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The chemokine microenvironment of Squamous Cell Carcinoma and its precursor lesions

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Abstract

Chemokines play a significant role in attracting immune cells to extravasate to areas of peak expression in response to various stimuli. *IL-8, MIP-1α* and *MIP-1β* are known to be highly expressed in multiple cancers, as well as benign skin conditions. Their role in cutaneous Squamous Cell Carcinoma has yet to be established. 67 lesions suspicious for keratinocytic malignancy were sampled from 37 patients and tested for chemokine expression via Flow Cytometry. Each lesion was measured for thickness, histopathological grading, and immune cell infiltration score. Expression of the chemokines *IL-8* and *MIP-1α* increased significantly with increasing lesion thickness ($p = 0.0003$***), histopathological grading ($p = <0.0003$***), and infiltration score ($p = <0.01$***). These data demonstrate the increase in proinflammatory chemokines with tumour progression which has not previously been described in cutaneous Squamous Cell Carcinoma.

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Publications during candidature

None.

Publications included in this thesis

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Commonly Used Terms and Abbreviations

UV = Ultraviolet
AK = Actinic Keratosis
IEC = Intraepidermal Carcinoma
SCCis = Squamous Cell Carcinoma in situ
SCC = Squamous Cell Carcinoma
cSCC = cutaneous Squamous Cell Carcinoma
HNSCC = Head and Neck Squamous Cell Carcinoma

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Introduction

While it is recognized that skin and particularly Squamous Cell Carcinoma (SCC) is highly infiltrated by immune cells, there remains conflicting data regarding the presence and relative significance of different immune cell subtypes. The changes in both tumour and peritumoral stroma that occur affect and are affected by chemical signals attracting different cells to the area. Extensive research has investigated the presence of, and prognosis associated with immune cell subsets existing in cutaneous disease and malignancy. Chemotactic cytokines (Chemokines) provide expression dependent signaling to induce migration of receptor positive immune cells (Chemotaxis). The presence of chemokines can be both a cause and consequence of the immune cell microenvironment of tumours, and can influence tumour rejection as well as progression and survival. This is generally thought to be due to epigenetic changes relating to apoptotic proteins, with propagation of tumour growth and metastatic potential via angiogenic and lymphaniogenic stimulation.

To date, very little data exists on the qualitative and quantitative nature of chemotactic cytokine (Chemokine) expression in cutaneous squamous cell carcinomas. The significant prognostic data relating to chemokine expression in Head/Neck SCC, melanoma, ovarian and pancreatic cancers warrants further research into their role in cutaneous SCC (cSCC).

This study aims to quantify the presence of chemokines in squamous cell carcinoma, by proposing the hypothesis:

The expression of IL-8, MIP-1α and MIP-1β increase with increasing tumour thickness of squamous cell carcinoma and its precursor lesions.

This data will be correlated with tumour thickness, the degree of immune cell infiltration, and histopathological subtype.

To achieve this, a broad review of the available and relevant literature was performed, with particular focus on the role of chemokines in the skin, and their significance in other human cancers.

Theoretical Foundation

The significance of chemokine over-expression correlating with worsening prognosis in other human cancers has been well established. Studies investigating the presence of IL-8 in melanoma; ovarian and pancreatic cancer cell lines demonstrate hypoxic induction in response to ischaemic conditions. Over-expression has been correlated with increased angiogenesis of new vessels, and blockade of IL-8 receptors reduces tumorigenicity, and improves tumour clearance[1,2]. The C-C Chemokines CCL3/CCL4 are over expressed by Myeloid-Derived Suppressor Cells (MDSCs) in lymphoma, and act to attract Reguatory T Cells (T-Reg) infiltration, a factor associated with worse prognosis in cSCC. A reduction in the CCR5 receptor for CCL3/CCL4 reduces T-Reg infiltration in murine models and would theoretically predict an improved prognosis[3,4].

Nature of the study

This study utilized samples from random patients with lesions clinically diagnosed as keratinocytic cancers suspicious for cSCC. Fundamental independent differences between patients were location of lesion, lesion thickness, histopathological grading, inflammatory cell infiltration. The dependent variable of chemokine expression was evaluated and analyzed for univariate relationship to the independent variables.

Assumptions

Patients were questioned in regard to their general health and in particular, any immunosuppressive conditions or medications that may impact on the outcomes of the study. No patients were taking immunosuppressant medications (although no data was collected on other medications), and it was assumed that any general medical comorbidities (Diabetes, Peripheral Vascular Disease) significantly impacted on immune behavior, as any significant comorbidities would preclude surgery.

Scope and Delimitations

The patients and skin lesions studied represent the majority of patients with cSCC disease across Australia. Patients were of the at risk age group (50+yrs old) for Non-Melanoma Skin Cancer (NMSC) (age was not limited), and had no major systemic disease that would have precluded surgical resection of these cancers. Although the plastic surgeon works in private practice, most patients were funded by DVA repatriation benefits and therefore socioeconomic disparity was not deemed significant.

Whilst skin types ranged from Fitzpatrick I-IV, the majority of patients displayed Type I-II skin, representing the highest risk population for NMSC. Gender bias was not examined nor controlled for, and the population represented a random sampling of patients in attendance for surgery.

Limitations

This study examined a random population presenting for care from a plastic surgeon, in a secondary referral process from general practitioners. The lack of adequate numbers of control (normal skin) samples from each patient, as well as examinable (abnormal, immunosuppressed) subjects limited the internal validity of the study. Being unable to provide a normal sample for each patient reduced the impact of quantification for each lesion, particularly in advanced disease where variance between samples was high. Although flow cytometry was repeated in duplicate for each sample, and the test repeated 3 different times with separate kits, the study lacked a secondary validation model to ensure external validity and reproducibility in quantifying chemokine expression.

Significance of Study

This study fills a gap in the scientific literature regarding the expression of chemokines in the skin throughout the development of cutaneous squamous cell carcinoma. This follows on from studies in other cancers such as Melanoma, Ovarian and Pancreatic Cancer, demonstrating the significance of *IL-8, MIP-1α and MIP-1β* in disease progression and worsening prognoses.

The results of this study will not directly influence change in clinical care. However, it does highlight the role of the clinician scientist in facilitating the link between clinical care and laboratory based research in human subjects, further demonstrating the potential for future collaborative endeavours.

Summary

This is a quantitative study, which answers the question "*does chemokine expression correlate with increasing tumour thickness in cutaneous squamous cell carcinoma*". This bridges a gap in the medical literature surrounding the behavior of the skin in response to increasing dysplastic keratinocytic burden and the inflammatory response to cancer.
The results of this study provide a quantitative measure of chemokine expression from normal skin through its dysplastic changes to invasive cSCC. Whilst this does not provide a qualitative measure of the cell types present in response to these conditions, it does give

an indication of the degree of inflammatory response present and correlates this with the thickness of each lesion.

Review of the Literature

Skin cancer is the most common malignancy in the world, accounting for 1 in every 3 cancers diagnosed[5]. The majority (96%) of these are Non-Melanoma Skin Cancers (NMSC), predominantly made up of Basal Cell Carcinomas (BCC) and Squamous Cell Carcinomas (SCC)[6]. Current estimates suggest treatments for NMSC across Australia have increased from 412,493 in 1997 to 767,347 in 2010 and are estimated to continue to increase. These figures accompany a significant financial imposition, estimated at $511 million per year and expected to rise to $1 billion AUD by the turn of the decade[7].

Despite public awareness campaigns leading to an increased public vigilance and lower threshold to seek treatment at earlier stages, the delay in diagnosis and late stage presentations force more invasive treatment and increase both patient morbidity and financial cost to the health system.

Of the NMSCs, lesions classified as SCC are believed to exist on a spectrum of disease from early, easily treatable scaly lesions, to large fungating tumours requiring complex and morbid surgical procedures to resect. The vast majority of these early stage lesions are rejected by the body's immune system, and only a small number progress to invasive disease[8]. This capacity for host defense is reversed in immunosuppressed patients, suggesting a significant role for the immune system in both tumour surveillance and rejection. Further research into the role of the immune system and its response to both early and late stage lesions is required to facilitate the development of treatments that could potentially assist the body in rejecting these cancers.

Histopathology of Squamous Cell Carcinoma

Non-Melanoma Skin Cancer is a broad based term referring to multiple subtypes of cancer that are primarily induced by UV radiation damage from excessive sunlight exposure. BCCs are the most common form of NMSC, and develop at a rate of 3.7:1 when compared to SCCs[9]. These tumors grow slowly, invade locally and very rarely metastasize to other areas.

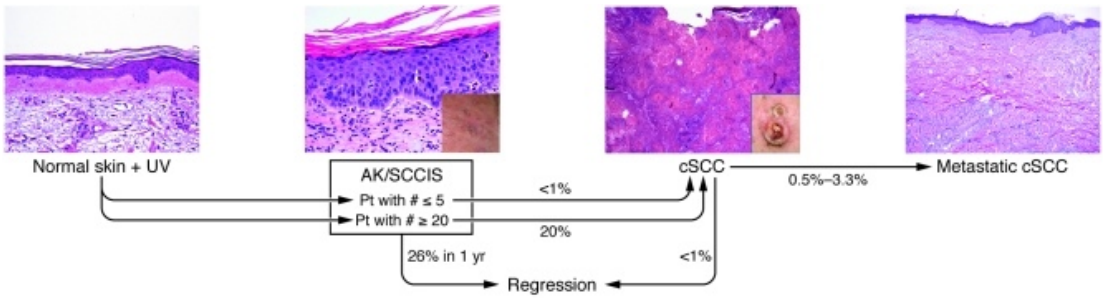


Figure 1. Annual risk of AK or multiple AK progressing to form cSCC or metastatic disease. Figure from ***Ratushny et al, 2012***[8,10–12]

SCCs are clinically and histopathologically distinct – being formed largely by progression from smaller lesions and deteriorating with further damage into more aggressive and morbid tumours capable of metastasis. Risk factors for NMSC are commonly excess sun exposure in individuals with fair skin that burns easily. Differences in the molecular pathogenesis of NMSC is evident in immunosuppressed patients, where the ratio of BCC:SCC is reversed to 1:2, and the risk of SCC increases 60-100 fold[9].

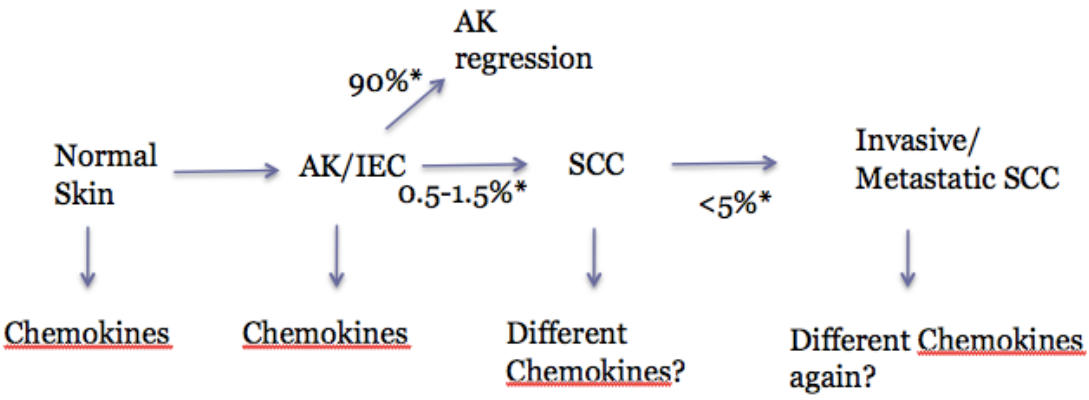


*Figure 2. Stylised representation of theory of chemokine expression during SCC development. Chemokine expression changes in the small numbers (0.5-1.5%) that progress to more invasive disease. *Data from Rigel et al., 2013*[13]

SCCs form on a spectrum of disease from early hypertrophic lesions such as Actinic Keratosis through to invasive cancers. Epidemiologic studies suggest only 0.5-1% of AK go on to form SCC, but between 40-60% of SCC are thought to have formed progressively from AK lesions. Clinically a large number of such lesions are treated without regard for tissue diagnosis by general practitioners in the community which may skew available

recorded data, however this still leaves a significant number of AK that are rejected by the patients immune response.

Significant changes in the phenotype of the immune microenvironment occur during this malignant transformation, and despite countless studies into genetic and structural makeup, there has been a paucity of research into the signaling proteins that facilitate these changes. Chemotactic cytokines (Chemokines) provide expression dependent signaling to induce migration of receptor positive immune cells (Chemotaxis).

