Aus der Universitätsklinik für Radioonkologie mit Poliklinik Tübingen

SDF-1/CXCR4 as prognostic markers for postoperative radiochemotherapy in head and neck cancer

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

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2018

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Tag der Disputation: 11.09.2018

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1 INTRODUCTION

1.1 OVERVIEW

Only about half of the patients affected by locally advanced head and neck squamous cell carcinoma (HNSCC) and treated with post-operative RT-CT are alive after five years, indicating a substantial unmet medical need for improvement. In addition to the already known HPV16 status, new biomarkers are currently investigated in order to personalize treatment options and improve patients' outcome. Evidence suggests that the chemokine pathway SDF-1/CXCR4 play a key role in tumour development, progression and therapies resistance. The present study explores the prognostic value of these two biomarkers in a cohort of more than two hundred HNSCC patients treated with post-operative RT-CT. The results of the present study have been published and the full text publication is attached to the thesis [1].

1.2 HEAD AND NECK CANCER

1.2.1 Epidemiology

Approximately 4% of the newly worldwide diagnosed malignant tumours are represented by head and neck cancers, with a large majority of squamous cell carcinomas [2]. Incidence of HNSCC varies geographically, even within the European Union, with countries showing higher incidence (e.g. Hungary, 23/100.000) and others much lower (e.g. Greece or Cyprus, 3/100.000). In Germany in 2012, HNSCC incidence was 14,4/100.000 and mortality 4,2/100.000 [3]. Almost 2/3 of the head and neck cancer patients are men, often with a history of alcohol and/or tobacco abuse. For this reason, patients with HNSCC have also a higher risk to develop other tumours, e.g. lung or oesophageal cancers. Even though tobacco and alcohol are the most common

risk factors, the incidence of HPV positive tumours especially in non-smoker/drinker population is arising. The human papilloma virus, mostly the HPV-16 type, through the interaction of the viral oncoproteins E6 and E7 with tumour suppressor genes Rb and p53, can lead to development of cancer lesions. HPV-related tumours are more often located in the oropharynx, respond better to radio-chemotherapy and have a more favourable prognosis.

1.2.2 Natural history and pattern of spread

The local spread of the primary tumour depends mostly on the anatomical site, with more common muscle invasion and spread along muscle of facial planes, while bone structures represent a barrier and are infiltrated only in the most advanced stages of disease. The presence of perineural or vascular invasion (PNI, VI) is recognised as risk factor for a poorer outcome. Predicting the risk and the pattern of lymph node metastasis are crucial for the therapy choice and planning. The lymph node spread depends on different factors, among them tumour localisation, histology, T stage, PNI and VI. Generally, lateralized lesions have higher probability to spread to the ipsilateral lymph nodes, while tumours closed to or crossing the middle line are more likely to spread ipsi- and contralaterally. The CUP syndrome (carcinoma of unknown primary), characterized by initial presentation of disease with pathologic lymph nodes without evidence of primary lesion, is not uncommon among HNSCC patients (ca. 2-9%). Distant spread represents a late event which is more related to the N stage (circa 20-30% probability by N2-N3 tumours) than to the T stage and with the lung being the most commonly involved organ [4, 5].

1.2.3 Diagnostic work-up

Initial signs and symptoms can vary, being dysphagia, weight loss, local pain and clinically visible lesions and adenopathies the most common. The local extent of disease is determined by the clinical examination with fibroscopy and

radiologically through computed tomography (CT) and magnetic resonance (MR). The MR, especially with contrast, allows high anatomical resolution: it defines the size of the primary tumour, its relationship with adjacent structures and gives information about the regional extent of disease. The CT-PET, through functional imaging, is able to identify with high sensitivity eventual nodal metastasis and has become a widely used tool for pre-treatment staging. Its key role has been demonstrated not only in the diagnostic work-up, but also to evaluate the response to the treatment [6]. Moreover, together with a chest-CT, PET-CT may detect distant metastasis. Before start of treatment, histology verification through biopsy is mandatory. Patients with CUP syndrome, additionally to the fine needle aspiration (FNA) or less frequent to the excisional biopsy, receive a complete endoscopic examination with multiple biopsies performance. Standard of care is also the HPV-DNA/p16 status. To decide the adequate treatment strategy a multidisciplinary team, formed by head and neck surgeons, radiation oncologists, medical oncologists, pathologists, radiologists, dentists, plastic surgeons and speech and swallowing therapists has to be involved.

1.2.4 Staging

The tumour stage helps in defining the therapeutic approach and predicting outcomes. It differs according to the involved site, but generally the T stage is based on the tumour size and the N stage on the size, number and localisation (ipsi- or also controlateral) of the pathologic lymph nodes. Stages I and II usually identify relatively small lesions without regional spread, while stages III and IV indicate loco-regionally advanced tumours or a metastatic disease. The present study was performed on the basis of the 7th TNM edition, that presents some differences respect to the latest 8th edition. Particularly, the new edition takes into account for the T staging of the oral cavity tumours, not only the tumour size, but also how deep the tumour infiltration is. For the pharynx cancers, the 8th edition distinguishes the oropharynx carcinomas HPV positive and HPV negative, reflecting the better outcomes of HPV positive tumour.

Regarding the N staging, the new edition separates the lymph nodes status in clinical (cN) and pathological (pN), considering not only the size and the laterality of the metastatic lymph nodes but also the presence of extranodal spread [7].

1.2.5 Treatment

1.2.5.1 General principles

HNSCC, excluding metastatic disease, can be treated with curative intent through surgery and/or radiotherapy. Chemotherapy alone is a palliative treatment, but it can be used as part of a curative strategy together with radiotherapy to increase the response rate and survival. Being the head and neck a region containing many structures for key functions as swallowing, breathing and speaking, the treatment choice should be based on the probability to achieve the highest tumour cure rate with the lowest morbidity rate, trying to avoid long-term functional (and cosmetic) deficits. Thus, patient's will and a multidisciplinary discussion play an essential role in defining the treatment strategy.

A surgical approach allows a pathological staging, a shorter treatment time, a limited non-tumoural tissue exposure to treatment and, by definition, a tumour resection (sometimes psychologically preferred by patients), but it can lead to permanent important dysfunctions. In cases where the oncological outcomes are assumed to be similar and surgery could inflict permanent functional defects, a radio (chemo) therapy is to be preferred. This is usually characterized by acute toxicities, but the late effects are often minimal if compared to a major surgery and functions as swallowing, breathing and speaking can be better preserved [5, 8]. While the management of the primary tumour lesion is relative straight forward (curative RT-dosis, i.e., according to a standard fractionation schedule, 66-70 Gy, or tumour excision in case of surgery), the management of

the neck is more complex. Different types of neck dissection as part of the surgical management can be performed. It includes radical neck dissection, modified neck dissection or selective neck dissection, according to the clinical initial disease presentation. Similarly, the radiation treatment of the neck, doses and volumes definition, is performed considering the macroscopic disease and the probability of the different lymph nodes levels to host microscopic metastasis, with the definition of usually two or three different target volumes.

1.2.5.2 Surgery

A head and neck surgeon with expertise in the field of oncology should perform the operation, planning the surgical procedure (tumour resection and reconstruction) to achieve tumour-free margins and the best possible functional and cosmetic outcomes. Usually, a surgical approach should be avoided when the tumour infiltrates structures like M. pterygoideus, base of skull, carotid arteries, skin, prevertebral fascia and nasopharynx [5]. En-block tumour resection with clear margins, defined as > 5 mm between the tumour and the resection margin, is the goal. Close margins (< 5 mm) and positive margins correlate with higher risk of relapse and should be avoided. In case of positive margins an adjuvant treatment with radio (chemo) therapy must be considered. Cranial nerves, if not macroscopically infiltrated and if preoperative functioning, should be preserved. As mentioned before, different types of neck dissection can be performed. A radical neck dissection removes the superficial and deep cervical fascia with all the lymph nodes from level I to level V, the sternocleidomastoid muscle, the omohyoid muscle, internal and external jugular veins, XI cranial nerve and submandibular gland. To avoid such a major surgery with subsequent important deficit and high risk of complications, when possible a modified neck dissection is performed, that spares the XI cranial nerve (type 1), the cranial nerve and the internal jugular vein (type 2), the cranial nerve, the jugular vein and the sternocleidomastoid muscle (Type 3 or functional neck dissection). In both the radical and the modified (inclusive the functional) neck dissection lymph nodes of level I to V are removed. A selective neck dissection

is characterized by the removal of the high-risk lymph nodes, usually level II and III always, sparing, according to the disease presentation, level I and/or IV and /or V. For lateralized lesions, only an ipsilateral neck dissection, sparing the contralateral neck, can be considered, while for more central tumours a bilateral dissection is indicated. Even by clinically N0 tumours, an elective nodal dissection has to be evaluated, being the estimated risk of microscopic metastasis less than 20% by T1 tumours, but between 20 and 30% by T2 tumours and more than 30% by T3-4 tumours [9]. By clinically N0 tumours, a selective neck dissection (levels I-III for oral cavity, levels II-IV for oropharynx, levels II-IV and eventually V) may be performed. By N1-N2 a selective or a comprehensive (modified or radical) dissection should be evaluated and by N3 tumours a comprehensive neck dissection is the surgical standard [10]. Relative new frontiers of the surgical management of the neck concern the use of the sentinel lymph node biopsy for early stage (T1-2) oral cavity tumours [5]. Neck dissection is also indicated by a rest tumour after curative RT (CT) therapy, assessed by CT or PET-CT [11].

1.2.5.3 Radiotherapy

Radiotherapy has been established for a long time as a curative treatment for HNSCC. During the last decades, advancements in treatment planning and delivery, together with the addition of chemotherapy and modified fractionation schedules, led to significant improvements of the oncological outcomes and minimization of late toxicity. Target volumes definition became (and is becoming) more and more precise through the incorporation of functional (PET-CT) and high resolution anatomical (MRI) information [12]. Intensity modulated RT (IMRT) techniques allow a better sparing of normal tissues and relatively new image guided RT (IGRT) methods, as cone beam CT, allow narrower margins for the definition of the target volumes. Together, IMRT and IGRT approaches led to a reduction of treatment-related toxicities. In the treatment planning phase, the gross tumour volume (GTV), when present, must be delineated for the tumour and for the nodal disease. A clinical target volume

(CTV) is then defined taking into account the risk of microscopic disease. Finally, the planning target volume (PTV) is determined considering the uncertainties due to patient's positioning and (even though in H&N region is limited) organ motion. According to standard fractionation schedules, a radical RT treatment is performed with 66-70 Gy to the macroscopic disease, 60-66 Gy to the high-risk volume (lymph nodes clinically negative but at high risk of microscopic metastasis) and 50-54 Gy to the low risk volume. In the adjuvant setting, usually RT doses range between 60-66 Gy to the high risk volume and 50-54 Gy to the low risk volume. A dose-volume histogram is carefully evaluated before treatment, to check the coverage of target volumes and the dose to organs at risks. Being the head and neck a region with many organs at risk, the final treatment plan is often a compromise between the optimal therapeutic doses and volumes and the probability of, especially late, toxicities (Figure 1.1).

