LEC sprouting, lymphatic patterning and valve formation. Further, an Ephrin-B2 mutation may produce ectopic smooth muscle coverage on cutaneous lymphatic endothelium. Similarly, acquired ectopic vascular smooth muscle cells in platelet-derived growth factor B (PDGF-B) knockout mice suggests that intact pericyte and vascular smooth muscle cell chemotaxis is essential to the regulation of mural cell vessel acquisition. Abnormal associations between smooth muscle actin-positive cells and lymphatic vessels have been associated with compromised tissue drainage in lymphoedema distichiasis. Lymphoedema-distichiasis (LD), a rare form of primary lymphoedema, presents with lymphoedema of the limbs, and is associated with a congenital abnormality in which aberrant eyelashes arise inappropriately from the site of the meibomian gland openings, often causing corneal irritation [50,51]. Bell R et al, examined 14 families with this rare genetic condition and found a small insertions or deletion in the 16q24.3 which is the forkhead transcription factor FOXC2 [52]. A forkhead box transcription factor (FOXC2) gene helps in the differentiation of collecting lymphatic and ducts and the differentiation of valves. Mutated mice that have a FOXC2 deficiency will have dysfunctional lymphatics that express several BEC markers, including PDGFB, are covered by smooth muscle cells and lack valves. The mucin-type sialoglycoprotein podoplanin, also known as T1A, T1A2, GP36, OTS-8 or

AGGRUS, is a transmembrane glycoprotein that controls podocyte shape and platelet aggregation. PDPN helps to promote LEC adhesion, migration and tubulogenesis formation. PDPN-knockout mice die perinatally and have malformed lymphatic vessels and diminished tissue drainage [43,53].

1.7 Lymphatic Patterns in Tumours

Several tumour types have been studied to identify lymphatic growth patterns and their metastatic potential. Zhang et al. used endothelial markers for lymphatic vessels to study lymphatic growth in cervical carcinoma. Zhang et al. found that cervical carcinomas displayed increases in lymphatic size and number and were more distorted in shape than normal cervical mucosa [54]. Further, peri-tumoural lymphatic vessels remain more dilated than intra-tumoural lymphatic vessels. This could be because the rapid proliferation of tumour cells places newly developed lymphatic vessels under high stromal hydrostatic or a mechanical external pressure. Additionally, Baek et al. showed that increased lymphatic vessel density (LVD) and VEGF-C expression were predictors of lymph node metastasis in supraglottic cSCCs [55].

It is in our prediction that the overall expression of different in differentiation of the cSCCs will influence the lymphatic vessels growth and pattern. From the literature, these influences are likely to be consequence of both chemical and mechanical exerted by the tumour cells.

1.8 The Mechanics of Tumours: External Pressures

Uncontrolled cancer cell proliferation is nonlinear, random and has a rapid growth pattern. As a tumour grows, it forms a packed network of cells that places stress on its surrounding environment. The externally applied stress is generated by the growing tumour mass pushing up against the normal host tissue. The normal tissue responds by resisting the tumour expansion and applying its own counter pressure. Due to the lack of organisation and structural support of the tumour cells, solid tumours often stiffen as they grow in the host tissue. Increases in the structural components of a tumour, particularly in cancer cells and the ECM constituents, can also contribute to the stiffening of the packed cells. Tumour stiffening grants it the property of being a solid mass. This is the only mechanical aspect that patients and clinicians can feel when examining tumours and it is this that allows them to differentiate tumours from the surrounding tissue.

In biomedical engineering, an elastic modulus is used to measure an object resistance to being deformed elastically when a stress is applied to it. Many researchers have used this to calculate the force of resistance that different type of tissues have and exert onto its surrounding. Typical elastic moduli of human breast cancer lesions have been measured in the range of 10.0–42.0 kPa (75.0–315.0 mmHg), one order of magnitude higher than that of the elastic modulus of normal breast (3.25 kPa/24.37 mmHg) [56]. For normal human brain tissue (grey and white matter) typical values of the elastic modulus are 2.0–6.0 kPa (15.0–45.0 mmHg) and for brain tumour tissue, the typical value is 35 kPa (262.5 mmHg) [57]. Tumour stiffening and the continued accumulation of forces by tumour constituents can displace the host tissue cells, organisational frameworks and surrounding structures, such as the vascular and lymphatic networks. This can disrupt normal cell function and the flow of interstitial fluid. The ability of the host tissue to withstand the external force and maintain its structural integrity plays an important role in the magnitude of tensile force applied in tumours [58]. Specifically, the state of stress in a tumour depends on the tumour-to-host relative stiffness (the stiffer the host tissue, the higher the stress in the tumour becomes up to the point where a plateau might be reached) [58]. However, *in vivo* experiments measuring stress within a tissue cannot yet be conducted, as the equipment and methods to conduct such an experiment are not yet available. Voutouri et al. and Stylianopoulos et al. used a mathematical module to estimate stress force and found that the externally applied stress at the interior of a tumour can be as high as 40 kPa (300 mmHg) [58,59]. Further, both radial and circumferential components of solid stress are compressive at the interior of a tumour; however, at the periphery of a tumour, radial stress diminishes and circumferential stress rapidly turns tensile [59–62]. These stresses are sufficiently high to compress or eventually cause the collapse of tumour vessels [59,63,64].

It can further be speculated that other structures within tumours (e.g., blood vessels) may also be affected. As vessels (lymphatic or vascular) closer to the centre of the tumour cell are inspected, the pressure should have a much greater effect. In the central region, these vessels are more likely to be stenotic or collapse and thus lose their functions entirely. Notably, if a proximal afferent vessel is hypo-perfused, the blood supply to a large number of distal vessels is reduced. The compression of tumour blood vessels can drastically reduce tumour perfusion. Preclinical studies in mice bearing breast and pancreatic tumours have shown that only 20–40 per cent of tumour blood vessels are perfused and that the rest are non-functional [65,66]. This could explain why, prior to the use of specific lymphatic biomarkers, many investigators have found and suggested that tumours do not possess a lymphatic supply. In addition, in cases in

which intratumoural lymphatics have been detected, they have been reported to be non-functional based on the results of dye uptake measurements [67]. Without vascular and lymphatic circulation to maintain the cell survival, this part of the tumour cell will undergo necrosis. This could explain the ulcerative appearance that has been observed around the centre of tumour specimens.

1.9 Microscopic Examinations

Recent research has focused on the modulating effects of the cellular and ECM components that exist in the tumour microenvironment on tumour progression [68]. Schoppmann et al. recently showed that tissue macrophages support the growth of a lymphatic network in the peritumoural area by expressing lymphatic growth factors. They proposed that the rationale for this was enhanced antigen presentation by blood monocyte-derived dendritic cells [69]. Many innate and adaptive immune cells can be found in the tumour microenvironment. For example, mast cells, which have a role in both innate and adaptive immune responses, are the first cells to infiltrate the tumour microenvironment and are known to deliver important regulatory molecules, such as proteolytic enzymes, cytokines, and growth factors. These molecules have a stimulatory role in angiogenesis and lymphangiogenesis, as they increase the release of lymphangiogenic factors (e.g., VEGF-A, -C, -D] and endostatin under hypoxic conditions [53,70–72]. This condition could stimulate tumour angiogenesis through proangiogenic molecules and thus result in an increase in both the lymphatic number and the size around the tumour cells.

1.10 Tumour Induced Vascular Endothelial Growth Factor Lymphangiogenesis

Metastatic cSCCs often spread via tumour-associated lymphatic vessels to sentinel and distant lymph nodes Sometimes, they may also spread to distant organs, preferentially to other sites of the skin and to the lungs, liver, brain and bone. The mechanism by which tumours spread via the lymphatic channel has been the subject of extensive discussion and research [73-76]. Experimental and clinical evidence suggests that the growth of tumour-associated lymphatic vessels (i.e., tumour lymphangiogenesis) is actively induced in many tumour types, including cSCC and promotes early lymph node metastasis [25,77]. The discovery and ability to analyse the molecular systems that control the formation of blood vessels and lymphatics has provided

great insights into the potential for angiogenesis and lymphangiogenesis (the growth of lymphatic vessels) to influence tumour growth and spread.

The VEGF family of ligands comprises a group of secreted glycoproteins. Various mammalian VEGF families have been defined and designated as placenta growth factors, VEGF-B, VEGF-C and VEGF-D, each encoded by separate genes. Vascular endothelial growth factors (i.e., VEGF-C and –D and the c-fos-induced growth factor [FIGF]) are key stimulators of lymphangiogenesis. They are the only member of the VEGF family that activate the VEGFR-3 that is then expressed in lymphatic endothelium. At least in part, the signals from VEGFR-3 are transduced via the protein kinase C-dependent activation of the MEK/ERK1/2 mitogen-activated protein kinase signalling cascade and induction of Akt1 phosphorylation. Similarly, it has been found that tumour cells also express VEGFR-3 in angiogenic blood vessel endothelia. Thus, signalling through VEGFR-3 can activate tumour angiogenesis [78].

During embryonic development, VEGF-C is essential to the formation of lymphatic vasculature; however, VEGF-D is not [79,80]. VEGF-C null mice exhibit oedema and die before birth. Conversely, mice heterozygous with VEGF-C deficiency develop chylous fluid in the abdomen after birth due to dysfunctions of the lymphatic vasculature [49]. A later study on the dermal lymphatic networks of mice showed that this factor is needed for the maintenance of calibre and the functional capacity of initial lymphatics [81]. In humans, both ligands can also bind to VEGFR-2 on blood vascular ECs to activate angiogenesis and to support primary tumour growth and metastasis [82]. An overly high expression VEGF-C or VEGF-D has been shown to increase VEGFR-3-mediated signalling, which in turn promotes tumour lymphangiogenesis. In various experimental mice studies, increases in the signalling pathway have resulted in enhanced lymph node and distant organ metastasis [83-85]. Further, by blocking the signalling pathway for VEGF-C and VEGF-D from binding to VEGFR-3, it has the ability to inhibit these processes [85,86]. A reduction in peritumoural lymphangiogenesis and lymph node metastasis has been demonstrated in a VEGF-D pancreatic tumour mice model [80].

The effects of VEGF-C deficiencies on tumour growth is not yet known. This is largely due to embryonic lethality, resulting from VEGF-C deletion. Conversely, an alteration in human VEGF-D expression has been found to either promote [85,87] or inhibit [88] angiogenesis and tumour growth. In a range of human cancers, expressions of VEGF-C or VEGF-D have shown a positive correlation with lymph node metastasis and poor patient outcomes [89–91].

Collectively these data indicate that both VEGF-C and VEGF-D may play major roles in tumour lymphangiogenesis and in metastatic spread to sentinel and distal lymph nodes and beyond. Initial studies in mice have led the way for a humanised version of the anti-VEGF antibodies, the efficacy of which has been demonstrated in phase III clinical trials in patients with metastatic colorectal cancer [92]. Additionally, several other studies of different tumours have shown an over expression of both VEGF-C and VEGF-D in most organs [85,90]. These studies have examined melanomas, and head and neck, cervical, endometrial, ovary, breast, thyroid, oesophageal, gastric, pancreatic, prostate and lung tumours [85,90].

The effects of VEGF-D on cutaneous tumour development has been observed in mice. In one study, mice were divided into two experiential groups: one with an overexpression of VEGF-D and another with VEGF-D knockout genes. They were then introduced to skin carcinogenic chemically-induced skin tumours (i.e., papillomas that progressed to cSCCs). It was found that the VEGF-D acted as a ligand for VEGFR-3. Thus, this receptor appears to activate both tumour lymphangiogenesis and angiogenesis. The number of peritumoural lymphatic vessels detected with lymphatic histochemical stains was significantly higher in the elevated VEGF-D transgenic mice than in the wild-type mice. The LVD was also about two times higher in the untreated VEGF-D overexpression mice skin than in the control skin. Conversely, the papillomas and cSCCs in the knockout mice showed a trend towards decreased tumour lymphangiogenesis, and in the untreated skin and in cSCCs. Further, they found that the metastatic dissemination into the regional lymph nodes was more increased in the transgenic mice than the wild-type mice. No regional lymph node spread was found in the VEGF-D knockout mice [93].

In a study of humans, Karatzanis et al. observed VEGF-D positivity in lymph node metastasis samples among patients with oral SCCs [94]. VEGF-C is more significantly associated with lymph node metastasis than VEGF-D. Hirota et al. also observed VEGF-D positivity in VEGF-C-expressing tongue SCCs. Increased VEGF-C and VEGF-D expression has been correlated with higher LVD and worse recurrence-free survival. Thus, evaluations of these markers should help to predict which patients will develop lymph node metastasis [95]. However, it should be noted that VEGF-D is downregulated in certain tumour tissues and the relationship between VEGF-D and lymphatic metastasis is still unknown.

Interestingly, another phenomenon was also observed in the human study: VEGF-D induced significant changes in the immune cells and cytokine profiles developing skin tumours. This

led to a shift from a protumoural Th2 to an antitumoural Th1/Th17 response and substantial regression in primary tumours in an early stage of carcinogenesis. This also raised the possibility that VEGF/VEGFR plays a role in tumour suppression and support that is beyond the level of VEGF-C or VEGF-D expression. A recent study showed that VEGF-D induces and a VEGFR-3 neutralising antibody suppresses the production of IL-6 by CD11c+ dendritic cells, resulting in enhanced anti-tumour immunity in an allogeneic hematopoietic stem cell transplantation model in mice [96]. Such results support the immunomodulative role of the VEGF-D/VEGFR-3 axis [96].

1.11 Lymphatic Vessels: Patterns and Tumours

The lymphatic system can be examined as small blind-ended vessels that have the function of absorbing fluids and cells in tissues. Initial lymphatics are characterised by short anchoring filaments that connect the abluminal membrane of LECs to the surrounding elastic fibres in the tissue. The tethering of LECs to elastic fibres and the unique 'button-like' cell–cell junctions between LECs allow for the formation of a one-way valve. The structure formed flaps along the vessel and opens so that fluid can enter, but not leave. Fluid is then transported into the deeper pre-collecting vessels and then into larger calibre lymphatic collecting vessels.

Research on the mechanisms governing lymphogenous spread has predominantly focused on the effects of lymphangiogenic growth factors on initial and pre-collecting lymphatics in and around primary tumours. Observations from animal models of VEGF-C driven lymphogenous metastasis indicate that the collecting lymphatic vessels that drain the primary tumour might play an active role in metastasis. It was found that the presence of VEGF-C promoted the enlargement of collecting lymphatics, which was attributed to the proliferation of LECs, and that this was associated with an increase of the total cross-sectional volumetric flow rate of the lymphatics [86,97]. Similarly, it has been shown that tumour-derived VEGF-D promotes the enlargement of tumour-draining collecting lymphatics and that this is required for the dissemination of cancer cells to sentinel lymph nodes (SLNs) and distant organs [98].

Studies of human malignant melanoma have shown that the parameters of lymphangiogenesis and lymphatic remodelling are directly correlated with patient outcomes. For example, clinical and histological analyses of primary melanomas with early lymph node metastasis and primary non-metastatic melanomas with similar parameters have shown a significantly higher incidence of intratumoural lymphatics in metastatic melanomas. It has also been shown to negatively

affect disease-free survival [99]. Within the primary melanomas which have metastasised, the tumour was found to have enlarged tumour-associated lymphatics and lymphatic vessel area. This has been shown to have significant association with poor disease-free and overall survival [99]. Unsurprisingly, given the findings, it has been suggested that tumour lymphangiogenesis could be used as a prognostic indicator of the risk of lymph node metastasis in cutaneous melanomas.

Similar findings have been reported for other types of tumours. In colorectal carcinoma, the immunohistochemical staining of resected specimens for the lymphatic vessel marker D2-40 showed a high lymphatic vessels density. This was found to have prognostic significance, as it was correlated with the metastasis of the regional lymph nodes and distant organs [27]. Lymph node metastasis is also a key prognostic factor in the survival of patients with breast cancer [100,101]. It is therefore thought that the lymph node spread of breast cancer cells is facilitated by lymphangiogenesis. Some studies contradict these results. However, these studies experienced difficulties in detecting dividing LECs. Other studies have found reduced LVD within breast cancer cells. These findings may be reflective of pre-existing lymphatic vessels that are associated with metastasis and not of new lymphatic vessels being generated through lymphangiogenesis. Interestingly, inflammatory breast cancer contains a significantly higher proportion of proliferating LECs than non-inflammatory breast cancer [102]. Thus, lymphangiogenesis might help to explain the propensity of inflammatory breast cancer and its propensity to have a worst prognosis than other types of breast cancer.

1.12 Vascular Endothelial Growth Factor D Increases Interstitial Permeability

Lymphatic vessels could also facilitate metastasis by increasing its vascular permeability or changing the adhesive properties or cytokine or chemokine expression patterns of the lymphatic endothelium. The VEGF-C and VEGF-D secreted by tumour cells could also have important effects on tumour interstitial fluid pressure. VEGF-C can increase vascular leakage, but not as efficiently as VEGF. However, it should be noted that a parallel increase in lymphangiogenesis could alleviate this effect [103,104]. The increased interstitial fluid pressure could be a significant determinant of tumour cell seeding into blood vascular and lymphatic circulation. Recent studies have shown that a proportion of the lumens of tumour blood vessels contain tumour cells.

1.13 Lymphatic Permeability

The invasion of cancer cells into lymphatic vessels plays an important role in tumour metastatic spread. This cascade includes cancer cell migration and adhesion to lymphatic vessels, which is in turn followed by invasion through vessel walls. The final result is a subsequent and systemic spread. The ability for cancer cells to invade lymphatic vessels is believed to be the result of the cancer and lymphatic vessel barrier functions being disrupted. Such disruptions promote cancer lymphatic vessel invasion and metastasis. In normal tissue, a lymphatic vessel is lined by continuous ECs that are attached to each other by tight junctions. In the early stages of cancer, the lymphatic vessel barrier function may be affected by alterations in cell shape and cell junctions (e.g., contractions or intercell gap formations).

1.13.1 Hyaluronan

To understand the effects of tumour cells on lymphatic vasculature permeability, an understanding of the role of hyaluronan (HA) is required. HA (also called hyaluronic acid) is a linear and negatively charged glycosaminoglycan polymer composed of repeating disaccharide units of glucuronic acid and n-acetylglucosamine. HA can exist at variable polymer lengths as it is being synthesised by transmembrane HA synthases (i.e., HAS1, HAS2 and HAS3) on the inner surface of a cell membrane and secreted into the ECM. The high molecular weight HA polymers that exist in the ECM become degraded into smaller-sized fragments by two types of hyaluronidases (Hyal-1 and Hyal-2). It can bind to all CD44 isoforms receptors on the surface of cells [20,24,25]. CD44 is a transmembrane glycoprotein that is over expressed in many types of cancerous cells, such as melanoma, breast, lung and colorectal cancer. Thus, CD44 is the major receptor of extracellular HA. Interestingly, a number of recent studies have associated CD44-HA interactions with tumour progression and metastasis.

HA participates in tissue remodelling and rapid cell proliferation in several physiological processes (e.g., embryonic morphogenesis and wound healing). HA expression is highly regulated by balancing the synthesis and degradation of physiological conditions. High levels of HA are found in human digestive cancers, including colorectal cancer, gastric cancer and pancreatic cancer [105]. Increased expressions of HA have been implicated in tumour cell growth, adhesion, migration, invasion, chemotherapy resistance, radiotherapy resistance,

angiogenesis and lymphangiogenesis in human digestive cancers. It is thought that HA-mediated signalling pathways play an essential role in these cancer progression events.

