Diploma thesis

The role of chemokine receptors in the progression of brain metastases in colon carcinoma and clear cell renal cell carcinoma

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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.

Hallersdorf, on the 05.07.2023 *Lina Maria Luisa Erlacher eh.*

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List of abbreviations

ccRCC clear cell renal cell carcinoma

GPCR G-protein-coupled receptor

CRC colorectal carcinoma

GPCR rhodopsin like G-protein-coupled receptors

qPCR.—quantitative Polymerase chain reaction

HE…hematoxylin eosin dye

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Zusammenfassung

Nierenzellkarzinome sind maligne epitheliale Tumore deren Inzidenz steigend ist. Es sind vor allem Männer im Alter von 50-69 Jahren davon betroffen, wobei das Auftreten in jüngeren Jahren bei familiären Formen zu finden ist. Risikofaktoren sind neben Lifestyle Faktoren kaum bekannt. Die Metastasierung erfolgt überwiegend hämatogen, 15% davon ins Gehirn. Zusätzlich findet sich in 20% der Fälle eine lymphogene Metastasierung. Der häufigste Subtyp der Nierenzelltumoren ist der klarzellige Typ – genau dieser wird in dieser Arbeit genauer beleuchtet.

Es werden ebenfalls Colonkarzinome thematisiert. Bei diesen ist die Inzidenz sehr stark von der Region abhängig, in Europa ist diese hoch und bildet eine der häufigsten tumorbedingten Todesursachen. Auch hier sind Männer häufiger betroffen, der Häufigkeitsgipfel bezüglich des Alters ist jedoch etwas nach hinten verschoben im Vergleich zum Nierenzellkarzinom. Es gibt verschiedene (genetische) Entstehungspfade – meist handelt es sich um Adenokarzinome. Der Prozess der Metastasierung erfolgt hauptsächlich lymphogen in regionäre Lymphknoten oder auch hämatogen z.b. in die Leber.

Die Behandlungsmethoden der Metastasen im Gehirn sind bei beiden Tumorentitäten stark begrenzt. Den Chemokinen und ihren Rezeptoren wird eine nicht zu vernachlässigende Rolle beim Metastasierungs-Prozess zugeschrieben.

Diese Diplomarbeit soll aufzeigen, welche Chemokinrezeptoren in Primärtumoren (klarzellige Nierenzellkarzinome und Adenokarzinome des Colons) sowie den korrelierenden Hirnmetastasen exprimiert werden. Das Wissen darüber kann einen positiven Einfluss auf die Entwicklung einer zielgerichteten Therapie (targeted therapie) haben, worüber neue therapeutische Optionen für dieses Patientenkollektiv entstehen könnten. Mittels RT-qPCR wurde die Expression der derzeit am besten charakterisierten Chemokinrezeptoren (CCR1-10, CXCR1-7, XCR1 und CX3CR1) auf den Tumorzellen dargestellt.

Alle getesteten Chemokinrezeptoren (CCR4,6,8,9 und CXCR4 und 6) wurden sowohl in den Primärtumoren als auch in den Metastasen der Entitäten exprimiert. In den Nierenzellkarzinomen wurde eine höhere relative Expression von CCR8, CCR6 und CCR9 in der Metastase festgestellt, beim Colonkarzinom hingegen ist CXCR6 signifikant in der

Metastase erhöht Daraus lässt sich die Schlussfolgerung ableiten, dass die genannten Chemokinrezeptoren im Prozess der Metastasierung eine Rolle spielen.

Abstract

Renal cell carcinoma is a epithial malignant carcinoma with increasing incidence. Patients are usually males between 50 and 69 years. The exception are younger patients who suffer from a familial form. There are no known risk factors beside the usually known Life-style ones. They metastasize mostly hematogenously, 15% of metastases are found in the brain. 20% of them also metastasize through/via the lymphatic system. The most common subtype of the renal cell carcinoma is the clear cell renal cell carcinoma (ccRCC) which is one focus of this thesis.

As ccRCC, colorectal carcinoma is also a subject of this thesis. It is one of the top malignant causes of death in Europe, the incidence is increasing but depends on geographical factors. Diagnosed patients are also usually older males. There are different (genetical) pathways causing progression of colorectal cancer, the most common entity are adenocarcinomas. They most likely metastasize into lymph nodes through the lymphatic system, but also hematogenously to the liver and further.

The treatments of metastases in the brain are really limited. Chemokines and their receptors most likely play an important but nearly unknown role in the process of metastasizing – it is the aim of this thesis to explore this role.

The focus of this thesis is to explore the expression of different chemokines and their receptors of the primary tumors (of ccRCC and colorectal Cancer) and its brain metastases. The gained knowledge could pave the way for new treatments like targeted therapies for terminal patients.

RT-qPCR was used to explore the expression of the most well characterized chemokine receptors (CCR1-10, CXCR1-7, XCR1 and CX3CR1).

The result of this thesis is, that the expression of all tested Chemokine receptors (CCR4,6,8,9 and CXCR4 and 6) can be found on all primary tumors and their metastases in the brain. In renal carcinomas there is a higher expression of CCR8,6 and 9 in the metastases. In colorectal carcinomas there is a significant higher expression of CXCR6 in the metastases.

1 Introduction

1.1 Colorectal Adenocarcinoma

Colorectal cancer is the second leading cause of cancer related death world wide, it's the third most common cancer. In 2018 there where about 1.8 million new cases of colorectal cancer and about 881,000 deaths worldwide. From an epidemiological point of view there is a lot of variation depending on the region. Genetic features have a huge impact on the prognosis as well as factors like gender and ethnical factors (1).
20% of patients with a new colorectal cancer diagnosis have a metastatic disease and another 25% will develop metastases (2).
Patients with a diagnosed metastatic disease have a 1 year survival rate of 70-75%, 3 years survival of 30-35% and a 5 years survival rate of about 20%. If the tumor of this metastatic disease is not treatable with surgery the first line therapy are systemic treatments as chemotherapy and targeted therapy as antibodies to cellular growth factors and/ or immunotherapy. Targeted therapies improve the survival, which shows the importance of analyzing the somatic variants (2). The variation of chemokine receptors maybe another factor for survival.