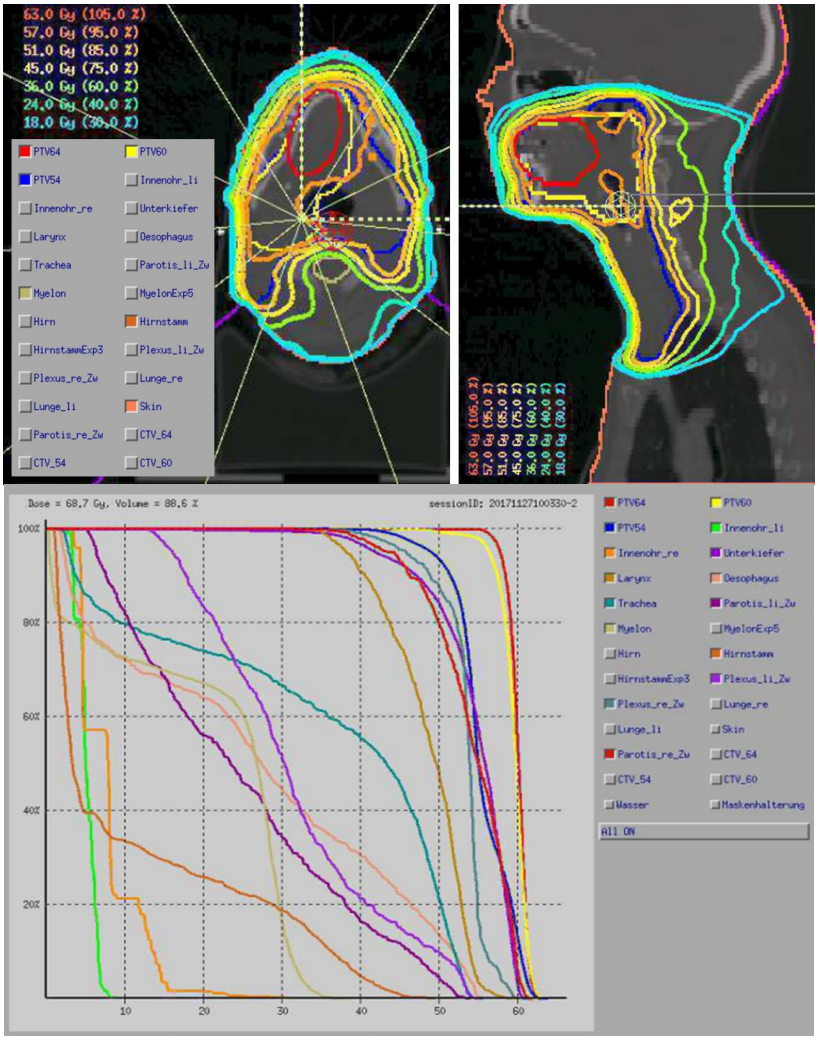

Figure 1.1. Example of an IMRT treatment plan for a HNSCC patient treated with adjuvant radiotherapy. Below, the dose-volume histogram. Different planning target volumes, with the relative prescribed dose, are defined, according to the risk of regional involvement (here, 54 Gy, 60 Gy and 64 Gy, respectively PTV54, PTV60 and PTV64). Organ at risk, such as the left parotid and the spinal cord are spared.

Chemotherapy applied concomitantly to radiation led, through a radiosensitizing effect, to significant improvements of loco-regional tumour control and survival of HNSCC patients and is now the standard of care for locally advanced tumours treated with primary RT and finds indications in different adjuvant scenarios. Since the present study was conducted on patients treated with adjuvant RT-CT, the next paragraph is focused on this combined approached without further description of primary RT-CT, palliative CT and target therapies.

1.2.5.4 Combined modalities: surgery and post-operative radio (chemo) therapy

For patients treated with surgery and present with high risk (more than 20%, i.e. extranodal spread, positive margins and/or multiple involved lymph nodes) for locoregional relapse after surgery alone, adjuvant RT must be considered. In this setting, two significant even though not randomized studies provided evidence in favour of a postoperative radiation treatment. In the study conducted by Huang end colleagues 441 HNSCC resected patients were retrospectively analysed. 125 of them were found to have extranodal spread (ECE) and/or positive resection margins. 71 patients were treated with surgery alone and 54 with surgery and adjuvant RT. The univariate and multivariate analysis showed a significant difference in locoregional control (at 5 years 59% vs 31%, p=0.0001) and adjusted survival (72% vs 41%, p=0.001) in favour of the adjuvant treatment [13]. In a second significant study, Lundal et al. retrospectively analysed a cohort of 95 HNSCC patients treated with surgery and adjuvant RT and, through a matched pair analysis with HNSCC patients treated with surgery alone, found significant differences in terms of recurrence in the dissected neck (RR=5.82; P=0.0002), recurrence in any side of the neck (RR=4,72), cancer-related death (RR=2.21; P=0.0052) and death from any cause (RR=1.67; P=0.0182) in favour of adjuvant RT [14]. Thus, adjuvant RT improves locoregional tumour control and survival and is therefore the standard treatment in HNSCC patients treated with surgery and at high risk of relapse.

The addition of CT to RT for patient with high-risk resected HNSCC was established later, though with higher level of evidence. Two randomized trials, EORTC [15] and RTOG [16], were published in 2004 in the New England Journal of Medicine and both showed a better loco-regional control and survival among patients treated with adjuvant RT-CT if compared with adjuvant RT alone. In the EORTC study, 334 patients were randomized between adjuvant RT alone (66 Gy standard fractionation) or RT-CT (66 Gy RT parallel to cisplatin 100 mg/m^2 every 21 days). With a median follow up of 5 years, fewer local and loco-regional relapses (31% after RT vs 18% after RT-CT, p=0.007), a better

progression-free survival (36% vs 47%, p=0.04) and overall survival (40% vs 53%, p=0.02) were shown in patients treated with adjuvant RT-CT. Acute toxicity was higher in the RT-CT group, but without significant differences in terms of severe late toxicity. In the RTOG study, a total of 459 were randomly treated with adjuvant RT or RT-CT, with the same RT and CT schedules as in the EORTC study. With a shorter follow-up (circa 46 months) similar results, especially for locoregional control and disease-free survival, could be shown, even though no difference in terms of overall survival was detected. A German trial, where 440 patients were randomly assigned to receive adjuvant RT (66 Gy standard fractionation) or RT-CT (additionally to RT Cisplatin 20 mg/m2 and 5 FU 600 mg/m2 on day 1-5 and 29-33) confirmed at 5 years a better loco regional tumour control (72% vs 89%, p=0,003) and progression free survival (50% vs 62%, p=0,024) for patients receiving the combined postoperative treatment, while no statistically significant differences where seen in terms of overall survival (49% vs 58%, p=0.1) [17]. Based on this evidence, high risk resected HNSCC are nowadays commonly treated with adjuvant RT-CT.

1.3 CHEMOKINE SIGNALING AND CANCER

1.3.1 Chemokines and their physiological role

Chemotactic cytokines (chemokines) are small secreted molecules that bind to seven-transmembrane-domain of G-protein-coupled receptors. On the basis of the position of the first two cysteine residues, they are classified into four groups: CC, CXC, CX3C and XC, and their receptors are named accordingly. More than 50 ligands and 18 receptors have been so far described [18]. One of the most conserved chemokine pathways is represented by CXCL12 (also known as stromal derived factor 1, SDF-1) and his receptors CXCR4-CXCR7, that was found even in simple vertebrates like jawless fishes [19]. The reason of such a high evolutionary conservation is the key role that SDF-1/CXCR4-CXCR7 and chemokines in general play in immune response and development.

Chemokines are produced by leucocytes, fibroblasts, endothelial cells and epithelial cells. They regulate both the innate (mediated by neutrophils, natural killer, dendritic cells and monocytes) and the adaptive (mainly T cells and B cells) immune response [20]. Through the so-called chemokine gradient, they attract immune cells to the site of inflammation. During embryogenesis, they are responsible for the migration of neurons, neural crest cells, germ cells, cardiomyocytes and hematopoietic stem cells. In addition, also in the adult, they play a crucial role in the angiogenesis process and their expression can be regulated by microenvironmental factors, like hypoxia. In fact, if in the embryonal development the axis SDF-1/CXCR4 is essential for the formation of the vascular system, inclusive big vessels like Aorta, in the adult SDF-1/CXCR4 expression is induced by hypoxia inducible factor 1α (HIF-1α) and vascular endothelial grow factor (VEGF) in hypoxic areas, leading to migration of the endothelial precursor cells to the hypoxic site [19]. The ligand binding determines the phosphorylation of the receptor and the activation of several intracellular pathways, ending up in the nucleus with the transcriptions of genes regulating the interaction cell-extracellular matrix, cell motility, invasion, proliferation and survival [21]. While CXCR7 can bind also to other ligands, CXCR4 is one of the very few receptors that bind only to one chemokine, SDF-1. Main pathways activated by SDF-1/CXCR4 are represented by PI3K/AKT and RAS/RAF/ERK. These are also ones of the most important pathways involved in cancer development and metastasis.

1.3.2 Chemokines network in cancer

CXCR4 expression has been observed in many haematological malignancies, such as AML, CML, B-ALL, C-ALL, follicular and non-Hodgkin lymphomas and multiple myeloma, as well as in solid tumours, like breast, prostate, thyroid, oesophageal, pancreatic, colo-rectal, ovarian, cervical, HNSCC, kidney, bladder, gliomas, sarcomas, melanoma and lung cancers [20, 22, 23]. The presence of chemokines in tumours has been described to regulate at least the

following key factors: infiltration of leucocytes and tumour immune response, angiogenesis, induction of proliferation and survival signals, tumour cell motility and migration [24].

1.3.2.1 Infiltration of leucocytes and tumour immune response

Immune system is crucial in preventing tumour development. Even in cancer patients, the presence of tumour infiltrating lymphocytes has been shown to correlate with better prognosis [25]. On the other hand, chronic inflammation may lead to tumour development. In chronic inflammatory processes, the prolonged presence of high chemokine concentrations activates macrophages releasing immunosuppressive factors like IL-10 and TGF-β [24]. In fact, if type I macrophages are activated in response to microbes and parasites and have also antitumoral activity through the production of reactive oxygen species, an aberrant expression of chemokines and their receptors causes the activation of type II macrophages, owing immunosuppressing properties, in particular through TGF-β mediated suppression of the T-cell related antitumoral activity [20]. Additionally, chemokine signalling promotes migration of myeloid-derived suppressor cells from the bone marrow and the spleen to the tumour. These cells contribute to suppress the antitumoral T-cell activity [20]. Moreover, T cell function is dependent on the antigen presentation by dendritic cells. SDF-1/CXCR4 signalling has been seen to downregulate dendritic cells motility and activity [24]. In addition to this “extrinsic pathway”, activated as a consequence of chronic inflammatory processes, tumour cells themselves are responsible of an “intrinsic pathway”, namely, due to different mutations and other types of genetic alterations, aberrant cells are able to produce chemokines and chemokines receptors creating an inflammatory tumour microenvironment that, as a loop, sustains itself independently of infectious conditions [26].

1.3.2.2 Angiogenesis

Different CXC chemokines promote angiogenesis, in an autocrine as well as paracrine manner. SDF-1 has been shown to be upregulated by HIF-1α, determining the recruitment of endothelial precursor cells to the hypoxic areas [19, 27]. VEGF is able to induce CXCR4 expression on endothelial cells precursors, rendering endothelial cells sensible to the chemokine gradients and therefore migrate and form new vascular structures [28]. Interestingly, in renal cell carcinomas with inactivating mutation of the von Hippel Lindau gene (tumour suppression gene inducing HIF-1α degradation under normal oxygen supply) HIF-1α is overexpressed and upregulates CXCR4 expression, that was seen to correlate with worse survival [29].

1.3.2.3 Induction of proliferation and survival signals

SDF-1/CXCR4 signal is mainly mediated by four intracellular pathways (Figure 2.1). The phosphorylated receptor activates the way of RAS/RAF and MAP kinases as well as of PI3K and AKT. These pathways are also stimulated by many grow factors and are often altered in cancers. After being activated by RAS/RAF, ERK translocates to the nucleus, where it promotes the transcription of genes responsible for cell survival, proliferation and migration [30]. Similarly, after the interaction with PI3K generated phospholipids, AKT brings the signal to the nucleus, where it is phosphorylated by PDK1/2 and determines the transcriptions of genes involved in cellular growth, survival and cell cycle progression [31]. Interestingly, hyperactivation of PI3K/AKT is also involved in mechanisms of resistance to radio and chemotherapy [32]. A third pathway activated by SDF-1/CXCR4 is mediated by IP3, that increases the intracellular calcium concentration. Calcium signalling has been shown to play a role in cell proliferation, death, and invasion [33], as well as interfering with the response of cancer cells to radiation [34]. Finally, β arrestin can be responsible of the internalization of CXCR4.

