# THE EFFECT OF SUBSTANCE P (SP) ON ADHESION OF JURKAT LEUKAEMIA CELLS AND SQUAMOUS CARCINOMA CELLS (SCC) TO VASCULAR ENDOTHELIAL CELLS AND ROLE IN METASTASIS

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# Dedication

"Read in the name of your Lord Who created. He created man from a clot. Read and your Lord is most Honourable, Who taught (to write) with the pen. Taught man what he knew not." **The Qur'an**

To My Mum, and Dad

"My parents are my backbone. Still are. They're the only group that will support you if you score zero or you score 40." **Kobe Bryant**

To my supervisors

"A true teacher would never tell you what to do. But he would give you the knowledge with which you could decide what would be best for you to do." **Christopher Pike, Sati**

To my wife

Thank you

# Declaration

I certify that this work contains no material that has been used for the award of any diploma or degree, or has been published before by another author except for where it is referenced.

I certify that the whole thesis contains findings that are from my own data and experiments and no results have been used except when needed for comparison purposes.

I give consent to James Cook University to make this thesis available on the web. I also consent to depositing the thesis in the University library under the provision of the *Copyright Act* 1968.

Moustafa Elhousiny

# Acknowledgment

To my advisory panel: it was a long, hard (nearly impossible) journey with more downs than ups. Thank you for taking the journey and putting up with me and all the obstacles in the way. I appreciate all your advice and help, and my hope is that this material is produced in a way that makes you proud.

For Prof Alex Lucas on behalf of the QTHA lab scientists: they were of great help to me in every single aspect of the experimental work from cell culture all the way up. Thank you for your assistance and great help.

# Statement of Contribution

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| Statistical assistance | Data organisation<br>Statistical analysis | Cairo University Centre for Statistical Studies |
| Editing assistance | Thesis editing and revision | Dr. John Cokley |

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# List of abbreviations

| | |
|---|---|
| BM | Basement membrane |
| CAM | Cell adhesion molecule |
| CCL3 | Macrophage inflammatory protein 1-alpha (MIP-1-alpha) |
| CCL20 | Macrophage Inflammatory Protein-3 (MIP3A) |
| CCR7 | Chemokine receptor 7 |
| CXCL1 | GRO1 oncogene, GROα, KC, neutrophil-activating protein 3 (NAP-3) |
| CXCL12 | Stromal cell-derived factor 1 |
| CXCR2 | Interleukin 8 receptor, beta |
| CXCR4 | Chemokine receptor type 4 |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EMT | Epithelial-mesenchymal transition |
| FGF | Fibroblast growth factor |
| GRO-1 | CXCL1 |
| HIF | Hypoxia inducible factor |
| ICAM | Intercellular adhesion molecule |
| IL-6 | Interleukin 6 |
| IL-18 | Interleukin 18 |
| LC | Leukocyte |
| LNM | Lymph-node metastasis |
| MVD | Microvascular density |
| MIP-3 | CCL20 |
| MET | Mesenchymal epithelial transition |
| MMP | Matrix metalloprotinease |
| NF-κB | Nuclear factor pathway protein complex that controls transcription of DNA |
| NSAID | Nonsteroidal anti-inflammatory drugs |
| OSCC | Oral squamous-cell carcinoma |
| PSGL-1, | P-selectin glycoprotein ligand-1 |
| ESL-1, | E-selectin ligand-1 |
| CD24 | Cluster of differentiation 24 or heat stable antigen CD24 |
| Sialyl Lewis | Tetrasaccharide carbohydrate |
| CD34 | Glycosylated transmembrane protein |
| MAdCAM-1 | Mucosal vascular address in cell adhesion molecule 1 |
| PDGF | Platelet-derived growth factor |
| SDF-1 | Stromal derived factor |
| STAT | Signal Transducer and Activator of Transcription |
| TNF-α | Tumour necrosis factor |
| VCAM | Vascular cell adhesion molecule |

# Abstract

Metastasis is the leading cause of fatality in 90% of cancers, and approximately 60% of all cancer cases will have either regional or distant metastasis at initial diagnosis. Global data shows that survival rates drop significantly with the advance of disease stage and metastatic activity; therefore, patients with localised disease have a far better prognosis than those with disseminated tumours. In this aspect, Leukaemia and oral squamous-cell carcinoma (OSCC) are invasive neoplasms. Both cancers are considered the worst prognosis cancers with an overall survival rate of 50% or slightly higher.

The accumulating research suggests that inflammation is more likely to act in favour of tumour initiation and metastasis. The striking similarity which exists between the out flux of tumour mass in the circulation and that of inflammatory exudates in wound healing points to the possibility that the same mediators might be utilised for both purposes. Substance P (SP) is a primary regulator of neurogenic inflammation mainly acting as a trigger for vasodilatation, plasma protein extravasation and leukocyte adhesion to vascular endothelial cells. SP has been proposed as a model that can explain the inflammation-tumour relationship. Therefore, the link between the role of SP and the extravasation of tumour cells into the circulation represents an attractive opportunity for unravelling the metastatic mechanisms.

We carried a systematic review which identified nine inflammatory mediators associated with metastasis. The 16 articles reported lymph node metastasis and one article reported both lymph node and distant metastasis. The inflammatory mediators identified were CXCR4 (six studies), CXCL12 (SDF-1), CCR7, IL-6 (two studies each), IL-18, CCL20 (MIP-3), CXCL1 (GRO-1), CCL3, CXCR2 (one study each). This review systematically summarises the evidence of the prognostic role of inflammatory mediators in predicting metastasis/metastatic stages in OSCC. Additionally, it compares the available evidence from clinical and experimental animal settings.

Our experimental results showed that SP increased the adhesion of Jurkat Leukaemia cell lines in a time-course treatment with a peak adhesion increase at three-four hours. SP increased the adhesion of H157, CAL27, and DOK cells to HUVEC endothelial cells ($P < 0.001$), significantly, in a time-dependent treatment with peak adhesion at three hours. It has been demonstrated that SP is expressed by several tumours and several roles have been proposed for its action in tumour growth and progression. Our study describes a new role for SP in stimulating an early onset adhesion of tumour-endothelial adhesion.

We found that treating endothelial cells with either stimulating factor or inhibitor produces more potent levels of adhesion which may 1) explain the organ-specific metastasis of cancers; and 2) highlight the active interaction of tumour-endothelial cells during adhesion. Moreover, our data suggests that inhibition of adhesion levels – achieved using cycloheximide, which blocks translation of messenger RNA on the cytosolic 80S ribosomes but does not inhibit the organelle protein synthesis – did not achieve higher levels such as the one inflicted with monoclonal antibodies. This might suggest that tumour cells highly express adhesion molecules as well as inflammatory receptors.

Our data also suggests that in Jurkat cells, CAL27 and BICR22, SP 1mcg/ml treatment has increased both CD 11 (not significant) and CD 49 ($P < 0.05$), but not CD15s expression in 1-48 hour time-scale treatment, as indicated by the FACS analysis. Anti-human monoclonal antibodies to VCAM or ICAM significantly inhibited adhesion levels to below the untreated baseline levels. The NK-1R antagonist was only effective in inhibiting adhesion molecule expression in Jurkat and CAL27. No other effect was noted in the other OSCC cell lines.

We hypothesised that adhesion molecules were the main requirement of the adhesion process, and adhesion molecule expression followed the pattern predicted which increased from no expression in the normal oral keratinocytes to the lymph node positive cell line H157 and declined in the metastatic cells BICR22, for the three adhesion molecules. The pre-cancer cell line DOK had an elevated expression profile which agrees with previous studies, suggesting an early invasive/metastatic phenotype in the tumour cycle.

Our results did not prove any significant effect for SP on the release of MMP-2 in either Jurkat cells or OSCC cell lines. This was against the predicted pattern in which we hypothesised that SP would trigger up-regulation of MMP enzymes to facilitate the transmigration of tumour cells through the endothelium.

The resulting model represents a novel approach in cancer treatment where the main target is prevention of metastasis. This paradigm shift has a powerful potential to develop effective anti-metastatic therapies through interfering with the metastatic cycle. The data resulting from the systematic review as well as the experimental findings can be integrated for future implementation in two main categories.

In conclusion, the study identifies a new role for Substance P in mediating an early onset adhesion of cancer cells to endothelial cells. The study also highlights the role of the NK-1R antagonist as a novel therapy in inhibiting this adhesion in those cell lines. A combined therapy of the NK-1R antagonist and monoclonal anti-adhesion molecule would be powerful in preventing the onset of metastasis. These primary data warrant further research through animal models to confirm this role for Substance P.

# Chapter 1 Introduction

Cancer remains a major health problem with "17.5 million cancer cases worldwide and 8.7 million fatalities" reported in 2016 according to GLOBCAN (1). Metastasis is the leading cause of fatality in 90 % of cancers, and approximately 60% of all cancer cases will have either regional or distant metastasis at initial diagnosis (2). Global data shows that survival rates drop significantly with the advance of disease stage and metastatic activity; therefore, patients with localised disease have a far better prognosis than those with disseminated tumours (2). The advancement of treatment modalities has increased the survival rates. Ironically, this has increased the possibility of recurrence (3). It is also estimated that a third of cancer survivors (even early-stage patients) will experience recurrence due to treatment failure to fully eliminate the disease (4-8).

In this perspective, oral squamous cell carcinoma (OSCC) (90% of all the oral cavity tumours) is a locally invasive neoplasm (4, 8). The rate of metastatic spread for OSCC is reported to be 65% for regional metastasis, 5-25% for distant metastasis and up to 50% of recurrence (9-11). OSCC remains one of the worst prognosis cancers with an overall survival rate of OSCC of 50% or slightly higher (12, 21). Patients with positive lymph-node involvement have a 50% reduction in the overall survival rate compared with that of their negative lymph node peers (13). Metastasis has a great impact on the prognosis of the cancer patients not only in terms of survival but also in recurrence which, in turn, reduces chances of survival. Even with new combination treatment modalities, those patients are at high risk of recurrence (14). Metastasis are not limited to late stages but can occur in patients with clinically negative lymph nodes as micro-metastatic foci that are difficult to detect with conventional methods and need highly specialised tools such as molecular assessment (15).

The reciprocal relationship between diagnosis, treatment and metastasis is a daunting task for clinicians to manage in the clinical settings. As far as the diagnostic methods fail to precisely predict metastatic lesions in the early stages, high-risk patients will be liable for more aggressive treatment (15). While early detection and prevention programs in most cancers prolonged survival time, they are also likely to increase recurrence by prolonging survival time (3). Recurrence is the end result of even the most aggressive therapeutic combination, and no improvement in loco-regional control has been attained in the recent past (4).

Recurrence requires re-treatment and relapsed patients will have the worst prognosis. Therefore, there is a need for extensive research to underpin the mechanisms of metastasis to explain these relapses and improve this outcome (142).

Metastasis is a precise biological process that consists of several successive stages and requires the collaboration of several signalling pathways and molecular components (150). Metastasis commences through the invasion of the tumour to the surrounding extracellular matrix (ECM) and basement membrane (BM). Then, the detached tumour mass enters the circulation, evades the immune response and adheres to the vascular endothelial cells most likely through adhesion molecules. Distant metastasis occurs when this mass extravasates (steps out of the circulation) and implants in a remote site (153, 158). Although the mechanisms behind local invasion have been extensively studied, the stages of distant metastasis, particularly extravasation and their mechanisms are poorly understood (154-157).

Cancer does not use novel mechanisms but rather implements existing biological processes in favour of its growth and progression (157, 215). With this concept in mind, observations from the attraction of the circulating leukocytes to the injured site in inflammation can unravel the extravasation process (157, 208). Accumulating research suggests that inflammation is more likely to act in favour of tumour initiation and metastasis (215). The extravasation of leukocytes in inflammation is well researched and the different stages they undertake have been identified (232). The process of extravasation in cancer and inflammation requires similar specific components, namely: the adhesion of migrating cells to the endothelium through their adhesion molecules; activation of these adhesion molecules; and the proteolytic action of matrix metalloproteinase (MMP) (156).

The adherence of leukocytes to endothelial cells is a prerequisite for the inflammatory reaction to occur. This adherence occurs mainly through a group of adhesion molecules and their receptors on both types of cells (155). There are two categories of these adhesion molecules: the endothelial cell adhesion molecules (CAM) and their binding ligands expressed on leukocytes (224). The pairing between the two categories causes a different degree of adhesion which results in firm adhesion and finally trans-endothelial migration

(230-233). The endothelial adhesion binding legends are not expressed in normal cells. They are reported as highly expressed in several malignant cell types, specifically highly metastatic tumours (208).

MMPs are a group of proteolytic enzymes whose main function is to degrade ECM protein (292). Up-regulated expression of MMP members is often associated with the presence of inflammation (293). Studies suggest that MMPs are necessary for all stages of the inflammation from tissue repair and foreign body elimination to activating cytokines (294). In addition, MMP has been implicated in cancer progression and invasion and is highly expressed in a variety of cancers including OSCC (297-302).

The striking similarity which exists between the outflux of tumour mass in the circulation and that of inflammatory exudates in wound healing points to the possibility that the same inflammatory mediators might be utilised in both processes (155). In this respect, Substance P (SP) is a primary regulator of neurogenic inflammation mainly as a trigger of vasodilatation, plasma protein extravasation and leukocyte adhesion to vascular endothelial cells (324-334). SP has been proposed as a model that can explain the inflammation-tumour relationship (352), therefore, the link between the role of SP and the extravasation of tumour cells into the circulation represents an attractive opportunity to unravel metastatic mechanisms.

**Key Points**

- Metastasis is a major health concern.
- The complexity of metastatic process.
- The need to reveal the mechanism of extravasation of tumour cells in the circulation.
- Substance P can provide the link between inflammation and cancer.
- Can cancer cells use the same mechanism to adhere to the endothelium to extravasate as in the inflammatory process?

# Chapter 2 Literature Review

## 2.1 Introduction

Oral squamous cell carcinoma (OSCC), the most common malignant neoplasms of the oral mucosal membrane, together with pharyngeal and laryngeal cancers, are collectively termed "head and neck cancers" (16). Oral cancer (ICD10 C00-C06) is considered the 6$^{th}$ most common cancer for both sexes, and the 10$^{th}$ most common cancer in males combined with oropharyngeal cancer (17-18). Oral cancer is the leading cancer type and accounts for more than a third of all cancers in South-East Asian regions such as India and Sri-Lanka (Figure 2.1-2.2) (19). OSCC, which represents 90% of all oral cavity tumours, is a highly invasive lesion with its average survival rate at 50% (18). These figures have remained the same since 2014 at the commencement of the thesis research.

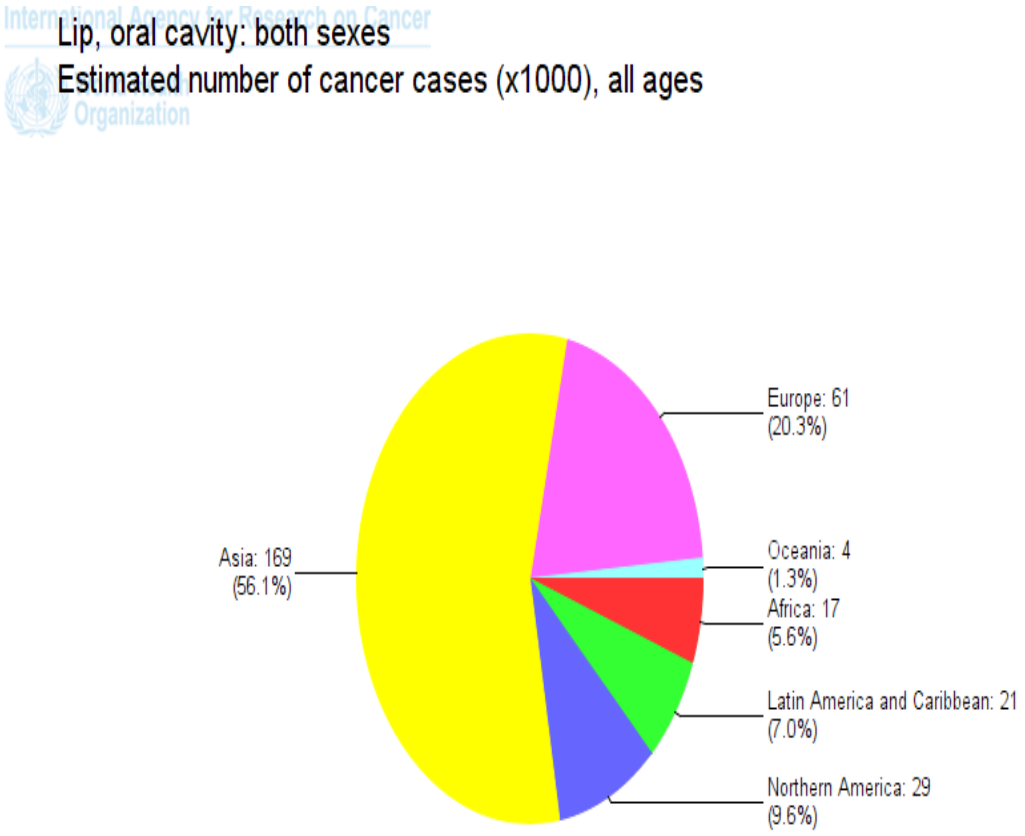



**Figure 2.1:** Worldwide estimated cases X 1000 of incidence of oral cancer in, Globocan.iarc.fr. Redirect [Internet]. 2018 [25 July 2018]. Available from: http://globocan.iarc.fr.

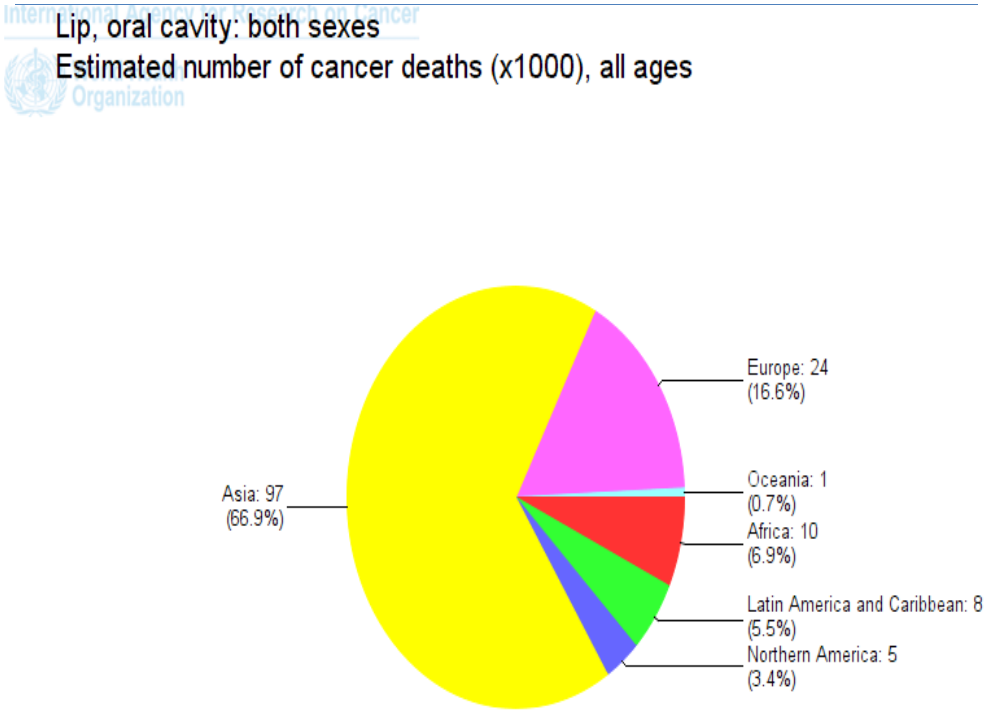



**Figure 2.2:** Worldwide estimated cases X 1000 of mortality of oral cancer in five Continents, Globocan.iarc.fr. Redirect [Internet]. 2018 [25 July 2018]. Available from: http://globocan.iarc.fr.

## 2.2 The effect of metastasis of oral cancer

### 2.2.1 Metastasis and Prognosis

More than 60% of oral cancer cases are diagnosed at advanced stages which decreases the survival probability significantly (Table 2.1, Figure 2.3-2.4) (20-21). The asymptomatic nature of oral cavity tumours and the delay in diagnosis/referral are the main factors contributing to the discovery of late-stage tumours that have already metastasised at the time of diagnosis (22-24).

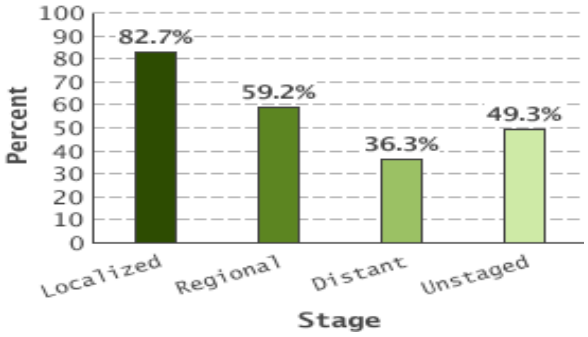



**Figure 2.3:** Percentage of survival by stage of disease SEER.

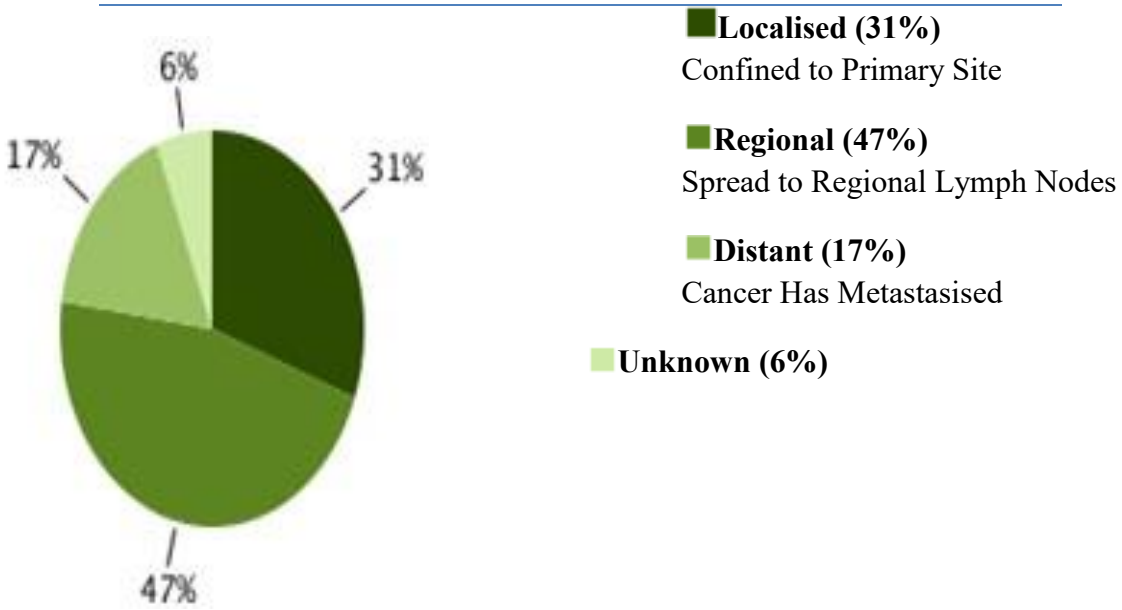



**Figure 2.4:** Percentage of Cases by Stage at Diagnosis; Oral cavity and Pharynx Cancer SEER.

| Stage | Description | 5-year survival rate (%) |
|---|---|---|
| **I** | The tumour is relatively small and contained within the organ it started in. | 80 |
| **II** | The tumour is larger than in stage 1 but the cancer has not started to spread into the surrounding tissues. | 66 |
| **III** | The cancer have started to spread into surrounding tissues and there are cancer cells in the lymph nodes in the area. | 46 |
| **IV** | The cancer has spread from where it started to another body organ. This is also called secondary or metastatic cancer. | 38 |

**Table 2.1:** Survival according to the stage of cancer, adapted from the Surveillance, Epidemiology, and End Results Program (SEER) National Cancer Institute.

| Article | Country | Year | Sample Size | 5-year survival rate (%) | | | | (%) of patients with Stage III, IV |
|---|---|---|---|---|---|---|---|---|
| | | | | Stage I | Stage II | Stage III | Stage IV | |
| Chen GS[24] | China | 1996 | 103 | 62 | 80 | **42** | **19** | **62.1** |
| Y.K Chen[25] | Taiwan | 1999 | 703 | 72.7 | 38.9 | **26.8** | **11.8** | **49.2** |
| J.A.Woolgar[26] | UK | 1999 | 200 | 85 | 90 | **82** | **42** | **68** |
| Lo WL[27] | Taiwan | 2003 | 378 | 75 | 65 | **49** | **30** | **47.1** |
| A. Chandu[28] | Australia | 2004 | 116 | 88.7 | 83.8 | **83.3** | **76.5** | **40** |
| Goto[29] | Japan | 2004 | 180 | 92.7 | 91.1 | **65.9** | **44.7** | **50** |
| Garzino-Demo[30] | Italy | 2006 | 245 | 79 | 78 | **48** | **42** | **44** |
| Lisetta Lam[31] | Australia | 2007 | 212 | >50 | >50 | **<40** | **<40** | **47** |
| Katayoun Sargeran[32] | Iran | 2008 | 470 | 51 | 44 | **13** | **12** | **50** |
| Paolo[33] | Italy | 2008 | 334 | 90.7 | 80 | **58.9** | **51.5** | **31.4** |

**Table 2.2:** Studies comparing survival of oral cancer patients according to stage status at the diagnosis.

The accumulating body of evidence is that the involvement of metastasis, whether regional or distant, is the most important prognostic factor in OSCC (27, 30, 33, 34-39) (Table 2.2). The presence of nodal metastases in oral malignancies significantly decreases both overall and disease-specific survival to less than half and increases the risk of recurrence and distant metastasis (40, 41). The metastatic potential of oral cancers varies according to the site of the primary lesion. In the buccal mucosa, the incidence of cervical metastasis is reportedly 16-43% (42, 43). On the tongue and the floor of the mouth, this percentage is higher, reaching 60% of the affected cases (44, 45). The cervical spread occurs in 19-37% of the cases where the hard palate, maxilla, or alveolus is the affected sites (46, 47).

The higher the clinical stage of lymph node (N1 “tumour spread to close or fewer lymph nodes”; N3 “tumour spread to distant or numerous lymph nodes”), the lower is the survival outcome of the patients (Table 2.3) (14, 36, 48-50). In one study of 524 patients with oral carcinoma of different sites, the 5-year survival period significantly dropped from a crude average of 44% for patients with N0, to 18% and 11% for N2 and N3 stages, respectively

(35). The study also demonstrated that despite the treatment modalities in place, the difference in survival between N-negative and N-positive group was significantly different, 65% and 38% respectively. Cervical metastasis affects survival and has a tremendous impact on recurrence and therefore the relapse-free survival. Tankéré et al. demonstrated in a study of 137 patients with OSCC that recurrence rate increased from 9% in N0 patients to 20% in the N-positive patient group and, more importantly, to 29% in N-positive patients with extracapsular spread (51). The study also identified that the increase in node stage (N0-N3) increased the rate of recurrence (31.2% in the N2 group and 57.1% in the N3 group) as well as decreasing the survival rate (44.7% in patients with N0, 37.7% in patients with N1, and 15.6% in patients with N2; none of the N3 patients survived past the two years).

| Study | Country | Year | Study Size | Survival Probability (%) | | | | Percentage of Lymph node incidence |
|---|---|---|---|---|---|---|---|---|
| | | | | N0 | N1 | N2 | N3 | |
| Kalnins[34] | US | 1977 | 340 | 75 | | **29*** | | 75 |
| Jones[35] | UK | 1993 | 524 | 44 | 29 | 18 | 11 | 24 |
| Noguchi[36] | Japan | 1999 | 136 | 91 | | **41*** | | 58 |
| Lo WL[27] | Taiwan | 2003 | 378 | 65.8 | 38 | 40 | 20 | 32 |
| Garzino-Demo[30] | Italy | 2006 | 245 | 74 | 39 | 34 | N/A | 29 |
| Paolo[33] | Italy | 2008 | 334 | 87.5 | 70.4 | 32 | N/A | 31.7 |
| Wang Ling[37] | China | 2013 | 210 | 53 | 41 | 29 | 0 | 42 |
| Geum[38] | Korea | 2013 | 37 | 92 | | **30*** | | 27 |
| Li[39] | China | 2013 | 155 | 47.36 | 27.48 | 15.55 | 0 | 40 |

**Table 2.3:** Comparison of survival outcomes between node-free and node-positive patients and the incidence percentage of lymph node metastases.

* Denotes average rate for all the node-positive stages. No details about specific stage.

The burden of metastasis in OSCC is magnified by the problem of extracapsular spread (ECS), the spread of tumour metastatic deposits outside the lymph node (52). Woolgar et al. assessed 200 patients with oral cancer, in which 99 (50%) had metastases subdivided into three groups of N0, intra-nodal metastasis and extracapsular metastatic spread. The 5-year survival rate was 81%, 64%, and 21%, respectively (26). In a second study, they assessed 173 patients with confirmed neck metastases, grouped into intra-nodal metastatic group (no ECS),

micro ECS, and macro ECS (53). The 1, 2, 3 and 5-year survival rates were 87%, 75%, 72% and 70% for Group 1 compared with a mean of 36% for Group 2, falling to 33% for the third group. The authors suggested the urgent need for a novel, thorough and reliable method for neck staging to detect these micro-metastatic deposits, as currently used routine clinical and radiological diagnostic tools usually tend to overlook such lesions.

ECS burden affects survival rate and has a great impact on both recurrence and rate of distant metastasis (54, 55). In a study of 400 patients with oral cancers grouped into N0 (*n*= 221), N+ECS- (*n*= 78) and N+ECS+ (*n*= 101) (56), the 5-year survival was 65%, 52%, and 23% for the three groups, respectively. The 5-year-free period from distant metastases, local recurrence, and neck recurrence was two times worse for the ECS+ group than for the other two groups. Similar results were obtained by Mayers et al., (57) where they estimated the rate of distant metastasis incidence rates to be three times higher for the ECS+ group than for the other two groups.

The incidence of cervical metastasis is not confined to late-stage tumours. Metastasis can be divided into clinical and subclinical (occult) with the latter reaching up to 30% of early stages of oral cancer (stage I, II) (10). These occult metastases can be further divided into micro-metastatic deposits (0.2-2mm) and isolated tumour cells that can be only detected using staining techniques or molecular tools (58). The incidence of occult metastatic deposits can be in the range of 11-44% in T1-T2 clinically negative lymph node patients and has a deterministic effect on the prognostic outcome of the disease (59-63). Ganly et al. demonstrated that patients with occult metastasis in early-stage OSCC have a mortality risk five times higher than those who do not (64).

Research suggests that micro metastases significantly decrease the survival rates and recurrence rates even when the most aggressive treatment modalities are in place (29, 65-66). Jang et al. found that 57% of early-stage oral cancer patients who presented with occult metastasis had decreased survival rates (24% compared with 66% for those with no micro-metastasis) despite receiving radiation treatment and neck dissection (67). Thiele et al. retrospectively studied 122 patients with early-stage oral cancer, all of whom underwent neck dissection and found 17 (13.9%) patients with occult metastasis (68). Even with neck

dissection, the disease-specific survival for those patients was down to 17.8% from 61.9% for the occult-free patients. These minimal foci escape treatment and flourish with time into overt metastases, which might explain the high recurrence rates of oral cancer especially in early stages (69). Despite the importance and effect of these micro-metastases on the prognosis, current diagnostic techniques cannot assess the neck nodal stage in patients with early stages of oral cancer with a high degree of reliability (70-71), and therefore, most surgeons resort to neck dissection procedures which might have a satisfactory advantage (72).

The final form of metastasis in OSCC is distant metastasis (DM) which has been reported in a range of 3-30% prevalence among different studies (73-74) and is steadily increasing in prevalence, despite the advancement of treatment modalities (21). The rate of distant metastasis at initial diagnosis of the oral tumours is estimated to be less than 3% (75-77). Moreover, when distant metastasis develops in patients, it usually occurs within two years of the discovery of the primary tumour (78-79). The rates of distant metastasis along the course of the disease range from 9-30 % (79-80), which might explain the huge fluctuation in the rate of metastasis reported by different studies. Another explanation might be that distant metastasis develops as a consequence of a regional metastasis in patients who fail to achieve loco-regional disease control after treatment (81-83). Shintani et al. found that a pathologically positive lymph node group had a threefold incidence of distant metastasis compared with their negative peers (50). Lim et al. concluded that patients with positive lymph nodes are seven times more likely to develop distant metastasis (84). Shingaki et al. divided the patient sample (*n*= 103) into two groups based on lymph node involvement (69). The rate of distant metastasis in the lymph node positive-group was 40% compared with 4% in the negative group.

Regardless of the actual percentage, the incidence of distant metastasis represents the end-point for the cancer patients with very minimal survival chances. Li et al. (85) reported 1, 3 and 5-year survival rates of 56.8%, 9.1% and 6.8%, respectively for patients with DMs. Only three out of 36 patients with distant metastasis survived to the second year. The most common sites for metastasis from OSCC tumours are: lung (>60%) followed by bone, liver, and skin (79, 86).

### 2.2.2 Metastasis and treatment

As discussed earlier, there is a high risk of metastasis in oral cancer, particularly nodal metastasis, both in advanced and early stages, due to the inability of conventional diagnostic methods in detecting early metastatic signs. This metastatic potential of OSCC is critical in the decision-making when choosing treatment, necessitating aggressive therapeutic modalities to ensure residual disease-free outcomes (87). The surgical resection of the primary tumour remains the mainstream treatment of oral neoplasms (88). Advanced malignant lesions (stage III/IV) are often treated with a combination of surgery with chemo or radiotherapy or palliative care in the inoperable cases (87). Success in managing oral neoplasms depends on treating the neck and ensuring no remaining cervical spread is left by performing one of the neck dissection alternatives (89).

#### 2.2.2.1 Neck dissection for early tumours

The basis of the decision on whether to conduct a block dissection or "observe and wait" for the clinically negative neck is not very clear, although there is some evidence supporting the treatment option (90-91). The risk of micro-metastasis is around 30% in early-stage cases, which significantly increases the risk of recurrence and reduces the chances of survival (59-63). It is argued that the "wait-and-see decision" is suitable when the risk is minimal and will unnecessarily cause morbidity to significant organs of patients, in comparison to neck dissection, which might not provide marked outcomes or advantages on survival (92-94). Meanwhile, multiple studies suggest that the benefits of treatment far outweigh the morbidity disadvantage and can be utilised to stage the neck pathologically (95-97). The decision to treat the neck or the observation policy is mostly made based on the risk of occult metastasis (~20%), depending on multi-factors such as the depth, site, and size of the tumour, and experience of the physician and institute (98). If current diagnostic methods remain unchanged, with no advance in the molecular detection tools, neck dissection with or without irradiation will still be the method of choice to treat the clinically negative neck in order to ensure the elimination of micro-metastasis and improve outcome.

Radical neck dissection (RND) remained the gold standard in treating regional spread in patients with head and neck cancer since its introduction by Crile et al. in the 19$^{th}$ century (99). The procedure involved removing major structures of the neck, including accessory

spinal nerve, internal jugular vein, and sternocleidomastoid muscle (Figure 2.5). Because of the significant morbidity of the procedure and the persistence of recurrence of tumours, oncology surgeons have sought alternative ways to dissect the neck that can preserve organs and function (100-103).

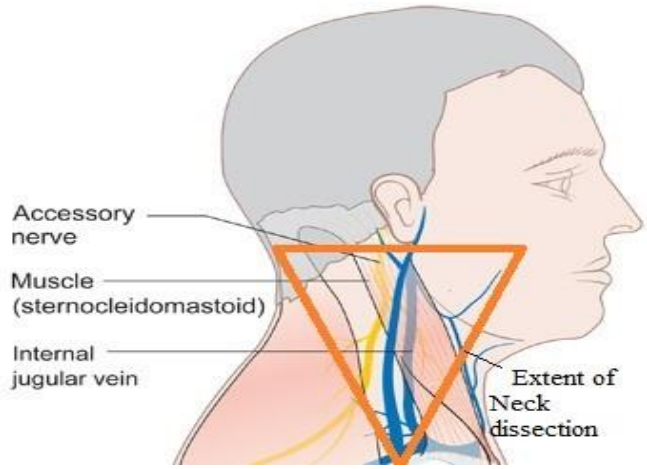



**Figure 2.5:** Extent of anatomical structures removed by radical neck dissection.

Neck dissection is suggested for elective treatment and staging of the clinically negative lymph node patients with stages I and II. However, the indications and advantages of this procedure in the head and neck area are under debate (104). Several retrospective studies and few RCTs have been conducted to identify the benefits of this invasive procedure (95, 111-112) (Table 2.4). A recent meta-analysis reported in favour of neck dissection, although several issues have been raised regarding the study design, statistical analysis and selection criteria, highlighting the need for proper randomised trials (105).

Careful insights into the studies performed revealed insignificant differences in survival or recurrence rates in the overall outcomes (106). Another important factor in the evaluation of neck dissection is that some studies reported similar rates of distant metastasis and recurrence with very poor salvage results compared with that of the non-treated groups (94, 107-110). Neck treatment is currently the only choice available for high-risk patients with some prognostic advantages. If the occurrence of micro-metastasis is the main prognostic indicator, then its prevention should be the aim of research efforts as it would be of a great benefit to cancer patients.

**Table 2.4:** Comparison of studies performed on the outcome of neck treatment through neck dissection or observation according to survival.

| Author | Study Sample | Study Type | Recurrence Treated Vs OBS % | DSS survival | Overall survival |
|---|---|---|---|---|---|
| Smith[92], 2004 | 171 | Retro | 20<br>7 | 96<br>92 | same |
| Kelner[97], 2014 | 172 | Retro | 7*<br>15 | 96*<br>85 | 87^<br>84 |
| Feng[96] | 229 | Retro | 9.6*<br>19.2 | 79.2*<br>61.9 | 20*<br>41 |
| Keski-Sa¨ntti[93], 2006 | 80 | Retro | 44*<br>20 | 63^<br>66 | 82^<br>77 |
| Liu[94], 2011 | 131 | Retro | 14.8<br>23.2 | NS | NS |
| Poeschl[106], 2012 | 86 | Retro | 16.6<br>18.4 | NS | NA |
| D'Cruz[110], 2009 | 359 | Retro | 5.7*<br>47 | 74^<br>68 | 60^<br>60 |
| Yuen[112], 2009 | 71 | RCT | 6<br>37 | 89^<br>87 | N/A |
| Fakih[111], 1989 | 70 | RCT | 30<br>58 | 63<br>52 | N/A |
| Klingerman[95], 1994 | 67 | RCT | 12<br>39 | N/A | 72*<br>49 |

**Abbreviations**

* Significant results

NS. Author claimed non-significant

^ Non-significant

N/A Data not available

DSS = disease-specific survival

Retro = retrospective.

RCT = randomised control trial

OBS = observation

#### 2.2.2.2 Treatment of advanced oral cancers

Patients with advanced stages of head and neck tumours were treated with surgery and radiotherapy or radiotherapy alone for inoperable tumours, which resulted in poor survival and severe morbidity. Therefore, multimodality therapy (surgery, chemo-radiotherapy) was introduced to improve the outcome and eliminate the microscopic spread of the disease (113-114). Loco-regional control, distant metastasis, overall survival and disease-specific survival are the end-point measures for studies evaluating the success of these treatments. Clinically, these end-points are not practical as patients with these advanced lesions are co-morbid. Therefore, tumour response (i.e. tumour shrinkage) has been set as an evaluation measure (115-116). Numerous studies have been published claiming significant gains in tumour control and survival. However, the results of these studies have to be taken with extreme caution (117). First, as mentioned earlier, these studies evaluated tumour response as the main outcome but this does not reflect the clinical outcome. Second, population-wide epidemiological studies showed a non-significant improvement in the outcome of advanced stage patients even with the introduction of these advanced multimodality strategies (21).

While it is out of the scope of this research to evaluate the huge number of studies comparing different treatments and outcomes, meta-analysis and systematic reviews have been conducted for informed decision-making. In 2000 Pignon et al. (118) performed a meta-analysis of 70 trials, including 10,741 patients, highlighting only 4% improvement in the survival of advanced-stage patients of head and neck cancers in favour of the multimodality treatment. Another comparison within the same meta-analysis showed that this humble improvement is warranted to some treatment regimens (concurrent chemotherapy with loco-regional treatment not adjuvant chemotherapy). In 2009, an updated version of the meta-analysis was been published with 24 new trials with similar results and no difference between outcomes in the previous two decades (119). Another meta-analysis claimed that induction chemotherapy (i.e. before treatment) resulted in 7% enhancement in the distant metastasis-free rate compared with loco-regional treatment alone: however, no effect on survival was achieved (120).