1.2 Clear cell renal cell carcinoma

Renal cell carcinoma a common urogenital cancer and due the risk factors which are mainly lifestyle depending it gets even more common. Besides a male gender this risk factors are hypertension, smoking, obesity and chronic kidney disease (3).
The mortality of renal cell carcinoma (RCC) in Europe is 2.6%, RCC makes up about 3-4% of solid tumors in adults (4).
Interestingly due the diagnostic tests (CT and MRT) the incidence increased over the last few years. Treatment includes a surgery like nephrectomy or partial nephrectomies. Additionally a targeted therapy and adjuvant therapies are done in patients with metastases. The way of metastasizing is mostly hematogenous. Liver metastases are the first metastases to appear, about 15% reach the brain which means a worse prognosis (3).

1.3 Chemokines

Chemokines are a subgroup of cytokines with the ability to induce cell migration another name for them is "chemotactic cytokines". They are known for playing an important role

for the immune system but also in cancer progression. There are about 50 different Chemokines known today. Their target receptor is a G-protein coupled receptor (5). These chemotactic cytokines regulate leukocyte migration – based on this function they can be characterized as either homeostatic or inflammatory. Homeostatic chemokines are important for movements (up and down the concentration gradient) of leukocytes in and out of secondary lymphoid organs and tissue all over the body. In contrast, inflammatory chemokines play a role in leukocyte migration everywhere where tissue is damaged for example in infections, wounds or inflammation process.
Furthermore, they are known for being part of the regulation of stem cell migration in embryogenesis (6). When chemokines activate their receptor on leukocytes they can cause a wide range of effects: differentiation, production of cytokines, proliferation, angiogenesis, survival, degranulation – some chemokines are also directly antimicrobial. Their impact is not limited to leukocytes, there is a wide range of other affected cell types: mesenchymal and epi- and endothelial cells, astrocytes and even neurons (7).

Chemokines are proteins each including four cysteine and disulfide bonds and their nomenclature is based on the spacing of the two first cysteines (1).
There are four different groups of chemokines naimed XC, CC, CXC, CX3C (7,8).
The cysteines of CXC are separated by an amino acid, in CX3C by three amino acids and in CC the cysteines are next to each other. Furthermore there are some chemokines not fitting into this nomenclature: XCL1 and XCL2 (1).
Until the Keystone Chemokine Symposium in 2000 the nomenclature of cytokines was very inconsistent, often the names were given based on the binding cell type. The “R” (receptor) in the old nomenclature was replaced for “L” (ligand) for better differentiation between the chemokines and the chemokine receptors (8).

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CHEMOKINES	COMMON OTHER NAMES
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
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

Table 1: Chemokine nomenclature (adapted from Bachlerie et al (9))

1.4 Chemokine Receptors

These receptors can be found on a big variety of different cells: cancer cells, endothelial cells, leukocytes and more (9).

Chemokine receptors are type A rhodopsin like G-protein-coupled receptors (10). GPCRs are membrane proteins that undergo structural changes after ligand binding which causes the activation of cytosolic signal pathways leading to a cellular response. There is a wide range of ligands e.g. ions, proteins and other small organic molecules (11).

When a chemokine binds to the GPCR an intracellular pathway activates the substation of GDP for GTP, which results in the dissociation of XY into numerous α- and βγ subunits leading into the activation or inhibition of intercellular pathways (e.g. inhibition of adenyl cyclase or activation of phospholipase C (12,13).

Another important example for a pathway which is activated by a chemokine is the activation of PI3K (phosphatidyl-inositol,3,4,5 triphosphate) This generates PIP3 (phosphatidyl-inositol,3,4,5 triphosphate) which plays a role in the emergence of cancer (14,15). If the concentration of PIP3 increases it will recruit proteinkinase B (AKT) to the cell membrane where it is activated. In a healthy cell this pathway regulates signaling for

apopotosis, cell proliferation and growth as well as cell metabolism, if it fails neoplastic cells can be the result (16).

Besides the GPCRs there are atypical chemokine receptors which decrease inflammation process by binding the chemokines without an effect of any pathway furthermore they create chemokine gradients (9).

Chemokine receptors are classified depending on the subgroup of chemokines that can bind them. Most chemokine receptors can recognize more than one chemokine but in most cases this is restricted to a chemokine subclass.

XC chemokines bind on XCR1, CXC chemokines bind on CXCR1-CXCR6, CX3C chemokine binds on CX3CR1 receptor and CC chemokines bind on CCR1-CCR10 receptors (9,10).

CHEMOKINE RECEPTOR	COMMON OTHER NAMES	LIGAND
G PROTEIN-COUPLED CHEMOKINE RECEPTORS		
CXCR1	IL8RA	CXCL5, CXCL6, CXCL8
CXCR2	IL8RB	CXCL1-3, CXCL5-8
CXCR3	IP10/Mig R	CXCL9-11
CXCR4	Fusin	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	

Table 2: Chemokine receptors and their ligants (adapted from Bachlerie et al (9))

1.5 The role of chemokines in cancer

Where cancer occurs there is always inflammation, in some cases even before the neoplastic cells occur, in others after that. This inflammatory microenvironment plays a role in survival and proliferation. It subverts the adaptive immune response and promotes metastases and angiogenesis. It also influences the response to therapies and hormones (17).

The inflammatory microenvironment is induced through the expression of chemokines and their receptors. They recruit stromal cells like lymphocytes, fibroblasts and endothelial cells, macrophages but also natural killer cells, eosinophiles, granulocytes and B cells (18). Chemokines are described as “a key determinant of the macrophage and lymphocyte infiltrate of human cancers” (19).

The different expression of chemokines and their receptors affect the cell types and number of the infiltrating cells (19).

Chemokines can also have beneficial impacts through are helpful over their effect on Lymphocytes (CD8+, T cells, natural killer cells and TH1-cells), suppressingtumorigenesis. The CXCR3 on their surface they follow the concentration to CXCL9 or CXCL10 (20).