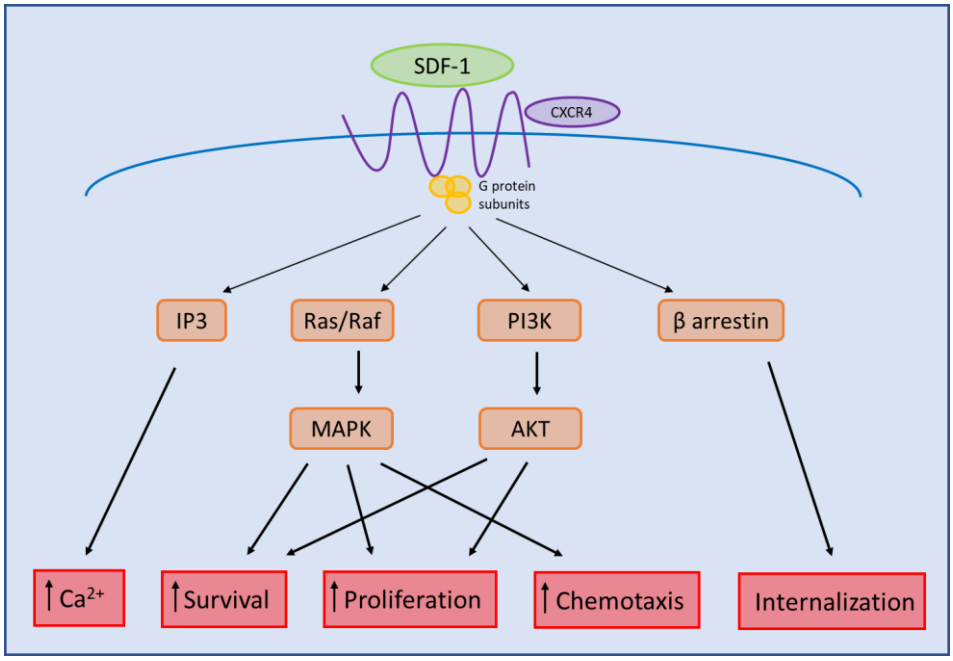

Figure 2.1 SDF-1/CXCR4 intracellular activated pathways.

1.3.2.4 Tumour cell motility and migration

Chemotaxis occurs when cells move from a low level of chemokines concentration to a higher one (chemokine gradient). In response to extracellular chemotactic molecules, cells with proper receptors are chemosensitized, namely, they generate podia (membrane cell extensions) in the direction of the movement. This asymmetric organization of the actin and myosin filaments renders the cells polarized, with a "front" towards the higher chemokines' concentration and a "back". The cell then detaches and trails through integrin mediated adhesion and contraction of the actin/myosin filaments (locomotion). Within tumour tissues, four different types of chemotaxis have been described. In the amoeboid migration, a single cell squeezes through gaps in the extracellular matrix (ECM). The mesenchymal migration is typical of cells undergoing epithelial-mesenchymal transition and is characterized by elongated cells that exploits the lysis of the ECM through metalloproteinases (MMP). Migration of multiple cells can occur through the so-called chain migration, in which the movement is led by elongated cells being at front and driving other cells that remain attached to the leader ones through cell-cell junctions. Another

possibility of group migration is the streaming modality, in which each cell moves actively but following each other through cell-cell junctions. Interestingly, it has been shown that the leader cell is not always a tumour cell, rather often a stromal cell of the tumour microenvironment able to degrade the ECM (e.g. macrophages) [35]. The main chemokine axis regulating chemotaxis in cancer is SDF-1/CXCR4. Tumour cells that, through gene mutations or in response to microenvironmental factors like hypoxia, overexpress CXCR4 respond to chemokine gradients and acquire the ability to migrate (Figure 2.2). Studying cancer cell lines and mouse models of different tumour types, such as ovarian, breast, prostate, renal, lung cancer and melanoma, cells modified to express high levels of CXCR4 have been shown to own a higher metastatic potential [18].

A last notably remark concerns the link between SDF-1/CXCR4 and cancer stem cells (CSCs). This chemokine axis is activated in CSCs of different tumour types and play a key role in clonogenicity and invasion potential as well as in resistance to treatment strategies as RT [36]. Self-renewal capacity of lung cancer stem cells has been shown *in vitro* and *in vivo* to be sustained by CXCR4, that, when blocked by the antagonist AMD3100, reduces the ability of lung cancer stem cells to form spheroid [37]. *In vitro* and *in vivo* studies showed also that prostate cancer cells CD133+/CD44+ (markers of CSC) adhere to the extracellular fibronectin under stimulation by SDF-1 and that prostate cancer stem cells proliferation is decreased when CXCR4 is inhibit by AMD3100 [38]. Similarly, SDF-1 has been seen to stimulate the formation of podia and migration in CD44+/CXCR4 positive HNSCC lines [39, 40].

1.3.3 SDF-1 and CXCR4 in head and neck cancer

1.3.3.1 Preclinical evidence

In vitro and *in vivo* studies have investigated SDF-1/CXCR4 mechanisms of actions in HNSCC. Under SDF-1 stimulation, CXCR4 positive oral squamous

cell carcinoma (OSCC) cell lines showed increased motility and invasion potential [41]. In different HNSCC cell lines, PI3K/AKT pathway was activated by SDF-1 in CXCR4 positive cells and migration and proliferation were shown to be enhanced; conversely, they were inhibited when CXCR4 was not functioning [42]. Invasion and metastasis mediated by NF-kB were enhanced by SDF-1/CXCR4 in OSCC cell lines [43]. Tumour cell invasion and migration through modulation of MMP expression induced by SDF-1/CXCR4 have been shown in different HNSCC cell lines [44-46]. Hints concerning the involvement of SDF-1/CXCR in the EMT through the activation of PI3K/AKT come from studies in OSCC [47] and xenograft mouse models [48]. In OSCC cell lines and in nude mice SDF-1 and CXCR4 were shown to be involved in development of lymph nodes metastasis through ERK1/2 or PI3K/AKT [49]. The same authors demonstrated SDF-1/CXCR4 involvement in the development of distant metastasis, through a paracrine action, which weas in contrast inhibited, with also a gain in survival of the mice, when AMD3100 was applied [50]. CXCR4 knockdown was seen to induce cell cycle arrest and apoptosis in OSCC with anti-proliferative and anti-invasive effect [51, 52].

1.3.3.2 Clinical studies

SDF-1/CXCR4 expression on tumour tissues derived from HNSCC patients has been described. Data indicate that this chemokine pathway correlates with enhanced metastatic potential and worse oncological outcomes [53]. Delilbasi et al. described CXCR4 expression in squamous cell carcinomas of the tongue from 23 patients, with higher expression in tumour cells of metastatic lymph nodes than in the primary tumour, while no expression was found in normal tongue tissue [54]. A significant correlation between CXCR4 expression and lymph node metastasis was found by Ishikawa and coll. among 90 patients affected by OSCC, 30% of those were CXCR4+ [41]. Katayama et al. showed that CXCR4 expression, which was identified in 29% of 56 OSCC patients, was stronger in patients with lymph node or distant metastasis and was a predictive

factor for worse cancer-specific survival (55% CXCR4 positive vs 85% CXCR4 negative patients at 5 years) [42]. Tan and coll. detected SDF-1 and CXCR4 in, respectively, 54% and 40% of 30 patients affected by carcinomas of the larynx/ hypopharynx and found a significant correlation between CXCR4 high expression and lymph node and distant metastasis [44]. Among 30 patients affected by OSCC Uchida and coll. saw a higher SDF-1 expression in the metastatic lymph node cells than in the primary tumour and showed that SDF-1 and CXCR4 were correlating with worse cancer-specific survival (25% SDF-1 positive vs. 71% SDF-1 negative patients and 39% CXCR4 positive vs. 71% CXCR4 negative patients) [50]. Lymph node and distant metastasis were found to correlate with CXCR4 expression also in 25 HNSCC patients by Ueda et al. [55]. More aggressive features (e.g. lymph node metastasis and more advanced TNM stage) were described by Yin and coll. in patients affected by OSCC [56] and by Tao and coll. in patients affected by nasopharyngeal carcinoma [57]. CXCR4 but not SDF-1 expression was found to be a predictive factor for overall survival among 47 patients with carcinoma of the tongue (24% for CXCR4 positive patients vs. 81% for CXCR4 negative patients at 5 years) [58]. Within 233 HNSCC patients analysed by Rave-Fränk et al. high CXCR4 expression was found predictive for reduced distant metastasis-free survival and disease-free survival but, in contrast, high SDF-1 expression was predictive for a better overall survival [59]. Clatot and coll. found among 71 HNSCC patients no correlation between CXCR4 and recurrence or survival and a poorer metastasis-free, disease-free and overall specific survival in patients with lower SDF-1 expression [60]. Leon et al., analysing 111 HNSCC patients, described a more complex pattern, with patients expressing high CXCR4 and low SDF-1 values having a significantly worse regional recurrence-free survival rates [61].

1.4 AIM OF THE STUDY

Though advancement in RT planning and delivery and melioration of the oncological outcomes through modified fractionation schedules and addition of

concomitant CT, overall survival (OS) after adjuvant RT-CT in HNSCC patients remains unsatisfactory. One reason might be represented by the fact that the treatment choice is nowadays mostly determined by clinico-histopathological factors, which are apparently not enough to predict patients' treatment response. New biomarkers are required for treatment individualization and the existing preclinical and clinical evidence suggest that SDF-1/CXCR4 axis is crucial for cancer development and progression. Moreover, this chemokine pathway may be involved in mechanisms that regulate tumour resistance to treatments, e.g. radiotherapy. Nevertheless, the published literature regarding the role of SDF-1 and CXCR4 in HNSCC patients is not conclusive, partially due to the small number of patients, the diversities in SDF-1/CXCR4 detection methods and because of the heterogeneity of patient and tumour characteristics, treatments and outcome parameters. Therefore, the goal of the present study was to explore the prognostic role of SDF-1/CXCR4 in a homogenous and large cohort of patients affected by locally advanced high-risk HNSCC treated with surgery and adjuvant CT-RT. The study was conducted retrospectively as part of a multicentre biomarker trial of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). The results of the study have been published [1].

2 MATERIAL AND METHODS

2.1 PATIENTS AND TREATMENT

The ethical committees of all the DKTK-ROG centres (Berlin, Dresden, Essen, Frankfurt, Freiburg, Heidelberg, Tübingen, Munich- Technische Universität and Ludwig-Maximilians-Universität) approved the study. Patients with locally advanced squamous cell carcinoma of the oral cavity, oropharynx or hypopharynx treated between 2004 and 2012 with surgery and adjuvant RT-CT were retrospectively reviewed. The postoperative treatment was administered because of the presence of one or more of the following risk factors: stage pT4, >3 positive lymph nodes, positive microscopic resection margins, extracapsular extension. According to standard guidelines, all patients received platinum-based CT and radiation treatment of the former primary tumour region and of the neck lymph nodes at risk regions. CT schemes, RT plans, formalin-fixed paraffin embedded (FFPE) tumour specimens as well as follow up data inclusive CT, MRI and/or PET images of the relapse, when present, had to be available. These data were collected centrally in the DKTK RadPlanBio Platform in Dresden. A period of 24 months was considered as minimum follow up. Finally, 221 patients were found to be eligible for inclusion.

2.2 TISSUE SAMPLES, DETERMINATION OF HPV 16 DNA AND P16

FFPE tumour materials retrieved after surgery were centrally (DKTK partner site Dresden) stained with haematoxylin and eosin for histology verification. Tissue microarrays (TMAs) were generated, having each core a diameter of 1-mm, and tumour content in each core was verified by expert pathologists. TMAs were sent to the DKTK partner centres for multibiomarker analyses.

For the analysis of HPV16 DNA and p16, samples with <10% squamous cell carcinoma content were excluded. 214 patients were analysed. According to the

manufacturer's instruction, HPV DNA was extracted from FFPE-sections, amplified by PCR and detected by hybridisation using QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, Germany), HotStarTaq Plus Master Mix (Qiagen GmbH) and LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, Germany), respectively. p16 expression was evaluated by immunohistochemistry and the staining was performed using the the CINtec Histology Kit (Roche mtm laboratories AG, Basel, CH), according to the manufacturer's instruction. Overexpression, criterium of positivity, was considered when the staining was positive in more than 70% of the tumour sample.