Furness et al. analysed 89 randomised studies on oral and oropharyngeal cancer and found that induction or concomitant chemotherapy plus surgery and radiotherapy produced a 10-20 % survival advantage in patients with advanced-stage neoplasms. However, the study did not show any significant improvement in terms of recurrent or metastatic control for these treatments (121). A recent study showed an improved outcome for patients with advanced oral cancer treated with multimodality in terms of recurrence, metastasis-free and overall survival compared with those treated with mono or dual therapies (122). The study revealed that 115 patients of 222 (51%) died due to OSCC-related causes with 58% survival advantage for surgery chemo-radiotherapy (S-CRT) group. Patients in this group achieved a 22% reduction in the recurrence rate, from 39%. The metastasis-free rate for S-CRT group was 84% but this was a secondary objective of the study and the comparison criteria were not clear.

The results confirm the notion that despite a slight improvement in survival, these treatment regimens still fail to prove any advantage in recurrent oral cancer. OSCC has a higher rate of recurrence even in patients who undergo treatment (8). Patients with recurrent diseases have a very poor prognosis, and even with the salvage treatment, the outcome remains unfavourable (123-124). The relative success in early detection and prevention of early-stage tumours has resulted in prolonged survival periods but with more recurrence and distant metastasis incidence rates observed (125).

The choice of treatment is currently determined using diagnostic tools available to identify the level of the cervical metastasis. Clinical examination alone is not capable of detecting occult metastasis; therefore, adjunctive methods such as imaging should be used to diagnose such lesions. The low specificity and sensitivity of these diagnostics compels clinicians to deal with the consequences of metastasis after it has occurred. Therefore, the management of OSCC patients with metastasis, or those in early stages with high risk of metastasis, requires invasive management approaches to ensure the best outcome. The complications of these treatment strategies (neck dissection, chemotherapy, radiotherapy) are numerous, resulting in morbidity, reduced functionality, and even patient mortality due to treatment complications (126). From an evolutionary point of view those treatments impose a selective pressure, which kills the weak cancerous cells while preserving the most aggressive clones which have

the ability to survive the treatment and further spread into other parts of the body (127). Also, the financial burden of such treatment is enormous on the health systems and individuals (128). Finally, neither of those modalities have brought significant improvements in patient survival or metastatic or recurrence control. The therapeutic approach for treating OSCC relies on the stage of the tumour, particularly the nodal status, which in turn defines the prognosis and outcome of the disease. Due to incomplete understanding of the metastatic process, those treatment modalities fail to establish full recovery and are confined to symptomatic relief for cancer patients.

In summary, the focus on exploring the mechanisms of cancer initiation progressed the state of knowledge about primary tumours but added only a little to our understanding of how the tumour can spread and result in death (146). Information available from this research influenced the advent of early detection programs and revolutionised treatment strategies leading to prolonged survival across most types of cancer (142). Unfortunately, due to the knowledge gap in understanding metastasis, we experience more relapses with cancer treatment (144). Metastasis has shifted to become the prime killer of the OSCC patient, not the primary tumour itself (125). The presence or risk of metastasis is the single indicator of patient outcomes and the most deterministic in electing the method of treatment (14).

Molecular diagnostics (e.g. PCR, DNA microarray) are revealing higher than ever metastatic rates, up to 30%, even in very early stages, with some new opinions that metastasis commences as early as the tumour itself (129). This spread makes it hard for any treatment regimen to effectively reach and target these disseminated cells, which explains the failure of treatments to fully eradicate the disease or establish recurrence-free outcome (130). What's more, the introduction of systematic combination therapies increases morbidity and has proven to be highly toxic, and even fatal to the host organism (126-127). Once metastasis has occurred, then the treatment of these disseminated tumours is either ineffective or confined to palliative treatment providing no clinical benefit to the quality of life of the patient. Accordingly, the potential lies in a preventive approach where interference with the critical stages in the metastatic process takes place before metastasis occurs. In "the battle against cancer", neoplastic initiatives have been in the lead and clinical oncology is often left to react

to the consequences. This approach would represent a paradigm shift, in which medical intervention can for the first time gain an advantage over cancer mechanisms.

If metastasis is of utmost clinical importance, why is this lag in understanding metastasis? And how can we interfere with this fierce process? The answer lies in that metastasis is an extremely complex process that results from the interplay between tumour and the host microenvironment. Only by understanding the molecular mechanism of the pathogenesis of metastasis, can we establish any basis for precise clinical models, which will allow the identification of the critical characteristic of those cells and this is a vital requirement for development of effective therapeutic modalities.

## 2.3 Extravasation in inflammation and metastasis

### 2.3.1 Evolutionary stages of metastasis

Metastasis, according to the progression model, was considered to be an end-point in the tumour genesis process (Figure 2.6) (131). Only after the primary tumour has formed then mutational changes take place in rare cell populations of the tumour bestowing full metastatic potential to those cells (131). Supported by several studies that revealed the existence of metastatic cell subpopulations within tumours, this was the prevailing model of metastasis for more than three decades (132-135).

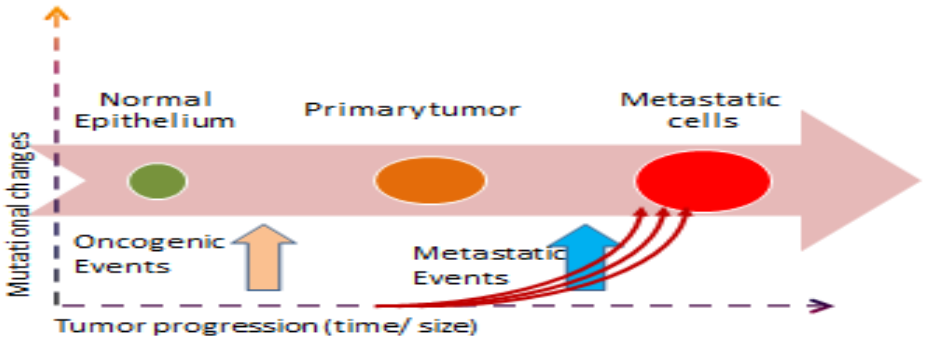



**Figure 2.6:** The diagram is showing the progression model of metastasis and the onset of metastatic changes in relation to the diagnosis.

However, the model failed to explain the origin of metastatic lesions with unknown primary tumour which makes up to 5 % of all metastasis (136). As the model suggests a pattern of continuous mutational changes throughout tumour cycle, it would be expected that the disseminated cells would have a higher metastatic potential than the primary tumour, which proven to be not the case clinically (137). The model prevailed for a long time focusing the research on eliminating the primary tumour, which was expected to annihilate the problem of metastasis as it is a very late sequela of the cancer formation process. This, once more, was clinically proven not to be the case (138).

The introduction of genome wide profiling techniques has identified a "metastatic signature profile" expressed by the bulk of tumour cells which stressed the importance of somatic mutations in the cancer evolution, but more importantly, pointed out that the metastatic ability is a common trait for most of the tumour cells (139-141). This clearly conflicted with the progression model leading to the emergence of an "early genetic model for metastasis" which denotes that metastasis is not only an early event in cancer, but might even be caused by the same oncogenic alterations that produced the primary tumour (Figure 2.7) (139).

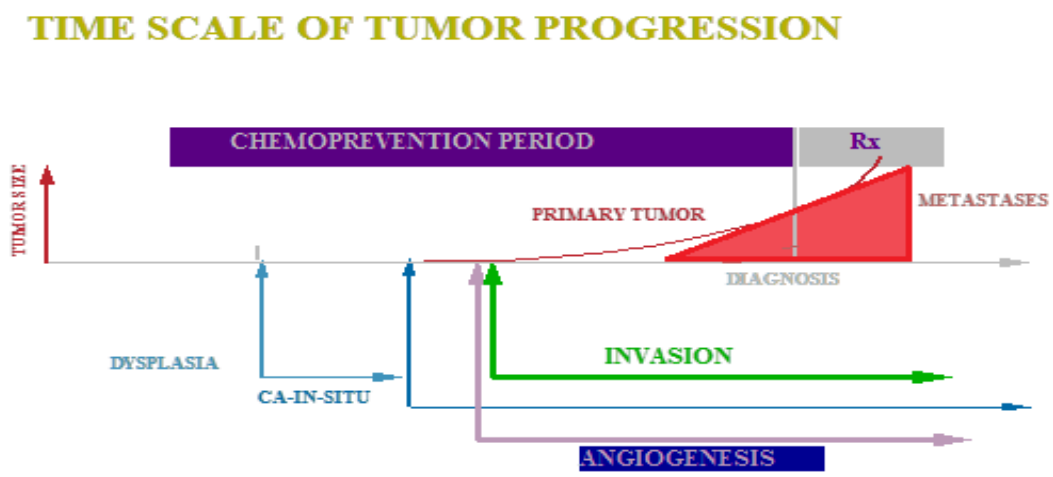



**Figure 2.7:** The diagram shows the early oncogenic/metastatic model, in which the tumour cycle progress from a carcinoma in situ, developing into the tumour. The model hypothesise that the oncogenic events that progress the tumour are themselves already establishing a metastatic genotype.

The current paradigm views metastasis as an evolutionary cycle starting with the initiation of the tumour governed by laws of selection and driven by genetic instability (Figure 2.8) (142). Cells with better survival advantages such as growth self-sufficient, resistant to inhibitory signals and apoptosis will be selected through the neoplastic transformation process to become the predominant population and form the primary tumour. These selected clones are characterised by a high degree of genetic instability which makes them prone to faster rates of genetic and epigenetic alterations, leading to the emergence of different sub-clones with different molecular prints and establishing the tumour's heterogeneous nature (143).

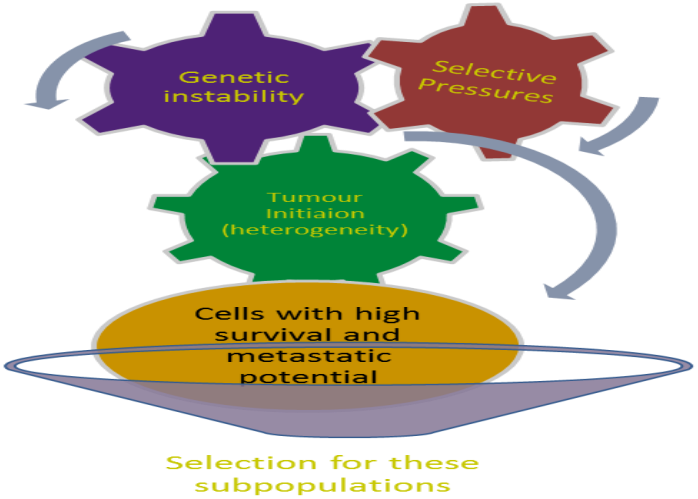



**Figure 2.8:** Relation of genetic instability, selective pressures, tumour heterogeneity and selection of metastatic cells.

Tumour heterogeneity (the presence of different sub clones with different molecular characteristics) driven by genetic instability due to loss of DNA repair mechanisms permits the early rise of new sub-populations with superior biological advantages (e.g. blood vessel formation), alongside an unprecedented ability for invasion and relocation to distant sites (143). The tumour is challenged by numerous internal (e.g. telomere attrition) and external (e.g. hypoxia) stresses which can potentially limit its growth (144). Selective pressure endorsed by these stresses leads to the selection of those metastatic clones with their higher survival and proliferation capabilities (145-146). These sub-populations are adapted to lead the tumour progression phase of the tumour as well as disseminating to other organs to signal the commencement of the malignant stage (147-148). This has been a topic of debate in the

oncology field where some argue that the systemic treatment is, in fact, a metastasis trigger in which it imparts a selective pressure on the tumour cells, killing only labile tumour cells. This allows for the most aggressive subpopulations to escape treatment and stay dormant. They are the treatment-resistant clones which will cause the spread of the disease.

Despite progressing our understanding of some aspects of the metastatic process, all these models fail to completely unravel the complexity of tumour dissemination (149). The transformation of these hypothetical models into clinical applications will require significant efforts through multi-institutional studies to pinpoint the complicated network of factors implicated in the process and distinguish suitable markers that can be of clinical value. Currently, a more robust approach is needed to improve patient outcome and quality of life. A more effective pathway is to review all stages of metastasis, identify the critical ones and intervene at this level to prevent the spread of the disease. This can be achieved through investigating molecules regulating this stage.

### 2.3.2 Clinical Stages of metastasis

Unlike the evolutionary cycle of metastasis, this phase can be detected clinically with more precision and accuracy. It starts with the separation of the cells from the original tumour, then colonising a secondary site by disseminating through blood or lymph vessels. For either route to produce effective metastasis, cancer cells must survive a marathon of several events where each event imposes a challenge to the metastatic process (150). Migrating cells have to acquire intrinsic traits as well as deploy extrinsic signals from their environment to direct the outcome of this marathon in their favour (151-153). Although all of these steps are necessary for the metastatic process, the actual success depends significantly on the rate-limiting step of extravasation (154-157). A myriad of molecules control each step but they can overlap to regulate the whole process and a clear distinction becomes very hard (158). In this section, we provide a review of the stages involved in this process (Figure 2.9) with a focus on the extravasation step, its significance and its key regulators.

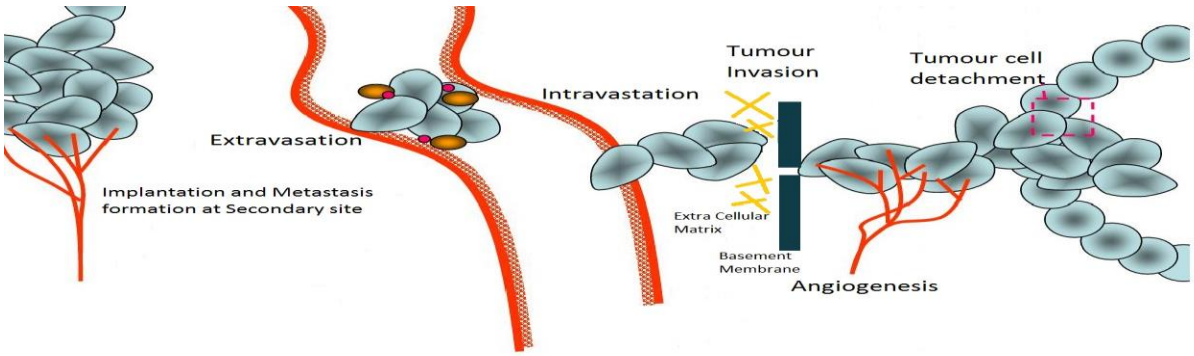



**Figure 2.9:** Schematic diagram of the clinical stages of metastasis (sequence right to left).

**Tumour detachment, invasion and epithelial-mesenchymal transition (EMT)**: In order for tumour cells to commence dissemination, a tumour has to lose its tight adhesion with its neighbours and gear into an invasive form, which is more mobile and can degrade any hindering structures in its way to access the blood vessels. Cadherin (the intercellular adhesion molecules) expression is found to be significantly diminished in several types of carcinomas associated with metastatic phenotypes (159). The loss or reduction of E-cadherin, which maintains intercellular junctions by adhering to actin cytoskeleton filaments via B-catenin, causes loss of cell attachment leading to "cadherin-switching" to N-cadherins which can trigger EMT (160).

EMT is the process through which the epithelial cell loses its characteristic epithelial molecular phenotype (expression of E-cadherins, Actin cytoskeleton, B-catenin) and acquires a mesenchymal phenotype (expression of vimentin, N-cadherin) (161-162). The process is akin to embryogenesis (e.g. neural crest cell migration) and is driven mainly through transforming growth factor (TGF-β) and the transcription factors snail and slug (163-165). EMT is a critical process in the metastatic cascade: first, by reversing back into MET, it helps the survival of the tumour at the distant site. Second, through EMT, tumour cells acquire a mesenchymal mobile phenotype as indicated by *in-vivo* microscopy, which revealed the

ability of tumour cells to migrate across the ECM (166). Finally, the induction of EMT promotes the release of MMPs, which can deconstruct and remodel ECM and BM, facilitating tumour migration (167).

**Neo-vascularisation and angiogenesis**: Neovascularisation involves a de-novo formation of blood vessels and occurs in the embryonic development stage (168). Angiogenesis is the formation of vascular structures from pre-existing ones and often takes place in the physiologic processes of wound healing and pregnancy (169). The increasing demand for nutrients and oxygen to fuel tumour growth and its separation from its original blood supply by expansion, dictate the adoption of angiogenesis mechanisms to guarantee the survival of tumour cells (170). Under normal physiologic conditions, a delicate balance exists between several pro/anti angiogenic factors. This balance is tipped in the case of the tumour towards "the angiogenic switch" (171-172).

The angiogenic switch allows a tumour to adopt both neo-vascularisation and angiogenesis to maintain its growth (150). In neo-vascularisation, the cancer reactivates the embryonic state to recruit endothelial precursors from bone marrow, inducing them to proliferate and configure a new vasculature. Tumours can also stimulate angiogenesis by attracting nearby endothelial cells, which mobilise towards the tumour bulk influenced by a gradient of angiogenic factors secreted by the tumour and its microenvironment, where they can reorganise and form new capillaries (173-174). Some tumour cells can form three-dimensional tubes mimicking blood vessels, with blood flowing into these tubes (175-176). Recent microscopic photos demonstrated that the blood vessels formed in the tumour are largely different from normal ones in terms of quality of vessel permeability, blood flow, and stability (177-178).

Insights from the physiologic processes where these new blood vessel formations take place revealed a set of regulating factors which proved to be the same in the tumour angiogenic switch (179-180). Pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) as well as inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) have been demonstrated to be the main modulators of angiogenesis. These molecules are triggered by a state of tissue hypoxia

endorsed by tumour overgrowth, which induces hypoxia inducible factor (HIF) to initiate the angiogenic cascade (181-182).

**Intravasation of metastatic cell foci** into lymph or blood vessels follows their detachment from the primary tumour. Studies on intravasation show that tumour cells acquire migratory behaviour that enables them to mechanically translocate and squeeze through the endothelial barrier (183). This includes the ability to orient themselves towards the blood vessels, change shape into a more rounded form, and the development of migrating processes that permit their locomotion (184-185). Several factors responsible for these mobility transformations have been identified, such as insulin-like growth factor and extracellular matrix molecules such as laminin and fibronectin (186-187). This process can be active due to the release of chemotactic peptide and the movement of cells towards this gradient (188). On the other side, a passive mechanism has been postulated, in which the mechanical stress in the tumour overgrowth breaks the fragile, immature, newly-formed vessels and pushes the tumour through these frail barriers (188). VEGF was shown to accompany tumour-associated angiogenesis causing formation of leaky vessels which provide easy access for intravasated cells (189).

**Extravasation (the escape of tumour cells from circulation or lymphatic vessels into tissue)** is a critical step in the metastatic cascade and leads to the implantation of the tumour mass at the distant site (156). Insights into the extravasation steps are driven from the studies of leukocyte interaction and migration during inflammation (155). The completion of this stage requires active adhesion between both tumour and endothelial cells through adhesion molecules and is aided by proteolysis enzymes (190, 155). Evidence suggests that the adhesion between tumour and endothelial cells during extravasation is mediated by the endothelial cell adhesion molecules and their ligands on tumour cells (155, 156). Extravasation, itself, is a multistage process encompassing several sub-steps:

1- Loose attachment of cancer cells to endothelial cells, which triggers rolling movement of tumour cells on the endothelium
2- Cells attach firmly to the endothelium which causes cell structural modifications allowing the cells to cross the endothelium in the third step (diapedesis).
3-

**Metastasis formation at secondary site**: Once metastatic cells exit the vasculature and settle into their new microenvironment, they are confronted with a new set of challenges. Most of these cells will undergo apoptosis or remain dormant and only a few will have the ability to adapt to the new niche (191). This adaptation to create a favourable site for tumour growth involves interaction between tumour cells and the host microenvironment (142, 192). The implantation of tumours at secondary sites is not merely a random process but is a "seed" and "soil" guided event (142).

Despite an incomplete understanding of the specific molecular mechanisms underlying this process, research efforts have suggested a few explanations such as the interaction of the tumour with its stromal components, which can trigger cell translocation into the new sites to modify the niche to accommodate the new metastasising cells (193). Other studies suggest that the different molecular signatures on the endothelium representing the tissue in which they reside might attract specific disseminated cells which possess similar signatures (194-195).

### 2.3.3 The significance of extravasation

The stages of the clinical metastatic cascade are crucial for the successful dissemination and implantation of the tumour at the secondary site. Failure of the completion of any stage entails the collapse of the overall process (192). However, only the completion of the so-called "rate-limiting steps", not all steps, will guarantee the success of metastasis (154). For instance, millions of cancer cells can be detected in the circulation of patients (even in patients with very small tumours or who have never developed an overt metastasis), yet less than 0.01% of these circulating cells can develop clinical metastasis (196-197). When the tumour cell is introduced into the circulation, it faces several stresses that diminish its survival chances, including the sheer force of blood flow, immune response and apoptosis signals induced by loss of cell contact as well as the rocking of cells against the wall of the blood vessels (198-199). This highlights the inefficiency of the metastatic process and denotes the importance of extravasation and subsequent colonisation as deterministic events defining the outcome of the whole process (138).

The ability of the specific tumour to extravasate determines its metastatic capacity. For example, colon cancer cell lines with higher extravasation rates displayed higher colonising

potential (200). In a separate study, only 0.05% of lung carcinoma cells injected intravenously (IV) were able to survive and colonise the lungs (201). Some tumours display higher extravasation rates, which might support the theory that tumour metastasis occurs because of mere mechanical entrapment (202-203). These results were challenged with the advance in the new intra-vital microscope, which showed that tumour cell arrest occurs in capillaries with diameters larger than that of the tumour cells (204). This new data does not disprove the importance of extravasation but rather denotes that the steps after extravasation are also rate-limiting steps.

The adhesion of the tumour cells to endothelial cells represents a milestone event in the extravasation process (204-205). The interaction of the endothelium with the neoplastic cells determines the physical point of cell exit, and hence can explain specific organ metastasis (206). In addition, this adhesion is of vital importance to the survival of tumour cells as it provides anchorage and, therefore, it prevents cell death and induces proliferation (207). In contrast to intravasation, which can be largely passive due to abnormal tumour vasculature, adhesion is a highly active and specific event (188, 155-157). The analogy between adhesion in inflammation and tumour extravasation provides a powerful tool to understand this process (208). These criteria accentuate the promise of the regulators of adhesion mechanism to develop therapies that can interfere effectively with the metastatic cycle (207, 209).

### 2.3.4 Role of inflammation in cancer and metastasis

Inflammation is a protective response by which the body can eliminate any harmful irritants (physical, chemical, microbial, etc.) (210). In addition, the commencement of inflammation triggers the repair process which is vital to restoring the function of organs and the homeostasis of the body (210). The strong relationship between cancer and inflammatory process has labelled cancer as the “wound that never heals” (211). Population-based studies revealed that 25% of all cancers are a result of inflammation, and almost every single cancer type is infiltrated or surrounded by inflammatory cells (212-213). The use of anti-inflammatory drugs to prevent the incidence of several types of cancer highlights the key role of inflammation in cancer (214). Hahn et al. identified inflammation as the “seventh hallmark of cancer” and recent research indicated that inflammation underlies several aspects of tumour growth, survival and, most importantly, metastasis (143).

The role of inflammation in several stages of cancer metastasis is supported by several studies (215). Research shows that *in-vitro* co-culturing of tumour cells and tumour-associated macrophages enhances the invasive capacity of neoplastic cells, which is mainly dependent on the secretion of MMPs (216-217). Other studies demonstrated that inflammatory cells can induce the expression of both MMP and angiogenic factors, which promotes angiogenesis, a crucial requirement in the invasion of tumours (218). Several signalling pathways such as nuclear factor kappa-light-chain beta (NF-κβ) and signal transducer and activator of transcription (STAT) have been identified to be regulators of both metastasis and inflammation (219-220). Inflammatory cytokines can increase the motility of metastatic cells through the induction of the EMT program (216, 221). Similarities between wound healing and tumour extravasation via identification of the inflammatory mediators that can increase the adhesion of either tumour or inflammatory cells to the endothelium provide a strong link between inflammation and metastasis (222-223). Advances in exploring these common mediators have the potential to be translated into clinical interventions that are more efficient in managing patients with metastasis.

### 2.3.5 Extravasation in inflammation and metastasis

The ultimate goal of the inflammatory process is to recruit the leukocytes to the site of inflammation so that they can combat the offending stimulus either by ingestion or by the appropriate antibody response (210). As mentioned previously, insights into malignant extravasation came from the recruitment of leukocytes in the inflammatory process. Initially, researchers considered the two processes to be identical (Figure 2.10, 2.11). However, new data from different well-documented *in-vivo* and *in-vitro* experiments have shown some differences (155). The main difference is that tumour cells disrupt the endothelium during its migration, unlike inflammatory cells, which preserve its integrity (224). The main similarity is that both processes are guided through localisation signals by the tumour, the leukocytes or the endothelium, which is an active promoter in both events (225). These localisation signals are mainly delivered by neurotransmitters such as Substance P (SP) or cytokines such as tumour necrosis factor (TNF-α). Although these mediators have been elaborated extensively in inflammation, their action in metastatic extravasation is yet to be identified (225-227). Another facet of similarity in both cascades is the use of MMP to ingest the endothelial basement membrane and facilitate the movement of cells during diapedesis, though the

mechanisms and regulators that induce their expression are not clear (303-304). Finally, a definite set of adhesion molecules controls the interaction between the tumour, the leukocytes and the vascular endothelium, resulting in the same sequence of adhesion to the endothelium leading to extravasation (208).

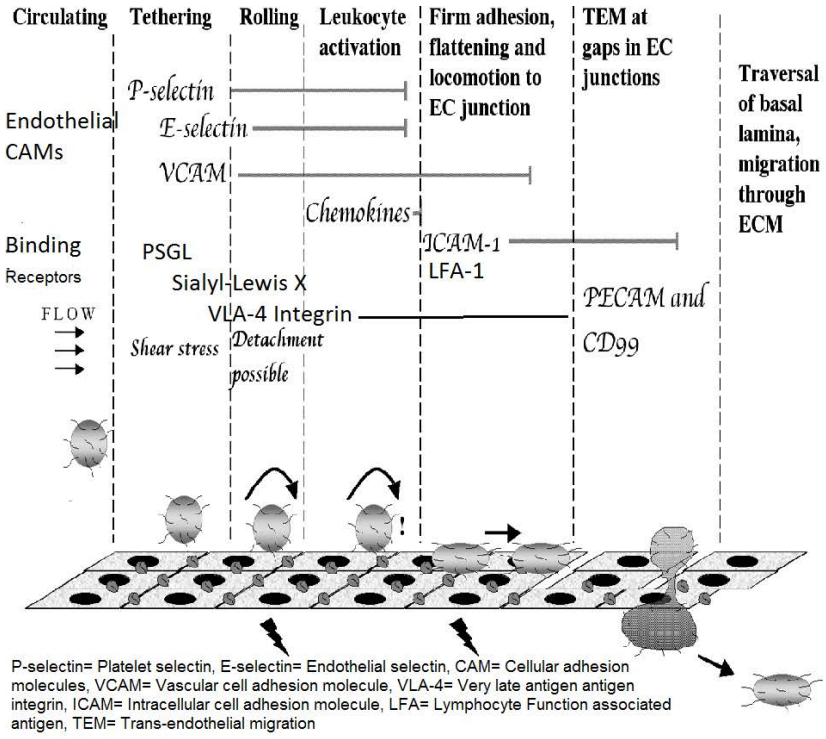



**Figure 2.10:** Stages involved in extravasation and the adhesion molecules responsible for each stage as has been investigated through inflammation research (232).

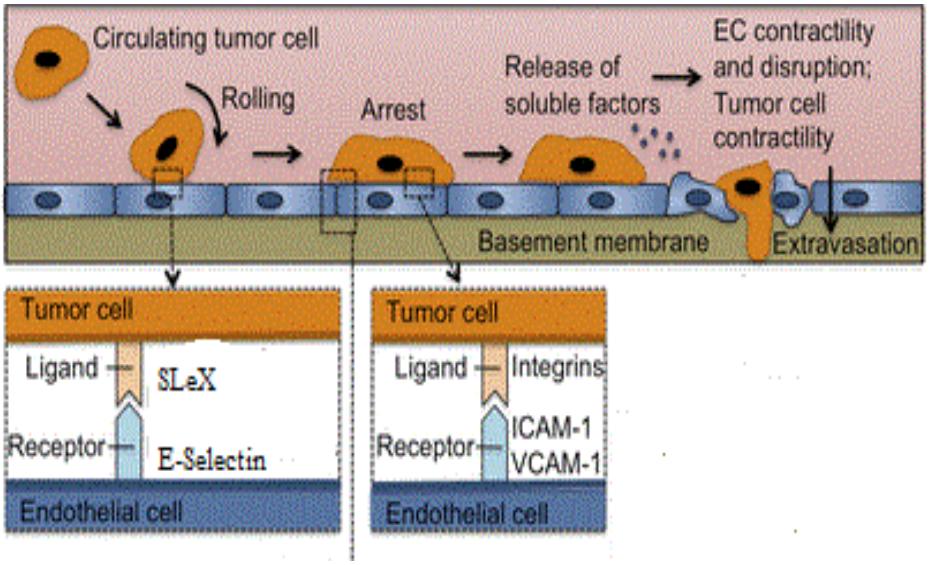



**Figure 2.11:** Suggested stages of tumour extravasation (206).

### 2.3.6 Adhesion molecules during stages of extravasation

Adhesion of the leukocyte/tumour cells to the endothelium is the initial critical step in the extravasation process. It is a critical requirement for cell migration through the vasculature and typically takes place in post-venule capillaries (228-229). The adhesion starts with a weak attachment (rolling) of the migrating cells of the endothelium, which in turn triggers firmer adhesion and subsequent crossing of the cell through the endothelial barrier (156).

Every step of the extravasation process is mediated through a group of adhesion molecules expressed on the migrating cells and their binding receptors on the endothelial cells (Table 2.5-2.6) (230-231). The majority of these adhesion molecules have been identified through extensive research in inflammation (232). However, their expression and action in tumours is not clearly defined. Two large families of molecules, the selectin and integrin, were found to be responsible for the adhesive interaction which takes place in extravasation (233). The binding of these adhesion molecules to their receptors controls the adhesion and can inflict morphological and functional changes on the cells involved in the adhesion.

**Table 2.5:** Endothelial adhesion molecules and their binding receptors.

| Endothelial adhesion molecule | Leukocyte receptor | Major function |
|---|---|---|
| P-selectin | Sialyl-Lewis X, PSGL-1 | Rolling neutrophils, monocytes and lymphocytes |
| E-selectin | Sialyl-Lewis X | Rolling, adhesion of neutrophils, monocytes and T cells to activated endothelium |
| ICAM-1 | CD11/CD18 (integrins) LFA-1, Mac-1 | Adhesion and transmigration of leukocytes |
| VCAM-1 | α4β1 (VLA4) (integrins) | Adhesion of eosinophils, monocytes and lymphocytes |
| GlyCam-1 CD31 (PECAM) | L-selectin CD31 | Lymphocyte homing and migration through endothelium |

**Table 2.6:** Endothelial cells adhesion molecules, their binding receptors in leukocyte and counterparts in tumour cells.

| Endothelial cell surface molecule | Leukocyte counter-receptor | Cancer cell counter-receptor |
|---|---|---|
| Selectins | | |
| E-selectin | PSGL-1; SLeA-, SLeX-glycoproteins | SLeA-, SLeX-glycoproteins |
| Immunoglobulins | | |
| ICAM | aLb2 (LFA-1); aMb2 (Mac-1) | aLb2 (LFA-1)?? |
| VCAM | a4b1 (VLA-4) | a4b1 (VLA-4) |

The prediction of this hypothesis proves correct in leukaemias and lymphomas where they have the highest metastatic rates (359). Taking into consideration the nature of the cell of origin for these neoplasms – as inflammatory cells (i.e. white blood cells and lymphocytes) – they are predisposed to express several adhesion molecules to bind with the endothelium in inflammatory conditions (360). This facilitates their interaction with the endothelium and further dispersion in distant organs. Therefore, these tumours represent an excellent model for the study and validation of understanding of the adhesion process in the spread of cancers.

#### 2.3.6.1 Rolling

Initial weak interactions between leukocytes and endothelial cells allow rolling on the endothelium (EC) and margination of leukocytes from the main blood stream in the blood vessels (156, 234). This repeated tethering of the leukocyte retracts the cell from the high-velocity centre of the blood vessel as well as keeping the migrating cell close to the endothelium, which prevents dislodging of the leukocytes (LC) (235). In cancer, research has not produced a clear demonstration of this stage, using intra-vital microscopy, unlike the distinct rolling motion of neutrophil which has been visualised *in-vitro* (236-237). Extensive experimental models of inflammation revealed that tethering of LC on EC is mainly mediated through selectin adhesion molecules. However, tumour cells do not express selectin; instead, they express their ligands (238).

Selectin is a family of vascular adhesion molecules, including E, P and L types that are expressed on the endothelium, platelet and leukocyte surfaces respectively (239). The studies show that P- and E-selectin are expressed in response to inflammatory mediators and are involved in the initial rolling of white blood cells on the surface of the endothelium at the site of the inflammation (234). This effect was inhibited in T cells by using anti-P or anti-E selectin, which in turn inhibited the migration of leukocytes into endothelial cells (240). Genetically engineered mice lacking the genes for P- and E-selectin demonstrated a lack of rolling of neutrophils on the endothelium and low extravasation capabilities (241).

Cancer cell interaction with endothelial cells, mediated via E-selectin, is important for metastasis (242-243). E-selectin was shown to be up-regulated in endothelial cells at the site of liver metastasis and the media from cancer cell lines induced the expression of E-selectin when added to endothelial culture (243, 244-245). Jiang et al. (246) demonstrated that a

higher incidence of lung metastasis from breast cancer cell lines was evident in inflammation-induced mice, dependent on higher E-selectin production by endothelial cells, and anti- E-selectin reduced this metastasis. The high expression of E-selectin in several invasive types of tumours such as breast, colon, ovarian and lung cancers suggested that E-selectin expression by tumours might contribute to the interaction of tumour cells and leukocytes during motion of tumour emboli in the circulation (247-250). A study by Ye et al. demonstrated that the expression of selectin on EC adjacent to the metastatic tumours was higher than that of the EC of the primary tumours (249).

Selectins can bind to a variety of ligands such as PSGL-1, ESL-1, CD24, sialyl Lewis-a, sialyl Lewis-x (SLe-X), CD34 and MAdCAM-1, all of which are expressed by leukocytes (238). The most common ligands for P-, L- and E-selectins are PSGL-1, ESL-1, sialyl Lewis-x, respectively, although binding to other ligands is not uncommon (251-252). PSGL-1, ESL-1, and sialyl Lewis-x are proteins or lipid cores attached to oligosaccharides epitope that works as the functional ligand and requires fucosylation for proper function (251). These ligands have been reported to be highly expressed in a variety of cancer types and this expression was correlated with a poor prognosis (253-254). In a study of 145 head and neck cancer patients by Gunawardena et al., SLe-X was a negative prognostic marker for survival in those patients (255). It has been demonstrated that colorectal and gastric cancer cells with high expression of the selectin ligands binds more efficiently and extravasate at higher rates than those with low expression (256). The use of an anti-SLe-X antibody inhibited the formation of metastasising tumour colonies, and the addition of a selectin antagonist to the chemotherapy regimen (cisplatin) has a cumulative metastatic inhibitory action (257). Cimetidine showed an inhibitory effect on metastasis in gastric cancer and this effect was thought to be due, in part, to blocking selectin/SLeX adhesion in those cells (258). These results, despite having inconclusive results due to the lack of understanding of the role of selectin and its ligand in metastasis, provide some evidence that interference with this interaction could be introduced in clinical trials to interfere with metastatic formations.

#### 2.3.6.2 Adhesion

Firm adhesion to the endothelial cells requires the activation of a group of endothelial cell adhesion molecules (CAM) to achieve tight binding with their receptors (integrins) for the

successful completion of the extravasation (Figure 2.12) (259). CAM categories that are known to participate in the adhesion process are: VCAM and ICAM-1 (related to Ig superfamily) (260-261). While VCAM is only expressed in inflammation, ICAM-1 is expressed in low-affinity concentrations on the surface of the resting endothelium and becomes up-regulated in response to an inflammatory mediator (262-263). Several studies have revealed a role for both molecules in leukocyte recruitment during a wide range of inflammatory diseases. Antibody blocking of ICAM-1 caused reduced infiltration of neutrophils and ICAM-1-deficient mice demonstrated the same signs, whereas VCAM knockout mice die during early embryogenesis. (264-265).

The involvement of these endothelial adhesion molecules is attracting attention recently in oncology research. The expression of VCAM and ICAM was shown to be higher in the tissues and serum of breast cancer patients (266-268). Other *in-vitro* studies demonstrated that ICAM and VCAM are key mediators of adhesion of endothelial cells and cancer cells (269-270). Contrary to this, other findings showed a lack of correlation between specific CAMs and the metastatic or adhesion potential of tumour cells (271-272). This conflict of results does not void the role of CAMs in the adhesion of cancer to endothelial cells, but rather it reflects an incomplete understanding of the adhesion process and different modulators and tissue response. Additionally, the type of assay used to assess the adhesion greatly influences the outcome and requires the development of more accurate and reproducible adhesion models for more validated results (273).

Integrins are complex molecules that mediate cell-ECM adhesion and control cell conformation and several cellular processes such as survival and proliferation through interaction with the cytoskeleton (274). There are 18 different alpha and eight beta chains which can form 24 heterodimers integrin receptors (274). Integrin molecules which are responsible for endothelial adhesion belong to the β1 and β2 sub-families. The αLβ2 (lymphocyte function associated antigen LFA-1) and αMβ2 (Mac-1) are both ligands for ICAM-1/-2 whereas, α4β1 integrin VLA-4 is the ligand of the vascular cell adhesion molecule (VCAM-1) (275).

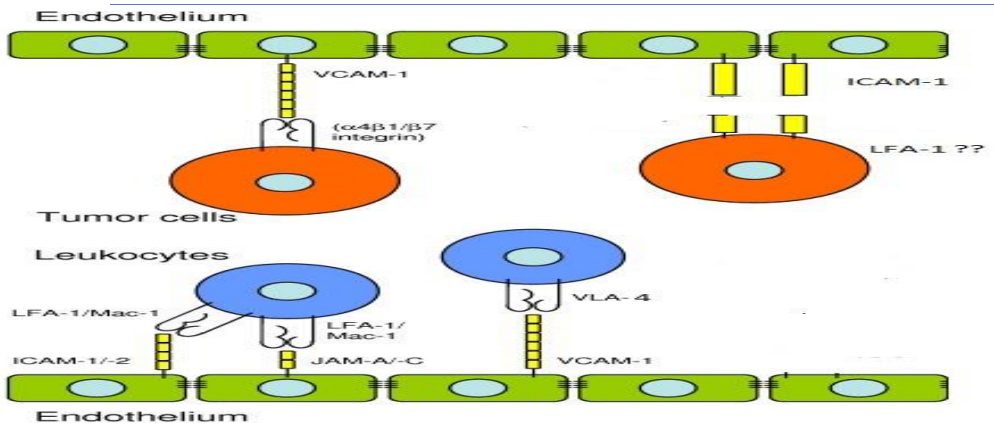



**Figure 2.12:** Molecules involved in the adhesion of tumour and leukocytes to endothelium (155).

Studies on melanoma identified VLA-4 integrin as the main molecule involved in binding with VCAM and it plays an important role in the endothelial adhesion and trans-endothelial migration (TEM) of the tumour cells during metastasis (276-277). Similar results were obtained *in-vitro* in brain metastasis, where metastatic brain cells injected in mice showed specific high expression of VLA-4 in the early stages of the tumour seeding (278). VLA-4 was shown to induce the adhesion with lymphatic endothelium *in-vivo* and promotes lymph node metastasis, therefore, it has been suggested that it can serve as a biomarker in lymph node metastasis (279). In oral cancer, few studies have been performed on the expression of VLA-4 either *in-vivo* or *in-vitro*. Songet et al. showed that Cal 27 oral carcinoma cell line can express VLA-4 and demonstrate strong adhesion with TNF-α activated endothelial cells. However, the study has not reported the basal expression levels of VLA-4 by OSCC cells (280).

Inhibiting of ICAM-1/LFA-1 axis prevented the formation of micro-metastasis in prostate cancer (281). In lung carcinoma, it was shown that the adhesion of tumour cell lines to the cultured endothelium was achieved through ICAM-1, both in static and shear flow. The monoclonal antibodies to these molecules significantly hindered this adhesion (282). However, there is a controversy about the presence of LFA-1 in tumour cells. Jiang et al., in their study of adenocarcinoma cell lines, demonstrated that ICAM-1 was expressed on the tumour cell proper of all the carcinomas, while its receptor LFA-1 was expressed only in

tumour infiltrating lymphocytes. Therefore, they suggested that the actual binding occurs between the tumour and the lymphocyte which facilitates the tumour trans-endothelial migration and evasion of the immune system (283). These findings were further validated with the lack of expression of LFA-1 in melanoma (highly metastatic tumour), however, the media of melanoma cell lines promoted tumour-endothelial adhesion and transmigration through LFA-1 interaction with its ligand ICAM-1 (284-285). Further studies are required to investigate the actual mechanism of LFA-1 in the adhesion of tumour cells.