Research has shown that HA regulates malignant tumour growth, invasion, and metastasis. The high molecular weight of HA contributes to space filling and acts as an inhibitor of vascular leakiness. A high molecular weight of HA plays a significant role in providing a protective barrier between vascular ECs and thus preventing the spread of cancer while a low molecular weight of HA exerts a disruptive effect on vascular integrity under cancer conditions [106,107]. Yu et al. showed that a low molecular weight of HA increased the permeability of LECs' monolayers and produced a significant increase in LECs' monolayers of permeability. It also induced the disruption of the Zonula Occludens-1 (ZO-1), an essential component of tight junctions. It is functionally critical in regulating the endothelial permeability barrier and is linked to the underlying actin cytoskeleton. They further found that a low molecular HA weight opens the ZO-1 intercellular junction and promotes actin stress fibre or paracellular gap formations. Thus, LMW-HA appears to disrupt the cancer lymphatic vessel barrier [108].

1.14 The Implications of Measuring Lymphatic Vessels in Primary Cutaneous Squamous Cell Carcinomas of the Head and Neck.

The major functions of the lymphatic network include filtering and destroying microorganisms and other foreign substances, absorbing fat from the small intestine and maintaining fluid balance. Since the time of Le Dran, a 16th century French surgeon who was the first to note that cancers of the breast that spread to axillary lymph nodes have significantly worse survival outcomes than those which localise only to the primary tumour [109], it has been believed that the initial spread of cancer cells can be observed histologically, as tumours escape beyond defined boundaries and invade local lymphatic vessels. In some cancers, lymph node metastasis is thought to occur even before primary tumours are clinically detectable and often heralds distant organ metastasis at the time of diagnosis. Thus, lymph node staging may alter the type and the timing of the treatment offered to patients.

It was once believed that less well-differentiated cSCCs were more likely to metastasise via direct invasions into the vascular/lymphatic system. More importantly, well-differentiated cSCCs were often thought to have good prognoses and thus were often treated more

conservatively. However, it has since been found that well-differentiated cSCCs can metastasise and less well-differentiated cSCCs can remain dormant. Peat et al. evaluated the risks associated with metastatic spread in cSCCs. They noted that one feature is the presence of poorly differentiated cSCCs. They also noted that over 15 per cent (i.e., 12 of 78) of metastasised SCCs originated from a well-differentiated subtype and that 33 per cent of the moderately or poorly differentiated subtypes did not metastasise [12].

Following the development of specific antibodies against LECs, the concept of lymphangiogenesis has been studied extensively. Many studies have shown that the formation of tumour-associated lymphatic vessels (lymphangiogenesis) has a strong influence over the growth and metastatic spread of cSCCs [55,110–115]. These lymphatic vessels are believed to act as conduits by which cancer cells can escape primary tumours. The development of lymphangiogenesis involves multiple steps, including EC migration, proliferation, cellular rearrangement and cell permeability [54,116]. All of these steps are attributable to the lymphangiogenic growth factors that are released by the tumour cells.

In cSCCs, an over expression of the immune modulators responsible for inducing lymphangiogenesis has been found. Kataru et al., Watari et al. and Schoppmann showed an increase in macrophage derived VEGF-C activity and considered its potential role in inflammatory lymphangiogenesis of cSCC [117–119]. Moussari et al. studied which gene expressions resulted in lymphogenesis. Specifically, they compared cSCCs to normal skin using the LYVE-1), reverse transcription (RT)-PCR and laser capture microdissection (LCM). Similar to previous studies, they found an increase in lymphatic vessels and detected expressions of endothelial growth factors in cSCC tissues [120]. However, in a clinical setting, such tests are expensive and time consuming. Several studies have sought to take a more direct approach to observe the lymphatic vessels in metastatic cSCC and used the monoclonal antibody against human D2-40. This antibody binds specifically to the endothelial wall of human lymphatic vessel unlike other agents that also bind to the vessel wall [121]. Toll et al. used D2-40 to identify lymphatic endothelial and found a higher uptake in metastatic cSCCs compared to non-metastatic cSCCs [38]. Similarly, several studies used D2-40 to enhance the detection of lymphatic invasion in several malignancies (e.g., breast, lung, oesophagus, colon, gastric and uterine tumours) [122–126].

These studies suggest that there is clear link between cSCCs and lymphangiogenesis (attributable to an overexpression of endothelial growth factors). Increases in

lymphangiogenesis and physiological changes can increase the risk of regional metastasis and thus distant metastasis. Thus, the presence of metastases worsens prognoses in terms of patients' overall and disease-free survival. Further, under current classification of cSCCs, tumours are subjectively subdivided according to cell morphology, architecture and differentiation. However, better prognostic subtypes (i.e., well-differentiated subtypes) may not necessarily lead to good outcomes for patients if a conservative approach is adopted. To date, no study has sought to directly examine expressions and changes in lymphatic patterns in different cSCC subtypes. This study is designed to perform an objective analysis of the lymphatic vessels with regarding to its features and quantities. We hypothesis that the differentiation subtype exerts influence over the lymphatic growth. This effect will result in a different lymphatic growth pattern. It is very likely that a high expression of lymphatic vessel in relation to numbers and density is more in line with poorer prognostic group (ie. a less well differentiated group). The lymphatic pattern within the metastasis group is only an observational secondary endpoint as there was not enough data to be analysed. However, it should be noted, that to design a comprehensive set of staging guidelines that are accurate, easily applied and efficient represents a tremendous challenge and a challenge to which there may be no perfect solution. This study is an observation study that examines the lymphatic patterns within the different Broder's cSCC's [38, 122-126].

Chapter 2: Materials and Methods

2.1 Introduction

In this chapter, the study's methodology is outlined. Specifically, details of the inclusion criteria for the selected samples are provided. Additionally, the process by which each specimen underwent immunohistochemistry examination is outlined. In the present study, the final specimen images were reconstructed for analysis after the biological staining had been completed.

This chapter begins by describing the technique and the computer program used to analyse each specimen's images. Next, the analyses of the specimens and their lymphatic structures are described. Finally, the static analysis process and the techniques adopted are outlined.

2.2 Ethical Approval

Prior to this research being conducted, ethical approval for this study was obtained from the Peninsula Health Human Research and Ethics Committee (EC00347), a part of the Victorian Government's Department of Health. An application form for a Victorian Specific Module (VSM) 2013 and a Victoria 2013 Site Specific Assessment (SSA) form were submitted to the committee. The application form had various requirements in relation to information privacy (Information Privacy Act 2000), health information (Health Records Act 2001), the use of ionising radiation (Radiation Act 2005 and Radiation Regulations 2007), the removal of human tissues (Human Tissue Act 1982) and the use of poisons or controlled substances (Drugs, Poisons and Controlled Substances Act 1981). The research also had to comply with the Guardianship and Administration Act 1986 and the Mental Health Act 1986. The Committee approved the application on 4 April 2013 (application number HREC/13/PH/1).

2.3 Patient Selection and Data Collection

2.3.1 Patient Selection

Patients who had undergone excisions of primary cSCCs of the head and neck region by the Plastic and Reconstructive Unit of Peninsula Health between 2007 and 2011 were identified on Peninsula Health's digital database. The patient identification process comprised two steps. First, the primary cSCC specimens stored at Peninsula Health's tissue bank (a part of the

Pathology Department) were identified. Second, all of the specimens had to have been stained and examined by qualified pathologists working for the institute during the stated period. The specimens were collected as part of patient medical management using a standard protocol of the Pathology Department. The Pathology Department of Peninsula Health assigned a key code for each specimen based on tumour type. Number coding was also assigned to identify patients. The information on the Pathology Department's database included specimen examination results, patients' name, dates of birth and hospital identification number. Information was stored in a digital database. Thus, both specimens and relevant information could be recalled using computer software.

A search was conducted using the keywords 'squamous cell carcinoma'. In response to the search, a total of 362 patients were identified as having a SCC of the cutaneous origin that had been excised by the Plastic and Reconstructive Unit from various regions of the body. The search was further narrowed to identify patients with primary cSCC of the head and neck region. The head and neck region were defined to include any lesions that originated superior to the clavicles, but not involving the oral mucosa, nasal passage or external meatus. In total, 82 patients were identified as having cSCC of the head and neck region. Only the head and neck region were included to eliminate potential bias for comparison. Different parts of the body have different lymphatic drainage patterns and therefore may have different lymphatic vessel pattern establish within it [127].

2.3.2 Data Collection

Once the specimens were identified in the Pathology Department's database, each patient's name, date of birth and hospital identification number were then used to obtain further clinical information from the Health Information Services Database. Clinical information was collected in relation to patients' age, gender, comorbidities, medications used, immunosuppression status, site of involvement and known distance metastasis (including regional nodes). Eight patients who had experienced recurrent lesions, multiple lesions or re-excisions were excluded from the study. Of the 82 patients identified, a total of 74 consecutive patients were included in the study.

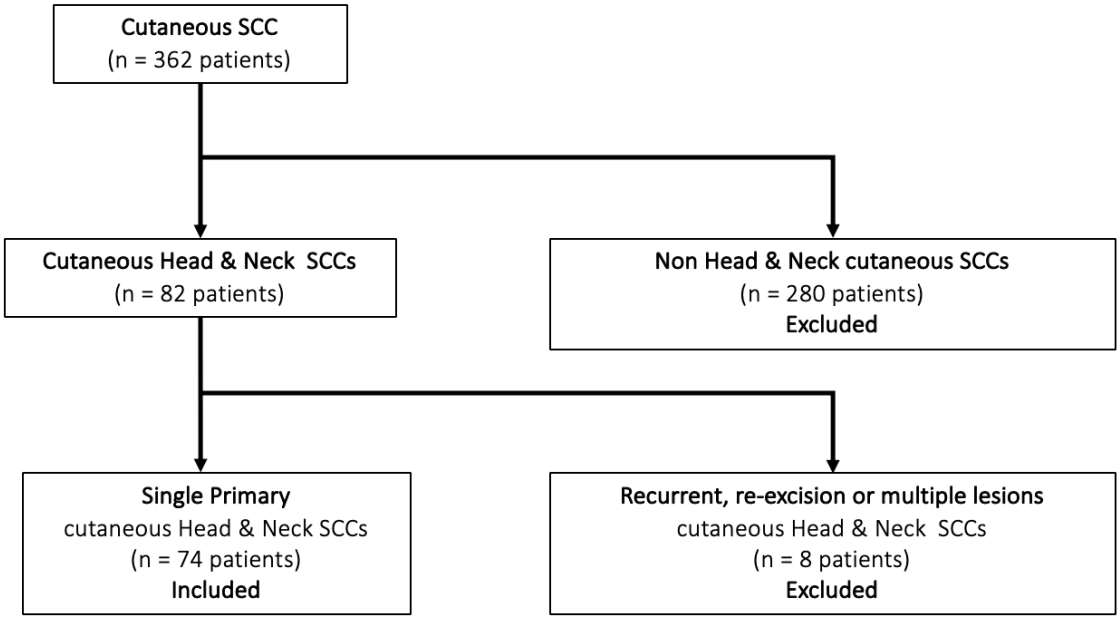

Figure 2. 1: Patients selection

2.4 Tissue Preparation

The original formalin-fixed paraffin-embedded specimen blocks of all the patients with cutaneous head and neck SCCs who had been diagnosed between January 2007 and December 2010 were retrieved from the Department of Pathology, Peninsula Health (Frankston Hospital), Victoria, Australia. A total of 74 specimens were recalled and randomly assigned numbers for identification. For the immuno-histochemical analysis, two slices of the central section of each specimen were taken for D2-40 and haematoxylin and eosin (H&E) staining. Tissue sections of 4 μm thickness were prepared on coated slides. Each slice was deparaffinised with deionised water and phosphate-buffered saline. Heat induced antigen retrieval was performed in a water bath. The slides were placed in a preheated Coplin jar containing Antigen Retrieval Citra Plus solution (BioGenex ™) and incubated at 58°C for 20 minutes. The slides were then allowed to cool at room temperature for 20 minutes and stained for examination. All the slices were prepared with the assistance and resources of Dorevitch Pathology laboratory.

2.4.1 The Haematoxylin and Eosin Staining Procedure

H&E staining was performed in accordance with the protocol of Frankston Hospital's Department of Pathology (see below).

2.4.1.1 The Reagents

The following reagents were used in the staining procedure:

- Harris Haematoxylin Hg++-free, acidified;
- Eosin-Y in 1 per cent alcohol;
- Ammonium Hydroxide, 0.3 per cent (or 1 per cent if the original stock is counted as 100 per cent);
- Ethanol;
- Xylene; and
- 10 per cent Formalin or 4 per cent Formaldehyde, or 4 per cent Paraformaldehyde (stable at 4^{o}C for one month).

2.4.1.2 The Procedure

The H&E staining procedure was carried out as follows:

1) The slides were immersed in filtered Harris haematoxylin for up to 2 minutes;
2) The slides were rinsed two times (or until water looks clean) with water for 1 minute each time;
3) The slides were immersed in 0.3 per cent ammonium hydroxide (10–20 dips); and
4) The slides were rinsed two times with water for 1 minute each time.

After the above steps had been carried out, each slide was observed under a microscope for blue intensity. If a slide was too light, the haematoxylin stain was repeated. However, if it was too blue/dark the slide was rinsed with 1 per cent hydrochloride to destain it until the correct level of blue intensity was achieved. The following process was used:

5) The slides were immersed in Eosin-Y (10 dips);
6) The slides were washed three times with water for 1 minute each time;
7) The slides were dehydrated as follows:
    a) In 70 per cent ethanol for 1 minute;

b) In 80 per cent ethanol for 1 minute;
c) In 95 per cent ethanol three times for 1 minute each time; and
d) In 100 per cent ethanol three times for 1 minute each time (or until the ethanol did not look pink);

8) The slides were immersed two times in xylene for 1 minute each time; and
9) The Permount ® (Fisher Chemical™) and cover slips were added.

Figure 2. 2: Paraffinised Tissue Block Slicing Process

2.4.2 D2-40 Staining Procedure

In relation to the D2-40 staining, the procedure was performed using an automated slide stainer in accordance with a programmable staining protocol. The prepared deparaffinised slides were each placed onto a Ventana™ device over a slide loading platform. The selected D2-40 stain solution was then placed on the stain dispenser loading dock where the solution was automatically dispensed. The antibody was then allowed to incubate at the temperature of 37^{o}C for a period of 32 minutes. Following the completion of this preparation process, the slides were rinsed with a wash buffer. Glass slips were then used to cover each slide. To ensure that any overly or inadequately stained slides could be identified, a normal tonsil tissue specimen was used in each group of stained slides as a control. The automated Ventana™ device allowed multiple specimen slides to be processed simultaneously. It also eliminated the need for any

manual processes and prevented any issues related to temperature dependencies or quality consistency from arising. All of the slides were treated with the condition and concentration of reagents to eliminate human error bias. The Ventana™ device was used and the Dorevitch pathology (Heidelberg, Victoria) protocol adopted to stain the specimens for lymphatic endothelium antibody D2-40 (see Table 5 and refer to the appendix, for full details of the protocol).

Table 2. 1: Protocol #641: Ultraviolet—Podoplanin (D2-40) Staining Protocol

Preparation	Cut a 3-4 μm section of formalin-fixed, paraffin-embedded tissue and place on positively charged slides; dry and fix for 20 mins at 58ºC
D2-40 concentration	0.10 μg/ml
Ventana staining procedure (UltraView™)	Load the slides, antibody and Ultraview™ detection kit dispensers onto the Benchmark instrument Select CC1, the standard pre-treatment One drop of D2-40 is applied automatically by the device Antibody incubation is set for 32 minutes at 37 ºC. Start the run When the staining run is complete, the slide is moved from instrument and rinsed with the wash buffer Cover the slide with glass slip

Figure 2. 3: An Automated Ventana™ Device

2.5 Haematoxylin and Eosin Analysis

Two trainee pathologists, blinded to the identity of each of the specimens, independently evaluated each slide. The H&E stains were examined under microscopes and characterised into subgroups based on their degree of differentiation (i.e., well-differentiated [21 cases], moderately differentiated [43 cases] and poorly differentiated [19 cases]). The grade of cSCC was determined according to the criteria set out in Table 2. If the two reviewers disagreed to the degree of differentiation of any of the specimens, a senior pathologist reviewed the specimens. The presence of perineural invasion was also noted.

2.6 Photographic Images and Image Reconstruction

2.6.1 Podoplanin (D2-40) Analysis

At the beginning of the session before the microscopic examination was conducted, the microscope, including the microphone lens, was cleaned with alcohol solution. To remove any debris that could affect the viewing process, each slide was also cleaned with alcohol solution and left to dry for 30 seconds. The slides were examined under a Leica™ Microsystems

(Wetzlar, Germany) microscope camera. A white balance was performed using the automated built-in software. Each slide was examined in its entirety under high-power fields (i.e., x 100). The focus of the lens was adjusted until optimal viewing was achieved on the camera displaced screen. The images were captured in a divided section for further image reconstruction. The complete images were then saved into a single folder and labelled according to their assigned identification number.

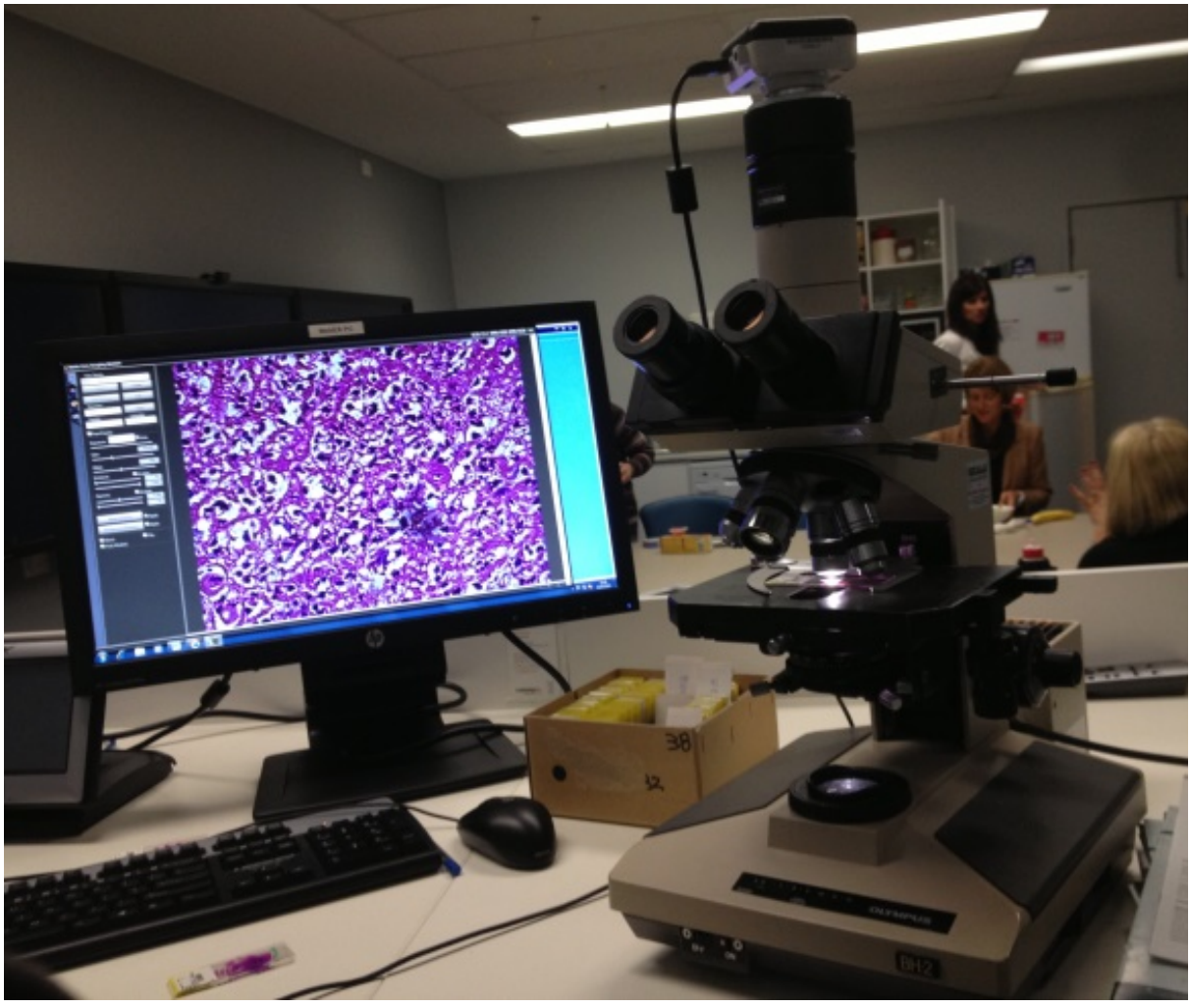

Figure 2. 4: Microscopic and Photographic Examination

Coral Paintshop Pro X5™ was used to reconstruct and examine the images captured of the specimens. Each image had an identifiable distinct landmark to allow the adjacent images to overlap. Once the reconstruction process was completed, a final image was stored as a single file image for further analysis.