2.3 STAINING, IMAGING AND SCORING SYSTEM

Immunofluorescence staining was used to evaluate SDF-1 and CXCR4 expression. The staining was established under supervision of an experienced pathologist, using positive (tonsil tissue) and negative controls (PBS instead of primary antibody and anti-IgG from the same specie). TMAs staining was performed following deparaffinization, through Xilol, rehydration, through graded series of ethanol, and epitope-retrieval technique, by application of citrate buffer retrivial solution for 30 minutes at 600 Watts. According to the manufactures instructions, TSATM Kit T20912 (containing goat anti-mouse IgG and tyramide labelled with Alexa 488, Life Technologies GmbH, Molecular probes, Invitrogen, Darmstadt, Germany) was used to detect SDF-1 and TSATM Kit T20922 (containing goat anti-rabbit IgG and tyramide labelled with Alexa 488, Life Technologies GmbH, Molecular probes, Invitrogen, Darmstadt, Germany) to detect CXCR4. Antibody dilutions were 1:100 for SDF-1 (mouse monoclonal, Clone 79018, R&D Systems, Minneapolis, USA) and 1:200 for CXCR4 (rabbit monoclonal [UMB2], Clone ab124824, Abcam, Cambridge Science Park Milton Rd, Milton, Cambridge, United Kingdom). Zeiss Axio Imager MI fluorescence microscope controlled by AxioVision 4.8 software (Carl Zeiss, Jena, Germany) was used to visualize the staining and obtain images of the TMAs. Whole TMA scans were performed using a motorised scanning stage and a monochrome

digital camera (AxioCam MRm, Carl Zeiss, Jena, Germany; Maerzhaeuser, Wetzlar, Germany, 400x (EC Plan Neofluar)). Different subcellular staining patterns, i.e. membrane and intracellular (including cytoplasmic and nuclear), could be identified and were confirmed by an expert pathologist. Criteria of positivity were the staining extent and intensity, evaluated only in the tumour areas. A staining intensity score with arbitrary thresholds was evaluated per each core as follow: negative (0), low (1), intermediate (2) and high (3). The score per each pattern type (membrane and intracellular) was calculated as the mean score of all the cores of the tumour specimen derived from each patient. At least one core having a score ≥2 (meaning a mean patient score >1) was considered as positive. This semi-quantitative analysis of the tissue staining was performed jointly by two observers blinded to the clinical characteristics and oncological outcome of the patients. In case of disagreement between the two observers a consensus was found.

2.4 STATISTICAL ANALYSIS

Events (relapse, metastasis and death) were calculated from the date of RT-CT start. Kaplan-Meier curves for loco-regional tumour control (LRC), distant metastasis free survival (DMFS) and OS were generated. Comparisons between staining results and clinico-pathological patients and tumours characteristics were performed using the Fisher's exact test. Prognostic parameters were evaluated using univariate and multivariate analysis (Cox model). Hazard ratios and 95% confidence intervals were calculated. P-values <0.05 were considered statistically significant and between 0.05 and 0.1 as a trend. No correction for multiple testing was performed. To test whether the results were affected by the fact that the tumour material was coming from different institutions, we performed an additional univariate analysis for LRC and calculated the hazard ration (HR) for the different DKTK partner centres. The results were then tested for heterogeneity using $\square^2$ test. The open-source software R (www.r-project.org, 3.2.3.) was used for statistical analyses, while

GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for graphical representation.

3 RESULTS

The median follow-up of the 221 patients included in the study was 47.3 months (range, 2.5-100 months). CT was administered with a median dose of 200 mg/m^2 and RT was consisting of a median dose of 50.4 Gy to the neck with a boost up to a median dose of 64 Gy to the former tumour region. The details for the entire population as well as the prognostic role of HPV16 have been reported by Lohaus and colleagues [62]. For the present study, some patients had to be excluded because of lack of tumour material. Finally, 201 patients were analysed for SDF-1 and 190 for CXCR4. For each patient, one to five cores were available. Patients and tumour characteristics as well as treatment details are reported in Table 3.1.

Table 3.1. Patients characteristics, SDF-1/ CXCR4 expression and treatment details for the 201 patients included in the present study [1].

Age (years)	57 (median)	24 (min)	76 (max)
Gender	Male	161	80,1%
	Female	40	19,9%
Site	Oral cavity	56	27,9%
	Oropharynx	116	57,7%
	Hypopharynx	29	14,4%
pT stage	T1	34	16,9%
	T2	92	45,8%
	T3	45	22,4%
	T4	30	14,9%
R status	Negative	113	56,2%
	Positive	87	43,3%
	Unknown	1	0,5%
ECE	Negative	95	47,3%
	Positive	106	52,7%
p16	Negative	121	60,2%
	Positive	75	37,3%
	Unknown	5	2,5%
HPV16 DNA	Negative	134	66,7%
	Positive	67	33,3%
icSDF1	Negative	148	73,6%
	Positive	53	26,4%
icCXCR4	Negative	135	67,2%
	Positive	55	27,4%
	Missing	11	5,5%
mSDF1	Negative	177	88,1%
	Positive	24	11,9%
mCXCR4	Negative	176	87,6%
	Positive	14	7,0%
	Missing	11	5,5%

		Median	Percentiles				Range	
			10%	25%	75%	90%	Min	Max
Cisplatin dose (mg/m²)		200	100	200	200	240	100	300
RT dose (Gy)	Boost volume	64,0	60,0	63,8	66,0	66,0	56,0	68,4
	Per fraction	2,0	1,8	2,0	2,0	2,1	1,8	2,2
	Adjuvant volume	50,4	50,0	50,0	55,8	60,0	46,8	66
	Per fraction	2,0	1,8	1,8	2,0	2,0	1,8	2,1
Time between surg. and RCT (weeks)		6,0	4,6	5,1	7,6	9,6	0,6	22,9
Overall treatment time of RCT (days)		44,0	41,0	43,0	47,0	51,0	31,0	57,0
Follow-up time (months)		46,2	9,7	30,1	60,5	70,4	2,5	100,1

Different staining intensity and subcellular staining patterns, namely membrane and intracellular, were observed (Figure 3.1).

Membranous SDF-1 (mSDF-1) positive staining was found in 24 (11.9%) tumours and membranous CXCR4 (mCXCR4) in 14 (7%). Intracellular SDF-1 (icSDF-1) positive staining was detected in 53 (26.4%) tumours and intracellular CXCR4 (icCXCR4) in 55 (27.4%) (Table 3.1). The relationship between icSDF-1 and icCXCR4 expression and clinic-pathological tumour characteristics are summarized in Table 3.2.

A higher icSDF-1 expression was present within grade 2 (G2), HPV16 DNA and p16 positive tumours, while icCXCR4 positive staining was associated with G2 tumours and lower pT-stages.

No significant correlations between mSDF-1 or mCXCR4 with LRC, DMFS or OS were found (Table 3.3). High icSDF-1 expression was associated with significantly lower LRC, among all patients and within the HPV16 DNA negative subgroup (HR 2.67, 95% CI 1.29-5.54 and HR 2.54, 1.19-5.4, respectively) (Table 3.3 and Figure 3.2). A trend towards lower LCR rates in icCXCR4 positive tumours was found (Table 3.3 and Figure 3.3). No correlation between icSDF-1 or icCXCR4 expression and DMFS or OS was found (Table 3.3). The

combined icSDF-1 and icCXCR4 expression showed a strong correlation with lower LRC (Table 3.3 and Figure 3.4).

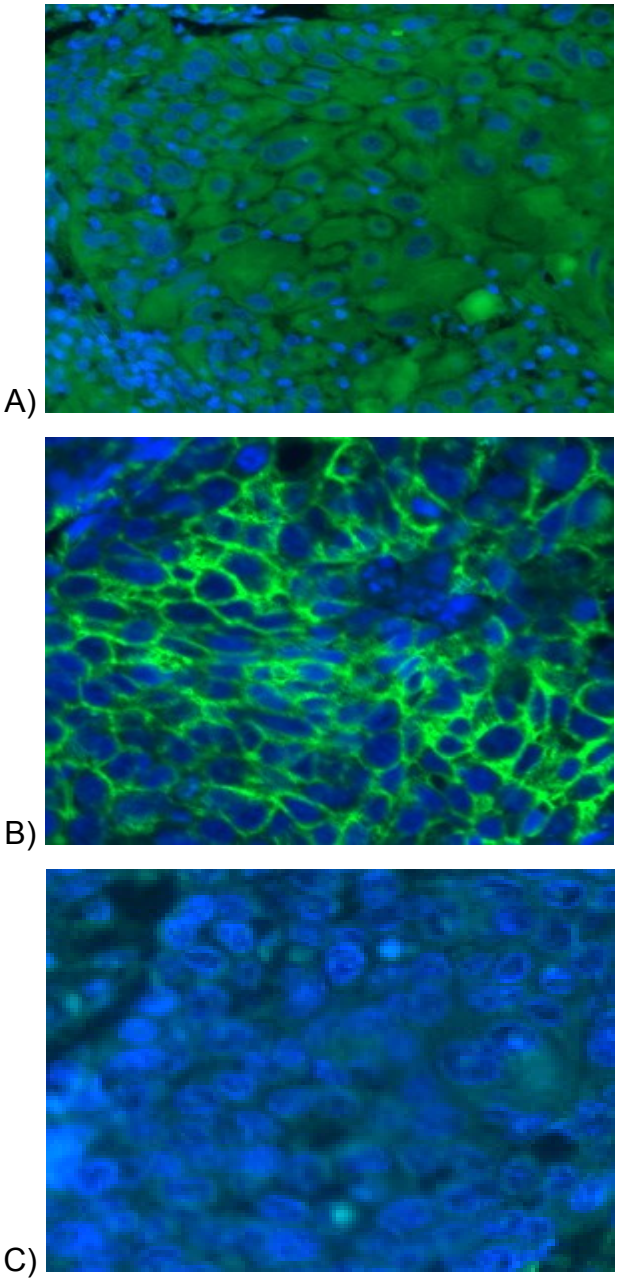

Figure 3.1. Representative images of SDF-1 immunofluorescent stained tumour sections. A) Intracellular staining (score 2). B) Membrane staining (score 3). C) Negative staining (score 0). SDF-1 is shown in green, DAPI in blue. Similar staining patterns and intensities were observed for CXCR4. Original image magnification: 400x [1].

Table 3.2. Clinico-pathological characteristics and icSDF-1/icCXCR4 expression. P-values for comparisons using the Fischer's exact test, significant p-values in bold [1].

	icSDF-1+		**icSDF-1 -**		**p**	**icCXCR4+**		**icCXCR4-**		**p**
Age										
< Median (57 y)	23	43%	75	51%	0,42	30	55%	65	48%	0,52
≥ Median	30	57%	73	49%		25	45%	70	52%	
Gender										
M	43	81%	118	80%	1,00	42	76%	110	81%	0,43
F	10	19%	30	20%		13	24%	25	19%	
Site										
Oral cavity	18	34%	38	26%	0,31	19	35%	34	25%	0,41
Oropharynx	26	49%	90	61%		29	53%	82	61%	
Hypopharynx	9	17%	20	14%		7	13%	19	14%	
pT stage										
pT1-2	33	62%	93	63%	1,00	27	49%	89	66%	**0,03**
pT3-4	20	38%	55	37%		28	51%	46	34%	
pN stage										
pN0-1	12	23%	38	26%	0,71	12	22%	36	27%	0,58
pN2-3	41	77%	110	74%		43	78%	99	73%	
Grading (3 missing)										
G1	0	0%	5	3%	**0,01**	2	4%	3	2%	0,08
G2	38	73%	75	51%		37	69%	71	53%	
G3	14	27%	66	45%		15	28%	59	44%	
Resection margin (1 missing)										
R0	32	62%	81	55%	0,42	32	59%	73	54%	0,63
R1	20	38%	67	45%		22	41%	62	46%	
ECE										
No	25	47%	70	47%	1,00	22	40%	66	49%	0,34
Yes	28	53%	78	53%		33	60%	69	51%	
HPV16 DNA										
Negative	41	77%	93	63%	0,06	40	73%	88	65%	0,39
Positive	12	23%	55	37%		15	27%	47	35%	
p16 (5/4 missing)										
Negative	39	76%	82	57%	**0,01**	37	69%	78	59%	0,25
Positive	12	24%	63	43%		17	31%	54	41%	
Smoking (69/67 missing)										
Yes	27	84%	90	90%	0,36	29	85%	80	90%	0,53
No (never)	5	16%	10	10%		5	15%	9	10%	