As dysplasia and cellular proliferation continues, it is plausible that expression of such proteins would alter and potentially increase with increases in cellular load – resulting in a cellular microenvironment, which would vary accordingly from other lesions along the spectrum.

Inflammatory Infiltration of Cancer

Infiltration of inflammatory immune cells as a result of chemotactic protein expression can influence dysplastic change in skin cells and drive malignant transformation[14]. Cellular inflammation results from multiple causes (microbial invasion; trauma; hypoxia; chemical irritation; genetic mutations) and can be either acute or chronic or a continuum of both, and following observations of malignancy arising from sites of chronic inflammation, is now thought to be a pre-requisite, rather than a consequence, of malignancy[15]. The prognostic significance of immune cell infiltration has been extensively studied in Colorectal Cancer and Melanoma and, whilst controversial, remains at the forefront of cancer research directed towards targeted therapies[16–18].

The immune system is heavily active in the skin, eliciting responses to microbial stimuli, trauma, and dysplastic change. Dendritic cells monitor for tumour antigens that are later presented via lymph drainage, to T and B lymphocytes[19].

Antigen presenting cells provide oncogenic stimulus for cytotoxic cells to extravasate: The production of CCL2/CCL3 by NK cells stimulates DCs to express CXCL9/CXCL10 and, in turn, attract CD8+ T Cells[20]. This effect appears to rely upon proximity to the tumour core

in cSCC, DCs from peritumoral skin were stronger stimulators of T-Cell proliferation compared to those more centrally located[21]

The effective response of immune cell infiltration is reduced in invasive cSCC, and they are capable of evading the effective immune response through multiple mechanisms[22]. Immune infiltration relies on gradient driven expression of chemotactic proteins and vascular extravasation for effect.
The prognostic significance of T-Cell infiltration is a source of contention that varies based on the tumour type. Infiltration of T-Regs is associated with better outcomes and overall longer survival in Colorectal Cancer[17,18,23]. This effect is reversed or not present in most other cancers, including cSCC, where their presence is associated with a worse prognosis. The presence of high intratumoral NK, and cytotoxic Th1 infiltration is associated with a favourable prognosis in the majority of human cancers[19].

Over 50% of T-Cell infiltrates in untreated cSCC are FOXP3+ T-Regs, and display minimal Ki-67 staining, suggesting migration and not local division in response to inflammatory stimuli[24]. Tumour cells act to reduce E-selectin expression, and prevent CLA+ T Cell extravasation in tumour parenchyma under normal and inflamed conditions. This change is reversed with application of the TLR-7 agonist Imiquimod. Tumours treated with Imiquimod display increased E-Selectin, and subsequently CLA+ CD8+ T Cell infiltration, and reduced FOXP3+ T-Reg infiltration[24,25]. Interestingly, FOXP3+ T-Reg infiltration into tumour core appears higher in immunosuppressed solid organ transplant recipients and is associated with a worse prognosis – an effect not seen in other cancers[18,19,24,26].

Overactivity of immune cells has been linked to chronic inflammation and disease in non-malignant conditions, and keratinocyte production of cytokines has been known to selectively recruit T cells to inflamed skin[27]. Cutaneous SCCs show a diverse population of T-Cells with low Ki-67, and varying CLA expression, suggesting migration from non-cutaneous sources[28]. CLA+ T-Cells are present in high levels in the skin and act as memory cells to perpetuate immune reactions to stimuli, and have been implicated in the development and severity of psoriasis. 95% of the CLA+, CCR4+, CCR6 T-Cells show Th1 polarization, and are present in a 7:1 ratio with Th2 cells[24].

Peritumoral Inflammatory Infiltration

The peritumoral inflammatory infiltrate demonstrates the microenvironmental changes that occur in response to chemokine expression without the impact of dysplastic and often hypoxic conditions more central to the tumour core. High Tumour Associated Macrophage (TAM) infiltration is correlated with increased TGF- β expression and Myeloid Derived Suppressor Cell (MDSC) presence[29,30]. These cells have been shown to prevent antigen specific T cell response and activation, particularly with regards to CD8+ T Cell function, and can furthermore regulate CCR5 dependent T-Reg infiltration via a CCL3/CCL4 dependent pathway[31–33].

The presence of TAMs, MDSCs, and T-regs inhibit T cell anti-tumor activity, are associated with worse prognosis, and can affect efficacy of chemotherapy and immunotherapy[32,34,35]. Interestingly however, Dendritic Cell presence is associated with improved prognosis. DCs attract CD8+ Cytotoxic T-lymphocytes (CTL), which attack tumour cells. Immature DCs are present in the tumour core, while mature DCs populate the peritumoral stroma, which may influence the capacity for CTL infiltration and effect[36,37].

The composition of immune cells in the tumour microenvironment differs between immunocompetent and immunosuppressed organ transplant recipients, and may impact on the differences in prognosis between these patient groups. The perineoplastic infiltrate is weighted towards Th2 dominance, inhibiting Th1 responses, which may result in peritumoral field change and permit higher recurrence rates, and more aggressive behavior. This is a consequence of reduced mRNA for Th1 and IL-17A, and contributes to a compromised local inflammatory response in the cSCC or Organ-Transplant Recipients (OTR)s[38]. Whilst fewer T-Regs generally correlates with improved prognosis, the use of the Calcineurin inhibitor Cyclosporine negatively impacts T-Cell generation[39].

In cSCC, TAM infiltration is increased via adhesion of monocytes to vascular endothelium, a process which is chemokine IL-8 dependent[40,41]. Increased TAM infiltration is associated with worse prognosis in over 80% of clinical studies, and subsequently, so is increased IL-8 expression[3].

The presence of neutrophils in peritumoral/tumoral stroma is associated with tumour cell death and improved prognosis. CXC chemokines such as IL-8 stimulate neutrophil

chemotaxis and infiltration. TGF-β inhibits Tumour-Associated neutrophil (TAN) infiltration and is associated with a worse prognosis for the patient[42–45]. NK cells accumulate in tissue when stimulated by C-C chemokines such as Monocyte Chemoattractant Protein-1 (MCP-1)[46]. Priming of NK cells by IL-18 likely induces them to increase expression of CCL2/CCL3, and attract DCs to the target area[37]. The presence of Tumour-Infiltrating Lymphocytes (TILs) constitutes improved prognosis for the many tumour types that display them[47]. They are attracted by expression of CCL2, CXCL16, CXCL9, CXCL10, and assist in inducing tumour regression via synergistic cytotoxic activity with CD4/CD8+ T cells[37,48–50]. Interestingly, nitrosylation (and functional inactivation) of CCL2 is associated with defective infiltration of TILs and worsened prognosis, indicating an important role in this chemokine for tumour survival[51].

Failures of primary activation of immune cells by chemokines will lead to altered immune cell infiltration, and alteration of the host response to tumour cells. The degree of cytokine expression along the spectrum of keratinocytic neoplasia has not been well established. Theoretically there should be a reduction in successful cell signaling from early AK (known to have high rates of spontaneous apoptosis and gross tumour death) to the invasive SCC where tumour cells have seemingly unregulated cellular growth.

The expression of chemokines in cSCC show increased proangiogenic and prolymphangiogenic factors, chemotactically attracting macrophages and circulating immune cells[3]. Ischaemia is a common feature in all invasive tumours that out-grow their blood supply, and thus require angiogenic stimulatory factors to maintain tumour growth. The expression of IL-8, Vascular Endothelial Growth-Factor (VEGF), Angiogenin, Fibroblastic Growth Factor (FGF) and Platelet Derived Growth Factor (PDGF) are inducible under hypoxic conditions. Anoxia induces transcriptional factor stability which in turn facilitates increased IL-8 expression[1]. Increased expression of these factors correlates with an increased microvessel density and subsequent improved blood supply to the tumour parenchyma[55]. The combination of an extrinsic pro-inflammatory microenvironment with intrinsically, genetically initiated pre-cancerous cells results in further alterations in gene expression, enhanced proliferation and resistance to apoptosis, and sustained angiogenesis of the tumour[52].

On a molecular level, multiple pathways have linked chronic inflammation with malignant progression. Proteins can be activated by extracellular signaling, growth factors (Epidermal Growth Factor, EGFR) and cytokines (IL-6; IL-16; IL-22) to regulate gene expression and mediate survival, proliferation, invasion and angiogenesis of cancers as reviewed by Clark in 2008[24]. Nuclear Factor of Activated T Cells (NFAT) acts to regulate inflammatory responses to stimuli via a wide range of pro-inflammatory cytokines, and has been experimentally induced by Ultraviolet-B (UV-B) wavelengths in HaCaT keratinocytes. The inhibition of this response results in reduced COX-2 protein induction and increased apoptosis in response to UV-B radiation[53]. Immune cell production of pro-inflammatory cytokines induce Signal Transducer and Activator of Transcription-3 (STAT3) and Nuclear Factor κβ (NF-κβ) – which have been shown to promote cell proliferation, inhibit apoptosis and contribute to cell survival in SCC cell lines[54–56]. The behavior of cytokines promoting tumour progression is further substantiated by enhanced tumour growth of IL-6 transfected human BCC, as well as malignant transformation associated with expression of G-CSF and GM-CSF in HaCaT keratinocytes[57]. Persistent autocrine and paracrine stimulation by CXC chemokines has previously been shown to promote pre-neoplastic progression to malignancy[58]. Receptor mediated tumour formation via chemokine expression has been associated with RAS mutations in response to pro-inflammatory cytokines IL-1; IL-6 and IL-11, and chemokine IL-8[59].

The concept of intrinsic genetic damage being propagated by extrinsic stimulation has been exhibited in aggressive ovarian cancers, where rapid tumour growth results in hypoxic conditions. This induces genetic transcription of hypoxia-inducible factors (HIF) that further upregulates the transcription of angiogenesis promoting factors, such as the chemokine Interleukin-8 (IL-8)[1].
This propagation of tumour growth and survival through genetic manipulation by cancer cells has not been studied in cutaneous SCC and subsequently warrants further investigation.

Cytokines and Chemokines

Cytokine and chemokine function is fundamental to immune responses in the skin. Keratinocytes are the major source of cytokine production in the epidermis and coordinate cellular proliferation, ECM growth, communication, inflammatory infiltration and

angiogenesis. When released in excess, they can produce and propagate acute and chronic disease in a complex positive feedback loop[54,60,61]. Subsequently, dysplastic and hyperplastic growth results in an increased keratinocytic load, and an increase in potential cytokine production.

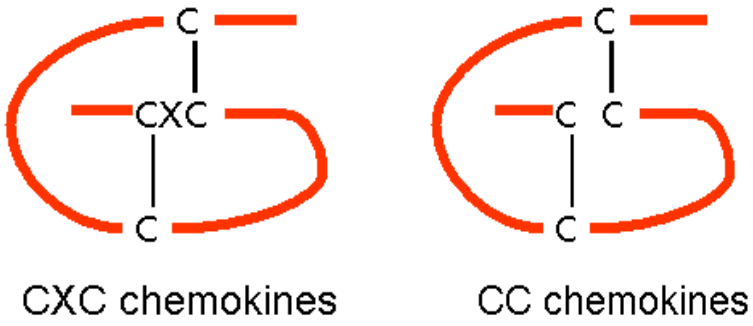


Figure 3. The structure of CXC and CC Chemokines[61].

Chemotactic cytokines stimulate the above functions in immune cells, and attract their migration in a concentration gradient dependent manner[62,63]. Cytokines are induced by exposure to irritants, exhibiting an important role in epidermal and specifically keratinocytic response to noxious stimuli and disease of the skin[64].

Chemokine expression can be elevated in response to tumour burden as well as inflammatory cell migration and subsequent local production. Breast cancer displays elevated levels of TNF, which is majorly produced by macrophages. When chronically produced, this contributes to tissue remodelling and stromal development necessary for tumour progression. This local migration has been demonstrated to not only correlate with the extent of lymphocytic infiltration (CCL2/CXCL8), but also to localize regionally within the tumour itself (CCL3/4)[54]. High levels of neutrophil infiltrate correlate with the expression of IL-10; IL-17; TNF- α and, TNF- β and are detected in the precancerous microenvironment[50,65–67].