CD8+ cells are a subclass of T-cells which present a glycoprotein as their cluster of differentiation, with their receptor they recognize a specific antigen presented by an antigen presenting cell. This group of cells includes cytotoxic T-cells but also regulatory T-cells, natural killer cells, thymocytes and dendritic cells (21) (22).

Another effect of the CD8+ cells is to induce apoptosis. A link was found between an increased number of them and decreasing rates of metastasis in colorectal cancer.

CD8+, NK and TH1 cells also follow TH17 cells which concludes in the mediation of anti-tumor effects. Furthermore TH17 cells also show chemokine receptors on their surface (CCR6 and CXCR4) (20).

Besides the mentioned cells, plasmocytic dendritic cells also have effects regarding malignacys. Its known that they do have a tumor promoting effect. They encourage the

development of regulatory CD8+ T-cells which inhibitis the activation of T-effector cells by myeloid dentritic cells T-effector cells would have an anti-tumor activity (23).

On the opposite, lymphocytes can also support/promote tumorigenesis.
T-regulatory cells suppress anti-tumor cell response especially in the tumor surroundings, which leads to supported tumor growth and progression.
T-regulatory cells express CCR4 on their surface and migrate to their ligand CCL22 which can be found in the tumor environment produced by the tumor itself and macrophages (24). They also follow CCL28 which is spilled by hypoxic tissue because of their CCR19 receptor (25).
TH22 cells are drawn to CCL20 to the tumor because of their CCR6, which can be found in colorectal cancer and other entities (26,27). TH22 cells promote tumorgenesis through IL22, which upregulates genes, the result is a higher potential for tumorgenesis (28).
T-regulatory cells also have CCR4 and follow CCL22 which is exposed by tumors and macrophages furthermore they promote tumorgenesis (24). The T-regulatory cells suppress the anti-tumor T cell response which creates an immunosurpressed environment. In this immunosuppressed environment the neoplastic cells can display their malignant potential (24).
Hypoxic tissue expresses CCL28, T-regulatory cells with CCR10 on their surface wander towards this chemokine (25). T-regulatory cells decrease the activity of the immune system resulting in a tumor promoting effect (24).
B-lymphocytes showed different results regarding tumor activity: In mouse models they appear as tumor promoting, in breast cancer patients they prolonged survival. On B-lymphocytes there is CXCR4 which is attracted by CXCL12 (20,29).

Antigen-presenting cells do have anti-tumor activity, promoting T cells but also pro-tumor activity over the tumor cells itself and over the tissue surrounding the tumor. In dendritic cells there is a link between their maturity and whether they act against or pro tumor. Older ones activate T-cells, so they support anti-tumor activity (30).
In the tumor environment there are mostly young dentritic cells (31). Young dendritic cells increase tumor progression because they strengthen the TH2-Immune response which inhibits anti-tumor effects (32). These young dentritic cells follow CCL20 expression from the tumor cells because they have CCR6 on their surface (33). CCL20 also has anti-tumor

effects. An overexpression of the chemokine leads to wandering of myeloid cells and also fastens their maturing process. This leads to an inhibition of tumor growth (20).

Tumor cells produce CCL2 which attracts macrophages who express CCR2 (23). In some cancer entities these macrophages are associated with a poor prognosis, they may inhibit T-cell activation (20). There is also a link between those macrophages and resistance to chemotherapy, metastastatic spread and progression of the cancer (20,34).
Macrophages in all of their different stages are monocytes and neutrophilic cells in different stadiums are granulocytes . These cell types are all myeloid derived suppressor cells (MDSC) (20,35). MDSC have effects on cancer cells and on immunosuppression. They are part of the microenvironment of tumors (35). These cells are attracted by CCL2, furthermore they may be attracted by CXCL5 (over CXCR2) and CXCL12 (over CXCR4) (20,36).
Tumor cells as well as myeloid cells express CXCL8 which is a ligand to CXCR1 and CXCR2. These receptors can be found on granulocytic MDSCs (37). The migration of granulocytic cells towards the tumor enhances tumor progression and angiogenesis (37,38).

CXCL12 is targeted by CXCR4, this receptor can be found on vascular endothelia and promotes angiogenesis. Furthermore, it can be expressed by tumor cells where it promotes proliferation, invasion and the process of metastasis (20). Cancer cells with CXCR4 on their surface show a higher potential for metastases and a lower sensibility to radiation (39).

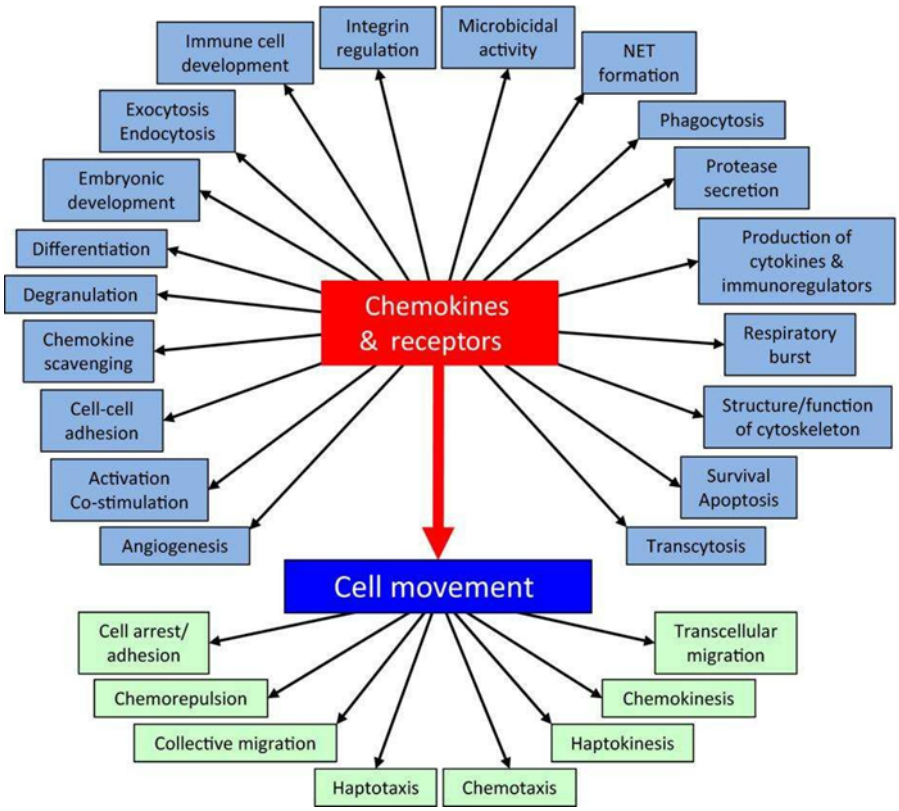
CXCR8 is also expressed by vascular endothelia and promotes angiogenesis by binding CXCL8 (40). This ligand also promotes invasion and metastastic spread of cancer cells expressing CXCR8 (41).