The importance of the CAM-integrin adhesion axis is that it can explain site preference of metastatic tumours, as studies on metastatic lymphoma and colon cancer cell lines showed preferred adhesion to hepatic endothelial cells (286-287). Additionally, the integration between different integrin members ensures that after the establishment of adhesion, the completion of the TEM will take place (157).

#### 2.3.6.3 Diapedesis

After the firm adhesion with endothelium, the migrating cell has to exit the vasculature. In the case of inflammation leukocytes leaves the endothelial layer intact, whereas tumour cells disrupt their integrity mainly due to their larger size and triggering aptopsis of endothelial cells (224). Two routes have been proposed for this step, the first of which is the para-cellular route where cells can move between neighbouring endothelial cells. The other route is the trans-cellular route in which cells migrate through the endothelial cells themselves (288). While both leukocytes and tumour cells can deploy both routes, different subpopulations adhere to specific routes and the reasons for this are yet to be identified (288). The adhesion step inflicts structural changes in the migrating cells, changing their shape into a drop shape as well as arranging the cytoskeleton of those cells (289-290). These changes also occur in the endothelial cells where an apparent change of vascular endothelial cadherin has been recorded as well as an increase in vascular permeability (289). Adhesion to endothelial cells triggers changes that activate PECAM and N-cadherin, which have been demonstrated to play a major role in this process (290). Once the migrating cells pass the endothelial layer, they are faced with two obstacles; basement membrane of the blood vessel and the extracellular matrix of the secondary site (291). Studies have shown that leukocytes utilise MMPs to degrade these components to advance to reach the site of inflammation (303-304).

On the other side, it is not clear whether cancer cells implement the same mechanism. This might seem instinctively mandatory, hence further studies are needed to investigate the role of MMP in tumour extravasation.

### 2.3.7 Action of MMP in extravasation and inflammation

MMPs are a group of proteolytic enzymes whose main function is to degrade the extracellular matrix (ECM) and basement membrane (BM) (292). A recent study suggested that the MMP role might not be restricted to matrix digestion, with new evidence demonstrating involvement in various processes associated with inflammation and cancer such as angiogenesis and phagocytosis (293). MMPs are highly expressed in any disease or condition that is associated with inflammation, therefore proteolysis is considered a hallmark of inflammatory process (294). Several MMP inhibitors are used as anti-inflammatory drugs in periodontal and vascular disease (295-296). Several models of MMP knockout mice have been established and each displays a dysregulation of a specific aspect of acquired immunity depending on the type of MMP deleted (293).

In cancer, MMPs are constantly expressed at the invasive front of the tumour, which indicates a strong correlation with invasion and metastasis (297-298). It is believed that MMPs can also regulate the pre-metastatic niche and play a key role in angiogenesis (298-299). MMPs are tightly linked to integrin, which maintains cell-ECM adhesion. Therefore, the interplay between both molecules is vital in tumour mass detachment and also in interaction with microvasculature and invasion in the distant site (300). Hong et al. (301) showed that high levels of MMP-2 and MMP-9 were related to the invasiveness and lymph node metastasis of OSCC. Similar results were achieved by Singh et al. (302) who showed a significant relationship between MMP-2 expression and tumour stage and differentiation and similar results for MMP-9. In addition, multivariate analysis revealed a strong correlation between MMP-2 expression and lymph node metastasis.

The contribution of MMP in the extravasation of leukocytes in inflammation has not been thoroughly researched, despite a role having been established for these enzymes in establishing a chemo-attractant gradient for immune cells which facilitates its migration (303-

306). Moreover, the relation of the inflammatory mediator's effect on MMPs as well as the relationship between MMPs and integrin adhesion molecules is not fully understood. The relationship between MMP and extravasation of tumour cells was suggested when carcinoma cells were injected into mice, where this showed invasive protrusions extending between endothelial cells in the lung microvasculature, pointing out the disruption of endothelial cells by a proteolytic action (307). Applying a gelatinase inhibitor shortly after the injection of melanoma cells in mice inhibited lung colonisation by these tumour cells (308). However, applying a broad-range MMP inhibitor did not affect the survival of the colonising cells (309). Notably, the survival of the cells was the outcome of the study, and so, the results of this study do not effectively evaluate the role of MMP in extravasation. Leroy et al. showed that MMP-2 is necessary for the endothelial transmigration of ovarian cancer cells (310). Voura et al. developed an *in-vitro* endothelial transmigration assay, which indicated that the proteolysis takes place at the tumour-endothelial interface during migration, mediated by the action of MMP-2/9. This was significantly reduced with the application of MMP inhibitors (311). Since the regulators of extravasation mechanism are poorly understood, the same will apply for MMP role in extravasation, and further studies will be needed to outline whether the role of MMP is regulated by the same factors that control the adhesion process.

### 2.3.8 Substance P in inflammation

Substance P (SP), the most potent member of the tachykinin family, was initially identified as an active neurotransmitter in primary sensory afferent fibres (312-315). The canonical pathway for SP is through binding to the G protein-coupled Neurokinin-1 receptor (NK-1R) (316-317), in which the SP/NK-1R ligand-receptor complex is formed, and then SP is degraded while NK-1R is recycled to the cell surface (318). The SP/NK-1R complex activates a different MAPK signalling cascade such as ERK and P38 MAPK, through forming a scaffolding complex with β-arrestin (319). Additionally, the SP/NK-1R complex can mediate other pathways such as the NF-kB pathways (320), and transactivates other tyrosine kinase receptors such as EGFR (321). SP contributes to the inflammatory process possibly through different mechanisms: I) release of inflammatory mediators such as cytokines and arachidonic acid derivatives; II) chemo-attractant to the immune cells; III) vasodilatation (VD) and leukocyte/endothelial cell adhesion to aid leukocyte recruitment to

the injured tissue; and IV) through angiogenesis (322) (for excellent reviews about SP please refer to 312-314).

SP was found to produce vasodilatation and neurogenic plasma extravasation in the rat trachea upon infusion or release, due to stimulating of peripheral sensory nerves (323). Mashito et al. (324) induced ovalbumin sensitisation in rat trachea, and three weeks later assessed bronco-alveolar lavage fluid resulting from the airway allergic response, where they found a very high concentration of SP. They concluded that elevated SP caused vascular permeability and eosinophils accumulation and was inhibited by using the Nk-1R antagonist CP 96,345 (324-325). Upon the injection of nano-concentrations of SP in the skin and conjunctiva, similar effects were obtained with concomitant distant flare and wheal formation (326-327). Furthermore, non-neuronal injection of SP caused post-venule vascular leakage (VL) and plasma extravasation in the gastrointestinal tract and conjunctiva (328-330). Not only did SP cause VL and VD, but in most cases this was accompanied by neutrophil or eosinophil adhesion or accumulation (324, 331). Hence the main function of the inflammatory response is to recruit inflammatory mediators and cells to the injury site. We can comprehend that SP can mediate inflammatory reaction via inducing vasodilatation of the vessels and plasma extravasation as a result of the sensory nerve stimulation. This is followed by leukocyte adhesion and migration through the endothelial barrier (332-334).

Despite evidence that SP is involved in the adhesion and migration of leukocytes during inflammation, the mechanism underlying this process is poorly understood and requires further investigation. It is well known that the up-regulation of adhesion molecules on the endothelial cells (EC) caused by the release of tumour necrosis factor and interleukin 1 (TNF-α, IL-1β), is a prerequisite for slowing of leukocytes (LC) (335-337) and subsequent adhesion of LC to EC and their further transmigration (288). SP is a potent and specific stimulus for the release of these specific cytokines (338-339). IL-1β and TNF-α were proved to up-regulate the expression of the selectin adhesion molecules which caused the rolling of leukocyte to the EC. It also induces the expression of ICAM and VCAM adhesion molecules, which are responsible for the tight binding with leukocyte integrins (340). The most likely explanation is that SP triggers the inflammatory pathway NF-KB through p38 MAPK, as the inhibitor of this specific pathway blocked the expression of the pro-inflammatory cytokines (341-342). Thus, SP can cause vascular permeability by inducing the release of cytokines,

which causes the up-regulation of vascular and leukocyte adhesion molecules and eventually transmigration of inflammatory cells to the site of injury.

### 2.3.9 Substance P in cancer and metastasis

High expression of SP and NK-1R receptor in various tumours including melanoma(343), human malignant glioma (344), retinoblastoma (345) and mammary carcinoma (346), both in the primary tumour and their metastatic counterparts, suggests an important role of SP in carcinogenesis (347). SP has been suggested to act as a mitogen for cancer progression (348) and even in the early malignant transformation (349). The role of SP in metastasis and invasion has not been fully investigated, although initial studies suggested that SP might trigger the metastasis of colon cancer (350-351). Taking in consideration the main roles of SP in inflammation as vessel permeability, adhesion and migration of leukocyte, it is logical to predict similar roles for SP in metastasis (352) via inducing the adhesion between the tumour and the endothelium.

SP was found to be significantly expressed in the tumour cell membrane, cytoplasm and nucleus of OSCC as well as the infiltrating lymphocytes of the neoplasms (353). The expression of SP was found to be significantly associated with the presence of dysplasia ($P$<0.001) and carcinoma *in situ* ($P$=0.021) which strongly suggested a role of SP in the early onset of oral oncogenesis (354). Moreover, SP IHC expression was significantly higher in kerato-odontogenic tumours and in oral lichen planus than in normal controls (355-356). The above evidence points out that SP might be a key player in tumour growth and progression, however, the role of SP in metastasis of OSCC has not been well investigated.

Two points are of concern here. Firstly, in the Brener et al. study the expression of SP was 58.1% ($n$= 43) in the peri-tumoural or intra-tumoural blood vessels and these observations have not been further studied (353). Secondly, Esteban et al. found that expression of SP was higher in 111 out of 114 cases of laryngeal carcinoma with 17 cases of metastases all of which expressed SP (+++/++++) (100%) (357).

The above discussion underscores a number of interesting findings: 1) dissemination of the tumour cells requires adhesion of tumour and endothelial cells mediated through their adhesion molecules and their legends; 2) SP is an inflammatory mediator that can produce vascular adhesion and extravasation of leukocytes through inflammation; 3) SP is highly

expressed in metastatic tumours and OSSCC in particular around vascular sites. “Can cancer implement the same inflammatory mechanism to adhere and metastasise to other organs of the body?” is a question supported by the recent finding that SP enhances breast cancer cell adhesion and migration through the blood-brain barrier (358). Furthermore, it constructs a hypothetical framework to consider a role for SP in metastases, through affecting the adhesion between tumour cells and the vascular endothelium, as a mechanism for extravasation of malignant neoplasms, leading to lymph node or distant metastasis.

**Key Points**

- Extravasation and adhesion of cancer to endothelial cells is a rate-limiting step in metastasis.
- SP is a key mediator of vascular permeability and extravasation.
- SP is highly expressed in cancer in particular metastatic tumours.
- Can cancer implement the same inflammatory mechanism to adhere and metastasise to other organs of the body via Substance P?

# Chapter 3 The prognostic role of inflammatory mediators in metastasis of oral squamous-cell carcinoma, comparison of clinical and experimental evidence: Systematic review

## 3.1 Introduction

Metastasis accounts for up to 90% of cancer fatalities. Ironically, the success in early detection and prevention of tumours has resulted in prolonged survival periods, with higher metastatic incidence rates observed (17, 361, 3). The rate of metastatic spread for OSCC is reported to be 65% for lymph node metastasis, 5-25% for distant metastasis and up to 50% rate for local recurrence (20, 362, 18).

The involvement of metastasis, as the most important prognostic factor (14, 10), significantly decreases the survival probability (21). This explains why OSCC remains one of the tumours with the worst prognosis, with an overall 5-year survival rate of around 50% (21). Moreover, metastasis is not limited to late stages but also can occur in patients with clinically negative lymph nodes as "micro-metastatic" foci. This foci can activate primary or secondary recurrence in OSCC patients even after they have been treated for the primary tumour (63).

OSCC patients with metastasis or those in early stages with a high risk of metastasis are subjected to invasive management approaches. However, such treatment strategies (neck dissection, chemotherapy, radiotherapy) are accompanied by significant morbidity, poor quality of life, and even mortality as a result of treatment complications (121). Finally, those modalities have not brought significant improvements in patient survival or metastatic or recurrence control (118).

## 3.2 Clinical stages of metastasis

For cancer cells to disseminate to a secondary site, they have to go through several stages termed the "metastatic cascade" (146, 142). Despite being an ineffective process, it is highly regulated by a complex network of mediators. First, the neoplastic cell detaches from its neighbouring cells and transforms into a mesenchymal, invasive form that degrades any hindering structures to access the blood/lymphatic vessels. This is achieved through structural changes of the cytoskeleton of the cells, in particular, the adhesion molecules associated with increases in production of matrix degrading enzymes (158). Second, the invading cell turns into a migratory pattern termed epithelial-mesenchymal transition (EMT) (163). The increasing demand for nutrient and oxygen to fuel tumour growth dictates the induction of angiogenesis (formation of new blood vessels) mechanisms by the tumour to guarantee its survival (172).

Formation of new blood vessels within the tumour provides access for the migrating cells to enter the main blood stream in a process termed "intravasation" (183). The escape of the tumour cells from the circulation or lymphatic vessels into the tissue (extravasation) is a critical step in the metastatic cascade and leads to the implantation of the tumour mass at the distant site (127).

### 3.3 Description of the intervention

Cancer does not use novel mechanisms but rather implements existing biological process in favour of its growth and progression (208). This fundamental concept is supported by insights into the different stages of cancer progression and the metastatic cascade (e.g. epithelial mesenchymal transition (EMT), angiogenesis, intravasation/extravasation) which reveal that most of these stages are identical to some inflammatory processes (215). The link between inflammation and cancer has been a topic of research in the recent years since Hahn et al. identified inflammation as a main cardinal sign of cancer (363). Although this research has revealed several aspects of inflammation in initiating and progressing neoplasms, it has not completely highlighted the role of inflammation as a main driver of metastasis (212, 213, 143).

### 3.4 Rationale

Metastasis has become the prime killer of cancer patients, rather than the original tumour itself (17, 361, 3). The presence or risk of metastasis is the most important indicator of prognosis and the most deterministic in selecting the method of treatment (21). Once metastasis has occurred, then the treatment of the primary tumour is either ineffective or confined to palliative treatment, providing no clinical benefit in prolonging survival. Although there has been progress in understanding the mechanisms of cancer initiation which resulted in prolonged survival periods across most types of cancer, relapses are seen clinically more often (3). This can be traced to the poor understanding of the key players in the interaction between the tumour and its microenvironment which predispose and establish the blueprint of the metastatic process.

Evidence of the key role of inflammatory mediators in tumour metastasis is paramount. However, using this evidence for clinical implementation is challenging, as most of the studies focus on other prognostic factors such as survival and treatment response. Systematic identification of the role of inflammatory mediators in each stage of metastasis is missing, which in turn, reduces the potential of clinical use of these factors as predictors of disease progression. Numerous experimental studies reported varying, and even conflicting, findings on the role of inflammatory mediators in metastatic development due to different tumour sites as well as different experimental conditions. The majority of these studies have not been validated by a clinical follow up, indicating a huge gap between the clinical findings and the experimental data. This systematic review aims to provide an evidence-based critical analysis of the clinical and experimental information. Integrating this vast amount of existing data will facilitate the development of more effective prognostic systems which can add clinical value to the current inflammation-based grading systems.

## 3.5 Methods

### 3.5.1 Objectives

The objectives of this systematic review are: 1) To examine the prognostic value of inflammatory mediator expression in relation to the metastatic spread of the tumour in clinical patient samples; 2) To assess the association of the expression/inhibition of the inflammatory mediator with promoting/inhibiting specific metastatic stages in experimental animal studies; 3) To outline the gap in the state of knowledge between clinical and experimental data; and 4) To establish a preliminary inflammatory mediator panel that can be used for accurate and reliable prediction of the risk of metastasis in cancer patients.

### 3.5.2 Search strategy

To achieve the objectives of the review, the search was designed to include two main groups. The first group reports the association of the expression of inflammatory mediators with the metastatic progression of OSCC tumours in clinical cases. The second group includes studies performed on animal models reporting the effect of over-expression/inhibition of an inflammatory mediator on tumour metastasis or one of the specific metastatic stages described above.

### 3.5.3 Literature search

The search strategy was developed according to guidelines outlined in the clinical practice guidelines devised by the Australian National Health and Medical Research Council (NHMRC). A systematic search was carried out on the PubMed database for articles published from January1980 to December 2017. To maximise the identification of relevant results, the initial search used truncated forms of the words “Inflammation”, “Cancer” and “Metastasis”. The search was further refined by crossing with the terms “prognosis”, “tumour progression” and “mediators”. Searches with the inflammatory mediators terms included “tumor necrosis factor-alpha”, “neuropeptide”, “chemokine”, “inflammatory cytokines”, “inflammatory mediators”, “kinins”, “substance P”, and “interleukin”. Terms were searched alone and then crossed with “expression” and the following terms: “tumor progression”, “metastatic cascade”, “metastatic stages”, “tumor invasion”, “epithelial-mesenchymal transition”, “tumor angiogenesis”, “tumor extravasation” “metastasis formation at secondary” (for full description of search strategy and MeSH headings, see Appendix2). The reference list of related reviews and potentially relevant articles was checked for further studies. Citation lists of relevant articles were screened manually to ensure sensitivity of the search strategy. Repeated articles showed on the citation manager were deleted. Relevant journals were identified and their index was searched manually to identify further important citations.

### 3.5.4 Selection criteria

The titles and abstracts of all articles identified through the electronic and hand searches were scanned independently and grouped into the clinical or the experimental group based on the search strategy. The scanned studies were passed through general eligibility criteria before being placed into their nominated groups. The general criteria for inclusion of primary studies in the clinical or experimental group were: 1) original paper; 2) abstract available; 3) clear reporting of association of inflammatory mediator expression with metastasis or role in specific metastatic stage; 4) sufficient key information as indicated by the score list; and 5) sufficient statistical data. General criteria for exclusion were: 1) duplicate; 2) non-original paper; 3) abstract unavailable; 4) missing key information or statistical data; 5) metastasis was not reported; and 6) haematological cancer. Articles in every group passed through specific inclusion/exclusion criteria as follows;

***Clinical search***: The selection was limited to human studies and included all longitudinal studies that presented data for progressing and non-progressing metastatic lesions. Case-control and cohort studies were included. Potential randomised controlled trials and cross-sectional studies were included if found.

Metastatic progression was defined as having a distant disseminated tumour, either nodal or distant, as a consequence of the tumour under investigation versus tumour with no spread followed over time. The inclusion criteria were as follows: 1) a clinical study on human subjects either from biopsy, surgical specimen or bodily fluid (saliva, blood) samples, where clear diagnosis and histo-pathological assessment of the case and control group had been performed; 2) the inclusion of a control group for comparison; 3) correlation of the investigated inflammatory mediator with the metastatic spread of the tumour is reported; 4) measurement of inflammatory mediator protein, mRNA or genetic expression; 5) the cancer was not associated with another pre-existing condition; 6) TNM was the standard staging system used for tumour staging in the patients; and 7) the patient at the time of the biopsy or sample had not undergone any chemo or radio therapy. Follow-up period and sample size were not a critical factor in inclusion the study.

Exclusion criteria for primary studies were: 1) duplicate studies; 2) non-human subject; 3) no control group; 4) case report studies; 5) review articles were excluded from the primary search but categorised for extraction of relevant articles; 6) no follow-up was reported; 7) other prognostic outcome (not metastatic progression) was reported; and 8) chemo/radiotherapy treatment of the control or the case subjects before the intervention (surgical resection was not an exclusion criteria).

***Experimental search***: The main criteria for including studies were: 1) experimental animal studies reporting the expression/inhibition of specific inflammatory mediators and their effects on a specific metastatic stage; 2) evaluating the potential association of the measurement of inflammatory mediators with the different stages of disease progression; 3) specific inhibition was carried out through the use of antibodies, gene knockdown, miRNA, or ablation experiments; and 4) NSAID was included in studies where it was used to inhibit the action of a specific inflammatory mediator.

Exclusion criteria were: 1) no control group; 2) the inflammatory mediator was expressed as a downstream to another molecule; and 3) only the expression was reported with no association with disease progression.

### 3.5.5 Data extraction

Once finalised, eligible articles were obtained and the data were extracted using a specific reporting form for each of the (clinical or experimental) groups. Data extraction forms for the clinical research recorded the following information; study title, score, inflammatory mediator, number of patients, number of controls, lymph node or distant metastasis, correlation with staging, correlation with specific metastatic stage, correlation with other prognostic factors, *in-vivo* validation and remarks. In the experimental search, the form contained the following information; article title, score, inflammatory mediator, type of animal, method of measurement, downstream pathway, cell of origin, inhibition method and remarks. The forms were prepared using Microsoft *Access* software to facilitate report preparation.

### 3.5.6 Critical appraisal of studies

The REMARK Quality Guideline Scale was used to assess the quality of the studies for the clinical search (364). The scale was modified to have a score of 16 for the included studies (Appendix 3). Animal research: Reporting *In-vivo* experiments (ARRIVE) guidelines were used to assess the quality of the animal studies (365). The original 20-item-checklist guideline was modified to meet the specific aims and objectives of the current review (Index 2). The modified 19-item list scored each item on a scale of [0-1] or [0-1-2].

## 3.6 Results

Initially, 5,278,203 articles were found through the general search with the truncated form of “Inflammation”, “Cancer”, and “Metastasis”. The search with the specific terms yielded a total of 277,974 papers. By scanning through the titles of those articles, 35,111 studies were identified as relevant for the review, 19,564 articles were excluded as being irrelevant, 1,345 systematic reviews and 116 meta-analysis studies were excluded but were further scanned for any potential articles, 14,086 research articles were assessed through the abstract, of which

3,492 and 3,712 articles were excluded for exclusion criteria and duplication, respectively. The remaining articles were divided into 3,103 clinical studies and 3,379 experimental studies.

I: Clinical study selection and characteristics: The flow diagram of the retrieved articles for the clinical search is shown in Fig. 3.1. In total, 78 articles were obtained for the final selection, 50 articles from the clinical search, and 28 from the experimental search. By examining the full text of the articles, 62 articles were excluded due to: full article not available; *in-vivo* study (moved to the experimental group); only the expression of the inflammatory mediator with no association to metastasis; not enough data (as per the scoring guideline); not statistically significant; outcome other than metastasis was reported; or the cases have a pre-operative chemo/radio treatment. Sixteen articles were eligible for the review and were scored accordingly (Fig. 3.3). The review identified nine inflammatory mediators associated with metastasis. Fifteen articles reported lymph-node metastasis and one article reported both lymph node metastasis and distant metastasis. Four articles were clearly associated with stages of metastatic cascade and five studies were validated with *in-vivo* studies. The inflammatory mediators identified were CXCR4 (six studies), CXCL12 (SDF-1), CCR7, IL-6 (two studies each), IL-18, CCL20 (MIP-3), CXCL1 (GRO-1), CCL3, CXCR2 (one study each).

II: Experimental studies selection and characteristics: The flow diagram of the retrieved articles for the experimental search is shown in Fig. 3.2. Fifteen articles were identified for review. Eleven articles were excluded due to: *in-vitro* experiments; non-specific inhibition; or other outcomes than metastasis. Four articles were included in the final review and have been scored (Table 3.1). Three inflammatory mediators have been identified as follows: CXCR4, CXCL12 and CXCR7. Three articles reported lymph node metastasis and one reported both LN and DM, with all experiments conducted in BALB Nude mice. Finally, two articles detailed the downstream pathway of the inflammatory mediator, and all the articles addressed specific metastatic stage (adhesion and extravasation).

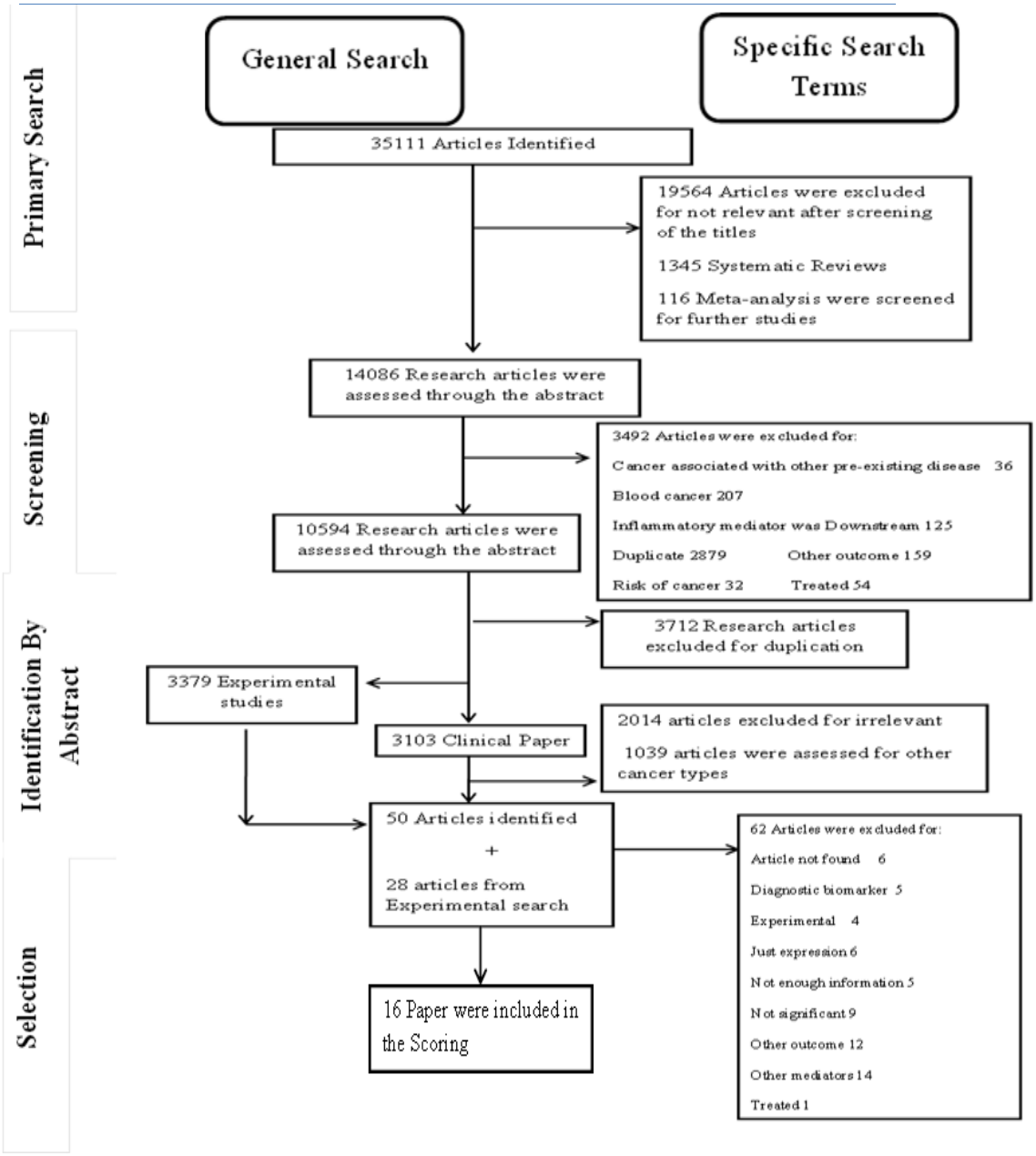



**Figure 3.1:** Flow diagram of clinical studies search and selection.

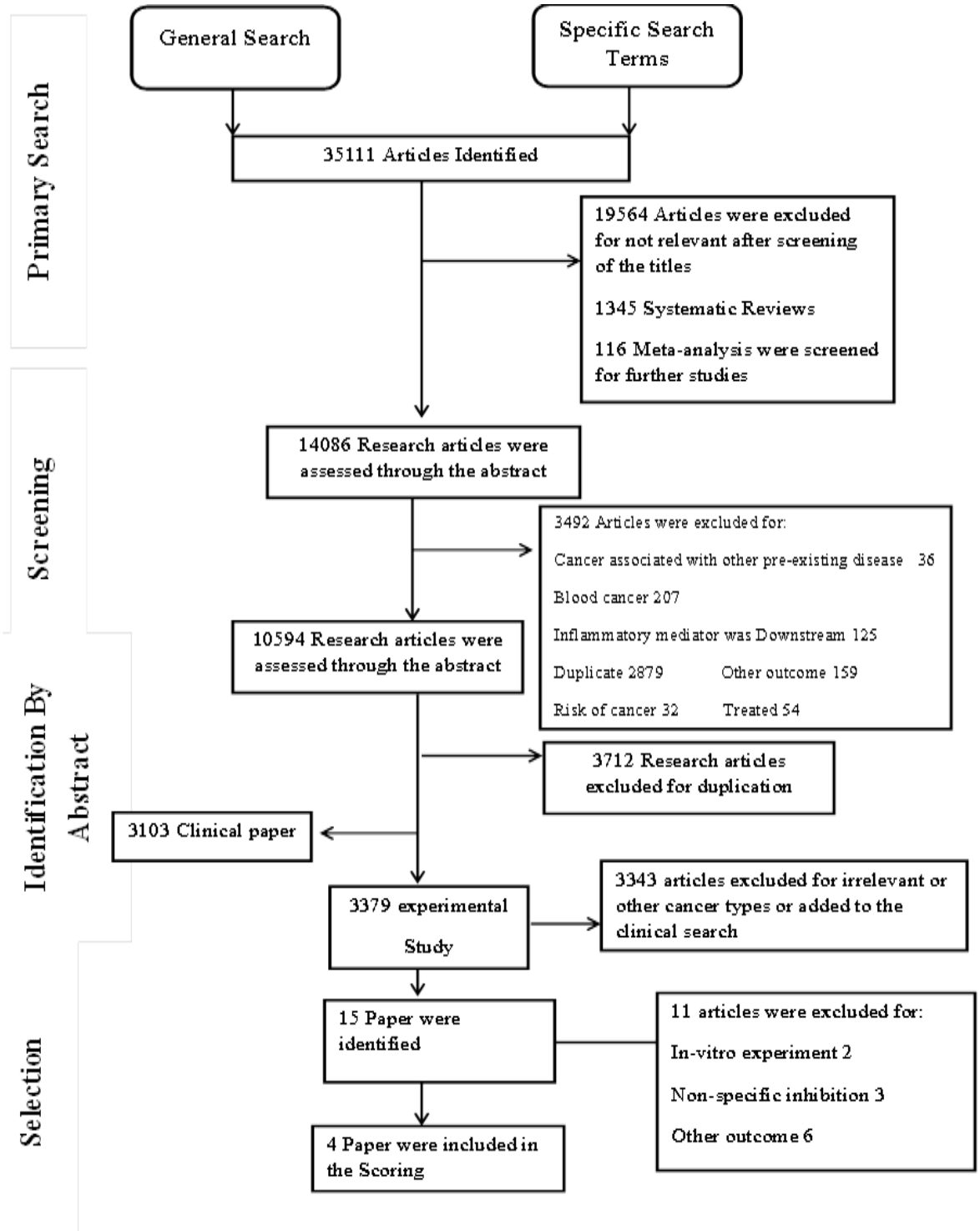



**Figure 3.2:** Flow diagram of experimental studies search and selection.

## 3.7 Discussion

Metastasis remains the major cause of fatality in cancer. Despite extensive research, little is known about the precise mechanism of this process (146). The link between metastasis and inflammation has been firmly established, however, an understanding of this interaction for clinical use remains obscured (366).

This review systematically summarises the evidence of the prognostic role of inflammatory mediators in predicting metastasis/metastatic stages in OSCC. Additionally, it compares the available evidence from clinical and experimental animal settings. The clinical search of the review identified nine inflammatory mediators associated with lymph node metastasis and one associated with distant metastasis. The experimental search identified three mediators, all of which were included in the clinical search. These inflammatory mediators are discussed below.

**CXCL12/CXCR4** axis is composed of CXCR4 polypeptide receptor that binds to its conical ligand CXCL12 (SDF-1) and is responsible for a variety of physiological processes e.g. lymphocyte homing, development, and vascularisation (367). It has been shown to be highly expressed by numerous types of tumours with a significant correlation with metastasis (367). Several mechanisms have been hypothesised to explain its role in metastasis. First, CXCR4 acts as a chemotactic factor which is expressed by tumour cells migrating towards an increasing CXCL12 gradient in the lymph nodes (368). Further evidence for this role is provided by Olivira et al. (369) who showed that non-metastatic lip SCC samples did not express either CXCL12 or CXCR4. Uchida et al., (373) – the only study that was validated with animal model – showed that CXCR4 has a similar expression both in tumour tissue and metastatic lymph node, whereas SDF-1 is higher in metastatic lymph nodes. In the animal models, they obtained similar results with heavier staining scores and more numbers of lymph node and lung metastases showed autocrine signalling, however, there was no comparison with a non-metastatic group.

Second, the activation of CXCR4 due to HIF production, that is induced by the limited supply of oxygen to the neoplastic cells, initiates a cascade of downstream pathways leading

to more invasive phenotypes (370). Third, Albert and Lee et al. (371, 372) suggested that CXCR4 is responsible for the induction of

| Author/Year | Score | IM | Type of Study | Method of Measure | No.Pt/No. of control | Corr. With LNM | Corr. With DM | Staging | Specific Metastatic stage | Other prognostic factors | In vivo validation | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Xia[40] 2015 | 13 | CCR7 | Retrospective | IHC | 60/20 | Yes | No | Yes | Lymphatic Density-VEGF | OS | Yes | |
| Qian[49] 2014 | 7 | CXCR2 | Retrospective | IHC | 36/49 | Yes | No | NA | NA | NA | Yes | |
| Tsai [46]2013 | 12 | IL-18 PM | retrospective | Real time PCR with Taqman | 567/559 | Yes | No | Yes | NA | NA | NA | inverse relation |
| Albert[33] 2012 | 13 | CXCR4 | Retrospective | IHC | 24/23 | Yes | No | Yes | EMT | OS | NA | |
| Chang[50] 2011 | 12 | MIP-3 (CCL20) | retrospective | IHC/PCR | 37/65 | Yes | No | NA | NA | DSS | NA | |
| Shimrki[44] 2011 | 9 | IL-6 | retrospective | IHC/PCR | 24/33 | Yes | No | NA | Lymphogenesis | NA | Yes | |
| Meng[32] 2010 | 9 | CXCR4 | Retrospective | IHC | 46/45 | Yes | No | Yes | NA | NA | NA | |
| Shang[41] 2009 | 11 | CCR7 | Retrospective | IHC | 39/46 | Yes | No | Yes | NA | NA | NA | |
| Lee[34] 2009 | 12 | CXCR4 | Retrospective | IHC | 26/48 | Yes | No | NA | Invasion-MMP | OS | NA | |
| Oliveira-Neto[31] 2008 | 9 | CXCR4 | NA | IHC/ PCR | 21/20 | Yes | No | NA | NA | NA | NA | |
| DE Oliveira-Neto[48] 2007 | 8 | CCL3 | NA | IHC | 10/10 | Yes | No | NA | NA | NA | NA | compares lymph node metastatic and non metastatic |
| Uchida[36] 2007 | 11 | CXCL12 | Retrospective | IHC | 30/30 | Yes | No | NA | NA | OS | Yes | |
| ISHIKAWA[30] 2006 | 8 | CXCR4 | NA | IHC | 39/51 | Yes | No | Yes | NA | NA | NA | |
| Shimtani[47] 2004 | 7 | GRO(CXCL1) | retrospective | IHC | 41/53 | Yes | No | NA | Angiogenesis | NA | NA | |
| Uchida[35] 2003 | 8 | CXCR4 | NA | IHC | 9/9 | Yes | No | NA | NA | NA | Yes | |
| Wang[45] 2002 | 13 | IL-6 | retrospective | IHC/ISH | 43/43 | Yes | Yes | NA | NA | OS | NA | inverse relation |

**Figure 3.3:** Articles included in the clinical search.

EMT phenotype as well as MMP2/9 expression. Fourth, Ucida et al. (373-375) suggested the CXCR4/SDF-1 axis activates the ERK/AKT/SRC family of kinases. However, the included articles (*n*=7 for clinical, *n*= 3 experimental) for CXCR4 and CXCL12 did not report the role

of these chemokines as a cancer stem cell niche (which has been reported in other studies and a role for which has been suggested in the invasive ability of tumours harbouring those mediators) and their role in metastasis. Only one experimental study reported the occurrence of distant metastasis in the animals. These results suggest a strong role for CXCR4/CXCL12 for prognosis and predicting lymph node metastasis in OSCC.

A hypothesis can be constructed that CXCR4 is up-regulated in tumour cells due to a hypoxic environment. This activates downstream pathways which endow tumours with EMT, invasive phenotype and mitogenic signalling. These cell populations mark a new stem cell-like niche with growth and migratory advantages, and therefore will be selected to migrate towards gradient of CXCL12 displayed by lymph nodes.

**Table 3.1:** Articles included in the Experimental Search.

| Auth. Year | Score | I.M | Type of Animal | Dowstream | Stage | Cell of Origin | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| Xia [366] 2015 | 12 | CCR7 | BALB mouse | NA | NA | Tumour Cell | Knock-down | T. met to LN (IM act on adhesion) |
| Uchida [377] 2007 | 11 | CXCL12 | BALB mouse | NA | NA | Tumour cells | Over-expression | LN and DM met (adhesion and extravasation) |
| Uchida [380] 2003 | 8 | CXCR4 | BALB mouse | Yes | NA | Tumour cells | NA | LN (adhesion extravasation and downstream pathway) |
| Uchida [375] 2004 | 13 | CXCR4 | BALB mouse | Yes | NA | Tumour cells | Over-expression | LN (adhesion and extravasation and pathway) |

* **Abbreviations:** DM: distant metastasis, IM: inflammatory mediator, LN: lymph node metastasis.

**CCR7** is a trans-membrane receptor that exerts its action by binding to its ligands CCL21/19 and it is expressed by many immune cells for homing and directional movement of these cells (376). CCR7 has been reported to be associated with lymph-node metastasis in several tumours (377). Similarly, Xia and Shang et al. described a significant association of this receptor with LNM with no distant metastasis (378, 379). Moreover, Adenoid cystocarcinoma, which commonly migrates through a hematogenous pathway, did not migrate to a gradient concentration of CCL21. The studies suggested an adhesion mechanism between CCR7 expressing tumour cells and lymph node cells, explaining the LNM tendency.

**IL-6** cytokine can exert either an inflammatory response through autocrine signalling to its IL-6R receptor or an anti-inflammatory effect through a paracrine signal via GP130 protein (380). Recent studies showed that IL-6 can activate several downstream pathways and it is correlated with several types of cancer (381). Due to the complex network activated by IL-6, it is difficult to pinpoint its role in metastatic process (381), however, Shinriki et al. showed a significant correlation with IL-6 and LNM as well as a correlation with VEGF-C and lymphogenesis suggesting a direct effect for IL-6 in the process of metastasis (382). Wang et al., was the only study to report an association of an inflammatory mediator with distant metastasis, though they did not report specific organ preference (383). More interestingly, there is a negative correlation between IL-6 mRNA and the presence of either LNM or DM. These conflicting results need further validation to pinpoint the actual role of IL-6.

**IL-18** is an IL-1 superfamily cytokine which is reported in the only study in the review to report genetic polymorphism (384). The results concluded that G/C heterozygous is a factor in increasing OSCC susceptibility though a protective factor against metastasis.

**CXCL1 (GRO-1)** Shintani et al. (385) found that GRO-1 is significantly correlated with LNM, MVD and leukocyte infiltration, which suggests a role for GRO-1 in attracting immune cells to the tumour for deploying in new vessel formation.

**CCL20 (MIP-3)** is a small peptide chemokine with a selective, pleiotropic ligand CCR6 (389). It has been found to be expressed in liver, mucosa-associated lymph tissue and other lymphoid tissue with a main function of recruiting CD34+ (389). CCL20 activates the conical

inflammatory pathway NF-kβ and might implement an autocrine manner via activation of ERK1/2-MAPK and PI3K pathways (390). This evidence is supported by high expression of CCL20 both by highly metastatic colorectal and lung tumours (389, 390). Chang et al. (388) showed that MIP-3α was significantly overexpressed by positive lymph node patients (*P=0.036*). This expression was demonstrated to be expressed by the tumour cells versus the normal epithelium. They applied *in-vitro* interfering RNA to CCL20 which inhibited the cancer cell migration and proliferation.

**CCL3** Macrophage inflammatory protein-1α is a member of the CC inflammatory chemokines. They are able to induce chemotactic mobilisation of monocyte-lineage cells and lymphocytes into inflammatory tissues as well as the proliferation of haematopoietic stem/progenitor cells (391). Only one study reported the expression of the inflammatory mediator CCL3 in metastatic lymph nodes and non-metastatic lymph nodes (386). They found that CCL3 was significantly higher in metastatic lymph nodes compared with non-metastatic lymph nodes of the same patients (*P=0.013*). In contrast, another study showed CCL3-secreting tumours resulted in similar increases in CD4+ and CD8+ T cells, resulting in immune activation against a murine colon tumour model CT26 (392). Those results might indicate a different role for CCL3 in neoplastic progression, depending on the stage of the neoplasm as well as the specific tissue microenvironment, which warrants further validation.