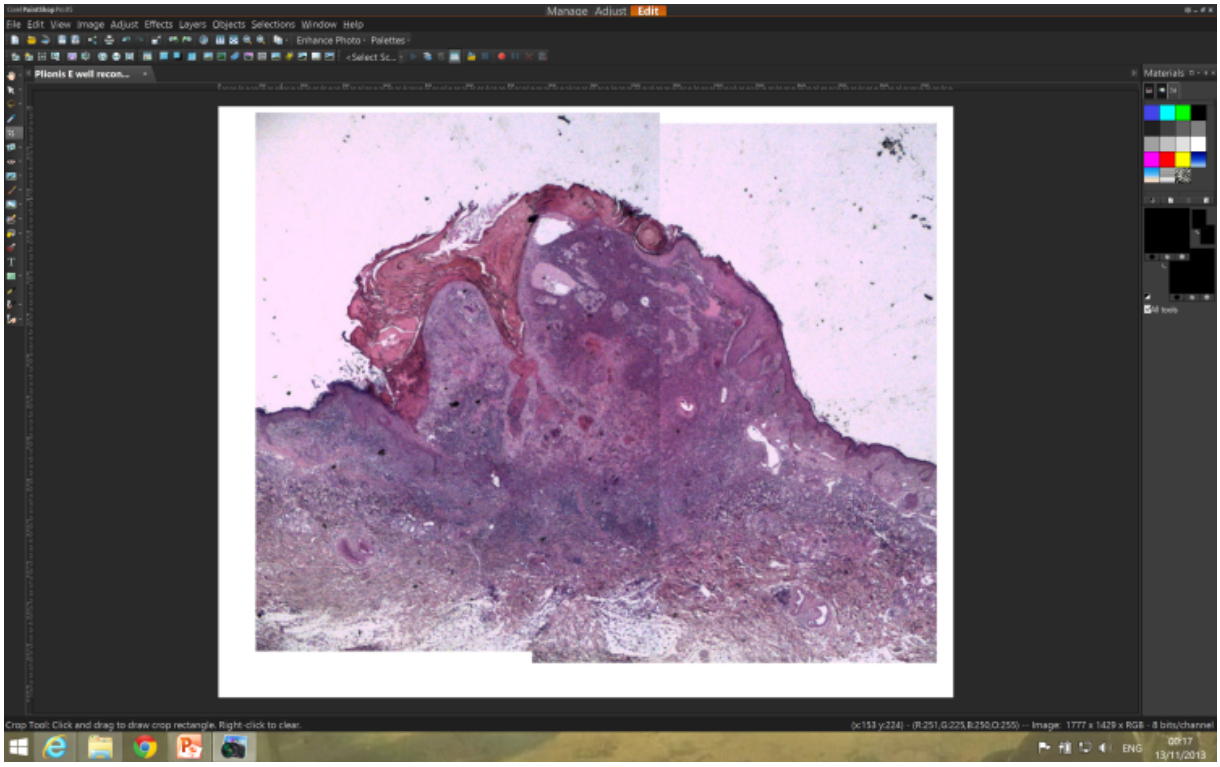

Figure 2. 5: Coral Paintshop Image Reconstruction (Haematoxylin and Eosin Stained)

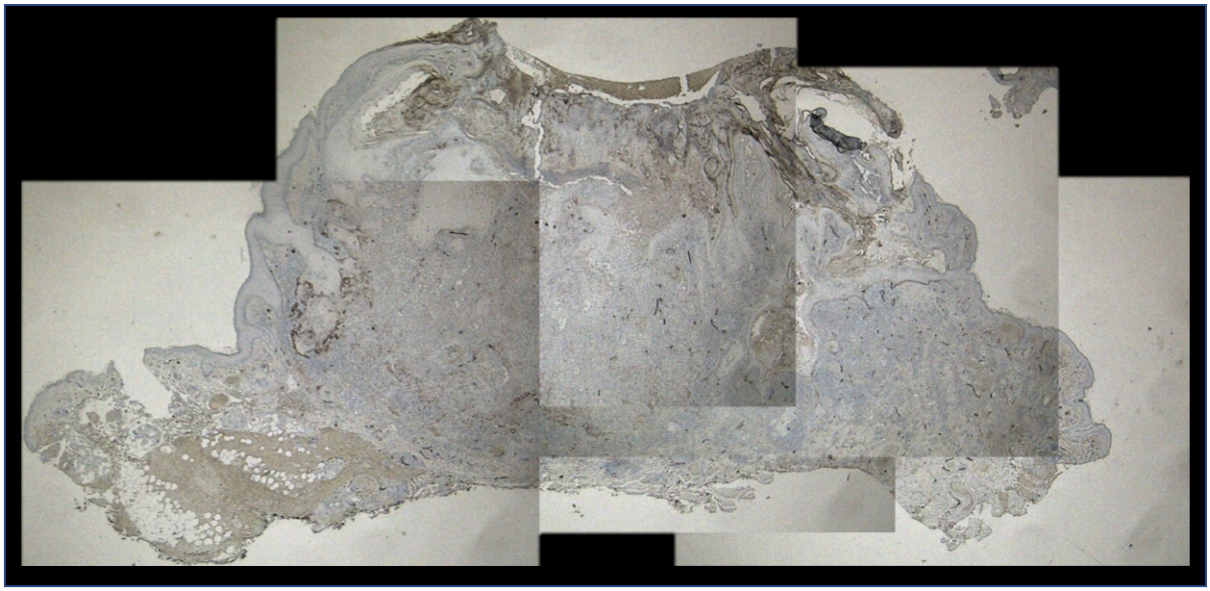

Figure 2. 6: Image Reconstruction (D2-40 Stained)

2.7 ImageJ Lymphatic Measurement

Each specimen image was divided into intratumoural and peritumoural areas. The peritumoural area was identified as the area 0.5 mm from the most peripheral cancer cell. Dark brown or

black highlighting (from the D2-40 stain) was used to identify the lymphatic vessels. The presence of any lymphatic invasions was also recorded. A specimen was recorded as displaying lymphatic invasion if there was a tumour cell within a lymphatic lumen.

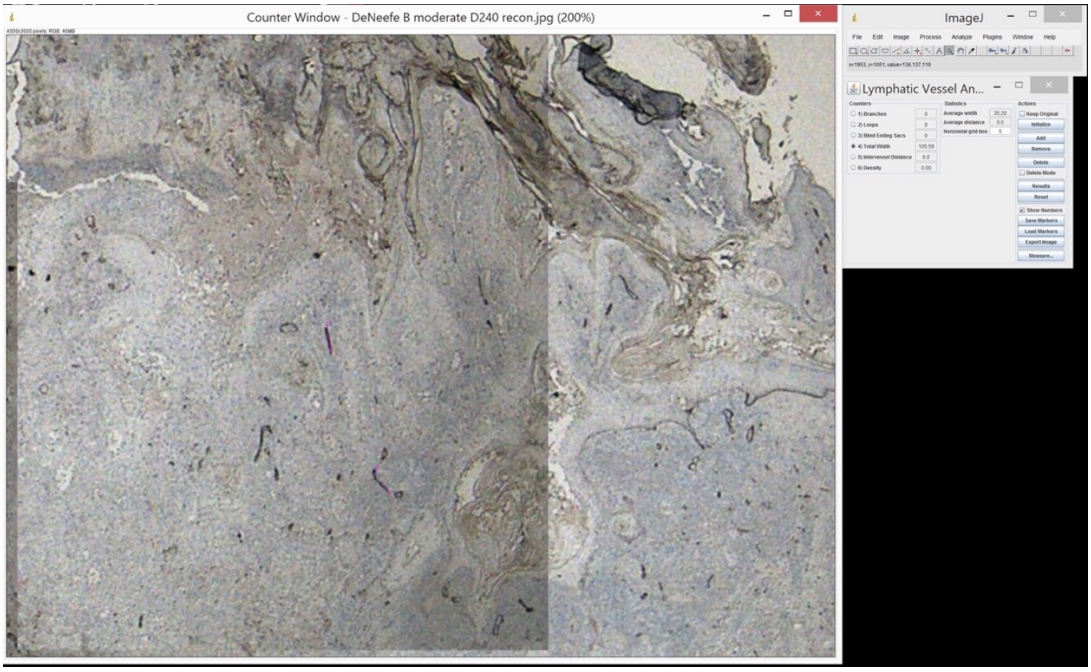

Figure 2. 7: ImageJ of a Lymphatic Analysis obtained using a Lymphatic Vessel Analysis Plug-in

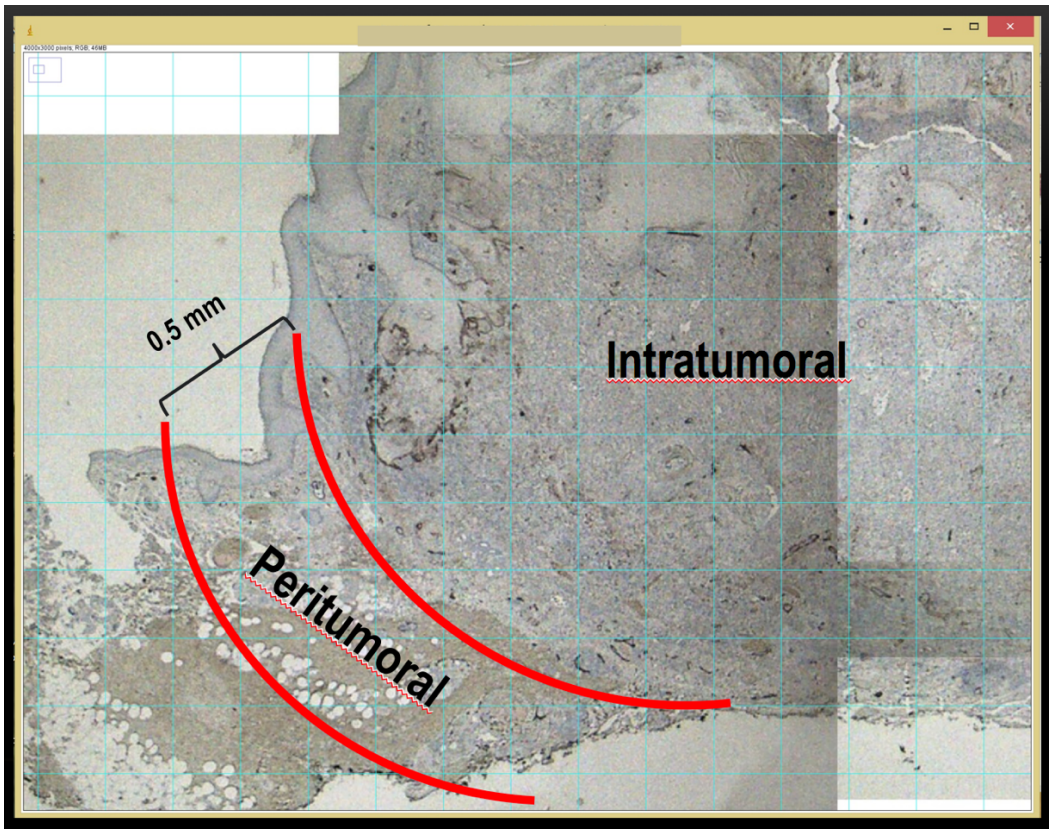

Figure 2. 8: ImageJ showing the Peritumoural and Intratumoural Areas

ImageJ (a Java-based image processing program) was used to analyse the images. Both an automated and semi-automated technique was used. After setting the scale for each image to its actual size, each image was converted into grey scale. The brightness and contrast for each image was then adjusted so that only the lymphatic vessels were highlighted. Each image was then sorted and converted into binary images to identify the automated specimen area and enable lymphatic quantity counts to be obtained. Each individual lymphatic vessel that had been selected for counting was then semi-automatically measured using a plug-in that had been specifically designed for lymphatic vessel analyses. This plug-in for Image J was created by a researcher in the team (R Shayan). The plug-in and its source code were accessed via an open source database and are available from the Melbourne Branch of the Ludwig Institute for Cancer Research (http://www.ludwig.edu.au/archive). The plug-in enabled the users to analyse each lymphatic vessel to ascertain the width of each vessel, the total width, the average width and the total cross-sectional area of each slide. The width of each lymphatic vessel was determined by measuring the longest diameter of the lumen. The total cross-sectional area is automated by the ImageJ counting the pixels within the lymphatic vessel after it was converted into a binary image. This was done as a two-dimensional construct using the same technique described by the Ludwig institute. The density of each lymphatic vessel was calculated by dividing the total area of a lymphatic vessel by its total lymphatic cross-sectional area which was the same techniques done by other validated and published papers [128-130]. Lymphatic cross-sectional area provides an information of how much lymphatic vessels occupy the tumour itself. The plug-in also allowed for the quantity of the lymphatic vessels to be counted manually by placing a lighted dot on each individual lymphatic vessels. This allowed the results to be double checked. This technique of lymphatic analysis followed the same established image analysis protocol by the Ludwig institute, Melbourne. The work has been validated and published Growth Factors Journal, 2007 [131].

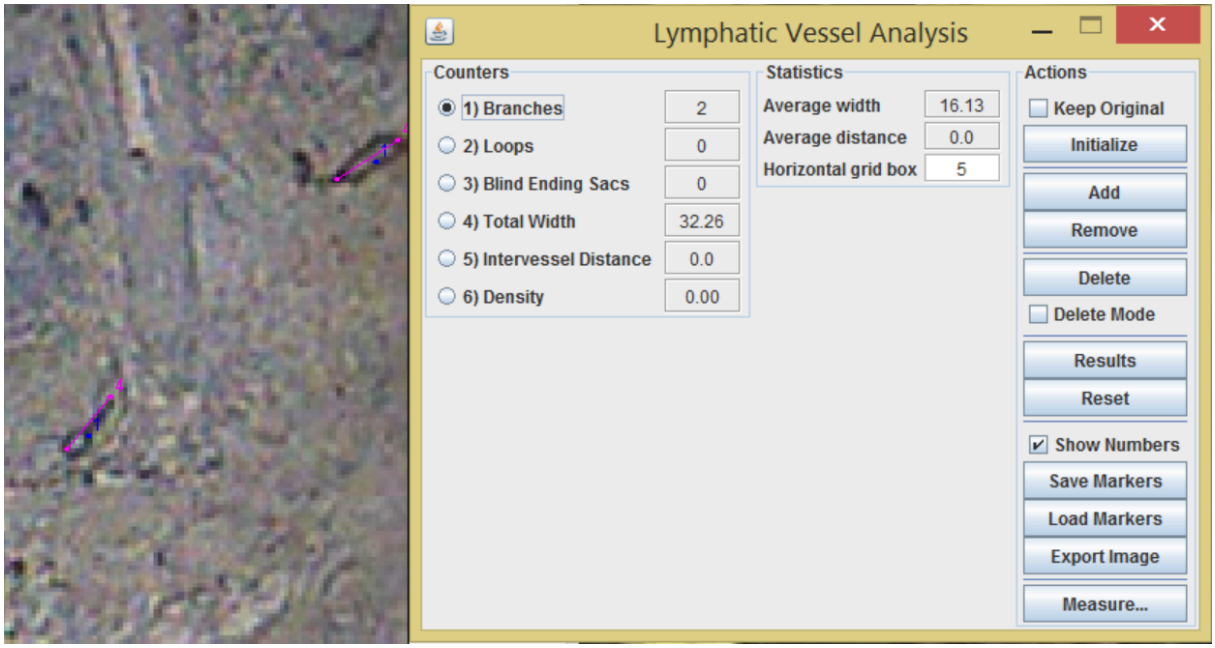

Figure 2. 9: Image J of a Lymphatic Vessel Analysis: Counting was undertaken by Marking the Vessels and Measuring the Distance. (A Pre-Binary Image was used to show the Contrast between the Vessels and the Specimen Tissue)