Structural Subgroup	Function	Examples	References
C-X-C	Important for neutrophil and/or T-lymphocyte selective accumulation in inflamed skin, angiogenesis/angiostasis	IL-8	Baggiolini et al., 1992[45]
		Gro-Alpha	Bornscheuer et al., 1996[68]
		IP-10	Waugh et al., 2008[44]
		MIG	Tensen et al., 1999[69]
		CXCL5	Park et al., 2002[70]
		CXCL9	Zhao et al., 2002[62]
		CXCL10	Lasagni et al., 2003[63]
		CXCL16	
C-C	Attract monocytes, T cell subsets, eosinophils and basophils; proinflammatory	MCP-1	Carr et al., 1994[71]
		MIP-1a	Kim et al., 1995[50]
		MIP-1b	Menten et al., 2002[65]
		MARC (MCP-3)	Jing et al.,2003[66]
		CCL20	Maurer et al., 2004[67]
		CCL21	Kitahara et al., 2012[72]
			Schlecker et al., 2012[73]
			Chandrasekar et al., 2013[33]
			Tsai et al., 2013[74]

Table 1. The C-C and C-X-C Chemokines

Keratinocytic tumours are capable of producing large quantities of cytokines, both by virtue of keratinocytic load and dysplastic genetic changes altering protein expression. The SCC microenvironment suppresses myeloid DC function via increased IL-10 and TGF- β expression, as well as higher VEGF-A likely as a response to hypoxia, and attempt at neoangiogenesis[21].

The capacity of chemokines to influence the tumour immunophenotype relates to their stimulatory role for immune cell migration and extravasation. Their structure is significant for receptor functionality, and stimulation of particular cell subsets that exhibit these. The structural differences are based on the presence of terminal Cysteine-Cysteine residues, and the substitution of a variable amino acid motif in the C-X-C subgroup (Figure 3), and have functional manifestations described in Table 1. Further specific structural differences appear to have impacts beyond chemotaxis, extending to trigger protein transcription and cellular behavioural shifts. The presence of a *Glutamine-Leucine-Arginine* (ELR+ motif) stimulates angiogenesis, and even its relative absence through overexpression of CXL9/CXCL10 (both ELR^-) has been noted to suppress this behavior in a murine Burkitt's lymphoma model[75,76]. The significance of angiogenesis to tumour survival and progression

is highlighted by the increase in microvessel density, neovascularization of peritumoral stroma, and worsened prognosis in Melanoma and Pancreatic Cancers associated with increased CXCL1/CXCL8 expression[77,78].

Non-Chemotactic functions of Chemokines

Beyond stimulating cellular extravasation and proliferation, Chemokines can influence the behaviour of endothelial and stromal tissues, and this effect is well demonstrated by VEGF, which acts to increase angiogenesis and vascular permeability. Expression is markedly increased in SCC compared to KA and BCC, and significantly correlated with degree of tumour differentiation[79,80]. It likely enhances tumour angiogenesis by increasing microvessel density, which is associated with increased expression of Vascular Endothelial Growth Factor (VEGF) mRNA, and is higher in SCC than BCC[81]. Prognostically, higher levels of VEGF and Microvessel Density (MVD) are associated with worse overall mortality in HNSCC, but interestingly not with risk of metastasis[82].

The level of IL-8 expression has been shown to impact growth potential of tumours. Human Ovarian Cancer cell lines constitutively producing IL-8 have a higher proliferative rate than those with lower levels[1]. This production is increased by the hypoxic conditions created by high turnover tumours that proliferate faster than their blood supply can support. Ovarian cancer cells display IL-8 mRNA and protein expression highest in areas adjacent to necrotic and hypoxic zones[1].

This stimulation of angiogenesis appears fundamental to tumour progression to invasive disease. In advanced disease, peripheral tumour cells divide and proliferate in concert with neoangiogenesis and the proliferation of new endothelial cells. Whilst the capacity of the tumour to induce angiogenesis has not previously correlated with malignant staging, its absence precludes the expansion of the tumour mass, regardless of the proliferative capacity of cells. This dependence on enhanced blood supply through increased MVD has been shown with VEGF, and appears particularly important for the progression of premalignant conditions to invasive disease[14]. Furthermore, the blockade of NF-κβ signaling suppresses IL-8 and VEGF expression and reduces ovarian cancer tumorigenicity, highlighting their implication in disease progression[2].

Interleukin-8

Interleukin-8 is a ELR+ C-X-C chemokine produced primarily by macrophage and mesenchymal cells in response to a range of factors: macrophage-cancer cell interaction; infection; inflammation; ischaemia; and, trauma[83].

It can also be produced by keratinocytes in response to the above, but also to UV light (280-320nm), explaining its presence in sunlight induced cancers[84,85].

Otherwise known as Neutrophil-Activating Peptide-1, the ELR+ CXCL8 (IL-8) is primarily produced by macrophage and mesenchymal cells in response to inflammatory stimuli[44,75]. This has been shown to stimulate haptotactic transendothelial migration via a CXCL1/CXCL2 dependent mechanism, both ex-vivo and in melanoma and prostate cancers[86–88]. This mechanism has been experimentally blocked via administration of anti-IL-8 antibodies with good effect ex-vivo, however more recent studies using anti-CXCL-1/-2 to reduce neutrophil migration in respiratory disease has also been promising[89].

Whilst well known to have significance in the activation of neutrophils, lymphocytes and macrophages, it has a significant role in cancer progression, angiogenesis and metastasis[90]. Studies of Non-Small Cell Lung Cancer demonstrate a dose dependent response to IL-8 via infiltrating TAM density and MVD, which correlated negatively with patient survival[91].

IL-8 expression is enhanced in response to ischaemia stimulated Hypoxia-Inducible Factor 1a gene transcription, commonly occurring in the peripheral margins of tumours outgrowing their blood supply due to rapid proliferation[1,92–95]. Anoxia induces an enhanced binding activity of transcriptional factors AP-1 and NF-Kκβ in aggressive melanoma cells lines, which in turn induces IL-8 expression[94,95]. This effect has been observed in rapidly growing ovarian cancer cells in response to hypoxia, and subsequent experimental blockade of IL-8 reduced tumorigenicity[1,2].

Macrophage Inflammatory Proteins-1α and -1β

The Macrophage Inflammatory Proteins-1α and -1β are related members of the C-C chemokine subfamily, and besides keratinocytes, in the skin are produced largely by Antigen Presenting Cells (APCs)[67]. They show differential signaling at CC-Receptors CCR1, CCR2, CCR3, and CCR5, based on their terminal NH(2) residues[66].

These proteins have Chemotactic/ Proinflammatory effects, roles in resistance to bacterial infections. They are up-regulated by IL-1ß, TNF-a, and down regulated by Tacrolimus, and PGE2[72,66,33]. Inhibition of expression by PGE2 (anti-inflammatory), suggests an inflammatory action further supported by in-vivo studies of dendritic cells stimulated with LPS and Peptidoglycan[66].

Studies of lymphoma models show high levels of CCL3/CCL4 expression by large populations of MDSCs accumulating in organs during tumour progression. Increased levels of Monocyte-MDSCs expressing CCL3/4 correlate with increased tumour-infiltrating T-reg presence. A deficiency in CCR5 (CCL3/CCL4 receptor) reduces T-reg infiltration and tumour growth[73]. A reduction in CCR5 ligands (CCL3/4) would predict a reduction in T-reg presence in peritumoral inflammatory infiltrate[40,73,38]. T-reg presence has been correlated with worse prognosis in cSCC, Therefore, on examination of the chemokine profile of advanced SCCs, one would expect to find increased CCL3/CCL4 levels, with increased T-reg presence, and a worse prognosis in these tumours.

Methods

Literature Review – a broad review of the literature was performed via Medline; bibliographic databases; academic abstracts; and, use of the University Library. Key search terms are listed in pre-text. Given the broad scope of the topic and minimal existing literature, no publication date limitations were put on the papers cited.

Population

Patients were recruited from the private practice of a single Plastic and Reconstructive Surgeon. Patients were included if they had skin lesions suspicious for keratinocytic malignancy of squamous differentiation. Patients were excluded if they displayed lesions that were either biopsy proven or clinically distinct Basal Cell Carcinoma or Melanoma. Initial conceptual plans to analyze the four major histopathological groups shown below led to a plan for 25 of each lesion subtype to be collected. After significant numbers of lesions being histopathologically different to preoperative clinical assessment – this number was not pursued, in an attempt to facilitate completion of the project.

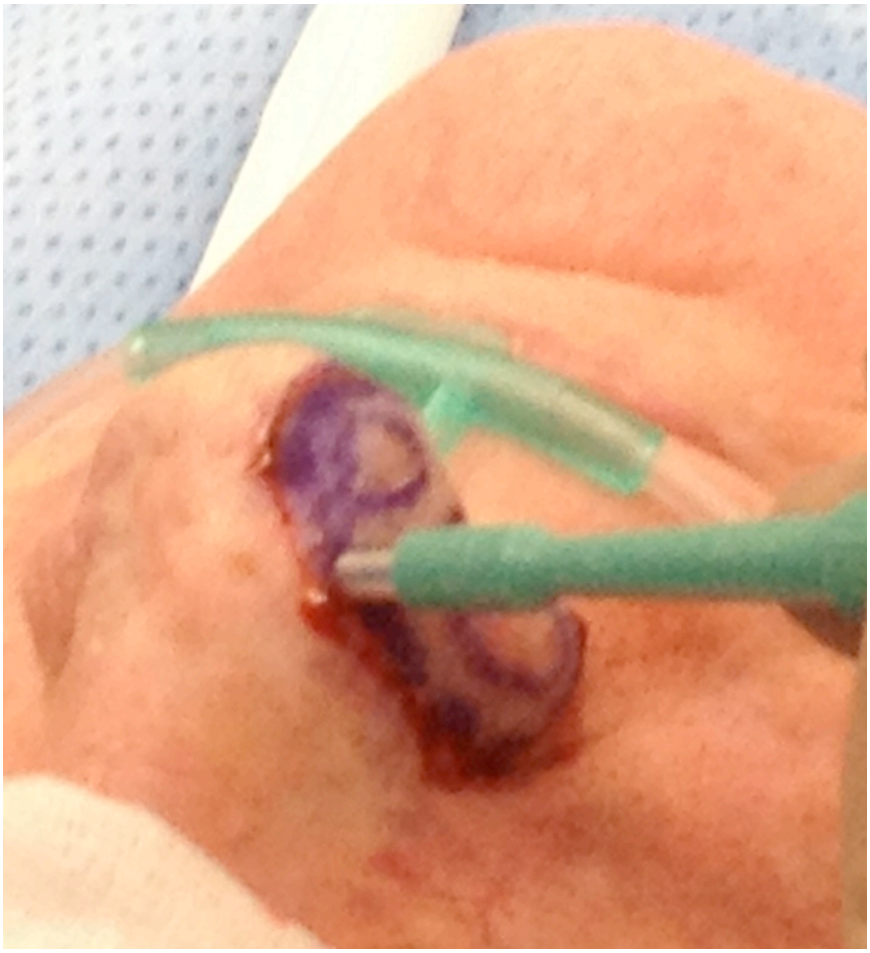


Figure 4. Peritumoral punch biopsy sampling technique.

Sampling/Sampling Procedures

After discussion between the Surgeon and involved Pathologist, lesions were sampled with a 4mm punch biopsy from either the 12 o'clock or 6 o'clock margin of the tumour (**Figure 4**). This was in an effort to preserve sound oncologic practice and not impact histopathological diagnosis or patient care. All pathology bloc specimens were then kept for a minimum of 5 years per Pathology Company policy.

Recruitment, Partcipation Data Collection

Patients were consulted on the day of their surgery preoperatively, and informed regarding the aims of the study, the techniques involved, and potential impact on pathological diagnosis.

Data Storage

De-identified data including patient information, consent forms, and specimens were kept on laboratory campus in labeled storage, and hard-drive format. This information will be stored for a minimum of 5 years per organizational policy.

Ethical Consent Process

Ethical applications were approved by the University of Queensland, and Greenslopes Hospital Ethics Committees, and were updated every 12 months in compliance with Codes of Conduct. The copies of patient consent and data forms are available in the appendix.