**CXCR2** is chemokine receptor that binds with high affinity with CXC chemokine ligands which contains the ELR positive motif (chemokines responsible for neutrophil chemo-attractant) (393). CXCR2 has been postulated to play a vital role in the progression of various cancers presumably by enhancing angiongenesis, chemoresistant as well as chemo-attractant characteristics (394). This is evidenced through the inhibition of CXCR2 either by knockdown, deletion, or suppressing its downstream resulted in suppression of metastasis and enhancement of chemotherapy efficacy (395). Qian et al. found a significant expression of CXCR2 in OSCC patients with cervical metastases. They attributed the role of CXCR2 in tumour progression and OSCC LN metastasis was mainly attributed to the ability to invade as well as modifying cytoskeleton structure (387).

It is well established from several studies that inflammatory processes in cancer can activate signalling pathways, which lead to the remodelling of the tumour microenvironment (396). This remodelling is accompanied by a profile of inflammatory mediators (e.g. cytokines or chemokines) that can be either a driver or an inhibitor of tumour progression. Hence the stage of the tumour (progressing or dormant) and the profile of the existing inflammatory mediators prognosticate the tumour progression and metastasis. Our review has identified two cytokines, three chemokine receptors and four chemokines. This profile was associated with cervical metastasis and three of those mediators were further validated through the experimental animal models.

It is not surprising that the majority of the panel is chemokines and their receptors with their well-known function as chemo-attractant for cell trafficking, homing and upregulating endothelial cell adhesion moelcules (397). CXCL12, CXCR4 and CCR7 are known to be of the basal (homeostatic) chemokines/receptors produced by different lymphoid organs and the receptors displayed by cells supposed to home to those organs. In particular, CCR7 is expressed by antigen presenting cells, whereas its ligand is expressed by lymph node (378). Meanwhile, CXCL12/CXCR4 is the main axis expressed by bone marrow niche for B-cell maturation. CXCR2, CCL3, CXCL1 and CCL20 are inflammatory chemokines that are produced in high quantities in inflammatory lesions to attract all types of leukocytes (386). Recent experimental investigations are revealing more roles for those mediators in early stages of metastasis, such as recruitment of myeloid stem cells for endothelial cell proliferation (e.g. CXCL12) (398). Others are suggested to be the regulators of the stem-cell nature of those cells (CCL3) or promote angiogenesis directly. IL-6 has been implicated in EMT program activation and circulating tumour cell seeding which explains its association with distant metastasis (398).

Cancer immunotherapy has achieved some success with some inflammatory mediators being used as an adjuvant to increase efficacy metastatic treatment (399). However, the complexity of the immunomodulators and their interaction within the tumour domain is challenging and will require time to unravel the precise mechanism and role for each tumour. Furthermore, the timing of the treatment for metastasis proves to be a critical factor and it has been shown that preventing metastasis in animal model was more effective than reversing the process

once it has been initiated (400). Therefore, we propose validating this panel with a cohort study for prediction of metastatic progression in OSCC high-risk patients such as recently diagnosed or precancerous patients.

The review supplemented the clinical data with experimental information which proved critical in overcoming the shortcomings of either the clinical or experimental studies. A profound disadvantage of the clinical studies is the difficulty in monitoring specific metastatic stages as well the uncontrolled experimental condition. While the experimental studies do not have such problems, they possess another set of issues such as having an immunocompetent model which is technically challenging and time-consuming. On the other hand, implementing an immunodeficient model represents a highly biased design. This highlights the need for new experimental models which are more robust, representative and experimentally reliable. The advent of new molecular biology tools and imaging will facilitate such models (e.g. microfluidic metastatic models).

A summary of the findings of the review includes: 1) there was an overwhelming amount of information about the involvement of inflammatory mediators in OSCC but this information is not in a systemic order to allow for better understanding of their specific role; 2) most of the studies did not take metastasis into account as a prognostic outcome but considered other prognostic points such as tumour stage or size; 3) there is a clear gap between clinical and experimental information, where most of the clinical studies are not supplemented with enough experimental data for validation and explaining the biological mechanism; 4) the reporting of the association of inflammatory mediators with metastasis lacks precise highlighting of their role in specific metastatic stages either in clinical or experimental research and some critical metastatic stages such as extravasation could not be detected at all in any clinical studies, reflecting the lack of proper experimental tools; and 5) it is more likely that a panel of mediators, rather than a single one, orchestrates the metastatic cascade.

Research in such areas is very cumbersome as most of the above-mentioned inflammatory mediators have redundant pathways and therefore underpinning their main role is a very challenging task. Moreover, most of these mediators have vital physiologic functions and

thus interfering with them may result in a catastrophic adverse effect to the cancer patients, which may partly explain the slow pace of research in this area.

Some limitation does exist with this review with the small number of studies. Additionally, some of the potential articles were difficult to obtain for the final analysis. However, the review represents a starting point for a new direction in researching the role of inflammatory mediators in the prediction and prevention of OSCC.

## 3.8 Conclusion

Metastasis is a fatal consequence of cancer and so a new paradigm needs to be followed in research. Evolutionary insights and experimental evidence are revealing an intricate relationship between cancer and its microenvironment with inherent weaknesses and strengths on the host and tumour sides. The review revealed a panel of inflammatory mediators, each responsible for a pathway/stage in metastasis. Despite requiring further validation, this panel can be implemented for the introduction of a new "Metastasis early monitoring and prediction" tool similar to cancer early-prevention tutorials. The review provides a panel of inflammatory mediators that can be used, after proper validation, in clinical settings in high-risk groups of patients and *in-vivo* to elucidate the specific correlation of these mediators with specific stages and their mechanisms. However, such validation will require a new and reliable experimental method that can avoid the high technicality of animal models, with the ability to detect precisely every stage of metastasis in a way that mimics the *in-vivo* environment.

# Chapter 4 Study hypothesis

## 4.1 Concluding remarks from the literature and systematic review

The previous two chapters discussed the overall picture of metastasis and its underlying factors and mechanisms. The findings from the two chapters can be summarised in a few points. Metastasis is the main cause of death in cancer patients and even with aggressive treatment in place, there is only a slight improvement in survival and patient outcome. Models of metastasis on an evolutionary basis fail to improve this outcome mainly due to a lack of understanding of the key players in the process. The systematic review pinpoints a panel of inflammatory mediators that are shown to be active players in the clinical and experimental studies. The literature review highlights these findings by revealing the accumulating evidence to understand metastatic mechanisms. Extravasation remains a rate-limiting step in metastasis, which requires inflammatory mediator, adhesion molecules, and proteolytic enzymes (e.g. MMP). The review also shows the inevitable role of inflammation as the main driver of metastasis and this is slowly being underpinned. Putting these pieces together, metastasis bears a striking similarity with inflammatory processes, in particular the extravasation step. Both processes need adhesion molecule expression, adhesion of the migrating cell to endothelium, and proteolytic enzymes to cross blood vessels. Moreover, inflammatory mediators can drive all these steps in both processes. The logical consequence here is to find the common mediators in both metastasis and inflammation, and by blocking them, we can interfere with the spread of tumours. That is what the systematic review shows. However, a clinical problem appears, in that the extracted inflammatory mediators are critical to physiological body functions, hence blocking them will cause more severe side effects than the treatment. Therefore, these mediators can be implemented for prognostic purposes but not for clinical intervention. With that in mind, the review sets a criterion for an effective intervention inflammatory mediator. It can be defined as a substance that mediates inflammatory adhesion, expressed in tumours, and, most importantly, its role is mainly pathologic. Therefore, antagonists to this mediator can prevent metastasis while not causing much harm to normal body functions.

## 4.2 Rationale of the study

Metastasis is the prime cause of mortality in 90% of cancer patients, particularly OSCC. The incidence of metastasis significantly reduces the prognostic outcome. More than half of neoplasms will have disseminated lesions at the time of primary diagnosis, which render

these cases with very poor survival rates. The high risk of cancer metastasis mandates aggressive therapeutic modalities in an attempt to prevent their occurrence. However, even with the most advanced treatments, little or no improvements have been achieved in the outcome of the most advanced-stage cancers. The complications of these treatments outweigh their benefits resulting in severe disfigurement, poor psychological wellbeing, poor quality of life, toxicity and even death to the patients. Recurrence is the end-result in most of these cases (even with multi-modality treatment) signalling the failure of those treatments.

Oral cancer is a highly invasive disease, which is often diagnosed in an advanced stage with a relatively poor prognosis. Furthermore, the burden of metastasis is evident even in early stages (despite high survival figures), in which the occurrence of occult metastasis significantly affects this outcome in terms of survival or recurrence. The current methods of detecting metastasis, whether the invasive lymph node biopsy or the traditional histologic assessment, fails to detect early metastatic lesions. The inefficacy in exterminating metastasis stems from the lag in understanding the complex network of mediators controlling the underlying steps. This is reflected in clinical practice with an absence of reliable diagnostic or prognostic markers that can be used to accurately predict the presence or absence of micro metastases or an effective treatment for a total elimination of metastasis. While the oncology researcher's main target is the early detection of primary tumours, longer survival periods are seen with more chances of metastasis and recurrence for those patients. In order to reduce the high mortality and morbidity rate of cancer patients, a better understanding is needed of molecular modulators, leading to metastatic seeding of the tumour cells and their homing in distant sites or lymph nodes.

Tumour cell extravasation is an important, rate-limiting step in the process of metastatic spread. This study investigates the mechanisms of extravasation, and examines the action of one potential mediator of this process, SP. The effects of antagonising the actions of SP, through treatment with a selective NK1 receptor antagonist, are examined at Jurkat leukaemia cells as well as different stages of oral cancer and pre-cancer cell cultures, and the effect of this treatment on the adhesion of each cell line to endothelial cells is monitored. This will add to the current state of knowledge about the unknown "rate-limiting" extravasation stage as

well as to an understanding of the role of inflammatory mediators and adhesion molecules in oral cancer metastasis. The study at this cellular level has the advantage of examining the relevant factors, thus, providing the raw data that can be integrated into a complex model that can be applied to *in-vivo* trials. The resulting model represents a novel approach in cancer treatment where the main target is prevention of metastasis incidence. This paradigm shift has a powerful potential to develop effective anti-metastatic therapies through interfering with the metastatic cycle.

### 4.2 Study hypothesis

SP increases the expression of adhesion molecules of leukaemia, oral squamous cell carcinoma cells and vascular endothelial cells so it facilitates the adhesion of invading tumour cells to the endothelial lining of the blood vessels which in turn enhances the metastatic potential of the tumour.

**STUDY QUESTION**

What is the effect of Substance P on the adhesion of leuakaemia and oral squamous cell carcinoma cells to vascular endothelial cells?

### 4.3 Aims of the study

The study's overall objective is to examine the role of SP and the adhesion molecules in the adhesion of oral cancer cells to endothelial cells and the subsequent effect on metastasis of the tumour. To obtain the information that can establish the model to answer the main study question, the study has six main aims.

Aim 1. To examine the adhesion of Jurkat cell lines to endothelial cells and to determine the effect of SP on this adhesion.

Aim 2. To examine the expression of adhesion molecules in Jurkat cell lines and to determine the effect of SP on this process.

Aim 3. To examine the effect of the SP on the release of MMP-2 in Jurkat leukaemia cell lines.

Aim 4. To examine the adhesion of different oral cell lines to endothelial cells and to determine the effect of SP on this expression.

Aim 5. To examine the expression of adhesion molecules in different oral cell lines and to determine the effect of SP on this process.

Aim 6. To examine the effect of SP on the release of MMP-2 in oral cell lines.

**4.3.1 Study:** To examine the effect of SP on the adhesion of Jurkat cell lines to the endothelial cells lines HUVEC.

**Study Question**

What is the optimal dose of Substance P required to produce early-response significant change in the adhesion of Jurkat cell and endothelial cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the adhesion basal level of Jurkat cell lines to endothelial cells?

b: Do different SP doses cause a change in the level of adhesion?

c: Is this change of adhesion level between Jurkat cell lines and endothelial cells significant?

**Hypothesis**

Substance P treatment of Jurkat cancer cell lines does change the level of its adhesion to endothelial cells.

Null hypothesis: Substance P treatment of Jurkat cancer cell lines does not change the level of its adhesion to endothelial cells.

**Aim**

1: Determine the basal level of adhesion of Jurkat cancer cell lines to the HUVEC cell lines.

2: Determine the effect of SP treatment on this basal level of adhesion.

**Materials and Method:**

1: Endothelial Cell Adhesion Assay Kit (Cat. No. ECM645, Millipore) are used as per manufacturer's protocol to measure the basal adhesion level of Jurkat cell lines to HUVEC endothelial cells.

2: This is repeated following the treatment of these cells with different doses of substance P.

3: The experiments are divided into two groups. The first group will have only the cancer cells treated according to the protocol. The second group will have only the endothelial cells treated according to the protocol.

4: Results are measured using a microplate reader and confirmed with the fluorescent microscope.

5: Determine whether there is a significant difference between the results before and after treatment using ANOVA with *P* value=0.05 considered statistically significant.

6: Optimise the assay procedures and controls.

**4.3.2 Study:** To examine the expression of cell adhesion molecules in Jurkat cell lines and to determine the effect of SP on this adhesion

**Study Question**

What is the optimal dose of Substance P required to produce an early-response significant change in the adhesion molecules of Jurkat cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the basal expression level of Jurkat cell lines?

b: Do the different SP doses cause a change in the level of expression of adhesion molecules?

c: Do the timings of SP treatment affect the level of adhesion molecule expression?

d: Is there a change in the expression of adhesion molecules before and after treatment? Is it significant?

**Hypothesis**

Substance P treatment of Jurkat cell lines does change the expression of the adhesion molecules.

Null Hypothesis: Substance P treatment of Jurkat cell lines does not change the expression of cell adhesion molecules.

**Aim**

1: Determine the basal level of expression of cell adhesion molecule (VLA-4, LFA, SLeX) in Jurkat E-6 cell line.

2: Determine the effect of SP treatment on this basal level of expression.

**Materials and Method:**

1: Use flow cytometry to measure the expression of these adhesion molecules in the Jurkat cell line before and after the treatment of these cells with different doses of SP.

2: Mean fluorescence intensity for each replicate with reference supplied by the antibody manufacturer which will be used for statistical calculation before and after treatment.

3: Determine whether there is a significant difference between the results before and after treatment using ANOVA with *P* value=0.05 with CI= 95% considered statistically significant.

**4.3.3 Study:** To examine the effect of SP on the release of MMP-2 in Jurkat cell lines.

**Study Question**

What is the optimal dose of Substance P required to produce an early-response significant change in the expression of MMP-2 in Jurkat cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the MMP-2 basal expression level of Jurkat cell lines?

b: Do different SP doses cause a change in the level of expression of MMP-2?

c: Do the timings of SP treatment affect the level of MMP expression?

d: Is there a change in the expression of MMP-2 before and after treatment? Is it significant?

**Hypothesis**

Substance P treatment of Jurkat cancer cell lines does change the expression of MMP-2.

Null hypothesis: Substance P treatment of Jurkat cancer cell lines does not change the expression of MMP-2.

**Aim**

1: Determine the basal level of expression of MMP-2 in Jurkat cell lines.

2: Determine the effect of SP treatment on this basal level of expression.

**Materials and Method:**

1: Total MMP-2 ELISA (Enzyme linked immunosorbent assay), to measure the basal expression of MMP-2 in Jurkat cell lines.

2: Using ELISA, to measure the expression of MMP-2 in these cell lines after the treatment of these cells with different doses of Substance P.

4: Determine whether there is a significant difference between the results before and after treatment using ANOVA with *P* value=0.05 considered statistically significant.

**4.3.4 Study:** To examine the effect of SP on the adhesion of different oral cell lines and endothelial cells.

**Study Question**

What is the optimal dose of Substance P required to produce significant change in the adhesion of OSCC and endothelial cell lines cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the basal adhesion level of each cancer cell line to endothelial cells?

b: Do different SP doses cause a change in the level of adhesion?

c: Is this change of adhesion level between cancer cell lines and endothelial cells significant?

**Hypothesis**

Substance P treatment of oral cancer cell lines does change the level of its adhesion to endothelial cells.

Null hypothesis: Substance P treatment of oral cancer cell lines does not change the level of its adhesion to endothelial cells.

**Aim**

1: Determine the basal level of adhesion of each (CAL 27, SCC 25, H157) oral cancer cell line, and BICR22 oral metastatic cell line to the HUVEC cell lines.

2: Determine the effect of SP treatment on this basal level of adhesion.

**Materials and Method:**

1: Endothelial Cell Adhesion Assay Kit (Cat. No. ECM645, Millipore) is used as per manufacturer's protocol to measure the adhesion level of CAL 27, SCC 25, H157 oral cancer cell lines, and BICR 22 oral metastatic cell line to HUVEC endothelial cells.

2: The same assay is performed following the treatment of these cells with different doses of Substance P.

3: The experiments are divided into two groups. The first group has only the cancer cells treated according to the protocol. The second group has only the endothelial cells treated according to the protocol.

4: Results are measured using a microplate reader and confirmed with the fluorescent microscope.

5: Determine whether there is a significant difference between the results before and after treatment using ANOVA with *P* value=0.05 considered statistically significant.

6: Optimise the assay procedures and controls.

**4.3.5 Study:** To examine the expression of cell adhesion molecules in different stages of oral cancer cell lines and to determine the effect of SP on this adhesion

**Study Question**

What is the optimal dose of Substance P required to produce an early-response significant change in the adhesion molecules of oral cancer cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the basal expression level of each oral cell line? Is there any difference between the expression of the adhesion molecules in normal and malignant cell lines?

b: Do the different SP doses cause a change in the level of expression of adhesion molecules?

c: Do the timings of SP treatments affect the level of adhesion molecule expression?

d: Is there a change in the expression of adhesion molecules before and after treatment? Is it significant?

**Hypothesis**

Substance P treatment of oral cell lines does change the expression of the adhesion molecules.

Null Hypothesis: Substance P treatment of oral cell lines does not change the expression of cell adhesion molecules.

**Aim**

1: Determine the basal level of expression of cell adhesion molecules (VLA-4, LFA, SLeX) in normal oral keratinocytes, DOK, (CAL 27, SCC 25, H157) oral cancer cell lines, and BICR22 oral metastatic cell lines.

2: Determine the effect of SP treatment on this basal level of expression.

**Materials and Method:**

1: Use flow cytometry to measure the expression of these adhesion molecules in the normal oral keratinocytes, DOK, (CAL 27, SCC 25, H157) oral cancer cell lines, and BICR22 oral metastatic cell lines before and after the treatment of these cells with different doses of SP.

2: Mean fluorescence intensity for each replicate with reference supplied by the antibody manufacturer which will be used for statistical calculation before and after treatment.

3: Determine whether there is a significant difference between the results before and after treatment using ANOVA with *P* value=0.05 with CI= 95% considered statistically significant

**4.3.6 Study:** examine the effect of SP on the release of MMP-2 in oral cell lines.

**Study Question**

What is the optimal dose of Substance P required to produce an early-response significant change in the expression of MMP-2 in OSCC and pre-cancer cell lines?

This main question can be subdivided into the following sub-questions:

a: What is the basal expression level of each cell line?

b-: Do different SP doses cause a change in the level of expression of MMP-2?

c: Do the timings of SP treatments affect the level of MMP-2 expression?

d: Is there a change in the expression of MMP-2 before and after treatment? Is it significant?

**Hypothesis**

Hypothesis: Substance P treatment of oral cancer cell lines does change the expression of MMP-2 molecules.

Null hypothesis: Substance P treatment of oral cancer cell lines does not change the expression of MMP molecules.

**Aim**

1: Determine the basal level of expression of MMP-2 in each of CAL 27, SCC 25, H157 oral cancer cell lines, and BICR22 oral metastatic cell lines.

2: Determine the effect of SP treatment on this basal level of expression.

**Materials and Method:**

1: Total MMP-2 ELISA (Enzyme linked immunosorbent assay), to measure the basal expression of MMP-2 in the (CAL 27, SCC 25, H157) oral cancer cell lines, and BICR 22 oral metastatic cell lines.

2: Use ELISA (section 5.9) to measure the change in expression level of MMP-2 in these cell lines after the treatment of these cells with different doses of Substance P.

4: Determine whether there is a significant difference between the results before and after treatment, using ANOVA with *P* value=.05 considered statistically significant.

## 4.4 Definitions of terminology

Early-response: defined as the response to treatment in time scale from 1- 48 hours.

Level of adhesion/expression of adhesion molecules: the quantitative measurement of these levels as shown by fluorescent methods such as plate reader for adhesion levels and flow cytometry as mean of events for cell population.

Basal level of adhesion: the expression or the level of adhesion in untreated cell lines (also defined as the control adhesion level).

# Chapter 5 Materials and Method

## 5.1 Cell lines

Cell culture technique is a tool that involves removal of cells from human or animal tissues and propagating them to obtain a continuous cell culture that can be utilised for research purposes. The majority of cells used in this thesis are continuous cell lines derived from tumours that are illustrated for each cell culture. Cell culture is one of the major tools used in molecular biology research, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, ageing), the effects of drugs and toxic compounds in the cells, mutagenesis and carcinogenesis. It is also used in drug screening and development, and large-scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. Another advantage with using cell culture is the ability to control experimental conditions and therefore to manipulate one specific experimental variable at one time. A major drawback is that cell culture does not measure the effect of microenvironments on the biological system and other tissue components. Nonetheless, it provides an inexpensive, quantitative tool to assess the initial effects of a drug or treatment before further proceeding with other expensive animal models or clinical trials.

Cells were selected to present the progressive stages of oral cancer as follows: HOK primary oral keratinocyte isolated from human oral mucosa was purchased from Sciencell®. CAL 27 (ATCC CRL- 2095) was obtained from tissue taken prior to treatment from a 56-year-old Caucasian male with a lesion in the middle of the tongue. SCC25 (ATCC CRL- 1628) tongue squamous carcinoma cell line is from a tongue carcinoma. Both were purchased from ATCC® cell lines and they are from negative lymph-node patients. DOK cell line was isolated from a piece of a dorsal tongue from a 57-year-old man with mild to moderate dysplasia. An H-157 cell line from oral squamous-cell carcinoma from a positive lymph node patient and BICR-22 cell line from OSCC lymph node metastases were purchased from Sigma-Aldrich®. The selection of the cells to represent the progressive stages of OSCC was intended as discussed earlier as this choice can monitor every stage within the tumour cycle. For example, the adhesion molecule expression as well as the adhesion of the tumour to the endothelial cells can be measured in every stage of the tumour development so as to identify how early those events are likely to occur in the tumour cycle. HUV-EC-C (ATCC CRL-

1730) human vascular endothelium, Jurkat Clone E6-1(ATCC TIB-152™) human acute cell leukaemia cell line lines were purchased from ATCC®.

## 5.2 Culture Media

Dulbecco modified eagle (DMEM) culture media, fetal bovine serum (FBS), endothelial cell growth supplement (ECG), L-glutamine, hydrocortisone, heparin sodium, and trypsin-EDTA were obtained from Sigma-Aldrich®. Eagles minimum essential medium (EMEM), F-12k medium, ATCC DMEM/F12 medium, vascular basal media, endothelial cell growth kit BBE, I-1640 media, and antibiotics were purchased from ATCC® cell lines. The oral keratinocyte medium was purchased from Sciencell®.

## 5.3 Treatment

Substance P acetate salt hydrate (SP) and [succinyl-ASP6,ME-PHE8] Senktide Substance P analogue were purchased from Sigma-Aldrich®. Tumour necrosis factor (TNF-α) was purchased from Sigma-Aldrich. The selective NK1 antagonist, CH123001 (N-(3,5-bis-trifluoromethyl-benzyl-N-methyl-6-4-methylpiperazin-1,4-(o-tolyl-nicotinamide), was provided by CH Biotech Pty Ltd, Sydney, Australia.

## 5.4 Cell culture

For SCC25 A 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate and supplemented with 400 ng/ml hydrocortisone and 10% FBS was used for culture.

CAL27 was cultured in Dulbecco's Modified Eagle's Medium with FBS to a final concentration of 10%.

DOK cells were cultured in DMEM + 2mM Glutamine + 5µg/ml Hydrocortisone + 10% FBS.

For HOK, OKM consists of 500ml of basal medium, 5ml of oral keratinocyte growth supplement and 5ml of penicillin/streptomycin solution.

Jurkat cells Clone E6.1 was cultured in RPMI 1640 + 2mM Glutamine + 10% FBS.

HUVEC-EC was cultured in F-12K Medium, with 0.1mg/ml heparin; 0.03-0.05mg/ml endothelial cell growth supplement (ECGS) adjusted to a final concentration of 10% FBS.

## 5.5 Culture method

All culture procedures were carried out under sterile conditions within a laminar flow hood, with all equipment sterilised in an autoclave and all solutions were either sterile filtered, autoclaved or obtained sterile. Cultures were incubated in a 37°C incubator with 5% $CO_2$ in air atmosphere. Most of the experimental procedures used cells in passage 3-9.

### 5.5.1 Resuscitation of frozen cells.

Upon receiving frozen cell ampoules, the cap of the ampoule was wiped with 70% alcohol immersed tissue in the laminar flow cabinet. The ampoule was quickly transferred into a water bath at 37C for 1-2 minutes. The ampoule was then wiped with 70% alcohol. The content of the ampoule was pipetted into a pre-warmed appropriate complete media in the culture flask. The cell density was adjusted according to the supplier data sheet. Cells were incubated in incubator at 37C and 5% $CO_2$ level. Media was changed the next day.

### 5.5.2 Subculturing Cells.

Once the cells achieved 70-80% confluence, they were split at a ratio recommended by the manufacturer. The culture medium was discarded and the cells were briefly rinsed with trypsin 0.25% / EDTA 0.2% for the specified time on the data sheet. Cells were observed under the light microscope until they became round in shape and detached. Once the majority of the cells detached from the culture vessel, growth media with trypsin inhibitor was added rapidly to stop the enzymatic actions. The contents of the flask were pipetted to a 15ml tube and centrifuged at 1000RPM for 5 minutes and the cells were resuspended in 5ml of fresh growth media. Cells were counted and then seeded in culture plates for subculturing or used for experimental procedures or aliquot and frozen at -80C. Every cell line has a cell record and log to describe each procedure and the number of passages and special requirements for this cell type. Cells were used in passage 3-9 for the experimental purposes.

**5.6 Flowcytometry Antibodies** Anti-integrin alpha 4 antibody CD49d PE (12-0499), Mouse IgG1 K isotype control (124714), Anti- CD11a antibody FITC (BMS102FI) were purchased from affymetrix eBioscience®. Anti CD15S Mouse anti human Alexa Flour 647 (563526), Alexa Flour 647 Mouse IgM isotype control was purchased from BD$^{R}$.

### 5.7 Adhesion molecule expression by flow cytometry

Flow cytometry measures single cells "flowing" through a detector system. The process begins with the selection of fluorescent-labelled antibodies specific to cell-surface markers used to characterise the cell population of interest. These cell surface markers are usually called cluster of differentiation (CD) markers. In our study, we used CD markers that represented antibodies to the adhesion molecules expressed by the cell.

The sample is processed with enzymatic degradation, centrifugation, and/or filtration to isolate the cells of interest, and the resulting cellular suspension is "stained" with fluorescent antibodies. The single cell suspension is then introduced into the flow cytometer into a cell-free buffer solution, called the sheath fluid, which flows towards a laser aimed at the solution's path (optic part of the flow cytometry). Because the flow of the liquid through the tubing is laminar, or sheet-like, and the diameter of the tubing narrows along its path, the cells are forced to line up single-file as they approach the laser. The fluorescent chemical bound to the antibody, called a fluorophore, is chosen based on the specific wavelength of laser present in each flow cytometer. If cells have the selected marker on the surface, the bound antibody-fluorophore will absorb the laser energy and subsequently release it in the form of a specific wavelength of light as the cells pass through the laser. The emitted light is detected by an optical system that is sensitive to various wavelengths, allowing for information on multiple surface markers to be read simultaneously and collected by an adjoined computer. Specialised software then can graphically represent the distribution of the labelled cell populations in one-, two- or three-dimensional formats. A variety of fluorochromes are available that individually emit light of specific, different wavelengths while absorbing light of the same wavelength. This is the basis for polychromatic flow cytometry, and it allows for a flow cytometry sample labelled with different fluorochrome–antibody complexes to be read simultaneously with one pass through the laser. This is the technique that was implemented in this study to enable the analysis of multiple cell adhesion molecules at once. Flow cytometry offers a powerful analytic tool that can provide precise

information about the expression of specific molecules on the cell. It also provides a high-throughput, quantitative technique that is less time-consuming and has more productivity. Some limitations exist with flow cytometry. Extensive data production makes analysis hard and time-consuming. Also, no information is available about the tissue structure. But for the purpose of this study, these limitations were of no great concern.

In general, cells were harvested after serum starvation with serum-free media for 12 hours before the beginning of treatment and washed as a single-cell suspension in 96-well round-bottomed microtiter plates. Density was adjusted to a concentration of >50,000 cells/ml then the media was discarded and the cells were twice washed and centrifuged in an ice-cold FACS Buffer.

An antibody cocktail was made at a 1:20 concentration with the FACS buffer with 50µL for each sample; 50µL of antibody cocktail was added and the sample was incubated for at least 30 minutes at room temperature or 4°C in the dark. The cells were washed with FACS buffer three times by centrifugation at 1500RPM for 5 minutes and resuspended in 200µl to 1ml of ice-cold FACS buffer. The cells were kept in the dark on the ice or at 4°C in a fridge until analysis.

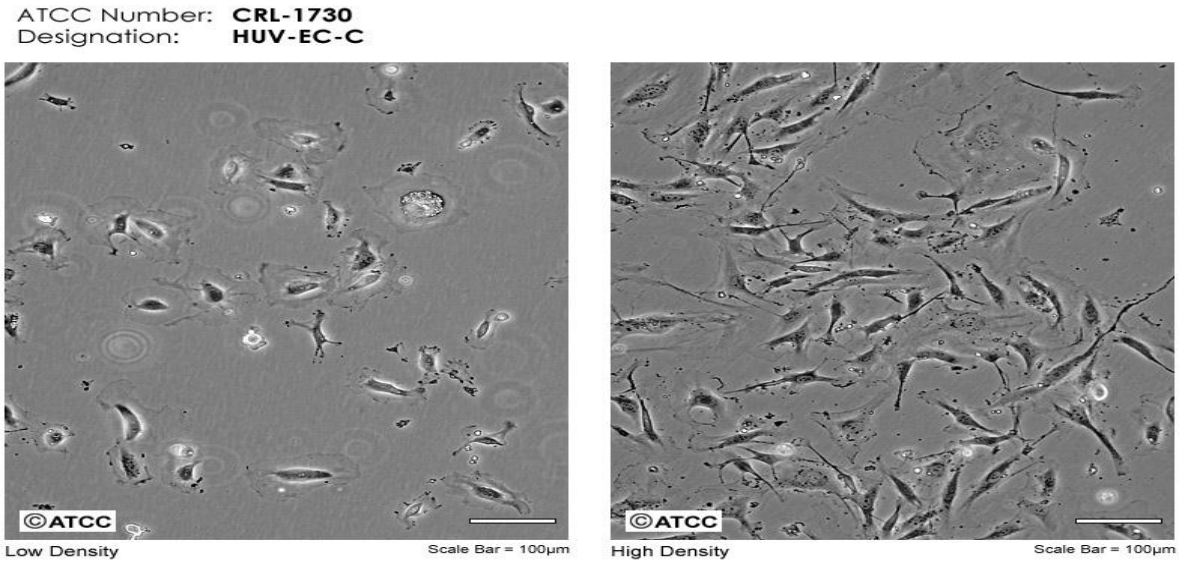



**Figure 5.1:** High and low density of HUVEC endothelial cell lines with permission of ATCC®.

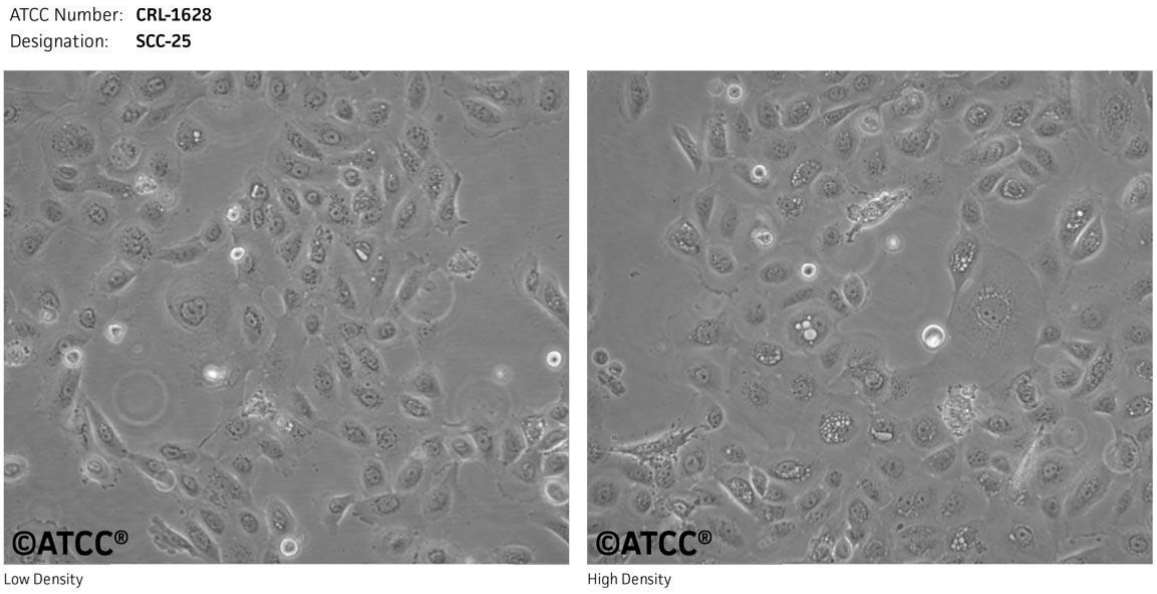



**Figure 5.2:** High and low density of SCC 25 oral squamous tongue cell lines grown in culture with permission of ATCC®.

## 5.8 Cell adhesion assay

The CHEMICON® Endothelial Cell Adhesion Assay kit (ECM645) is designed to test a variety of cell types that interact with activated or inactivated endothelial cell layers. The CHEMICON® Endothelial Cell Adhesion Assay kit includes a 96-well tissue culture treated fluorescence microtiter plate and reagents that allow for large-scale screening and quantitative comparison of multiple samples/cell line adhesion to an endothelial monolayer. Once endothelial cells are seeded in the tissue culture plate, they can be treated with cytokines, chemokines, transcription regulators or selective adhesion inhibitors. The protein synthesis inhibitor Cycloheximide and the RNA synthesis inhibitor Actinomycin D are provided as controls for adhesion protein expression studies.

The viable cell fluorescent compound Calcein-AM® is provided for labelling test cells and quantifying their adhesion by comparing bound versus unbound cells. The kit includes three Mouse anti-human monoclonal mAbs for adhesion blocking studies of ICAM-1, VCAM and E-selectin endothelial cell surface markers. These may be used to verify changes in expression levels of adhesion molecules, or effects of integrins upon endothelium activation.

This simple and high-throughput kit format allows for the monitoring of the effects of a variety of conditions and compounds on endothelial adhesion. CHEMICON® also offers a variety of antibodies to leukocyte adhesion molecules and surface antigens.

ECM645 was obtained from Merckmillipore®. Briefly, HUVEC cells were grown as normal to 80% confluent. Cells were then harvested (see subculture methods 5.5.2) after serum starvation, then seeded at a density of 50,000 in a 96-well plate or 10,000 in a 384-well plate. The plate was incubated for 48 hours at 37C in a $CO_2$ incubator and cells were verified microscopically to ensure cell viability. In all the experiments of adhesion, half of the endothelial cells were treated or inhibited where the media and the dose were adjusted to the desired concentration and time. Tumour cell lines were grown to 80% confluent, harvested and viability determined. Cells were seeded in plates at 200,000 cells/ml for a 96-well plate and 50,000 for a 384-well plate and the appropriate treatment was applied to each well. After treatment, both the endothelial cells and tumour cell media were discarded and tumour cells were treated with Calcein-AM to a final concentration of 2.5µM. Cells were then incubated for 30 minutes and centrifuged at 400Xg for 5 minutes. Cells were washed three times in PBS buffer until all the remnant solution was removed. Cells were suspended in 1-5ml of assay buffer. Tumour cells from each well were pipetted with the media to the corresponding well of endothelial on the fluorescent plate. Cells were covered and incubated for 30 minutes. The majority of the solution was then aspirated and discarded. Nonspecific binding was removed by washing each well 2-3 times with the assay buffer. After washing, the plate was read with a fluorescence plate reader using 485/530nm excitation/emission filter sets. Experiments were done in four replicates or duplicates and repeat.

The experimental design was divided into two groups as in the following diagram;

| Group/Experiment | Tumour treated group | Endothelial treated group |
|---|---|---|
| Sample size *n*=20 | When incubating the tumour and endothelial cells only the treatment has been applied to the tumour cells | The same protocol but in this group the treatment has been applied to the endothelial cells. |

| | | |
|---|---|---|
| Negative control | Untreated endothelium/untreated cancer cells | Untreated endothelium/untreated cancer cells |
| Experimental group (stimulation) | Untreated endothelium/treated cancer cells with SP | Endothelium treated with SP/untreated cancer cells |
| Experimental group (inhibition) | Untreated endothelium/treated cancer cells with NK-1R inhibitor | Endothelium treated with NK-1R inhibitor/untreated cancer cells |
| Experimental group (inhibition) | Untreated endothelium/treated cancer cells with (monoclonal antibody) | Endothelium treated with monoclonal antibody/untreated cancer cells |
| Positive control | Untreated endothelium/treated cancer cells with TNF | Endothelium treated with TNF/untreated cancer cells |

Cell adhesion was measured as a percentage of the basal adhesion according to the following:

$$\frac{\text{Particular cell adhesion plate reader value}}{\text{Basal value plate value (non-treated cell adhesion value)}} \text{ X } 100$$

## 5.9 Quantikine total MMP-2 ELISA kit

**Description of the procedure:** The kit utilises an enzyme-linked immunoassay sandwich technique. The antibody used is a monoclonal epitope highly specific to MMP-2 which has been coated on the microplate. The proper samples and controls are added to the wells of the plate and washed after binding to MMP-2 and then a secondary antibody is added and then washed. Finally, the substrate is added for colour development. The ELISA assay has a very high sensitivity to detect very low concentrations of the antigen represented in the biological

system. It also plots those concentrations against a standard curve of serial dilutions of a conjugate protein supplied by the manufacturer. This data is plotted with the log of the total antigen concentration versus the optical density reading of the plate reader for the sample. The kit was obtained from R&D® Systems. Media from treated and untreated cells are collected and stored at -20C. 50 µL of assay diluents R1-116 is added to each well. A 50 µL of standard, control or sample is added to each well and incubated on an orbital microplate shaker (0.12") orbit at 500RPM. After incubation, each well is washed with wash buffer three times using a squeeze bottle. A 200 µL of Total MMP-2 conjugate is added to each well and incubated for another two hours. The washing procedure is repeated after incubation. A 200 µL of substrate solution is added to each well and incubated for 30 minutes at room temperature in the dark. A 50 µL of stop solution is added to each well until the colour changes to yellow. Immediately the optical density of each well is determined using a microplate reader set to 450 nm and wavelength correction to 540-570 nm. Cells are either untreated, treated with SP or TNF-α for three hours. Samples were run in duplicates and repeated.

Experiment Design

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Standard curve (MMP-2 recombinant protein) X 2 rows | 32 ng/ml | 16 ng/ml | 8ng/ml | 4ng/ml | 2ng/ml | 1ng/ml | 0.5ng/ml |
| Experiment | -ve CNT Clear media X2 and repeat | EXP Media of untreated cells X2 and repeat | EXP Media of untreated cells X2 and repeat | EXP Media of cells treated with SP for 3 hrs X2 and | EXP Media of cells treated with SP for 3 hrs X2 and | EXP Media of cells treated with TNF for 3 hrs | EXP Media of cells treated with TNF for 3 hrs X2 |

repeat repeat X2 repeat and repeat and repeat

**5.10 Fluorescent microscope.** The technique of fluorescence microscopy is an essential tool in biology and the biomedical sciences, as well as in materials science due to attributes that are not readily available in other contrast modes with traditional optical microscopy. The application of an array of fluorochromes has made it possible to identify cells and sub-microscopic cellular components with a high degree of specificity amid non-fluorescing material. The fluorescence microscope is capable of revealing the presence of a single molecule. Through the use of multiple fluorochromes, labelling different probes can simultaneously identify several target molecules simultaneously. Although the fluorescence microscope cannot provide resolution below the diffraction limit of specific specimen features, the detection of fluorescing molecules for a single molecule is achieved with a satisfactory degree with the fluorescent microscope. The fluorescent microscope used is Zeiss Axio Imager M1 and the imaging system (camera) is a CRI Nuance multispectral imaging system. The experimental design and protocol was similar to section 5.8. Cell adhesion assay was performed on an 8-well glass chamber for visualising the cell adhesion under the fluorescent microscope.