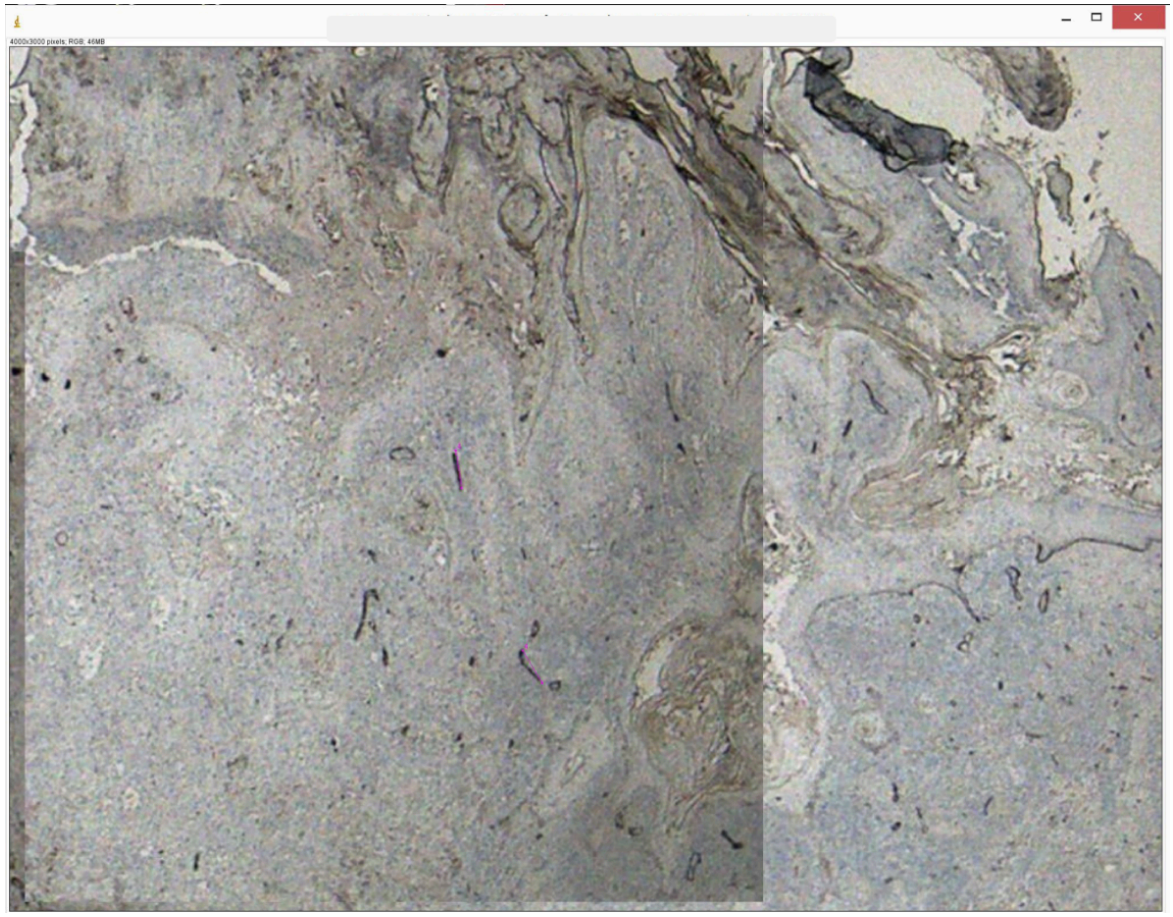

Figure 2. 10: ImageJ of a Pre-Binary Image

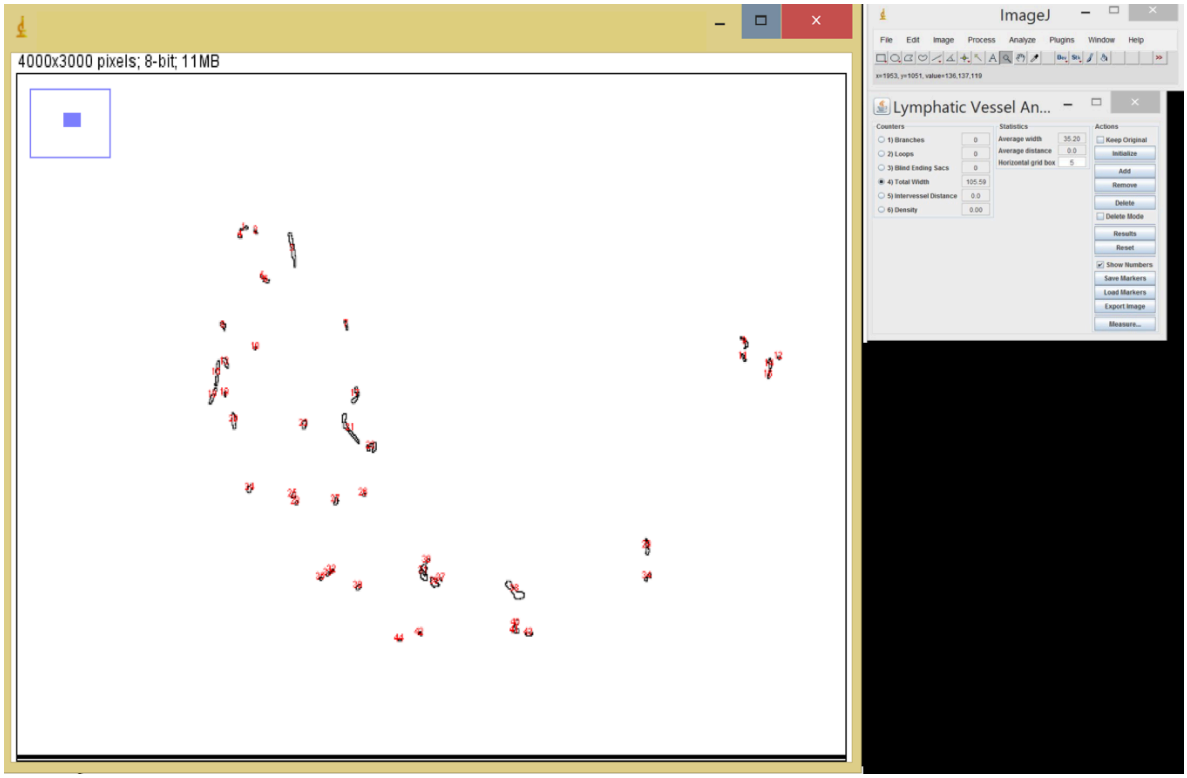

Figure 2. 11: Image J of a Binary Image (obtained using a Lymphatic Vessel Analysis Plug-in)

2.8 Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 20.0 (Chicago, Ill., USA). The following non-parametric tests were undertaken: Chi-square, Mann-Whitney and Kruskal-Wallis tests. The following parametric test were undertaken: Paired-sample t-tests and a one-way analysis of variance (ANOVA). Across all the tests, a two-sided p-value of < 0.05 was considered statistically significant. A syntax was generated for each order function.

Three data sets were created. Overall the results were as follows: well-differentiated cSCC results, moderately differentiated cSCC results and poorly differentiated cSCC results. The variable set was a plug-in. The variable view icon for each individual parameter is shown in the data variable diagram. The qualitative nominal data (i.e., gender and comorbidity data), the ordinal data (i.e., age and differentiation data) and the binary data (i.e., comorbidity and lympho-vascular data) were assigned a string type and measured in scale. The quantitative discrete data (i.e., lymphatic numbers) and the continuous data (i.e., the geometry of the lymphatic vessels) were assigned a numeric type and measured in scale. Each data section was then imported from a Microsoft Excel spreadsheet.

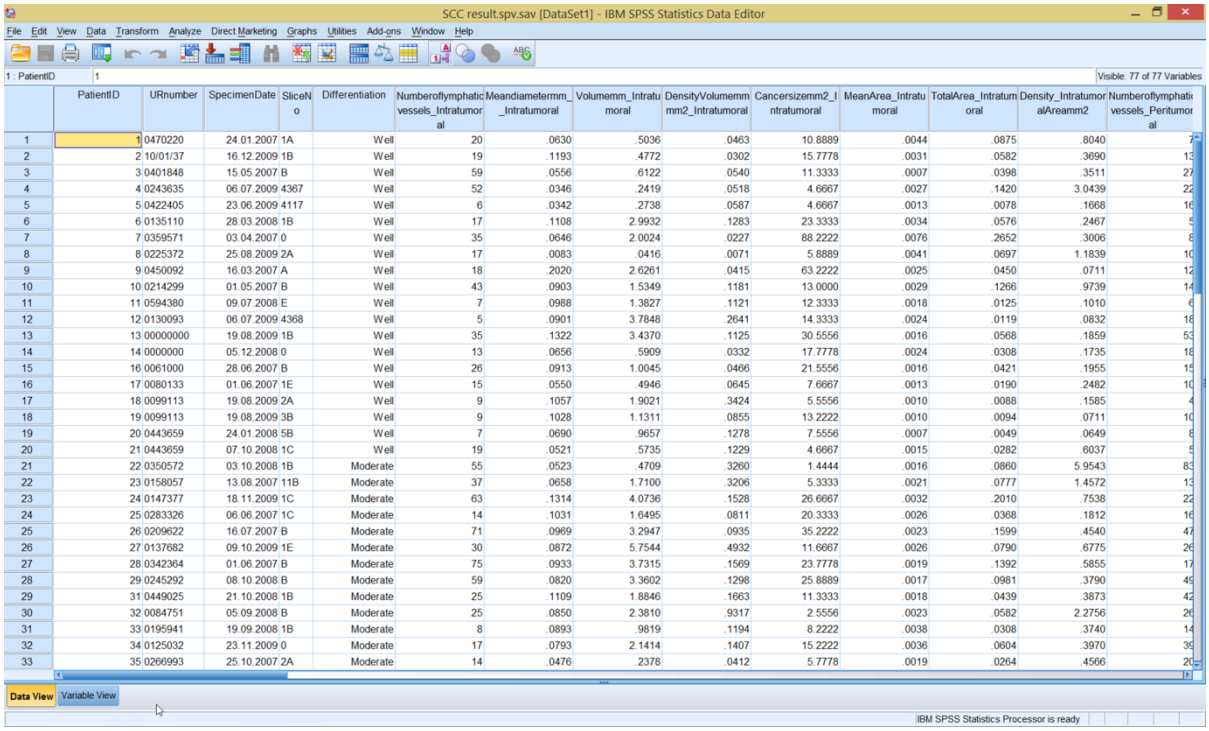

Figure 2. 12: SPSS imported data table including histological feature of the specimen and the lymphatic pattern.

2.8.1 Preliminary Analyses

The descriptive statistics for categorical variables were obtained by analysing the descriptive statistics and any frequencies in the data. Frequency results and percentages were generated. In relation to the continuous variables, descriptive statistics were used rather than frequencies so that information could be obtained about the total number, minimum, maximum, mean, standard deviation, skewness (i.e., symmetry of the distribution) and kurtosis (i.e., 'peakedness' of the distribution).

It should be noted that skewness values provide an indication of the symmetry of any distribution. A value of 0 represents a perfectly normal distribution. Positive skewness values indicate scores clustered to the left of the lower values. Negative skewness values indicate a clustering of scores on the right-hand side of the graph. Conversely, kurtosis provides information about the level of a peak on the distribution of a curve. Again, a value of 0 indicates a perfect distribution. Positive kurtosis values indicate a distribution that is clustered in the centre and has long thin tails. A value below 0 indicates a distribution that is relatively flat (i.e., a distribution that has too many cases in the extremes).

2.8.2 Assessing Normality

A normal distribution curve was used to assess the data in respect of its degree of differentiation, demographics and comorbidities. Similarly, each subgroup (i.e., each differentiation type) was analysed to examine their lymphatic geometry. The 'analyse function' button was first selected at the top menu, next 'descriptive statistics' were selected and then 'explore' was selected. The variables of interest were moved into the 'dependent list' box. The 'statistic' function button allowed for descriptive and outliers to be chosen. A histogram was created at this point by choosing the 'plots' option. A summary of means, 95 per cent confidence intervals, minimums, maximums, variances, standard deviations, range skewness, kurtosis and distribution curves in the form of a histogram, a Q-Q plot and a boxplot were generated using the drop-down menu.

2.8.3 Analysis of Parametric and Non-Parametric Statistical Technique

There are two main groups in statistics: parametric and non-parametric. Many people view parametric tests as the more powerful analytical technique; however, the data must meet many conditions if it is to be examined accurately (see discussion below).

2.8.3.1 Parametric Tests: Paired-Samples T-tests and One-way Analyses of Variance

Parametric tests are used to ascertain whether the distribution of scores in a population follows a normal distribution curve. However, parametric tests can also be used when each group has more than 15 in its sample size. In this study, a paired-samples t-test was used to make a direct comparison between the two groups (i.e., to compare the well-differentiated and less well-differentiated groups). One-way ANOVAs are used when a population has a normal distribution or otherwise has a sample size of greater than 15. In the present study, this technique was used to compare the three differentiated groups.

The functional input for analysis was chosen by selecting 'Compare Means' from the 'analyse' menu. Next, the appropriate statistical analysis techniques were selected. In this study, the following tests were chosen: paired-sample t-tests and a one-way ANOVA. The comparison variables were assigned as either 'variable 1' or 'variable 2' for the analysis.

2.8.3.2 Non-Parametric Tests: Chi-square, Mann-Whitney and Kruskal-Wallis

Non-parametric tests will be used rather than parametric tests in certain conditions (e.g., to compare normal [categorical] data if the sample size is small). It should be noted that non-parametric tests tend to be less sensitive and can fail to detect differences between groups that actually exist. The non-parametric tests were largely used to analyse the metastatic and non-metastatic groups. In the present study, Chi-square and Mann-Whitney tests were used to compare the two groups. Conversely, the Kruskal-Wallis test was used to compare the three differentiation subgroups.

To run the non-parametric test in SPSS, the 'analyse' function first was chosen from the top menu. Next, the 'non-parametric tests' option was selected. Several analytical techniques were then selected from the 'legacy dialog' options. Similar to the parametric tests, the variables being compared were then chosen and designated variable numbers.

2.8.4 Generating Graphs

SPSS was used to generate various graphs to represent different data. From the top menu, the 'graphs' function was selected. Next, the 'legacy dialogs' function was chosen. Finally, different types of graphs (e.g., box, bar, line, pie, scatterplot, matrix scatterplot and boxplot) were selected.

Chapter 3: Results

In this chapter, the results of the cSCC specimen analyses are presented. First, details about patients' demographics and the general morphological features of the cSCCs are provided. Next, the analyses of the lymphatic vessels across the three differentiation groups are examined. Notably, the following areas were examined: i) the intratumoural area (i.e., the area within the tumour); ii) the peritumoural area (i.e., the area surrounding the tumour); and iii) the overall area (i.e., both the intratumoural and peritumoural areas). Following this, comparisons are made across each of the three differentiation groups. Finally, comparisons are made between the metastatic and non-metastatic groups.

3.1 Introduction

Over a 24-month period, 74 patients were identified as being eligible to participate in this study based on cSCC specimen examinations. All of the cSCCs were taken from the head or neck region. To prevent any possibility of misrepresentations in the data and because it would be difficult to determine risk associations, each cSCC specimen was excised from a different patient (i.e., no specimen was excised from the same patient). Of the 74 cSCC specimens, 21 (i.e., 25 per cent) had well-differentiated cSCCs, 34 (i.e., 52 per cent) had moderately differentiated cSCCs and 19 (i.e., 23 per cent) had poorly differentiated cSCCs. The majority of the specimen has clear margins of at least 3 mm; however, for the specimens that had close margins (i.e., margins less than 3 mm, but more than 1 mm), further excisions were performed. No residual tumours were observed in any of the subsequent specimens.

3.2 Demographics

3.2.1 Overview

Of the 74 patients, a majority (i.e., 56) were male and 18 were female. Thus, the ratio of women to men was approximately 3:1. The mean age of the patients was 73.48 years (45–97: ±12.17 years). Of the patients, 12 were diabetic, 39 had ischaemic heart disease (IHD), 10 had a medical condition or were taking medication that resulted in immunosuppression and 22 were classified as current smokers or smoked up until the final 3 months before their surgeries.

3.2.2 Cutaneous Squamous Cell Carcinoma Subtypes

The male to female ratios were similar across the three groups. The ratio of males to females was 15 males to 6 females in the well-differentiated cSCC group, 26 males to 8 females in the moderately differentiated cSCC group and 15 males to 4 females in the poorly differentiated cSCC group. The subgroup analysis revealed that there were no statistically significant differences between the cSCC gradings (p-value = 0.85, Kruskal-Wallis test).

The mean age of the patients at the time of cSCC excision was similar across all of the groups. The mean ages of the patients in the well-differentiated group, moderate differentiated group and poorly differentiated group were 72.29 (45–97: ±13.69), 76.79 (55–94: ± 9.62) and 71.37 (39–91: ± 13.21) years, respectively (the p-value was non-significant at 0.20, One-way ANOVA). No statistically significant differences were found in relations to comorbidities or smoking habits across any of the groups. Table 6 provides a summary of patients' demographics.

3.3 Characteristics of Tumours

The size of each tumour was determined by using the maximum horizontal and vertical distances of the tumour to calculate the tumour area. In this study, the mean tumour sizes were 18.30 mm^2 in the well-differentiated cSCC group, 25.73 mm^2 in the moderately differentiated cSCC group and 33.55 mm^2 in the poorly differentiated cSCC group. No statistically significant differences were found among the three groups in relation to size (p-value = 0.23, One-way ANOVA).

The DOI was measured. The results showed that patients in the well-differentiated cSCC group had the lowest invasion mean across the three groups with an average DOI of 2.59 mm (1.33–8.33 ± 1.47). Patients in the moderately differentiated cSCC group had a mean DOI of 3.06 mm (1.33–8.3 3 ± 1.59). While patients in the poorly differentiated group had a mean DOI of 3.44 mm (1–7.67 ± 2.03). No statistically significant differences were found between the maximum DOIs across the three groups; however, the DOI did appear to increase in the less well-differentiated cSCC groups (i.e., the moderately and poorly differentiated cSCC groups). Overall, five (i.e., 6.76 per cent) of the specimens displayed perineural invasion. Of these, two (i.e., 5.88 per cent) were specimens from the moderately differentiated cSCC group and three (15.79 per cent) were specimens from the poorly differentiated cSCC group. No statistically

significant differences were found when comparisons were made among each of the subgroups. A comparison between the well-differentiated cSCC group to the less well-differentiated groups (i.e., the moderately and poorly differentiated cSCC groups) also yielded no statistically significant difference (p-value = 0.32, Kruskal-Wallis test). Table 6 includes a summary of the specimen features.

Table 3. 1: Patients' Demographics and Features of Tumours

	Well (N = 21)	**Moderate (N = 34)**	**Poor (N = 19)**	**P-value**
Gender				0.8488
Female	6	8	4	
Male	15	26	15	
Age	72.29 (45–97: ± 13.69)	76.79 (55–94: ± 9.616)	71.37 (39–91: ± 13.21)	0.2025
Comorbidity				
DM	4	6	2	0.3347
IHD	12	18	9	0.8254
Immunosuppression	1	7	2	0.2257
Smoker (within the last 3 months)	4	12	6	0.4313
cSCC size (mm^2)	18.30 (4.67–88.22: ± 20.64)	25.73 (1.44–95.11: ± 22.94)	33.55 (3.22–114.60: ± 39.78)	0.2267
cSCC DOI (mm)	2.58 (1.33–8.33: ± 1.47)	3.06 (1.33–8.33: ± 1.59)	3.44 (1–7.67: ± 2.02)	0.5874

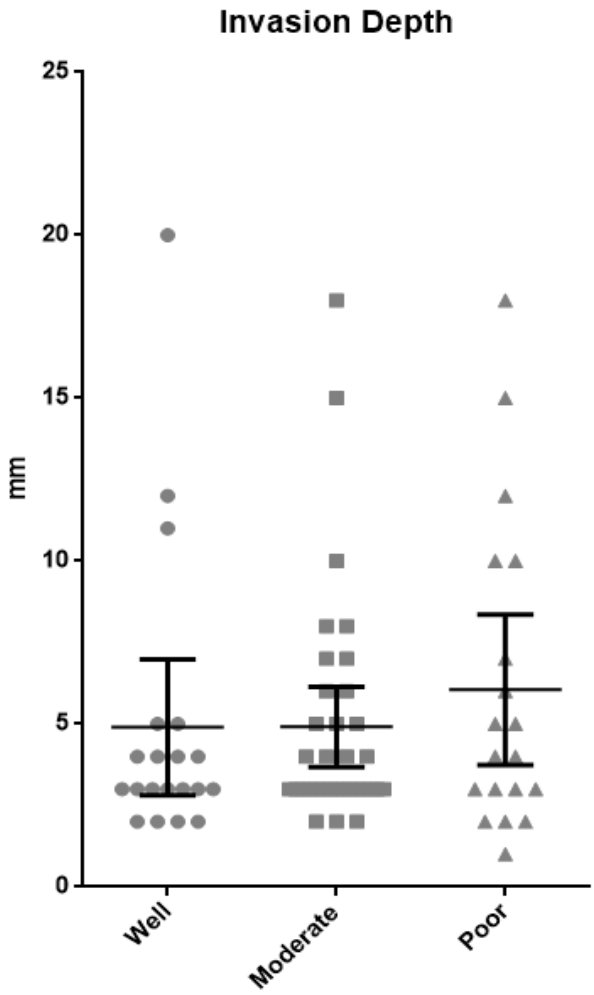

Figure 3. 1: Depth of tumour invasion by differentiation.

3.4 The General Morphology of Lymphatic Vessels

After the D2-40 staining, the lymphatic vessels could be identified under a high-power light microscope. The positively stained vessels had a brown-yellow appearance and each vessel wall (a single layer cell) was thin. Large nuclei appeared as dark brown stains.