CCL25 encourages tumor cells with CCR9 on their surface, which leads to metastasis towards tissue with this ligand. For example melanoma cancer cells expressing CCR9 metastasize into the small bowel which distributes CCL25 (42). CCR9 is known for more cancer relevant effects: cancer cells with a high expression are linked to chemoresistance (43).
CCL25 promotes expression of matrix metalloprotease which is linked to tumor progression (44). Matrix metalloprotease 9 (MMP9) acts as a tumor promoting factor and

is induced by CCL2, CCL3 and CCL5. Leucocytes, which can be found in the tumor microenvironment moved there through attraction by chemokines express these ligands and show their effect on tumor cells with the matching receptor over the introduction of MMP9. Leucocytes are also responsible for a local immunosuppression around the tumor and they produce growth factors (45,46).

CCL5 and CCL2 promote epithelial mesenchymal transition (EMT, is an important process of metastasis), tumor cell proliferation and survival of malignant cells. Another relevant chemokine ligand is CCL18. The effect of this ligand is dependant on the type of cancer. In pancreatic, ovarian and breast cancer it is promoting EMT, meanwhile in gastric cancer it is linked to a higher rate of survival (20).

Figure 1: functions of the chemokine network (7)



1.6 Chemokines and the process of metastasizing

Some organs are more likely to get metastases: liver, brain, lymph nodes, lungs and bone marrow, while others are rarely affected, for example kidneys and skin to name some (47). CXCL12 is present in lung, brain, bone marrow and lymph nodes. Tumor cells upregulate the matching CXCR4 receptor which leads to a migration process to these tissues. This

plays a role in metastasizing of colorectal carcinoma, melanoma breast, ovarian and lung carcinoma (48). It is known that CXCL12 is involved in proliferation and promoting growth in some malignancies like non-small-cell lung cancer (49).

The process of metastasizing in tumors is a complex one with certain steps that must be fulfilled.
It starts with a failing of the so called cellular senescence, which describes the fact that healthy cells are mostly hindered from going into a cycle of growth and division. It is to prevent uncontrolled division, a failing of that is one of the character points of a malignant cell. Chemokines and their receptors might be part of the protection mechanisms to prevent a decoupling of that protection mechanisms.
If CXCR2 expression on a cell ceases, the cellular senescence is increased as well as the repair mechanisms of damaged DNA, which can result in malignancy. When CXCR2 is increased, it leads to premature cell senescence (50).

The chemokine network can also activate the MAPK/ERK signalling pathway, which is a direct stimulator of tumor cell proliferation.
For the entities lung carcinoma and melanomas there is known that chemokines CXCL1, CXCL2, CXCL3 promote growth (48).

The next characteristic of a malignant cell is the ability to loosen from the primary tumor and migrater through the basement membrane. This process is described as EMT (epithelial-mesenchymal-transformation). This describes the change of a malignant epithelial cell into more of a mesenchymal cell to overcome this last layer and metastasize throughout the body (51). A 2011 study shows a positive link between CXCL8 and the corresponding receptor (CXCR1) and EMT(52). The transforming growth factor β (TNF-β) may help in this process through upregulation of CXCR4 (53).

A chemokine pathway which showed to have an impact on metatstazing is the CCL19 and CCL21 are the ligands to CCR7 pathway. CCR7 in a physiological setting is responsible for the migration of activated DC (dendritic cells) and native T-cells into lymph nodes where the activation of the adaptive immune system happens. Some tumor cells use this pathway to metastazise into lymph nodes. Some of these entities are lung cancer, head and

neck cancer, oesophageal and gastric cancer as well as melanomas (54–59). There is already some more research done regarding chemokines and metastasizing of melanomas. Another known chemokine which plays a role in metastasizing of melanomas is CCL25 which is the ligand to CCR9 (42) The interaction between them plays a role in mucosal immunity and T-cell development. CCL25 can be found in the thymus and in the small intestine (60). Melanomas express CCR9 which is linked to metastases in the small bowel (42). Even more chemokines are found to play a role in progression of melanomas. CXCL9, CXCL10, CXCL11 might play a role in spreading of melanoma into lymph nodes (receptor CXCR3) (61). CXCR3 can also be found on lung cancer (59).

All of this known pathways of chemokines in progression and metastasizing of cancer might have the potential to change the future treatment of cancer. Especially the connection of chemokines and brain metastases is yet not well understood. It is a fact that brain metastases are linked to a higher rate of cancer mortality, but the pathway of how a malignancy can spread into the brain is not completely understood yet.
What is known yet is that interestingly NSCLC with brain metastases have an overexpression of CXCR4 in their metastasis (ligand CXCL12) (62,63).
Furthermore in breast cancer there is some research showing a link of CXCR1 and brain metastasis and there is a link for this metastasis in melanomas and CCR4(64,65).

2 Colorectal adenocarcinoma and chemokines

2.1 CC-Chemokines and Colorectal Adenocarcinoma

CCL4 expression is upregulated in colorectal cancer compared to normal colon tissue Furthermore, in non-metastatic carcinomas the expression is even higher. A study from 2021 found that a low expression of CCL4 which was found in a metastatic state, lowers the cancer-specific survival rate by 30%, this shows the relevance of certain chemokines for the prognosis of cancer (66).
A blockade of CCR5 has anti-tumor effects through their influence on macrophages (leads to repolarisation). This receptor can be found on tumor cells, lymphocytes and myeloid cells (67). The matching ligand CCL5 can be found in a higher than normal concentration in colorectal malignant tissue (68).

