Thesis

The Role of Chemokine Receptors in Salivary Gland Tumors

submitted by

Victoria Ruhdorfer

in partial fulfillment of requirements for the degree of

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University of Graz

executed at the

Diagnostic and Research Institute (D&F) of Pathology

under supervision of

Priv.-Doz^{in} Dr.^{in} med. univ. Ariane Aigelsreiter

Dr.^{in} med. univ. Marlene Leoni

Graz, Datum 21.02.2025

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Furthermore, I hereby declare that if artificial intelligence (AI) tools were used for the generation and/or correction of certain text passages in the creation of this work, such employment was conducted in compliance with ethical principles, academic integrity, and the regulations of my university. Additionally, it was ensured that this usage was transparently disclosed and appropriately attributed.

Graz, Datum 21.02.2025 Victoria Ruhdorfer m.p.

Acknowledgment

First and foremost, I would like to thank my supervisors, Priv.-Doz.^{in} Dr.^{in} med. Univ. Ariane Aigelsreiter and Dr.^{in} med. Univ. Marlene Leoni for all their help and support and for giving me the chance to work in a field I´ve been so interested in since I was a young adult.

I would also like to thank Priv.-Doz. Mag. rer. nat. Dr. scient. med. Alexander Deutsch, for all the help with the statistical analysis.

I am deeply grateful for the support I received from Silvia Schauer and Dr. med. Raphael Gassner, who taught me all I needed to know about the practical lab part of my thesis.

Finally, I would like to thank my partner, Claus Trost, and my parents for always supporting me.

Thank you!

Zusammenfassung

Hintergrund

In dieser Diplomarbeit wurde das Chemokinrezeptorprofil von Speicheldrüsenkarzinomen ermittelt, um Unterschiede in der Expression von Chemokinen zwischen Normalgewebe und Tumorgewebe zu finden. Ziel war es Daten zu generieren, die die Grundlage einer möglichen zielgerichteten Therapie von Speicheldrüsenkarzinomen in der Zukunft darstellen könnten. Mittels Immuntherapie könnte die Tumorprogression und/oder Rezidive positiv beeinflusst beeinflusst werden. Des Weiteren wurde erwartet mit den Ergebnissen die grundlegende molekulare Pathologie von Speicheldrüsenkarzinomen besser zu verstehen.

Methoden

In dieser retrospektiven empirischen Arbeit wurden 70 in Paraffin eingebettete Patient*innenproben labortechnisch aufgearbeitet (Extraktion von RNA) und mittels qPCR ausgewertet. Das Chemokinrezeptorprofil wurde anschließend statistisch ausgewertet. Bei der Auswertung wurde das Speicheldrüsentumorgewebe mit Gewebe von gesunden Tonsillen und Lymphknoten verglichen. Es wurden plattenepitheliale mit drüsigen Speicheldrüsentumor statistisch verglichen. Die Daten wurden anschließend mittels einer Heatmap graphisch dargestellt. Des Weiteren wurde anhand dieser Daten eine Survival-Analyse durchgeführt.

Ergebnisse

Obwohl es in der wissenschaftlichen Literatur Publikationen gibt, die eine erhöhte Expression von CXCR4, CXCR7, CCR4 oder CCR7 in Speicheldrüsentumoren beschreiben, konnte in dieser Diplomarbeit keine solche erhöhte Expression gefunden werden. Auch beim direkten Vergleich von drüsigen Phänotypen von Speicheldrüsentumoren und plattenepithelialen Phänotypen wurden keine statistisch signifikanten Unterschiede gefunden. Eine Survival Analyse konnte zeigen, dass Patienten*Innen mit Speicheldrüsentumoren mit einer niedrigen Expression in CCR6 und CCR10 eine höhere Sterberate aufwiesen.

Schlussfolgerung

Es konnten keine statistisch relevanten Ergebnisse in dieser Diplomarbeit gefunden werden. Allerdings zeigte die Analyse der Daten eine gesteigerte Sterberate bei Patient*innen welche Tumore mit niedriger CCR-Genexpression für CCR6 oder CCR10 aufwiesen. Mehrere

andere Studien haben Hinweise gefunden, dass eine niedrige Expression von CCR6 ein prognostischer Faktor für eine niedrige Überlebensrate sein könnte. Aus diesem Grund sollten die gefundenen Ergebnisse in größeren Kohorten analysiert und validiert werden.

Abstract

Background

In this thesis the chemokine receptor profile of salivary gland tumors was investigated to find differences in the expression of chemokines between normal tissue and tumor tissue. This data could be of significance for the development of a targeted therapy through immunotherapy to influence tumor progression and/or tumor relapse. Furthermore, the molecular pathology of salivary gland tumors could be better understood.

Methods

In this retrospective study, 70 paraffin-embedded patient samples were processed in the laboratory. RNA was extracted and analyzed via qPCR. The chemokine receptor profiles were statistically analyzed . The salivary gland tumor tissue was compared to healthy tissue from tonsils and lymph nodes. Additionally, squamous and glandular salivary gland tumor tissue was statistically compared. The data were plotted in a heatmap, and a survival analysis was performed.

Results

Although scientific literature describes an increased expression of CXCR4, CXCR7, CCR4 or CCR7 in salivary gland tumors, no such increased expression could be found in this thesis. When directly comparing squamous and glandular phenotypes of salivary gland tumors, no statistically significant differences could be found. The survival analysis showed that patients with salivary gland tumors that had a low expression in CCR6 and/or CCR10 have a higher death rate.

Conclusion

No statistically significant results were found in this thesis. However, the survival analysis showed an increase in the death rate of patients with tumors with low CCR-gene expression for CCR6 and CCR10. There are several other studies suggesting that a low CCR6 expression might be a prognostic factor for poor survival. Therefore, this result should be further studied in larger cohorts.

Table of Contents

Acknowledgment 3
Zusammenfassung 4
Abstract 6
Table of Contents 7
List of Abbreviations 8
List of Figures 9
List of Tables 11
1 Introduction 12
1.1 Salivary Gland Carcinoma 12
1.2 Chemokines 15
1.3 Chemokine Receptors 17
1.4 The Functions of the Chemokine Network 19
1.5 Chemokine Network and Cancer 21
1.6 Chemokines and Salivary Gland Carcinoma 22
2 Material and Methods 24
2.1 Tissue Samples 24
2.2 RNA Isolation 25
2.3 cDNA-Synthesis 25
2.4 Real-Time PCR 25
2.5 Statistical Analysis 27
3 Results 29
3.1 Chemokine Receptor Expression Pattern 29
3.2 Relative Expression of CC Chemokine Receptors in salivary gland carcinoma. 29
3.3 Relative Expression of CXC Chemokine Receptors in salivary gland carcinoma 30
3.4 Chemokine Receptor Expression Patterns 31
3.5 Survival analysis 32
4 Discussion 35
5 Conclusion 37
References 38
6 Appendix 45

List of Abbreviations

ACKR	atypical chemokine receptor
C	Cystein
cDNA	complementary DNA
CKR	chemokine receptor
CCKR	conventional chemokine receptor
FFPE	formalin-fixed paraffin-embedded
GDP	guanosine diphosphate
GTP	guanosine triphosphate
MDSCs	myeloid-derived suppressor cells
MECs	mucoepidermoid carcinoma
NK	natural killer cells
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-qPCR	real-time quantitative PCR
SGC	salivary gland carcinoma
SGT	salivary gland tumor
TAM	tumor-associated macrophages
TAN	tumor-associated neutrophils
TH1	T-helper cell 1
TME	tumor microenvironment

List of Figures

Figure 1: Mucoepidermoid carcinoma. Tumor tissue is present in a major part of the picture with a small portion of non-neoplastic salivary gland tissue (left). 13
Figure 2: Adenoid cystic carcinoma. . In this picture, the acinar cell carcinoma has undergone a high-grade transformation. 14
Figure 3: Acinar cell carcinoma. Tumor tissue can be seen in most of the picture. In the left upper corner, a small portion of non-neoplastic salivary gland tissue can be seen 14
Figure 4: Squamous cell carcinoma. In this picture, the tumor is infiltrating the salivary gland tissue. 15
Figure 5: Chemokine structure. (A) Schematic indicating the relationships between conserved cysteine residues (C) and their corresponding intrachain disulfide bridges. The schematic depicts four classes of chemokines, including C-, CC-, CXC-, and CX3C-. **(B)** The schematic representation of human CXCL5 is an ELR+ chemokine that is pro-inflammatory and pro-angiogenic. The ELR motif is positioned N-terminal to the CXC consensus sequence (35). 16
Figure 6: Chemokine Family and Their Cognate Receptors. Most CC (blue) and CXC (green) chemokines demonstrate the capacity to bind numerous receptors, while a singular receptor can bond with multiple chemokines. Additionally, decoy receptors (black) can interact with multiple chemokines. On the other hand, a minority of receptors (red) only have one ligand (44). 18
Figure 7: Functions of Chemokines and their Receptors. The biological processes regulated by chemokines and their receptors are presented in light blue boxes arranged clockwise in alphabetical order (starting from the bottom left) around the central 'Chemokines & Receptors' box. "Cell movement" is the primary biological process regulated by chemokines and their receptors. The figure shows green boxes that depict chemokine-mediated cell arrest and adhesion, as well as various migratory behaviors that fall under chemokine control (26). 21
Figure 8: CCR tested. None of the tested CCRs showed statistically significant results (p-value shown in each subfigure). The ordinate shows the relative expressions of the chemokine receptor given with its CCR number in the sub-heading of the subfigure (a-h). 30
Figure 9: CXCR tested. None of the tested CXCR showed statistically significant results (p-value shown in each subfigure). The ordinate shows the relative expressions of the chemokine receptor given with its CXCR number in the sub-heading of the subfigure (a-c). 31
Figure 10: Hierarchical clustering based on the mRNA expression levels using ΔCT-values. The different entities include ACC (Adenoid cystic carcinoma, brown), AciCC (Acinic cell carcinoma, khaki), BCC (Basal cell adenocarcinoma, grey), EMC (Epithelial-myoepithelial carcinoma, dark blue), MEC (Mucoepidermoid carcinoma, blue), SCC (squamous cell carcinoma, red) and SDC (salivary duct carcinoma, light blue). Shades of red display a low chemokine receptor expression, whereas shades of blue indicate a high chemokine receptor expression. The black and grey columns display a division in squamous and glandular phenotype. 32
Figure 11: Survival analysis of high vs low CCR6 expression over several years. There are two strata, high CCR6 expression and low CCR6 expression, that represent the two sample groups that are being compared. The number of patients at risk represents the number of samples (respective patients) that are still being followed. This number decreases due to

patient that no longer made use of checkups, patients who were no longer advised to seek medical help due to remission and patients who unfortunately died. 33
Figure 12: Survival analysis of high vs low CCR10 expression over several years. Similar to Figure 11 there are two strata, high CCR10 expression and low CCR10 expression, that represent two sample groups that are being compared. The number of patients at risk represents the number of samples (respective patients) that are still being followed. This number decreases due to patient that no longer made use of checkups, patients who were no longer advised to seek medical help due to remission and patients who unfortunately died. 34

List of Tables

Table 1: Chemokines in humans (adapted from Zlotnik et al.(36) and Bachelerie et al.(37)) 16
Table 2. Chemokine receptors in humans (adapted from Zlotnik et al.(36) and Bachelerie et al.(37)) 19
Table 3: Nucleotide acid sequence of primers used in the real-time PCR 26
Table 4: Concentration of the primers used in the experiment 27
Table 5: High vs low expression of chemokine receptor expression in different entities of salivary gland tumors. The various entities include ACC (Adenoid cystic carcinoma), AciCC (Acinic cell carcinoma), BCC (Basal cell adenocarcinoma), EMC (Epithelial-myoepithelial carcinoma), MEC (Mucoepidermoid carcinoma), SCC (squamous cell carcinoma) and SDC (salivary duct carcinoma). 32

1 Introduction

1.1 Salivary Gland Carcinoma

Salivary glands originate from an epithelial placode during embryonic development between the 4th and the 12th week (1). Myoepithelial cells regulate secretory release (2). Inner epithelial cells differentiate into acini or ducts (3). The striated ducts and the following conducting portion are supported by basal cells (4). The produced saliva is essential for the digestion process, speech, taste, and protection of the soft oral tissues and teeth (5,6).