## 5.11 Statistical analysis

Student t-test was employed to determine the significance of the mean of two groups. ANOVA was used to determine the mean of more than two groups. The SPSS software package was used as the statistical analysis software. $P<0.05$ was considered statistically significant.

## 5.12 Research highlight

The research was carried out in two main studies which represent chapters 6 and 7. The first study (chapter 6) investigated:

1. The effect of SP and the NK-1R antagonist on the adhesion between Jurkat and endothelial cells.

2. The effect of SP and the NK-1R antagonist on the expression of the adhesion molecules (VLA-4 integrin, SLeX and LFA) in Jurkat cell line.

3. The effect of SP and NK-1R antagonist on the expression of MMP-2 in Jurkat cells.

The study in chapter 7 investigated the same effects of SP and its antagonist in different cell lines representing different stages of tumour development (section 5.1). In each study the effect of the treatment or the antagonist was assessed through time-dependent manner and dose-dependent manner. For the adhesion assays, early response was defined as 1-4 hours of treatment, while in FAC analysis the time of treatment was assessed at 1-48 hours. In the adhesion experiments, there were two groups where the treatment/antagonist was allocated to either tumour cells (group 1) or the endothelial cells (group 2) as mentioned in the experimental design. Finally, chapter 8 is the discussion and the synthesis of all the experimental research data as well as the systematic review and position the results of the thesis in the current literature to outline the potential and future significance.

# Chapter 6 Substance P (SP) increases the adhesion of Jurkat cell line to human umbilical vein endothelial cells (HUVEC) through upregulating adhesion molecules; implication for metastasis

**Background**

Leukaemia is a highly metastatic neoplasm with the lowest prognosis among all cancers, which can drop to 27% survival rate. Despite decades of research into the biophysics of this process, little insight has been obtained into the actual key players of metastasis. Metastasis is a multi-step process where the tumour cell has to detach from the primary neoplasm, invade the surrounding tissues, enter the circulation, exit the circulatory system and finally implant in the new site. The whole metastatic success depends on the ability of neoplastic cells to interact and cross the endothelial barrier in the rate-limiting step (extravasation). The remarkable similarity between outflux of cells in inflammation and cancer points to the possibility of a common inflammatory mediator driving both processes.

**Aims**

The study examines the effects of SP on tumour-endothelial adhesion between Jurkat cells and HUVEC cell line. In addition it examines the role of SP in upregulating the expression of the adhesion molecules in Jurkat cell line. The study also examines the effect of SP on the expression of MMP-2 in these cells.

**Methods**

Flow cytometry was used to measure the level of expression of the adhesion molecules. Adhesion assay 645 ECM was used to measure the adhesion of Jurkat cell line to HUVEC cells. The MMP-2 ELISA kit was used to measure the expression level of MMP-2 in Jurkat cell line.

**Results**

Our results showed that: 1) SP and TNF-α increased the adhesion of Jurkat cells in a time-course treatment with a peak adhesion at 3 hours; 2) this adhesion was proportional to the log concentration of SP from 100 ng/ml ($10^{-8}$) M to 1 mcg/ml ($10^{-7}$) M; 3) NK-1R inhibitor reduced the adhesion in a time and dose-dependent manner; 4) the HUVEC treated group had a higher values of adhesion as well as lower values of adhesion inhibition than the Jurkat treated group; 5) SP 1mcg/ml ($10^{-7}$) M or TNF-α 100 ng/ml ($10^{-9}$) M treatment has increased both CD 11 (not significant) and CD 49 (significant), but not CD15s expression in 1-48 hour timescale treatment, as indicated by the FACS analysis; 6) NK-1R inhibited CD49 expression stimulation in Jurkat cell lines at a dose-dependent curve with concentrations of 100 ng/ml,

300 ng/ml, 750 ng/ml and 1 mcg/ml ($10^{-6}$-$10^{-7}$) M, respectively; 7) Anti-human monoclonal antibodies to VCAM or ICAM significantly inhibited the adhesion levels to below the untreated baseline levels; 8) The treatment of Jurkat cell lines with SP or TNF-α for 3 hours did not significantly affect expression of MMP-2. However, the treatment of the Jurkat cell line with SP treatment for 1 hour significantly decreased this expression significantly $P$= 0.003.

**Introduction**

Metastasis is the main cause of death of cancer patients. A systematic review of the global burden of cancer shows 17.5 million cancer cases worldwide and 8.7 million deaths, annually (401). Approximately 90% of cancer mortality rates are due to metastasis. Leukaemia is a highly metastatic neoplasm with the worst prognosis among all cancers, which can drop to 27% survival rate (359). Despite decades of research into the biophysics of this process, little insight has been obtained into the actual key players of metastasis (402).

Metastasis is a multi-step process where the tumour cell has to detach from the primary neoplasm, invade the surrounding tissues, enter the circulation, exit the circulatory system and finally implant in the new site (403). The migrating tumour cells face critical events (e.g. immune attacks in the blood vessels) leading to the death of most of them (403). The whole metastatic success depends on the ability of neoplastic cells to interact and cross the endothelial barrier in the rate-limiting step (extravasation) (404). Several studies elucidated variety of molecules – mainly adhesion molecules – as effectors of the tumour-endothelial interaction; however, the exact drivers and their role in the interaction remain poorly understood (233).

The extravasation step requires active interaction between the migrating cell and the endothelium through changes in the expression of the adhesion molecules on both cells (208). This is most likely mediated by an inflammatory mediator expressed in the area where extravasation takes place. Finally, proteolytic enzymes such as MMPs facilitate the physical invasion of the cell across the endothelial barrier (405). This remains the main scheme for cellular extravasation, however, the drivers and the exact mechanisms requires further investigations. Recent research points to the same inflammatory mediators controlling the extravasation events in both metastasis and inflammation (215).

Substance P was initially identified as a neurotransmitter in primary sensory afferent fibres, and the most potent member of the tachykinin family (312-315). The canonical pathway for SP is through binding to the G protein-coupled NK-1 receptor (316-317) in which the SP/NK-1R ligand-receptor complex is formed, then SP is degraded while NK-1R is recycled to the cell surface (318). SP contributes to the inflammatory process possibly through different mechanisms: 1) release of inflammatory mediators such as cytokines and arachidonic acid derivatives; 2) as a chemo-attractant to the immune cells; 3) vasodilatation (VD) and leukocyte/endothelial cell adhesion to aid the facilitation of leukocyte recruitment to the injured tissue through angiogenesis (322) (for excellent reviews about SP please refer to 312-314).

The high expression levels of SP and the NK-1R receptor in various tumours including melanoma (343), human malignant glioma (344), retinoblastoma (345) and mammary carcinoma (346), both in the primary tumour and their metastatic counterparts, imply an important role of SP in carcinogenesis (347). SP has been suggested to act as a mitogen for cancer progression (348) and even in the early malignant transformation (349). The role of SP in metastasis and invasion has not been fully investigated, although initial studies suggested that SP might trigger metastasis of colon cancer (350-351). Taking into consideration the main roles of SP in inflammation (vessel permeability, adhesion and migration of leukocyte) it is logical to predict similar roles for SP in metastasis (352). This study examines the role of SP in upregulating the expression of the adhesion molecules in both Jurkat cells and HUVEC endothelial cells, in addition to examining the effect of SP on tumour-endothelial cell adhesion. The effect of SP on the expression of MMP-2 in those cells is also investigated.

## Materials and Methods

**Cell lines:** HUV-EC-C (ATCC CRL-1730) human vascular endothelium, Jurkat Clone E6-1 (ATCC TIB-152$^{TM}$) human acute leukaemia cell lines were purchased from ATCC®.

**Culture Media:** Dulbecco modified eagle (DMEM) culture media, foetal Bovine serum (FBS), endothelial cell growth supplement (ECGS), L-glutamine, hydrocortisone, heparin sodium, and trypsin-EDTA were obtained from Sigma-Aldrich®. Eagles Minimum Essential

Medium (EMEM), F-12k medium, ATCC DMEM/F12 medium, vascular basal media, endothelial cell growth kit BBE, RPMI-1640 media and antibiotics were purchased from ATCC$^{R}$ cell lines.

**Treatment:** Substance P acetate salt hydrate (SP) and [succinyl-ASP6, ME-PHE8] Senktide substance P analogue were purchased from Sigma-Aldrich®. Tumour necrosis factor-α (TNF-α) was purchased from Sigma-Aldrich. A selective NK1 receptor antagonist, CH123001 (N-(3,5-bis(trifluoromethyl)benzyl)-N-methyl-6-(4-methylpiperazin-1-yl)-4-(o-tolyl)nicotinamide) was a gift from CH Biotech Pty Ltd, Sydney, Australia.

**Cell culture** Jurkat cells Clone E6.1 was cultured in RPMI 1640 + 2 mM Glutamine + 10% FBS.

HUVEC-EC was cultured in F-12K Medium, with 0.1 mg/ml heparin; 0.03-0.05 mg/ml adjusted to a final concentration of 10% FBS.

**Flow cytometry Antibodies:** Anti-integrin alpha 4 antibody CD49d PE (12-0499), Mouse IgG1 K isotype control (124714), Anti-CD11a antibody FITC (BMS102FI) were purchased from Affymetrix Bioscience®. Anti-CD15S Mouse anti-human Alexa Flour 647 (563526), Alexa Flour 647 Mouse IgM isotype control purchased from BD®.

**Adhesion molecule expression by flow cytometry:** In general, cells were harvested after treatment and washed as a single cell suspension in a 96-well, round-bottomed microtiter plates and adjusted to a concentration of >50,000 cells/ml, then the media was discarded and the cells were washed twice and centrifuged on ice-cold FACS Buffer. An antibody cocktail was made at 1:20 concentration with the FACS buffer with 50 µL for each sample. 50 µL of antibody cocktail was added and the sample was incubated for at least 30 minutes at room temperature or 4°C in the dark. The cells were washed three times with FACS buffer by centrifugation at 1500 RPM for 5 minutes and resuspended in 200 µl to 1ml of ice-cold FACS buffer. Cells were kept in the dark on ice or at 4°C in a fridge until analysis ($n$=3).

**Cell adhesion assay:** ECM645 was obtained from Merckmillipore®. Briefly, HUVEC cells were grown as normal to 80% confluent. Cells were then harvested (see subculture methods) then seeded at a density of 50,000 in a 96 well-plate or 10,000 in a 384-well plate. In all, half

the endothelial cells were treated or inhibited where the media and the dose were adjusted to the desired concentration and time. Cancer cells were seeded at 200,000 cells/ml for the 96 well-plate and 50,000 for the 384-well plate. The appropriate treatment was applied to either the endothelial or the tumour cells according to the experimental protcol. After treatment, tumour cells were treated with Calcein-AM to a final concentration of 2.5 µM. Cells were then incubated for 30 minutes and centrifuged at 400X g for 5 minutes. After washing the plate was read with a fluorescence plate reader using 485/530 nm excitation/emission filter sets. Sample size ($n$= 20).

**Quantikine total MMP-2 ELISA Kit** was obtained from R&D Systems®. Cells were either untreated, treated with SP or TNF-α for 3 hours. Samples were run in duplicates and repeated. Media from different treated and untreated cells were collected and stored at -20C. A 50 µL of standard, control or sample were added to each well and incubated on orbital microplate shaker (0.12") orbit at 500 RPM. A 200 µL of Total MMP-2 conjugate was added to each well and incubated for another 2 hours. A 200 µL of substrate solution was added to each well and incubated for 30 minutes at room temperature in the dark. Immediately the optical density of each well was determined using a microplate reader set to 450 nm and wavelength correction to 540-570 nm.

**Fluorescent microscope:** The same protocol for the adhesion assay was followed for validation of adhesion results with the fluorescent microscope with the use of an 8-well chamber slide ($n$=20). The microscope used was a Zeiss Axio Imager M1 with an imaging system (camera) CRI Nuance multispectral imaging system.

**Statistical analysis:** Student t-test was employed to determine the significance of the mean of two groups. ANOVA was used to determine the mean of more than two groups. The SPSS software package was used as the statistical analysis software. $P<0.05$ was considered statistically significant.

## Results

### Effect of SP on the adhesion of Jurkat-HUVEC

The effect of SP on the adhesion of Jurkat cells to HUVEC cells was evaluated through the adhesion assay by co-incubating the two cell lines. The experimental design involved two groups wherein the first group, tumour cells, were treated with SP, while in the second group;

the endothelial cells were treated with SP. Both groups were treated with a time course from 1-4 hours (SP peak threshold activity is 60-180 minutes) (Figure 6.1- 6.2). Data are presented as per cent of adhesion, calculated as follows: Sample adhesion value/ control adhesion value X 100, where control adhesion was measured in the absence of any treatment of the tumour or endothelial cells. Two concentrations of SP were utilised 1 mcg/ml ($10^{-6}$) M and 100 ng/ml ($10^{-8}$) M. Both concentrations of SP significantly increased the adhesion of Jurkat cells to HUVEC as measured by the mean fluorescence intensity of the well compared with the baseline adhesion level of the untreated cells. In the first group, Jurkat cells that were treated with 100 ng/ml ($10^{-8}$) M of SP for 1, 2, 3 and 4 hours reached a maximum of adhesion at 3 hours while treatment with 1 mcg/ml ($10^{-6}$) M reached a maximum at 2 hours. The second experimental group, where the HUVEC cells were treated with 1 mcg/ml or 100 ng/ml of SP for the same time course, had similar time-dependent curves. In both groups, HUVEC treated groups had slightly higher adhesion values. Both treatment concentrations significantly increased the adhesion of Jurkat cells to HUVEC endothelial cells ($P<0.001$). These values were significant for both treatment groups; the tumour treated and the HUVEC treated group.

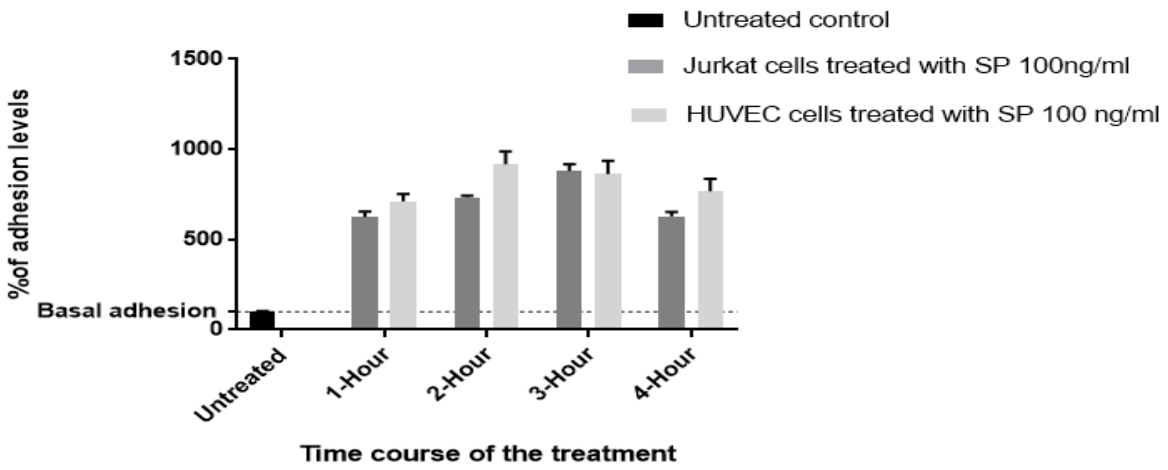



**Figure 6.1:** Effects of SP on Jurkat cells adhesion to HUVEC. Two experimental groups in which Jurkat cells were treated with 100 ng/ml ($10^{-8}$) M, where the other group had HUVEC cells treated the same. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion. Statistical analysis established the following significance: $P<0.001$ vs control for all the time points for either group, however, the group in which HUVEC cells were treated had slightly higher values of adhesion levels.

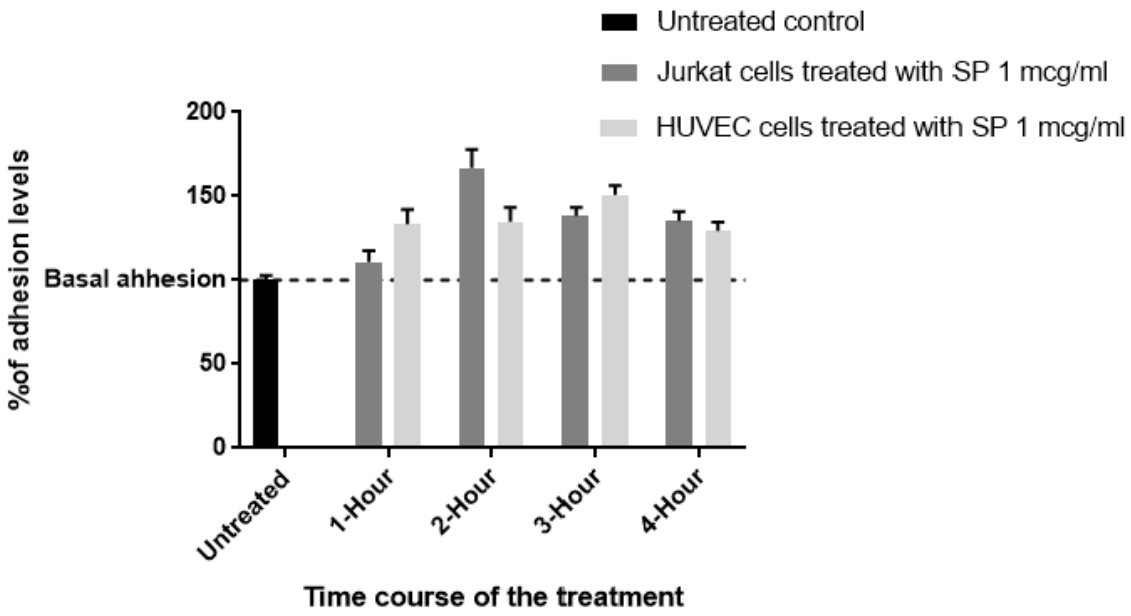



**Figure 6.2:** Figure 6.2: Effects of SP on Jurkat cells adhesion to HUVEC. Two experimental groups in which Jurkat cells were treated with 1 mcg/ml ($10^{-6}$) M, where the other group had HUVEC cells treated with the same treatment. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion. Statistical analysis established the following significance: $P<0.001$ vs control for all the time points for either group, except of the 1-hour time point in the Jurkat treated group.

### Comparison of the effect of SP and TNF-α on Jurkat-HUVEC adhesion

Next, we compared the effect of TNF-α 100 ng ($10^{-9}$) M, as a positive control, on the adhesion of Jurkat to HUVEC cells at the time points 3 and 4 hours of treatment with 1 mcg ($10^{-6}$) M. All of the treatments increased the adhesion levels of Jurkat cells to HUVEC (Figure 6.3).

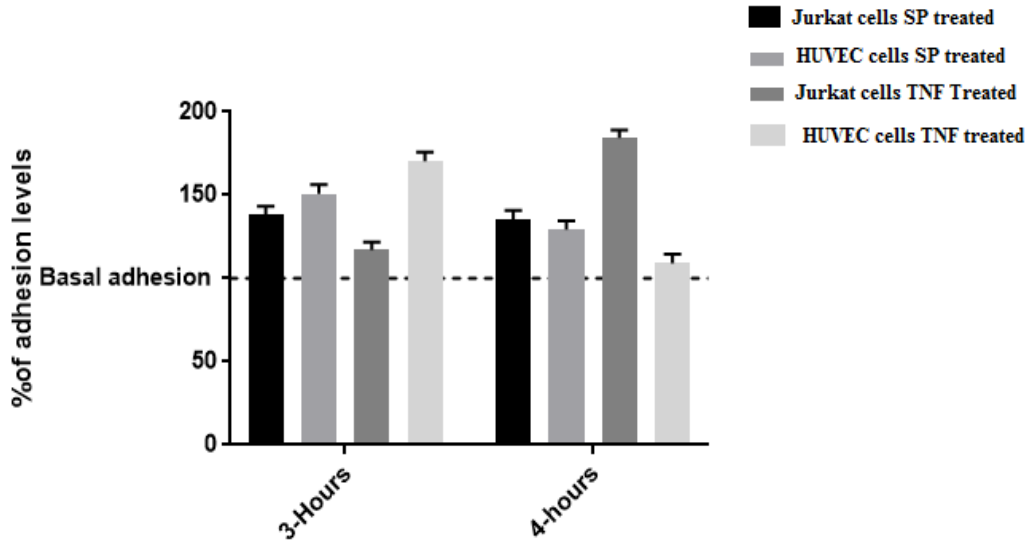



**Figure 6.3:** Comparison of the effect of SP and TNF-α as a positive control on the adhesion of Jurkat cells to HUVEC. The first two groups were treated with SP ($10^{-6}$) M, where the other two groups were treated with TNF-α ($10^{-9}$) M at time points 3 and 4 hours. All values are significant at ($P<0.001$), except for Jurkat cells treated with TNF at 3 hours and HUVEC cells treated with TNF-α at 4 hours.

**Effect of SP receptor (NK-1R) antagonist**

A time-course inhibition assay was performed with the same time points 1-4 hours, where cell adhesion was inhibited with NK-1R antagonist. The NK-1R antagonist significantly reduced the adhesion level, where this adhesion followed a dose-dependent curve through the two concentrations of inhibitor which mirrored the concentration of SP used for the treatment. In each group, Jurkat or HUVEC cells were incubated with NK-1R inhibitor 100 ng/ml or 1 mcg/ml for 1, 2, 3 or 4 hours and then incubated with the corresponding SP concentration for the same time point. In the Jurkat cells treated group (Figure 6.4), the use of NK-1R antagonist (1 mcg/ml and 100 ng/ml) was significant in inhibiting the adhesion of Jurkat cells to HUVEC cells significantly ($P<0.001$) at 2 hours. In the HUVEC treated group (Figure 6.5) with NK-1R antagonist (same concentrations), the inhibition effect was significant ($P<0.001$) in all the time points except at 3 and 4 hours with 100 ng/ml of NK-1R. SP inhibition of the HUVEC had a higher effect of inhibiting the tumour-endothelial adhesion.

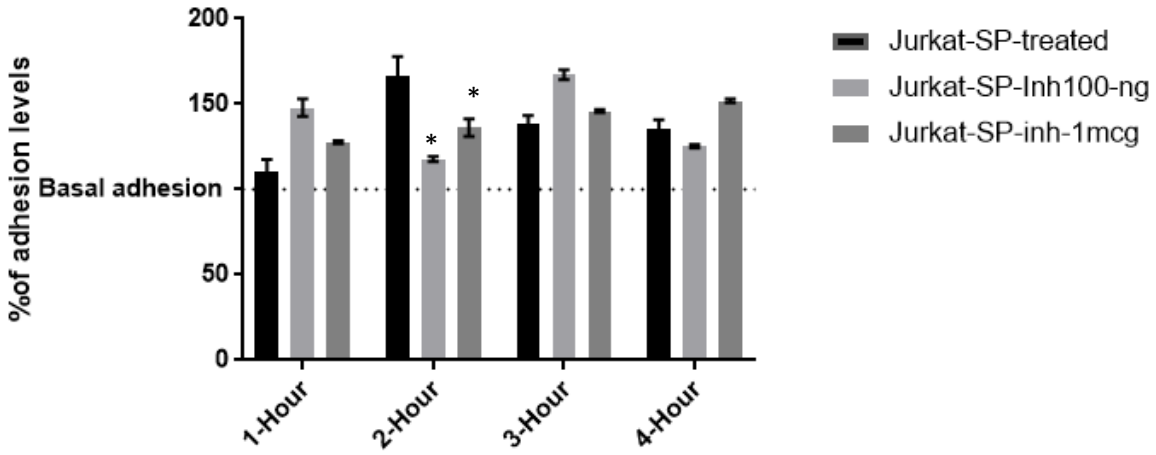



**Figure 6.4:** Effect of the NK1 receptor antagonist on the adhesion effects evoked by SP treatment. Jurkat cells were incubated for one time point from 1-4 hours with the antagonist (10−6, $10^{-8}$) M, then was incubated with the same SP concentration for the same time point. Asterisks mark statistically significant inhibition values (*P<0.01).

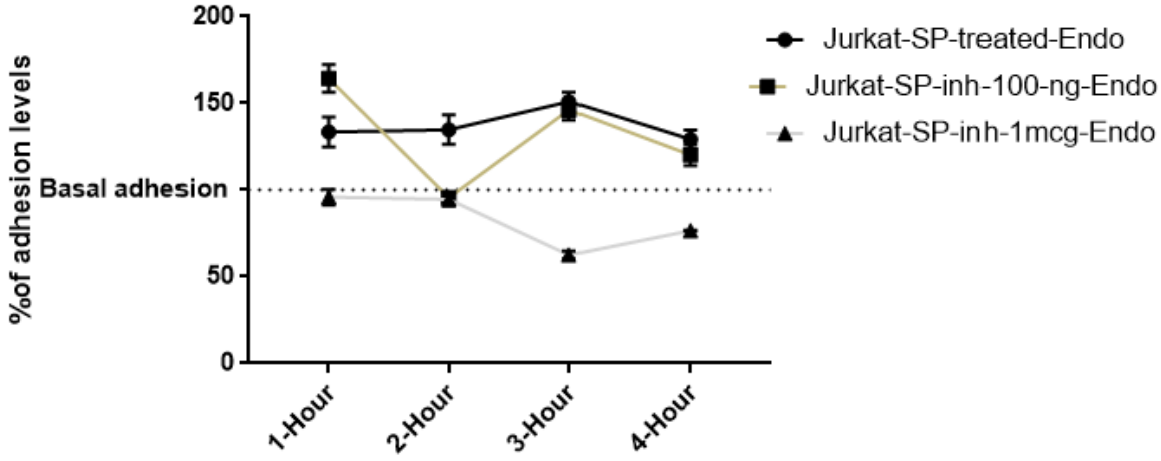



**Figure 6.5:** Effect of the NK1 receptor antagonist on the adhesion effects evoked by SP treatment. HUVEC cells were incubated for one time point from 1-4 hours with the antagonist (10−6, $10^{-8}$) M, then was incubated with the same SP concentration for the same time point.

**Effect of adhesion molecule Monoclonal Antibodies on the adhesion**

Finally, we performed an anti-human monoclonal antibody inhibition assay. Briefly, either tumour or HUVEC cells was treated with SP 1 mcg ($10^{-6}$) M or TNF-α 100 ng ($10^{-9}$) M for 3 hours and then co-incubated with mouse anti-human monoclonal antibodies VCAM 50 µg/ml, ICAM 50 µg/ml, or cycloheximide 50 µg/ml for 30 minutes as per assay protocol. Cycloheximide treated cells (tumour or endothelial) had significantly reduced adhesion levels triggered by SP treatment. However, they remained higher than the untreated basal adhesion levels. Monoclonal antibody to VCAM and ICAM significantly reduced the adhesion to levels beyond the basal adhesion levels of the untreated cells (Figure 6.6).

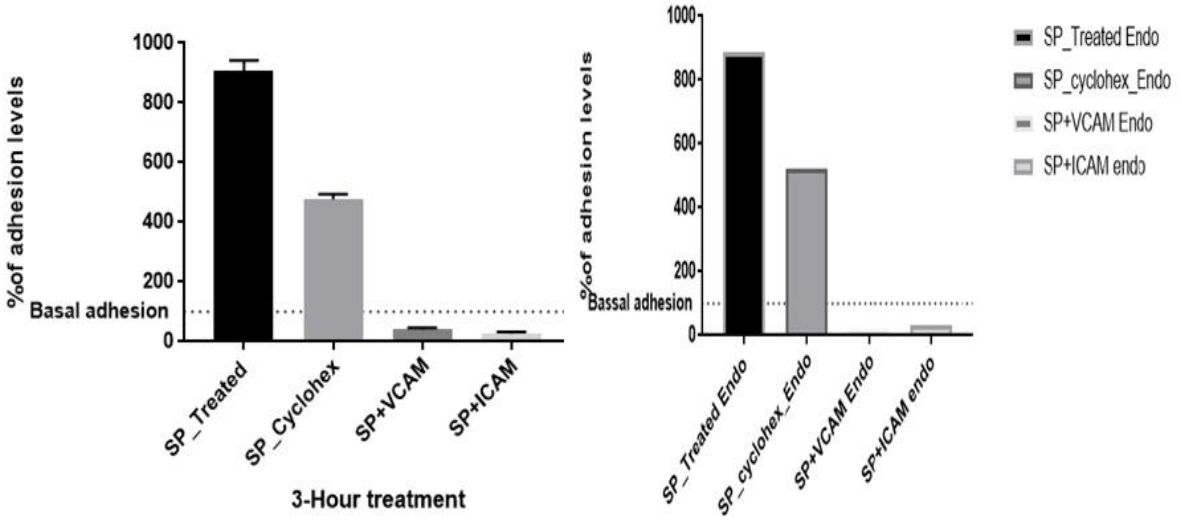



**Figure 6.6:** Inhibition of the adhesion triggered with SP treatment. Jurkat (left) or HUVEC (Endo) (right) cells were incubated with monoclonal antibodies or cycloheximide then incubated with SP 100 ng/ml for 3 hours. All the treatments significantly inhibited the adhesion as compared to the treated levels or the control (basal levels) ($P<0.001$). Note: All columns have error bars; however, due to the small ratio of some error bars in relation to column, they do not appear on the graph.

**Effect of SP treatment on the adhesion molecule expression**

Next, we tested the effect of SP and TNF-α on the expression of the adhesion molecules, using flow cytometry: CD11 (LFA), CD49 (VLA-4) and CD15S (Sialy Lewis) both on a

time-course at 1, 2, 3, 4, 24 and 48 hours as well as a dose-response curve for NK-1R inhibitor at 100, 300, 750 ng/ml, 1mcg/ml and 10 mcg/ml ($10^{-5}$-$10^{-8}$) M.

**CD11:** Both SP and TNF-α increased the CD 11 expression. However, it was not significant. The optimal increase in the expression was at 3-hour treatment of SP and 24-hour treatment of TNF-α. The NK-1R antagonist at 1mcg concentration at 4 hours reversed the expression back close to the untreated levels (Table 6.1, Figure 6.7).

**Table 6.1:** The effect of SP 1 mcg/ml and TNF 100 ng/ml on the expression of CD 11 in Jurkat cells. Data are represented as measured by the fluorescence intensity of the treated samples against the untreated levels of expression *n*=3.

| CD11 JURKAT_SP 1 mcg | P-Value | CD11 Jurkat TNF 100 ng | P-Value |
|---|---|---|---|
| JURKAT_SP_1 Hr | 0.925 | 1 Hr | 1.00 |
| JURKAT_SP_2Hr | 0.996 | 2 Hr | 0.85 |
| JURKAT_SP_3Hr | 0.459 | 3 Hr | 0.71 |
| JURKAT_SP_4Hr | 0.544 | 4 Hr | 0.44 |
| JURKAT _SP_24Hr | 0.897 | 24 Hr | 1.00 |
| JURKAT_SP_48Hr | 0.896 | 48 Hr | 0.21 |

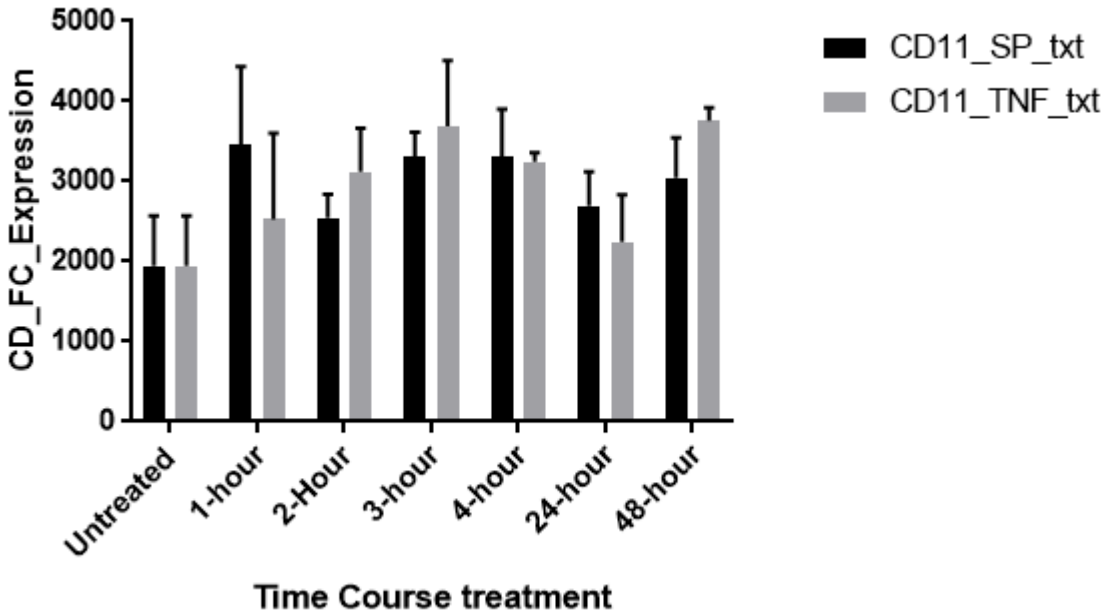



**Figure 6.7:** The effect of SP 1mcg or TNF-α 100 ng/ml on the expression of CD11 (LFA) on a time-course treatment from 1-48 hours. Data are represented as mean of fluorescence intensity.

**CD15s (Sialyl Lewis x):** Neither SP or TNF-α treatment affected the expression of CD15s expression. The untreated expression of CD15s molecule was relatively low at a mean value of 64.9 (Table 6.2).

**Table 6.2:** Flow cytometry mean expression levels of CD15s in Jurkat cells treated with SP 1mcg/ml for different time points in comparison to untreated Jurkat cells expression of CD15s.

| CD15s_JURKAT_ | *P*-Value |
|---|---|
| CD15s_JURKAT_SP_2Hr | 0.05 |
| CD15s_JURKAT_SP_3Hr | 0.58 |
| CD15s_JURKAT_SP_4Hr | 0.89 |
| CD15s_JURKAT_SP_48Hr | 0.48 |

**CD49 (VLA-4 integrin):** SP treatment has significantly increased the expression of VLA-4 in a time-course treatment at 1, 2, 3, 4, 24, 48 hours (Figure 6.8). The peak of the expression increase was at 3-4 hours with ($P$ = 0.02, 0.03) respectively, and the expression assumed a plateau curve after this time point (Table 6.3). Next, inhibition of CD49 expression using NK-1R inhibitor at doses of 1mcg, 750 ng, 300 ng, and 100 ng produced a dose-response curve (Figure 6.9). Jurkat cells were incubated with NK-1R antagonist for 1, 2, 3, 4, 24 hours then cells were incubated with the 1 mcg/ml then analysed for FACS expression of CD49.

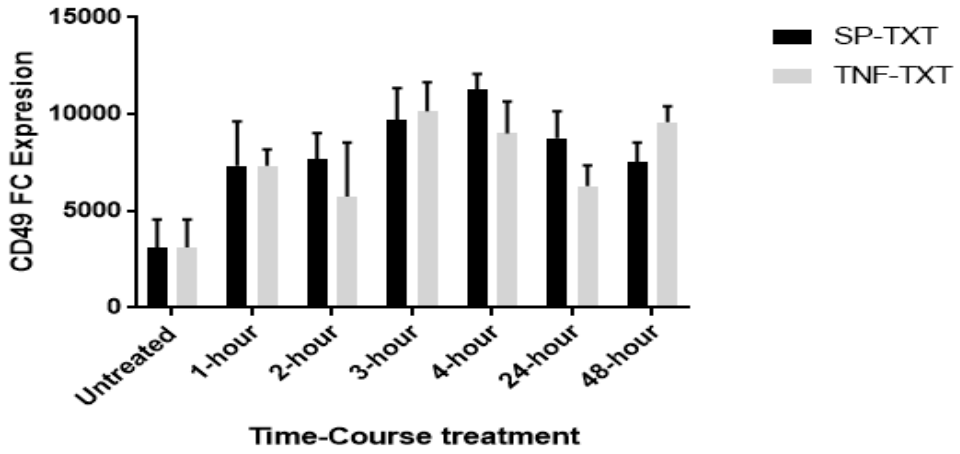



**Figure 6.8:** The effect of SP 1mcg or TNF-α 100 ng/ml on the expression of CD49 (LFA) on a time-course treatment from 1-48 hours. Data are represented as a mean of fluorescence intensity.

**Table 6.3:** CD49 FAC expression SP treatment values.

| CD49_JURKAT_Untreated | *P*-Value |
|---|---|
| CD49_JURKAT_SP_1Hr | 0.03 |
| CD49_JURKAT_SP_2Hr | 0.01 |
| CD49_JURKAT_SP_3Hr | 0.02 |
| CD49_JURKAT_SP_4Hr | 0.03 |
| CD49_JURKAT_SP_12Hr | 0.04 |
| CD49_JURKAT_SP_48Hr | 0.05 |

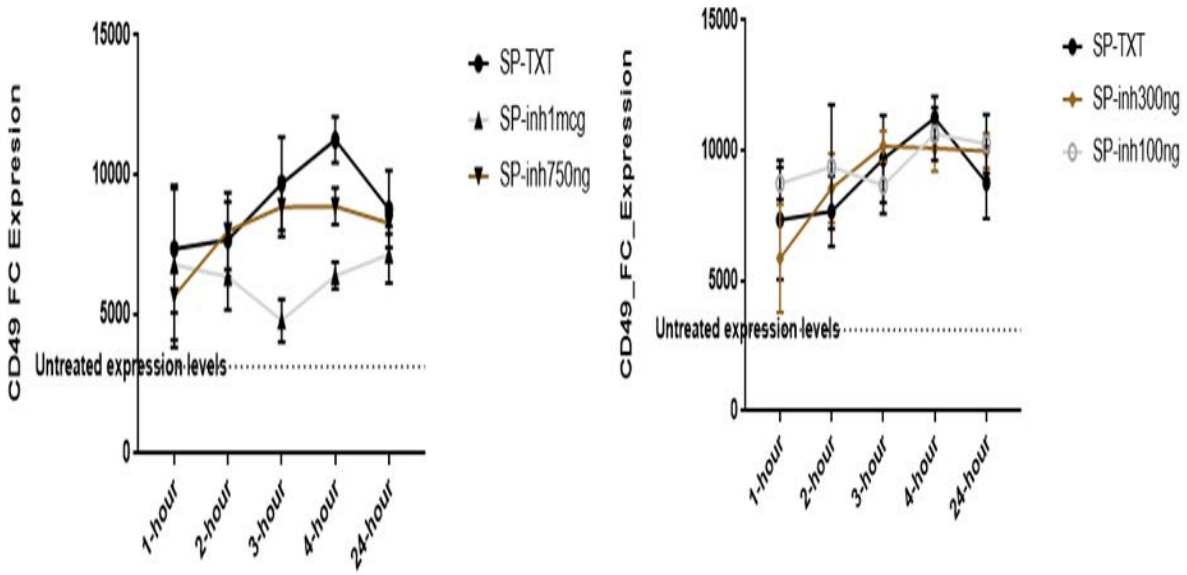



**Figure 6.9:** Effect of the NK1 receptor antagonist on the expression of the adhesion molecule CD49. Jurkat cells were incubated with each concentration of the antagonist for 1, 2, 3, 4, 24 hours, then incubated with the corresponding concentration of SP. Data are expressed as a mean of the fluorescence intensity.

**Table 6.4:** Comparison of the effect of the inhibitor NK-1R on the expression of CD49 at time points 1-4 hours in a dose-dependent manner with 1 mcg/ml, 750 ng/ml, 300 ng/ml, 100 ng/ml. Data are represented as a mean of the fluorescence intensity of the inhibitor sample versus the SP treatment at that time point. * denotes significant <0.05.

| NK-1R antagonist *P*-Value | 1 mcg/ml | 750 ng/ml | 300 ng/ml | 100 ng/ml |
|---|---|---|---|---|
| **1-Hour** | 0.96 | 0.58 | 0.66 | 0.34 |
| **2-Hour** | 0.43 | 0.81 | 0.52 | 0.48 |
| **3-Hour** | *0.002 | 0.13 | 0.74 | 0.11 |
| **4-Hour** | <*0,001 | *0.002 | 0.08 | 0.56 |

**Fluorescence Microscope**

Using the same protocol, we investigated the effect that adhesion of human Jurkat cells to HUVECs with SP activation on a chamber glass with the fluorescence microscope. The result showed that the adhesion of Jurkat cells increased significantly with SP stimulation for 3 hours (Figure 6.10). In contrast, when cells were treated with NK-1R inhibitor for 3 hours, they showed significant reduction of Jurkat cells adhering to ECs.