In liver metastasis from colorectal adenocarcinoma there is an elevation of CCL21/CCR7, while it was not raised in hepatocellular carcinoma itself. In contrast, the opposite was the case for CCL20/CCR6 (69).The data for CCL20/CCR6 regarding colorectal Adenocarcinoma is inconsistent, another study found a potential raised level of this axis (70). This is supported by a study which found that a coexpression of CCL20 and CXCL8 in CRC promotes metastasis by introducing EMT via the PI3K/AKT-ERK1/2 signalling. A coexpression of CCL20 and CXCL8 in CRC indicates a poorer prognosis (71). This study may explain why the data of previous studies regarding CCL20 is inconsistent, as it may depend on the co-expression of different chemokines, which again demonstrates the complexity of this system.

Regarding CCR1 in CRC a recent study from 2022 which tested Fucoxanthin in a mice model, found that this drug has down regulating effects on the chemokine which furthermore could be a target for new treatment (72).

CCL28 (matching CCR3) is increased in CRC compared to normal colon tissue, in comparison there was no difference in CCL28 expression in rectal tumors and the normal comparison tissue. CCL28 plasma levels in patients with colon carcinoma was higher than in rectal carcinoma. This study from 2006 which did research on the expression of CCL28

in CRC suggests a difference of chemokine expression even depending on the localisation of carcinomas of the colon (73).
CCR9 and its matching ligand CCL25 may inhibit metastasis and invasion of CRC. CCR9 was expressed in a way higher level in colon adenomas compared to invasive CRC (74). Another positive prognostic factor might be a higher expression of CCL18 (75).

2.2 CXC-chemokines and colorectal adenocarcinoma

There is data that shows an increased expression of CXCL11 in colorectal adenocarcinoma. A difference in between colon cancer and rectal cancer was found regarding the association with the prognosis: in rectal cancer an upregulation worsens the prognosis, while the opposite is the case for colon carcinomas (76).

CXCL1 expression is upregulated in colorectal cancer compared to normal tissue and cancer cells induce microvascular endothelial cell migration with this chemokine. PG E2 induces CXCL1 which results in increased tumor growth and angiogenesis. CXCL1 could also activate oncogenes in CRC, which worsen the prognosis. A high expression of this chemokine results in a poorer prognosis in metastatic CRC (77).

The overexpression of CXCL1-3, 5 and CXCL8 in CRC tissue compared to normal colon tissue is known. The expression varies regarding the stage for CXCL1,2,3 and CXCL9-11. CXCL4 and CXCL9-11 are in a positive correlation to the prognosis (75).
The overexpression (in CRC) of CXCL1-5, 8-11 as well as CXCL13,14,16 is supported by another study. Furthermore, the mentioned study done in 2021 which aim was research at the CXC expression in CRC, showed an association between the expression of CXCL1,2,3,9,10,11 and tumor stage. A significance in expression was found regarding the coexpression of CXCL16 with NRAS, KRAS and EGFR (78).

CXCR4 on the primary tumor of CRC not only correlates with the response to first line chemotherapy but it can also function as a prognostic factor according to studies (79)(81). CXCR4 presence on tumor cells significantly enhance metastasis into the liver which results in a poorer prognosis (80) (81).
EMT and progression of colorectal carcinoma is increased by CXCL12/CXCR7 (82).

CXCL17 in lymph node metastases of CRC worsens the prognosis (83).

Higher expression = positive survival impact	Higher expression = negative effect on survival
CCL4	CCR5/CCL5
CCR9-CCL25 (blockes metastasis)	CCL21/CCR7 (metastasis)
	CCL20 + CXCL8 (promotes metastasis)
CXCL11 in colon carcinoma	CXCL11 in rectal carcinoma
CXCL4	CXCL1
CXCL9	CXCR4
CXCL10	CXCL12/CXCR7
	CXCL17 on tumor cells in lymph nodes

Table 3: what is known from previous studies regarding tumor effects of chemokines in colorectal cancer

3 Clear cell renal cell carcinoma and chemokines

The mortality of renal cell carcinoma (RCC) in Europe is 2.6%, RCC makes up about 3-4% of solid tumors in adults (4).

3.1 CC-Chemokines and Clear cell renal cell carcinoma

The subgroup of the CCR chemokines are known for their role in regulation of the immune system but besides that there is some knowledge regarding their influence of malignancy. CCR5 is important in movement of monocytes, macrophages, T cells and leukocytes in general. There is data, that shows a possible association between high CCR5 expression in clear renal cell carcinoma and a high T-stadium of the primary tumor, and also higher rates of postoperative recurrence and mortality (84).

Another study supports that targeting CCR5 in BAP1-mutant clear cell renal cell carcinoma could be beneficial (63).

CCR4 has also been a point of interest for previous studies: CCR4 is correlated with a more aggressive ccRCC course (85). In a mouse model CCR4 was the target of an antagonistic antibody with the result of an antitumor effect. An inhibition of this receptor did not show a reduction of leukocytes around the tumor, but the phenotype of the myeloid cells as well natural killer cells was altered, Th1-cytokine levels were reduced. The level of immature myeloid cells and the level of chemokines in the blood was reduced as well. Thus, the adaptive immune system and CD4+ cells are necessary components of the treatment of the tumor with an anti-CCR4 antibody (86).

The 190AA genotype of CCR2 in ccRCC is linked to decreased survival and could be used as a prognostic factor (87).

The grade of malignancy seems to correlate with the presence of CCR3 (88).

CCL1 is a ligand to CCR8, this axis mediates Th2 cell and Treg cell recruitment in chronic inflammatory diseases like asthma. Tumors with CCR8 expression show an increased tendency in ccRCC to spread into lymph nodes. An expression of this receptor is linked to a decreased risk of recurrence of this malignancy (4).