There are three pairs of major salivary glands, including the parotid, sublingual, and submandibular glands, as well as numerous minor salivary glands which are distributed throughout the oral cavity. Porcheri et al. describes tumors in salivary gland tissue as frequently benign (7). Malignant tumors that occur in salivary glands are primarily carcinomas (epithelial differentiation) and show very heterogeneous features and occasional neuroendocrine differentiation (8). Malignant salivary gland tumors (SGTs) account for about 3-10% of all head and neck cancers, with males slightly more affected (9–11). Sung H et al. estimated that 53.583 new cases of salivary gland cancer were diagnosed worldwide in 2020 (12). Thus, SGTs account for 0.3% of all cancer cases (12).

The incidence peaks between the ages of 30 to 60 years (4, 9).The most common site for malignant salivary gland tumors are the minor glands with a described percentage of 47% (14). Malignant tumors found in the parotid gland account for about 42% of all salivary gland tumors, which are followed by submandibular gland neoplasms, which account for 10% of cases (14). 70-90% of tumors affecting sublingual glands are malignant, while almost half of the tumors of the minor salivary glands are malignant (15).

The etiology of salivary gland tumors is predominantly described by the multicellular theory, according to which each tumor type develops from a specific differentiated cell originating within the salivary gland (16,17). In 2023, the World Health Organization (WHO) acknowledged 21 types of malignant salivary gland cancer (18).

The most common malignant salivary gland tumors include mucoepidermoid carcinoma (Figure 1), adenoid cystic carcinoma (Figure 2), acinic cell carcinoma (Figure 3), polymorphous adenocarcinoma, squamous cell carcinoma (Figure 4), non-Hodgkin lymphoma, and pleomorphic adenoma (18–21). Furthermore, salivary glands are a site for tumorigenic cells to establish metastases (7).

Clinical manifestations, histology, and response to therapy in salivary gland tumors differ and may vary according to the TNM stage. In addition, some tumors have prognostic

markers such as histologic grade, histologic type, and genetic translocations (14). Common salivary gland neoplasms depend on signaling pathways that play a central role in their development (22,23).

The initial treatment for salivary gland tumors typically involves surgery, followed by adjuvant radiation therapy (18, 24).

An overview of different tumor tissues is given in Figure 1 to 4. The images were taken via an optical microscope.

In Figure 1 a typical mucoepidermoid carcinoma is presented. There are three main cell types in mucoepidermoid carcinoma: mucin-producing cells, squamous cells and intermediate cells. There may be both cystic or solid patterns (8).

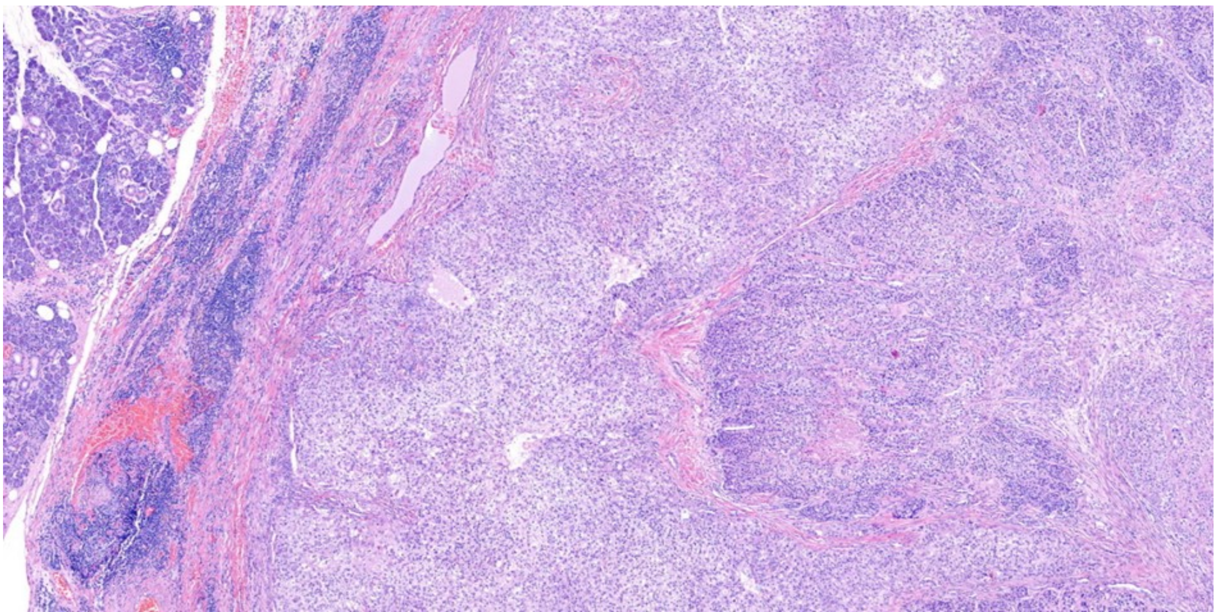

Figure 1: Mucoepidermoid carcinoma. Tumor tissue is present in a major part of the picture with a small portion of non-neoplastic salivary gland tissue (left).

In Figure 2 an adenoid cystic carcinoma is shown. This specimen of tumor is composed of epithelial and myoepithelial neoplastic cells. There are various patterns that this tumor can present itself in, including tubular, cribriform and solid forms (8).

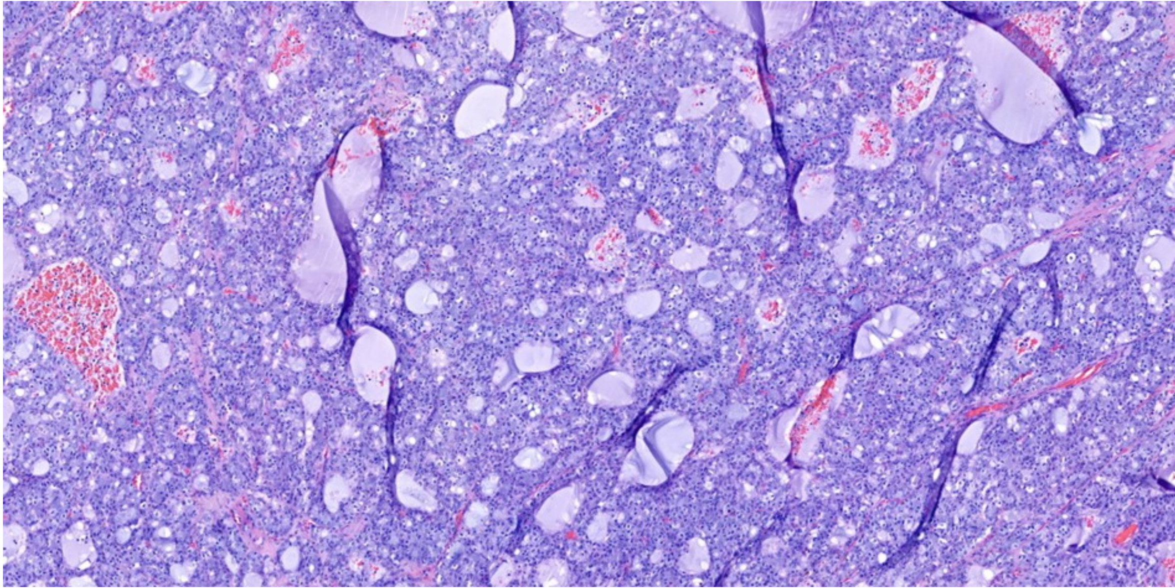

Figure 2: Adenoid cystic carcinoma. . In this picture, the acinar cell carcinoma has undergone a high-grade transformation.

An acinar cell carcinoma is presented in Figure 3. Acinar cell carcinoma are neoplasms composed of cancer cells with acinar features (8).

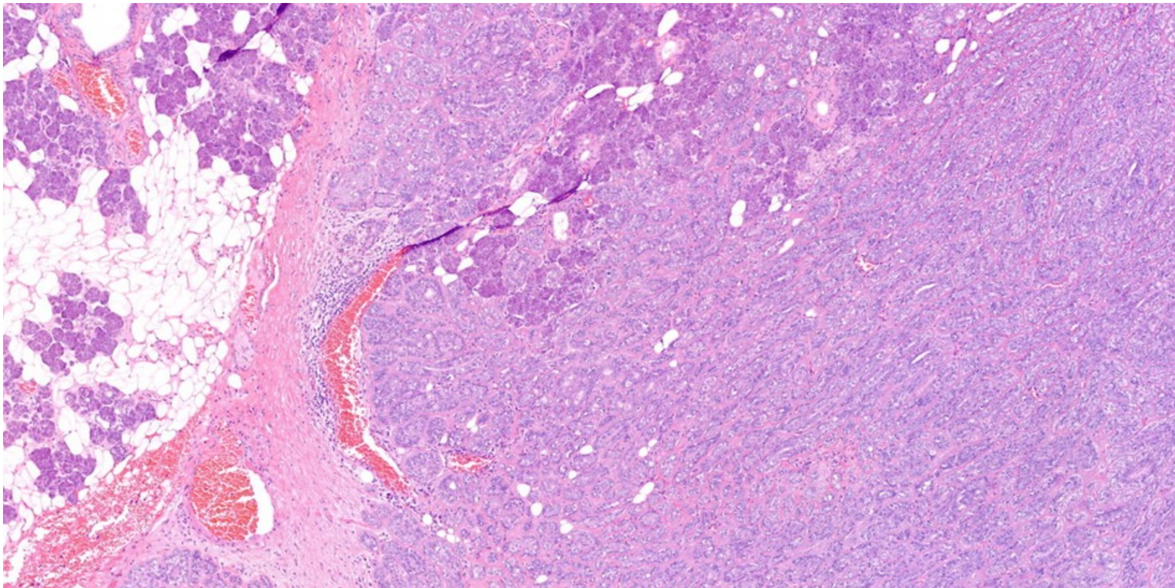

Figure 3: Acinar cell carcinoma. Tumor tissue can be seen in most of the picture. In the left upper corner, a small portion of non-neoplastic salivary gland tissue can be seen

Figure 4 shows a squamous cell carcinoma. This kind of tumor is typically moderately to well-differentiated and presents itself with infiltrative growth, desmoplasia and infiltration of periglandular soft tissue (8).

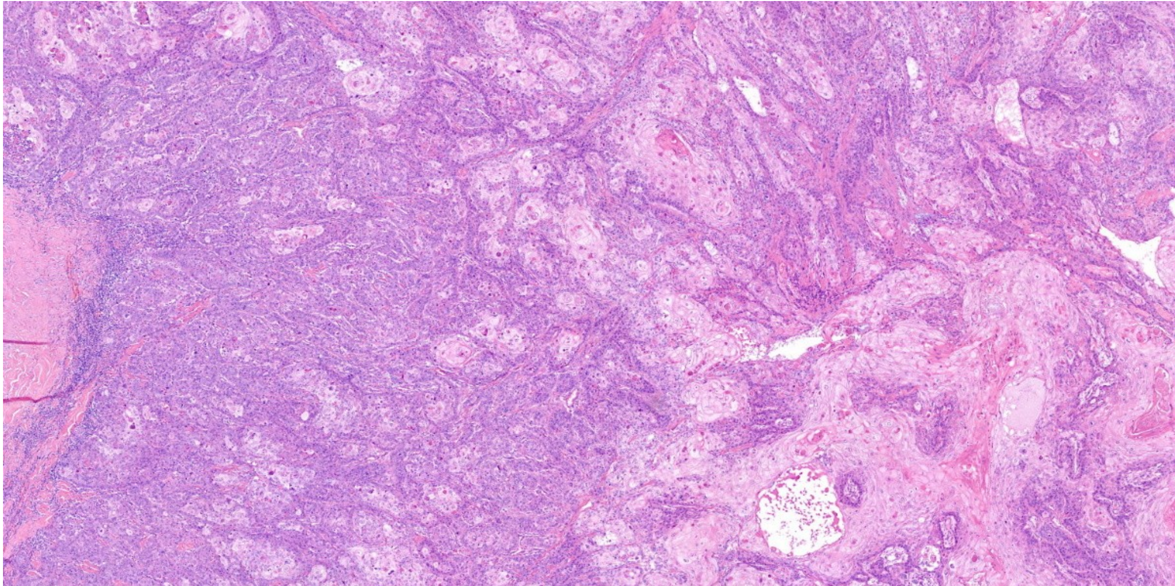

Figure 4: Squamous cell carcinoma. In this picture, the tumor is infiltrating the salivary gland tissue.