Lesion Assessment

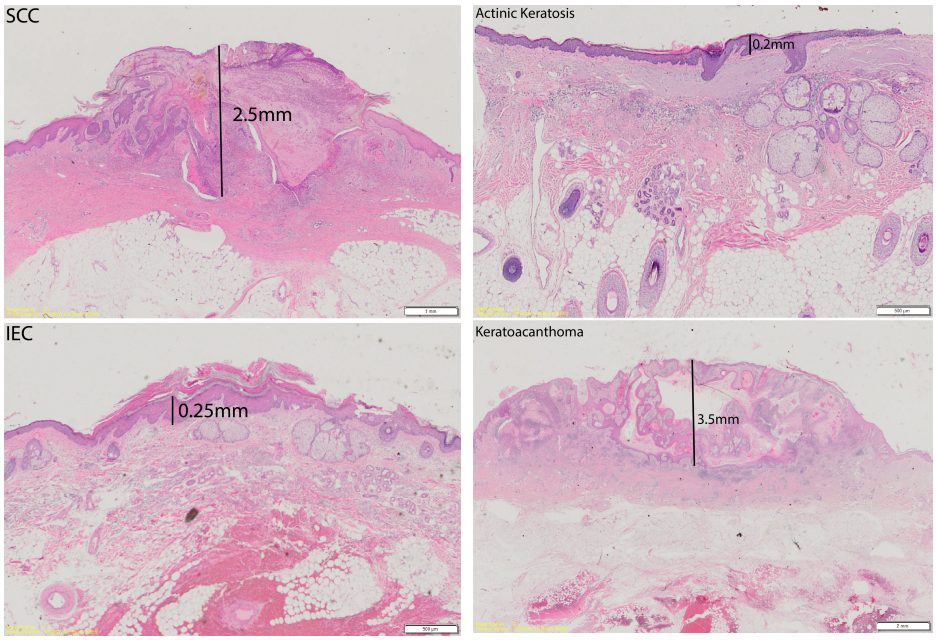


Figure 5. Histopathological grading and thickness of lesions.

Lesion H&E histopathology slides scanned at 20X magnification using the Olympus VS120 slide scanner, and analyzed using Aperio image software. Thickness was measured from the granular layer to the deepest part of tumour. Histopathology reports were gathered from private pathology laboratories, and were confirmed by re-analysis by a Consultant Dermato-pathologist (HS).

Immune Cell Grading

Immune cell infiltration was measured visually by duplicate histological assessment of H&E paraffin mounted slides of specimens (HS & MY), and scored from 0 to 3 for presence and density of immune cell infiltrate, utilizing a modified immunoscore for skin cancer[96,97].

Specimen Collection / Processing

Consented patients underwent surgery per normal oncologic practice. Excised specimens were biopsied using a 4mm punch biopsy (Figure 5). These specimens were transferred to 500µl cold PBS (< 4 degrees celsius) containing a broad-spectrum non-toxic protease inhibitor cocktail (EDTA-free “Mini-complete protease inhibitor cocktail” from Roche). Samples were kept fresh and cold during transit. Within 2 hours of receipt, these samples were dry-weighed, homogenized into solution (Figure 6) and deep frozen (-80 degrees Celsius).

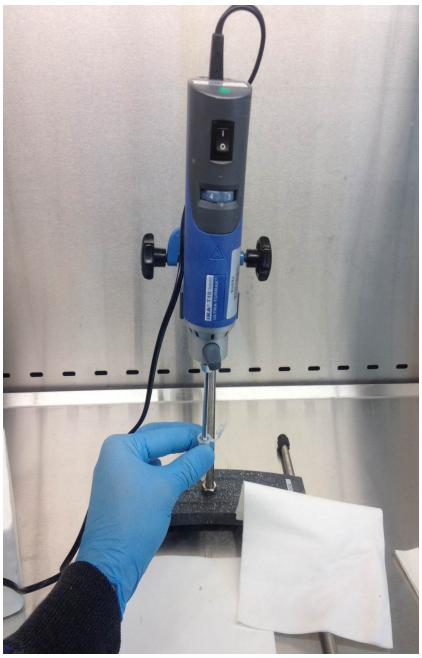


Figure 6. samples were homogenized into solution within their 500µl aliquots.

To standardize samples, lesions were weighed, and the concentration of chemokine in pg/ml via flow cytometry was analyzed. Each specimen was dry-weighed before homogenization into solution, and precipitates were tagged with the Multiplex chemokine assay (*LEGENDplex*™ (Fig 7)) for the 13 chemokines listed in **Appendix 1**. Results from FACS were standardized based on weight into pg/g of tissue for comparison.

Initial pilot study utilized a multiplex immunoassay with antibodies to IL-8; CCL3; CCL4; G-CSG; MIG; MCP-1 from **eBioscience** (Affymetrix, San Diego, USA). After production of this kit was ceased, the second and third validation studies used a dual bead multiplex immunoassay against human chemokines IL-8; IP-10; CCL3; CCL4; CCL11; TARC; MCP-1; RANTES; MIG; ENA-78; GROα; I-TAC from **BioLegend**, San Diego, USA (Figure 7.). Homogenized cells in the PBS-protease inhibitor solution were tagged with biotinylated detection antibodies. These were then sandwiched with capture-bead Streptavidin-phycoerythrin antibodies (SA-PE) to bind the biotinylated antibodies to enhance the fluorescence signal, and then analyzed using a Flow Cytometer (**FACSAria III Beckton Dickinson, NJ, USA**). All specimens were tested in duplicate. The concentration of each chemokine was then determined using a known standard curve from the LEGENDplex™ data analysis software.

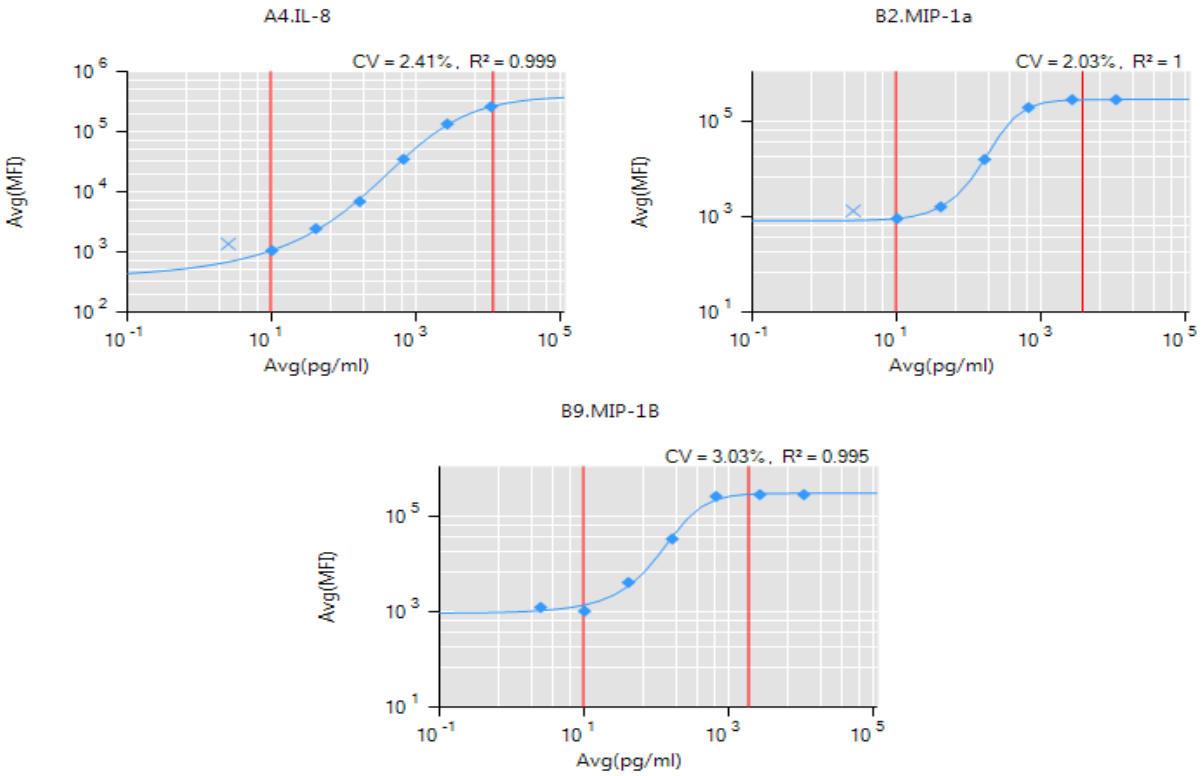


Figure 7. FACS Standard Curves – BioLegend LEGENDPlex Chemokine Assay

Statistical Analysis

Data obtained from flow cytometric analysis are expressed as mean ± standard deviation in pg/ml. This was converted by specimen weight to a pg/g value for relative comparison. Due to significant skewing of data, these values were logarithmically transformed.

Lesion thickness was expressed continuously in millimetres, and subsequently a Pearson correlation test was used to interpret relationship between these two factors. Given the difficulty in diagrammatic representation of this data, a line of best fit was chosen via linear regression.

Differences in expression between the histopathological subgroups, infiltration scores and body regions were determined by one-way ANOVA and Holm-Sidak multiple comparison test. A value of $p < 0.05$ was deemed significant. Multivariate analysis was performed using Linear Regression using **Prism** software (La Jolla, CA, USA).

All statistical methods were validated by an independent biostatistician (QFAB, A Bernard).

RESULTS

Patient demographics

Histopathology	Non-dysplastic	14
	AK	11
	IEC/ SCC in situ	18
	SCC	25
	Total	67
	Age range (mean)	45-96 (78.45)
	Gender	51M : 16F

Table 2. Patient demographics + Histopathology

A total of 33 patients ranging in age from 45-96 years old (mean 78 years old) provided 67 specimens for analysis. Non-dysplastic samples were comprised of 3 non-sun-exposed skin from a single patient; 5 samples of sun-exposed non-dysplastic tissue from a single patient who was SCC free; and 6 samples of non-dysplastic scar from re-excised tissue – at sites of previous SCC excision. 16 patients had multiple lesions (>2), and of these, 5 patients had >5 lesions sampled. There was a significant gender disparity – most probably due to the 51 specimens coming from 24 men. 11 of these men had >2 lesions, averaging 3.45 lesions per patient (total of 38 from 11 men). Of the women, 8/16 specimens were from 2 patients with normal skin specimens. The other 8/16 lesions came from 7 women and subsequently displayed a lower tendency to have more lesions. There was a disparate number of more invasive SCC lesions of the samples taken, which represents a sampling bias, resulting from patients referred for a higher level care from a specialist surgeon for more aggressive tumours, and not for less invasive AK/IEC/SCC in situ lesions.

Chemokine expression increases with increasing lesion thickness

Samples ranged in thickness from 0.1mm to 7.5mm (mean 1.175mm +/- 1.597). This was inclusive of dysplastic and normal skin. The expression of IL-8 and MIP-1α correlated significantly with increasing thickness (Pearson coefficient r = >0.4, p = 0.0003***), whilst MIP-1β did not. Thicker lesions contain higher numbers of keratinocytes and subsequently possess more potential for increased chemokine expression. This difference in correlation is potentially due to the pro-inflammatory impact of IL-8 and MIP-1α, also correlating strongly with an increase in inflammatory infiltration (Fig 8).

Despite significant individual correlation tests, Multiple linear regression modelling showed a significant correlation of chemokine expression with lesion thickness (p = 0.017) but not infiltration scoring (p = 0.147).

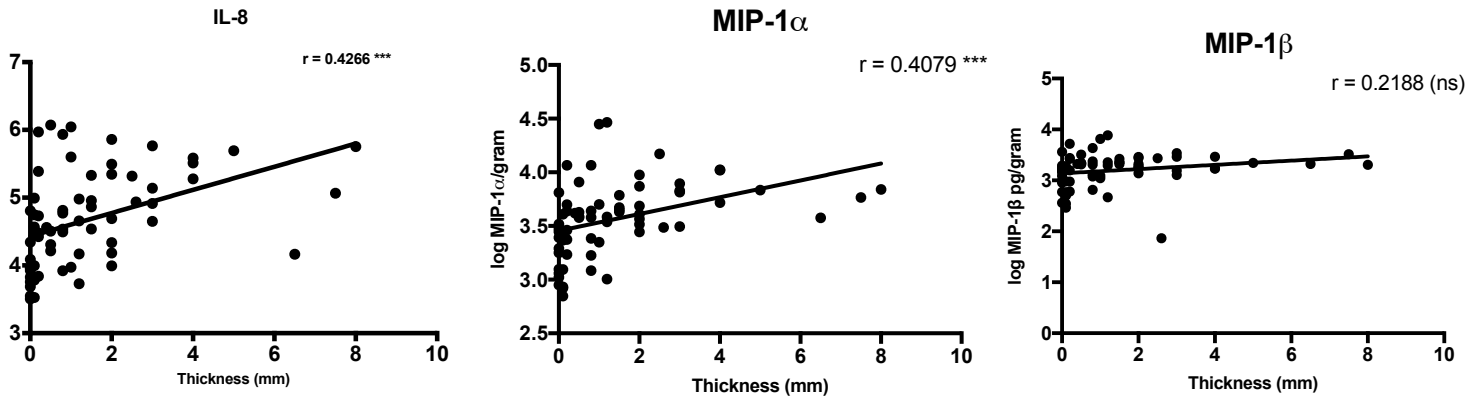


Figure 8. Correlation plots of log Chemokine expression vs lesion thickness. Pearson coefficient r on figure. Dots represent individual specimens with no differentiation between histopathological groups. (n = 67)

Histopathological severity impacts chemokine expression

Worsening histopathology was significantly associated with increasing chemokine expression in IL-8 and MIP-1α, but not MIP-1β (Fig 9). This was again likely a result of the increased presence of tumour-progressive cell types, secreting factors such as IL-8 that promote angiogenesis and assist in evading apoptosis. This increase with worsening histopathological grading supports the model of keratinocyte:cancer cell interactions increasing chemokine expression – more dysplastic cells potentiate the release of proteins for various roles[54,60,98].