A potential target in this malignancy to reduce tumor growth, angiogenesis as well as macrophage infiltration is an inhibition of CCL2 (89).

CCL5 shows potential as a target for therapy of ccRCC and also as a prognostic factor, as an increased expression indicates a worse prognosis (90).

A highly expressed CCL4 in ccRCC is linked to a poorer prognosis (91).

3.2 CXC-chemokines and clear cell renal cell carcinoma

CXCL12 is the ligand to CXCR4 and CXCR7, this axis can be found in different types of cancer. These receptors can be linked to increased ccRCC tumor size and CXCL12 to a higher tumor grade. Mortality is linked to tumor size in this case, which concludes in a link between the expression of these receptors and mortality. The survival decreases by 1.3 times with every centimetre of the tumor size of the ccRCC (92). CXCR4 and CXCR7 can be used as a prognostic predictor in ccRCC (93). This is also supported by a study that targeted not only these two receptors but also CXCR6. High levels of CXCR6 have also been observed in ccRCC and are linked to a worse survival rate. CXCR2 is linked to a better survival (94).

Higher expression = better prognosis	**Higher expression = worse progonosis**
CCL1-CCR8	CCR5
CXCR2	CCL5
	CCL4
	CCR3
	CCL2
	CXCR6

Table 4: what is known it literature about chemokines and ccRCC

4 Material and methods

4.1 Tissue samples

From the Biobank Graz obtained tissue samples starting from 2012 of colorectal adenocarcinoma and clear cell renal cell carcinoma were used.
In forehand the ethical approval was obtained from the local ethics committee. (31- 144 ex 18/19).

The used control tissue were lymph nodes and tonsils acquired from autopsies from the Department.
Paraffin-embedded tissue samples of 15 brain metastases of the clear cell renal cell carcinoma and 8 clear cell renal cell carcinomas were obtained. Additionally, samples of 7 colorectal adenocarcinoma as well as 8 of their brain metastases were acquired . Healthy surrounding tissue samples were used for comparison. A microdissection as descripted below was done.
With a microtome this formalin-fixed-paraffin-embedded (FFPE) tissues were cut with a disposable blade. To ensure that there is the malignant tissue on the slides and to disguingish between that and the surrounding healthy tissue, the first cut of each block was used for HE staining. The same thing was done to the last cut, to be sure that there is still malignant tissue.
The microscopic verification was done by our supervisor Dr.in med.univ. Marlene Leoni.
The thickness of the sections was 10 µm, but depending on the tissues size of the tissue the total thickness was about 50 µm for the further process.
On some samples there was no surrounding tissue present, those cuts were used in whole for the RNA isolation and were put directly into extraction tubes.
On the slides where surrounding tissue was present it was microdissected. The microdissection was done by using a sterile, disposable blade and a single-use toothpick.
The whole process was done with special attention to cleaning the microtome and all other tools with Thermo Scientific™ RNase AWAY™ (Thermo Fisher Scientific Inc., Waltham, MA, USA). In between new samples the water was changed as well as the gloves.

4.2 RNA Isolation

The RNA isolation has been performed with Relia Prep™ FFPE Total RNA Miniprep System (Promega, Madison, WI, USA) using the manufacturer's protocol (TM353 Revised 12/15).
The result of the RNA isolation was diluted in 30 µL nuclease-free water. The quantity of the RNA was then measured by using the Thermo Scientific™ NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the NanoDrop 1000 operating software, version 3.8.1.
Until further processing the RNA was stored at -80°C.

4.3 cDNA synthesis

For this process the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to revision 11 of the manufacturer's protocol was used with 1 µL random hexamer primer. The used total reaction volume was 20µL.
The UNO96 thermocycler from VWR International GmbH (Darmstadt, Germany) has been used for cDNA synthesis. Cycling 21 conditions for cDNA synthesis were 25°C for 5 minutes, 42°C for 60 minutes, and the termination of the reaction happened at 70°C for 5 minutes.

4.4 Real time PCR

A two-step approach was used for reverse transcription -quantitive real time PCR (RT-qPCR) because the RNA concentration in some samples was low.
The first step was to test the chemokine receptor expression of all 19 receptors on one of each sample type (one CRC and its brain metastasis and one renal cell carcinoma and its brain metastasis) and on the control tissue. Followed by a melt curve analysis which showed unspecific amplification of CCR1, CCR5, CCR10, CXCR1, CXCR2, and CX3CR1. This receptors where excluded from further testing.
13 chemokine receptos (CCR2-CCR4, CCR6-CCR9, CXCR3-CXCR7, and XCR1) remained and where tested on five samples of each type and on control tissues.
The receptors which could be found in the tumor samples were then tested on all samples.

The used primers were ordered from Ingenetix GmbH (Vienna, Austria).The exeption of that were CCR1, CCR6, CCR8, CCR10, CXCR4, and CX3CR1, which have been obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany).

Table 5 shows the primers for the chemokine receptors and their sequence.

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 5 Nucleotide acid sequence of primers used in the real time PCR

For the PCR the cDNA was diluted to 1:20 with nuclease free water.

For RT-qPCR, the Luna® Universal qPCR Master Mix M3003 (Applied Biosystems, Foster City, CA, USA) with a modified protocol was used. 10 µL was the total reaction volume, containing 4 µL cDNA and 5 µL of Master Mix with varying forward and reverse primer concentrations.

The best concentration of the primer was determined before on tonsil and lymph node tissue.

Table 6 shows the concentration used in the experiment for the respective primer

CHEMOKINE RECEPTOR	CONCENTRATION OF FORWARD AND REVERSE PRIMER
CCR1	**100 nM**
CCR2	**250 nM**
CCR3	**250 nM**
CCR4	**500 nM**
CCR5	**125 nM**
CCR6	**250 nM**
CCR7	**500 nM**
CCR8	**125 nM**

CCR9	250 nM
CCR10	125 nM
CXCR1	250 nM
CXCR2	250 nM
CXCR3	125 nM
CXCR4	100 nM
CXCR5	500 nM
CXCR6	500 nM

Table 6 Concentration of the used primers

The used thermocycling conditions were 50°C for 2 minutes and 95°C for 10 minutes for polymerase activation and 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds. Upon PCR, a melt curve stage, fluorescence data was capured during gradual temperature increase from 60°C to 95°C. It has been carried out to distinguish specific amplicons from nonspecific amplification products.