1.2 Chemokines

Chemokines (short for chemoattractant cytokines) are small signaling proteins secreted by different cell types such as stem cells, stromal cells, and several immune cells (25, 26). Chemokines are primarily known for their role in mediating immune cell trafficking and lymphoid tissue development (27, 28). Chemokines act through binding G-protein-coupled chemokine receptors on cell surfaces, resulting in diverse receptor-ligand interactions (e.g., chemotaxis, leukocyte migration, inflammation). They are involved in both physiological and pathological immune responses (29). Table 1 gives an overview of the more than 50 human chemokines, 19 different chemokine receptors, and four atypical chemokine receptors known today (30, 31). Chemokines can be categorized into four subfamilies (C, CC, CXC, CX3C) based on the location of the cysteines (C) in their amino acid sequences, and X denotes any amino acid residue (30–32) (Figure 5). Figure 5 shows the typical structure of chemokines. In chemokine nomenclature, an L for “ligand” is added to the subfamily-name, followed by a number according to the time of discovery of the gene (Table 1) (33, 34). Chemokines are the largest subfamily of cytokines (28).

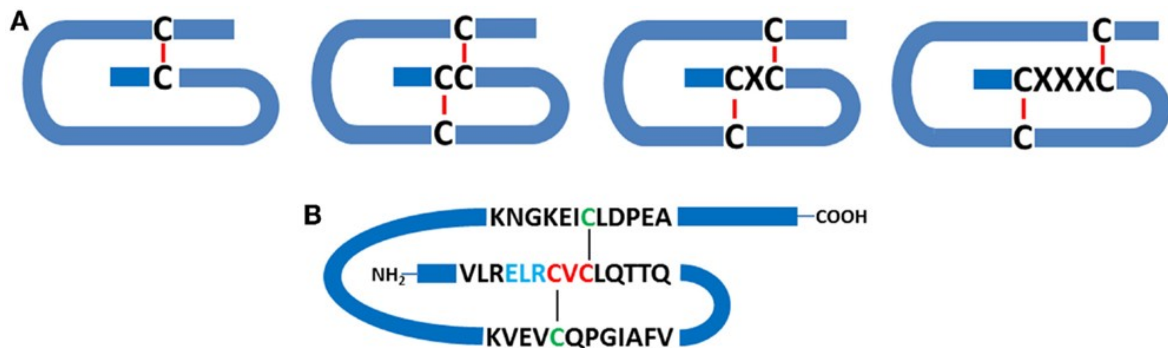

Figure 5: *Chemokine structure. (A) Schematic indicating the relationships between conserved cysteine residues (C) and their corresponding intrachain disulfide bridges. The schematic depicts four classes of chemokines, including C-, CC-, CXC-, and CX3C-.* ***(B)*** *The schematic representation of human CXCL5 is an ELR+ chemokine that is pro-inflammatory and pro-angiogenic. The ELR motif is positioned N-terminal to the CXC consensus sequence (35).*[1]

Table 1: Chemokines in humans (adapted from Zlotnik et al.(36) and Bachelerie et al.(37))

CHEMOKINES	SYNONYMS
CXC-CHEMOKINE FAMILY	
CXCL1	GROα, MGSA
CXCL2	Groβ, MIP-2α
CXCL3	Groγ, MIP-2β
CXCL4	Platelet Factor-4
CXCL5	ENA-78
CXCL6	GCP-2
CXCL7	NAP-2
CXCL8	IL-8
CXCL9	Mig
CXCL10	γIP-10
CXCL11	I-TAC
CXCL12	SDF-1α
CXCL13	BLC
CXCL14	BRAK
CXCL16	SR-PSOX
CC-CHEMOKINE FAMILY	
CCL1	I-309
CCL2	MCP-1
CCL3	MIP-1α
CCL4	MIP-1β
CCL5	RANTES
CCL7	MCP-3
CCL8	MCP-2

[1] Reprinted from Frontiers in Immunology, Volume 6, S. E. Sahingur, W. A. Yeudall, Chemokine Function in Periodontal Disease and Oral Cavity Cancer, with CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

CCL11	Eotaxin
CCL13	MCP-4
CCL14	HCC-1
CCL15	HCC-2
CCL16	HCC-4
CCL17	TARC
CCL18	PARC
CCL19	ELC
CCL20	MIP-3α, LARC
CCL21	SLC
CCL22	MDC
CCL23	MPIF-1
CCL24	Eotaxin-2
CCL25	TECK
CCL26	Eotaxin-3
CCL27	CTACK
CCL28	MEC
C-CHEMOKINE FAMILY	
XCL1	Lymphotactin α
XCL2	Lymphotactin β
CX3C-CHEMOKINE FAMILY	
CX3CL1	Fractalkine

1.3 Chemokine Receptors

Chemokine receptors are seven-transmembrane receptors that are coupled to G-proteins (28,38). They have an N-terminus on the cell surface, three extracellular and three intracellular loops, and a C-terminus in the cytoplasm (26). Most chemokine receptors can bind to multiple ligands (CXCR, CCR, XCR, and CX3CR), and multiple chemokines bind to the same receptor, which is described in Figure 6. Chemokine receptors are named based on the chemokine class they can bind (34). CXCR1-CXCR6 binds CXC chemokines, CCR1-CCR10 binds CC chemokines, XCR1 binds the XC chemokine, and CX3CR1 binds the CX3C chemokine (34). In addition, chemokines can also bind to various chemokine receptors, resulting in a complex level of interaction (Table 2) (34, 39).

Chemokine receptors can be divided into two main categories: typical and atypical (40). Typical chemokine receptors belong to the G protein-coupled receptor (GPCR) superfamily and primarily play a role in inflammation (40). They are predominantly expressed by immune cells and are involved in immune cell movement (40). On the other hand, atypical

chemokine receptors coordinate the trafficking of immune cells through chemokine scavenging via the beta-arresting pathway (41). These receptors are expressed in blood and lymphatic endothelial cells, among others (40).

When a chemokine binds to the extracellular section of a chemokine receptor, it induces a conformational change in the receptor's tertiary structure (42, 43). Subsequently, the receptor's intracellular portion binds to and activates G proteins. This leads to the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by the activated G proteins, which dissociate into a and o subunits (42, 43). Chemokine receptors can couple with different G a isotypes, resulting in downstream effector activation or inhibition (27).

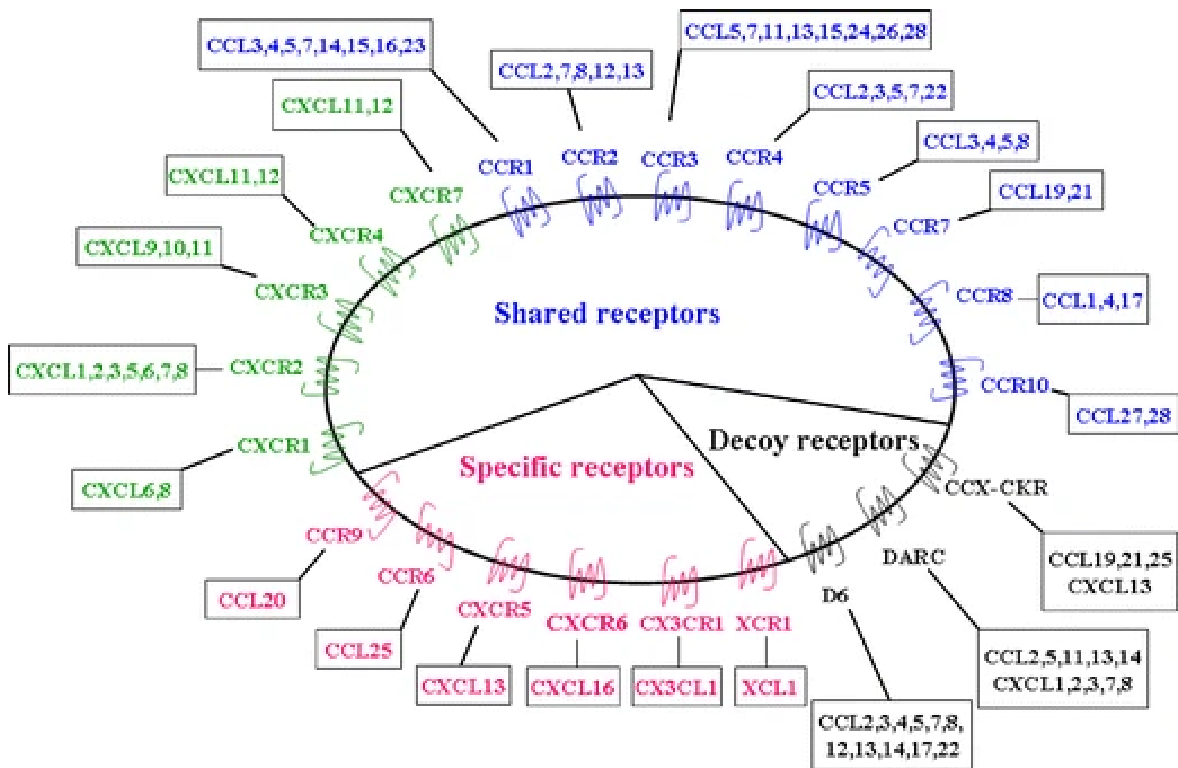

Figure 6: Chemokine Family and Their Cognate Receptors. Most CC (blue) and CXC (green) chemokines demonstrate the capacity to bind numerous receptors, while a singular receptor can bond with multiple chemokines. Additionally, decoy receptors (black) can interact with multiple chemokines. On the other hand, a minority of receptors (red) only have one ligand (44).[2]

[2] Reprinted from CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression, X. Sun, G. Cheng, M. Hao, J. Zheng, X. Zhou, J. Zhang, R. S. Taichman, K. J. Pienta, J. Wang, Cancer Metastasis Review, 4, Springer US, 2011, reproduced with permission from SNCSC

Table 2. Chemokine receptors in humans (adapted from Zlotnik et al.(36) and Bachelerie et al.(37))

CHEMOKINE RECEPTOR	SYNONYMS	CHEMOKINE LIGAND
G PROTEIN-COUPLED CHEMOKINE RECEPTORS		
CXCR1	IL8RA	CXCL5, CXCL6, CXCL8
CXCR2	IL8RB	CXCL1-3, CXCL5-8
CXCR3	IP10/Mig R	CXCL9-11
CXCR4	Fusion	CXCL12
CXCR5	BLR-1	CXCL13
CXCR6	BONZO, STRL33	CXCL16
CCR1	CC CKR1, MIP-1α/RANTES R	CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23
CCR2	CC CKR2, MCP-1-R	CCL2, CCL5, CCL7, CCL8, CCL13, CCL16
CCR3	CC CKR3, Eotaxin receptor	CCL4, CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28
CCR4	CC CKR4	CCL17, CCL22
CCR5	CC CKR5	CCL3, CCL4, CCL5, CCL7, CCL14, CCL16
CCR6		CCL20
CCR7	EBI-1, BLR-2	CCL19, CCL21
CCR8		CCL1, CCL18
CCR9		CCL25
CCR10		CCL27, CCL28
XCR1		XCL1, XCL2
CX3CR1	Fractalkine receptor	CX3CL1
ATYPICAL CHEMOKINE RECEPTORS		
ACKR1	DARC, Duffy	CXCL5, CXCL6, CXCL8, CXCL11
ACKR2	D6	CCL2-CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22
ACKR3	CXCR7, RDC1	CXCL11, CXCL12
ACKR4	CCRL1, CCX-CKR, CCBP2, CCR11	CCL19, CCL21, CCL25
CCRL2	ACKR5, CKRX, CRAM-A, L-CCR, CRAM-B	CCL19
PITPNM3	ACKR6, Nir1	

1.4 The Functions of the Chemokine Network

Conventional chemokine receptors can trigger cell migration after ligand binding (31). Cells expressing chemokine receptors migrate toward higher chemokine concentrations, matching their ligand within human tissue, also called chemotaxis (45). Chemokine receptors are multifunctional and regulate not only the chemotaxis of leukocytes but also their survival, migration rate, endocytosis, differentiation, and cytoarchitecture (32) (Figure 7).