A comparison was made of the lymphatic vessels with cSCC in the intratumoural and peritumoural areas. Notably, the morphology of the lymphatic vessels differed between the two areas in the same tumour specimens. In the peritumoural area, the lymphatic vessel lumens tended to remain open and had a large nucleus that extruded towards the lumen face. Conversely, the lymphatic vessels located in the intratumoural area were often irregular in shape and had lumen that appeared flattened and constricted. The stained nuclei could be observed, but were not clearly distinguishable from the collapsed endothelial walls.

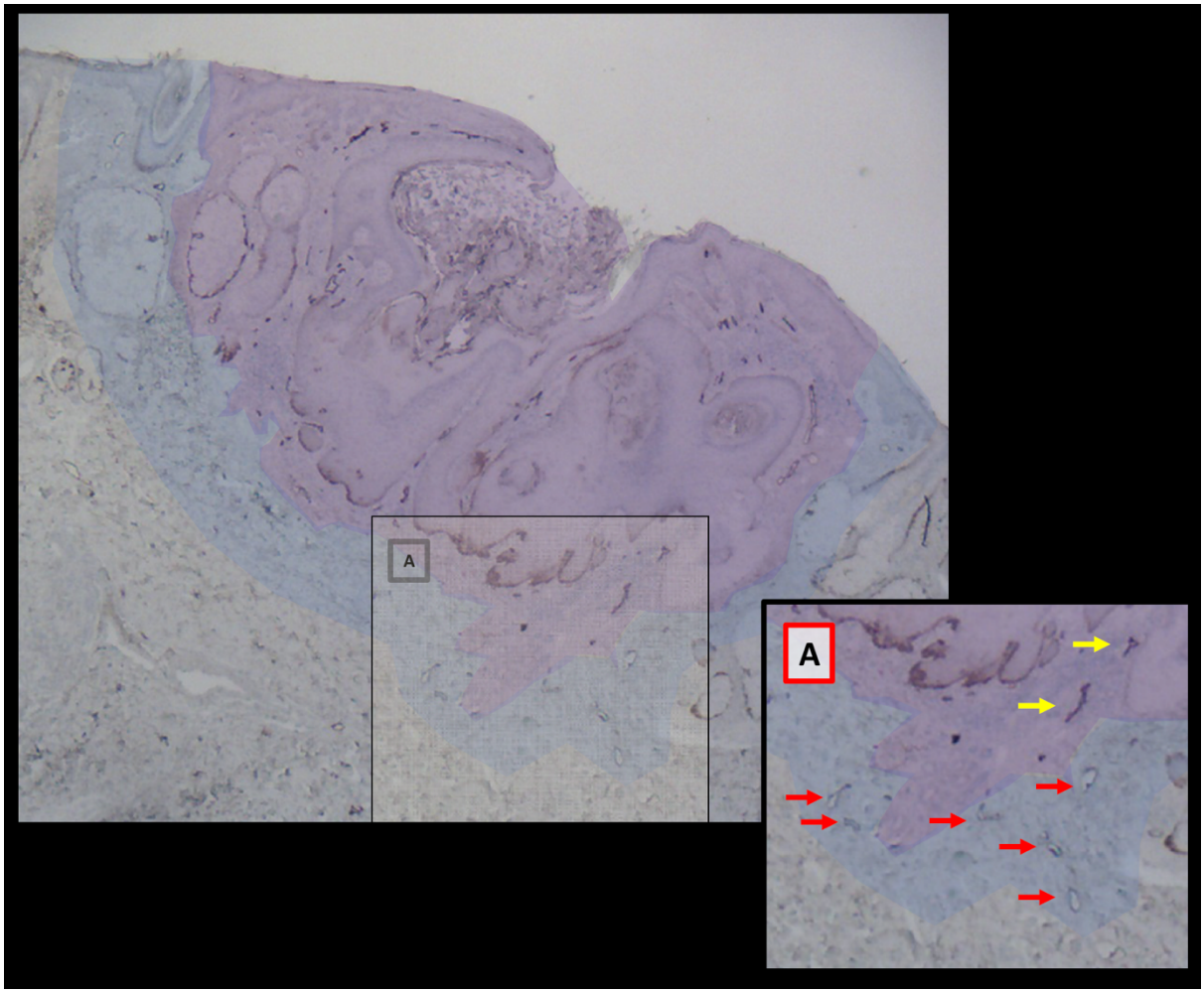

Figure 3. 2: More Collapsed Lymphatic Vessels in the Intratumoural Area (Highlighted in Purple) than the Peritumoural Area (highlighted in blue); intratumoral lymphatic vessels are shown with yellow arrows and peritumoral lymphatic vessels are shown with red arrows.

3.5 Lymphatic Patterns

Analyses were undertaken of the lymphatic patterns present in the intratumoural area, the peritumoural area (i.e., those 0. 5 mm from the most peripheral cancer cell) and both these areas.

3.5.1 Lymphatic Vessel Quantity

3.5.1.1 Intratumoural Area

The quantity of lymphatic vessels in the intratumoural area was statistically significant different (p-value = 0.001, one-way ANOVA) between the three differentiation groups. Additionally, there was an upward trend towards a higher quantity of lymphatic vessels in less well-differentiated cSCC specimens. The mean number of lymphatic vessels in the

intratumoural area was 14.76 (4–42 ± 9.41) in the well-differentiated cSCC group, 36.18 (5–109 ± 22.15) in the moderately differentiated cSCC group and 36.26 (7–115 ± 27.15) in the poorly differentiated cSCC group. Thus, it appears that the less well-differentiated tumours had more lymphatic vessels.

3.5.1.2 Peritumoural Area

Similarly, the lymphatic vessel quantities present in the groups were compared in relation to the peritumoural region. A higher number of lymphatic vessels were present within the 0.5 mm adjacent to the tumour edge (p-value = 0.03, one-way ANOVA). The number of lymphatic vessels present also appeared to increase as the tumours became less well differentiated. The mean number of lymphatic vessels in the peritumoural area was 11.33 (4–25 ± 5.82) in the well-differentiated cSCC group, 16.97 (5–53 ± 10.32) in the moderately differentiated cSCC group and 18.68 (10–37 ± 9.21) in the poorly differentiated cSCC group.

3.5.1.3 Overall Area

Given the results of the above two analyses, it was unsurprising that combining the two areas (i.e., the intratumoural and peritumoural areas) revealed similar results. The mean number of lymphatic vessels when the two areas were combined was 25.90 (13–54 ± 11.92) in the well-differentiated cSCC group, 53.12 (14–144 ± 27.74) in the moderately differentiated cSCC group and 53.89 (20–145 ± 30.58) in the poorly differentiated cSCC group. Thus, the poorly differentiated cSCC group had the highest total number of lymphatic vessels when the two areas were combined. The differences in the overall total lymphatic vessel quantities were found to be statistically significant. Specifically, the less well-differentiated cSCC groups had higher numbers of lymphatic vessels than the other groups (p-value = 0.0005, one-way ANOVA).

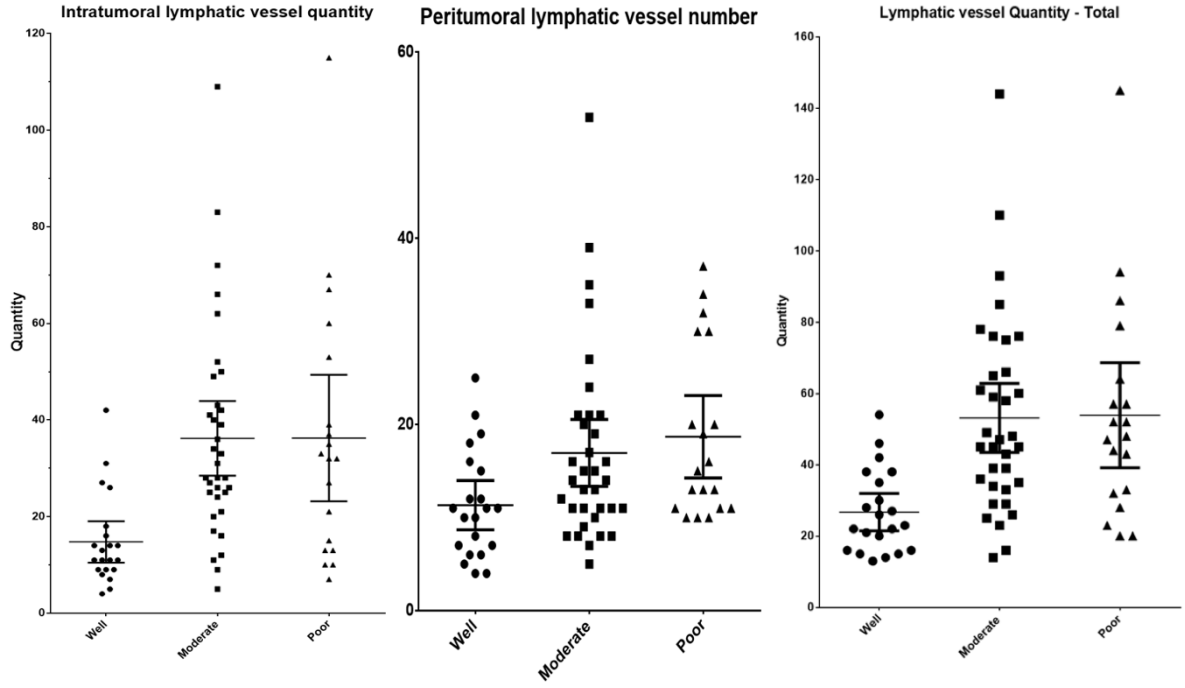

Figure 3. 3: Number of Lymphatic vessels in intra-tumoural, peritumoral and both regions.

3.5.2 Lymphatic Vessel Diameter

No lymphatic vessel is a true circle. Thus, the measurement of a lymphatic vessel has to be taken from the longest diameter. As described above in the Methodology section, ImageJ software was used to measure the lymphatic vessels. The specimens were divided into groups based on the degree of differentiation and then subdivided into intratumoural, peritumoural and overall areas so that further comparisons could be drawn.

3.5.2.1 Intratumoural Area

In relation to the intratumoural area, no statistically significant differences were found between the three groups (p-value = 0.4817, one-way ANOVA). However, specimens in the moderately and poorly differentiated cSCC groups had higher mean maximum diameters than specimens in the well-differentiated cSCC group. The mean lymphatic vessel maximum diameter of specimens in the well-differentiated group was 0.08 (0.01–0.2 ± 0.04) mm. Conversely, specimens in the moderately differentiated group had a mean lymphatic vessel maximum diameter of 0.12 (0.05–1.06 ± 0.17) mm, a diameter much larger than that of the well-differentiated group. Similarly, specimens in the poorly differentiated group had a mean maximum diameter of 0.11 (0.05–0.17 ± 0.04) mm.

3.5.2.2 Peritumoural Area

Interestingly, the findings differed when the area adjacent to the tumours themselves were analysed. In the peritumoural area, the lymphatic vessels were larger in specimens in the less well-differentiated cSCC groups. Further, the difference was statistically significant (p-value = 0.0002, one-way ANOVA). An upwards trend was also identified in which diameters increased as cSCCs became less well-differentiated. Specimens in the well-differentiated cSCC group had a mean lymphatic maximum diameter of 0.08 (0.01–0.19 ± 0.08) mm. A higher mean lymphatic vessel maximum diameter of 0.10 (0.05–0.17 ± 0.03) mm was observed in specimens in the moderately differentiated cSCC group. While specimens in the poorly differentiated cSCC group had a mean lymphatic maximum diameter of 0.12 (0.05–0.17 ± 0.03) mm. Further analyses revealed a linear correlation whereby the diameter of lymphatic vessels increased with the less well-differentiated characteristics of tumours. The comparison of the well-differentiated cSCC group to the less well-differentiated cSCC group was found to be statistically significant (p-value = 0.001, paired-sample t-tests). Similarly, a p-value of 0.0004 was found when a direct comparison was made between the poorly differentiated cSCC group and the moderately and well-differentiated cSCC groups.

3.5.2.3 Overall Area

The intratumoural analysis revealed no statistically significant differences in the sizes of the lymphatic vessels; however, an analysis of the overall area (i.e., an analysis of both the intratumoural and peritumoural areas) revealed a statistically significant correlation whereby the size of the lymphatic vessels size was larger in less well-differentiated cSCCs. Combining the intratumoural and peritumoural areas revealed an increase in the size of the lymphatic vessels (p-value = 0.003, one-way ANOVA). The well-differentiated group had a mean lymphatic vessel maximum diameter of 0.08 (0.01–0.20 ± 0.04) mm. A larger diameter was observed in the moderately differentiated group, which had a mean lymphatic vessel maximum diameter of 0.10 (0.05–0.14 ± 0.02) mm. Of the three groups, the poorly differentiated cSCC group had the largest lymphatic vessel diameter mean with a mean lymphatic vessel maximum diameter of 0.11 (0.06–0.17 ± 0.03) mm. Further analyses were undertaken to compare the well-differentiated group to the moderately and poorly differentiated groups combined. A statistically significant increase was found between the groups (p-value = 0.004, one-way ANOVA). A comparison of the poorly differentiated cSCC group to the well- and moderately differentiated cSCC groups combined revealed that the specimens in poorly differentiated

group had large lymphatic vessel diameters (p-value = 0.005, one-way ANOVA). These results are similarly to those found for specimens in the peritumoural area and suggest that the diameters of lymphatic vessels are larger in less well-differentiated tumours.

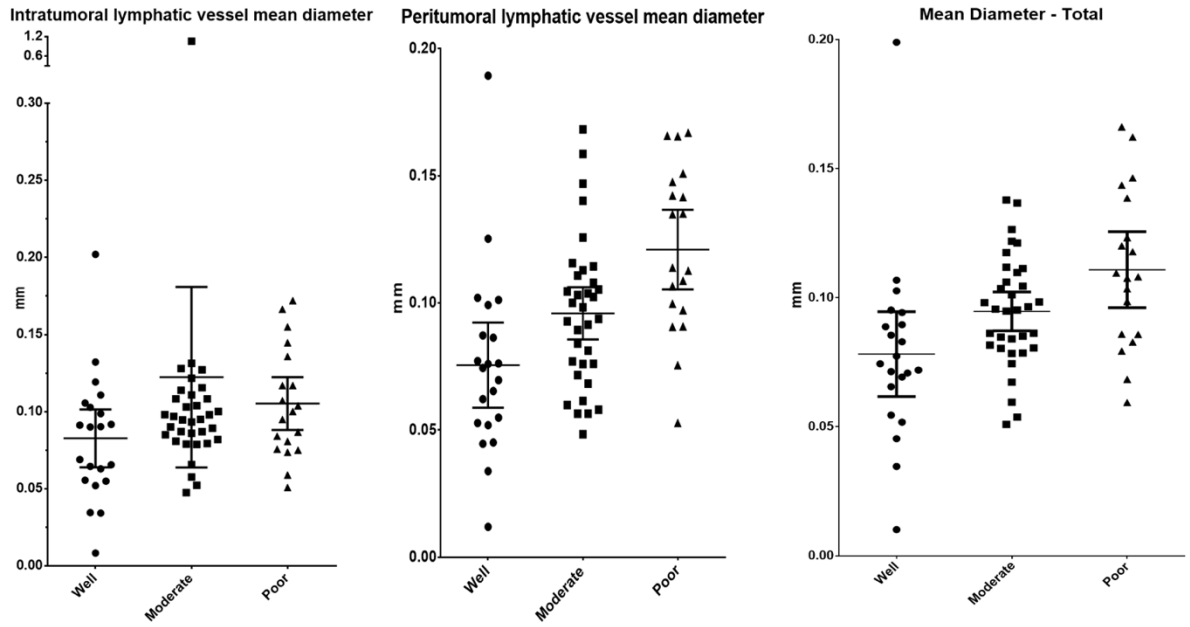

Figure 3. 4: Mean Lymphatic vessels maximum diameter in intra-tumoural, peritumoral and both regions. NB: error bar indicates one standard deviation.

3.5.3 The Cross-sectional area of Lymphatic Vessels

The size and the quantitative value of the lymphatic vessels in the cross-sectional specimens provided insights into the effects of differentiation on lymphatic patterns. However, a simple increase in number or size alone has a less pronounced effect on lymphatic flow than an increase in both parameters. Simply put, multiple small water pipes or a few large pipes have less drainage capacity (in terms of cross-sectional area) than multiple large pipes.

In a Newtonian fluid, it is well understood in fluid mechanics that there is an inverse relationship between flow and radius of the pipe. This is explained by the use of Poiseuille law. As the radius of the pipe increases, the cross-sectional volumetric flow rate increase provided that the pipe length, fluid viscosity and pressure difference between the two ends are constant which is true in living lymphatic vessel. The opposite is also true, a decrease in pipe radius (small lymphatic vessel will result in a decrease flow). Hence, a higher flow cross-sectional area across the lymphatic channel in a larger diameter. Algebra work on Poiseuille law is shown below.

$$\Delta P = \frac{8\mu LQ}{\pi R^4}$$

$$\pi R^4 = \frac{8\mu LQ}{\Delta P}$$

$$R = \frac{8\mu LQ}{\Delta P}$$

ΔP = Pressure difference between the two ends
L = Length of the pipe
μ = Viscosity
Q = volumetric flow rate
R = Radius

The total cross-sectional area was analysed. The total cross-sectional area of each specimen was calculated by multiplying the total diameter by the total numbers of lymphatic vessels within a specimen:

$$V = \mathrm{N} \cdot \mathrm{D}$$

where V = lymphatic cross-sectional area, N = the number of lymphatic vessels and D = the diameters of the lymphatic vessels.

3.5.3.1 Intratumoural Area

In relation to the intratumoural area, no statistically significant difference in the cross-sectional area of the intratumoural lymphatic vessels was found across the three differentiation cSCC groups (p-value = 0.1216, one-way ANOVA). However, a direct comparison between the well-differentiated cSCC group and the less well-differentiated groups (i.e., the moderately and poorly differentiated groups), revealed a statistically significant increase in cross-sectional area (p-value = 0.05, paired-sample t-tests). The mean lymphatic cross-sectional area in the well-differentiated cSCC group was 1.33 (0.04–3.79 ± 1.09) mm. The moderately differentiated group had the highest mean lymphatic cross-sectional area of all the three groups with a mean of 5.04 (0.24–54.70 ± 9.12) mm. The poorly differentiated group had a lower vessel cross-sectional area compared to the moderately differentiated group, but with a mean lymphatic cross-sectional area of 3.74 (0.59–10.92 ± 2.94) mm, it still had a higher cross-sectional area than the well-differentiated group. This could be because a lymphatic vascular collapse

occurred as a result of the highly distraught architecture of the tumours (see the discussion section below).

3.5.3.2 Peritumoural Area

A trend towards an increase in lymphatic cross-sectional area was observed in the peritumoural area. This was unsurprising, given that both the quantity and diameters of the lymphatic vessels in the peritumoural area increased as the tumours became less well-differentiated. This change was statistically significant (p-value = 0.001, one-way ANOVA). In the well-differentiated group, the mean lymphatic cross-sectional area within the peritumoural area was 0.94 (0.10–4.74 ± 0.10) mm. In the moderately differentiated group, the mean lymphatic cross-sectional area was 1.64 (0.24–4.31 ± 1.03) mm. With a mean of 2.34 (0.58–5.24 ± 1.41) mm, the poorly differentiated group had the largest lymphatic cross-sectional area in the peritumoural area. Thus, less well-differentiated tumours were observed to have larger lymphatic cross-sectional areas in the peritumoural area.