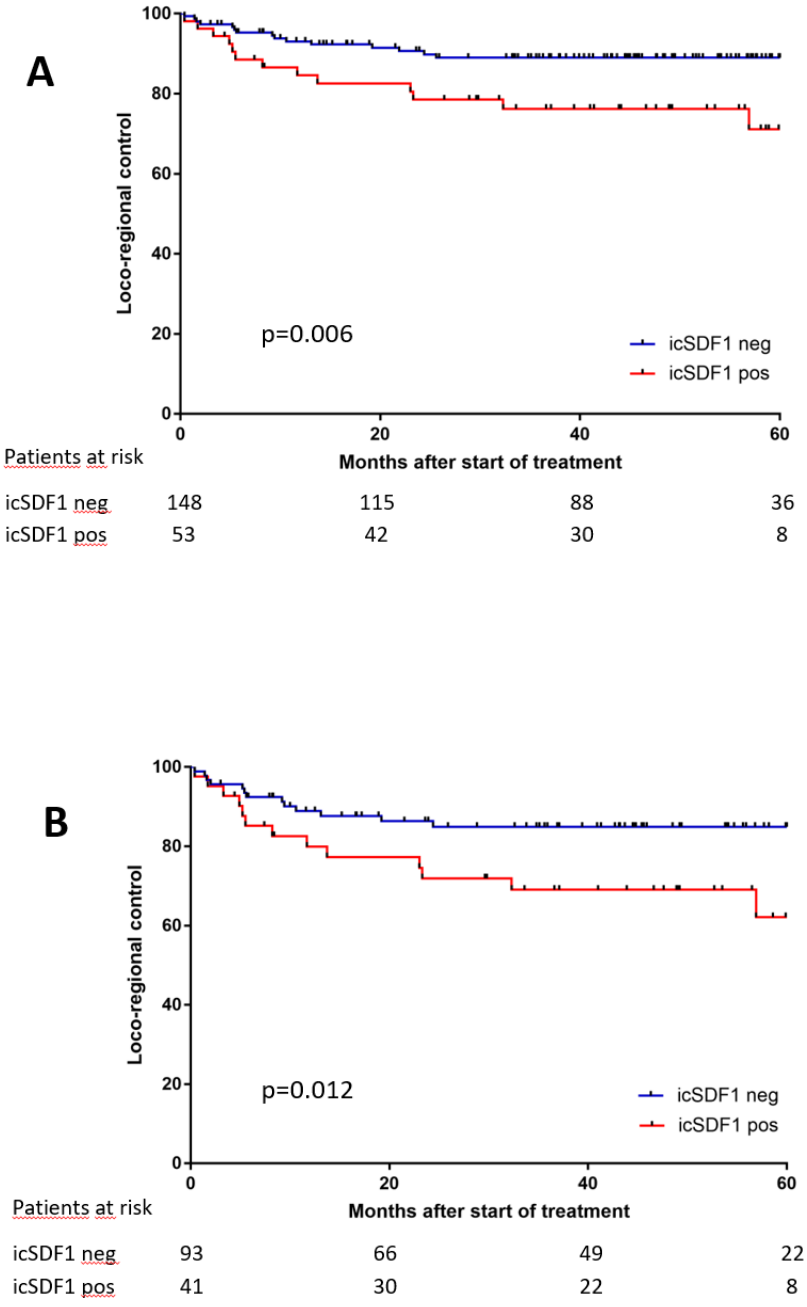

Figure 3.2: Kaplan-Meier curves for locoregional tumour control in all patients (A) and patients with HPV16 DNA negative tumours only (B), according to the SDF-1 intracellular expression [1].

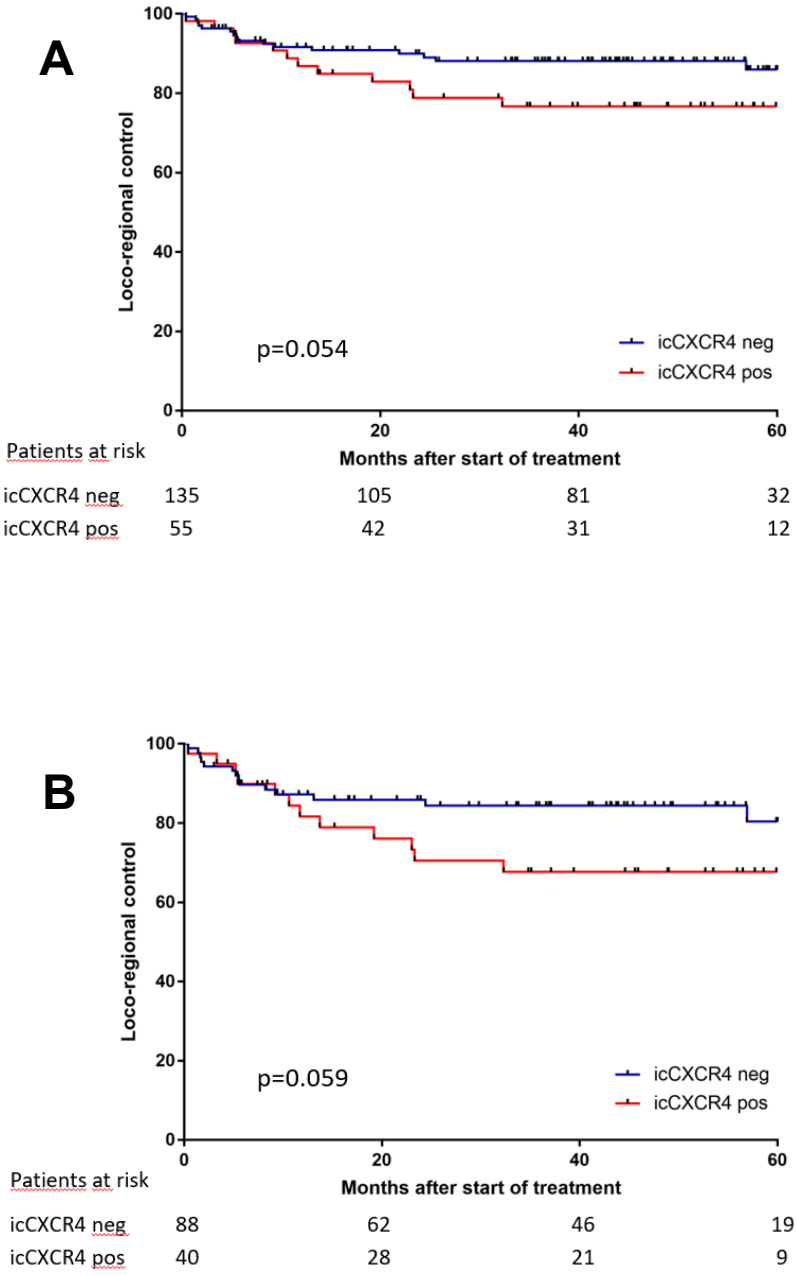

Figure 3.3: Kaplan-Meier curves for locoregional tumour control in all patients (A) and patients with HPV16 DNA negative tumours only (B), according to the CXCR4 intracellular expression [1].

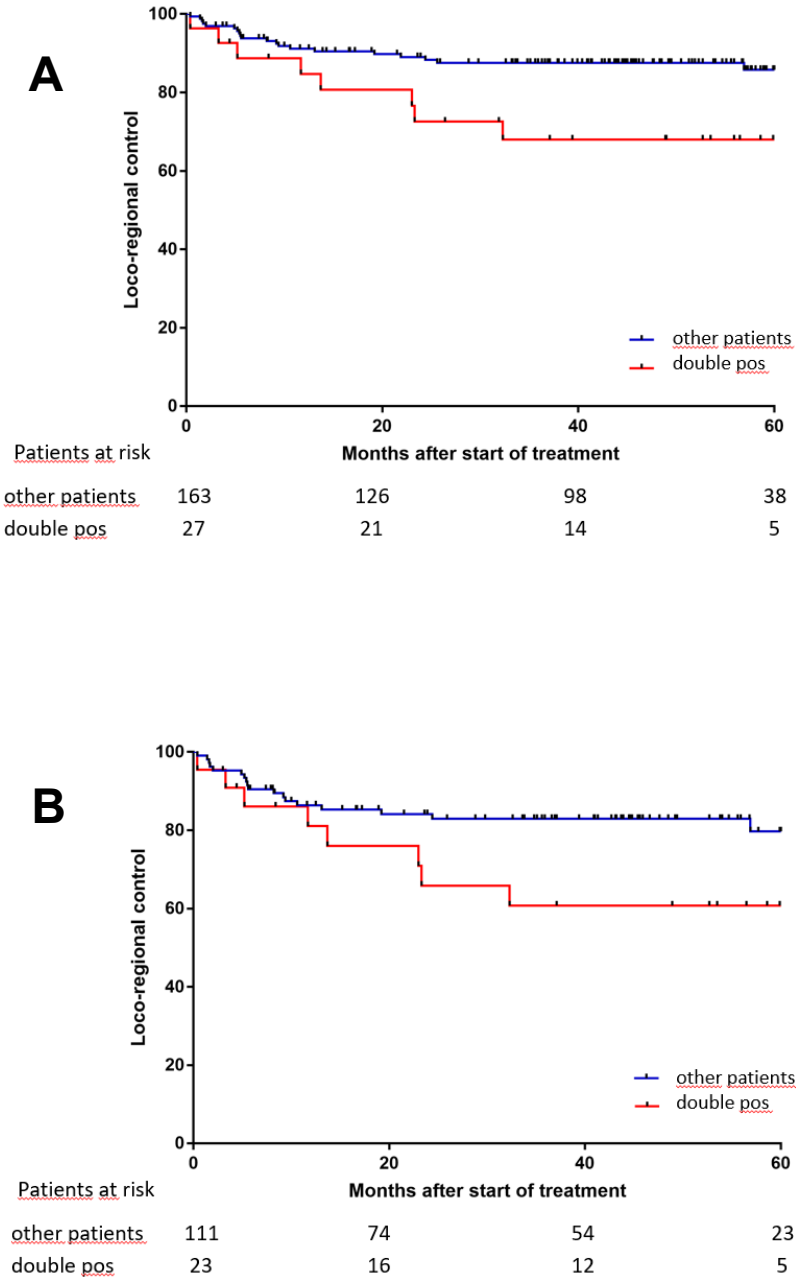

Figure 3.4: Kaplan-Meier curves for locoregional tumour control in all patients (A) and patients with HPV16 DNA negative tumours only (B) according to the SDF-1 and CXCR4 intracellular coexpression (double positive patients).

Table 3.3. Univariate analysis in all patients and patients with HPV16 DNA negative tumours only. Significant results in bold [1].