In addition, the four different atypical chemokine receptors coordinate the transport of immune cells through chemokine scavenging (31). Atypical chemokine receptors (ACKR1-

4) are incapable of triggering G-protein-dependent signaling in response to binding of their ligands (46). Atypical chemokine receptors are internalized after binding to their ligands (47,48). These receptors are able to transport chemokines across cellular borders (49). For instance, if a pathogen is detected in the bloodstream, the chemokine can enter the circulation, which then allows lymphocytes to examine the blood (49). Additionally, atypical receptors collaborate with standard receptors to organize chemotaxis (49).

Chemokines can be divided into the following three groups: inflammatory (or constitutive), homeostatic (or inducible), and dual-type (homeostatic-inflammatory or constitutive-inducible) (32). This classification is based on their expression and their function. Homeostatic chemokines play a role in physiological cell migration and homing, whereas inflammatory cytokines are secreted at sites of inflammation to attract effector cells to the inflamed area (50).

Chemokines and their receptors play an important role not only in inflammation but also in infection, tissue injury, allergy, cardiovascular diseases, and malignant tumors (51). Chemokine receptors may potentially facilitate metastasis of tumors through adherence of tumor cells to endothelium, extravasation from blood vessels, metastatic colonization, angiogenesis, proliferation, and protection from the host response via activation of key survival pathways (44, 52, 53). Targeting chemokine receptors has shown promising results, such as preventing or reducing immunosuppressive cells from entering the tumor microenvironment, lessening the metastatic potential of tumor cells, and in some cases, even reducing tumor cell viability (41).

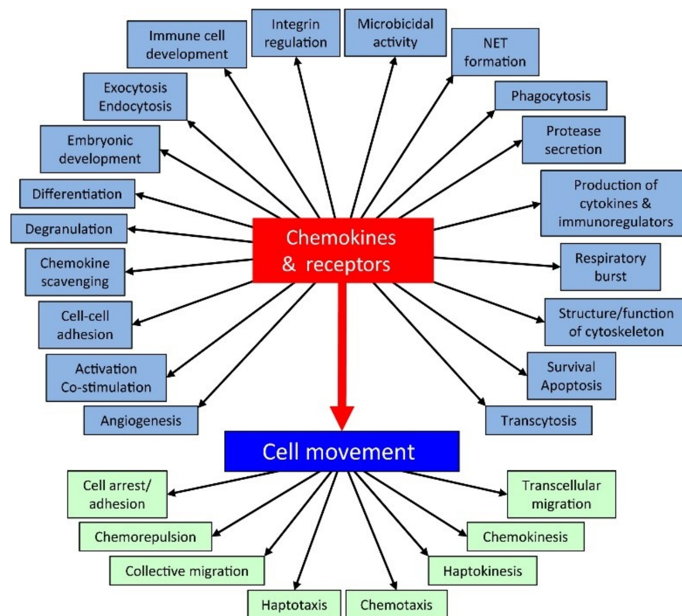

Figure 7: Functions of Chemokines and their Receptors. The biological processes regulated by chemokines and their receptors are presented in light blue boxes arranged clockwise in alphabetical order (starting from the bottom left) around the central 'Chemokines & Receptors' box. "Cell movement" is the primary biological process regulated by chemokines and their receptors. The figure shows green boxes that depict chemokine-mediated cell arrest and adhesion, as well as various migratory behaviors that fall under chemokine control (26). [3]

1.5 Chemokine Network and Cancer

When discussing malignant tumors, it is not solely referred to a group of tumor cells but also to the adjacent tissue comprised of stromal and immune cells (30). This tumor microenvironment is the primary site for interactions between immune cells and tumor cells (30). Within this environment, chemokines are expressed by tumor cells and other cells, including immune and stromal cells (30). In response to particular chemokines, distinct subtypes of immune cells move into the tumor microenvironment and control tumor immune responses (54). In addition to immune cells, chemokines can impact tumor cells and endothelial cells to regulate tumor cell proliferation, cancer stem cell characteristics, cancer invasiveness, and metastasis (55). Therefore, chemokines affect both tumor immunity, directly and indirectly, thereby influencing disease progression and therapeutic outcomes (55, 56). Chemokines can impact immune responses in both primary tumors and metastatic sites (30). The same chemokine systems can contribute to both anti- and pro-tumor immune responses (57). Slight initial variations in anti-tumor and pro-tumor responses may become amplified with time, potentially contributing to the formation of the tumor´s immune context (57). Although chemokine signaling plays a critical role in recruiting immune cells with

[3] Reprinted from The FEBS Journal, Volume285, Issue16, C. E. Hughes, R.. J. B. Nibb, A guide to chemokines and their receptors, with CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

antitumor effects, including CD8+ T cells, T Helper 1 (TH1) cells, and natural killer cells (NK), alterations in the secretion of chemokine ligands and the expression of chemokine receptors are frequent occurrences within the TME (58, 59). Chemokines frequently shape the microenvironment of tumors into a state conducive to tumor growth (60). This often triggers the recruitment of pro-tumor immune cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TAN), tumor-associated macrophages (TAM), and regulatory T-cells (Treg cells) (59, 61, 62). The proliferation of these cells during disease progression suppresses effector lymphocytes, which has been linked to a poorer prognosis in patients with different cancer types (59, 61, 62). The migration of immune cells into the tumor microenvironment and the expression of chemokines and their receptors are unpredictable (63). This is because the microenvironment is heterogeneous and lacks a clear structure (63). Therefore, the TME composition differs among tumors of the same type as well as within a single tumor (63). Manipulation of chemokine-chemokine receptor signaling pathways can alter a tumor's immune and biological phenotypes, improving the efficacy of immunotherapy (30). Chemokines are known to affect the mobility of various cells, suggesting a role in metastasis (41). In order to develop effective cancer treatments, it is crucial to have a deep understanding of which cell types express each chemokine receptor, the role or function of these receptors in different settings, and the identification of TME changes upon inhibition (41). Multiple drugs have shown promising results, including preventing or reducing the infiltration of immunosuppressive cells into the TME, decreasing the metastatic potential of tumor cells, and, in some cases, reducing tumor cell viability (41). This is important because numerous immunotherapies are believed to fail due to solid tumors being plagued with immunosuppressive TMEs (41).

1.6 Chemokines and Salivary Gland Carcinoma

As researchers gained a greater understanding of the importance of the TME in cancer biology, cancer research and treatment shifted from a focus on cancer cells to a focus on the TME (64). This same trend is observed in SGCs.

CXCR4 and CXCR7 are among the most extensively researched chemokine receptors in the literature (65). Phattarataratip et al. described that both CXCR4 and CXCR7 expressions are upregulated in SGTs and identified a significant correlation between high CXCR4 expression and increased pathological grade of MECs (mucoepidermoid carcinoma) (65). Uchida et al. found a frequent increase in the expression of CXCR4 mRNA and protein in salivary gland carcinoma cell lines (74). CXCR4 and CXCR7 have the same ligand,

CXCL12, also referred to as stromal-derived factor 1 (SDF-1) (65). In physiological settings, the CXCR4/CXCR7/CXCR12 axis is imperative for the development of lymphoid tissue and leukocyte trafficking, and thus, CXCL12 is commonly expressed in the human body (65). The CXCL12 binding to CXCR4 or CXCR7 seems to be involved in cancer cell movement toward target organs during metastasis and triggers various downstream effectors engaged in cancer survival and proliferation (65–67). Previous research has demonstrated that CXCR4 silencing in MEC cell lines resulted in decreased cell proliferation, chemotaxis, adhesion, and invasion in vitro (68). These results indicate that CXCR4 may be critically important for MEC progression. The correlation between elevated CXCR4 expression and high tumor grade has also been observed in other cancer entities, including head and neck squamous cell carcinoma, urothelial carcinoma, chondrosarcoma, and breast cancer (69–72). Sun et al. (44) suggest that CXCR7 plays a fundamental role in regulating immunity, angiogenesis, and stem cell trafficking, as well as mediating organ-specific cancer metastases (44,73). Uchida et al. found that CXCR4 expression was present solely in SGC cell lines exhibiting high tumorigenic potential, whereas CXCR7 expression was observed in all SGC cell lines in cultured human SGC cell lines (74). Consequently, CXCR7 expression could indicate the existence of differentiated populations without tumorigenic potential in cultured SGC cell lines (74). Coexpression and heterodimerization of CXCR4 and CXCR7 on the same cells enhances CXCL12-induced signaling when compared to cells that only express CXCR4 (75).

Haghshenas et al. found a significantly higher expression of CCR4 and CCR7 mRNA in malignant SGT-tissue compared to benign tissues (73). Furthermore, a higher CCR7 expression in late tumor stages was indicated (73).

In conclusion, malignant and benign SGTs present distinctive chemokine and chemokine receptor patterns that are associated with their biological and clinical behavior. The significantly increased expression of CXCR4 and CXCR7 in malignant SGTs could play a central role in malignant transformation (69, 73, 74). This knowledge may potentially be used in future research on cancer immunotherapy.

2 Material and Methods

2.1 Tissue Samples

About 70 paraffin-embedded patient samples of benign (pleomorphic adenoma, Warthin tumor) and malignant salivary gland tumors dating back to 2012 have been obtained from the archives of the Medical University of Graz´s Biobank (FFPE material). Upon excluding 40 samples due to a lack of tumor tissue amount, 35 malignant samples were analyzed (17 squamous cell carcinomas, 5 acinic cell carcinomas, 3 mucoepidermoid carcinomas, 3 epithelial-myoepithelial carcinomas, 3 adenoid cystic carcinomas, 2 salivary duct carcinomas, 2 basal cell carcinomas). The sample number has resulted from systematic case count consultation. Salivary gland tumor entities were classified according to the current WHO classification of salivary gland tumors (8).

Tissue fixed in formalin and embedded in paraffin was cut using a microtome with a disposable blade. In each case, the first and last cut was used for HE staining to ensure the presence of tumor tissue in all cuts. Dr.in med. univ. Marlene Leoni, who supervised the project, verified the original diagnosis and the existence of tumor tissue under the microscope. For each sample, about ten serial cuts were made with the microtome with a thickness between 5 to 8 µm, which added up to a total thickness of approximately 40 to 60 µm for further processing (excluding two cuts for HE staining). In samples exclusively containing malignant tumor tissue, the microtome cuts were transferred directly into Eppendorf tubes. Samples containing non-malignant surrounding tissue were manually macroprepared using a single-use toothpick under sterile conditions.

Between each sample, the workspace and all tools were cleaned using Thermo Scientific™ RNase AWAY™ (Thermo Fisher Scientific Inc., Waltham, MA, USA). Disposable tools such as blades and gloves, as well as the water used at the microtome, were constantly exchanged. Ethical approval was obtained from the Ethics Committee of the Medical University of Graz (ethical application 35-354 ex 22/23).

Tonsils served as the control tissue in this study. The tissue used for this purpose was obtained from autopsies performed at the local Institute of Pathology of the Medical University of Graz. Unfortunately, it has not been possible to retrieve a matched set of samples from the same patients.

2.2 RNA Isolation

Total RNA was extracted from the tissue samples using the Relia Prep™ FFPE Total RNA Miniprep System (Promega, Madison, WI, USA) according to the manufacturer´s instructions (TM353 Revised 12/15). The isolated RNA was diluted in 40 µl of nuclease-free water, and its quantity was measured using the Thermo Scientific™ NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and version 3.8.1 of the NanoDrop 1000 operating software. The isolated RNA was stored at -80°C until further use.