3.5.3.3 Overall Lymphatic cross-sectional area

The overall mean of the total lymphatic cross-sectional area was significantly higher in the less well differentiated group compared to well differentiated group (p-value = 0.0003, one-way ANOVA). Increases in lymphatic cross-sectional area were associated with less well-differentiated tumours. The mean lymphatic cross-sectional area was 2.27 (0.14–7.36 ± 1.78) mm in the well-differentiated cSCC group. The moderately differentiated cSCC group had a mean lymphatic cross-sectional area of 5.23 (0.71–15.80 ± 3.20) mm. The poorly differentiated cSCC group had the highest overall lymphatic cross-sectional area of the three groups with a mean of 6.08 (1.59–15.89 ± 3.81) mm. A direct comparison between the well-differentiated and the other two groups revealed a statistically significant increase in lymphatic cross-sectional area in the latter group (p-value < 0.001, paired-sample t-tests).

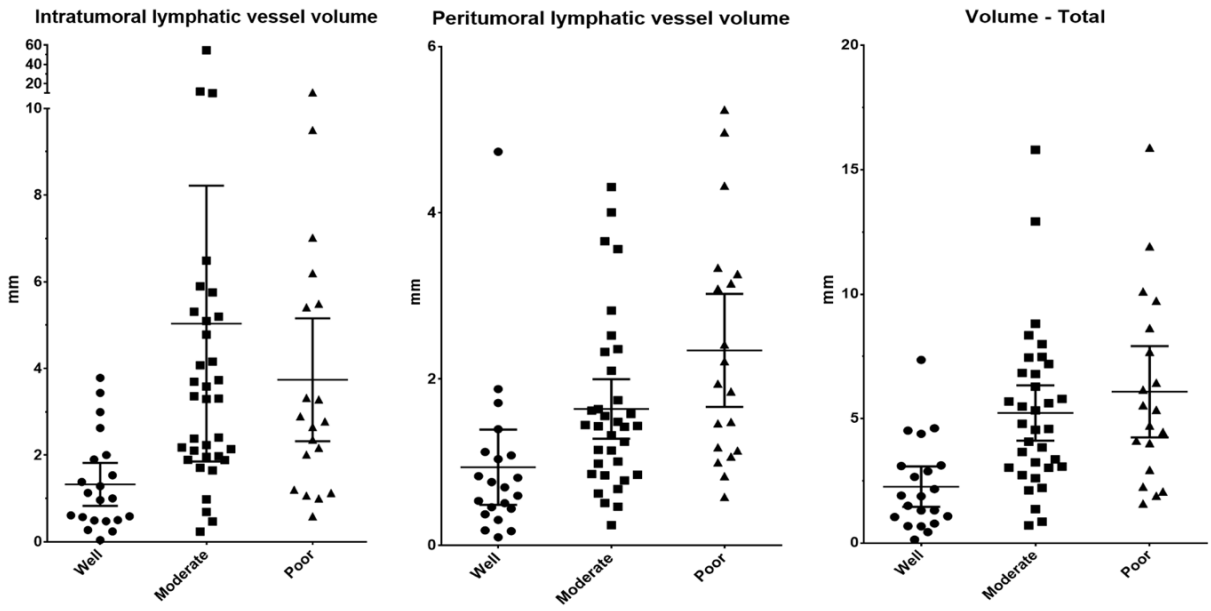

Figure 3. 5: Mean Lymphatic vessels cross-sectional area in intra-tumoural, peritumoral and both regions. NB: error bar indicates one standard deviation.

3.5.4 Lymphatic Vessel Density

Another factor that may have interfered with the results were the sizes of the tumours themselves. The larger the tissue, the more structure (e.g., lymphatic vasculature) is required to maintain its survival. To remove any errors related to tumour size, the LVDs of specimens were calculated. LVD was calculated by dividing the lymphatic cross-sectional area (mm) with the tumour size (mm^2):

$$\rho = \frac{V}{S}$$

where ρ = lymphatic density, V = lymphatic cross-sectional area and S = tumour size.

3.5.4.1 Intratumoural Area

Again, an increase in intratumoural area in a less well-differentiated group was found to be statistically significant (p-value = 0.01, one-way ANOVA). Higher densities were also found in the less well-differentiated groups. The mean intratumoural lymphatic density was 0.10 (0.01–0.34 ± 0.08) mm/mm^2 in the well-differentiated group. A higher lymphatic density was observed in the moderately differentiated group, which had a mean of 0.24 (0.04–0.93 ± 0.20) mm/mm^2. The poorly differentiated group had the highest mean tumour size (despite not being

statistically significant, as stated above) and the highest density of all the groups. It had a mean intratumoural density of 0.32 (0.08–0.87 ± 0.32) mm/mm^2.

3.5.4.2 Peritumoural Area

Similar findings were found when measuring lymphatic density in the peritumoural area. Lymphatic density increased as the tumours became less well-differentiated. The analysis revealed statistically significant differences among the three groups (p-value < 0.001, one-way ANOVA). Direct comparisons were made between the well-differentiated group and the less well-differentiated groups (i.e., the moderately and poorly differentiated groups) and the poorly differentiated group and the less poorly differentiated groups (i.e., the well- and moderately differentiated groups). The results revealed a statistically significant increasing trend towards the less well-differentiated groups (with p-values of 0.002 and < 0.001, respectively, paired-sample t-tests). In the well-differentiated group, the mean lymphatic density in the peritumoural area was 0.34 (0.06–0.73 ± 0.20) mm/mm^2. The mean lymphatic density in the moderately differentiated group was 0.51 (0.08–1.28 ± 0.29) mm/mm^2. The poorly differentiated group had the highest mean lymphatic density at 0.78 (0.28–1.77 ± 0.43) mm/mm^2.

3.5.4.3 Overall Area

The overall total density (derived by combining the intratumoural and peritumoural areas) also revealed a statistically significant increase in the density of specimens in less well-differentiated cSCC groups (p-value = 0.001, one-way ANOVA). A statistically significant linear increase was found in the lymphatic density of specimens when a direct comparison was made between the well-differentiated and less well-differentiated cSCC groups (p-value = 0.001, paired-sample t-tests) and the poorly differentiated and less poorly differentiated cSCC groups (p-value = 0.011, paired-sample t-tests). The mean overall area of lymphatic density for the well-differentiated group was 0.13 (0.02–0.34 ± 0.08) mm/mm^2. In the moderately differentiated group, the mean lymphatic density was 0.25 (0.05–0.64 ± 0.16) mm/mm^2. However, the poorly differentiated group had the highest mean of lymphatic density at 0.32 (0.08–0.87 ± 0.21) mm/mm^2.

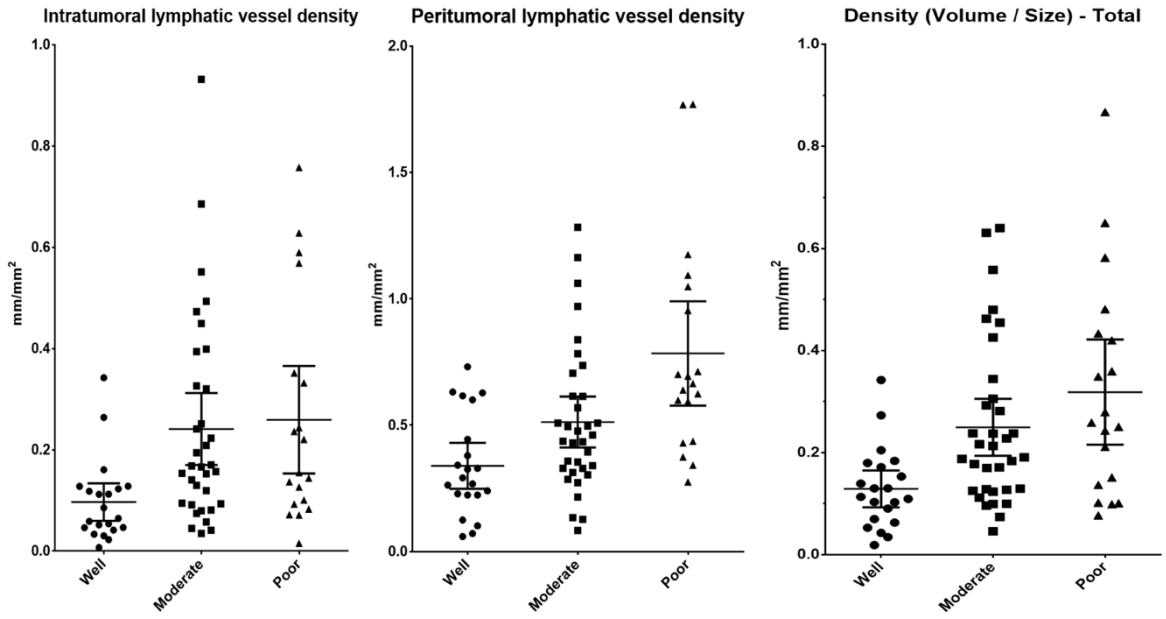

Figure 3. 6: Lymphatic vessel density in intra-tumoural, peritumoral and both regions. NB: error bar indicates one standard deviation.

3.5.5 Plain Language Summary

As stated above, the majority (i.e., 75.68 per cent) of the patients in this study were male; however, the distribution was evenly spread across the three differentiation groups. There were no statistically significant differences among the groups in relation to the demographic parameters (i.e., age, comorbidity and smoking status). Five specimens showed cutaneous perineural invasion (i.e., two specimens in the moderately differentiated group and three in the poorly differentiated group). In relation to size, no statistically significant differences were found among the three differentiation groups (p-value = 0.23, one-way ANOVA). However, there was an increase in the number of larger sized tumours in the less well-differentiated groups. Similarly, in relation to DOI, no statistically significant differences were found among the three differentiation groups (p-value = 0.59, one-way ANOVA). However, an increase in DOI was observed in the less well-differentiated group. Examining the overall morphology of the specimens, it appears that the D2-40 had a higher uptake in the less well-differentiated group, which was suggestive of an increase in number, size and density.

Microscopic analyses revealed statistically significant differences in lymphatic quantities in the intratumoural, peritumoural and overall areas (p-values < 0.05, one-way ANOVA). Statistically significant increases in lymphatic size were observed in the less well-differentiated group in both the peritumoural and overall areas. Interestingly, no statistically significant

differences were observed among the three groups in the intratumoural areas. Of the three groups, the moderately differentiated group had the largest mean maximum diameter and the poorly differentiated group had the second largest mean maximum diameter. This could be because the poorly differentiated cSCCs had a highly disrupted cellular architecture that caused the lymphatic vessel diameters to collapse.

Similar results were found in relation to the lymphatic cross-sectional areas of the three differentiation groups. An increasing trend towards higher lymphatic cross-sectional area was observed in the peritumoural and overall areas (p-values = 0.001 and < 0.001, respectively, one-way ANOVA); however, this increasing trend towards a higher lymphatic cross-sectional area only occurred in the less well-differentiated groups (p-value = 0.12, paired-sample t-tests). Again, the moderately differentiated group had the highest lymphatic vessel cross-sectional area and the well-differentiated group had the lowest lymphatic vessel cross-sectional area. Similar to the explanation given for the collapse of diameters, an extrinsic mechanical force could explain these findings (see the discussion section below). An analysis of the density (i.e., the cross-sectional area of a lymphatic vessel in each tumour area) revealed an increasing trend towards the less well-differentiated groups in the three areas (i.e., the intratumoural, peritumoural and overall areas). This trend was found to be statistically significant with p-values of 0.01, < 0.001 and < 0.001, respectively (one-way ANOVA).

3.6 Metastasis and Squamous Cell Carcinomas

Within the cohort, a total of nine (i.e., 10.8 per cent) patients demonstrated pathological and radiological evidence of distant metastasis. None of the specimens of the metastatic patients displayed any associated perineural invasion. Thus, none of the metastatic patients were immunocompromised. All of the metastatic patients were male and had a mean age of 71.78 (58–87 ± 10.71). Six (i.e., 66.67 per cent) of the metastatic patients had moderately differentiated cSCCs of the head and neck region and three (i.e., 33.33 per cent) metastatic patients had poorly differentiated cSCCs. None of the patients in the well-differentiated group were metastatic.

3.6.1 A Comparison of the Metastatic and Non-Metastatic Groups

3.6.1.1 Intratumoural Area

In terms of the number of lymphatic vessels, an examination of the intratumoural area revealed no statistically significant differences among the groups (p-value = 0.15, paired-sample t-tests). The mean number of lymphatic vessels was higher in the metastatic group than the non-metastatic group. The metastatic group had a mean number of 52.00 (20–109 ± 35.84) lymphatic vessels and the non-metastatic group had a mean number of 36.18 (5–109 ± 22.15) lymphatic vessels. There was no statistically significant difference in the size of the lymphatic vessels in the intratumoural area. The mean size of the lymphatic vessels in metastasis group was 0.11 (0.9–0.12 ± 0.01) mm and the mean size in non-metastatic group was 0.12 (0.05–1.06 ± 0.17) mm (p-value = 0.89, paired-sample t-tests). There were no differences between the lymphatic cross-sectional areas of the two groups (p-value = 0.87, paired-sample t-tests). The mean lymphatic cross-sectional area for the metastatic group was 5.69 (1.96–11.80 ± 4.23) mm and the mean cross-sectional area of the non-metastatic group was 5.04 (0.24–54.70 ± 9.12) mm. There was no statistically significant difference between the metastatic and non-metastatic groups in relation to intratumoural lymphatic density (p-value = 0.38, paired-sample t-tests). The mean lymphatic density of the metastatic and non-metastatic groups was 0.17 (0.02–0.25 ± 0.09) mm/mm^2 and 0.24 (0.04–0.93 ± 0.20) mm/mm^2, respectively. However, the graph shows that each individual metastasis specimen had a higher intratumoural number, diameter, cross-sectional area and density than the mean of the well-differentiated group (as denoted by the orange dotted line).

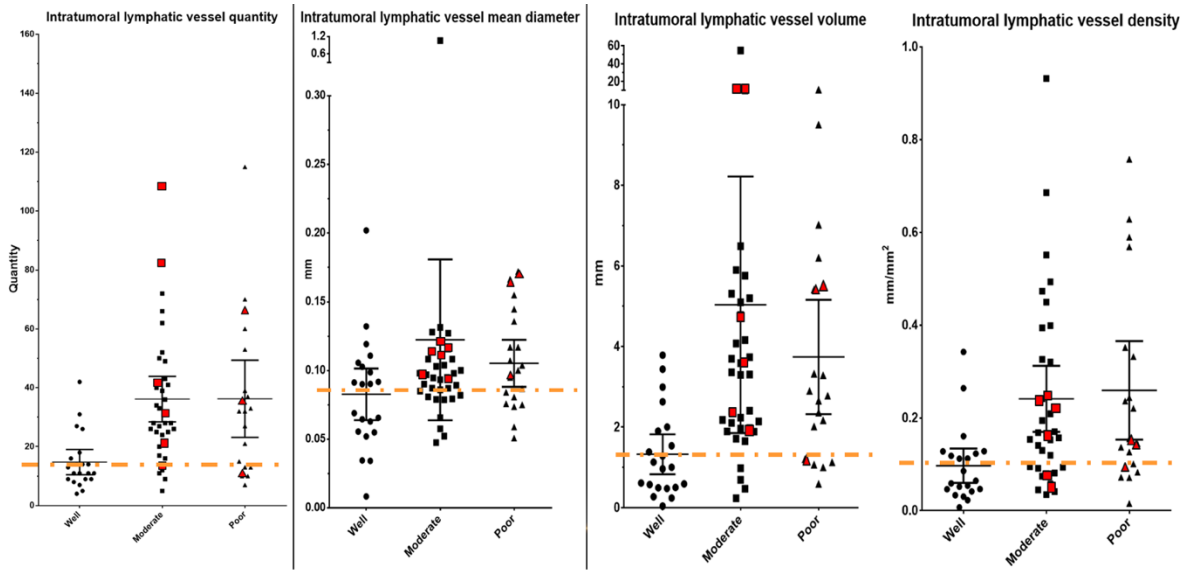

Figure 3. 7: Comparison of Metastatic (red dots) and non-metastatic (black dots) cSCC lymphatic vessels within intratumoral region. Orange line indicated mean lymphatic feature of non-metastatic group. NB: error bar indicates one standard deviation.

3.6.1.2 Peritumoural Area

The analysis of the peritumoural area yielded different results to those found in the analysis of the intratumoural area. In terms of the number of lymphatic vessels, the p-value of 0.056 (paired-sample t-tests) approached statistical significance. Again, the mean lymphatic number was higher in the metastatic than the non-metastatic group. The metastatic group had a mean lymphatic number of 26.17 (11–39 ± 12.17) vessels and 16.94 (5–59 ± 10.32) vessels. The mean maximum diameters of the lymphatic vessels were similar across both groups (p-value = 0.73). The mean lymphatic maximum diameter was 0.10 (0.08–0.11 ± 0.01) in the metastatic group and 0.10 (0.05–0.17 ± 0.01) in the non-metastatic group. A statistically significant higher in lymphatic cross-sectional area was observed in metastatic group (p-value = 0.03, paired-sample t-tests). The mean lymphatic cross-sectional area of the metastatic group was 2.69 (0.85–4.00 ± 1.34) mm and the mean lymphatic cross-sectional area of the non-metastatic group was 1.64 (0.24–4.31 ± 1.03) mm. The metastatic group also had a higher peritumoural lymphatic density than the non-metastatic group (p-value = 0.04, paired-sample t-tests). The mean lymphatic density was 0.79 (0.34–1.28 ± 0.35) mm/mm^2 in the metastatic group and 0.51 (0.08–1.28 ± 0.29) mm/mm^2 in the non-metastatic group. Again, each individual metastatic specimen a had higher peritumoural number, diameter, cross-sectional area and density compared to the means of the well-differentiated group (as denoted by the orange dotted line).

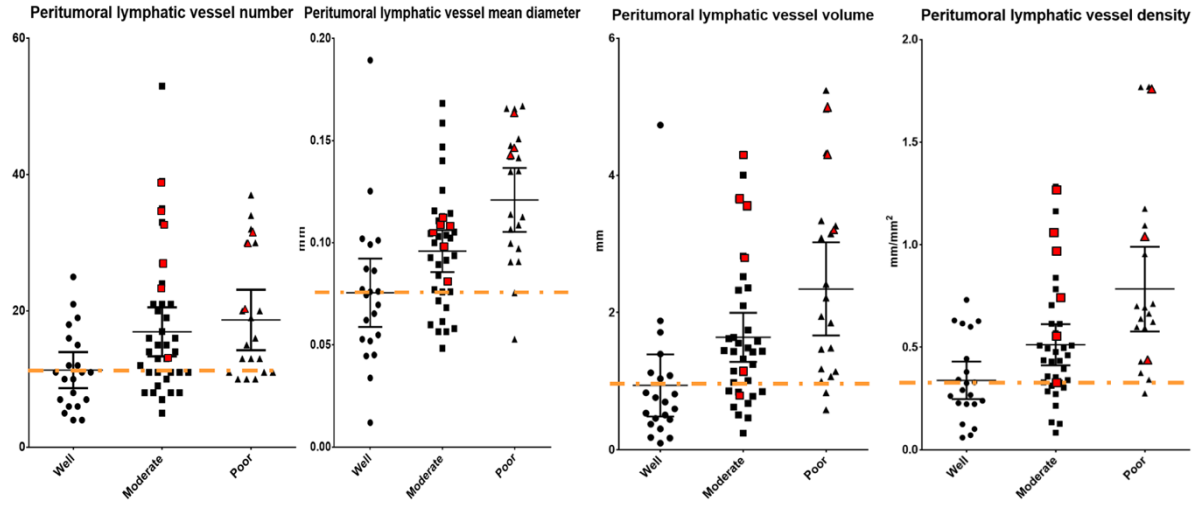

Figure 3. 8: Comparison of Metastatic (red dots) and non-metastatic (black dots) cSCC lymphatic vessels within Peritumoral region. Orange line indicated mean lymphatic feature of non-metastatic group. NB: error bar indicates one standard deviation.