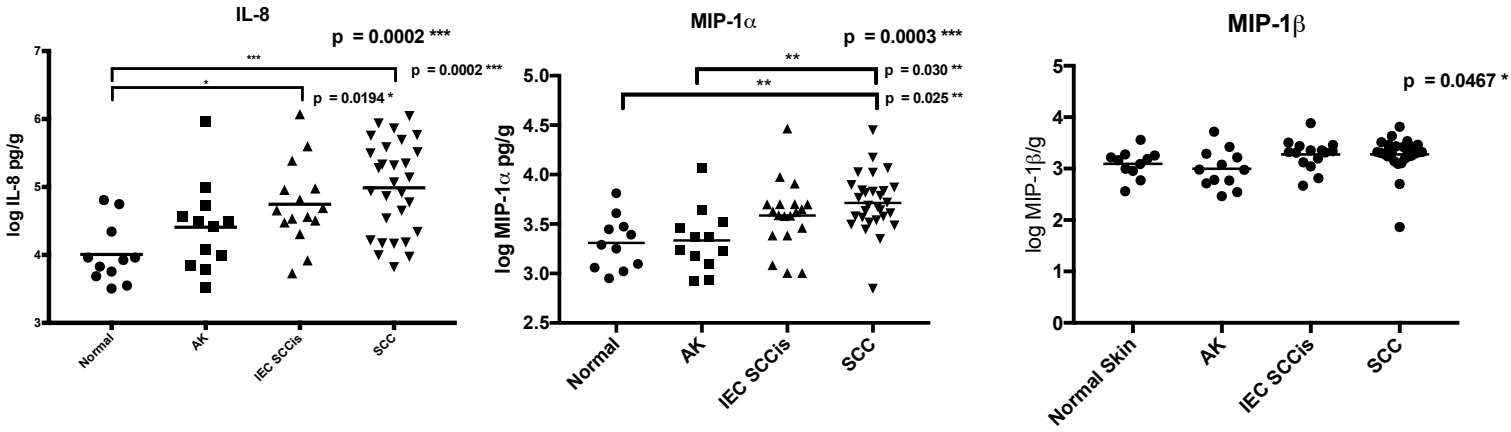


Figure 9. Chemokine expression vs Histopathological stages (Mean value (straight line) + Kruskal-Wallis One-Way ANOVA p values on figure). Dots in each column represent samples from each histopathological group on X axis. (n = normal skin (14); AK (11); IEC/SCCis (18); SCC (25)).

SCC is thicker and more infiltrated by Immune cells

Immune cell infiltration was graded on a scale from 0-3 (mean of 1.7716).

Lesions were significantly thicker the more dysplastic they were, most significantly seen in the invasive SCC group (Fig 10).

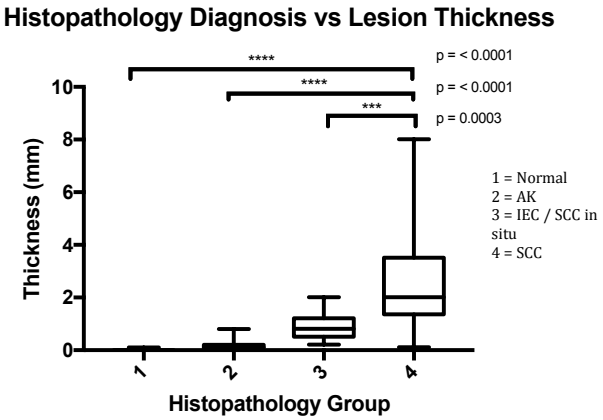


Figure 10. Histopathology Group vs Lesion Thickness. Box & whiskers plot graphically represented, with Kruskal-Wallis One-Way ANOVA p values on figure. (n = normal skin (14); AK (11); IEC/SCCis (18); SCC (25).

Thicker lesions were however not more infiltrated – this effect was maximal with lesions graded 1-2 (p = 0.0134*). This likely reflects the more infiltrated AK/IEC/SCC in situ lesions displaying immune cell attempts at clearance of the disease (Fig 11). Multiple linear regression modeling failed to establish a significant relationship between infiltration (p = 0.15) and histopathology (p = 0.4) with increasing lesion thickness. By failing to link

the two, the univariate anaylsis of histopathology and increasing lesion thickness supports histopathological grading and prognostic scores for cSCC.

From a prognostic standpoint, increasing thickness and worsening histopathology is correlated to increased risk of distant metastasis and a reduced overall survival[99]. Identifying the significance of chemokine expression in relation to prognostic outcome would provide a significant basis for further investigation into potential therapeutic targets for these patients.

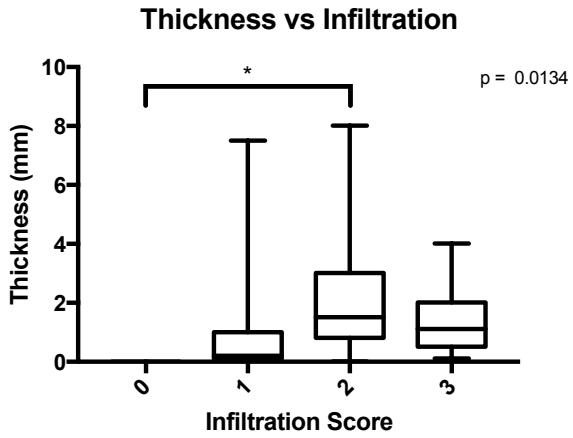


Figure 11. Thickness of lesions according to infiltration scoring. Box & whiskers plot graphically represented, with Kruskal-Wallis One-Way ANOVA p value on figure. Infiltration score (n = 0 (7), 1 (21), 2 (19), 3 (20)

Infiltration score correlates strongly with chemokine expression

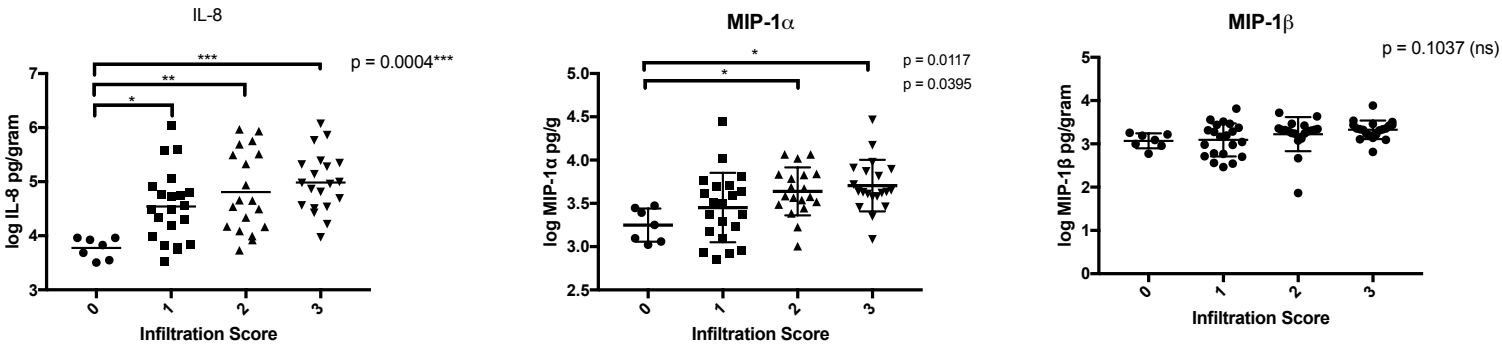


Figure 12. Chemokine expression by infiltration scoring. Mean value + SEM superimposed on values with Kruskal-Wallis One-Way ANOVA p value on figure. P value (= <0.01; ** = <0.001, *** = <0.0001). Infiltration score (n = 0 (7), 1 (21), 2 (19), 3 (20)*

One-Way ANOVA of increasing infiltration scoring correlated with the expression of IL-8 and MIP-1α, but not MIP-1β (Fig 12). More inflamed lesions produced higher amounts of the two more pro-inflammatory chemokines. These severely inflamed lesions did not necessarily make up the highest histopathological grading, the highest mean infiltration score was recorded in IEC/SCC in situ lesions (p= <0.0001***) (Fig 13).

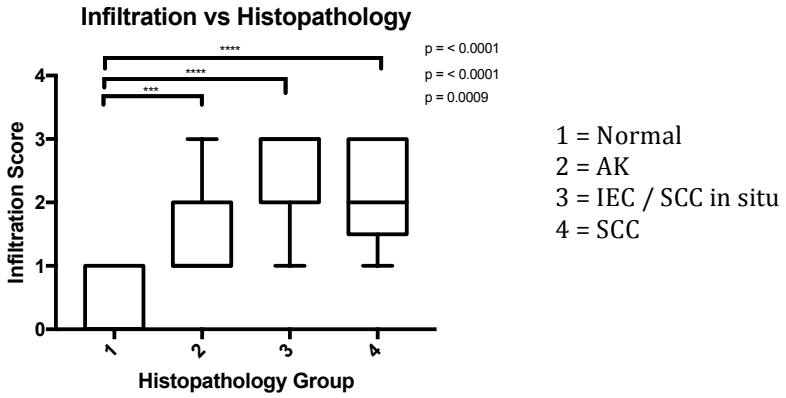


Figure 13. Infiltration scoring of histopathological groups. Box & whiskers plot of range & mean with Kruskal-Wallis One-Way ANOVA p values superimposed on figure. (n = normal skin (14); AK (11); IEC/SCCis (18); SCC (25)).

Tumour location does not impact lesion thickness or infiltration score

Specific tumour location was sub-group analyzed by physical region. No significant differences in chemokine expression, tumour thickness, or immune infiltration existed between regions. There was no difference in the expression in sun damaged vs non-sun damaged skin samples, likely due to a low sample size. Despite an anatomical difference in the thickness of the dermis and structural skin unit across these regional locations, no significant difference in chemokine expression was evident across the three groups in One-Way ANOVA (p values [IL-8 = 0.1627; MIP-1 α = 0.6437; MIP-1 β = 0.6997]).

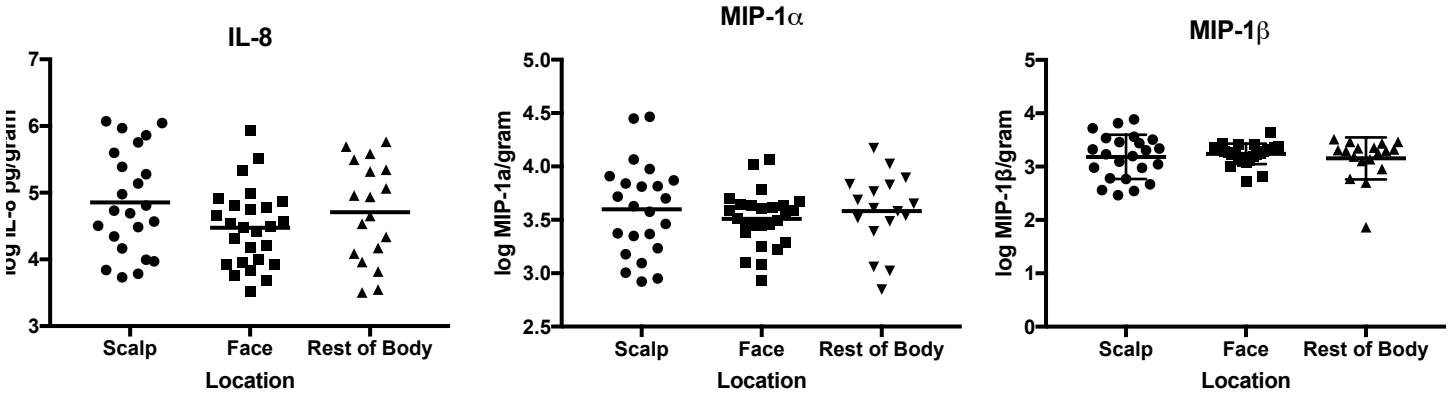


Figure 14. Chemokine expression of lesions from different tumour locations. Mean graphically displayed on figure. (N = scalp (22), Face (26), Rest of Body (18)).