2.3 cDNA-Synthesis

For the synthesis of cDNA, the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used according to the manufacturer´s instructions (revision 11). 1µL of a random hexamer primer was used, resulting in a total reaction volume of 20 µL. To synthesize the cDNA, the UNO[96] thermocycler from VWR International GmbH (Darmstadt, Germany) has been used in the cycles 25°C for 5 minutes, 42°C for 1 hour and lastly 70°C for 5 minutes.

2.4 Real-Time PCR

74 samples of malignant salivary gland tumors were prepared, including microtome cutting, macrodissection, RNA isolation and cDNA synthesis. Due to limited available tissue, the expression of 19 chemokine receptors was tested on five randomly chosen samples. After assessing these results, the CC chemokine receptors CCR5 and CCR7, the CXC chemokine receptors CXCR1, CXCR2, CXCR3 and CXCR5, the CX_3C chemokine receptor CX_3CR1, and the XCR chemokine receptor XCR1 were excluded from further analysis due to unspecific results. The remaining 11 chemokine receptors were tested on all 74 samples (the CC chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR6, CCR8, CCR9, CCR10, the CXC chemokine receptors CXCR4, CXCR6 and CXCR7). Unfortunately, 39 samples did not contain enough RNA to obtain usable results. The primers were purchased from two companies: Ingenetix GmbH (Vienna, Austria) and Eurofins Genomics Germany GmbH (Ebersberg, Germany). In Table 3, the primers for each chemokine are listed according to their sequence. The cDNA was prepared for Real-Time PCR by dilution at a 1:20 ratio in nuclease-free water. 6 µL of the Luna® Universal qPCR Master Mix M3003 (Applied Biosystems, Foster City, CA, USA) with varying forward and reverse primer concentrations was combined with 4µL cDNA. Table 4 shows the concentration of the forward and reverse primers for each chemokine receptor. RT-qPCR stands for quantitative reverse transcription

polymerase chain reaction and is a technique used to detect and quantify RNA. RT-qPCR was performed in triplicates using the QuantStudio™ Flex Real-Time PCR System in combination with the QuantStudio™ Real-Time PCR Software Version 1.3 (Applied Biosystems, Foster City, CA, USA). GAPDH and h-bactin served as housekeeping genes, which are reference genes to compare the cycle number in relation to the genes of interest. The following thermocycling conditions were applied: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds each, and 60° for 30 seconds. During the RT-qPCR, a melting curve was captured at a temperature increase from 60°C to 95°C in order to distinguish specific from nonspecific products. The threshold for undetermined values was set to 40 cycles, meaning that if the PCR reaction did not reach the detection threshold within 40 cycles, RNA was either at a very low level or undetectable in the tested sample. Based on triplicate measurements, relative expression was calculated using the 2-$\Delta\Delta CT$ method. The 2-$\Delta\Delta CT$ method is an approach for analyzing relative gene expression data from quantitative PCR experiments. The geometric mean values of the C_t values of h-bactin and GAPDH were used for calculation. Samples with C_t values larger than 35 in the reference genes were excluded because of inaccuracy. Formalin-fixed paraffin-embedded tissues of tonsils and lymph nodes collected in autopsies were used to calibrate the results. This tissue was prepared the same way as the samples were prepared and alike the geometric mean value of C_t values was calculated.

Table 3: Nucleotide acid sequence of primers used in the real-time PCR

GENE	PRIMER FORWARD	PRIMER REVERSE
CCR1	GACTATGACACGACCACAGAGT	CCAACCAGGCCAATGACAAATA
CCR2	GATGAATGGGAGTGAGGGATAGTG	GAGCCCTTTGCTTCACCTTTG
CCR3	CAACATCTACCTGCTCAACC	GCCAAAAACCCAGTTATGCC
CCR4	TAATATTGCAAGGCAAAGACTATTCC	GCGATTTACTCCATCAGCCAGTA
CCR5	GATTGATTTGCACAGCTCATCTG	TGTCATAGATTGGACTTGACACTTGA
CCR6	CCTGACTTGCATTAGCATGGA	GCGGTAGTGTTCTGGATCGG
CCR7	GGGCACAGCCTTCCTGTG	CCACCACCAGCACGCTTT
CCR8	CTGTCTGACCTGCTTTTTGTCT	CCACTTTGCACATTACAGTCCC
CCR9	GACTTCACAAGCCCTATTCCTAACA	AAGTCAAGTGAAGTTGAAGTTAACGTAGTCT
CCR10	GCAAACGCAAGGATGTCGC	CGTAGAGAACGGGATTGAGGC
CXCR1	CTCCTACTGTTGGACAC	ACATGTCCTCTTCAGTTTC
CXCR2	AGGTGTCCTACAGGTGAAAAG	AATCTTCAAAGCTGTCACTCTC
CXCR3	CAGCCCAGCCATGGTCCTTG	GGAAGAGCTGAAGTTCTCCAG
CXCR4	GGGCAATGGATTGGTCATCCT	TGCAGCCTGTACTTGTCCG
CXCR5	CAGCCATGAACTACCCGCTAA	CCAATCTGTCCAGTTCCCAGA
CXCR6	AGAGCAGCAGTGAAAACAAG	ACAAAAGTCAAGCCCCAAG
CXCR7	CTACACGCTCTCCTTCATTTAC	TATTCACCCAGACCACCAC

CX3CR1	AGTGTCACCGACATTTACCTCC	AAGGCGGTAGTGAATTTGCAC
XCR1	CCATCGTGGTGGCCTACTTC	CGCAGCTCCGGATGATCT

Table 4: Concentration of the primers used in the experiment

Chemokine Receptor	Concentration of forward and reverse primer
CCR 1	100 nM
CCR 2	250 nM
CCR 3	250 nM
CCR 4	500 nM
CCR 6	250 nM
CCR 8	125 nM
CCR 9	250 nM
CCR 10	125 nM
CXCR 4	100 nM
CXCR 6	500 nM
CXCR 7	500 nM

2.5 Statistical Analysis

To perform a statistical analysis, SPSS Version 27 (SPSS Inc, Chicago, IL, USA) as well as the nonparametric Mann-Whitney U test were used. The Mann-Whitney U test is a statistical test used to compare two independent samples. In the analysis, *p*-values smaller than 0.05 were considered statistically significant. The *p*-value indicates the likeliness of the investigated data occurring by chance in a range from 0 to 1. Whereas 0 means the samples are perfectly independent and 1 refers to a perfectly dependent variable. A *p*-value smaller than 0.05 is typically considered statistically significant, though this can vary. The *p*-value means that there is a chance of less than 5% that the two variables are dependent on one another. Thus, the smaller the *p*-value, the stronger the evidence for a significant result is. In this thesis, the Mann-Whitney U-test was used to analyze chemokine receptor expression levels with significant differences in their expression. Hierarchical clustering was used to group samples with similar expression patterns based on mRNA expression levels. ΔCT values were calculated using the heatmap.2 function of the R package "gplots" (77) (R version 3.6.3) to perform the clustering (78). The calculation was performed by subtracting the *CT*-value of a reference sample from the *CT*-value of the sample of interest. The *Z*-score was applied to standardize the data. It is calculated using

$$z = \frac{x - \mu}{\sigma} \quad (1)$$

where *x* is the value being evaluated (individual sample), μ is the mean of the samples and

σ is the standard deviation of the samples. In the presented case, the Z values are calculated with x representing the measured samples of malignant tissue and μ and σ were taken from healthy tissue of tonsils and lymphoid nodes, prepared in the same way as the malignant tissue respectively. A hierarchical clustering algorithm was applied to group similar samples. Furthermore, a survival analysis was calculated by using the R package "survival" (79). The results were analyzed using the Kaplan-Meier method and compared with the log-rank test. The resulting curves were plotted by using the R package "survminer" (80).

3 Results

3.1 Chemokine Receptor Expression Pattern

In the first testing phase, 19 chemokine receptors were tested on five randomly chosen samples of malignant salivary gland tumors. Upon evaluating these results, the CC chemokine receptors CCR5 and CCR7, the CXC chemokine receptors CXCR1, CXCR2, CXCR3 and CXCR5, the CX_3C chemokine receptor CX_3CR1, and the XCR chemokine receptor XCR1 were excluded from further analysis because of the nonspecific nature of the PCR analysis. In the second testing phase, with the remaining chemokine receptors, the CC chemokine receptor CCR 1, CCR2, CCR3, CCR4, CCR6, CCR8, CCR9, CCR10, the CXC chemokine receptors CXCR4, CXCR6, and CXCR7, testing was performed on the entire samples of the study.

3.2 Relative Expression of CC Chemokine Receptors in salivary gland carcinoma

The relative expressions of CC chemokine receptors in glandular phenotypes of salivary gland tumors were compared to their counterparts in squamous phenotypes of salivary gland tumors in Figure 8. There was no statistically significant difference in the expression of the tested CC chemokine receptors (CCR 1, CCR2, CCR3, CCR4, CCR6, CCR8, CCR9 and CCR10) (p-values <0.05 were considered significant).

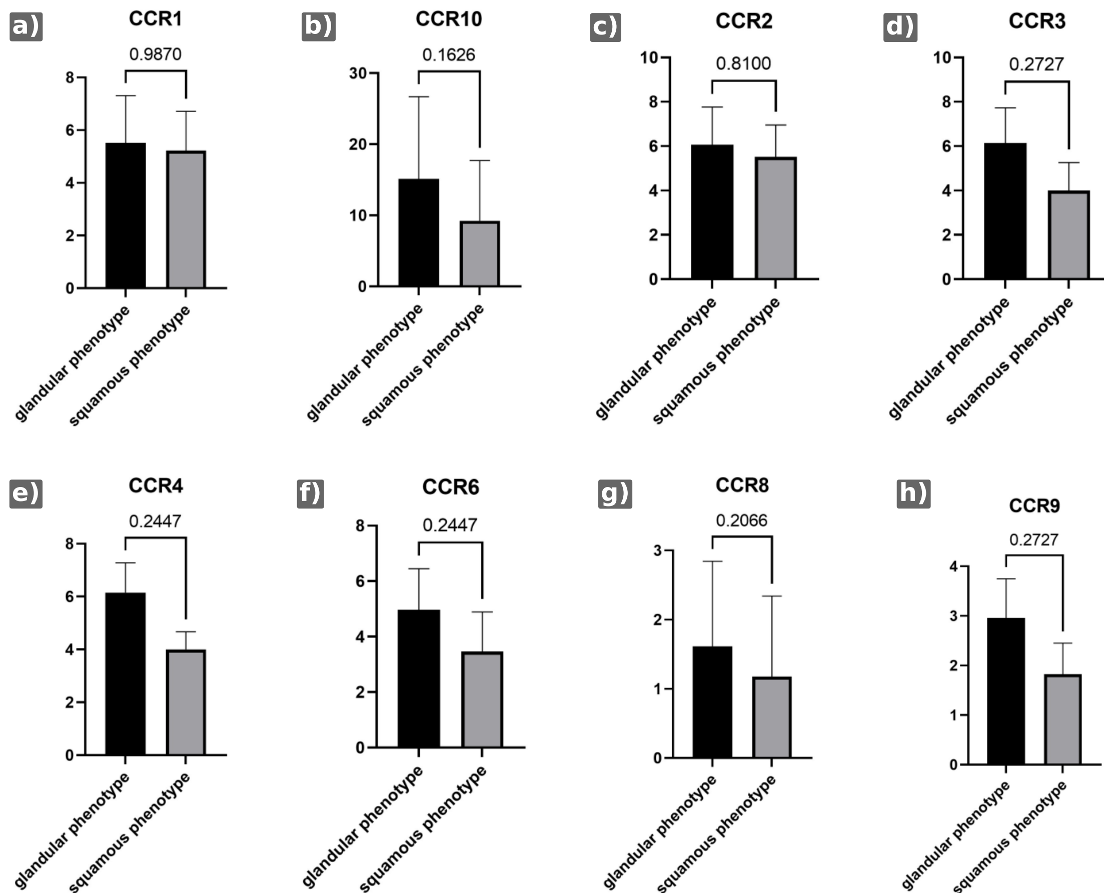

Figure 8: CCR tested. None of the tested CCRs showed statistically significant results (p-value shown in each subfigure). The ordinate shows the relative expressions of the chemokine receptor given with its CCR number in the sub-heading of the subfigure (a-h).