RT-qPCR was performed on the QuantStudio™ 7 Flex Real-Time PCR System and by the usage of the QuantStudio™ Real-Time PCR Software Version 1.3 of Applied Biosystems (Foster City, CA, USA).

GAPDH and ACTB were used as reference genes to be able to compare the cycle number in relation to the target genes.

The only samples which were included had GAPDH and ACTB Ct values less than 32.

For calculations the geometric mean of the Ct value of GAPDH and ACTB.

The used calibrators where 22 FFPE tonsil and lymphnode tissues which where processed as above and their geometric mean was used for calculation.

The relative expression was determined in triplets and calculated based on the $2^{-\Delta\Delta CT}$ method (95).

A P value less than 0.05 was considered significant. The statistic was done in Microsoft excel.

5 – Results

In the first trials the expression of certain receptors CCR2, CCR3, CCR5 and CCR10 as well as the expression of CXCR3, CXCR5, CXCR7, CX3CR1 and XCR1 was tested on tumor tissue. Due to inconsistent mRNA expression they have been excluded from the main experiment. The main experiment focussed on CCR8 CCR4, CCR6, CCR9, CXCR4, CXCR6.

5.1 Renal carcinoma

For analysis of RCC/ccRCC the following samples were used: 8 primary tumors, 15 brain metastases, 7 surrounding renal tissue samples and 2 surrounding brain tissue.

5.1.1 Chemokine expression pattern of renal carcinoma experiment

The table shows the expression pattern of the chemokine receptors on the clear cell renal cell carcinoma. The expression levels of all of the tested receptors were increased in the metastases compared to the primary tumor tissue, as is shown in table 7.
Regarding CCR8, CXCR4 and CCR6, it showed a similar level of expression when compared the renal tissue and the primary tumor .For this sample size which was quite low, CCR4 and CXCR6 were not expressed by surrounding brain tissue.

pos vs tested	CCR8	CCR4	CCR6	CCR9	CXCR4	CXCR6
Met.	14//15	11//15	12//15	10//15	14//15	9//15
prim Tumor	6//7	3//7	4//7	5//7	7//7	6//7
kidney	6//7	6//7	5//7	5//7	7//7	2//7
brain	2//2	0//2	1//2	2//2	2//2	0//2

Table 7: Expression pattern of chemokines in ccRCC

5.1.2 Relative Expression in renal carcinoma experiment

As is shown in figure 2, the CC receptors expression levels were higher than those for the CXR receptors in all tissues. All tested CC receptors showed the highest expression in the metastases of renal cell carcinoma. The expression of CCR8 (p= 0,073), CCR6 (=0,052) and CCR9 (p=0,076) and CCR4 (p=0,329) did not differ significantly between primary tumor and metastasis. We got the same result for CXCR6 (p=0,099) but here the primary tumor had a higher expression and CXCR4 (p=0,275). There was no significant difference in the expression of CXCR4 (p=0,275) in the primary tumor and the metastasis. There was no expression of CCR4, CXCR6 in the healthy brain tissue. CCR6 and CXCR4 were the only receptors which were expressed at greater levels in the surrounding renal tissue compared to the primary tumor. This was also the case for the comparison of brain tissue and metastasis regarding CXCR4.