Tumour location did not significantly correlate with infiltration score (Pearson r = 0.04696) (Fig 15b). Theoretically a reduction in the capacity of poorly vascularized tissue in the feet and legs of patients with peripheral vascular disease may impact on relative immune response, no difference was seen in the specimens. This was a sample bias, due to the majority of significant lesions referred to private Plastic Surgeons being on the Head/Neck and cosmetically sensitive areas. More variation in response, particularly on the lower limbs in an older cohort of patients would be expected.

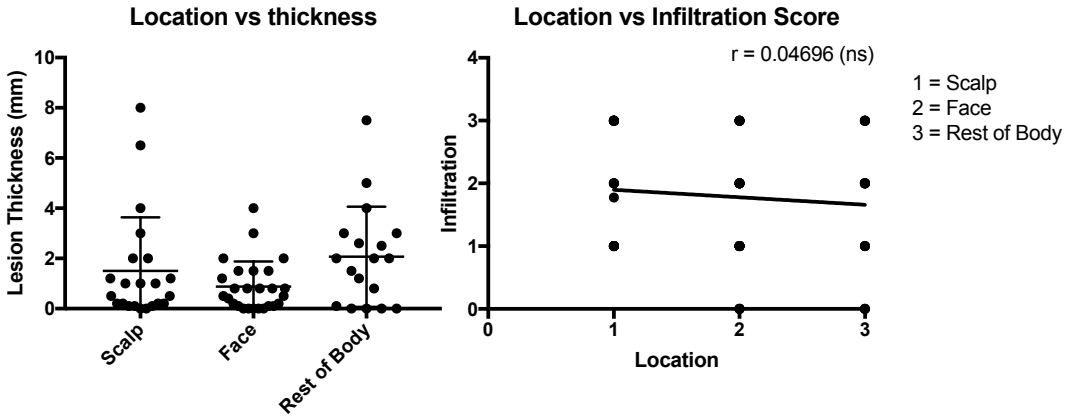


Figure 15. a) Lesion thickness relative to regional location. B) Lesion infiltration score relative to body location. Pearson Correlation r value superimposed on figure. (N = scalp (22), Face (26), Rest of Body (18)).

Intrapatient Variability of Chemokine Expression

While lesion thickness, immune infiltration and dysplastic change have attempted to analyze the impact of these factors; they have been approached as if each lesion was from a discrete patient. **Table 2** describes the patient cohort, and the large number of patients with more than 2 lesions sampled at a time. As a significant number of patients had multiple lesions, and the majority of those were males, there exists a high risk of sample bias. To remove the possibility that 1 patient may have consistently high levels of a chemokine or chemokines and skew the results, an analysis of intrapatient variability was undertaken.

5 separate patients had over 5 lesions each excised, the results of which are listed in Appendix 2. As these patients did not have individual controls, and had a variety of numbers of each type of lesion, no statistical tests were uniformly available to compare each group. Consideration was made towards converting data to Z scores in order to compare the mean data – however sample size remained to small and inconsistent for comparison. What can be established from the visual representation of the results is the high degree of variability between different specimens in the same patient. Figure 17 displays the high variation between lesion thickness and chemokine expression in all three groups in a single patient. These 5 specimens represented 3 of the histopathological groups as shown in the legend. Tissue histopathologically described as normal had similar output to that of AK/IEC/SCC in situ lesions. This may reflect sampling bias, as no normal tissue was intentionally excised from this patient, and may have clinically represented a re-excision site of a previously biopsied specimen that, whilst having no malignancy, represented an area of post-operative inflammation. The comparison between histopathological groups is displayed in Appendix 2. There was no significant difference in the chemokine expression of lesions in each patient, suggesting this may have more to do with individual patient reaction to inflammatory or cancer stimuli, however without truly representative control samples of non-damaged tissue from each patient this cannot be formally commented on.

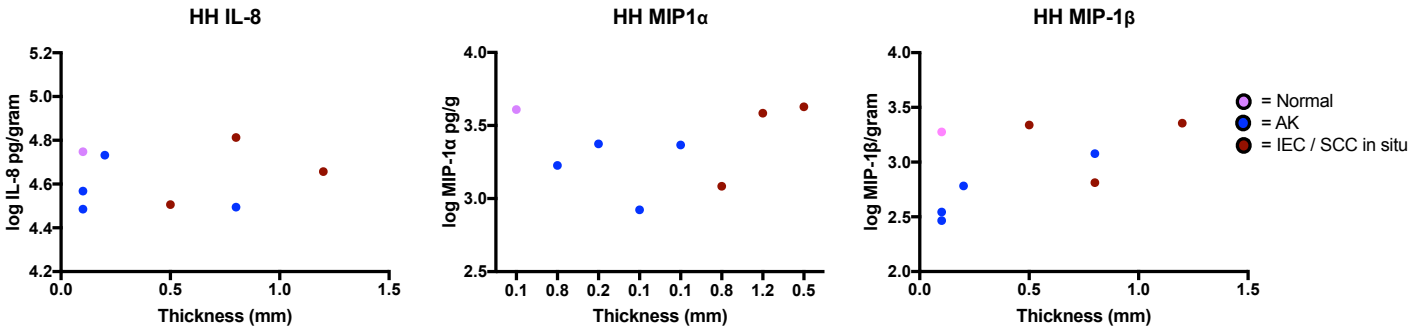


Figure 16. Intrapatient variation of chemokine expression in lesions of varying thickness.

Discussion

Tumour thickness was significantly correlated with increasing chemokine expression amongst all three groups (Fig 8). This is likely a demonstration of increased keratinocytic translation of chemotactic proteins in response to an increased local tumour burden in these lesions, and mimics the theory of increasing chemokine expression by keratinocytes due to hyperkeratosis often seen in keratinocytic dysplasia, and also due to keratinocyte: Cell interaction causing positive feedback and increased expression[54,60,98]. Peripheral ischaemia and resulting hypoxia resulting from rapidly proliferating tumours have been shown to reactively express pro-angiogenic chemokines and growth factors[1,78,95]. As IL-8 has been previously shown to increase in hypoxic tissue in response to an increase in HIF-1α expression, the increase demonstrated by the thicker lesions in this study support this model, despite significant variation in expression with progression[1,93,100].

Theoretically a thicker lesion, with increased dysplastic change, would have a higher element of tumour burden and immunogenic stimulus to support thicker inflammatory infiltrate. Whilst lesions with an infiltration score of 2 were significantly thicker than those scoring 0 (Fig 11, $p = 0.0134$). This relationship did not correlate in higher infiltration scores of 3. Inflammation is a feature common to advanced tumours – a chronic inflammatory cell infiltrate has been described both in peripheral stromal tissue and within the body of the lesion in a variety of cancers[101]. There is a significant inflammatory response evident in progressive stages of AK/IEC/SCC in situ. Inflammation is associated with the progression of AK to invasive SCC[102,103]. This inflammation is more than likely a pre-requisite rather than a result of cancer, and can be associated with cellular attraction and growth factors necessary for chemotaxis, activation and proliferation of immune cells[15,54,104].

Increasing dysplastic change across the groups was shown to significantly ($p = <0.0001$) correlate with both increasing inflammatory infiltrate and lesion thickness (Fig 10,13), which mimics previous reports in the literature in both OTR and immunocompetent populations[57]. The loss of a homeostatic chemokine expression has previously been shown to result in unregulated immune cell infiltration and failure of organization[105].

It is likely that increased expression of IL-8 in cSCC may result in increased infiltration of leukocytes that potentially will produce their own growth factors and chemokines, creating a positive feedback cycle, which results in angiogenesis, cell proliferation, and expansion of the tumour mass.

The inflammatory infiltrate increase does not initially support the idea of increased inflammation rejecting the growth of AK. This effect can be induced by topical therapies such as Diclofenac and Imiquimod. Both AK and SCC commonly display an interface dermatitis characterized by CTL infiltration[106]. This inflammatory stimulus in AK has been shown to precede malignant transformation to SCC and represents an attempt at immune rejection of these lesions. 50% of inflamed, clinically diagnosed but tender and erythematous AK are actually histologically SCC. Attempts to induce cytotoxic rejection fail, and these lesions display significant inflammation displayed by higher levels of CD3+; CD4+; CD8+ lymphocytes when compared with asymptomatic AK[103].

Capacity to mount a significant immune response in the case of immunosuppressed patients factors heavily in both the clinical and pathological patterns of disease seen in this cohort. Post solid-organ transplantation, risk of cSCC increases by 65-100 fold, and the ratio of BCC:SCC reverses from 3:1.7 to 1:2[9]. Increased ratios of T-Reg:CD8+ has been shown to contribute to more aggressive cSCC growth in OTRs[107,108]. High proportions of FOXP3+ T-Regs (>50% of T cells) are present in SCC – and much more than is seen in AK lesions[109].

Fundamentally, Clark et al demonstrated 50% of T-Cell infiltrate in untreated SCCs were FOXP3+ T-Regs, that displayed minimal Ki-67 staining, and suggests these cells are recruited, and not produced locally. Tumours pre-operatively treated with the immune-modulator Imiquimod showed reduced FOXP3+, CD39, CD73, IL-10 and TGF- β levels – down staging their inflammatory profile[28].

Prognostically, the literature provides varied outcomes with regards to immune infiltrate phenotype. Gonzalez-Garcia et al demonstrated that increased density of inflammatory infiltrates is correlated with protection from development of local recurrences and improved survival in HNSCC, and this was mimicked by Tosolini in regards to these cell populations in Colorectal Cancer[18,110]. This contrasts from data by Kosmidis et al, who found reduced

FOXP3, TGF-B, and CD4 mRNA in OTR SCCs, and increased local recurrence in aggressive tumours[39]. Studies of colorectal cancer have shown survival benefits with increased FOXP3+ T-Reg infiltration[17], and more relevantly – reduced infiltration of FOXP3+ in cutaneous SCC correlates with worse prognosis[57]. Conversely – Gerber et al demonstrated a negative prognostic value of FOXP3 expression in melanoma, independent of other significant factors[111].

Immune cell migration in response to chemokine expression has been both demonstrated and rejected experimentally and clinically in other tumours[40,46,98]. Despite our data supporting an increase in inflammation with increased dysplasia (Fig 13) we have not examined for cellular composition beyond histological examination. **Figure 12** demonstrates significant correlation between inflammation and chemokine production of MIP-1α and IL-8, and a lack of the same in MIP-1β. However we are unable to infer where the chemokine production is coming from, inflammatory cells or tumour burden. The increase in chemokine production without relative increase in immune infiltration may relate to recently described atypical chemokine receptor function at sub-receptor CCR-D6, which suppresses inflammatory response. This was not examined for in this study, however may represent a target for future research[51,112]

Increasing thickness correlated significantly ($p < 0.05$) with IL-8 expression when analysing immune infiltration scores separately across the groups (Fig 8, Fig 12). While variance was significant across all groups, multiple comparison tests failed to demonstrate significant differences in MIP-1β between infiltration scores. IL-8 and MIP-1α support a worsening pathological diagnosis being correlated with a higher immune infiltration and, separately, a thicker lesion correlating with a denser infiltration and higher chemokine expression. Mühleisen et al showed an increase in the thickness of inflammatory infiltrates with progression from IEC to invasive SCC[57].

One potential cause for a lack of significant correlation between increased MIP-1β and effective immune infiltration is the effect of protein aggregation observed in MIP-1α. At a concentration of 100ug/ml, MIP-1α exists as a dodecamer in physiological buffers. Sequential dilution progressively breaks up the aggregate via tetramers and dimers to the monomeric form. Aggregation of MIP-1a is a dynamic and reversible process and it appears that the chemokine is exclusively in the monomeric form at physiologically

relevant concentrations (less than 100ng/ml). While a plateau or decrease in expression was not observed in MIP-1α, the two proteins contain 58% identical amino acids, and theoretically may be at risk of the same phenomenon[113]. This effect has not been described in IL-8 before.

Conclusions

The expression of the chemokines *IL-8 and MIP-1α* correlates with increasing thickness, infiltration and histopathological severity in cSCC. These effects were not demonstrated with *MIP-1β*.

Invasive cSCC lesions were thicker and more infiltrated by immune cells than their precursor lesions ($p = 0.005^{***}$). IEC/SCC in situ lesions were the most highly infiltrated, which likely represents an attempt by the immune system to reject these malignant cells, and which process has failed in the more invasive cSCC lesions. The continuing increase in *IL-8 and MIP-1α* seen with increasing histopathological severity may be a consequence of keratinocyte: cancer cell interactions stimulating increased expression. However, the increase in size of more advanced lesions causes greater areas of ischaemia and induces the transcription of HIF-1*α,* which in turn stimulates IL-8 expression in an attempt to increase neoangiogenesis and improve blood supply.