3.3 Relative Expression of CXC Chemokine Receptors in salivary gland carcinoma

In Figure 9, CXC chemokine receptor expressions of glandular phenotypes of salivary gland tumors were compared to their counterparts in squamous phenotypes of salivary gland tumors. No statistically significant difference in the expression of the tested receptors (CXCR4, CXCR6, and CXCR7) were found. This is similar to the tested CC chemokine receptors described in Figure 8.

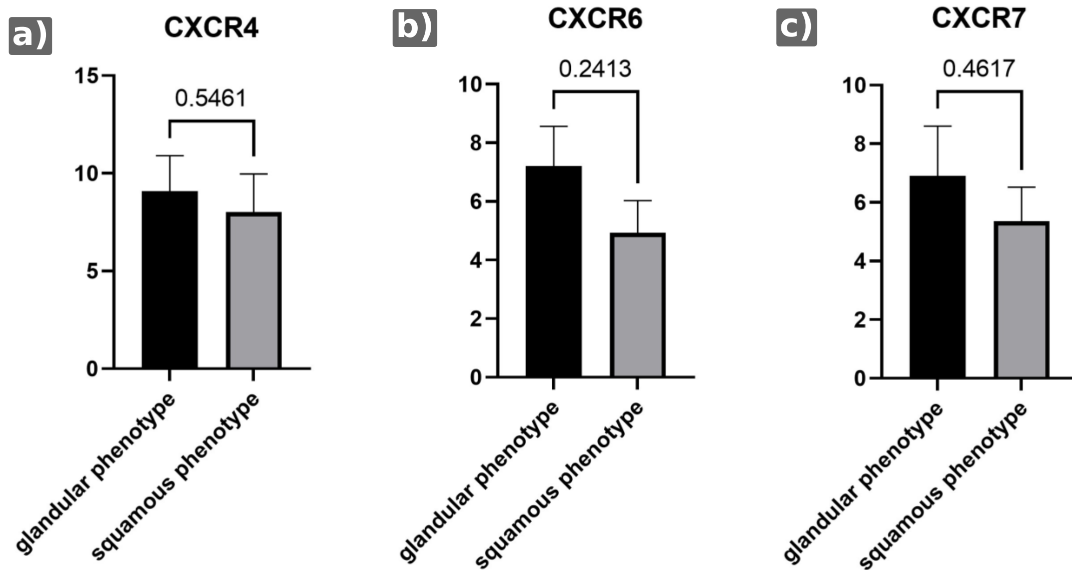

Figure 9: CXCR tested. None of the tested CXCR showed statistically significant results (p-value shown in each subfigure). The ordinate shows the relative expressions of the chemokine receptor given with its CXCR number in the sub-heading of the subfigure (a-c).

3.4 Chemokine Receptor Expression Patterns

To further explore the chemokine receptor expression patterns of malignant salivary gland tumors, a heatmap based on the mRNA expression levels using ΔCT-values of the analyzed chemokine receptors was assembled. As shown in Figure 10, the samples can be divided into two clusters based on the Z-score. One cluster displays a low chemokine receptor expression whereas the other cluster displays a high chemokine receptor expression. Table 5 shows which entity of salivary gland tumors has a high versus low expression of chemokine receptors.

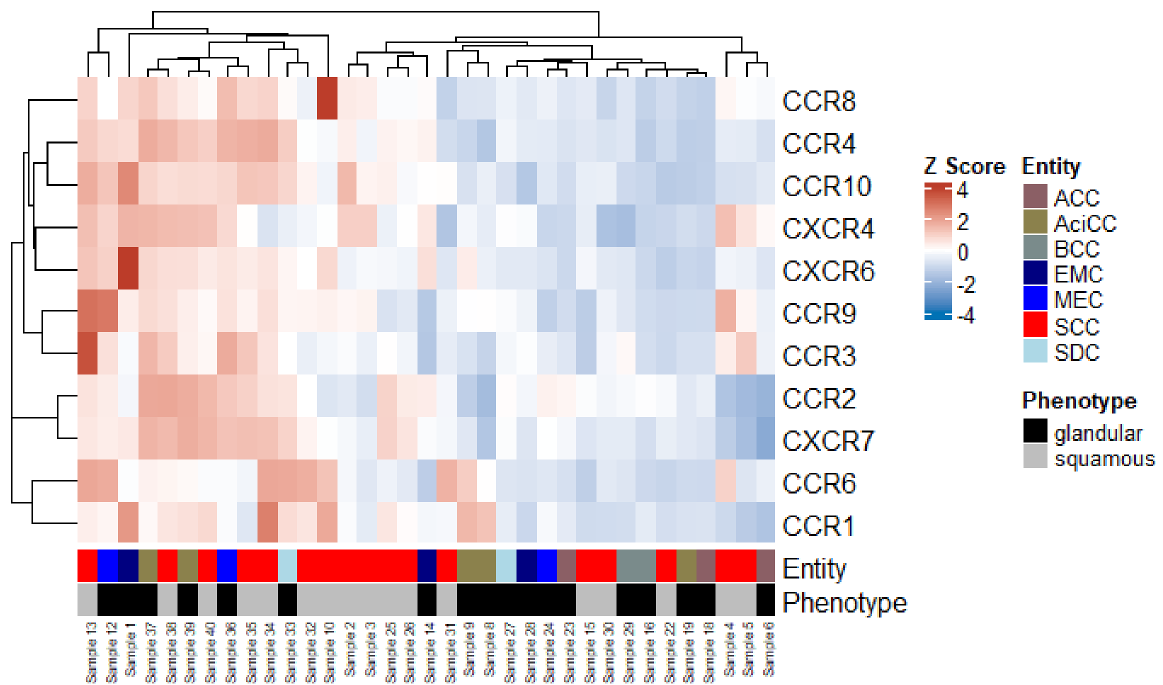

Figure 10: Hierarchical clustering based on the mRNA expression levels using ΔCT-values. The different entities include ACC (Adenoid cystic carcinoma, brown), AciCC (Acinic cell carcinoma, khaki), BCC (Basal cell adenocarcinoma, grey), EMC (Epithelial-myoepithelial carcinoma, dark blue), MEC (Mucoepidermoid carcinoma, blue), SCC (squamous cell carcinoma, red) and SDC (salivary duct carcinoma, light blue). Shades of red display a low chemokine receptor expression, whereas shades of blue indicate a high chemokine receptor expression. The black and grey columns display a division in squamous and glandular phenotype.

Table 5: High vs low expression of chemokine receptor expression in different entities of salivary gland tumors. The various entities include ACC (Adenoid cystic carcinoma), AciCC (Acinic cell carcinoma), BCC (Basal cell adenocarcinoma), EMC (Epithelial-myoepithelial carcinoma), MEC (Mucoepidermoid carcinoma), SCC (squamous cell carcinoma) and SDC (salivary duct carcinoma).

Entity	Low chemokine receptor expression (red)	High chemokine receptor expression (blue)
ACC	0	3
AciCC	2	3
BCC	0	2
EMC	1	2
MEC	2	1
SCC	7	10
SDC	1	1

3.5 Survival analysis

In Figures 11 and 12 the Kaplan-Meier plots are presenting the survival analysis of samples with high versus low CCR-gene expressions. Only patients with available survival data were used in the analysis. Over time, patients either experienced death or were censored due to remission or because they did not further make use of medical help (no follow up data available). This reduces the number of patients at risk as time passes. To analyze the influence of magnitude of the gene expression, the patient data were divided into two groups (also called strata). The cutoff between the strata was defined via median of the respective

gene expression. For both figures the blue stratum (low gene expression) starts decreasing while the red stratum (high gene expression) stays at a constant level. This indicates that the event of interest (death) is occurring more frequently in low CCR-gene expression compared to high CCR-gene expression for both CCR6 and CCR10.

The *p*-value indicates if there is a statistical difference between the survival in two groups (high and low expression) that are being compared. Despite being over the commonly used *p*-value of 0.05 for both Figure 11 and 12, the figures were found worth observing. They were reported since they were significantly lower than all other gene expressions investigated in this study while being reasonably close to 0.05, indicating that future experiments might be able to find a statistically significant difference.

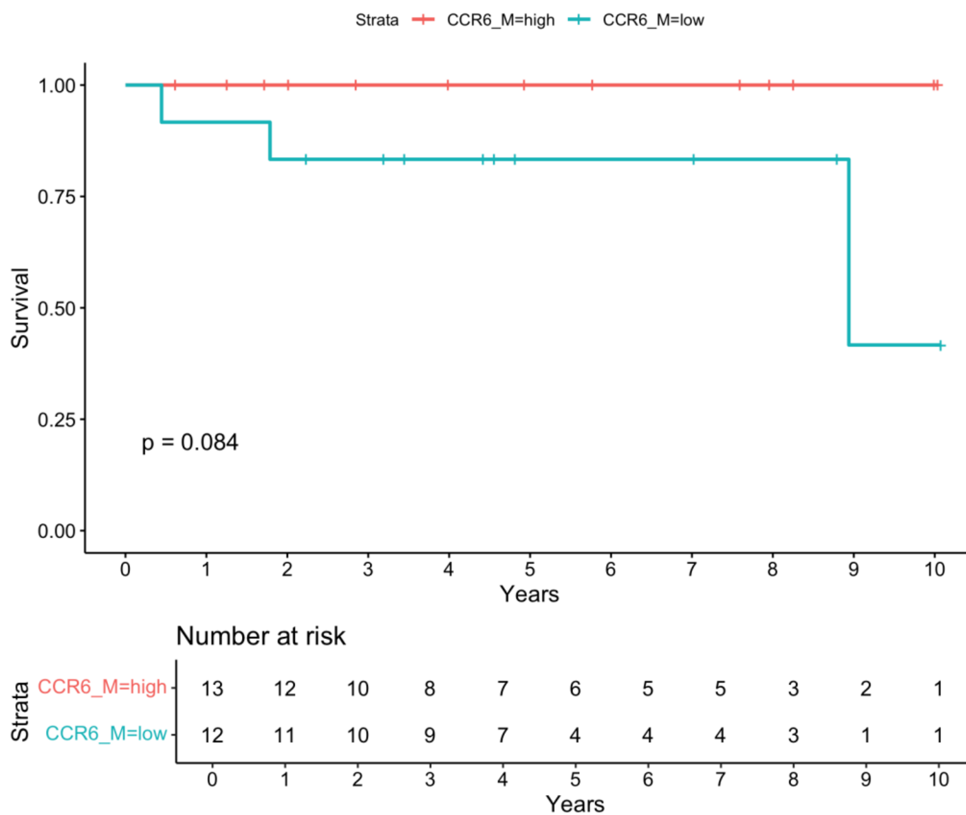

Figure 11: Survival analysis of high vs low CCR6 expression over several years. There are two strata, high CCR6 expression and low CCR6 expression, that represent the two sample groups that are being compared. The number of patients at risk represents the number of samples (respective patients) that are still being followed. This number decreases due to patient that no longer made use of checkups, patients who were no longer advised to seek medical help due to remission and patients who unfortunately died.