N=201 (all patients)	**LRC**				**DM**				**OS**			
	HR	CI lower	CI upper	p	HR	CI lower	CI upper	p	HR	CI lower	CI upper	p
Age (< Median 57y vs. ≥ Median)	2,68	1,21	5,91	**0,015**	1,39	0,73	2,64	0,310	1,43	0,87	2,35	0,158
Gender (M vs. F)	0,62	0,27	1,39	0,242	1,66	0,65	4,26	0,289	0,80	0,45	1,43	0,446
Tumour Site (OP vs. OC+HP)	0,37	0,17	0,77	**0,009**	0,34	0,17	0,66	**0,001**	0,55	0,34	0,90	**0,017**
pT stage (pT3-4 vs. pT1-2)	2,75	1,32	5,75	**0,007**	2,24	1,18	4,24	**0,013**	2,48	1,51	4,05	**0,000**
pN stage (pN 2-3 vs. pN 0-1)	1,33	0,54	3,28	0,532	3,02	1,07	8,52	**0,036**	1,10	0,62	1,93	0,752
Grading (G1 vs. G2 vs. G3)	0,57	0,28	1,16	0,121	0,92	0,51	1,69	0,793	0,75	0,47	1,21	0,240
Resection margin (R1 vs. R0)	1,07	0,51	2,22	0,867	1,09	0,57	2,06	0,797	1,12	0,68	1,83	0,655
ECE (yes vs. no)	1,63	0,77	3,46	0,203	2,49	1,19	4,86	**0,014**	1,70	1,03	2,83	**0,040**
p16 (pos. vs. neg.)	0,22	0,08	0,62	**0,005**	0,21	0,08	0,53	**0,001**	0,34	0,19	0,63	**0,001**
HPV16 DNA (pos. vs. neg.)	0,13	0,03	0,54	**0,005**	0,38	0,17	0,87	**0,024**	0,31	0,16	0,61	**0,001**
Smoking (yes vs. never)	1,53	0,36	6,62	0,566	4,13	0,56	30,47	0,164	2,57	0,79	8,34	0,115
mSDF1 (pos. vs. neg.)	0,81	0,25	2,68	0,729	1,28	0,54	3,07	0,578	0,82	0,37	1,80	0,623
mCXCR4 (pos. vs. neg.)	2,13	0,74	6,14	0,162	1,10	0,34	3,59	0,871	1,72	0,78	3,78	0,180
icSDF1 (pos. vs. neg.)	2,67	1,29	5,54	**0,008**	1,02	0,50	2,10	0,955	1,17	0,69	2,01	0,561
icCXCR4 (pos. vs. neg.)	2,02	0,97	4,21	0,059	1,07	0,53	2,16	0,856	1,26	0,75	2,13	0,384
icSDF1+icCXCR4 (pos. vs. neg.)	2,87	1,30	6,29	**0,009**	0,77	0,27	2,15	0,606	1,15	0,59	2,27	0,681

N=134 (HPV16 neg. patients)	**LRC**				**DM**				**OS**			
	HR	CI lower	CI upper	p	HR	CI lower	CI upper	p	HR	CI lower	CI upper	p
Age (< Median 57y vs. ≥ Median)	2,38	1,06	5,34	**0,035**	1,31	0,65	2,66	0,453	1,44	0,84	2,49	0,187
Gender (M vs. F)	0,63	0,28	1,45	0,277	1,98	0,69	5,65	0,204	0,90	0,48	1,69	0,754
Tumour Site (OP vs. OC+HP)	0,68	0,31	1,48	0,327	0,53	0,25	1,13	0,102	0,86	0,50	1,47	0,572
pT stage (pT3-4 vs. pT1-2)	2,69	1,24	5,81	**0,012**	1,95	0,96	3,97	0,063	2,05	1,20	3,51	**0,009**
pN stage (pN 2-3 vs. pN 0-1)	1,46	0,59	3,62	0,417	3,94	1,20	12,96	**0,024**	1,38	0,74	2,58	0,313
Grading (G1 vs. G2 vs. G3)	0,68	0,32	1,45	0,321	0,93	0,47	1,86	0,837	0,62	0,36	1,06	0,081
Resection margin (R1 vs. R0)	1,16	0,54	2,48	0,702	1,07	0,53	2,17	0,858	1,03	0,60	1,75	0,925
ECE (yes vs. no)	1,62	0,75	3,50	0,223	2,63	1,21	5,71	**0,015**	1,94	1,11	3,37	**0,019**
Smoking (yes vs. never)	0,89	0,20	3,93	0,874	Cox PH model did not converge				2,15	0,50	9,29	0,303
mSDF1 (pos. vs. neg.)	0,96	0,23	4,04	0,950	1,69	0,59	4,83	0,329	0,93	0,34	2,59	0,897
mCXCR4 (pos. vs. neg.)	1,24	0,37	4,14	0,722	1,03	0,31	3,39	0,963	1,24	0,53	2,90	0,622
icSDF1 (pos. vs. neg.)	2,54	1,19	5,40	**0,016**	0,94	0,43	2,05	0,883	1,11	0,63	1,95	0,729
icCXCR4 (pos. vs. neg.)	2,04	0,96	4,33	0,065	1,13	0,53	2,41	0,757	1,27	0,73	2,20	0,405
icSDF1+icCXCR4 (pos. vs. neg.)	2,52	1,13	5,60	**0,024**	0,76	0,26	2,17	0,605	1,04	0,52	2,07	0,910

In the multivariate analysis icSDF-1 was confirmed as strong negative independent prognostic factor for LCR among all patients and within the subgroup of HPV16 negative patients (Table 3.4). CXCR4 was not significantly correlated with LCR at the multivariate analysis and the combined expression, even though statistically significant, did not outperformed SDF-1 expression alone.

Table 3.4. Multivariate analysis in all the patients and patients with HPV16 DNA negative tumours only. Significant results in bold [1].

N=134 (HPV16 DNA neg. patients)	**LRC**			
	HR	CI lower	CI upper	p
Age (< Median 57y vs. >= Median)	3,12	1,37	7,13	**0,007**
Tumour Site (OP vs. OC+HP)	0,70	0,32	1,56	0,386
pT stage (pT3-4 vs. pT1-2)	2,45	1,12	5,35	**0,025**
icSDF1	2,99	1,33	6,75	**0,008**
icCXCR4	1,22	0,55	2,74	0,621
	LRC			
	HR	CI lower	CI upper	p
Age (< Median 57y vs. >= Median)	3,11	1,35	7,14	**0,007**
Tumour Site (OP vs. OC+HP)	0,72	0,32	1,59	0,410
pT stage (pT3-4 vs. pT1-2)	2,51	1,15	5,47	**0,021**
icSDF1 + icCXCR4	2,87	1,25	6,59	**0,013**

N=201 (all patients)	**LRC**			
	HR	CI lower	CI upper	p
Age (< Median 57y vs. ≥ Median)	3,33	1,48	7,47	**0,004**
Tumour Site (OP vs. OC+HP)	0,63	0,29	1,38	0,246
pT stage (pT3-4 vs. pT1-2)	2,28	1,08	4,81	**0,031**
HPV16 DNA (pos. vs. neg.)	0,20	0,04	0,87	**0,032**
icSDF1 (pos. vs. neg.)	2,72	1,24	5,93	**0,012**
icCXCR4 (pos. vs. neg.)	1,13	0,52	2,46	0,764
	LRC			
	HR	CI lower	CI upper	P
Age (< Median 57y vs. ≥ Median)	3,33	1,48	7,50	**0,004**
Tumour Site (OP vs. OC+HP)	0,64	0,29	1,42	0,273
pT stage (pT3-4 vs. pT1-2)	2,31	1,09	4,88	**0,028**
HPV16 DNA (pos. vs. neg.)	0,19	0,04	0,86	**0,031**
icSDF1 + icCXCR4 (pos. vs. neg.)	2,66	1,18	6,01	**0,018**

No significant heterogeneity of the effect of icSDF-1 and icCXCR4 expression on LRC was observed between the different DKTK institutions (p-value of 0.25 and 0.33, respectively; Table 3.5).

Table 3.5. Analysis of the effect of icSDF-1 and icCXCR4 expression on locoregional control and relative HR values for the different DKTK centres. The results were tested for heterogeneity using χ^2 test and revealed a p-value of 0.25 and 0.33 for icSDF-1 and icCXCR4 respectively, i.e. the null hypothesis that the HR are identically distributed over the centres is not to be rejected.

				icSDF1 pos		icSDF1 neg		
Center	HR	CI		Events	Total	Events	Total	
1	Cox PH model did not converge			2	4	0	15	
2	3,119	0,437	22,27	2	9	2	22	
3	6,502	0,5826	72,57	2	5	1	22	
4	1,9094	0,5384	6,772	4	10	6	29	χ^2=6.5787 , p=0.25
5	1,7321	0,09752	30,76	1	4	1	12	
6	4,706	0,4229	52,37	1	4	2	24	
7	1,1962	0,1243	11,52	1	6	3	21	
8	Cox PH model did not converge			1	11	0	3	
Overall	2,6733	1,29	5,54	14	53	15	148	

				icCXCR4 pos		icCXCR4 neg		
Center	HR	CI		Events	Total	Events	Total	
1	Cox PH model did not converge			2	4	0	15	
2	3,46	0,49	24,59	2	7	2	22	
3	Cox PH model did not converge			3	9	0	14	
4	0,66	0,14	3,11	2	9	8	27	χ^2=3.438 , p=0.3289
5	Cox PH model did not converge			0	3	2	12	
6	0,63	0,06	6,90	1	12	2	16	
7	2,87	0,40	20,41	2	7	2	20	
8	Cox PH model did not converge			1	4	0	9	
Overall	2,02	0,97	4,21	13	55	16	135	

4 DISCUSSION

4.1 SDF-1/ CXCR4 EXPRESSION AS NEGATIVE PROGNOSTIC FACTOR

In the present study, we investigated the prognostic value of the expression of the chemokine SDF-1 and his receptor CXCR4 in, to our knowledge, the largest and most homogeneous cohort of patients affected by locally advanced HNSCC treated with surgery and adjuvant RT-CT. The results have been published [1]. Our analysis indicates that patients with SDF-1 positive tumours have poorer LRC rates, while the impact of CXCR4 was not confirmed at the multivariate analysis. Interestingly, the prognostic value of SDF-1 on LCR was shown also within HPV negative tumours, suggesting SDF-1 as potential biomarker for future treatment modification in a known higher risk population, as HPV negative patients in comparison to HPV positive patients are. These results suggest that SDF-1 might play a role in conferring to the tumour cells resistance to postoperative RT-CT.

We observed a membrane and an intracellular (cytoplasmic and nuclear) expression on SDF-1 and CXCR4, in linear with other studies. In HNSCC tumour specimens, Faber and colleagues noticed that CXCR4 and SDF-1 were expressed in the membrane and in the cytoplasm of the tumour cells [39]. Interestingly, in our study, the membrane expression had no prognostic value, while the intracellular expression in SDF-1 and, even though not confirmed in the multivariate analysis, of CXCR4 were negatively correlating with LCR. This might open to the speculation that SDF-1/CXCR4 play a significative role after their internalisation in the cytoplasm and translocation to the nucleus. Nevertheless, the value of the subcellular location of SDF-1/CXCR4 remains a matter of discussion. Some authors could detect SDF-1 and CXCR4 in the nucleus of tumour cells in patients-derived colorectal tumour tissues and found that their nuclear expression was a negative factor for patients' survival [63-65]. On the other hand, studies conducted on lung cancer patients showed a positive correlation between the nuclear SDF-1/CXCR4 expression and the

clinical outcome, while the cytomembrane staining had a negative prognostic role [66, 67].

The prognostic value of SDF-1 and CRCXR4 in cancer patients has been described in different studies. In a meta-analysis comprising more than five thousand patients with different tumour histologies, Zhao and colleagues found that patients with CXCR4-positive tumours had a shorter progression free survival and overall survival in comparison with CXCR4 negative tumours [68]. Specifically regarding HNSCC, some authors have already investigating the role of SDF-1/CXCR4. Leon and coll. collected evidence from more than one hundred patients, including laryngeal cancers, treated with surgery and adjuvant RT or RT-CT or primary RT or RT-CT [61]. Low SDF-1 was found a negative prognostic factor for LRC, DMFS and OS and low SDF-1 and high CXCR4 was prognostic for more regional failures. Similarly, Clatot et al. identified low SDF-1 expression as a negative prognostic factor for LRC, DMFS and OS analysing 71 patients affected by tumour of the oral cavity, pharynx and larynx and treated with adjuvant RT or RT-CT, while they didn't find any prognostic role of CXCR4 [60]. A study with a large cohort of 233 patients treated with primary RT or RT-CT, published by Rave-Fraenk and coll., showed that patients with high SDF-1 expressing tumours had better OS while CXCR4 expression was prognostic for worse DMFS. No correlation with LRC was found [59]. Albert and coll. found that among 47 patients treated with surgery for a tumour of the mobile tongue, CXCR4 correlated with high-grade tumours, lymph node metastases, and microscopic nerve invasion and was predictive for worse OS, but this result was not confirmed in the multivariate analysis. No correlation between SDF-1 expression and OS was found [58]. Among 30 patients affected by oral squamous cell carcinomas, Uchida et al. found that SDF-1/CXCR4 overexpression in lymph node metastases was predictive for poor OS [50]. Katayama and coll. evaluated 56 patients affected by oral cavity tumours and treated with neoadjuvant RT-CT, surgery or RT alone and observed that CXCR4 positive tumours was prognostic for poor OS and cancer specific survival [42]. Altogether, these studies offer a conflicting evidence regarding the prognostic role of SDF-1 and CXCR4 in HNSCC. Some of them are based on

relatively small patients' cohort [42, 50, 58] or include patients with tumour stage from I to IV and treated with different approaches (surgery alone, RT or RT-CT alone, surgery and adjuvant or neoadjuvant RT or RT-CT). SDF-1/CXCR4 expression was sometimes assessed through RT-PCR and sometimes through immunohistochemistry. In some studies HPV status was not determined [42, 50, 58] or was reported only for some patients and no correlation with SDF-1/CXCR4 expression was described [60, 61]. In the study published by Rave-Frank and coll. p16 positive tumours showed on average a higher SDF-1/CXCR4 expression in comparison with p16 negative tumours, while in our study SDF-1 was on average lower in p16 positive tumours [59]. The differences between these studies and our results might have different explanations, such as different patients' cohorts and methodological aspects, as follow. Many of the mentioned studies were conducted on patients affected by tumours of the oral cavity, that is less than one third of our cohort; moreover, some of them included laryngeal cancers, that we in contrast excluded. Differences in stage distribution and therapeutic management might also contribute to explain some discrepancies. Additionally, we used immunofluorescence and not immunohistochemistry or RT-PCR to detect SDF-1 and CXCR4. In contrast with these studies, our results are based on a large and homogeneous cohort of patients enrolled in different german centres with well-defined inclusion and exclusion criteria and centrally revised clinical and follow up data. This might overcome potential bias, e.g. cohort effects, methodological problems and differences in treatment modalities. Besides these strength points, the current study presents some weaknesses. In particular, patients were collected retrospectively and the staining analysis was performed with arbitrary-defined thresholds. Additionally, even though the staining analysis was performed by two observers who were blinded to patients' characteristics and outcomes, results might be affected by intrinsic errors of a subjective and not computational analysis.