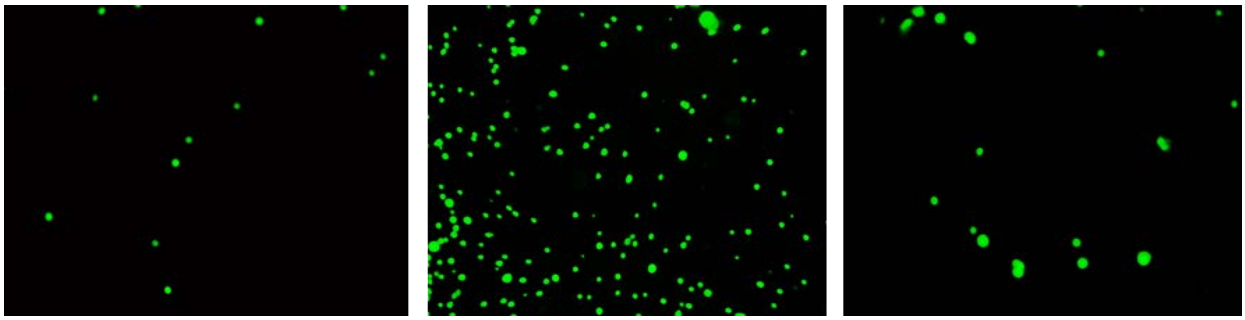


**Figure 6.10:** Fluorescence microscope image for Jurkat cell adhesion to HUVEC with SP treatment and SP inhibitor effect on the adhesion: (right to left) A: Untreated Jurkat adhesion to HUVEC; B: Jurkat SP-activated 3-hour adhesion to HUVEC; C: Inhibition of Jurkat adhesion HUVEC by NK-1R.

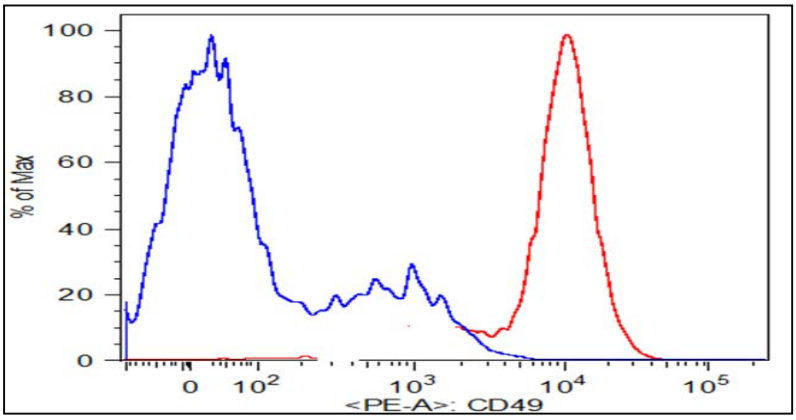



**Figure 6.11:** Flow cytometric analysis of SP-induced expression of cell-surface VLA-4. For FACS experiments, a PE-conjugated anti-VLA-4 antibody binding to HUVEC suspensions was detected.

**Effect of SP treatment on MMP-2 expression**

Finally, the effect of SP and TNF-α treatment on the release of MMP-2 was measured using an ELISA technique, comparing untreated Jurkat cells MMP-2 levels to those of the same cell line treated with SP 1 mcg for 1 and 3 hours. The basal (untreated) expression of MMP-2 was positive at1.87 ng/ml. Neither of SP 1 mcg/ml or TNF 100 ng/ml treatment at 3 hours significantly affected the expression with $P = 0.87, 0.50$, respectively. However, SP treatment for 1 hour was significant $P = 0.003$.

## Discussion

Blood cancers remain a challenge to the current therapeutic modalities because of their inherent capacity to metastasise (359). Recent studies have supported an important role for inflammation in cancer initiation, progression and metastasis. In addition, emerging studies highlight inflammation as a driver of the different stages of metastasis, such as tumour-endothelial cell adhesion (215). However, the current literature has not identified the particular mechanism of the relationship of inflammation and metastasis or the precise effect of inflammatory mediators on the adhesion and extravasation of tumour cells. Therefore, our study aimed at: 1) exploring the effect of the inflammatory mediator SP on the adhesion of Jurkat leukaemia cells to HUVEC cells; 2) the effect of SP on the expression of adhesion molecules in Jurkat cells; and 3) investigating the effect of SP on MMP-2 release by tumour cells.

Metastasis starts with the tumour cell loosely adhering to neighbouring cells, invading the surrounding tissues, gaining access to blood vessels, and finally adhering and exiting the blood vessels to implant in secondary sites (208). The success of metastasis is dependent on the rate-limiting step of extravasation. Leukaemias and lymphomas, being already in the blood, bypass the need for the rest of the steps before extravasation, as well as expressing a variety of adhesion molecules and inflammatory mediator receptors for normal function. This facilitates the migration of such cells and may account for the high metastatic potential of blood malignancies (359).

The extravasation stage is crucially important not only for the migration of the tumour but also for the survival of the migrating cell. Adhesion of tumour cells to the endothelium withdraws the cell from the mainstream in the blood vessel and provides protection against

immune attack (208). Therefore, this adhesion and subsequent exiting has to happen in an acute phase similar to an inflammatory process. Dianzani et al. reported that SP increased the adhesion of neutrophils to HUVEC cells in a time/dose-dependent curve at 5-40 minutes (331). In line with these data, our results (to the best of our knowledge) showed that: 1) SP and TNF-α increased the adhesion of Jurkat cells to HUVEC cells in a time-course treatment with a maximum adhesion at 3 hours (Figure 6.1, 6.2); 2) this adhesion was proportional to the log concentration of SP from 100 ng to 1 mcg; 3) treatment with NK-1R inhibitor reduced the adhesion in a time and dose-dependent manner; 4) the HUVEC-treated group had higher values of adhesion as well as lower values of adhesion inhibition than the Jurkat-treated group (treating the Jurkat with SP before mixing with HUVECs).

Stucki et al. reported a static quantitative adhesion assay of leukaemic cells to endothelial cells at 0-24 hours, where they found that the significant increase in adhesion occurred by three times at 3 hours up to 24 hours (360). The authors suggested a similar mechanism for leukaemic cells to produce inflammatory stimulating factors that can accumulate more tumour cell adhesion to the endothelium. We did not found any studies have reported less than 48-72 hours tumour-endothelial adhesion except for inflammation studies in the literature. It is logical to consider that a cancer cell with multiple genetic aberrations and high mutation levels could acquire a mutated phenotype that allows these cells to mimic an acute adhesion onset similar to that of inflammatory reaction (bearing in mind that cells of origin for blood malignancies are inflammatory cells).

Continuing the previous list, our study showed that: 5) SP 1 mcg or TNF-α 100 ng treatment has increased both CD11 (not significant) and CD49 (significant) but not CD15s expression in 1-48 hour timescale treatment, as indicated by the FACS analysis; 6) NK-1R inhibited CD49 expression stimulation in Jurkat cell lines at a dose-dependent curve with concentrations of 100 ng, 300 ng, 750 ng and 1 mcg; 7) Anti-human monoclonal antibodies to VCAM or ICAM significantly inhibited adhesion levels to below the untreated baseline levels. Zepeda-Moreno et al. reported that SDF-1 increased the expression of LFA-1 in Jurkat cells (406). Similarly, Stucki (360) reported that adhesion to TNF-α activated endothelium was dependent on members of the immunoglobulin superfamily ICAM-1 and VCAM-1 in a manner similar to normal leukocyte trafficking. However, they postulated that the adhesion was mainly dependent on the selectin family (406). This does not contradict our

results as it might be due to tissue specific factors (authors used different cell line), as well as the authors having concluded that while the selectin family mediates rolling activity on the endothelium, the main adhesion drivers are the ICAM-β1 integrin.

Interestingly, we found that treating endothelial cell with either stimulating factor or inhibitor produces more potent levels of adhesion which may explain the organ-specific metastasis of cancers. It also highlights the active interaction of tumour-endothelial cell during adhesion (404, 208). Moreover, our data showed that inhibition of adhesion levels achieved using cycloheximide, which blocks translation of messenger RNA on cytosolic 80S ribosomes, but does not inhibit organelle protein synthesis, did not achieve higher levels such as the ones inflicted with monoclonal antibodies which might suggest that tumour cells highly express adhesion molecules as well as inflammatory receptors.

Together with these findings, our results, provided a link of inflammation with metastasis, and suggested that SP and TNF-α may serve as an inflammatory mediators triggering early onset of cancer–endothelial cell adhesion.

Adhesion of tumour cells to endothelial cells requires subsequent steps to promote tumour cell migration and implantation in the secondary site. MMP group of enzymes have been implicated for facilitating the tumour cell migration and invasion of the endothelial barrier (405). In our study Jurkat cell line had a high basal level of expression of MMP-2, however, the treatment of SP or TNF-α for 3 hours did not significantly affect this expression. More importantly, the treatment of the cell line with SP treatment for 1 hour significantly decreased this expression significantly ($P = 0.003$). Li et al., reported a significant effect of SP on the expression of MMP-2 on mRNA level (407). These results are counterintuitive as if the adhesion is to peak at 2 to 3 hours, then MMP-2 release should be following this adhesion. This conflict may be due to either tissue factors or multiple functions for MMP-2 in tumour environment which will require further study to validate those results.

Our study showed that SP has the ability to mediate the adhesion between Jurkat cell lines and HUVEC cell lines and that integrin activation seems crucial for this adhesion. Despite the static and *in-vitro* nature of the study, this requires further validation with *in-vivo* models;

these observations increase our understanding of cancer cell biology. Importantly, they also suggest a new therapeutic potential for SP antagonists against leukaemic cell adhesion.

# Chapter 7 Substance P (SP) increases the adhesion of oral cancer cell lines (OSCC) to human umbilical vein endothelial cells (HUVEC) through upregulating adhesion molecules; implication for metastasis

**Background**

OSCC is a locally invasive lesion with a best survival rate of 50%. Interaction of cancer cells with endothelial cells plays an important role in both tumour angiogenesis and metastasis. The similarity between tumour and leukocyte extravasation indicates similar localisation signals through common mediators of inflammation and cancer. These localisation signals are mainly delivered by neurotransmitters such as SP or cytokines such as TNF-α, and although these mediators have been elaborated extensively in inflammation, their actions in metastatic extravasation is yet to be identified. Taking in consideration the main role of SP in inflammation (vessel permeability, adhesion and migration of leukocytes) and the up-regulated expression in OSCC, our study hypothesised that SP might play a role in the adhesion of OSCC cells to HUVEC cells.

**Methods**

HOK primary oral keratinocytes, CAL 27, SCC25, DOK, H157 and BICR22 OSCC cell lines were used for the experiments as well as HUVEC endothelial cell lines. Adhesion molecule expression was measured by flow cytometry. The ECM645 adhesion assay was obtained from Merckmillipore® for quantification of tumour-endothelial cell adhesion. A Quantikine total MMP-2 ELISA kit was obtained from R&DSystems® for measurement of MMP-2 expression in OSCC cell lines.

**Results**

Our results showed that: 1) treatment with SP and TNF-α increased the adhesion of H157, CAL27 and DOK cells to HUVEC endothelial cells in a time-dependent manner with a maximum adhesion at 3 hours; 2) the endothelial-treated group had more adhesion values than the tumour-treated groups; 3) NK-1R inhibitor reduced the adhesion in a time and dose-dependent manner from 1-4 hours with a peak suppression of 3 hours in H157 and DOK, while the peak of inhibition in CAL27 cell was at 2 hours treatment; 4) monoclonal anti-adhesion antibodies inhibited the adhesion between OSCC and HUVEC; 5) the expression of each adhesion molecule assumed an increasing expression according to malignant potential; 6) SP time treatment increased CD49 adhesion molecule with a peak of 3 hours as well as CD11 and CD49 in both SCC25 and BICR22, respectively; 7) SP treatment increased MMP-2 release at 3 hours in each cell line; however, this increase was not significant.

## The burden of metastasis in OSCC

The metastatic potential of OSCC has shaped the extent of treatment strategies necessitating aggressive therapeutic modalities to eliminate metastasis and ensure residual disease-free outcome (16, 18, 146, 408). The increasing rates of metastatic spread seen in clinical settings and detected by molecular diagnostics necessitates a new paradigm of cancer treatment approaches (125, 129). Furthermore, the scientific literature showing a failure of combination systematic therapies to produce any significant improvement of patient outcome or quality of life (126-127, 130).

The initial success of cancer immunotherapy highlights the efficacy of a novel approach to replace the traditional therapies as well as provide better understanding of the disease (215). This approach is based on looking into critical biologic mechanisms hijacked by cancer for the favour of its growth (225). By identifying those process, interfering with them will provide potential targets for metastatic therapies. A major critical as well as common mechanism, is inflammation and the role of its mediator in different aspect of cancer stages (409).

SP was found to be significantly expressed in the tumour cell membrane, cytoplasm and nucleus of OSCC as well as the infiltrating lymphocytes of the neoplasms (353). The expression of SP was found to be significantly associated with the presence of dysplasia and carcinoma *in situ*, which strongly suggested a role for SP in the early onset of oral oncogenesis (354). Taking into consideration the main role of SP in inflammation (vessel permeability, adhesion and migration of leukocyte) and the up-regulated expression in OSCC, our study hypothesised that SP might play a role in the adhesion of OSCC cells to HUVEC cells. Therefore, our study aimed to examine the role of SP in stimulating OSCC-HUVEC adhesion as well as investigating the role of SP in upregulating the adhesion molecule expression of OSCC cell lines. The study also examined the effect of SP on tumour-endothelial adhesion and the effect of SP on the expression of MMP-2.

### Materials and Methods

**Cell lines:** Cells were selected to represent the progressive stages of oral cancer as follows: HOK primary oral keratinocyte isolated from human oral mucosa was purchased from

Sciencell®. CAL 27 (ATCC CRL- 2095), SCC25 (ATCC CRL- 1628), DOK, H-157 and a BICR-22 were used. A HUV-EC-C (ATCC CRL-1730) human vascular endothelium was purchased from ATCC®.

**Culture Media:** Dulbecco modified eagle (DMEM) culture media, foetal bovine serum (FBS), endothelial cell growth supplement (ECG), L-glutamine, hydrocortisone, heparin sodium, and trypsin-EDTA were obtained from Sigma-Aldrich®. Eagle's minimum essential medium (EMEM), F-12K medium, ATCC DMEM/F12 medium, vascular basal media, endothelial cell growth kit BBE, RPMI-1640 media, and antibiotics were purchased from ATCC® cell lines. Oral keratinocyte medium was purchased from Sciencell®.

**Treatment:** Substance P acetate salt hydrate (SP) and [succinyl-ASP6, ME-PHE8] Senktide Substance P analogue were purchased from Sigma-Aldrich$^{R}$. Tumour necrosis factor (TNF-α) was purchased from Sigma-Aldrich®. A selective NK1 receptor antagonist, CH123001 (N-(3,5-bis(trifluoromethyl)benzyl)-N-methyl-6-(4-methylpiperazin-1-yl)-4(otolyl)nicotinamide) was a gift from CH Biotech Pty Ltd, Sydney, Australia.

**Flow cytometry antibodies:** Anti-integrin alpha 4 antibody CD49d PE (12-0499), Mouse IgG1, K isotype control (124714), Anti-CD11a antibody FITC (BMS102FI) were purchased from Affymetrix eBioscience®. Anti-CD15S Mouse anti-human Alexa Flour 647 (563526), Alexa Flour 647 Mouse IgM isotype control was purchased from BD®.

For a full description of experimental design and methodology, please refer to chapter 5.

## Results

### Effect of Substance P on OSCC/HUVEC adhesion

We examined the effect of SP on the adhesion of OSCC to HUVEC using the Tumour-Endothelial Adhesion Assay 645 (Merckmillipore®). The protocol used is listed in the Material and Methods chapter (section 5.8). Briefly, the experimental design was established to use cells representing the different stages of cancer progression. Therefore, the cells used were DOK (pre-cancer), CAL 27 and SCC25 (negative lymph node OSCC cell lines), H157 (positive lymph node OSCC), and BICR22 (lymph node metastases). All the treatments were done in two main groups. The first group had the treatment applied to the cancer cells, whereas the second group had HUVEC cells treated with the experimental reagents. The

results showed that SP treatment increased the adhesion in H157, DOK and CAL27, whereas no effect was observed in either SCC25 or BICR22 (Table 7.1). SP treatment 1 mcg/ml ($10^{-6}$) M significantly increased the adhesion of OSCC cell lines in a time-course treatment from 1 to 4 hours (Figure 7.1-7.4). In both H157 and CAL 27 cells, the adhesion levels were higher for the HUVEC treated group. Both groups had ($P$ = 0.001).

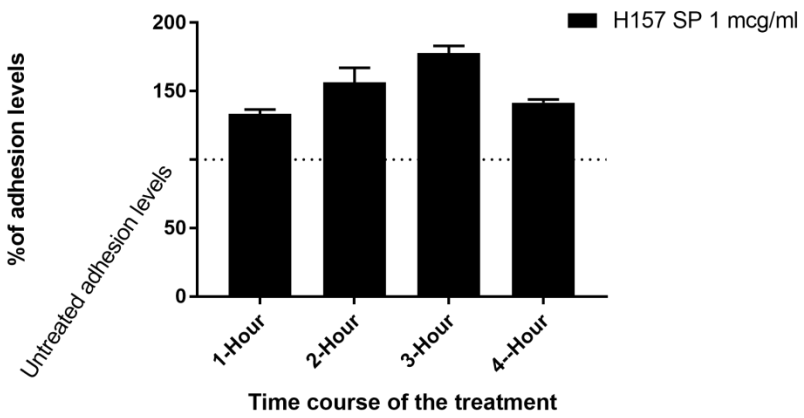



**Figure 7.1:** Effects of SP 1 mcg/ml ($10^{-6}$) M on H157 cells adhesion to HUVEC. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion. Statistical analysis established the following significance: P<0.001 vs control (untreated adhesion levels) for all the time points.

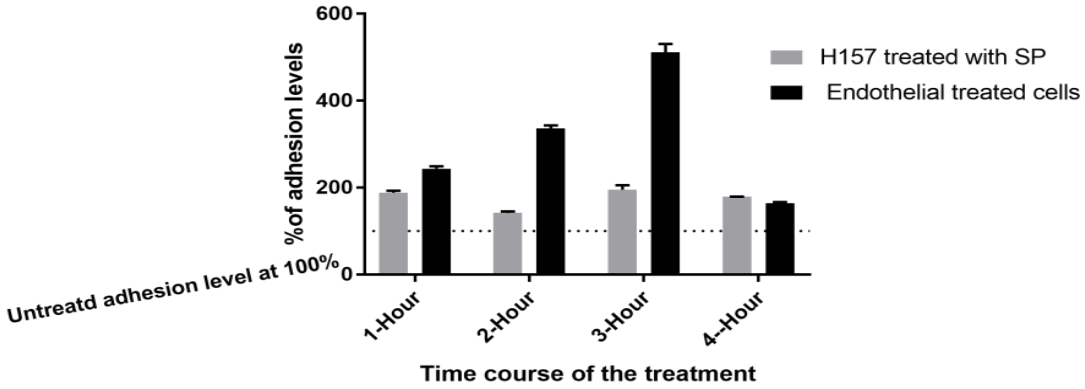



**Figure 7.2:** Effects of SP on H157 cells adhesion to HUVEC. Two experimental groups in which H157 cells were treated with 1 mcg/ml ($10^{-6}$) M, where the other group had HUVEC cells treated with the same treatment. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion. Statistical analysis established the following significance: P<0.001 vs control for all the time

points for either group, however, the group in which HUVEC cells were treated had higher values of adhesion levels.

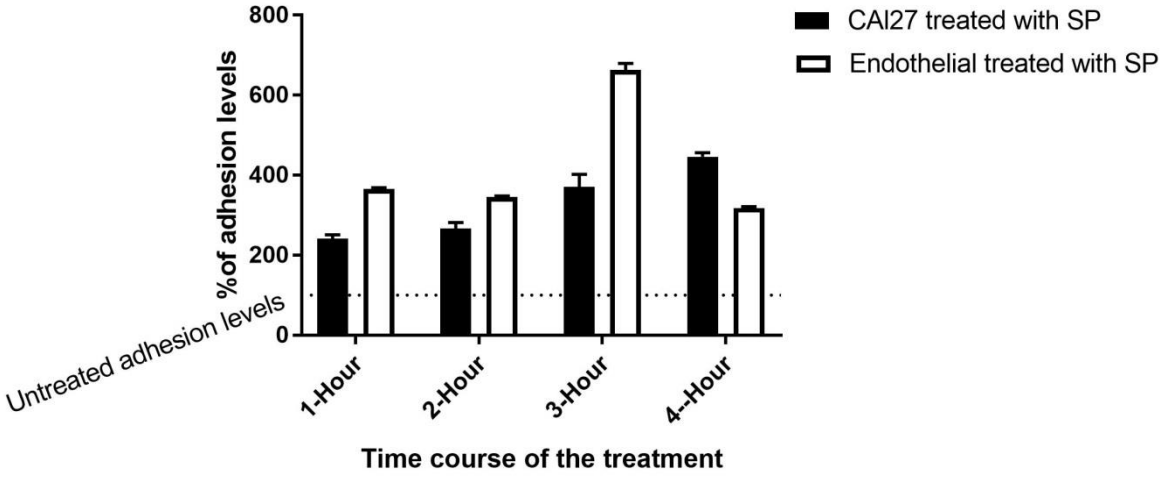



**Figure 7.3:** Effects of SP on CAL27 cells adhesion to HUVEC. Two experimental groups in which CAL27 cells were treated with 1 mcg/ml ($10^{-6}$) M, where the other group had HUVEC cells treated with the same treatment. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion. Statistical analysis established the following significance: $P<0.001$ vs control for all the time points for either group, however, the group in which HUVEC cells were treated had higher values of adhesion levels.

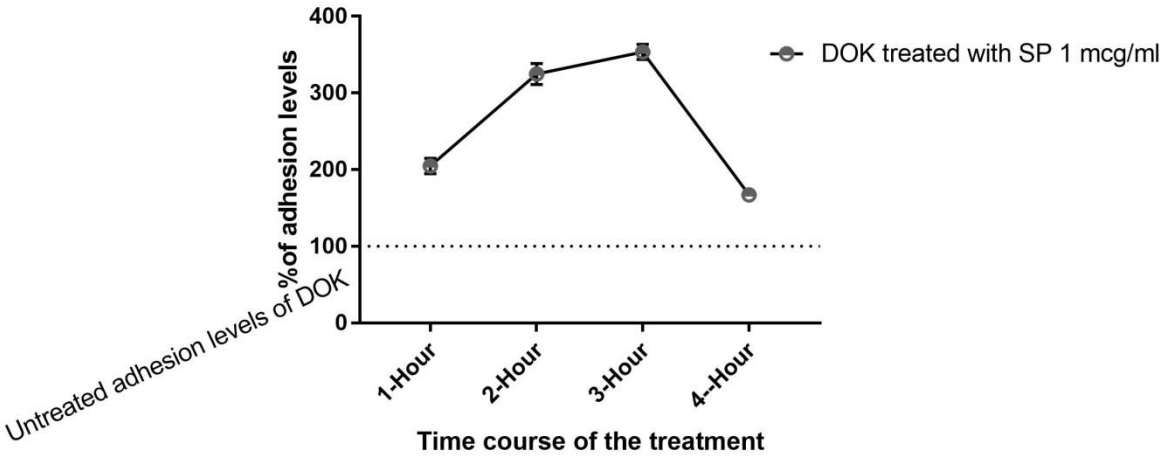



**Figure 7.4:** Effects of SP treatment 1 mcg/ml ($10^{-6}$) M on DOK cells adhesion to HUVEC. Treatment time was 1-4 hours and then both cells were incubated according to the assay protocol. Data are expressed as per cent of adhesion levels. Statistical analysis established the following significance: P<0.001 vs control for all the time points.

**Table 7.1:** Shows the *P*- value for adhesion levels of SCC 25 and BICR22 OSCC cell lines to HUVEC endothelial cells at 1, 2, 3 and 4 hour-time when treated with SP 1 mcg/ml.

| Cell Line | Time of Treatment/*P* Value for adhesion | | | |
|---|---|---|---|---|
| | 1-Hour | 2-Hour | 3-Hour | 4-Hour |
| SCC25 | 0.84 | 0.21 | 0.59 | 0.95 |
| BICR22 | 0.44 | 0.95 | 0.95 | 0.33 |

**Comparing SP and TNF-α effect on Jurkat adhesion to HUVEC**

Next, we compared the effect of SP and TNF-α treatment (as a positive control) on the levels of adhesion in OSCC lines to HUVEC. The results showed that both SP 1mcg ($10^{-6}$) M and TNF-α 100 ng ($10^{-9}$) M stimulation for 3 hours significantly increased the adhesion levels of H157 and CAL 27 to HUVEC (Figure 7.5).

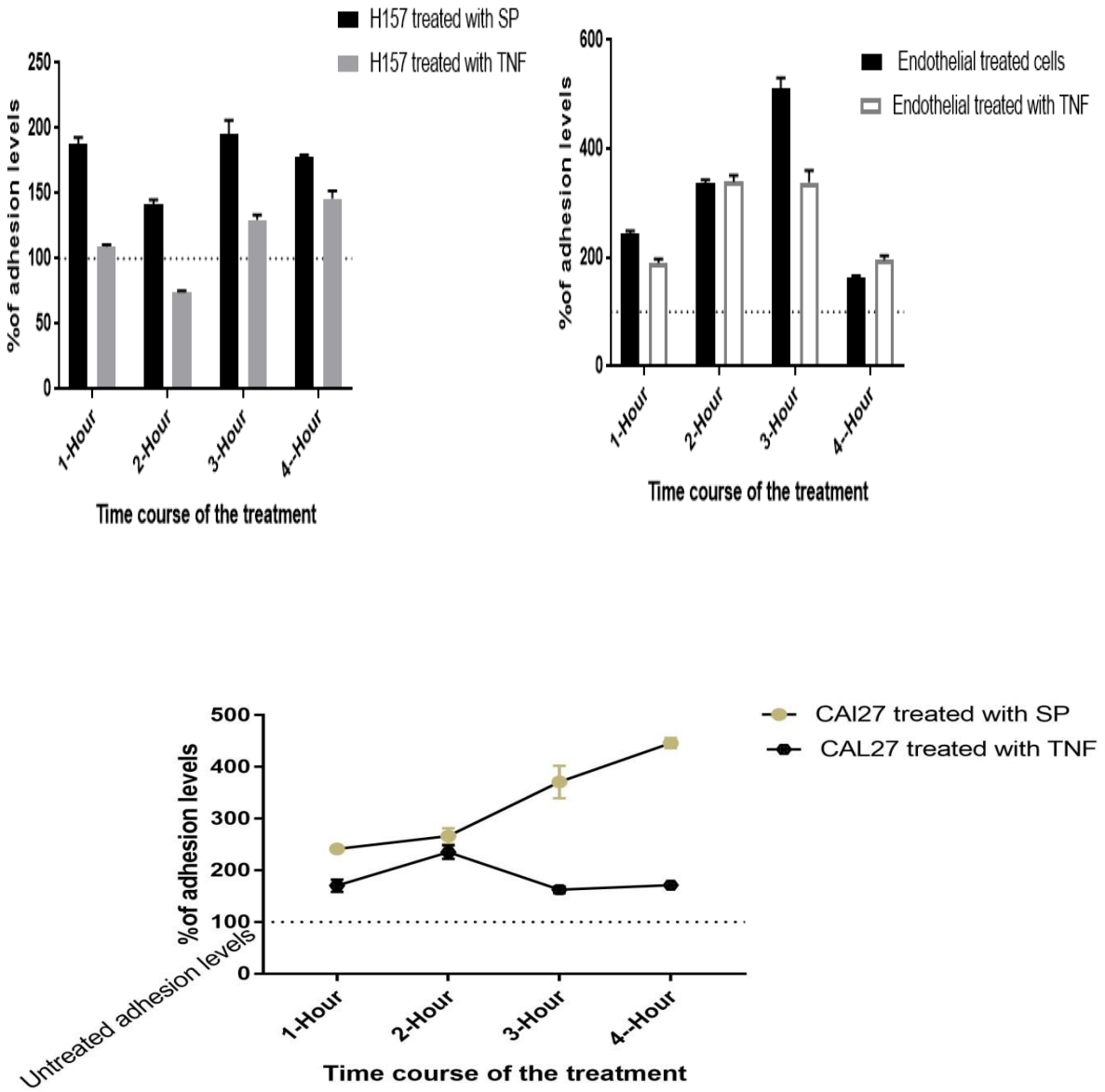
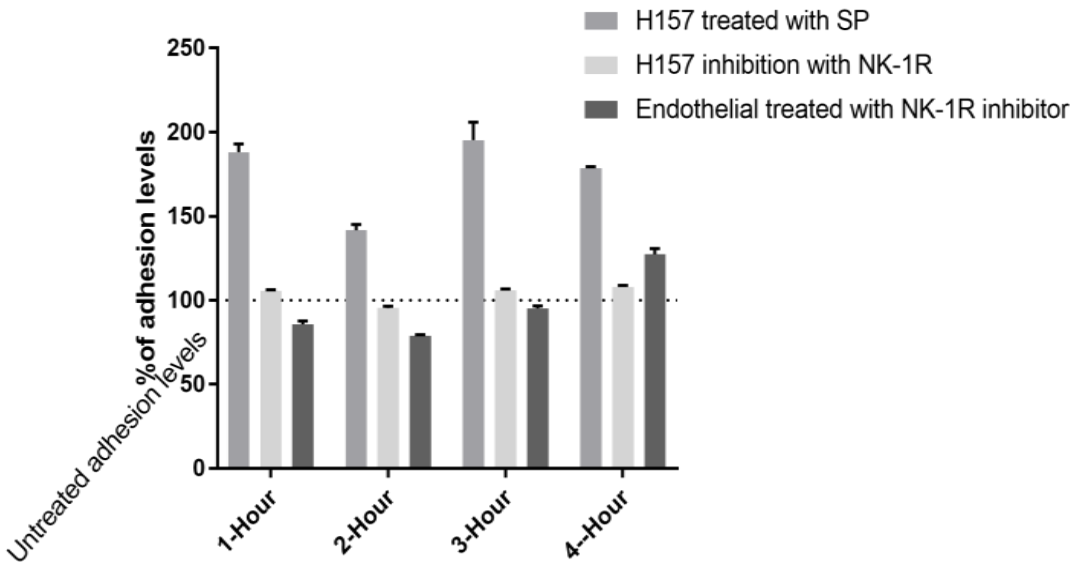



**Figure 7.5:** H157 (top) and CAL 27 (bottom) treated with SP 1mcg ($10^{-6}$) M or TNF-α 100 ng ($10^{-9}$) M at 1-4 hours adhesion levels to HUVEC. Data are represented as per cent of adhesion levels vs control adhesion (untreated adhesion levels). *P*= 0.001 for all treatments. Error bars might be smaller than some data points.

### Effect of SP receptor (NK-1R) antagonist

To investigate the effect of NK-1R on inhibiting the adhesion levels triggered by SP stimulation, we applied NK-1R antagonist to either the tumour cells or HUVEC. The data showed that the NK-1R antagonist significantly inhibited adhesion levels, reversing back to untreated baseline adhesion levels. Treating of H157 cells with SP treatment 1 mcg/ml for 1,

2, 3 or 4 hours then treating the cells with 1 mcg/ml of the NK-1R antagonist for the same time point significantly inhibited (*P=0.001*) the adhesion levels with HUVEC cells at all the time points 1-4 hours (Figure 7.6 top). Additionally, treating the HUVEC cells with SP 1 mcg/ml at 1, 2, 3 or 4 hours, then adding NK-1R, inhibited adhesion levels compared with the HUVEC treated with SP only (*P=0.001*) for all time points (Figure 7.6 bottom). The adhesion levels suppression induced by treating the HUVEC cells with NK-1R inhibitor had lower values of expression than H157 cells treated with NK-1R at 1, 2 and 3 hours. In CAL27 cells, application of NK-1R to the cells significantly inhibited the adhesion levels compared with the same cells treated with SP only, with a maximum value of suppression at the 2-hour time point (Figure 7.7). Also, the HUVEC cells treated with inhibitor suppressed adhesion significantly as compared to HUVEC cells treated with SP only, reaching a maximum at 3 hours (*P=0.001)* (Figure 7.8). The suppression of those adhesion levels brought back the adhesion levels triggered by the SP treatment to values below the untreated adhesion threshold. Similarly, adhesion of DOK cells to HUVEC was significantly suppressed by the application of NK-1R to DOK cells with a maximum at 3 hours (Figure 7.9).



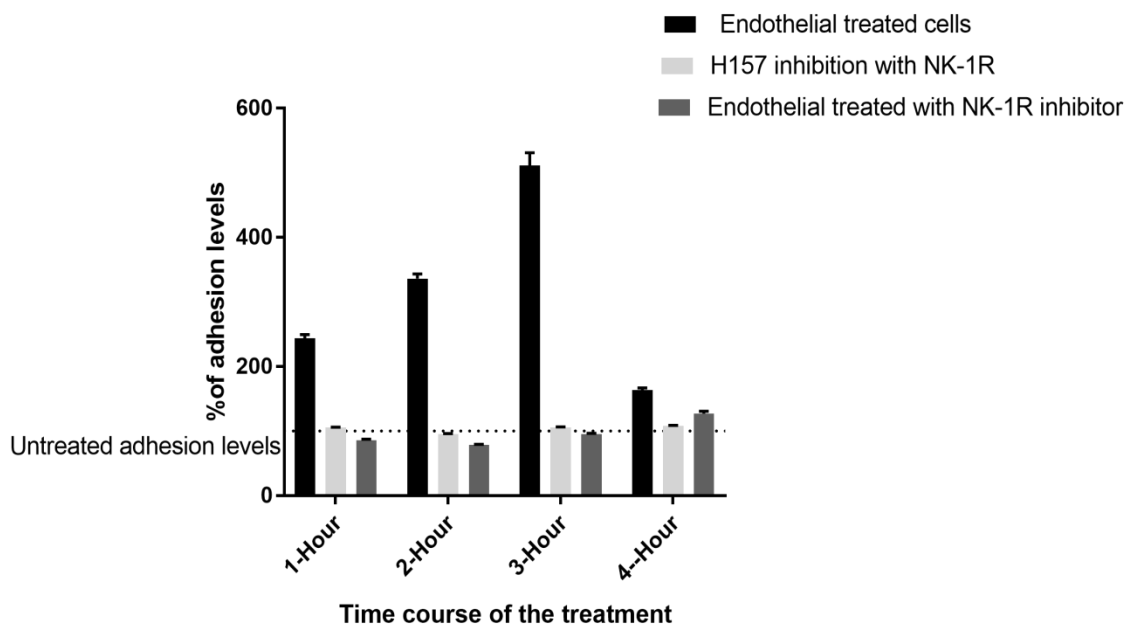



**Figure 7.6:** Effect of NK-1R antagonist on the inhibition of adhesion in H157 to HUVEC cells. Top figure compares the effect of SP 1 mcg/ ml treated H157 with the effect of the NK-1R 1 mcg/ml inhibitor applied to either H157 or HUVEC. The bottom figure compares the same inhibitor treatment in both groups with SP 1 mcg/ml HUVEC treated cells. Data represented as per cent of adhesion levels vs control adhesion (untreated). P=0.001 for all the time points (as a mean difference between SP treated and NK-1R inhibited).

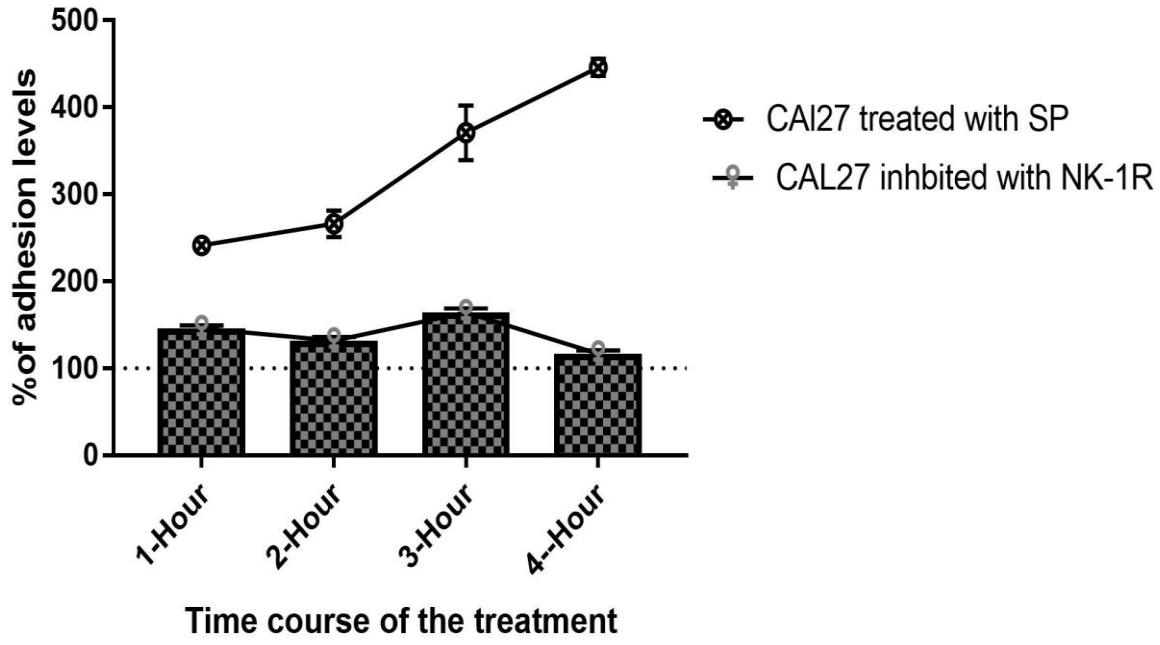



**Figure 7.7:** Effect of NK-1R antagonist on the inhibition of adhesion in CAL27 to HUVEC cells. The figure compares the effect of SP 1 mcg/ ml treated H157 with the effect of the NK-1R 1 mcg/ml inhibitor applied to CAL27. Data represented as per cent of adhesion levels vs control adhesion (untreated). *P=0.001* for all the time points (as a mean difference between SP treated and NK-1R inhibited).

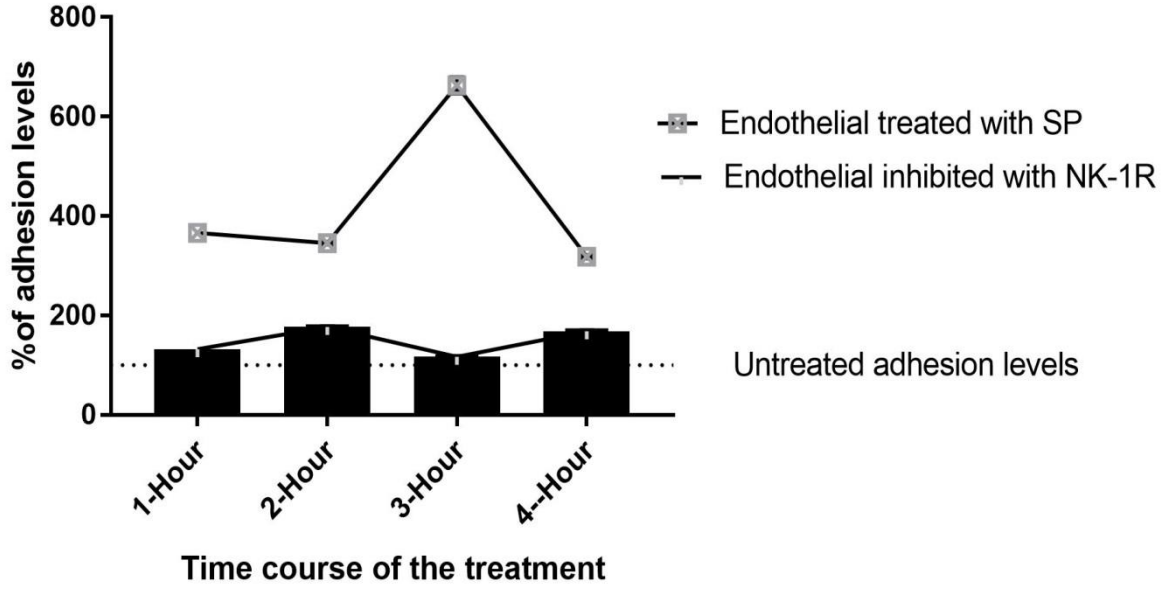



**Figure 7.8:** Effect of NK-1R antagonist on the inhibition of adhesion in CAL27 to HUVEC cells. The figure compares the effect of SP 1 mcg/ ml treated HUVEC cells with the effect of the NK-1R 1 mcg/ml inhibitor applied to HUVEC. Data represented as per cent of adhesion levels vs control adhesion (untreated). *P=0.001* for all the time points (as a mean difference between SP treated and NK-1R inhibited).