3.6.1.3 Overall Area

The overall area analysis (i.e., of the intratumoural and peritumoural areas) showed that the metastatic group had a higher number of lymphatic vessels than the non-metastatic group; however, the difference was not statistically significant (p-value = 0.07, paired-sample t-tests). The metastatic group had a mean number of 73.78 (36–144 ± 36.60) lymphatic vessels and the non-metastatic group had a mean number of 53.12 (14–144 ± 27.74) lymphatic vessels. The difference in the sizes of the lymphatic vessels between the two groups was statistically significant (p-value = 0.01, paired-sample t-tests). The metastatic group had a larger mean vessel maximum diameter of 0.12 (0.08–0.17 ± 0.03) mm than the non-metastatic group, which had a mean vessel maximum diameter of 0.10 (0.05–0.14 ± 0.02) mm. In terms of total cross-sectional area, the metastatic group had a higher mean lymphatic total cross-sectional area than the non-metastatic group (p-value = 0.02). The metastatic group had a total mean lymphatic cross-sectional area of 8.34 (3.03–15.80 ± 4.07) mm and the non-metastatic group had a total mean lymphatic cross-sectional area of 5.23 (0.71–15.80 ± 3.20) mm. Despite differences in overall lymphatic size and cross-sectional area and a trend towards a higher number when tumour size was taken into account, the differences were not statistically significant (p-value = 0.75, paired-sample t-tests). Additionally, the metastatic group had a lower lymphatic cross-sectional area per tumour size with a mean lymphatic density of 0.23 (0.04–0.35 ± 0.10) mm/mm^2. The mean lymphatic density in the non-metastatic group was 0.25 (0.05–0.64 ± 0.16) mm/mm^2. Each individual metastatic specimen had a higher intratumoural and peritumoural number, diameter, cross-sectional area and density compared to the means of the specimens in well-differentiated group (as denoted by the orange dotted line). Thus, similar findings were observed in the overall area.

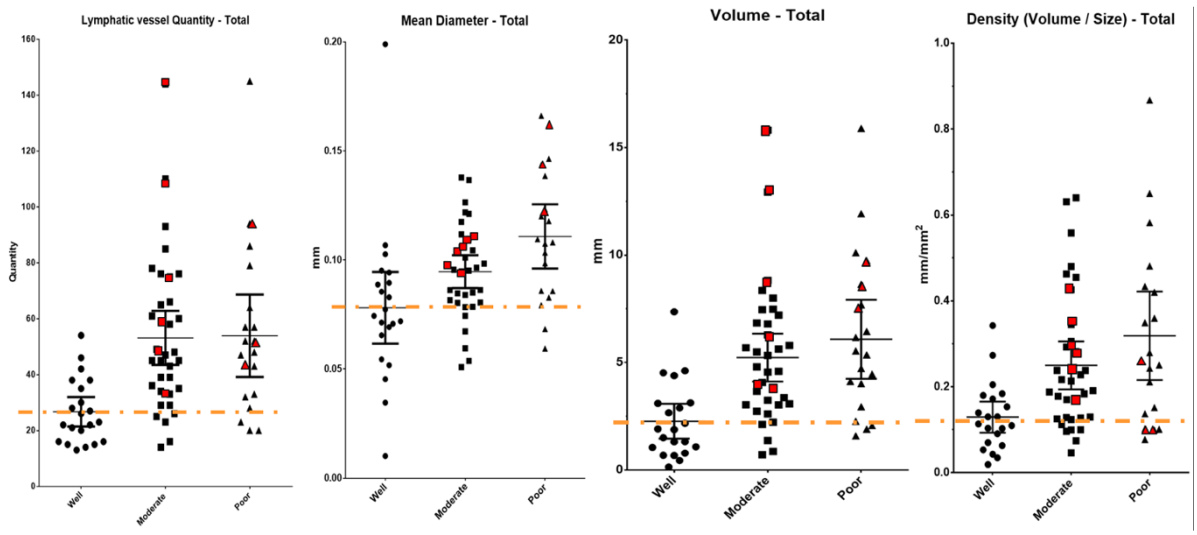

Figure 3. 9: Comparison of Metastatic (red dots) and non-metastatic (black dots) cSCC lymphatic vessels within both intratumoral and peritumoral region. Orange line indicated mean lymphatic feature of non-metastatic group. NB: error bar indicates one standard deviation.

3.6.2 Plain Language Summary

The subgroup analysis, which examined the effect of metastatic conditions on the lymphatic patterns, yielded interesting results. The two groups shared similar demographic characteristics. Metastatic spread was only observed in patients in the less well-differentiated groups (i.e., five patients in the moderately differentiated group and three patients in the poorly differentiated group). A direct comparison between metastasis and non-metastasis of the same differentiation degree revealed no statistically significant difference in the intratumoural region. However, the mean quantitative number of lymphatic vessels was higher in the metastatic group. A similar result was also observed when examining the area surrounding the tumours themselves. However, this difference was not statistically significant. An overall analysis of both regions showed that the metastatic group had a higher number of lymphatic vessels and a p-value that approached significance (p-value = 0.07, Mann-Whitney tests). The size and cross-sectional area of the lymphatic vessels were found to be higher in the metastatic group (p-value = 0.01 and p-value = 0.02; respectively, Mann-Whitney test). The overall lymphatic density (i.e., total surface area of the lymphatic vessel compared to the tumour specimen) was similar between the two groups. Thus, it appears that the metastatic spread of a tumour is associated with increased lymphatic growth patterns in terms of size and number, but not the cross-sectional surface area. Additionally, such changes were more apparent when both the intratumoural and peritumoural areas were combined.

The results did show a trend toward an increase in lymphatic vessels in the metastasis tumour, particularly around the tumours themselves. It may be that vascular and lymphatic growth factors are released by tumours to maintain their high metabolic processes. Further, an increase in lymphatic structure provides more opportunities for a tumour to invade through the lymph channel and thus can result in distant disease deposits (i.e., metastasis). The results of this study are discussed further in the next chapter.

Chapter 4: Discussion

In this chapter, a number of theories on how cSCCs and other types cancer metastasise are presented with a particular focus on lymphatic spread. The theory of direct lymphatic invasion has largely been replaced by the theory of lymphangiogenesis. This chapter outlines current understandings and research on how growth factors are released by tumours and their effects on lymphatic vessel morphology. Also considered are the findings of the present study in relation to other research. Finally, this chapter examines the impact of the present study and its potential use in the development of a new additional prognostic indicator that help determine cSCC metastatic potential.

4.1 Introduction

Non-melanoma skin cancer is the most common type of cancer and cSCC is the second most common type of cancer to affect Caucasians. In the United States, the condition accounts for approximately 10,000 deaths annually [77,132]. In Europe, it has an incidence rate of 16 per 100,000 people [133] and it should be noted that the incidence rate continues to rise. In 2008, the Australian Department of Health reported that the lifetime risk of cSCC is as high as 9 to 14 per cent among men and 4 to 9 per cent among women (percentages that equated to an estimated 138,000 new cSCC cases that year).

Cutaneous squamous cell carcinomas of the head and neck region are associated with a substantial risk of metastasis [12]. Tumorigenesis is a multi-step process. Several risk factors have been linked to the progression of tumorigenesis, including smoking, alcohol consumption and viral infections. Each of these factors can influence or promote genetic alterations in skin cells. Normal cutaneous tissue becomes invasive cSCC by proceeding through hyperplasia, mild/moderate/severe dysplasia and micro-invasions of carcinoma. Carcinoma cells exhibit an alteration of cell morphology that involves the formation of keratin pearls, intercellular bridges, plemorphism (i.e., changes in both the size and shape of the cell and/or nuclei), all of which lead to the loss of cellular organisation. These characteristics are currently used to subcategorise cSCCs as well, moderately or poorly differentiated. It is also believed that the less well-differentiated cSCCs have the ability to invade the vascular and lymphatic system, which may result in new carcinoma at sites distant from the point of origin (via the metastasis process). The process of metastasis is a major prognostic factor used to determine treatment,

monitoring and survival of each patient. Overall, the survival rate for non-metastatic cSCC is approximately 95 per cent; however, if a nodal metastasis is found, the survival rate decreases to 46.7 per cent and the rate of disease-free survival to 58.2 per cent [134].

Tumour-associated blood vessels not only nourish the primary tumour, but also serve as important routes for metastasis. The levels of tumour angiogenic factors have frequently been measured and have been shown to correlate with cancer prognoses [135]. However, only half of all malignancies are thought to use the blood vasculature as the primary route for tumour spread. The remaining half of malignancies, including head and neck SCC, largely metastasise via the lymphatic system, via a route that is presently not well understood. It was once believed that less well-differentiated cSCCs were more likely to metastasise via a direct invasion into the vascular/lymphatic system. More importantly, a well-differentiated cSCC is thought to have a good prognosis and will often be treated more conservatively. However, it has been found that some well-differentiated cSCCs do metastasise and less well-differentiated cSCCs can remain dormant. Peat et al. evaluated the risks associated with metastasis in cSCCs. They noted that one feature is the presence of poorly differentiated cSCCs; however, they also noted that over 15 per cent (i.e., 12 of 78) of metastasised SCCs originated from a well-differentiated subtype and that 33 per cent of moderate or poorly differentiated subtype did not metastasise.

Following the development of specific antibodies against proteins unique to LECs, the formation of tumour-associated lymphatic vessels (i.e., lymphangiogenesis) has been studied extensively. Many studies have shown that lymphangiogenesis has a strong effect over the growth and metastatic spread of cSCCs [55,67,105,111–115]. These lymphatic vessels are believed to act as conduits by which cancer cells can escape primary tumours. The development of lymphangiogenesis involves multiple steps, including EC migration, proliferation, cellular rearrangement and cell permeability [116]. All of these steps are attributable to the lymphangiogenic growth factors that are released by the tumour cells.

Several types of tumour have been studied to identify lymphatic growth patterns and their metastatic potential. Zhang et al. used endothelial markers for lymphatic vessels to study lymphatic growth in cervical carcinoma [116]. They found that cervical carcinomas showed increases in lymphatic size, number and distortion of shape compared to normal cervical mucosa [116]. In addition, they found that peritumoural lymphatic vessels remain more dilated than intratumoural lymphatic vessels that tend to collapse. This could be because the rapid proliferation of tumour cells places newly developed lymphatic vessels under high stromal

hydrostatic pressure. Further, Baek et al. showed that increased LVD and VEGF-C expression are predictors of lymph node metastasis in supraglottic cSCCs [55].

Enhanced expressions of an immune modulator that induces lymphangiogenesis has been found in cSCCs. Kataru et al., Watari et al. and Schoppmann et al. have shown an increase in macrophage derived VEGF-C activity and suggested that it has a role in the inflammatory lymphangiogenesis of cSCC [117–119]. A study by Moussari et al. also reported an increase in lymphatic vessels and expressions of genes associated with lymphogenesis. Moussari et al. used LYVE-1 to identify the lymphatic vessel cells that were harvested by LCM and subjected to (RT)-PCR to measure the expression of endothelial growth factor genes [120]. However, in a clinical setting, such tests are expensive and time consuming. Several studies have sought to take a more direct approach to observe the lymphatic vessels in metastatic cSCCs and used the monoclonal antibody against human D2-40. This antibody binds specifically to the endothelial wall of human lymphatic vessel unlike other agents that also bind to the vessel wall [121]. Toll et al. used D2-40 to identify lymphatic endothelial and found a higher uptake in metastatic cSCCs compared to non-metastatic cSCCs [38]. Similarly, Kreppel et al. used D2-40 to enhance the detection of lymphatic invasion in several malignancies (e.g., breast, lung, oesophagus, colon, gastric and uterine tumours) [122-126, 136].

These studies suggest that there is a clear link between cSCCs and lymphangiogenesis (attributable to an overexpression of endothelial growth factors). Increases in lymphangiogenesis and physiological changes can increase the risk of regional metastasis and thus distance metastasis. Thus, the presence of metastasis worsens prognoses in terms of patients' overall survival and disease-free survival. Further, under current classifications of cSCCs, tumours are subjectively subdivided according to cell morphology, architecture and differentiation. The relationship between subtype and outcome is not robust. Consequently, many patients may be either be over-treated when aggressive treatment is not necessary or miss vital treatment if an overly conservative approach is adopted. To date, no study has sought to directly examine expressions and changes in lymphatic patterns in different cSCC subtypes. Many studies on changes in lymphatic patterns in the presence of regional lymph nodes have stated that metastasis is an indication of a poor prognosis. The following discussion points are largely based on a study that examined lymph node metastasis as a prognostic indicator.

4.2 The General Morphology of Lymphatic Vessels

From our study, the overall morphology of the lymphatic vessels of cSCCs within the intratumoural and peritumoural areas tend to contrast with the patency of the lumen. The lymphatic vessels within the peripheral region of a tumour have more open characteristics than those located inside the tumours themselves. This is true regardless of the degree of differentiation of a tumour. The lymphatic vessel also has a large nucleus that extrudes towards the lumen face that is easily identifiable. Conversely, most of the lymphatic vessels that are located in the intratumoural area are more distorted. They are often irregular in shape and have flattened and constricted lumens. A stained nucleus can be observed, but may not always be clearly visible due to the collapse of the endothelial wall.

This feature suggests the ingrowing of a new lymphatic vessel towards the tumour. They remain open to allow for the transportation of essential material to and from the tumour. This lymphatic channel plays an essential role in maintaining the high metabolic process that allows tumour cells to grow and survive. However, the lymphatic vessels incorporated within the tumour structure (i.e., in the intratumoural area) tend to have a flattened appearance. The cell structure of cSCCs is often highly deformed and (unlike normal skin cells, the cells of cSCCs are tightly packed). These packed cells start to exert an external pressure on lymphatic vessels that in turn alters their morphology. This affect can be observed macroscopically. As a tumour grows, it has the potential to obstruct other structures, such as vital organs, vessels and lumens (e.g., the airway, the oesophagus, the auditory canal and nerve fossa). The obstructed lumens become stenotic and eventually lose their ability to function. Thus, it is not surprising that the same phenomenon exists at the microscopic level.

4.3 Lymphatic pattern within different regions.

In this study, the degree of cSCC differentiation was compared by examining the number of lymphatic vessels and their size, cross-sectional area and density across three different areas (i.e., the intratumoural, peritumoural and overall areas). Statistically significant differences were found in the lymphatic patterns in the peritumoural and overall areas, but not in the intratumoural area. In the intratumoural area, apart from the number of lymphatic vessels, a trend towards an increase in the less well-differentiated groups was found, but no statistically significant difference was found. The two areas (i.e., the intratumoural and peritumoural areas)

were examined individually. To date, no previous research appears to have examined the different degree of cSCC differentiation and its effects on lymphatic vasculature.

4.3.1 The Intratumoural Area

The mean numbers of the lymphatic vessels were significantly higher in the moderately and poorly differentiated groups than in the well-differentiated group. Other parameters, such as lymphatic vessel size, cross-sectional area and density, did not show any statistically significant differences; however, an increasing trend was recorded. Further, the moderately differentiated groups had higher means than that of the poorly differentiated group across all four parameters. These findings are similar to other studies that have mostly have found no statistically significant differences in terms of size, number and density. One study on tongue SCC compared hyperplasia and malignant tumour, but found no statistically significant differences. However, a subgroup analysis of the malignant tongues revealed that the worse prognosis group in relation to DOI (> T2) did show higher LVD compared to the less aggressive group. However, nodal metastasis and 5-year mortality did not differ significantly [137]. Similarly, in another oral SCC study, no statistically significant differences in LVD were found between the degrees of differentiation (i.e., well, moderate and poor). However, a univariate analysis of higher intratumoural LVD showed a lower 5-year survival rate and locoregional recurrence [138]. These studies imply that intratumoural lymphatic vessel have a functional role in malignancy; however, the mechanism is still unknown.

Currently, no consistent conclusion about the differences between intratumoural LVD and normal LVD can be drawn. Agarwal et al. and Van der Auwera et al. claimed that the intratumoural LVD of breast cancer was lower than that of normal or benign breast lesions. Conversely, other studies have found there no differences between the two. These contradicting results might be attributable to the different locations of the tumour lymphatic vessels referred to as ‘intratumoural lymphatic vessels’. Van der Auwera et al. defined intratumoural lymphatics as any vessels within the tumour area, either in the inner core or periphery [139,140]. Conversely, another study defined intratumoural lymphatics as only the vessels present among tumour cells. While Mohammed et al. defined intratumoural lymphatic vessels as the vessels within the inner two-thirds of the core of the tumour lesion [41].

Additionally, most of the studies in literature were observational and had relatively small sample sizes. Due to the lack of data on intratumoural LVD, recall and selection biases are

inevitable. Additionally, the unmeasured or inadequately measured factors (e.g., patient sources, histological types, antibody categories and antibody dilutions) could confound the final results. Different studies used different methods to count the lymphatic vessels, including the number of different hotspots, magnification (e.g., 100×[11], 200×[31], 400×[17]) and different measuring units (e.g., vessels/mm^2, vessels/area). The present study used a larger sample size and to represent each individual specimen, examined entire tumour specimens at their largest cross-sectional areas. Such techniques could help to eliminate any potential confounding factors from interfering with the results. Further, previous studies have examined non-cSCCs or malignancies of other types. However, it appears that no other study has examined cSCCs of the head and neck region.

4.3.2 The Peritumoural Area

It has long been observed that lymphatic vessels at tumour borders (i.e., in the peritumoural area) often have larger and more open lumen than those located centrally within the tumour. Intratumoural lymphatic vessels have a reticular architecture with numerous tiny and ill-defined lumens. Intratumoural lymphatic vessels are also typically collapsed and non-functional because of the high interstitial pressure within the tumours. Conversely, peritumoural lymphatic vessels are typically patent, dilated and functional. In one study of colorectal tumours, the central tumoural lymphatic vessels had no cavities and non-functional streaks, but the lymphatic vessels in the peritumoural areas had larger lumens that were hollow and oval in shape. In prostatic adenocarcinoma, an increased peritumoural area means that LVD is associated with lymphovascular invasion and positive lymph node metastasis. D2-40 expression is associated with high-grade tumours, lymphovascular invasion, positive lymph node metastasis and higher mean peritumoural LVD. Increases in levels of D2-40 may suggest different mechanisms of lymphatic vessel invasion whereby the biomarkers are related to poor prognoses [141]. Another study performed D2-40 immunostaining on the head and neck tumours, including on samples from oral cavities, oropharynges and larynxes, of an Italian population and showed that high peritumoural LVD predicts poor survival [112]. Conversely, another study performed D2-40 immunostaining on the head and neck tumours, including on samples from oral cavities, lower lips and larynxes, of a Greek population and found that peritumoural LVD had no predictive role [113].

In relation to colorectal tumours, Chen et al. examined LMVD in 352 primary rectal cancer cases and across 34 local recurrent specimens. They reported that peritumoural LVD was a

significant independent predictor of local recurrence [142]. They speculated that the invasion and metastasis of cancer cells is a multi-step process that involves the following successive abnormal changes in lymphatic vessels: the cancer cells migrate into nearby lymphatic vessels, the cancer cells are transported through the lymphatic system and finally, the cancer cells escape from the lumen of the lymphatic system into the distant tissues.