4.2 MECHANISMS OF TUMOUR AGGRESSIVENESS AND RADIOTHERAPY RESISTANCE IN SDF-1/ CXCR4 POSITIVE TUMOURS

The chemokine pathway represented by SDF-1/CXCR4 have been described in tumours of different type [20, 22, 23], inclusive HNSCC [53]. Many of the biological mechanisms underlying these clinical observations have been described. In general, SDF-1/CXCR4 axis is involved in stimulating tumour cell survival, proliferation and chemotaxis [69] and the evidence that not only tumour cells, but also cells of the tumour microenvironment such as fibroblasts, endothelial and tumour infiltrating leucocytes can produce chemokines and express their receptors, triggers the idea of the tumour tissue as a complex system that self-promotes growth, local invasion and metastasis [18]. More specifically, proliferation signals mediated by MAPK and AKT, increased integrin and TNF-α expression, enhanced MMP activity, induced angiogenesis and modulation of immune cells have been described as mechanisms underlying the actions of SDF-1/CXCR4 in tumours [24]. These factors might explain the more aggressive features of SDF-1/CXCR4 positive tumours *per se*. On the other hand, the poorer LRC rates that we observed among SDF-1/CXCR4 positive patients after post-operative RT-CT might also be explained by a higher radiation resistance of these tumours. SDF-1/CXCR4 induced radiation resistance may be supported by different factors, i.e. AKT mediated signaling, ion channels modification, bone-marrow derived immune cells and mesenchymal cells migration, regulation of focal adhesion kinases and CD8 positive T cells activity, as the following evidence has shown. One of the intracellular pathways activated by SDF-1/CXCR4 is represented by PI3K/AKT. I*n vitro* and in *in vivo* studies have shown that constitutively activated AKT favours a quicker repair of the DNA double strand breaks induced by radiation, conferring to the tumour cells radiation resistance properties [32]. Another possible mechanism underlying radiation resistance in SDF-1/CXCR4 positive tumours is the modification, through IP3, of ion channels activity, which has been found to regulate the formation of free radicals in the radiation induced DNA damage process [34]. As observed in human squamous cell carcinoma xenografts, after radiation, the chemokine gradient created by SDF-1/CXCR4

induces migration of bone-marrow derived immune cells such as CD11b positive myelomonocytes to the tumour, contributing to radio-resistance [70]. The presence of CD11b positive myeloid-derived suppressor cells under high SDF-1 concentration was associated with faster tumour growth in $p53^{null}$ mice [71] and a reduced p53 activity has been shown to stimulate the migration of mesenchymal stromal cells from the bone marrow to the tumour through higher SDF-1 level [72]. SDF-1/CXCR4 might influence the treatment response through regulation of the expression of focal adhesion kinases (FAK), as suggested by studies conducted on breast cancer cells [73] and on three-dimensional grown human HNSCC, where FAK overexpression induced by SDF-1 was responsible for radiation resistance through activation of ERK and AKT [74]. Additionally, in squamous cell carcinomas, FAK, under high chemokines concentrations, was responsible for inhibition of the anti-tumoural CD8 positive T cell activity [75]. CD8+ T cells proliferation and actions are enhanced by anti-programmed death-ligand 1 (PD-L1) drugs and some studies suggested a synergistic effect of anti-PD-L1 immunotherapy and anti-SDF-1/CXCR4 target therapies [76, 77].

Different factors are known to regulate the tumour response to fractionated RT, i.e. tumour cells repopulation, reoxigenation, redistribution, repair of the radiation induced-DNA damage and intrinsic radiation sensitivity. Even though during RT the tumour volume may change, these variations depends mainly by cells that do not have clonogenic properties, which are the majority of the tumour cells. The goal of RT is to avoid the repopulation of the few tumour cells that might be responsible for a recurrence, namely cancer stem cells (CSCs) [78]. If the quantitative transplantation assay remains the goal to prove if cells are CSCs or not, some proteins expressed on the cell surface have been described and are now commonly accepted as CSCs markers, e.g. CD133, CD44, CD29 [79]. Interestingly, evidence indicates CXCR4 as a potential biomarker for radioresistant cancer stem cells [36]. It has been found overexpressed in glioma stem cells and, together with SDF-1, in perihypoxic gliomas areas, contributing to tumour cells aggressiveness and resistance to RT-CT induced cell death [80]. Faber et al. observed that high SDF-1

concentrations stimulated the formation of podia in CD44 and CXCR4 positive HNSCC cells, indicating that SDF-1/CXCR4 axis might play a key role in the interaction between CSCs and their supportive cells in the CSC niche [40]. Additionally, oxygen availability is required for tumour growth and relapse. After irradiation, sprouting angiogenesis from adjacent vessels is inhibited and the formation of new vascular structures is dependent on circulating bone marrow derived cells (BMDCs). Experimental gliomas and prostate tumour models have shown that in a post RT-hypoxic tumour, HIF-1α stimulates SDF-1 production, which in turn promotes, as already mentioned, the migration of BMDCs to the tumour, contributing to reoxigenation and tumour growth [81, 82]. Moreover, through pro-proliferation signals such as those transcripted by NFkB under the stimulation of SDF-1/CXCR4 [43], after RT, quiescent tumour cells might be triggered to re-entering in the cell cycle and proliferate. Besides playing a role in tumour cell repopulation, reoxigenation and maybe redistribution, we have already seen how SDF-1/CXCR4 can interfere with the ability of the tumour cell to repair the DNA DSBs induced by RT though AKT [32], another crucial factor for the tumour response to radiation.

4.3 SDF-1/ CXCR4 AS TARGETABLE PATHWAY

Given the crucial role that SDF1 and CXCR4 play in cancers, multiple agents targeting this pathway have been developed [83]. The most studied is AMD3100, a pharmacological antagonist of CXCR4 (pubchem CID 65015; also known as Plerixafor, Mozobil; Sanofi SA, Paris, France). Preclinical studies, including in HNSCC models, showed a decreased cell proliferation, motility, invasion and a reduced metastatic potential under treatment with AMD3100 [48, 69, 84, 85]. Importantly, an increase in response to RT was observed after administration of CXCR4 antagonist in xenograft breast, lung and glioblastoma tumours [86-88]. In clinical studies, some CXCR4 antagonists were used as bone marrow stem cells mobilizers and were employed either alone or in combination with other systemic therapies for diseases like lymphoma,

leukaemia and myeloma [89, 90]. *In vitro* and *in vivo* studies suggest that treatment with CXCR4 antagonists favours the response to chemotherapeutic agents also in solid tumours, i.e. prostate cancer and glioblastoma [91, 92]. A phase I trial investigating the effect of a CXCR4 peptide antagonist (LY2510924) in combination with the anti-PD-L1 antibody Durvalumab (MEDI4736) in in advanced refractory solid tumours is ongoing (ClinicalTrials.gov identifier: NCT02737072). Another phase 1/2 study employing the CXCR4 inhibitor USL311 alone or in combination with Lomustin in patients affected by advanced solid tumours or relapse/recurrent glioblastoma is currently ongoing (ClinicalTrials.gov identifier: NCT02765165). Altogether, evidence supports the design of future studies, in which CXCR4 antagonists might be investigated as radiosensitisers in patients affected by solid tumours, inclusive HNSCC, and treated with RT or RT-CT.

4.4 SDF-1/ CXCR4 AND BIOLOGY-DRIVEN INDIVIDUALIZED RADIOTHERAPY

Tumour stage is the main factor determining treatment choice in HNSCC patients. Many biomarkers have been identified and tested, but haven't reached a clinical application yet. Since biological interpatient, but also intrapatient, differences are responsible for treatment resistance and recurrence, to incorporate biological information into treatment strategies is crucial to improve patients' outcome. Importance and efficacy of biology-driven RT were shown among HNSCC patients in the last decades, by applying accelerated fractionated RT schedules, using hypoxia modifiers and adding concomitant CT to RT. Historically, standard fractionated RT alone was known as curative treatment for HNSCC, but with survival rates of approximately 30% at five years [93]. Alternative fractionations, i.e. accelerated and accelerated hyperfractionated schedules, with or without concomitant boost, reduce the overall treatment time and compensate the tumour (stem) cells repopulation. Applied to patients in clinical studies, they have been seen to correlate with

better oncological outcomes, by higher acute but similar late toxicity [94-96] and became the standard of care in many institutions worldwide. Hypoxic tumour cells are known to be more radioresistant and the presence of hypoxic tumour regions is crucial for RT response and tumour control, especially in HNSCC [97]. Efforts to reduce the hypoxic tumour content have been made, e.g. through the addition of hypoxia-modifying drugs as Nimorazole. A better response was observed, but their use is still limited [98]. Approaches of dose-escalation on the hypoxic areas have been tested and appear promising [99]. Among, HNSCC, the significance of finding new biomarkers is exemplified by HPV16. Since its importance as prognostic factor was established, new trials have been opened to investigate the possibility of a de-escalation of the RT dose (e.g. De-escalation of adjuvant radio (chemo) therapy for HPV-positive head and neck squamous cell carcinomas: a phase I study to reduce late toxicity, ClinicalTrials.gov identifier: NCT03396718). Patients' stratification on the basis of prognostic and predictive biomarkers may lead in the future to individualized-biology driven RT.

The DKTK-ROG aims to identify the most significant biomarkers as basis for RT interventions and, within this project, we conducted the present hypothesis-generating study, investigating the prognostic potential of SDF-1/CXCR4 in HNSCC patients. Our exploratory results needed validation, which we performed in a cohort of HNSCC patients treated with primary RT-CT [100]. With the same methods of the present study, we could confirm the negative prognostic role of SDF-1 but also of CXCR4 for LRC. Additionally, we observed that these two biomarkers were correlating with a poor OS. Analysing the tumour material of the HNSCC patients included in the retrospective postoperative and primary cohorts, other partner groups within the DKTK-ROG showed the prognostic role of HPV16 DNA, p16 expression, CD8+ tumour-infiltrating lymphocytes, expression of cancer stem cells markers, PD-1/PD-L1 expression, Heat shock protein 70, tumour-infiltrating NK cells, hypoxia-associated gene signatures and genetic alterations [62, 101-108]. The DKTK-ROG is currently running the HNprädBio study, where the prognostic value of SDF-1/CXCR4 and other biomarkers might be validated in prospectively

enrolled HNSCC patients. Moreover, an analysis of all these promising biomarkers is currently ongoing, aiming to reveal the most promising ones and design future biology-driven interventional trials.