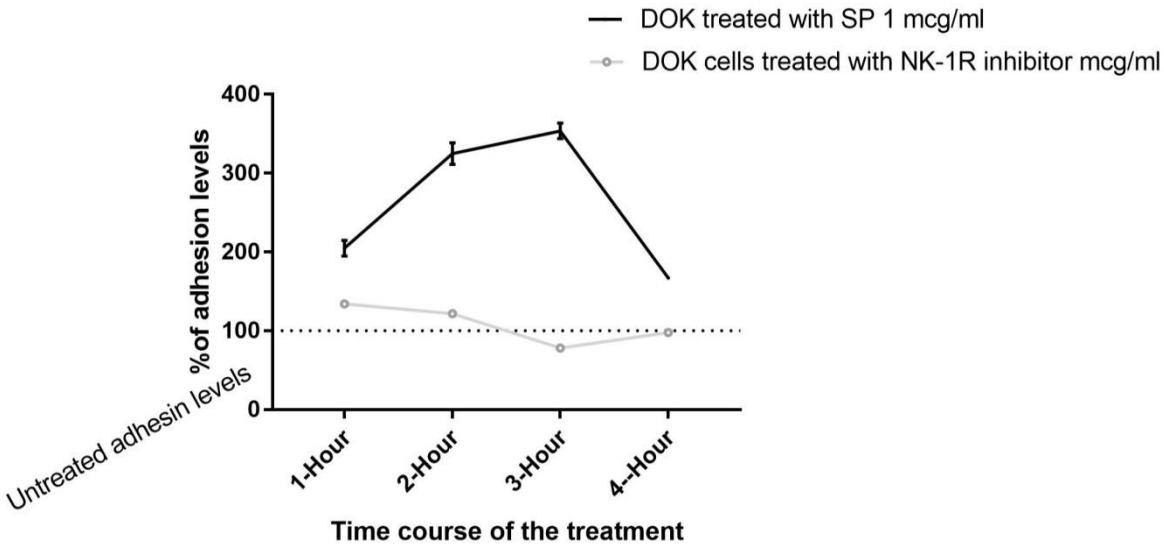



**Figure 7.9:** Effect of NK-1R antagonist on the inhibition of adhesion in DOK to HUVEC cells. The figure compares the effect of SP 1 mcg/ ml treated DOK with the effect of the NK-1R 1 mcg/ml inhibitor applied to the same cells. Data represented as per cent of adhesion levels vs control adhesion (untreated). P=0.001 for all the time points (as a mean difference between SP treated and NK-1R inhibited). Some error bars are smaller than the

corresponding data points and therefore they might not appear on the graph (All data points are represented as “Mean, SEM”).

Next, we performed a dose-response curve to investigate the effect of different concentrations of the NK-1R antagonist of the level of the adhesion. The NK-1R antagonist was used at concentrations of 100 ng, 300 ng, 750 ng and 1 mcg at time point 3-hour treatment (as this point was the average maximum for adhesion and inhibition for H157 and DOK). Application of increasing doses of NK-1R inhibitor to H157 cells produces a significant suppression of the adhesion which assumed a bell-shaped curve, with the maximum inhibitory point at 750 ng/ml and then the curve assumed a plateau (Figure 7.10 top). A similar curve was observed in DOK cells (Figure 7.10 bottom).

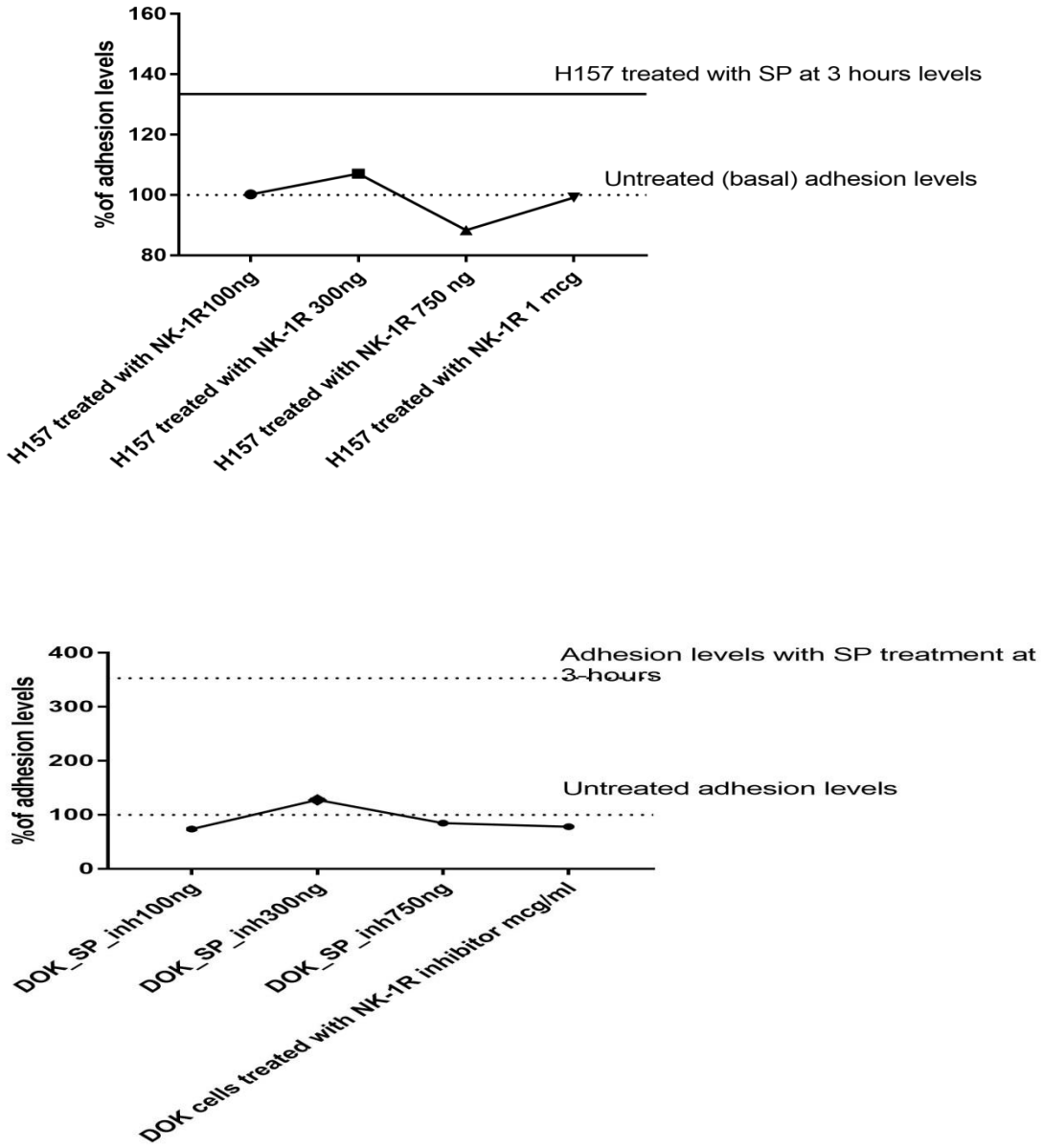
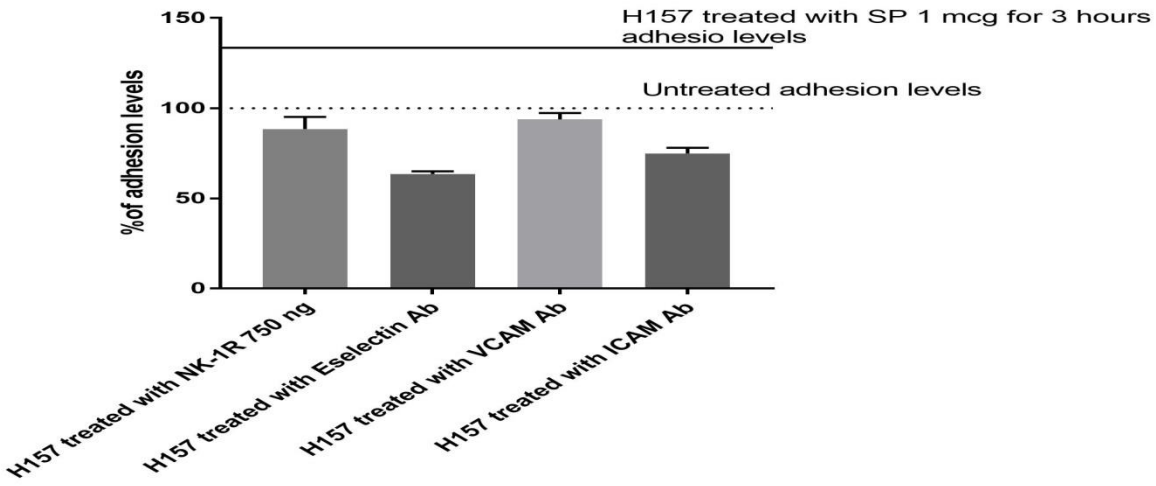



**Figure 7.10:** Dose-response curve of NK-1R antagonist at concentration 100 ng, 300 ng, 750 ng and 1mcg. Top: H157 cells adhesion levels at 3 hours showing SP stimulation levels above the untreated levels and the different concentration inhibition of adhesion by NK-1R, reversing the adhesion back to untreated basal levels. Bottom: DOK cells adhesion levels at 3 hours showing SP stimulation levels above the untreated levels and the different concentration inhibition of adhesion by NK-1R, reversing the adhesion back to untreated basal levels. Data are represented as per cent of adhesion levels versus control adhesion (untreated levels).

**Effect of adhesion molecule anti-Human Monoclonal Antibodies on the adhesion**

To investigate whether the monoclonal antibodies to the adhesion molecules had an effect on the adhesion levels stimulated by SP treatment, we used the adhesion monoclonal antibodies to E-selectin, VCAM and ICAM to test whether they can block the adhesion initiated with SP. Our data showed that in general, monoclonal antibodies to adhesion molecules suppressed the adhesion levels back to the untreated levels. However, the results were different for each cell line. In H157 cells, the maximum inhibitory effect was achieved with anti-E selectin followed by anti-ICAM which produced adhesion levels much lower than the threshold of the basal adhesion levels, while similar levels was observed for NK-1R inhibitor 750ng as well as anti-VCAM antibody at levels close to the basal untreated adhesion levels (Figure 7.11). In DOK precancer cells, the situation was completely reversed with the most potent adhesion suppressors NK-1R and anti-VCAM Mab.



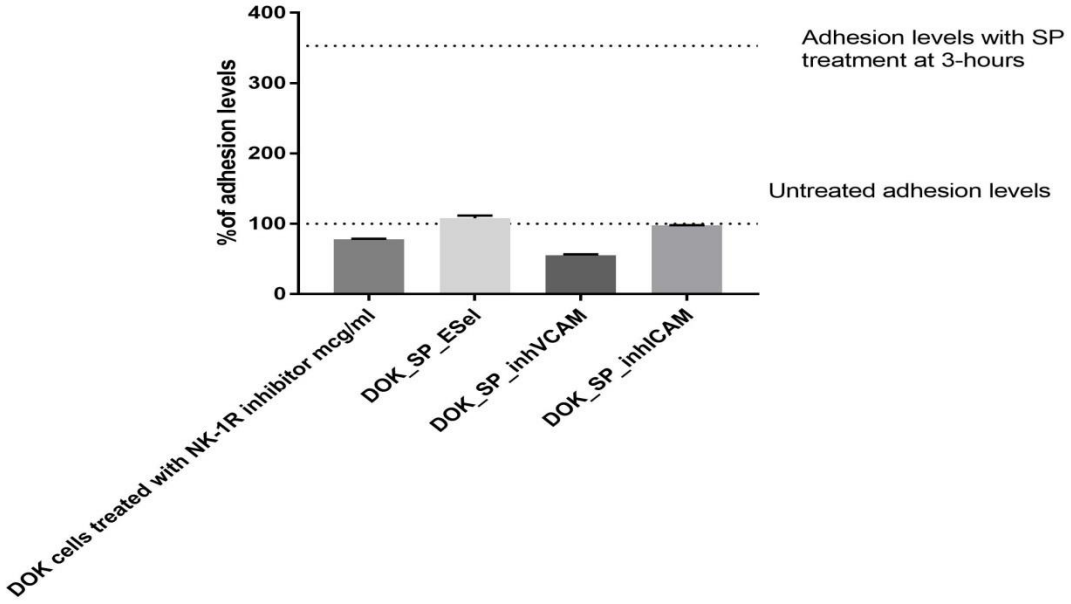



**Figure 7.11:** Comparing the effect of adhesion molecule monoclonal antibodies on the adhesion levels triggered by SP treatment to the NK-1R antagonist at time point 3 hours. Top: H157 cell line. Bottom: DOK cell lines.

**Effect of SP treatment on the adhesion molecule expression**

We hypothesised that surface adhesion molecules should be the main player in the adhesion event and that the increase in cell adhesion is attributable to induction of adhesion molecule expression on stimulated cells by either SP or TNF-α treatment. We used flow cytometry to analyse the expression of adhesion ligands (CD49, CD15s, CD11) for HUVEC adhesion receptors VCAM, E-selectin and ICAM, respectively in each cell line.

**Basal levels of adhesion molecule expression in OSCC**

First, we investigated the initial levels of those expression molecules in each cell line, according to the malignant potential, form normal, precancer, primary tumour (with/without lymph node metastases) to metastases. The untreated levels of adhesion molecule expression were plotted according to the mean value of the experimental samples for CD49, CD15s and CD11, respectively (Figure 7.12-7.14). Our results showed that each adhesion molecule expression increased significantly with the increase in the malignant potential of the cell line, ranging from a negligible expression in the normal oral keratinocyte to the more aggressive

H157 cell line with positive lymph node, and then declined in the metastases cell line BICR22.

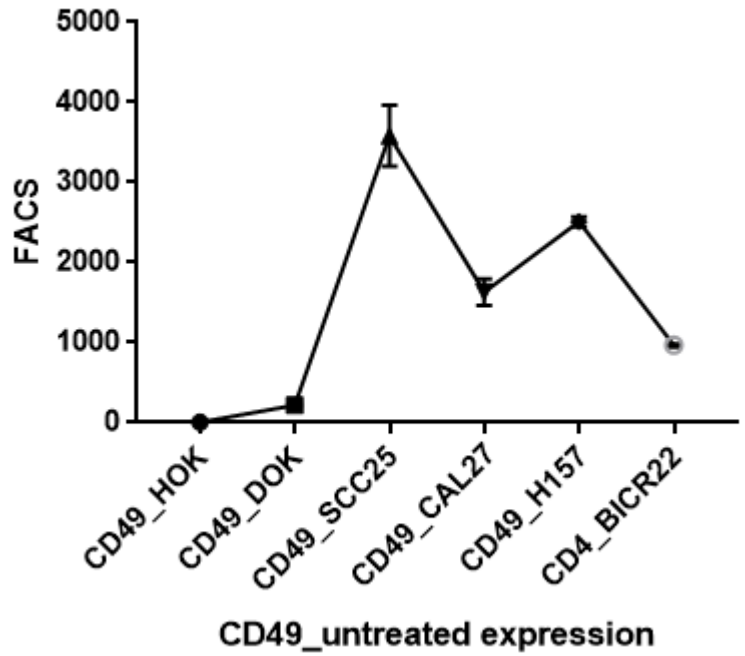



**Figure 7.12:** Untreated CD49 adhesion molecule expression levels in different stages of OSCC cell lines as illustrated by mean intensity using FACS analysis.

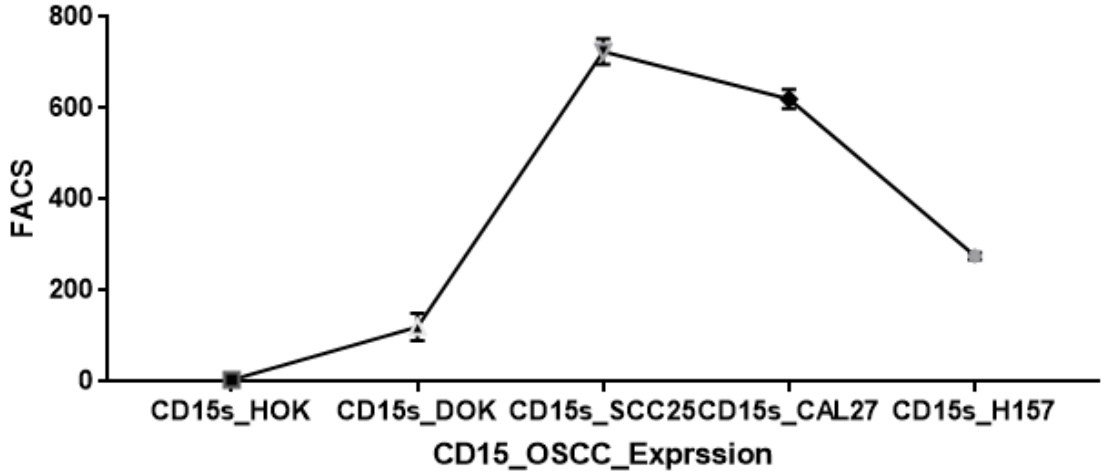



**Figure 7.13:** Untreated CD15s adhesion molecule expression levels in different stages of OSCC cell lines as illustrated by mean intensity using FACS analysis.

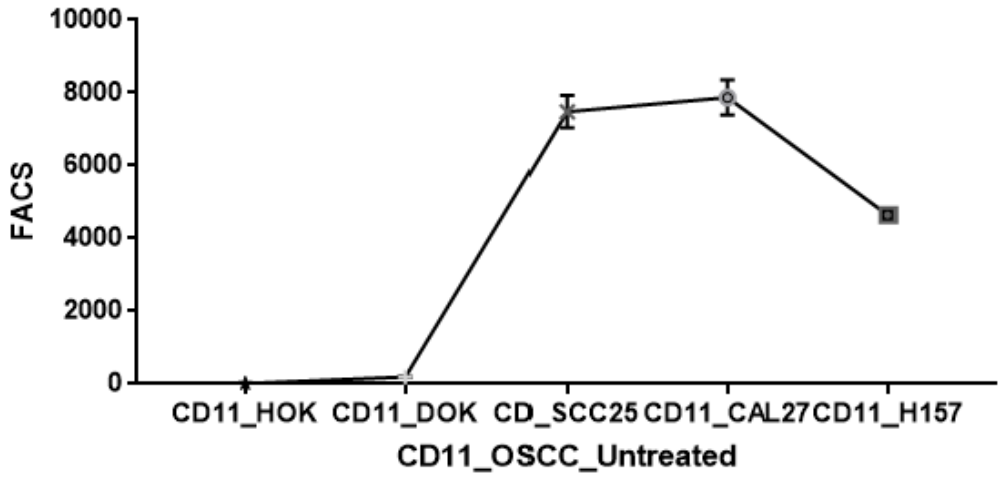



**Figure 7.14:** Untreated CD11 adhesion molecule expression levels in different stages of OSCC cell lines as illustrated by mean intensity using FACS analysis.

**Effect of SP treatment on the expression of adhesion molecules**

Our hypothesis was that if SP increased adhesion levels and, given the trend of the expression of adhesion molecules in the different cell lines, SP treatment may trigger expression of these adhesion molecules. Therefore, we performed a time-course treatment of SP 1mcg at 1-36 hours in each cell line and analysed the expression of each adhesion molecule in each cell line using the FACS cell sorter. Our results showed that SP treatment increased the expression of CD49 in CAL27 in a time-course treatment (Figure 7.15).

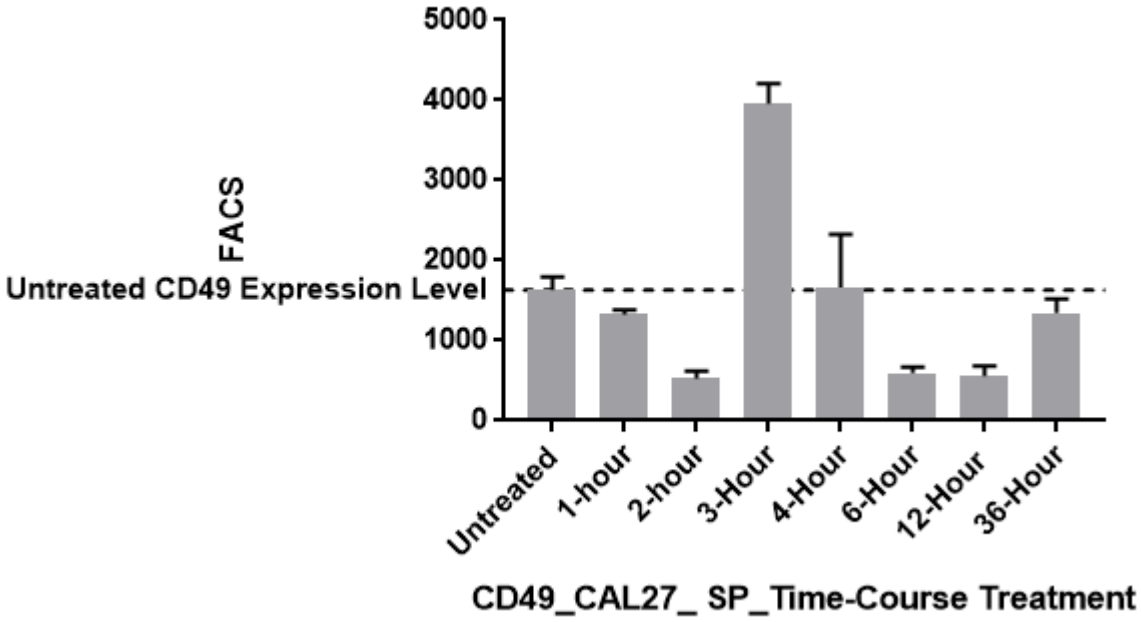



**Figure 7.15:** SP time-treatment for CAL27 cell line, showing the untreated expression level of CD49 (VLA-4).

The peak of expression increase was at 3 hours treatment and then the expression reverted to untreated levels. The 3-hour treatment significantly increased the expression of both CD11 FITC and CD49 molecules as indicated by FACS analysis in both SCC25 and BICR22, respectively (Figure 7.16).

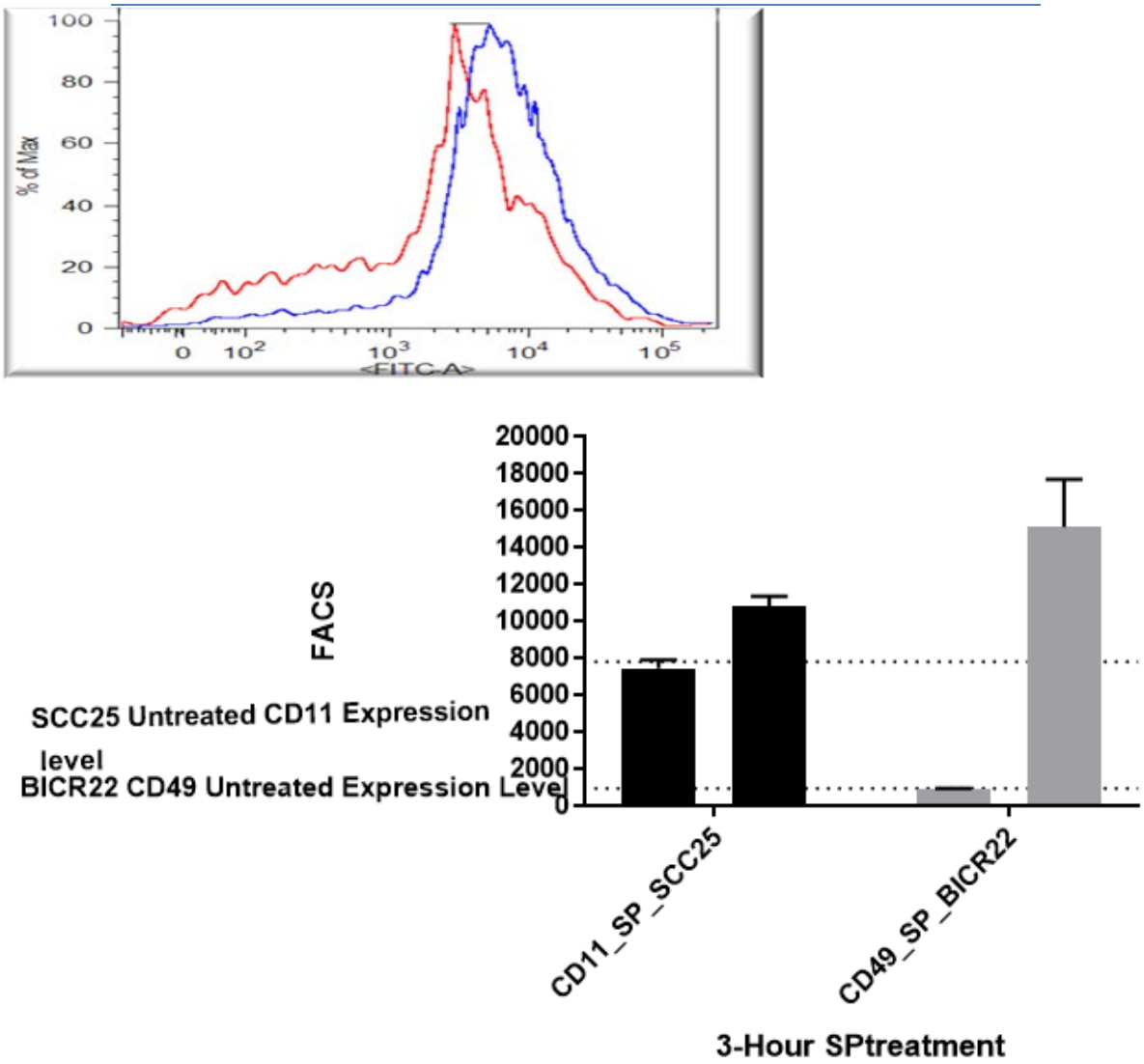



**Figure 7.16:** SP treatment effect on OSCC cell lines adhesion molecule expression; Top: FACS histogram showing CD11 FITC in SCC25 before and after treatment. Bottom: Treatment effect of SP at 3 hours on the expression of adhesion molecules CD11 and CD49 in both SCC25 and BICR22 OSCC cell lines, indicating the untreated levels.

### Comparing SP and TNF-α effect on the expression of adhesion molecules

We performed a comparison of the effects of SP (1mcg) and TNF-α (100ng) on the expression of adhesion molecules in different cell lines (Figure 7.17).

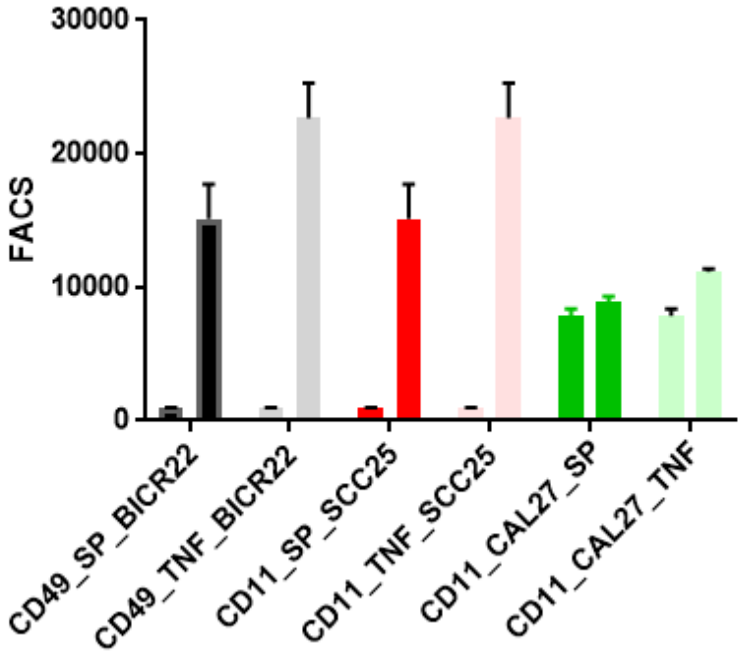



**Figure 7.17:** Comparison of SP and TNF effect on expression of different OSCC cell lines at 3-hour treatment.

**The effect of the NK-1R antagonist on the expression of adhesion molecules stimulated by SP treatment**

Finally, we investigated the effect of the NK-1R antagonist on the expression of adhesion molecules in OSCC cell lines, as we predicted that it would negate the stimulation caused by SP treatment 1 mcg. The inhibitor produced an inhibitory effect on CD49 (VLA-4) integrin in CAL27 at 3 hours adhesion in which it reverted the expression levels back to untreated levels (Figure 7.18). The NK-1R antagonist inhibited the effect of TNF-α stimulation to CD49 in the BICR22 cell line ($P$=0.002).

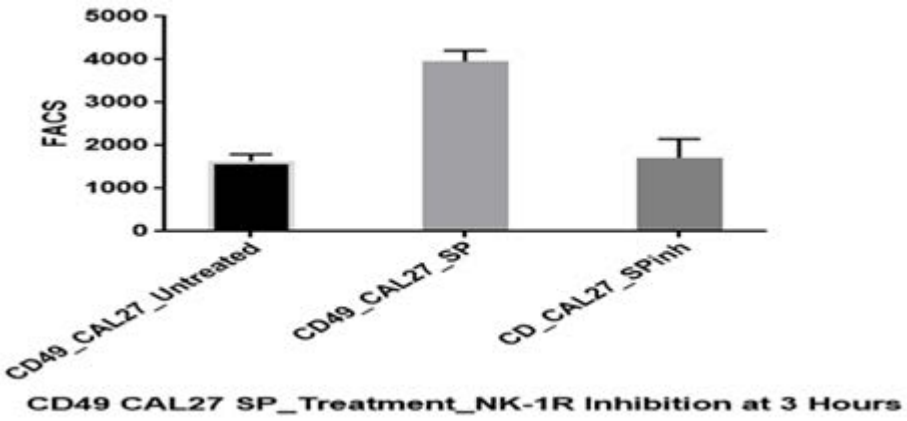



**Figure 7.18:** The figure shows the expression of CD49 in CAL27 cells (untreated levels, treated for three hours, and inhibited with NK-1R antagonist) as shown by the FACS analysis.

**Fluorescence microscope**

Using the same protocol as the endothelial adhesion assay, we investigated the effect of SP and its inhibitor NK-1R antagonist on the adhesion of DOK and H157 OSCC cell lines to HUVECs on a chamber glass with the fluorescent microscope. The result showed that the adhesion of both cell lines to HUVEC increased significantly with SP stimulation at 3 hours (Figure 7.19-7.21). By contrast, when cells were treated with NK-1R inhibitor for 3 hours, it showed significant reduction of this adhesion to ECs ($n$=20).

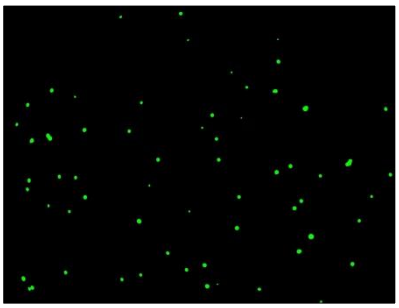


Figure 7.19: Untreated basal adhesion levels of H157 to HUVEC cell lines

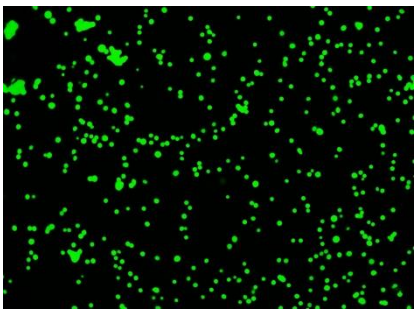


Figure 7.20: SP treatment at 3 hours adhesion levels of H157 to HUVEC

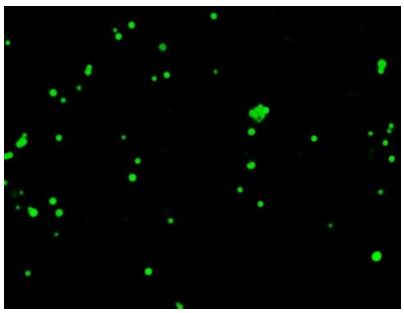


Figure 7.21: Inhibition of adhesion of H157 to HUVEC by NK-1R antagonist

**Effect of SP and TNF-α on the release of MMP-2**

Finally, we investigated the effect of SP and TNF-α treatment on the release of MMP-2 in OSCC cell lines. The experimental design involved measuring MMP-2 levels at basal (untreated levels) as well as after treatment at the 3-hour time point using the ELISA technique. Neither SP nor TNF-α treatment significantly affected the expression (results

shown in table 9). However, some interesting findings have been obtained. Firstly, the untreated expression of OSCC cell lines assumed an increasing expression of MMP-2 with the exception of DOK premalignant cells, which remarkably showed higher baseline expression of MMP-2 at a mean 1.76 ng/ml. This expression was proportional to the malignant potential of the corresponding cell lines. Secondly, SP treatment significantly decreased the expression of MMP-2 in BICR22 metastatic cell line $P = 0.003$.

**Table 7.2:** MMP-2 ELISA measurement in OSCC cell lines.

| Cell Line | Basal Expression | Treatment/*P* Value |
|---|---|---|
| DOK | ++<br>1.76 ng/ml | SP 0.36<br>TNF 0.82 |
| CAL27 | +<br>1.91 ng/ml | SP 0.45<br>TNF 0.12 |
| SCC25 | ++<br>1.51 ng/ml | SP 0.92<br>TNF 0.98 |
| H157 | +++<br>2.23. ng/ml | SP 0.32<br>TNF 0.23 |
| BICR22 | +++<br>1.85 ng/ml | SP 0.04*<br>TNF 0.78 |

## Discussion

Metastasis, the main cause of cancer patient deaths, starts with the tumour cell losing adhesion with its neighbouring cells, invading the surrounding tissues, gaining access to blood vessels, and finally adhering to and exiting the blood vessels to implant in secondary sites (402). Interaction of cancer cells with endothelial cells plays an important role in both tumour angiogenesis and metastasis (410). Recent studies support an important role for inflammation in cancer initiation, progression and metastasis. In addition, emerging studies highlight inflammation as a main driver of the different stages of metastasis, such as tumour-endothelial cell adhesion (416). However, the current literature has not identified the particular mechanism of the relationship of inflammation of metastasis or the precise effect of inflammatory mediators on adhesion and extravasation of tumour cells.

Our results showed that: 1) SP and TNF-α increased the adhesion of H157, CAL27 and DOK cells to HUVEC endothelial cells in a time-course treatment with a peak adhesion at 3 hours; 2) the endothelial treated group had higher adhesion values than the tumour treated groups; 3) NK-1R inhibitor reduced adhesion in a time and dose-dependent manner; and 4) Monoclonal anti-adhesion antibodies inhibited adhesion between OSCC and HUVEC.

These observations demonstrate a new observation of cancer-endothelial cell adhesion, occurring in the acute phase, which may be of fundamental importance to the process of metastasis. Firstly, early tumour-endothelial cell adhesion may contribute to enhanced tumour cell survival via bypassing immune attack and loss of attachment (233, 261). Secondly, it provides tumour cells with an early seeding capacity. In fact, early adhesion has been found to increase the metastatic capability of cancer cells (403). Cancer-endothelial cell adhesion has been speculated to be related to cancer progression and metastasis via a number of studies. However, the mechanisms of adhesion between cancer cells and endothelial cells and its main drivers remain unclear.

There has been a lack of research into the triggers and mechanisms underlying cancer-endothelial adhesion in an acute phase. Inflammation is the main characteristic of the tumour microenvironment and plays multifaceted and critical roles in every aspect of tumour development and progression (411). In the present study, we demonstrated for the first time that the inflammatory mediators, SP 1 mcg/ml and TNF-α -α 100 ng/ml were involved in oral cancer-endothelial cell adhesion in acute phase manner with a peak of 3 hours *in-vitro*.

It is well established that SP acts as a mitogenic stimulator for tumour growth and proliferation of tumour cells (20-22). Brener et al. described SP expression in infiltrating lymphocytes in 81.6% (*n*=71) and in peritumoural or intratumoural blood vessels in 58.1% (*n*=43), where they proposed a diffusion mechanism for SP from lymphocytes to the parenchyma of the tumour (353). Similarly, Munoz et al. identified NK-1R receptors in smooth muscle of the blood vessels surrounding the primary malignant melanoma (412). Similar results were obtained in leukaemia cell lines (349). In all those studies, the NK-1R antagonist inhibited the growth of tumour cells, suggesting a potent mitogenic action for SP/NK1R complex interaction, where several mechanisms have been proposed. The SP/NK-1R complex activates different MAPK signalling cascades such as ERK and P38 MAPK, through forming a scaffolding complex with β-arrestin (319). Additionally, the SP/NK-1R

complex can mediate other pathways such as the NF-kB pathways (413), and transactivates other tyrosine kinase receptors such as EGFR. In one recent study, Carotid artery injection of Walker 256 cells (for experimental metastasis induction) increased SP reactivity in coincidence with the primary tumour invasion of brain microvessels (414). These results can be explained in the context of SP acts as a mitogenic inducer of a mutant phenotype that selects for more invasive subpopulations of tumour cells with enhanced growth and migratory abilities that follow a concentration gradient, providing a pathway for these cells' invasion.

Based on previous observation that the close contact and adhesion of cells is a prerequisite for cell transmigration (261), we hypothesised that adhesion molecules are the main requirement of the adhesion process. Firstly, the adhesion molecule expression followed a predicted pattern which increased from no expression in the normal oral keratinocytes to the lymph node positive cell line H157 and declined in the metastatic cell lines (for the three adhesion molecules). The precancer cell line DOK had an elevated expression profile which agrees with previous studies, suggesting an early invasive/metastatic phenotype in the tumour cycle (415).

Although SP treatment increased a specific adhesion molecule as observed by FACS, the VLA-4 (α4β1 integrin) pathway was the main molecule affected with the SP treatment and may mediate the enhanced oral cancer-endothelial cell adhesion in this study. Rebhun et al. reported that B16α4+ tumours generated lymph node metastases in 80% of mice and that melanoma cells that were deficient in α4 integrins (B16α4−) were nontumorigenic (279). Okahara et al. showed that interaction between VLA-4 on tumour cells and VCAM-1 on endothelial cells is implicated in progression of metastasis *in-vivo* (416). Collectively, these data show that the α4 integrin expressed by cancer cells contributes to tumorigenesis and may also facilitate metastasis to regional lymph nodes by promoting stable adhesion of tumour cells to the lymphatic vasculature. It has been reported that cancer cell-endothelial adhesion is thought to be regulated by the mechanical properties of the cancer cells and also by the specific expression of various adhesion molecules and/or ligands to adhesion molecules on the surface of cancer cells and endothelial cells induced by inflammatory mediators (417-419). Thus, these results provided preliminary evidence for the adhesion of oral cancer cells to endothelial cells, suggesting a role for VLA-4 in this adhesion.

Our study reports a number of interesting findings; firstly that DOK precancer cells treatment with SP has produced adhesion results comparable with H157 positive lymph node cells. This indicates an early invasive phenotype in the tumour life cycle predisposing to an early metastatic potential. Secondly, although NK-1R inhibitor suppressed the adhesion, it was not as effective in suppressing the adhesion molecule expression, except in CAL 27 and BICR22. In addition, the monoclonal human antibodies to adhesion molecules had a profound inhibitory effect on the adhesion. Those results will require further investigation to underpin the exact mechanism of the tumour-cell interaction. Thirdly, the endothelial treated group with either SP or NK-1R inhibitor had more significant values of adhesion stimulation or adhesion suppression than the tumour-treated group in most cases. We suggest that this may be explained in the context of organ-specific metastasis. Finally, the NK-1R antagonist inhibited not only SP treatment but also TNF-α treatment which might be due to transactivation or for the suppression of downstream pathways, as SP and TNF-α are known to activate downstream NF-Kβ pathway (413).

Adhesion of tumour cells to endothelial cells requires subsequent steps to promote tumour cell migration and implantation in the secondary site. The MMP group of enzymes has been implicated in facilitating the tumour cell migration and invasion of the endothelial barrier (405). In our study, OSCC cell lines had a varying basal level of expression of MMP-2, which was directly proportional to its malignant potential. However, the treatment of SP or TNF-α did not significantly affect this expression. More importantly, the treatment of the BICR 22 cell line with SP treatment for 3 hours significantly decreased this expression significantly $P = 0.04$. Additionally, the DOK premalignant cell line had a high expression of MMP-2 which again suggests an early metastatic potential in tumour cycle (415). Li et al. reported a significant effect of SP on the expression of MMP-2 on mRNA level (407). This data will require further investigation to validate those results.

**Limitations**

The current study was performed *in-vitro,* using OSCC cell lines and this approach, despite providing an easy experimental approach, neglects the tumour microenvironment. Also, there is a limitation in the small sample size but the study provides a preliminary dataset that can be used for further validation in either animal or clinical studies.

In conclusion, the present study inflammatory mediator SP could promote oral cancer-endothelial cell adhesion. The SP enhanced oral cancer-endothelial cell adhesion can occur in an early onset and might be mediated through the VCAM-1/VLA-4 pathway. This adhesion was significantly suppressed with monoclonal antibodies to adhesion molecule as well as the NK-1R antagonist. These findings may provide insights into tumour microenvironment interaction and reveal new therapeutic targets for cancer prevention and treatment.