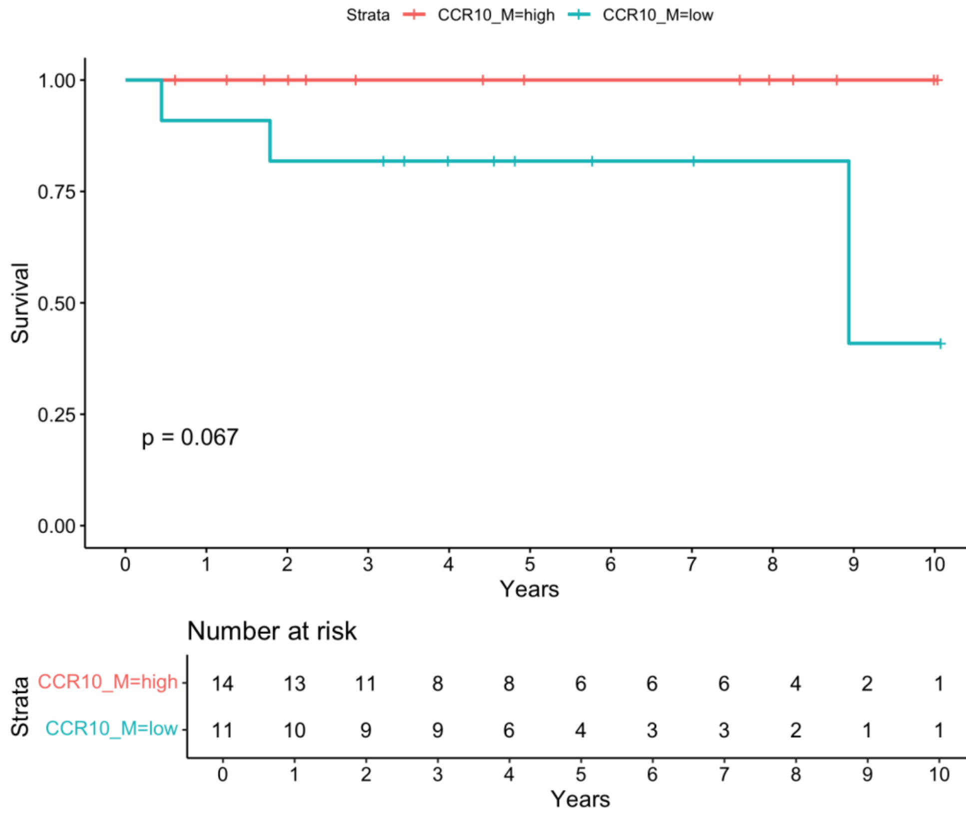

Figure 12: Survival analysis of high vs low CCR10 expression over several years. Similar to Figure 11 there are two strata, high CCR10 expression and low CCR10 expression, that represent two sample groups that are being compared. The number of patients at risk represents the number of samples (respective patients) that are still being followed. This number decreases due to patient that no longer made use of checkups, patients who were no longer advised to seek medical help due to remission and patients who unfortunately died.

4 Discussion

In this thesis, chemokine receptor profiles were researched to compare the expression of chemokines in normal- versus tumor tissue in salivary gland tumors. SGTs account for 0.3% of all cancer cases (12). Treatment typically involves surgery followed by adjuvant radiation therapy (18, 24).

Chemokines are small signaling proteins that play a role in mediating immune cell trafficking and lymphoid tissue development both in physiological and malignant processes (25–29). Thus, chemokines are a potential lead for new strategies in the treatment of salivary gland carcinomas.

CC chemokine receptors CCR 1, CCR2, CCR3, CCR4, CCR6, CCR8, CCR9, and CCR10, as well as CXC chemokine receptors CXCR4, CXCR6, and CXCR7 were tested on a sample size of 40 salivary gland tumor tissues.

Phattarataratip et al. described that both CXCR4 and CXCR7 expressions are upregulated in salivary gland tumors (65). Uchida et al. found a frequent increase in the expression of CXCR4 mRNA and protein in salivary gland carcinoma cell lines (66). Further research indicates that the expression of CXCR4 contributes to the metastatic potential of salivary gland carcinomas (66). Haghshenas et al. found increased expression levels of CCR4 and CCR7 in salivary gland carcinoma, indicating that these chemokine receptors are involved in the malignant transformation (74). These mechanisms could play a central role in malignant transformation and could be starting points for the development of new therapies.

Contrary to the above-described papers, no statistically significant increase of CXCR4, CXCR7, CCR4 or CCR7 could be found in this work. Nevertheless, the survival analysis showed an increase in the death rate of patients when their tumors had a low CCR-gene expression for CCR6 and CCR10. Nurzat et al. showed that a high CCR6 expression in cutaneous melanoma is associated with a good prognostic outcome through immune responses (80). This could imply that a low expression of CCR6 might be a prognostic marker for a worse outcome. Wang et al. suggest a loss of expression of CCR6 in combination with an up-regulation in CCR7 during the metastatic process in squamous cell carcinoma of the head and neck (81). A similar effect is described in dendritic cells after antigen uptake or other stimuli that activates their maturing (81). CCR6 expression in dendritic cells then gets lost while CCR7 is being upregulated (81).

No studies are known up to date, which suggest a raised death rate in tumors with low CCR10 expression. Contrary, there are multiple studies associating poor survival rates in patients with carcinoma with high CCR10 expression (82,83).
Zhu et al. suggested that the sample size in the search for chemokine- and chemokine receptor-based signatures may have an influence on finding statistically significant results, based on their comparison of differently sized cohorts (84).
An additional complicating factor is that tumors are very heterogenic and there are time-dependent chemokine expression changes which requires further research (85). Furthermore, as already mentioned above, the same chemokine systems can contribute to both anti- and pro-tumor immune responses (57). In conclusion, more studies are required in order to achieve the development of a targeted therapy through immunotherapy for salivary gland carcinomas.

5 Conclusion

Despite no statistically significant results being found in this thesis, it still provides a guide for further research, preferably with larger sample sizes. Such further research should be conducted to reinvestigate the findings of the presented survival analysis. The analysis showed an increase in the death of patients for tumors with low CCR-gene expression for both CCR6 and CCR10. There are multiple other studies suggesting that a low CCR6 expression might be a prognostic factor for poor survival rates. Therefore, this result should not be discarded yet further studied. Especially since no other studies are known to the author, which suggest that a low CCR10 expression in tumors might lead to a raised rate in deaths.

To conclude, it can be said that further studies need to be conducted to find out if a bigger sample size shows a clearer correlation as the results in this thesis are close to being statistically significant.

References

1. Jiménez-Rojo L, Granchi Z, Graf D, Mitsiadis TA. Stem Cell Fate Determination during Development and Regeneration of Ectodermal Organs. Front Physiol. 2012;3:107.

2. Gervais EM, Sequeira SJ, Wang W, Abraham S, Kim JH, Leonard D, et al. Par-1b is required for morphogenesis and differentiation of myoepithelial cells during salivary gland development. Organogenesis. 2016 Oct;12(4):194–216.

3. L C, M G, As T. Fgf10 and Sox9 are essential for the establishment of distal progenitor cells during mouse salivary gland development. Development (Cambridge, England) [Internet]. 2017 Jun 15 [cited 2022 Jun 7];144(12). Available from: https://pubmed.ncbi.nlm.nih.gov/28506998/

4. M S, Jp K. Current update on established and novel biomarkers in salivary gland carcinoma pathology and the molecular pathways involved. European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery [Internet]. 2009 Mar [cited 2022 Jun 8];266(3). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b55a005c.han.medunigraz.at/19052760/

5. R M. Role of saliva in the maintenance of taste sensitivity. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists [Internet]. 2000 [cited 2022 Jun 7];11(2). Available from: https://pubmed.ncbi.nlm.nih.gov/12002816/

6. Amano O, Mizobe K, Bando Y, Sakiyama K. Anatomy and histology of rodent and human major salivary glands: -overview of the Japan salivary gland society-sponsored workshop-. Acta Histochem Cytochem. 2012 Oct 31;45(5):241–50.

7. Porcheri C, Mitsiadis TA. Physiology, Pathology and Regeneration of Salivary Glands. Cells. 2019 Aug 26;8(9):E976.

8. Skalova A, Hyrcza M, Mehrotra R. WHO Classification of Tumours Editorial Board. Head and neck tumours [Internet]. Lyon, France; 2022 [cited 2023 Aug 30]. Available from: https://tumourclassification.iarc.who.int/chapters/52

9. Mh A. Salivary gland tumors in an Iranian population: a retrospective study of 130 cases. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons [Internet]. 2007 Nov [cited 2025 Jan 26];65(11). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w01aa.han.medunigraz.at/17954313/

10. Fp F, V CM, Op de A, Al R, Mc T, Ag B, et al. Clinicopathologic analysis of 493 cases of salivary gland tumors in a Southern Brazilian population. Oral surgery, oral medicine, oral pathology and oral radiology [Internet]. 2012 Aug [cited 2025 Jan 26];114(2). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w01aa.han.medunigraz.at/22769409/

11. Fa de O, Ec D, Ct T, Aa M, Ec de A, C AR, et al. Salivary gland tumor: a review of 599 cases in a Brazilian population. Head and neck pathology [Internet]. 2009 Dec [cited

2025 Jan 26];3(4). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w01aa.han.medunigraz.at/20596844/

12. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021 May;71(3):209–49.

13. Ja P, P C. Incidence rates of salivary gland tumors: results from a population-based study. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery [Internet]. 1999 Jun [cited 2022 Jun 8];120(6). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b55a005c.han.medunigraz.at/10352436/

14. Alsanie I, Rajab S, Cottom H, Adegun O, Agarwal R, Jay A, et al. Distribution and Frequency of Salivary Gland Tumours: An International Multicenter Study. Head and Neck Pathology. 2022 May 27;16(4):1043.

15. Guzzo M, Locati LD, Prott FJ, Gatta G, McGurk M, Licitra L. Major and minor salivary gland tumors. Critical Reviews in Oncology/Hematology. 2010 May 1;74(2):134–48.

16. Sonawane SG, Sachdev SS, Sardar MA, Chettiankandy TJ, Nayyar V, Ankit A. Pathogenesis of salivary gland neoplasms: The concepts of histogenesis and morphogenesis. JGOH. 2023 Jun 26;6:59–65.

17. Current Status of Histogenetic and Morphogenetic Concepts of Salivary Gland Tumorigenesis [Internet]. [cited 2025 Jan 26]. Available from: https://journals.sagepub.com/doi/epdf/10.1177/10454411930040050201

18. Yan K, Yesensky J, Hasina R, Agrawal N. Genomics of mucoepidermoid and adenoid cystic carcinomas. Laryngoscope Investigative Otolaryngology. 2018 Feb;3(1):56.

19. E E, Sm K. Salivary gland stem cells: A review of development, regeneration and cancer. Genesis (New York, NY : 2000) [Internet]. 2018 May [cited 2022 Aug 25];56(5). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b5590008.han.medunigraz.at/29663717/

20. V M, S R, St R, C C. Squamous cell carcinoma of submandibular salivary gland: A rare case report. Journal of oral and maxillofacial pathology : JOMFP [Internet]. 2014 May [cited 2022 Jun 8];18(2). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b5u50008.han.medunigraz.at/25328317/

21. Wm M, Cm M, Jw W, Rs M, Np M. Salivary gland pleomorphic adenoma. American journal of clinical oncology [Internet]. 2008 Feb [cited 2022 Aug 25];31(1). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b5590008.han.medunigraz.at/18376235/

22. Qu J, Song M, Xie J, Huang XY, Hu XM, Gan RH, et al. Notch2 signaling contributes to cell growth, invasion, and migration in salivary adenoid cystic carcinoma. Mol Cell Biochem. 2016 Jan;411(1–2):135–41.

23. Chen W, Cao G, Yuan X, Zhang X, Zhang Q, Zhu Y, et al. Notch-1 knockdown suppresses proliferation, migration and metastasis of salivary adenoid cystic carcinoma cells. J Transl Med. 2015 May 20;13:167.

24. Np J, Jr B. Postoperative Management of Salivary Gland Tumors. Current treatment options in oncology [Internet]. 2021 Sep 2 [cited 2022 Jun 8];22(3). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b57c0000.han.medunigraz.at/33560478/

25. Schall TJ, Proudfoot AEI. Overcoming hurdles in developing successful drugs targeting chemokine receptors. Nat Rev Immunol. 2011 May;11(5):355–63.

26. Ce H, Rjb N. A guide to chemokines and their receptors. The FEBS journal [Internet]. 2018 Aug [cited 2023 Aug 30];285(16). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b53311c2.han.medunigraz.at/29637711/

27. Rot A, Von Andrian UH. C HEMOKINES IN I NNATE AND A DAPTIVE H OST D EFENSE : Basic Chemokinese Grammar for Immune Cells. Annu Rev Immunol. 2004 Apr 1;22(1):891–928.