4.5 CONCLUSION

In our study, we demonstrated the negative prognostic role of the chemokine pathway SDF-1/CXCR4 in patients affected by locally advanced HNSCC treated with surgery and adjuvant RT-CT. Our data are still exploratory and need to be validated in a prospectively collected patients' cohort. Nevertheless, they appear promising and support further investigations of the SDF-1/ CXCR4 axis in HNSCC patients, aiming to stratify patients on the basis of biomarkers expression, for a biologically individualized RT-CT. Moreover, it represents a potential therapeutic target to overcome treatment resistance.

5 SUMMARY

Outcomes of patients affected by locally advanced head and neck squamous cell carcinomas (HNSCC) and treated with surgery and adjuvant radio-chemotherapy are still unsatisfactory, being the overall survival (OS) still settled around 50% at five years, with poor outcomes especially among HPV negative patients. Therefore, patients' stratification based on biomarker profiles is required for personalised therapies. SDF-1/CXCR4 is known as the most important chemokine pathway involved in tumour development, progression and metastasis. Nevertheless, no clear data exist regarding the role of SDF-1/CXCR4 among HNSCC patients. The present study aims to investigate the prognostic value of SDF-1 and CXCR4 in a large and homogeneous cohort of HNSCC patients treated with post-operative RT-CT, as part of a multicentre biomarker study within the German Cancer Consortium Radiation Oncology Group. The results of the study have been published [1].

221 patients affected by stage III and IVA HNSCC of the oral cavity, oropharynx and hypopharynx were treated with surgery and adjuvant RT-CT. Tumour microarrays with the post-surgical tumour material were generated and stained for SDF-1 and CXCR4 using immunofluorescence. 201 patients were analysed for SDF-1 and 190 for CXCR4.

The univariate and multivariate analyses showed that higher SDF-1 intracellular expression significantly correlated with poor locoregional control (LRC) in the whole patients' cohort as well as in the HPV16 negative patients. Higher CXCR4 intracellular expression correlated with poor LRC in the univariate analysis, but this trend was not confirmed in the multivariate analysis. Not high SDF-1 nor high CXCR4 expression correlated with distant metastasis free survival or OS.

In summary, we could show for the first time in a large and homogeneous cohort of HNSCC patients treated with adjuvant RT-CT that SDF-1 correlates with worse LRC. These results are exploratory and need to be validated

prospectively, but support further investigation of SDF-1/CXCR4 as potential biomarker for treatment individualization and as a target to overcome resistance to RT.

6 ZUSAMMENFASSUNG

Die Prognose von Patienten mit lokal fortgeschrittenen Plattenepithelkarzinomen im Kopf-Hals-Bereich, die mit einer chirurgischen Intervention und einer adjuvanten kombinierten Radiochemotherapie behandelt werden, ist mit einer 5-Jahres-Überlebensrate von 50% noch nicht zufriedenstellend. Dies gilt insbesondere für HPV negative Patienten. Der Stratifizierung von Patienten mit Hilfe von Biomarker-Profilen kommt daher eine besondere Bedeutung für personalisierte Therapien zu. SDF-1/CXCR4 ist dabei der wichtigste Chemokin-Pathway in der Tumorgenese, Tumorprogression und Metastasierung. Nichtsdestotrotz liegt keine klare Datenlage zur Rolle des SDF-1/CXCR4-Pathways bei Plattenepithelkarzinom-Patienten im Kopf-Hals-Bereich vor. Die vorgelegte Studie untersucht den prognostischen Wert von SDF-1 und CXCR4 in einer großen und homogenen Kohorte von Plattenepithel-Karzinomen im Kopf-Hals-Bereich, die mit einer postoperativen kombinierten Radiochemotherapie im Rahmen einer multizentrischen Biomarker-Studie der Deutsches Konsortium für Translationale Krebsforschung- RadioOncology Group (DKTK-ROG) behandelt wurden. Die Ergebnisse wurden publiziert [1].

Insgesamt 221 Patienten mit Mundhöhlen-, Oro- und Hypopharyxkarzinomen im Stadium III und IVa wurden mit einer chirurgischen Intervention und einer adjuvanten Radiochemotherapie behandelt. Tumor-Microarrays vom Resektionsmaterial wurden untersucht und eine Immunfluoreszenz-Färbung spezifisch für SDF-1 und CXCR4 durchgeführt. 201 Patienten wurden für SDF-1 und 190 für CXCR4 untersucht.

Univariate und multivariate Analysen zeigten, dass eine höhere intrazelluläre Expression von SDF-1 signifikant mit einer schlechteren lokoregionären Kontrolle (LRC) sowohl in der gesamten Kohorte als auch in der Gruppe der HPV-negativen Patienten korreliert war. Eine höhere intrazelluläre Expression von CXCR4 korrelierte mit einer schlechteren lokoregionären Kontrolle in der univariaten Analyse; dieser Trend konnte in der multivariaten Analyse jedoch nicht bestätigt werden. Weder eine hohe SDF-1- noch eine hohe CXCR4-Expression korrelierten mit Metastasen-freiem Überleben oder Gesamtüberleben.

In der vorliegenden Studie konnten wir zum ersten Mal in einer großen und homogenen Kohorte von Patienten mit Plattenepithelkarzinomen im Kopf-Hals-Bereich, die mit Operation und postoperativer Radiochemotherapie behandelt wurden, zeigen, dass SDF-1 mit einer schlechteren lokoregionären Kontrolle korreliert. Diese Ergebnisse sind explorativ und sollten im Verlauf weiter validiert werden. Dennoch sind SDF-1 und CXCR4 vielversprechende Kandidaten als potentielle Biomarker für die Individualisierung von Therapien und als Ansatzpunkt zur Überwindung von Strahlenresistenz.

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8 ERKLÄRUNG ZUM EIGENANTEIL DER DISSERTATIONSSCHRIFT

Die Arbeit wurde in der Klinik für Radioonkologie am Uniklinikum Tübingen unter Betreuung von Prof. Dr. D. Zips und Dr. A. Menegakis durchgeführt.
Die Konzeption der Studie erfolgte durch Prof. Dr. D. Zips und Dr. A. Menegakis.
Die Versuche wurden nach Einarbeitung durch Dr. A. Menegakis von mir eigenständig durchgeführt.
Die statistische Auswertung erfolgte nach Anleitung durch Dr. D. Mönnich durch mich.
Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.
Diese Arbeit wurde im Rahmen einer multizentrischen Biomarker-Studie des Deutsches Konsortium für Translationale Krebsforschung- RadioOncology Group (DKTK-ROG) durchgeführt. Der Material, den in Tübingen von mir analysiert wurde, wurde in acht DTKT-ROG partner Institutionen, die in diesem Manuskript aufgelistet sind, gesammelt.
Die Publikation [1], welche auf den Daten dieser Doktorarbeit basiert, wurde von mir unter Anleitung von Herrn Prof. Dr. Zips und Dr. A. Menegakis selbständig verfasst. Die Korrektur des Manuskripts erfolgte durch Herrn Prof. Dr. Zips, Dr. A. Menegakis, Dr. D. Mönnich und die anderen Autoren, die in der Publikation und in diesem Manuskript aufgelistet sind.

Tübingen, den 08.03.2018 Chiara De-Colle

9 PUBLICATION

The results of the present study have been published this year in the following original article:

Authors: C. De-Colle, D. Mönnich, S. Welz, S. Boeke, B. Sipos, F. Fend, P.S. Mauz, I. Tinhofer, V. Budach, J.A. Jawad, M. Stuschke, P. Balermpas, C. Rödel, A.L. Grosu, A. Abdollahi, J. Debus, C. Bayer, C. Belka, S. Pigorsch, S.E. Combs, F. Lohaus, A. Linge, M. Krause, M. Baumann, D. Zips and A. Menegakis

Title: SDF-1/CXCR4 expression in head and neck cancer and outcome after postoperative radiochemotherapy

Journal: Clinical and Translational Radiation Oncology

Year: 2017

10 AKNOWLEDGMENTS

I would like to thank Prof. D. Zips for the time and passion he dedicated to this project. A huge thank goes to Dr. A. Menegakis, whose experience and brilliant ideas could solve problems and finalize this work. Together, Prof. D. Zips and Dr. Menegakis taught me radiobiology and opened my mind to translational research.

Thanks to Dr. D. Mönnich, for helping me with the statistical analysis.

I would like to thank the laboratory of Prof. H.P. Rodemann, where I performed the experiments during the first months. Another special thanks to the group of research of the Centre for Ophthalmology, for allowing me to use their microscope.

Last but not least, thanks to my family and friends, for their support during these years of research work.

11 CURRICULUM VITAE

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Muttersprache	Italienisch
Weitere Sprache(n)	Englisch und Deutsch, C1 Niveau

PUBLIKATIONEN

- "SDF-1/CXCR4 expression is an independent negative prognostic biomarker in patients with head and neck cancer after primary radiochemotherapy". C. De-Colle, A. Menegakis, D. Mönnich et al. Radiotherapy and Oncology (2017) article in press
- "Ex vivo γH2AX radiation sensitivity assay in prostate cancer: Inter-patient and intra-patient heterogeneity". C. De-Colle, A. Yaromina, J. Hennenlotter et al. Radiotherapy and Oncology (2017) 3, 386-394
- "SDF-1/CXCR4 expression in head and neck cancer and outcome after postoperative radiochemotherapy". C. De-Colle, D. Mönnich, S. Welz et al. Clinical and Translational Radiation Oncology (2017) 5, 28-36
- "Insufficiency bone fracture after pelvic radiotherapy: retrospective evaluation of risk factors and dose-volume relationship analysis". A. Ruggieri, C. Airaldi, B. Baiotto, E. Gino, L. Bianco, M. Stasi, C. De-Colle and M.G. Ruo Redda. European Journal of pharmaceutical and medical research (2017) 4(1), 172-178
- "Hypofractionation with no boost after breast conservation in early stage breast cancer patients". F. Arcadipane, P. Franco, C. De-Colle et al. Medical Oncology (2016) 33:108
- "Residual γH2AX foci after ex vivo irradiation of patient samples with known tumour-type specific differences in radio-responsiveness". A. Menegakis, C. De-Colle, A. Yaromina et al. Radiotherapy and Oncology 116 (2015) 480-485
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PRAESENTATIONEN

- "Ex vivo γH2AX radiosensitivity assay in prostate cancer patient tumour samples. Evaluation of inter-patient and intra-patient heterogeneity". C. De-Colle, A. Yaromina, H. Thames et al. 26° Symposium experimentelle Strahlentherapie und klinische Strahlenbiologie. Tübingen, 09-11 Februar 2017
- "Factor 2.5 radiosensitivity difference determined by ex vivo γH2AX assay in prostate cancer patients". C. De-Colle, A. Menegakis, A-C. Müller et al. ESTRO 35 Congress. Turin, 29 April - 3 Mai 2016
- "Ex vivo γH2AX assay in prostate cancer samples reveals substantial differences in intrinsic radiation sensitivity". C. De-Colle, A. Menegakis, A-C. Müller et al. 25° Symposium experimentelle Strahlentherapie und klinische Strahlenbiologie. Dresden, 11-13 Februar 2016
- "The volume effect in radiotherapy". C. De Colle, invited lecture on the 1st advanced AIRB course on radiobiology, Brescia, 13 Oktober 2015

- "γH2AX as a biomarker of radiation sensitivity in ex vivo irradiated samples from tumour patients" C. De Colle, J. Hennenlotter, M. Scharpf et al. 24° Symposium experimentelle Strahlentherapie und klinische Strahlenbiologie.Hamburg, 26-28 Februar 2015
- "Accelerated Partial Breast Irradiation (APBI) with High Dose Rate Brachitherapy (HDR-BT): the Turin experience". C. De Colle, S. Gribaudo, P. Rovea et al. XXIV National AIRO Congress. Padova, 8-11 November 2014
- "Surgical resection versus radiotherapy after CDDP/ Docetaxel induction chemotherapy in locally advanced Non Small Cell Lung Cancer (NSCLC)". C. De Colle, P. Bironzo, M. Levis et al. XXII AIRO Congress. Rom, 17-20 November 2012
- "Impact of Four Dimensional Computed Tomography (4D-CT) in the treatment planning of Stage IA and IB NSCLC patients treated with SBRT: University of Turin experience". C. De Colle, S. Badellino, C. Piva et al. IV AIRO Young National Congress. Rom, 25 Juni 2011