Whilst tumour location did not significantly impact chemokine expression, this may represent a sampling bias, as patients referred for Plastic Surgery to remove these cancers are more likely to have lesions in cosmetically sensitive areas such as the Head/Neck and Face removed, and this was reflected in the regional breakdown. The lack of lower limb lesions in an elderly age group represents a further sampling bias. The prevalence of peripheral vascular disease and poor blood supply to limbs has an unknown impact on the capacity of immune cells to mount a response to cancer cells by chemokine expression. Despite patients being screened for significant medical comorbidities, studies of chemokine expression in cutaneous atopic and inflammatory conditions requires further comparison between the levels expressed between these and malignant diseases.

This study is generalizable for future studies to draw conclusions from – the high ratio of males: females is representative of the epidemiology of disease in the population, and the multiplicity of lesions on males is unfortunately endemic of this gender. The lack of control samples, and immunosuppressed patients represents a limitation which should be factored in to future research on this topic.

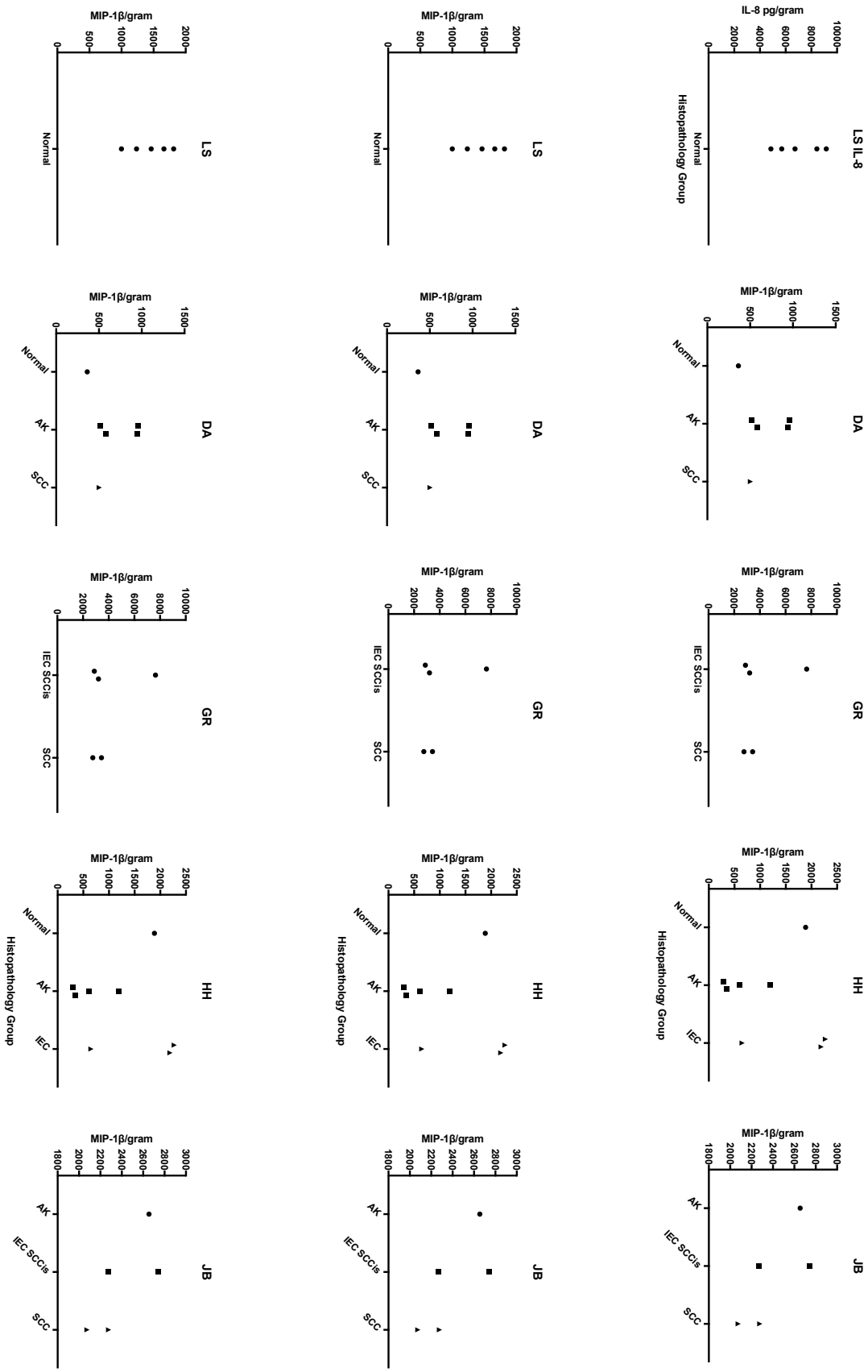
This study has effectively answered the question of whether or not chemokine expression in cutaneous SCC is affected by tumour thickness in a quantitative manner. However, qualitatively, the functional impact on tumour immune phenotype relative to the attracting chemokines is yet to be established. Given the known prognostic implications of chemokine expression and tumour cellular composition in other cancers, future studies should aim to identify the cell types present in response to chemokine expression.

Appendix 1

Prof Soyer Path	Thickness (mm)	Infiltration	Body Region	IL-8 pg/gram	MIP-1a/gram	MIP-1b/gram	IP-10/gram	Eotaxin/gram	TARC/gram	MCP-1/gram	RANTES/gram	MIG/gram	ENA-78/gram	MIP-3a/gram	GROa/gram	I-TAC/gram
AK	0.1	1	face	181.41	211.88	167.2086257	**24601.23**	542.87	1152.32	1557.93	4489.30	1207.94	72.22317303	784.19	700.4963204	1072.565463
AK	0.1	1	scalp	1317.88	329.26	202.1937086	**31012.83**	1355.75	6581.13	4389.90	6169.29	4375.41	87.33443709	2581.54	1800.910596	2586.92053
AK	**0.1**	1	scalp	4545.27	258.93	179.7938903	**50187.15**	1143.72	5596.43	4814.50	5342.47	14965.59	77.65918292	5201.88	2918.476261	2857.747516
AK	0.2	1	scalp	304.26	499.16	235.3649723	**52302.58**	1265.48	2144.54	2436.28	5435.56	10001.20	101.6622501	1848.47	2519.633823	1309.56396
AK	0.2	2	scalp	381065.05	3405.31	554.6574288	**59297.15**	6243.65	8681.68	9303.31	9989.99	13068.90	73392.22479	8013.47	16084.68052	3389.915319
AK	0.1	1	scalp	28029.29	1319.91	188.2829061	**78062.63**	1750.24	2214.88	12817.31	5694.16	18791.10	568.5103103	5548.47	2812.29524	1751.011756
AK	0.1	1	scalp	13255.50	402.48	208.7160863	**113380.26**	963.90	1302.29	8268.32	7795.56	28680.62	404.8280282	8358.26	2117.282632	2278.359325
AK	0.2	1	scalp	25942.79	407.41	323.0820106	**216839.29**	786.71	2168.65	5765.21	316.14	42765.87	139.5502646	6807.54	2460.978836	2747.354497
AK	0.2	3	face	3382.91	1357.29	548.241206	**502924.62**	6816.58	16264.32	16301.51	19800.00	130622.61	282.9145729	8669.85	7089.949749	24192.46231
AK (could be re-excision)	0.1	2	leg	856.95	570.18	328.0725319	**26505.71**	1508.39	524.85	3372.73	9309.27	3041.97	141.7058428	4282.74	1374.412357	2900.268637
Hyperplastic AK	0.1	3	face	107082.74	1528.44	661.926309	**65207.50**	5017.13	5368.13	16008.08	38434.07	21288.95	1021.008403	25123.46	10355.52683	6796.703297
IEC	0.4	3	face	21237.93	1086.79	413.6325148	**119894.58**	5316.26	4488.57	37926.33	19844.62	45267.57	1277.730737	12433.95	4417.442845	6923.793395
IEC	0.5	3	scalp	482694.78	5029.25	3838.127743	**105783.03**	8049.24	4215.02	12579.23	14625.06	62919.06	5988.785958	6810.82	60480.7411	7589.468552
IEC	1.2	3	scalp	38028.06	1778.70	3099.489796	**638278.06**	4943.24	4039.54	28493.62	55460.46	750734.69	277.4234694	10998.09	20343.75	60670.91837
IEC	2	3	scalp	20652.28	1623.10	900.6079027	**274127.66**	26154.10	2896.96	123227.05	39482.07	118072.04	5564.133739	1706.08	13314.28571	10231.61094
IEC	1.2	2	face	28353.35	720.64	324.1539482	**332057.07**	969.14	2092.24	4403.12	2739.55	26455.54	140.0132714	2532.18	9353.019244	1919.708029
IEC	0.5	3	scalp	14603.26	517.56	408.4448161	**287981.61**	1852.42	719.06	5573.16	10987.88	66541.81	338.6287625	1142.98	1711.120401	2078.595318
IEC	0.8	3	face	36676.06	2477.99	262.2114868	**146126.41**	3316.69	15408.21	16867.15	5473.43	35498.12	794.1492217	7450.89	6097.42351	8263.01664
IEC	0.8	3	face	116229.21	1269.54	271.7663421	**234252.57**	2494.85	5465.92	15180.53	6349.65	31150.49	526.286509	8726.56	7876.216968	5270.375522
IEC	0.2	3	scalp	141121.66	710.88	233.0629771	**15610.21**	2372.14	1009.30	10697.28	9436.31	6397.19	1104.007634	4333.02	10702.52863	1475.429389
IEC	0.5	1	face	19296.95	827.48	372.9007634	**37992.75**	2850.38	3727.48	4850.38	19358.40	10212.21	365.2671756	13945.04	6514.885496	3816.030534
IEC (Bowen's Disease)	0.8	3	arm	16232.26	790.66	366.2955687	**130077.28**	3001.09	3630.87	10288.15	18888.02	46096.05	408.6444008	17679.33	4380.048024	11263.91618
massive cluster	6	1	leg	141409.21	1205.93	517.2048703	**85316.57**	4944.42	1832.19	12235.57	16716.78	8615.14	1049.232398	5408.68	12552.67337	3824.775013
Norma (AK) at edge	0.1	1	face	6181.26	287.95	287.9457707	**221542.59**	1005.60	1751.25	3963.45	4541.70	15991.45	791.0403772	1529.91	1206.307103	1577.365164
normal	0.1	1	scalp	25064.17	304.15	480.0946548	**44422.33**	988.59	3771.99	9928.45	15582.13	21468.96	269.0701559	6000.84	3450.02784	2934.716036
Normal (inflamed excision	0.5	2	face	196990.82	8904.94	1432.470588	**83344.47**	7170.59	23676.47	53247.06	48068.47	38987.29	43360.47059	6778.59	27319.76471	4056.941176
(severe elastosis, AK at th	0.1	1	scalp	34155.61	657.73	319.3854201	**101898.99**	1729.00	5298.79	12294.21	29801.24	69127.82	1160.183066	12050.67	1338.01896	8905.524681
normal skin	0.1	0	thorax	385.85	511.07	284.5078626	**11696.85**	1668.61	4195.40	3596.10	16075.71	752.77	122.8887595	1193.65	1685.497962	962.7256843
normal skin	0.1	0	thorax	207.91	362.82	208.7606838	**7180.77**	1145.51	801.92	1352.14	1981.41	585.68	90.17094017	808.55	1788.675214	542.0940171
normal skin	0.1	0	thorax	264.98	545.48	266.0675381	**4268.52**	314.00	675.93	784.04	1342.32	698.26	114.9237473	618.19	1114.651416	448.8017429
normal skin	0.1	0	face	449.94	422.72	286.0070258	**8060.01**	1206.67	626.76	2689.70	3478.92	393.74	123.5362998	596.02	1198.185012	469.8477752
normal skin	0.1	0	face	506.32	301.08	301.0785824	**8891.83**	2128.20	1579.35	2954.39	6236.98	203.39	130.046225	1118.95	1976.271186	1539.291217
normal skin	0.1	0	face	761.96	493.39	307.6196474	**25172.23**	2268.89	3637.28	4189.55	7283.06	1006.30	188.6020151	868.39	1770.780856	1085.012594
normal skin	0.1	0	face	473.94	916.54	368.9577039	**13921.83**	1696.37	1408.23	3572.89	5707.70	682.40	159.3655589	1313.07	1545.694864	725.0755287
normal skin	0.1	0	face	340.51	459.82	269.8149682	**11566.14**	1643.19	3832.64	3629.94	12653.69	1007.46	228.9422811	714.72	1130.350732	665.5619994
scar	0.1	1	face	11506.15	1012.84	522.7394329	**75623.33**	4316.75	6423.76	9644.19	14473.52	14425.90	258.4269663	13394.33	9704.654896	4336.543606
Scar	0.1	1	leg	179935.84	9874.77	2300.617957	**167178.12**	10816.43	9965.47	73685.39	36193.75	92290.80	34655.21628	18085.61	42530.17085	7064.703744
SCC	2	2	leg	12453.49	987.77	277.9516358	**78533.43**	2409.39	13660.88	10998.29	11006.83	9678.81	1545.376956	8456.90	6017.923186	5272.830725
SCC	1.5	2	head	5136.31	1122.83	376.2033115	**45673.85**	2946.86	1651.14	3229.50	10458.22	9922.22	180.9780516	10328.07	4017.712745	6355.025029
SCC	3	1	face	31110.54	2689.39	336.4325069	**28187.33**	896.35	2864.33	9141.87	23636.71	10602.96	1380.853994	4013.43	3047.520661	1948.347107
SCC	2	3	scalp	397593.17	1307.23	392.3694779	**228052.21**	4112.85	1620.88	62953.41	10557.03	30479.52	9626.907631	4295.18	35718.4739	2691.566265
SCC	3	3	scalp	97423.02	3259.09	1299.358517	**163621.53**	6718.82	1803.99	16374.55	21993.23	74810.41	1915.894512	3893.80	35170.70563	5537.419815
SCC	1	1	scalp	380441.10	3636.53	1798.630137	**456995.43**	9098.63	6573.52	33204.57	29348.86	208659.82	7725.570776	30478.54	71974.88584	12222.83105
SCC	1.5	2	face	19146.69	2838.68	509.4076655	**348717.77**	6966.55	4831.71	10045.99	20769.34	169835.89	605.5749129	5489.90	5924.390244	14456.44599
SCC	1	3	scalp	238.80	274.19	224.5460814	**25678.92**	233.05	275.57	2428.87	4254.42	5539.42	96.98919789	1407.72	940.7032866	955.8722133
SCC	3	3	leg	237868.09	1083.37	371.4560308	**240466.12**	3758.77	1622.06	26491.35	23312.59	101476.21	1622.537242	2475.25	3650.408457	9823.882749
SCC	2	3	arm	59242.78	707.52	254.2284673	**89112.67**	3866.77	1236.79	7196.46	13095.50	9482.96	1252.14676	9549.05	3929.221962	4231.850117
SCC	2	1	face	4082.72	549.07	289.6531278	**44827.16**	1880.82	5014.53	3737.03	4315.15	9254.37	125.111177	8059.29	3676.845538	3380.670027
SCC	2.6	2	leg	253580.90	2010.73	1097.688498	**275404.51**	23667.31	20018.44	95812.60	24732.25	366128.23	4274.35333	8877.00	12096.58778	61866.81343
SCC	2	2	face	5271.53	1889.24	999.6527778	**100604.86**	10191.32	7079.86	10307.29	21324.65	21662.50	1020.486111	24105.21	13328.125	11957.29167
SCC	0.5	3	face	351.26	352.71	352.7075812	**352.71**	366.06	332.85	352.71	345.13	238.27	152.3465704	3907.58	1477.617329	2780.505415
SCC	0.8	2	face	391616.69	8125.00	6716.376582	**395893.99**	7365.51	5469.94	26074.76	66369.46	526309.34	6798.259494	7571.60	29943.43354	92584.6519
SCC	5	2	arm	201507.63	17505.19	4893.751272	**162412.78**	4118.66	2723.39	82519.23	12213.11	112235.70	38809.89212	20440.26	45664.15632	12913.69835
SCC (IEC at the edge)	4	3	scalp	160894.13	5671.45	1482.240437	**341810.11**	4554.64	2286.89	22991.80	27062.84	192739.41	1079.918033	10209.70	8146.174863	29997.60929
SCC in situ	0.2	1	face	4361.36	2145.92	394.9070331	**404535.17**	8010.51	5227.16	10012.13	17665.32	71831.85	208.1649151	3274.05	2561.843169	34556.99272
SCC in situ	0.2	1	thorax	522.28	850.46	430.9660344	**441473.31**	5062.64	3275.25	5128.80	7854.43	90735.77	186.1490957	2543.45	2693.868549	14648.43405
SCC in situ	0.8	2	face	650.33	378.98	223.7233799	**47725.21**	1373.94	803.98	2589.88	2944.36	6765.51	96.63384474	1994.50	2275.704145	1114.266087
SCC in situ/AK	1	1	scalp	187182.27	751.18	324.8251087	**189226.70**	3661.75	2944.03	7131.78	13292.87	115211.95	638.3059179	8444.70	5225.373417	16463.98185
nsitu & scar (re-excision o	1.2	2	scalp	318.21	276.03	195.2828303	**48124.33**	2071.76	1351.19	3686.39	12563.86	7488.31	405.9564261	3986.81	818.1091345	3433.739756
SCC-Keratocaanthoma	1.2	2	leg	2117.03	614.07	231.4617389	**96550.82**	1460.79	3201.37	13505.80	10780.62	10470.50	99.97630893	1724.95	2019.189765	2516.939114
SCC(good tumour volume	6.5	2	scalp	6529.10	2936.92	434.0293203	**55689.03**	4604.62	2686.81	10069.30	33826.30	12076.85	723.6783652	4113.73	5558.418481	4361.172812
SCC/ Acanthoma	7.5	1	arm	55925.81	1352.90	441.6129032	**92251.94**	19403.23	3187.74	121968.06	20590.32	18913.55	2141.935484	12898.39	8812.903226	4743.548387
SCC/ Acanthoma	2.5	3	arm	96697.76	5146.22	2144.537815	**280341.74**	5186.27	3796.36	67122.69	96722.41	372691.88	4217.927171	10465.55	20628.57143	64649.57983
superficial scar	0.1	1	leg	49.03	47.85	27.99426934	**3729.68**	289.89	113.64	278.74	405.96	398.54	12.09169054	541.75	246.7908309	351.4040115
Superficial SCC	1.5	3	face	43000.00	3095.86	345.5960382	**76804.03**	2215.78	5100.11	7022.64	17832.68	29119.21	1235.939158	5977.36	8342.058719	3130.173329
Superficial SCC (Bowens)	0.1	1	thorax	2797.91	235.61	139.9312518	**13298.63**	145.23	2522.77	2294.61	5653.82	1351.76	60.44113435	675.74	586.221713	526.4967058
Well Differentiated SCC	0.8	1	face	36029.37	894.77	407.6823757	**42146.55**	3888.96	4687.54	27060.04	20933.83	7185.60	1813.105229	8342.48	3103.292447	4216.26856