In relation to breast cancer, Norhisham et al. examined LVD in 58 consecutive breast cancer specimens using D2-40 and CD34 (for blood vessel staining). They found that the total LVD was associated with grade (p-value = 0.018) and increased in higher grade tumours. Peritumoural LVD was significantly associated with distant metastasis (p-value = 0.049). Interestingly, they also found that blood vessel density was not associated with all of the clinical criteria [143]. Zhang et al. performed a current meta-analysis of 28 studies with an overall population of 2,920 breast cancer patients. Their analysis revealed a triangle-like relationship between LVD, lymphovascular invasion, and lymph node metastasis in breast cancer. Peritumoural LVD also has the most robust correlation with LVI and LNM, while intratumoural LVD and total LVD have a relatively weak correlation. However, it should be noted that the p-values in this study only showed a trend towards an increase and were not statistically significant (peritumoural p-value = 0.711 and intratumoural p-value = 0.806). However, the researchers concluded that the results demonstrated that both LVD and LVI are valuable predictors of the LNM occurrence in breast cancer [54].

In relation to cutaneous melanomas, similar findings were demonstrated (i.e., lymphatic vessels were visible in all tumour masses and their peritumoural area). The lymphatic vessels had thin walls that consisted of a single layer of ECs. Again, in the intratumoural area, the lymphatics were smaller, often compressed and stenotic and the lumens were collapsed or partially collapsed. Conversely, in the peritumoural region, the lymphatic vessels were larger, and had more dilated open lumens. LVD was higher in the peritumoural area than in the intratumoural area; however, this difference was not statistically significant (p-value = 0.892). The peritumoural region in the metastatic melanomas showed a trend towards higher LVD than that of non-metastasising melanomas, but this difference was not statistically significant (p-value = 0.139). This was independent of depend of melanoma invasion. Shields et al. found a statistically significant difference in LVD in the peritumoural area in metastasising and non-metastasising melanomas. No statistically significant difference in LVD in the intratumoural area was found. In Shields et al.'s study, LVD in the peritumoural area was significantly higher

in metastasising melanomas [144]. In Massi et al.'s case-control study, LVD in both the peritumoural and intratumoural area was significantly higher in melanomas with SLN metastasis [145]. Several other studies [99,146] have shown that peritumoural LVD is significantly higher in melanomas with lymph node metastasis than in non-metastasising melanomas.

4.3.3 Lymphatic Vessels: Number and Size

In the present study, there was a statistically significant difference in the number of lymphatic vessels in all three regions examined and an increased number of lymphatic vessels in the less well-differentiated groups. Similar findings were observed when the sizes of the lymphatic vessels were examined. The lymphatic vessels in the peritumoural and overall areas were much larger in less well-differentiated groups. However, the size of the lymphatic vessels in the intratumoural area did not differ among the three groups. Thus, it appears that lymphatic vessels tend to dilate and increase in number as they approach a tumour. As a tumour grows larger, the lymphatic vessels are enveloped. The density of the tumour and its distorted cell architecture exert an external pressure over the lymphatic vessels that ultimately obstructs these vessels, causing an increase in the number of vessels; however, it should be noted that these vessels have smaller diameters.

To date, most of the research on the lymphatic changes that occur in the presence of tumours have reported on lymphovascular density rather than size and number. This may be due to an assumption of the correlation from one to another. Some studies have examined inflammatory cells and their effects on lymphatic vessel proliferation. For example, Kunstfeldet al. found an increase in the size and number of lymphatic vessels in inflammatory mice skin changes after delayed hypersensitive reactions. The inflammatory changes were induced by applying a tropical solution comprising 2 per cent oxazolone in acetone/olive oil (4:1 vol/vol). Both the number and size of the lymphatic vessels increased (p-value = < 0.01). Further, wild-type mice and VEGF transgenic mice were also subjected to an inflammatory induced therapy, after which a prolonged tissue oedema was demonstrated macroscopically. A microscopic examination was undertaken on day seven, which again showed that both the number and size of the lymphatic vessels were significantly larger in transgenic mice than in wild-type mice (p-value = < 0.01). A computer-assisted morphometric vessel analysis confirmed that on average, the lymphatic sizes of the vessels were larger in VEGF transgenic mice than in wild-type mice (935.2 ± 80.7 m^2 vs 592.6 ± 62.2 m^2; p-value = < 0.01, respectively) [147].

Another hallmark of inflammatory reaction is vascular leakage with consecutive plasma extravasation in the early phase. Kunstfeld et al. used an intravenous injection of Evans blue dye at 24 hours or at seven days after the elicitation of delayed-type hypersensitivity (DTH) reactions to demonstrate an increase in lymphatic permeability during the inflammation phase in VEGF-A transgenic mice. They found associated increased vascular permeability. The amount of extravasated dye was quantified in the mice's ear skin and served as a parameter for vessel leakage. The baseline extravasation of dye in the non-inflamed ears of VEGF-A transgenic mice (34.2 ± 6.5 ng/mg) was significantly higher than that in the non-inflamed ears of wild-type mice (7.0 ± 2.0 ng/mg; p-value = < 0.01). At 24 hours after the induction of the DTH reactions, the vascular leakage of wild-type mice (71.2 ± 19.3 ng/mg) was more than 10 times higher and the vascular leakage of VEGF-A transgenic mice was 11.5 times higher (395 ± 143.8 ng/mg) than the vascular leakage of vehicle-treated ears (p-value < 0.01). Seven days after the elicitation of the DTH reactions, vessel leakage in the inflamed ears diminished in both wild-type mice (15.5 ± 6.6 ng/mg) and VEGF-A transgenic mice (132.5 ± 39.4 ng/mg;); however, the levels were still much higher in VEGF-A transgenic mice. VEGF-A transgenic mice showed prolonged ear swelling and an enhanced and persistent enlargement of lymphatic vessels that was still detectable after one month. These findings indicate that persistently increased fluid accumulation contributed to the extended inflammatory response in the VEGF-A transgenic mice [147]. This study demonstrated the ability for interstitial fluid transition between intraluminal and extraluminal of lymphatic vessels. Such shifts in oncotic and osmotic pressure have been shown to drive or be assisted by the presence of VEGF and the VEGF-R family and thus may contribute to lymphatic metastasis.

4.4 Summary

The present study demonstrated the effects of tumour cells on the expressions and patterns of lymphatic vessels. The degree of cSCC differentiation was shown to express different degree of lymphatic development in relation to size, diameter, cross-sectional area and density. It may be that these changes are secondary to the expression of various growth factors and their receptors (e.g., VEGF-C, VEGF-D, VEGFR-3 and HA). The presence of intratumoural lymphatic vessels was identified using D2-40 staining. A comparison of the less well-differentiated cSCC group to the well-differentiated cSCC group showed that in the intratumoural region, there were significant increase in the number of lymphatic vessels, but not in their size. This finding was consistent with previous research. As stated above, it is likely

that the exertional external force of a tumour causes lymphatic vessel stenosis. Conversely, the peritumoural and overall areas were found to have a higher number of lymphatic vessels. These lymphatic vessels were also larger in diameter and higher in concentration (i.e., LVD). Such features are likely to result in the less well-differentiated cSCC groups having a higher chance of lymphatic metastasis. Comparisons of metastatic cSCCs and non-metastatic cSCCs showed increasing trends in relation to number, size, cross-sectional area and LVD; however, these findings were not statistically significant. This may be due to the secondary to low yield in the number of metastatic specimens' sample size.

Chapter 5: Conclusions and Future Research

5.1 Summary of Research

Currently, cSCCs are classified according to Broder's classification. Classifications are based on the observations of a reviewing pathologist, who examines the degree of disorganisation in the cell architecture, the presence of keratinising pearls, the degree of keratinisation, pleomorphism and the presence of intercellular bridges. The technique is subjective and thus classifications may vary among different examiners. Classifications are used to provide prognoses and predict tumour behaviours. Less well-differentiated types of tumours are more aggressive and likely to result in worse outcomes. Thus, more aggressive treatments are normally suggested to treat poorly or moderately differentiated cSCCs than those that would be suggested to treat well-differentiated cSCCs. More aggressive treatments may include wider margins, deeper resection plains and the use of adjuvant treatments (i.e., chemotherapy and radiotherapy).

The development of distant metastasis is considered a poor prognostic indicator. It has long been believed that this process was caused by lymphatic spread. New lymphatic specific biomarkers (e.g., D2-40 and LYVE-1) have given researchers the ability to visualise and examine lymphatic vessels more closely. Researchers now have a much better understanding of tumour cell interactions and their influence on lymphangiogenesis.

Within the literature, a less well-differentiated cSCC subtype is associated with a higher incidence of lymphatic spread. Also, the presence of regional and distant metastasis has been found to increase LVD and its characteristics (i.e., size and number). It is thus reasonable to consider whether a degree of differentiation could also interfere with lymphatic pattern growth. To date, it appears that no study has sought to examine the link between the degree of cSCC differentiation and its influence on lymphatic patterns.

This study showed that similar to the lymphatic pattern spread of distant metastasis, there is an increase in an overall lymphatic activity (i.e., larger sizes, greater numbers and higher LVDs) in less well-differentiated groups. This is likely to be secondary to the propensity for a more aggressive type of cSCC to express more chemical signals and thus generate and maintain new lymphangiogenesis. The chemical signalling pathway has been examined in various metastatic tumours. An increase in inflammatory mediators (e.g., cytokine and chemokine), growth

factors (e.g., VEGF-C and VEGF-D) and its receptor (i.e., VEGFR-3) was observed in tumour cells. Further, new lymphatic vessels were generated within and around these tumour cells. The chemical growth factors interact with the lymphatic vessel lining, which allows for an increase cell permeability and provides another pathway for cancer cell to metastasise.

Further examination of lymphatic vessel patterns in intratumoural (within the tumour) and peritumoural (around the tumour) areas revealed different results. Within the intratumoural area, the increase in the quantity of lymphatic vessels is similar to the increase in the peritumoural area, but the lymphatic vessels in the intratumoural area tend to be smaller in size. Intratumoural lymphatic vessels are flat or stenotic. Conversely, peritumoural lymphatic vessels are patent and dilated. Again, this finding supports previous research that examined various other metastatic types of cancer. This lymphatic vessel pattern could be explained by the stress force exerted by a densely packed and disorganised tumour cell. The external pressure force is able to overcome the intra-luminal pressure, causing lymphatic vessels to partially or totally collapse. Studies on the lymphatic flows of tumours have concluded that some lymphatic vessels collapse because they lack the capacity to maintain the lymphatic flow. Thus, lymphatic spread is more likely to occur at the periphery of a tumour than within a tumour. The present study showed that lymphatics within the peritumoural region increase in number, size and density in the less well-differentiated groups. This implies a higher chance of lymphatic spread via the peripheral pathway. There is an increase in lymphatic vessel surface area contact for less well-differentiated tumours to interact.

5.2 Research Implications

The current classification of cSCC is highly subjective and based on an observational technique. Thus, cSCC differentiations may vary from pathologist to pathologist. Further, despite the ability to classify the degree of differentiation according to cell formation and architecture, neither has any direct correlation to lymphatic spread. An additional ability to quantify and measure lymphatic vessels accurately could improve determinations of distant metastatic potential. Well-differentiated cSCCs, which should have better prognoses, can metastasise (although, no well differentiated cSCC was seen to have metastases in this study). Conversely, poorly differentiated cSCCs remain dormant. A poor prognostic lymphatic pattern should be included in routine histological examinations to help the clinicians determining the adjuvant treatment to lower the risk of lymphatic spread. Clinicians should focus on the peritumoural area, as it is this area that has the highest risk of potential spread. A cSCC with a

high lymphatic number, density and dilated and patented vessel characteristics should be considered as having a high potential metastatic risk particularly in the less well differentiated group.

5.3 Research Limitations

This appears to be the first study to examine the correlation of cSCC differentiation on lymphatic vessels. Lymphatic vessels were examined using routine histological slices. A central portion of the largest cross-sectional area with the highest DOI was used to represent the entire tumour specimen. A well-known LEC biomarker was used as an immunological stain to identify the presence and characteristics of each lymphatic vessel. This technique enables the specimens to be examined in a two-dimensional form. However, a few issues arise in relation to this technique.

Firstly, only a representative sample of the whole tumour was able to be examined using this technique. Thus, it was assumed that the concentration of the lymphatic vessel was uniform across the specimen and if it was not, that it would be mostly concentrated in the largest cross-sectional area. Further, it was assumed that the highest concentration of the lymphatic vessels would occur in the centre of all of the tumour specimens.

Secondly, by examining the specimens in a two-dimensional fashion, no distinctions could be made between any lymphatic vessels that had turned on themselves or between two distinct vessels. This may have interfered greatly with the final lymphatic number count. Additionally, the slicing of the lymphatic vessel only occurred at the diagonal plane and did not occur at the true cross-sectional plane. This may have distorted the true diameters of the lymphatic vessels. To obtain a more ideal measurement of lymphatic vessels, fluoresce dye in combination with LVYE-1 biomarker should be used. However, the technique would have required the use of entire tumour specimens and the complete destruction of the tissue. Further, while this technique provides more accurate results, it is impractical in clinical practice. It is very unlikely that in day-to-day clinical work, a single stain will be used on one specimen. Thus, three-dimensional examinations of specimens are unfeasible. Further, it is an expensive procedure to perform and is not available in all pathology laboratories. Given this limitation, it was decided that a standard slicing technique would be more practical in clinical settings. A large number of specimens were used to improve the accuracy of the study and address any confounding biases.

5.4 Future Research

The presence of lymphatic vessels is a morphological characteristic of a complex cSCC histopathological profile. The true mechanics of lymphatic spread extends beyond the ingrowth of lymphatic vessels towards and within tumour cells. The recent identification of co-receptors and novel signalling complexes suggests a greater complexity than previously thought. A number of factors (many of which have been mentioned in previous sections) appear to play a more crucial role, including fibronectin, ß1 intergrin, VEGFR-3, tyrosine kinase, intergrin alpha9, LYVE-1, hyaluronan, D2-40, VEGF-C and VEGF-D. A better understanding of and an ability to examine vascular endothelial growth factors could enable the quantity of tumour expressions to be tested. This could also increase the accuracy of predicting the metastatic ability of individual tumours. Additionally, the presence of certain inflammatory markers and high molecular weight HAs could be used to block the spread of tumour cells via lymphatic channels. Measuring these levels could also help to predict the protective potential of such tumours. An ability to measure these factors could help by providing an even more accurate picture of tumour behaviours. This in turn could assist in determining appropriate treatments and individualising treatments for specific target groups. Further, the quantification of these markers should eventually help to determine which target therapy should be used to minimise the malignancy potential of the tumour.

This study is a retrospective observational study that examined the correlation between the degree of differentiation and lymphatic vessel patterns. There are limitations to the study which can be improved by performing a prospective study with patients' outcome follow up. Nevertheless, the study helps identifying the correlation and suggested a potential link that guides further research.

Appendix.

A) Dorevitch pathology: The Ventana™ device protocol for D2-40 specimens stains.

Protocol # 641 : uv -D2-40(Podoplanin) (04/10/2012)

Procedure: U ultraView DAB (v1.02.0018)

BenchMark ULTRA IHC/ISH Staining Module

DOREVITCH PATHOLOGY, 18 BANKSIA RD HEIDELBERG, 3084 VIC

Step No	Procedure Step
1	Enable Mixers
2	Warmup Slide to [72 Deg C], and Incubate for [8 Minutes] (Baking)
3	Disable Mixers
4	[Set temperature to 72°C for Default]
5	Warmup Slide to [72 Deg C] from Medium Temperatures (Deparaffinization)
6	Incubate for 4 Minutes
7	Apply EZPrep Volume Adjust
8	Rinse Slide With EZ Prep
9	Apply EZPrep Volume Adjust
10	Apply Coverslip
11	Rinse Slide With EZ Prep
12	Apply EZPrep Volume Adjust
13	Apply Coverslip
14	Rinse Slide With EZ Prep
15	Apply Depar Volume Adjust
16	Apply Coverslip
17	Enable Mixers
18	Disable Slide Heater
19	Pause Point (Landing Zone)
20	[Short - 8 Minute Conditioning]
21	Rinse Slide With EZ Prep
22	Apply Long Cell Conditioner #1
23	Apply CC Coverslip Long
24	[Set temperature to 95°C for Default]
25	Warmup Slide to [95 Deg C], and Incubate for 8 Minutes (Cell Conditioner #1)
26	Apply Cell Conditioner #1
27	Apply CC Medium Coverslip No BB
28	Apply CC Medium Coverslip No BB
29	Apply Cell Conditioner #1
30	Apply CC Medium Coverslip No BB
31	[MILD]
32	Apply Cell Conditioner #1
33	Apply CC Medium Coverslip No BB
34	Apply Cell Conditioner #1
35	Apply CC Medium Coverslip No BB
36	Apply Cell Conditioner #1
37	Apply CC Medium Coverslip No BB
38	Apply Cell Conditioner #1
39	Apply CC Medium Coverslip No BB
40	Disable Slide Heater
41	Incubate for 8 Minutes
42	Rinse Slide With Reaction Buffer
43	Adjust Slide Volume With Reaction Buffer
44	Apply Coverslip
45	Rinse Slide With Reaction Buffer

* one drop is one reagent dispense

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Page 1 of 2

Protocol # 641 : uv -D2-40(Podoplanin) (04/10/2012)

Procedure: U ultraView DAB (v1.02.0018)

BenchMark ULTRA IHC/ISH Staining Module

DOREVITCH PATHOLOGY, 18 BANKSIA RD HEIDELBERG, 3084 VIC

Step No	Procedure Step
46	Adjust Slide Volume With Reaction Buffer
47	Apply Coverslip
48	Pause Point (Landing Zone)
49	Warmup Slide to 36 Deg C
50	Rinse Slide With Reaction Buffer
51	Adjust Slide Volume With Reaction Buffer
52	Apply One Drop of UV INHIBITOR, Apply Coverslip, and Incubate for 4 Minutes
53	Rinse Slide With Reaction Buffer
54	Adjust Slide Volume With Reaction Buffer
55	Apply Coverslip
56	Warmup Slide to 36 Deg C, and Incubate for 4 Minutes
57	Rinse Slide With Reaction Buffer
58	Adjust Slide Volume With Reaction Buffer
59	Apply One Drop of [Podoplanin/D2-40] (Antibody), Apply Coverslip, and Incubate for [0 Hr 32 Min]
60	Rinse Slide With Reaction Buffer
61	Adjust Slide Volume With Reaction Buffer
62	Apply Coverslip
63	Warmup Slide to 36 Deg C
64	Rinse Slide With Reaction Buffer
65	Apply 100ul + VA Reaction Buffer
66	Apply One Drop of UV HRP UNIV MULT, Apply Coverslip, and Incubate for 8 Minutes
67	Rinse Slide With Reaction Buffer
68	Adjust Slide Volume With Reaction Buffer
69	Apply Coverslip
70	Rinse Slide With Reaction Buffer
71	Adjust Slide Volume With Reaction Buffer
72	Apply Coverslip
73	Rinse Slide With Reaction Buffer
74	Adjust Slide Volume With Reaction Buffer
75	Apply One Drop of UV DAB and One Drop of UV DAB H2O2, Apply Coverslip, Incubate for 8 Minutes
76	Rinse Slide With Reaction Buffer
77	Adjust Slide Volume With Reaction Buffer
78	Apply One Drop of UV COPPER, Apply Coverslip, and Incubate for 4 Minutes
79	Rinse Slide With Reaction Buffer
80	Apply Coverslip
81	Disable Slide Heater
82	Rinse Slide With Reaction Buffer

* one drop is one reagent dispense
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