# Chapter 8 General Discussion

## Background

The last decade witnessed a revolution in the treatment of tumours. The information gained from the sheer effort of researchers worldwide led to the introduction of new therapeutics (2). However, the burden of metastasis assumed an increasing pattern alongside with a failure of treatment to provide a complete remission or control over the spread of the tumours (2). This metastatic potential is variable for different neoplasms, and some types of cancers (e.g. oral cancer and leukaemia) are highly invasive (12, 21). Therefore, in order to improve patient outcome a clear understanding of metastasis and its main mediators is critical (420).

Metastasis is a precise biological process consisting of several successive stages and requires the collaboration of several signalling pathways and molecular components (150). Metastasis commences through the invasion of the tumour to the surrounding extracellular matrix (ECM) and basement membrane (BM). Then, the detached tumour mass enters the circulation, evades the immune response and adheres to the vascular endothelial cells most likely through adhesion molecules. Finally, distant metastasis occurs when this mass extravasates and implants in a remote site (421-422). Although the mechanisms behind local invasion have been extensively studied, the stages of distant metastasis, particularly extravasation and their mechanisms are poorly understood (154-156, 423).

The accumulating research is suggesting that inflammation is more likely to act in favour of tumour initiation and metastasis (215). The extravasation of leukocytes in inflammation is well researched, and the different stages they follow have been identified (232). The process follows the main theme that cancer does not utilise novel mechanisms, but rather uses the existing biologic process in its favour. Both cancer and inflammation requires specific components, namely adhesion of migrating cells to the vascular endothelium through their adhesion molecules, activation of these adhesion molecules, and proteolytic action of matrix metalloproteinase (MMP) (156). The adherence of leukocytes to endothelial cells is a prerequisite for the inflammatory reaction. This adherence occurs mainly through a group of adhesion molecules and their receptors on both types of cells (155).

The striking similarity which exists between the outflux of tumour mass in the circulation and that of inflammatory exudates in wound healing points to the possibility that the same mediators might be utilised for both purposes (155). In this aspect, SP is a primary regulator of neurogenic inflammation, acting mainly as a trigger for vasodilatation, plasma protein extravasation and leukocyte adhesion to vascular endothelial cells (324-333, 424). SP has been proposed as a model that can explain the inflammation-tumour relationship (352), therefore, the link between the role of Substance P and the extravasation of tumour cells into the circulation represents an attractive opportunity for unravelling the metastatic mechanisms.

## Aims

The aims of the study included, firstly, performance of a systematic review to assess the association of the expression/inhibition of inflammatory mediators with the promotion/inhibition specific metastatic stages in clinical and experimental animal studies. Despite advances in the diagnostic molecular techniques, early detection of metastasis is still cumbersome (129). In addition, the emerging data describe a strong relationship between inflammation and cancer but these data remain of little value as they have not been translated for clinical use. Therefore, investigating the prognostic role of inflammatory mediators in OSCC has the potential to establish a preliminary inflammatory mediator panel that can be used for accurate and reliable prediction of patients with a high risk of metastasis.

The second aim was to investigate the role of SP in the adhesion of OSCC as well as Jurkat cells to the HUVEC endothelial cells. Adhesion of tumour cells to endothelial cells represents a milestone event in the extravasation process (204-205). The interaction of the endothelium with the neoplastic cells determines the physical point of cell exit and hence can explain specific organ metastasis (33). In addition, this adhesion is of vital importance to the survival of the tumour cell as it provides anchorage and, therefore, it can prevent cell death and induces proliferation (425).

Thirdly, we planned to investigate the effect of SP on the expression of adhesion molecules in OSCC as well as Jurkat cells. The steps of the adhesion process are mediated through a group of adhesion molecules on the migrating cells and their binding receptors on the endothelial cells (230, 426). Two large families of molecules – the selectins and integrins – are involved in the adhesive interaction which takes place in extravasation (233). The binding of these adhesion molecules to their receptors not only controls the adhesion but can inflict

morphological and functional changes to the cells involved in the adhesion and therefore represent a potential therapeutic opportunity for oncology research.

The fourth aim was to investigate the effect of SP on the release of MMP-2 in OSCC as well as Jurkat cells. MMP-2 are constantly expressed at the invasive front of the tumour, which indicates a strong correlation with invasion and metastasis (297-298). It is believed that MMPs can also regulate the pre-metastatic niche and play a key role in the angiogenesis (298-299). Other aspects of MMP in inflammation have not been thoroughly researched, such as the contribution of MMP in the extravasation of leukocytes, despite a role having been established for these enzymes in establishing a chemo-attractant gradient for immune cells which facilitates migration (303-306). Another aspect that has not been fully investigated is the relation of the inflammatory mediator's effect on these molecules and the relationship between MMPs and integrin adhesion molecules.

## Principal Findings

I: The review identified nine inflammatory mediators associated with metastasis. The 16 articles reported lymph node metastasis and one article reported both LN and DM. Four articles were clearly associated with stages of metastatic cascade and five studies were validated with *in-vivo* studies. The inflammatory mediators identified were CXCR4 (six studies), CXCL12 (SDF-1), CCR7, IL-6 (two studies each), IL-18, CCL20 (MIP-3), CXCL1 (GRO-1), CCL3, and CXCR2 (one study each).

This review systematically summarises the evidence of the prognostic role of inflammatory mediators in predicting metastasis/metastatic stages in OSCC. Additionally, it compares the available evidence from the clinical and experimental animal settings. The review is an effort to bridge the gap that was identified between the theoretical knowledge and the clinical implementation. The main aim of the review was to establish the key role of inflammation in metastasis progression as well as identifying the tumour-specific inflammatory markers that can be of potential to be used for diagnostic/prognostic purposes.

**CXCR4** is a polypeptide GPCR that binds to its conical ligand **CXCL12** and is responsible for a variety of physiological processes, e.g. lymphocyte homing, development, and vascularisation (46). It has been shown to be highly expressed by numerous types of tumours

with a significant correlation with metastasis (367). Several mechanisms have been proposed to explain its role in metastasis, mainly as a chemotactic factor which is expressed by tumour cells migrating towards an increasing CXCL12 gradient in the lymph nodes (368, 373). In addition, CXCR4 is responsible for the induction of the EMT phenotype as well as MMP2/9 expression as suggested by Albert and Lee et al. (371, 372). The CXCR4/SDF-1 axis activates the ERK/AKT/SRC family of kinases and is a major haempoetic homing factor for multipotent stem cells (372-374). However, the included articles (*n*=7 for clinical, *n*= 3 experimental) for CXCR4 and CXCL12 did not report the role of those chemokines as stem cell niches and their role in metastasis. We proposed that CXCR4 is up-regulated in tumour cells due to a hypoxic environment, and these cell populations marking a new stem cell niche acquire EMT, invasive phenotype and mitogenic signalling which gives this niche growth and migratory advantage and will be selected to migrate towards gradient of CXCL12 displayed by lymph nodes. These results suggest a strong role for CXCR4/CXCL12 for prognosis and predicting lymph node metastasis in OSCC.

**CCR7** is a trans-membrane receptor that exerts its action by binding to its ligands CCL21/19 and it is expressed by many immune cells for homing and directional movement of these cells (376). CCR7 has been reported to be associated with lymph node metastasis in several tumours (377). Similarly, Xia and Shang et al. describe a significant association of this receptor with LNM and LVD with no distant metastasis (378-379). Moreover, Adenoid cystocarcinoma, which commonly migrates through a haematogenous pathway, did not migrate to gradient concentration of CCL21. The studies suggested an adhesion between CCR7 and lymph node cells explaining the LNM tendency.

The **IL-6** cytokine can exert either an inflammatory response through autocrine signalling to its IL-6R receptor, or an anti-inflammatory effect through a paracrine signal via GP130 protein (380). Recent studies showed that IL-6 can activate several downstream pathways and is correlated with several types of cancer (381). Due to the complex network activated by IL-6, it is difficult to pinpoint its role in metastatic process. However, Shinriki et al. showed a significant correlation with IL-6 and LNM as well as a correlation with VEGF-C and lymphogenesis, suggesting a direct effect for IL-6 in the process of metastasis (382). Wang et

al. was the only study to report an association of the inflammatory mediator with distant metastasis, though it did not report specific organ preference (383). More interestingly, the relationship was a negative correlation between IL-6 mRNA and the presence of either LNM or DM. These conflicting results need further validation to pinpoint the actual role of IL-6.

The review showed that the research efforts provided an overwhelming amount of information about the involvement of inflammatory mediators in OSCC as well as other cancers (the rest of the systematic review identified other mediators in another 20 most common cancers which will be published in a series). However, the lack of follow up clinical trials to supplement the experimental studies has led to a clear gap between the experimental knowledge and the clinical translation into reliable bedside markers for prognostic or diagnostic value. Furthermore, the current understanding of metastasis and its mediators and stages still in early phase, in fact, the critical metastatic stages such as extravasation could not be detected at all in any clinical studies and this reflects a lack of proper experimental tools. It was clear from the review that it is also more likely that a panel of mediators, rather than a single one, orchestrates the metastatic cascade. Also, the systematic review revealed the inadequacy of the current research tools to investigate metastasis where the animal models represent expensive and time-consuming methods. The disadvantages include the lack of biologic simulation due to the difference between the patient and the host species. Due to these differences, inoculating such animals with tumours from human subject causes rejection of tumours by the immune system of the host. Using the knockout models does not fully represent the proper microenvironment response. All these factors results in the lag we see clinically in the understanding of the metastatic process. This in turn reflects the urgent need to develop in-vitro models that are more reproducible and precisely presenting the actual metastatic process and its dynamics in the specific patient tissue environment to gain potential information.

These three inflammatory mediators, after validation, can be adapted to develop a reliable method to accurately predict the risk of metastasis in high-risk patients. The information provided, as well as the advancement of new techniques such as microfluidic metastasis platforms, can be integrated to yield a reproducible metastatic model that can successfully replace the current experimental model based on a patient sample.

II: Our results showed that SP and TNF-α increased the adhesion of Jurkat cells in a time-course treatment with a peak adhesion increase at 3-4 hours. SP and TNF-α increased the adhesion of H157, CAL27 and DOK cells to HUVEC endothelial cells in a time-course treatment with a peak adhesion at 3 hours. It has been demonstrated that SP is expressed by several tumours and several roles have been proposed for its action in tumour growth and progression (343-347). Our study describes a new role for SP in stimulating early onset adhesion of tumour-endothelial adhesion.

These results agree with data showing a similar action for SP in neutrophil adhesion (331). Moreover, they explain an array of previous studies reporting SP/NK-1R complex expression in the peritumoural vessels in several tumours (349, 352, 412). Stucki et al. reported a static quantitative adhesion assay of leukaemic cells to endothelial cells at 0-24 hours, where they found that the significant increase in adhesion occurred by three times at 3 hours up to 24 hours (360). The authors suggested a similar mechanism for leukaemic cells to produce inflammatory stimulating factors that can accumulate more tumour cell adhesion to the endothelium. Reviewing the literature, we found that very few studies have reported a 48-72 hours adhesion effect, except for inflammation studies. The study also showed a significant inhibitory action of the NK-1R antagonist on the adhesion levels stimulated by SP and in some cases with TNF-α (though it remains to be investigated) which provides further evidence of the key role of SP in tumour-endothelial adhesion.

We found that treating endothelial cells with either stimulating factor or inhibitor produces more potent levels of adhesion which: 1) may explain the organ-specific metastasis of cancers; and 2) highlight the active interaction of tumour-endothelial cell during adhesion. Moreover, our data showed that inhibition of adhesion levels achieved using cycloheximide – which blocks translation of messenger RNA on the cytosolic 80S ribosomes but does not inhibit the organelle protein synthesis – did not achieve higher levels such as the one inflicted with monoclonal antibodies. This might suggest that tumour cells highly express adhesion molecules as well as inflammatory receptors.

Previous research has shown that SP is a potent mitogenic factor (356). Our results while proposing a new role for SP, it can be integrated with all the previous research to highlight SP as a model for the role of inflammation in metastasis. This can be viewed as SP, as an

inflammatory mediator, is utilised by tumour to increase its growth and proliferation. In doing so, SP works under selective pressure to induce a mutant phenotype selected for its invasive and migratory abilities. These new mutant phenotypes have the capacity to follow a concentration gradient and establishing metastasis. It is logical to consider that a cancer cell with multiple genetic aberrations and high mutation levels can acquire a mutated phenotype that allows these cells to mimic an acute adhesion onset similar to that of inflammatory reaction.

III: Our data showed in Jurkat cells, CAL27, and BICR22, SP 1 mcg or TNF-α 100 ng treatment has increased both CD 11 (not significant) and CD 49 (significant) but not CD15s expression in 1-48 hour timescale treatment, as indicated by the FACS analysis. Moreover, anti-human monoclonal antibodies to VCAM or ICAM significantly inhibited adhesion levels to below the untreated baseline levels. The NK-1R antagonist was only effective in inhibiting adhesion molecule expression in Jurkat and CAL27. No other effect was noted in the other OSCC cell lines.

Previous studies have reported the effect of several inflammatory mediators on the adhesion molecules and, in turn, on the tumour-endothelial adhesion and metastasis (411). As we hypothesised that adhesion molecules are the main requirement of the adhesion process, the adhesion molecule expression followed a predicted pattern which increased from no expression in the normal oral keratinocytes to the lymph node positive cell line H157 and declined in the metastases for the three adhesion molecules. The precancer cell line DOK had an elevated expression profile which agrees with previous studies, suggesting an early invasive/metastatic phenotype early in the tumour cycle.

Although SP treatment increased a specific adhesion molecule in each cell line as observed by FACS, the VLA-4 (α4β1 integrin) pathway appears to be the main molecule affected by the SP treatment and may mediate the enhanced cancer-endothelial cell adhesion in this study. This is in line with previous reports that showed the importance of VLA-4 in adhesion and metastasis, both *in-vitro* and *in-vivo* (416-418). Thus, these results provided preliminary evidence for the adhesion of oral cancer cells to endothelial cells, suggesting a role for VLA-4 in this adhesion.

Collectively, these data show that the α4 integrin expressed by cancer cells contributes to tumorigenesis and may also facilitate metastasis to regional lymph nodes by promoting stable

adhesion of tumour cells to the lymphatic vasculature. It has been suggested that cancer cell-endothelial adhesion is thought to be regulated by the mechanical arrest of the cancer cells (202-203). The current study reveals an active role of adhesion molecules and/or ligands to adhesion molecules on the surface of cancer cells and endothelial cells induced by inflammatory mediators.

Two findings reported in the study are of important significance: First, DOK (dysplastic cell line) showed high adhesion value to HUVEC endothelial cells as well as expressing high level of the adhesion molecules. Oral keratinocyte does not express adhesion molecules such as VLA-4 (integrin) except for the very basal cells attaching to the basement membrane. Instead, they demonstrate the expression of cytokeratin filament which anchors to the cadherins transmembrane proteins to form desmosomes junctions. These junctions are particularly important in the oral epithelium to withstand frictional pressures. The expression of integrin adhesion molecules is limited to the basal cells to bind to the laminin (a component of the basement membrane). The expression of integrin has been linked to motility and its expression by the precancer clones indicates that those early clones are selected for its invasive abilities. This has important implication to support the model that metastasis is a separate disease and its evolution commences early in the tumour cycle. This can reflect the emergence of new approaches for metastasis prevention and development of anti-metastatic vaccines targeting high-risk patient groups.

The second remark is that the endothelial-treated group with either SP or NK-1R inhibitor had more significant values of adhesion stimulation or adhesion suppression than the tumour-treated group in most cases. We suggest that this may be explained in the context of organ-specific metastasis. This might suggest the production of specific factors by the endothelium of the distant site which attract specific tumours (might be of similar embryogenic origin). Although this theory (142) has been proposed long time ago, a complete understanding of the factors and its relation remains to be investigated. However, this highlights the important of endothelium as an active participant in the process and that any metastatic therapy should not ignore the role of the endothelium of the distant site.

IV: Our results did not prove any significant effect for SP on the release of MMP-2 in either Jurkat cells or OSCC cell lines. This was against the predicted pattern in which we

hypothesised that SP would trigger up-regulation of MMP enzymes after adhesion to facilitate the further transmigration of tumour cells through the endothelium. Previous studies had supported this prediction (405-407) A small sample size, another effective MMP member e.g. MMP-9, or simply a tissue specific factor, might explain these results. SP treatment in the Jurkat cell line for 1 hour significantly increased the MMP-2 expression, while on the other side, 3-hour treatment of BICR22 significantly decreased this expression. Those results remain unexplained and warrant a further research for validation. Finally, as predicted, the expression of basal level MMP-2 increased in OSCC proportional to the malignant potential with a peak at BICR22 with the exception of DOK cells, which remarkably, showed a higher MMP-2 expression. This provides more evidence of early metastatic subpopulations in the tumour life cycle.

In summary, metastasis represents a clinical challenge to researchers and oncologist. Little improvements are gained with in cancer patients once the tumour has spread to other organs. Despite decades of research and clinical trials, the results are not yet promising. This lag is mainly attributed to unclear understanding of the actual dynamics of this process. Another reason is medical paradigm that is directing the oncology research considering only treating metastasis after its commencement (which is a medical ultimate goal). The formidable challenge of cancer necessitates new way of thinking. One of the most important changes to the current way of looking at cancer and metastasis is to start thinking of metastasis as an "early separate disease" which can be prevented. However, this requires changing the timing of intervention to as early stage as patients with pre-cancer lesions. Another aspect is the understanding that tumours utilise the same pathways and mechanisms of the normal physiologic functions which, if properly understood and implemented, would be a safer and successful alternative in treatment and diagnostic settings. One of these important pathways that is deployed by cancer is inflammatory mediators which continue to emerge as potent driver of cancer spread and progression. Investigating these mediators and their role in tumour progression will yield potential targets that can be implemented for new anti-metastatic therapy.

## Significance

Metastasis is the prime cause of fatality in 90% of cancer patients. The incidence of metastasis significantly reduces the prognostic outcome. More than half of neoplasms will

have disseminated lesions at the time of primary diagnosis, which render these cases with very poor survival rates. The high risk of cancer metastasis mandates aggressive therapeutic modalities in an attempt to prevent their occurrence. However, even with the most advanced treatments, no or little improvements have been achieved in the outcome of the most advanced stage cancers.

The resulting model represents a novel approach in cancer treatment where the main target is prevention of metastasis incidence. This paradigm shift has powerful potential in the development of effective anti-metastatic therapies through interfering with the metastatic cycle. The data resulting from the systematic review as well as the experimental findings can be integrated for future implementation in two main categories.

First, as the systematic review shows that the animal experiments are expensive and time-consuming, the problem can be overcome with the establishment of 3D microfluidic devices that can mimic the biological system, with representation of almost all the involved physiologic factors implicated in the cancer dissemination process. These devices are facilitated by advances in nanotechnology as well as imaging techniques. A miniature microfluidic device designed with a sample of patient tissue, including the blood vessels and expected destination of metastasis, can be an effective tool in early diagnosis of the potential for metastasis through measuring the effect and expression of various inflammatory factors that can predispose to cancer spread. The tool is significant in respect of ultimate personalised diagnosis of the particular patient in terms of his own immune response and tumour microenvironment.

Second, the implementation of such diagnostic tools will reveal the obscure inflammatory mediator network and, therefore, accumulation and better understanding of such information can pave the way for implementation of “Metastasis Early Prevention and Detection” programs. These programs could cause a shift in the scientific paradigm for metastasis management as a separate entity parallel with the primary tumour, but not strictly the same disease. These programs will specially target high-risk group patients in particular as well as precancer, recently diagnosed (before the initiation of treatment), high-risk of genetic susceptibility and patients who have successfully completed their treatment for primary tumours. This will include identifying personalised mediators in those patients and placing them on those vaccines for metastasis prevention for a period of time that can be determined

through clinical trials. This will greatly reduce the risk of metastasis in those patients and thus improve the overall survival of those patients.

## Future research

The research results provide a starting point to pursue an investigation in the role of inflammatory mediators in the metastatic progression of tumours through intervening with the adhesion with the endothelium. The preliminary data shows the feasibility of validating those results through animal models and clinical trials. The confirmation of these results will require the validation with dynamic vascular systems (such as in animals or artificial dynamic vascular devices) to represent the actual physiologic blood circulation. This will enable the visualisation and the proper quantification of the effect of such mediators on the adhesion of tumour cells and the subsequent metastasis. Once these results have been validated and confirmed through these animal experiments, research then can proceed in three directions:

I- Follow up with clinical trials where these mediators can be used in the intervention for high-risk patient groups outlined above as metastatic vaccine to prevent the occurrence of metastasis in those patients.

II- implementation of these inflammatory mediators resulted from the systematic review to develop a reliable experimental/prognostic tool which integrate the patient own tissue in a miniature devices that can be used to predict the possibility of metastatic progression in the patient with high degree of accuracy and examine the specific mediators responsible for this progression.

III- Investigating other tumour types and identifying the specific inflammatory mediator responsible for progressing each tumour type as well as investigating the relation of the circulating tumour cells with cancer stem/initiating cells.

## Conclusion

The systematic review identified nine inflammatory mediators associated with metastasis from the clinical and experimental studies which can be further validated for a diagnostic panel implementation for the early detection of metastasis. Our results showed that SP and TNF-α increased the adhesion of Jurkat cells in a time-course treatment with a peak adhesion increase at 3-4 hours. Similarly, SP and TNF-α have increased the adhesion of H157, CAL27

and DOK cells to HUVEC endothelial cells in a time-course treatment with a peak adhesion at 3 hours. Our data showed in Jurkat cells, CAL27, and BICR22, SP 1mcg or TNF-α 100ng treatment has increased both CD11 (not significant) and CD49 (significant) but not CD15s expression in 1-48 hour timescale treatment, as indicated by the FACS analysis. Moreover, anti-human monoclonal antibodies to VCAM or ICAM significantly inhibited the adhesion levels to below untreated baseline levels. The NK-1R antagonist was only effective in inhibiting adhesion molecule expression in Jurkat and CAL27. Our results did not prove any significant effect for SP on the release of MMP-2 in either Jurkat cells or OSCC cell lines.

The study results highlights two main concepts; first, the quest for search of alternative targets via biologic body mechanisms, in particular inflammation promise potential therapies. Second, metastasis should be investigated as a separate disease entity rather than a end result of the cancerous process, and it might commence as early as the tumour itself. With this two concepts in mind, a new role for substance P has been described. This inflammatory mediator can be used in the intervention/prevention of metastasis.

The results of the study can be integrated to provide a model for early prediction as well as prevention of metastatic spread which can be implemented in high-risk group cancer patients. This will advance our knowledge about the metastatic process as well as giving advantage to the clinicians. Additionally, it can help as a guide to investigating other tumours, searching for similar panels that can be of high value as they would be more tumour specific. Finally, it provides a rationale as well as an evidence for the implementation of “metastasis early prevention” programs.

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# List of Presentations and Publications

## Presentations:

Elhousiny, M. (September, 2014). *The effect of Substance P on adhesion/metastasis of oral cancer.* Presented at Annual Scientific Meeting of the ANZ Division, Brisbane, Australia.

Elhousiny, M. (November, 2014). *The effect of substance P (SP) on adhesion of squamous carcinoma cells (SCC) to vascular endothelial cells and role in metastasis.* Presented at North Queensland Festival of Life Sciences, Division of Tropical Health & Medicine. The Northern Clinical Training Network, Townsville Hospital and Health Service.

Elhousiny, M. (March, 2015). *The effect of substance P (SP) on adhesion of squamous carcinoma cells (SCC) to vascular endothelial cells and role in metastasis.* Poster session at QIMR institute National Research 2015. Brisbane, Australia.

Elhousiny, M. (April, 2017). *The effect of Substance P (SP) on the adhesion of squamous carcinoma cells (SCC) and Jurkat cells to vascular endothelial cells and role in metastasis.* Presented at Cancer Metastasis Through The Lymphovascular System: Biology & Treatment 7th International Symposium - April 20-22, 2017 San Francisco, CA, USA.

## Publications in progress:

Elhousiny, M. Miller, K. Ariyawardana, A. Nimmo, A (20118). *The prognostic role of inflammatory mediators in metastasis of oral squamous cell carcinoma, comparison of clinical and experimental evidence: Systematic review.* Clinical and Experimental Metastasis.

Elhousiny, M. Miller, K. Ariyawardana, A. Nimmo, A (20118). *Substance P (SP) increases the adhesion of Jurkat cells to Human Vascular Endothelial Cells (HUVEC) Through upregulating adhesion molecules: Implication for Metastasis.* Leukaemia Research.

Elhousiny, M. Miller, K. Ariyawardana, A. Nimmo, A (20118). *Substance P (SP) increases the adhesion of Oral Cancer Cell Lines (OSCC) to Human Vascular Endothelial Cells (HUVEC) Through upregulating adhesion molecules: Implication for Metastasis.* Oral Oncology.

# Appendix

**Appendix 1**

Guidelines for classifications of neck dissections (Robbins, K T. 2001)

| **CONCEPTUAL GUIDELINES FOR NECK DISSECTION CLASSIFICATION** |
|---|
| 1. Radical neck dissection is considered to be the standard basic procedure for cervical lymphadenopathy. All other procedures represent one or more alterations of this procedure. |
| 2. When the alteration involves preservation of one or more nonlymphatic structures routinely removed in the radical neck dissection, the procedure is termed modified radical neck dissection. |
| 3. When the alteration involves preservation of one or more lymph node groups routinely removed in the radical neck dissection, the procedure is termed selective neck dissection. |
| 4. When the alteration involves removal of additional lymph node groups or non-lymphatic structures not typically removed in the radical neck dissection, the procedure is termed an extended radical neck dissection. |

**Appendix 2**

**Query Terms and Results on PubMed**

| Search | Query | Items found |
|---|---|---|
| #118 | Search (neurokinin) AND metastasis | 2263 |
| #115 | Search (interleukin) AND metastasis | 4236 |
| #114 | Search (chemokine) AND metastasis | 3070 |
| #113 | Search ("inflammatory cytokines") AND metastasis | 315 |
| #112 | Search (("expression of  neurokinin") AND metastasis) AND prognosis | 1 |
| #111 | Search (("expression of  neurokinin") AND cancer) AND prognosis | 6 |
| #110 | Search ("expression of neurokinin") AND metastatic stages | 2 |
| #109 | Search ("expression of neurokinin") AND metastasis | 13 |
| #108 | Search (neurokinin) AND Metastasis formation at secondary site | 2 |
| #107 | Search (neurokinin) AND tumour Extravasation | 10 |
| #106 | Search (neurokinin) AND tumour angiogenesis | 16 |
| #105 | Search(neurokinin) AND epithelial-mesenchymal transition | 0 |
| #104 | Search (neurokinin) AND tumour invasion | 15 |
| #103 | Search (neurokinin) AND metastatic stages | 4 |
| #102 | Search (neurokinin) AND metastatic cascade | 1 |
| #101 | Search neurokinin and tumor progression ("neurokinin a"[MeSH Terms] OR "neurokinin a"[All Fields] OR "neurokinin"[All Fields]) AND ("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("disease progression"[MeSH Terms] OR ("disease"[All Fields] AND "progression"[All Fields]) OR "disease progression"[All Fields] OR "progression"[All Fields]) | 33 |
| #100 | Search "expression of neuropeptide" | 1434 |
| #99 | Search neuropeptide | 8249 |
| #70 | Search (((((expression of(interleukin) AND metastasis) AND prognosis))) | 385 |
| #69 | Search ((((expression of(interleukin) AND cancer) AND prognosis)) | 1675 |
| #68 | Search (expression of interleukin) AND metastasis | 1855 |
| #67 | Search (expression of interleukin) AND metastatic stages | 95 |
| #66 | Search (interleukin) AND Metastasis formation at secondary site | 84 |
| #65 | Search (interleukin) AND tumour Extravasation | 261 |
| #64 | Search (interleukin) AND tumour angiogenesis | 2579 |
| #63 | Search (interleukin) AND epithelial-mesenchymal transition | 345 |
| #62 | Search (interleukin) AND tumour invasion | 1778 |
| #61 | Search (interleukin) AND metastatic stages | 282 |
| #60 | Search (interleukin) AND metastatic cascade | 153 |
| #59 | Search (interleukin) AND #1 | 801 |
| #58 | Search expression of interleukin | 109839 |
| #57 | Search interleukin | 276059 |
| #56 | Search (((expression of chemokine) AND metastasis) AND prognosis) | 507 |
| #55 | Search (((expression of chemokine) AND cancer) AND prognosis) | 1106 |
| #54 | Search (expression of chemokine) AND metastatic stages (("gene | 68 |

| | | |
|---|---|---|
| | expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("chemokines"[MeSH Terms] OR "chemokines"[All Fields] OR "chemokine"[All Fields])) AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND stages[All Fields]) | |
| #53 | Search (chemokine) AND Metastasis formation at secondary site | 100 |
| #52 | Search (chemokine) AND tumour Extravasation ("chemokines"[MeSH Terms] OR "chemokines"[All Fields] OR "chemokine"[All Fields]) AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND Extravasation[All Fields]) | 157 |
| #51 | Search (chemokine) AND tumour angiogenesis ("chemokines"[MeSH Terms] OR "chemokines"[All Fields] OR "chemokine"[All Fields]) AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("Angiogenesis"[Journal] OR "angiogenesis"[All Fields])) | 1988 |
| #50 | Search (chemokine) AND epithelial-mesenchymal transition ("chemokines"[MeSH Terms] OR "chemokines"[All Fields] OR "chemokine"[All Fields]) AND ("epithelial-mesenchymal transition"[MeSH Terms] OR ("epithelial-mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields] OR ("epithelial"[All Fields] AND "mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields]) | 253 |
| #49 | Search (chemokine) AND tumour invasion | 1602 |
| #48 | Search (chemokine) AND metastatic stages ("chemokines"[MeSH Terms] OR "chemokines"[All Fields] OR "chemokine"[All Fields]) AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND stages[All Fields] | 129 |
| #47 | Search (chemokine) AND #2 | 87 |
| #46 | Search (chemokine) AND #1 | 3152 |
| #45 | Search expression of chemokine | 41487 |
| #44 | Search chemokine | 84418 |
| #43 | Search (((expression of cytokines) AND metastasis) AND prognosis)(("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("cytokines"[MeSH Terms] OR "cytokines"[All Fields])) AND ("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) AND ("prognosis"[MeSH Terms] OR "prognosis"[All Fields]) | 1414 |
| #42 | Search (((((expression of cytokines) AND cancer)) AND cancer) AND prognosis) (("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("cytokines"[MeSH Terms] OR "cytokines"[All Fields])) AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) AND ("prognosis"[MeSH Terms] OR "prognosis"[All Fields]) | 4704 |
| #41 | Search ((expression of cytokines) AND cancer) (("gene expression"[MeSH | 51915 |

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|---|---|---|
| | Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("cytokines"[MeSH Terms] OR "cytokines"[All Fields])) AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) | |
| #40 | Search "expression of cytokines" | 234020 |
| #39 | Search (("inflammatory cytokines") AND tumour progression) "inflammatory cytokines"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("disease progression"[MeSH Terms] OR ("disease"[All Fields] AND "progression"[All Fields]) OR "disease progression"[All Fields] OR "progression"[All Fields])) | 1024 |
| #38 | Search (("inflammatory cytokines") AND metastatic cascade) "inflammatory cytokines"[All Fields] AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND cascade[All Fields]) | 47 |
| #37 | Search (("inflammatory cytokines") AND metastatic stages)"inflammatory cytokines"[All Fields] AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND stages[All Fields]) | 38 |
| #36 | Search (("inflammatory cytokines") AND tumour invasion) "inflammatory cytokines"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND invasion[All Fields]) | 246 |
| #35 | Search (("inflammatory cytokines") AND epithelial-mesenchymal transition) "inflammatory cytokines"[All Fields] AND ("epithelial-mesenchymal transition"[MeSH Terms] OR ("epithelial-mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields] OR ("epithelial"[All Fields] AND "mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields]) | 75 |
| #34 | Search (("inflammatory cytokines") AND tumour angiogenesis) "inflammatory cytokines"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("Angiogenesis"[Journal] OR "angiogenesis"[All Fields])) | 313 |
| #33 | Search (("inflammatory cytokines") AND tumour Extravasation) "inflammatory cytokines"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND Extravasation[All Fields]) | 38 |
| #32 | Search (("inflammatory cytokines") AND Metastasis formation at secondary site) "inflammatory cytokines"[All Fields] AND (("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) AND ("metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "formation"[All Fields]) AND ("secondary"[Subheading] OR "secondary"[All Fields] OR "neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields]) AND site[All Fields]) | 5 |
| #31 | Search "inflammatory cytokines" | 32637 |
| #30 | Search ((((("inflammatory mediators") AND expression)) AND metastatic stages) ("inflammatory mediators"[All Fields] AND ("gene | 4 |

| | | |
|---|---|---|
| | expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields])) AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND stages[All Fields]) | |
| #29 | Search ((((("inflammatory mediators") AND expression)) AND metastasis) ("inflammatory mediators"[All Fields] AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields])) AND ("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) | 61 |
| #28 | Search (((((("inflammatory mediators") AND expression)) AND metastasis) AND "metastatic stages") Schema: all | 0 |
| #27 | Search (((((("inflammatory mediators") AND expression)) AND metastasis) AND "metastatic stages") | 0 |
| #26 | Search (("inflammatory mediators") AND tumour progression) "inflammatory mediators"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("disease progression"[MeSH Terms] OR ("disease"[All Fields] AND "progression"[All Fields]) OR "disease progression"[All Fields] OR "progression"[All Fields])) | 466 |
| #25 | Search (("inflammatory mediators") AND metastatic cascade) "inflammatory mediators"[All Fields] AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND cascade[All Fields]) | 30 |
| #24 | Search (("inflammatory mediators") AND metastatic stages) "inflammatory mediators"[All Fields] AND (("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND stages[All Fields]) | 15 |
| #23 | Search (("inflammatory mediators") AND tumour invasion) "inflammatory mediators"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND invasion[All Fields]) | 99 |
| #22 | Search (("inflammatory mediators") AND epithelial-mesenchymal transition) "inflammatory mediators"[All Fields] AND ("epithelial-mesenchymal transition"[MeSH Terms] OR ("epithelial-mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields] OR ("epithelial"[All Fields] AND "mesenchymal"[All Fields] AND "transition"[All Fields]) OR "epithelial-mesenchymal transition"[All Fields]) | 24 |
| #21 | Search (("inflammatory mediators") AND tumour angiogenesis) "inflammatory mediators"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("Angiogenesis"[Journal] OR "angiogenesis"[All Fields])) | 142 |
| #20 | Search (("inflammatory mediators") AND tumour Extravasation) "inflammatory mediators"[All Fields] AND (("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND Extravasation[All Fields]) | 24 |
| #19 | Search (("inflammatory mediators") AND Metastasis formation at secondary site) "inflammatory mediators"[All Fields] AND (("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) | 2 |

| | | |
|---|---|---|
| | AND ("metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "formation"[All Fields]) AND ("secondary"[Subheading] OR "secondary"[All Fields] OR "neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields]) AND site[All Fields]) | |
| #18 | Search ((((("inflammatory mediators"))) AND expression) AND metastasis "inflammatory mediators"[All Fields] AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) | 61 |
| #17 | Search (((((("inflammatory mediators"))) AND expression) AND cancer) AND prognosis "inflammatory mediators"[All Fields] AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields]) AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) AND ("prognosis"[MeSH Terms] OR "prognosis"[All Fields]) | 34 |
| #16 | Search ((((("inflammatory mediators"))) AND expression) AND cancer ("inflammatory mediators"[All Fields] AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields])) AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) | 613 |
| #15 | Search ((("inflammatory mediators"))) AND expression | 6846 |
| #14 | Search ((("inflammatory mediator"))) AND metastasis "inflammatory mediator"[All Fields] AND ("neoplasm metastasis"[MeSH Terms] OR ("neoplasm"[All Fields] AND "metastasis"[All Fields]) OR "neoplasm metastasis"[All Fields] OR "metastasis"[All Fields]) | 20 |
| #13 | Search ((("inflammatory mediator"))) AND cancer "inflammatory mediator"[All Fields] AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) | 246 |
| #12 | Search metastasis | 276521 |
| #11 | Search (("inflammatory mediator")) AND cancer "inflammatory mediator"[All Fields] AND ("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]) | 246 |
| #10 | Search cancer | 3229556 |
| #9 | Search "inflammatory mediators" | 18150 |
| #8 | Search Metastasis formation at secondary site | 2405 |
| #7 | Search tumour Extravasation | 3082 |
| #6 | Search tumour angiogenesis | 45654 |
| #5 | Search epithelial-mesenchymal transition | 12471 |
| #4 | Search tumour invasion | 84361 |
| #3 | Search metastatic stages ("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND cascade[All Fields] | 16368 |
| #2 | Search metastatic cascade("secondary"[Subheading] OR "secondary"[All Fields] OR "metastatic"[All Fields]) AND cascade[All Fields] | 3467 |

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|---|---|---|
| #1 | Search tumour progression ("tumour"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "tumor"[All Fields]) AND ("disease progression"[MeSH Terms] OR ("disease"[All Fields] AND "progression"[All Fields]) OR "disease progression"[All Fields] OR "progression"[All Fields]). | 186639 |
| | Search (inflam*) AND metastasis | 8141 |
| | Search (inflam*) AND metastas* | 9912 |
| | Search inflammation | 513890 |
| | Search (inflam*) AND cancer | 97238 |
| | Search metastasis | 278648 |
| | Search metastas* | 346994 |
| | Search metastas | 4 |
| | Search cancer | 3248111 |
| | Search inflam* | 775265 |

**Appendix 3**

Scoring Checklist Items for Experimental Studies

| | |
|---|---|
| **Title** | **0** |
| **Provide as accurate and concise a description of the content of the article as possible** | 1<br>2 |
| **Ethical statement**<br>**Indicate the nature of the ethical review permissions, relevant licences and national or institutional guidelines for the care and use of animals, that cover the research.** | 0<br>1 |
| **Study design**<br>**The number of experimental and control groups** | 0<br>1 |
| **Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when** | 0<br>1 |
| **The experimental unit (e.g. a single animal, group or cage of animals** | 0<br>1 |
| **Experimental procedures**<br>**For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example:**<br>**0. No 1.yes** | 0<br>1 |
| **Experimental animals 8**<br>**For each experimental group, provides information about medical condition and (mean) age. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knockout or transgenic), genotype, health/immune status, drug or test naïve, previous procedures method of tumour inoculation, types of animals used (SCID, transgenic, knockdown, etc.), method of carcinogenic induction, type of tumour, etc.**<br>**0. No 1.yes** | 0<br>1 |
| **Sample size 9**<br>**a. Specify the total number of animals used in each experiment, and the** | 0<br>1 |

| | |
|---|---|
| **number of animals in each experimental group.**<br>**0. No 1.yes** | |
| **b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.**<br>**0. No 1.yes** | 0<br>1 |
| **Indicate the number of independent replications of each experiment, if relevant.**<br>**0. No 1.yes** | 0<br>1 |
| **Experimental outcomes 12**<br>**Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).**<br>**0. No 1.yes** | 0<br>1 |
| **Statistical methods 13**<br>**Provide details of the statistical methods used for each analysis.** | 0<br>1 |
| **Baseline data 14**<br>**Provide clear information of**<br>**a- Any pre-experiment measurements.** | 0<br>1 |
| **Measurement of inflammatory mediator/s expression (in carcinogenesis induced)** | 0<br>1 |
| **The role of the mediator in inducing specific metastatic stage** | 0<br>1 |
| **The producing cell of origin for the mediator** | 0<br>1 |
| **The downstream pathway for the mediator to achieve its action** | 0<br>1 |
| **The method of inhibition** | 0<br>1 |

## Appendix 4

REMARK modified score checklist for the Clinical studies

| | |
|---|---|
| Introduction | |
| **1. State the marker examined, the study objectives and any prespecified hypotheses.** | 0-1 |
| Materials and Methods | |
| Patients | |
| **2. Describe the characteristics (e.g. disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria. (0,1)** | 0-1 |
| | |
| Specimen characteristics | |
| **4. Describe type of biological material used (including control samples), and methods of preservation and storage. (0,1)** | 0-1 |
| Assay methods | |
| **5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point. (0,1)** | 0-1 |
| Study design | |
| **6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease or age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time. (0,1)** | 0-1 |
| | |
| Statistical analysis methods | |
| **10. Specify all statistical methods, including details of any variable selection procedures and other model-building** | 0-1 |

| | |
|---|---|
| **issues, how model assumptions were verified, and how missing data were handled. (0,1)** | |
| | |
| Results | |
| Data | |
| **12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events. (0,1)** | 0-1 |
| **13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values. (0,1)** | 0-1 |
| Analysis and presentation | |
| **14. Show the relation of the marker to standard prognostic variables. (0,5)** | |
| **A. correlation with LN** | 0-1 |
| **B. correlation with DM** | 0-1 |
| **C. correlation with other prognostic factor** | 0-1 |
| **D. correlation with staging** | 0-1 |
| **E. correlation with metastatic stage (vessel density or invasion)** | 0-1 |
| **F. validation with *in-vivo* experiment** | 0-1 |
| **15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analysed. (0,1)** | 0-1 |
| **16. For key multivariable analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model. (0,1)** | 0-1 |