Appendix 1. Chemokine analysis (pg/ml) for samples studied. Corresponding lesion Thickness and Infiltration scores in table. De-identified source data stored on private locked server

Appendix 2


IL-8 pg/gram
LS IL-8
Histopathology Group
Normal
MIP-1β/gram
LS
DA
GR
HH
JB
AK
SCC
IEC SCCIs
IEC

Appendix 3

CONSENT FORM

(THE CYTOKINE MICROENVIRONMENT OF SCCs AND PRECURSOR LESIONS)

1. I, the undersigned ..... hereby consent to my involvement in the above study.

2. The procedure involving the excision and pathological examination of your lesion will proceed as per Dr Lewandowski's regular practice.
   The research will involve:
   Complete pathological examination of the excised specimen. Any remaining tissue fragments will be transferred to the Translational Research Institute at the Princess Alexandra Hospital campus.
   Here, the sample, and the minimal amount of patient information will be processed and prepared for the tests required for immune analysis.
   The information collected from this study will contain no identifiable individual patient data.

3. I acknowledge that the nature, purpose and contemplated effects of the study so far as it affects me have been fully explained to me by the research worker and my consent is given voluntarily. I have also read and understand the Patient Information Sheet.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the study was explained to me.

6. I am informed that no information regarding my medical history will be divulged and the results of any tests involving me will not be published so as to reveal my identity.

7. I understand that my involvement in the study will not affect my relationship with my medical advisers in their management of my health. I also understand that I am free to withdraw from the study at any stage without my future treatment being affected.

8. I understand that where biological material is collected, it may be stored and used for future research purposes either with my further consent or (in circumstances where my further consent cannot be obtained or is impractical to obtain) with the further specific approval of a hospital ethics committee set up in accordance with the NHMRC guidelines".

9. I give permission for the release of information regarding progress in this study to the study centre, on the understanding that while the study centre will keep confidential results under my name, no published study will identify me in any way.

10. I authorise the Greenslopes Private Hospital to allow access to relevant medical records to the investigators from the University of Queensland School of Medicine.

11. I have been told that this study has been approved by the Ethics Committee at Greenslopes Private Hospital.

Signed ..... **Date .....**

Appendix 4

GREENSLOPES PRIVATE HOSPITAL

PATIENT INFORMATION SHEET

THE PURPOSE OF THE STUDY

- Australia has the highest rate of non-melanoma skin cancer in the world. The Cancer Council Australia's Clinical Guidelines Network estimates that the combined cost of Squamous Cell Carcinoma (SCC) and Basal Cell Carcinoma treatment together involves the greatest cost of cancer care in Australia, totalling $A345 million per year, with approximately 130,000 new cases of SCC each year. While SCC is rarely fatal if treated early, the disease is especially prone to recurrence as a result of irreversible sun-damage to the skin, and is particularly prevalent in immunosuppressed transplant recipients, who suffer a 10-100 fold increased risk of developing SCC when compared with the general population[1]. Patients endure significant disfigurement, both from the disease and from its management. There is a critical need for more effective prevention and treatment strategies to enable oncologists to provide long-lasting protection from tumour formation and/or recurrence.
- There are many different early stage lesions that can progress to form Squamous Cell Carcinoma. Of these, the 'earliest' stage is thought to be Actinic (Solar) Keratosis. Unlike late stage or invasive SCC, the vast majority of AK lesions in immune eompetent patients eventually regress, with an estimated 1-10% of AK lesions progressing to invasive SCC.
- Recent studies have shown that as the immune system can fight off the majority of AKs, there are drugs such as Diclofenac and Imiquimod, which can help stimulate the body to eradicate these lesions.
- The role of the immune system is clearly very important in fighting skin cancers, but to date is relatively poorly understood.
- Understanding the role of the immune system will thus help us to better manage early AK lesions, and reduce the progression of these early lesions into more invasive and destructive SCCs.
- Cells of the skin produce specific proteins in small amounts, known as cytokines. These are signalling markers that allow cells to communicate with one another. By identifying the cytokine environment within lesions from AK to late stage invasive SCC, it is hoped that new and improved approaches for the diagnosis of precursor lesions will be developed.
- It is hoped then, that by understanding the cytokine profile of early and later stage cancers, we may be able to predict which lesions will regress through the bodys own immune system, and which lesions will progress to more invasive SCC – allowing us to more appropriately treat each type at an earlier stage.
- To do this, the tissue fragments remaining from your excised skin cancer will undergo Flow Cytometry, and cytokine-assay to look for the specific immune cells and proteins we are looking for.

Your Role:

- This is a research study looking at excised skin lesions after they have been clinically and pathologically examined.
- Your consent is required to allow research to be conducted on your skin cancer after it has been looked at by the pathologist.
- Your refusal/acceptance to consent in the study will not affect your treatment by any of your treating doctors.
- You will not be identified by any of the information collected, and will not be contacted by the study group unless you wish to be.
- You will be required to fill in an questionnaire relating to your sun exposure, immune status and general medical health.
- If at any time you do not feel comfortable with your tissue being used for research purposes, you may withdraw your consent from the study and have your tissue removed from use.

- This research is for the completion of a Master of Philosophy project conducted by Andrew Lewandowski from the University of Queensland School of Medicine. The project will be funded by the University of Queensland Dermatology Research Centre and the Translational Research Institute. The researchers will not receive financial incentive for their part in the study.

- Dr Lewandowski will be aware of your participation in the project, however as the research will not impact on your treatment, there will be no impact on your clinical relationship with him or the other doctors involved in your care.

- Your tissue sample and information will be de-identified to protect your privacy once it has reached the research facility.

- While the information collected about you and your tissue specimen will be de-identified, it is important that you are aware that it may be viewed, and used by the Greenslopes Hospital Staff as a means of auditing and regulation of studies that are conducted on the hospital campus.

- You will not be contacted again by the study group at the conclusion of your clinical treatment by Dr Lewandowski. If you wish to contact the study group or to receive notification of the results and publication of any data that comes as a result of your involvement in the study, please contact the Principal Investigator:

    - Andrew Lewandowski
    - andrew.lewandowski@uqconnect.edu.au
    - 0406646695

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