28. Griffith JW, Sokol CL, Luster AD. Chemokines and Chemokine Receptors: Positioning Cells for Host Defense and Immunity. Annu Rev Immunol. 2014 Mar 21;32(1):659–702.

29. Cecchinato V, Martini V, Pirani E, Ghovehoud E, Uguccioni M. The chemokine landscape: one system multiple shades. Front Immunol. 2023 May 11;14:1176619.

30. Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat Rev Immunol. 2017 Sep;17(9):559–72.

31. Mollica Poeta V, Massara M, Capucetti A, Bonecchi R. Chemokines and Chemokine Receptors: New Targets for Cancer Immunotherapy. Front Immunol. 2019 Mar 6;10:438073.

32. Beyond Chemoattraction: Multifunctionality of Chemokine Receptors in Leukocytes. Trends in Immunology. 2017 Dec 1;38(12):927–41.

33. Zlotnik. Chemokines: A New Classification System and Their Role in Immunity. Immunity. 2000 Feb 1;12(2):121–7.

34. Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, et al. International Union of Pharmacology. XXII. Nomenclature for Chemokine Receptors. Pharmacol Rev. 2000 Mar 1;52(1):145–76.

35. Se S, Wa Y. Chemokine function in periodontal disease and oral cavity cancer. Frontiers in immunology [Internet]. 2015 May 5 [cited 2023 Aug 30];6. Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b53311c2.han.medunigraz.at/25999952/

36. A Z, O Y. Chemokines: a new classification system and their role in immunity. Immunity [Internet]. 2000 Feb [cited 2023 Aug 29];12(2). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b533105c.han.medunigraz.at/10714678/

37. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, et al. International Union of Basic and Clinical Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. Ohlstein EH, editor. Pharmacol Rev. 2014 Jan;66(1):1–79.

38. Gustavsson M, Zheng Y, Handel TM. Production of Chemokine/Chemokine Receptor Complexes for Structural Biophysical Studies. Methods Enzymol. 2016;570:233–60.

39. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, et al. International Union of Basic and Clinical Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. Ohlstein EH, editor. Pharmacol Rev. 2014 Jan;66(1):1–79.

40. Torphy RJ, Yee EJ, Schulick RD, Zhu Y. Atypical chemokine receptors: emerging therapeutic targets in cancer. Trends in Pharmacological Sciences. 2022 Dec;43(12):1085–97.

41. Kraus S, Kolman T, Yeung A, Deming D. Chemokine Receptor Antagonists: Role in Oncology. Curr Oncol Rep. 2021 Nov 1;23(11):1–10.

42. Selective G Protein Coupling by C-C Chemokine Receptors. Journal of Biological Chemistry. 1996 Feb 23;271(8):3975–8.

43. Differential Coupling of CC Chemokine Receptors to Multiple Heterotrimeric G Proteins in Human Interleukin-2-Activated Natural Killer Cells. Blood. 1996 May 15;87(10):4255–60.

44. X S, G C, M H, J Z, X Z, J Z, et al. CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. Cancer metastasis reviews [Internet]. 2010 Dec [cited 2022 Aug 23];29(4). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b5si006a.han.medunigraz.at/20839032/

45. Märkl F, Huynh D, Endres S, Kobold S. Utilizing chemokines in cancer immunotherapy. Trends in Cancer. 2022 Aug;8(8):670–82.

46. Szpakowska M, D'Uonnolo G, Luís R, Alonso Bartolomé A, Thelen M, Legler DF, et al. New pairings and deorphanization among the atypical chemokine receptor family — physiological and clinical relevance. Front Immunol. 2023 Apr 20;14:1133394.

47. Ulvmar MH, Hub E, Rot A. Atypical chemokine receptors. Exp Cell Res. 2011 Mar 10;317(5):556–68.

48. Hewit KD, Fraser A, Nibbs RJB, Graham GJ. The N-terminal region of the atypical chemokine receptor ACKR2 is a key determinant of ligand binding. J Biol Chem. 2014 May 2;289(18):12330–42.

49. MD AWB, FRCPath REOFP, Broide DH, FMedSci STHMDs, MD LBB, PhD GKKHM, et al., editors. Middleton's Allergy 2-Volume Set: Principles and Practice. 9th ed. Edinburgh London New York: Elsevier; 2019. 1840 p.

50. BLANCHET X, Langer M, Weber C, Koenen R, von Hundelshausen P. Touch of Chemokines. Frontiers in Immunology [Internet]. 2012 [cited 2023 Aug 31];3. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2012.00175

51. Raman D, Sobolik-Delmaire T, Richmond A. Chemokines in health and disease. Exp Cell Res. 2011 Mar 10;317(5):575–89.

52. A review on CXCR4/CXCL12 axis in oncology: No place to hide. European Journal of Cancer. 2013 Jan 1;49(1):219–30.

53. T K, St H. Chemokines, chemokine receptors, and cancer metastasis. Journal of leukocyte biology [Internet]. 2006 Apr [cited 2022 Aug 23];79(4). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b550002e.han.medunigraz.at/16478915/

54. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer. 2004 Jul;4(7):540–50.

55. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer. 2005 Apr;5(4):263–74.

56. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 Pathway Blockade for Cancer Therapy: Mechanisms, Response Biomarkers and Combinations. Science translational medicine. 2016 Mar 3;8(328):328rv4.

57. Chemokines and the immune response to cancer. Immunity. 2021 May 11;54(5):859–74.

58. Wei S, Kryczek I, Zou W. Regulatory T-cell compartmentalization and trafficking. Blood. 2006 Jul 15;108(2):426–31.

59. Varn FS, Wang Y, Mullins DW, Fiering S, Cheng C. Systematic Pan-Cancer Analysis Reveals Immune Cell Interactions in the Tumor Microenvironment. Cancer Research. 2017 Mar 14;77(6):1271–82.

60. Bule P, Aguiar SI, Aires-Da-Silva F, Dias JNR. Chemokine-Directed Tumor Microenvironment Modulation in Cancer Immunotherapy. IJMS. 2021 Sep 10;22(18):9804.

61. Zhang S, Ma X, Zhu C, Liu L, Wang G, Yuan X. The Role of Myeloid-Derived Suppressor Cells in Patients with Solid Tumors: A Meta-Analysis. PLOS ONE. 2016 Oct 25;11(10):e0164514.

62. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol. 2006 Apr 1;6(4):295–307.

63. Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. Cell Communication and Signaling. 2020 Apr 7;18(1):59.

64. Egal ESA, Scarini JF, De Lima-Souza RA, Lavareze L, Fernandes PM, Emerick C, et al. Tumor microenvironment in salivary gland carcinomas: An orchestrated state of chaos. Oral Oncology. 2022 Apr;127:105777.

65. E P, K D. Expression of C-X-C motif chemokine receptors 4 and 7 in salivary gland neoplasms. Archives of oral biology [Internet]. 2017 Nov [cited 2023 Jan 12];83. Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b54j0141.han.medunigraz.at/28772134/

66. D U, N K, M K, G O, T T, H N, et al. Expression and function of CXCR4 in human salivary gland cancers. Clinical & experimental metastasis [Internet]. 2013 Feb [cited 2023 Jan 11];30(2). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b54j0044.han.medunigraz.at/22847686/

67. Salazar N, Muñoz D, Kallifatidis G, Singh RK, Jordà M, Lokeshwar BL. The chemokine receptor CXCR7 interacts with EGFR to promote breast cancer cell proliferation. Molecular Cancer. 2014 Aug 28;13(1):198.

68. Luker KE, Luker GD. Functions of CXCL12 and CXCR4 in breast cancer. Cancer Letters. 2006 Jul;238(1):30–41.

69. Wen DS, Zhu XL, Guan SM, Wu YM, Yu LL, Wu JZ. Silencing of CXCR4 inhibits the proliferation, adhesion, chemotaxis and invasion of salivary gland mucoepidermoid carcinoma Mc3 cells in vitro. Oral Oncology. 2008 Jun;44(6):545–54.

70. Meng X, Wuyi L, Yuhong X, Xinming C. Expression of CXCR4 in oral squamous cell carcinoma: correlations with clinicopathology and pivotal role of proliferation. Journal of Oral Pathology & Medicine. 2010;39(1):63–8.

71. Batsi O, Giannopoulou I, Nesseris I, Valavanis C, Gakiopoulou H, Patsouris ES, et al. Immunohistochemical Evaluation of CXCL12–CXCR4 Axis and VEGFR3 Expression in Primary Urothelial Cancer and its Recurrence. Anticancer Research. 2014 Jul 1;34(7):3537–42.

72. Bai S, Wang D, Klein MJ, Siegal GP. Characterization of CXCR4 Expression in Chondrosarcoma of Bone. Archives of Pathology & Laboratory Medicine. 2011 Jun 1;135(6):753–8.

73. Salvucci O, Bouchard A, Baccarelli A, Deschenes J, Sauter G, Simon R, et al. The role of CXCR4 receptor expression in breast cancer: a large tissue microarray study. Breast Cancer Res Treat. 2006 Jun 1;97(3):275–83.

74. Haghshenas MR, Ashraf MJ, Khademi B, Ghaderi A, Erfani N, Razmkhah M. Chemokine and chemokine receptor patterns in patients with benign and malignant salivary gland tumors: a distinct role for CCR7. European Cytokine Network. 2017 Mar;28(1):27–35.

75. F S, C B, L MM, M M, Rm R, M L, et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proceedings of the National Academy of Sciences of the United States of America [Internet]. 2007 Nov 9 [cited 2023 Sep 22];104(37). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b58p0b03.han.medunigraz.at/17804806/

76. Galili T. talgalili/gplots [Internet]. 2024 [cited 2025 Jan 30]. Available from: https://github.com/talgalili/gplots

77. R: The R Project for Statistical Computing [Internet]. [cited 2025 Jan 29]. Available from: https://www.r-project.org/

78. Therneau T. therneau/survival [Internet]. 2025 [cited 2025 Jan 30]. Available from: https://github.com/therneau/survival

79. Kassambara A, Kosinski M, Biecek P, Fabian S. survminer: Drawing Survival Curves using “ggplot2” [Internet]. 2024 [cited 2025 Jan 30]. Available from: https://cran.r-project.org/web/packages/survminer/index.html

80. Y N, D D, J H, F Z, Z L, Y H, et al. Prognostic biomarker CCR6 and its correlation with immune infiltration in cutaneous melanoma. Frontiers in oncology [Internet]. 2023 Apr 25 [cited 2025 Jan 27];13. Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w03e4.han.medunigraz.at/37182147/

81. Wang J, Xi L, Hunt JL, Gooding W, Whiteside TL, Chen Z, et al. Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. Cancer Res. 2004 Mar 1;64(5):1861–6.

82. Chen L, Liu X, Zhang HY, Du W, Qin Z, Yao Y, et al. Upregulation of chemokine receptor CCR10 is essential for glioma proliferation, invasion and patient survival. Oncotarget. 2014 Jun 26;5(16):6576–83.

83. E MT, Mj CO, A R, Mj A, Se S, Sa A, et al. Roles of CCR10/CCL27-CCL28 axis in tumour development: mechanisms, diagnostic and therapeutic approaches, and perspectives. Expert reviews in molecular medicine [Internet]. 2022 Sep 26 [cited 2025 Jan 27];24. Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w0403.han.medunigraz.at/36155126/

84. W Z, C W, S H, S L, S Z, D Z, et al. Chemokine- and chemokine receptor-based signature predicts immunotherapy response in female colorectal adenocarcinoma patients. Scientific reports [Internet]. 2023 Apr 12 [cited 2025 Jan 26];13(1). Available from: https://pubmed-1ncbi-1nlm-1nih-1gov-10013b56w01fc.han.medunigraz.at/38049474/

85. Kohli K, Pillarisetty VG, Kim TS. Key chemokines direct migration of immune cells in solid tumors. Cancer Gene Ther. 2022 Jan;29(1):10–21.

6 Appendix

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author used the Microsoft Word spelling and grammar checker in combination with Grammarly (v1.2.124.1571) and human proof reading in order to improve readability. After using these, the author reviewed and edited the content as needed and took full responsibility for the content of the publication.