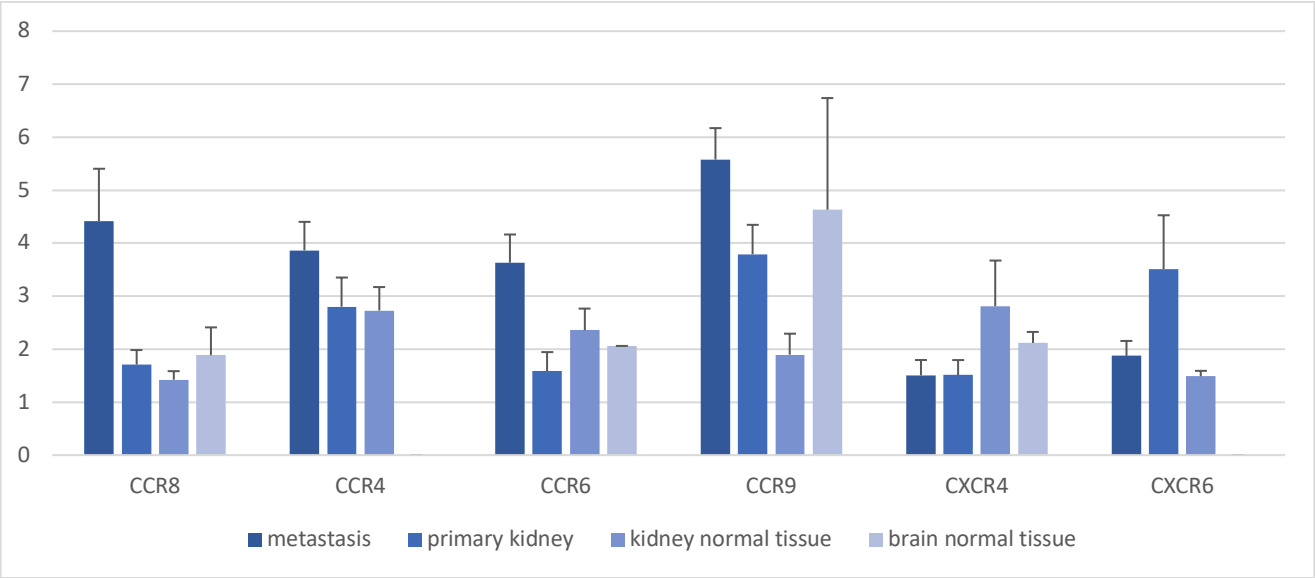


Figure 2: Relative Expression of the chemokines in ccRCC

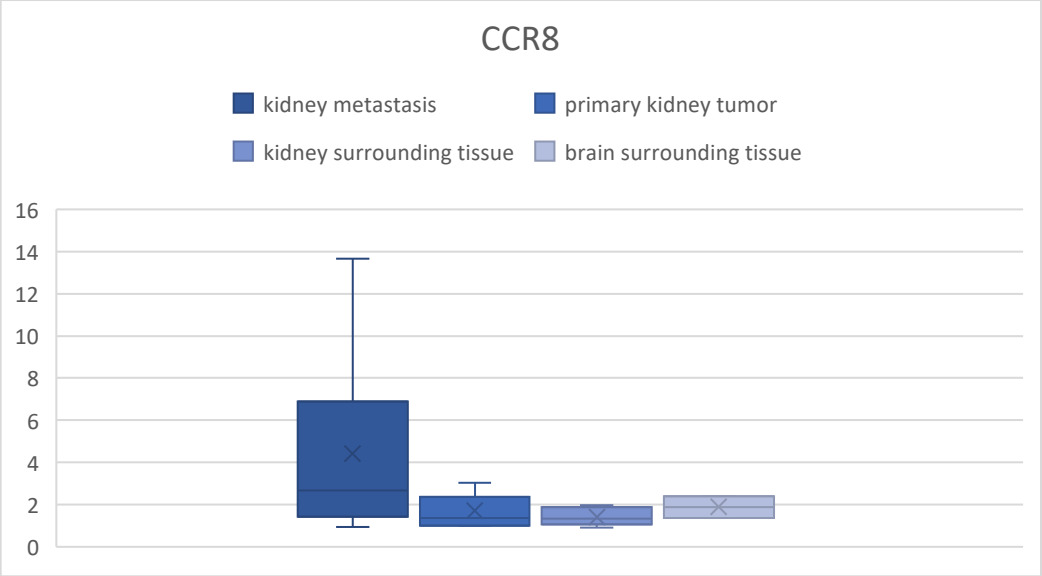


Figure 3 Relative expression of CCR8: each Box shows a different sample type

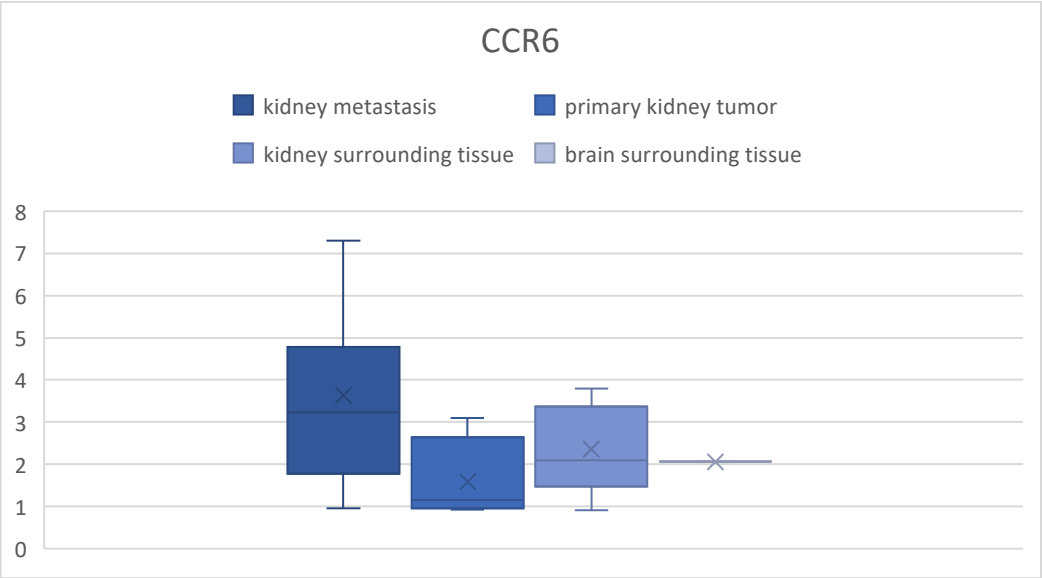


Figure 4: Relative expression of CCR6: each Box shows a different sample type

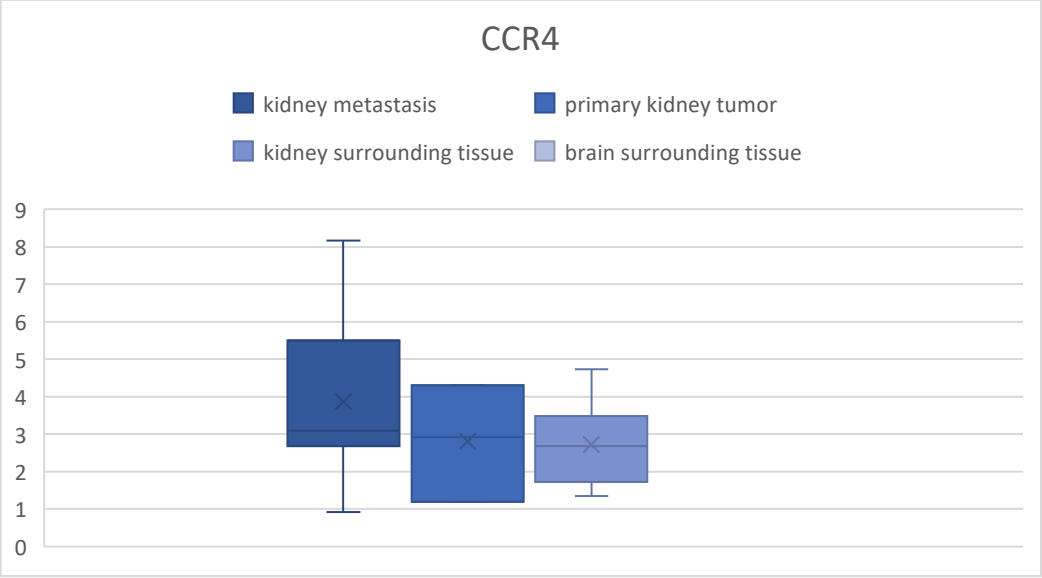


Figure 5: Relative expression of CCR4: each Box shows a different sample type

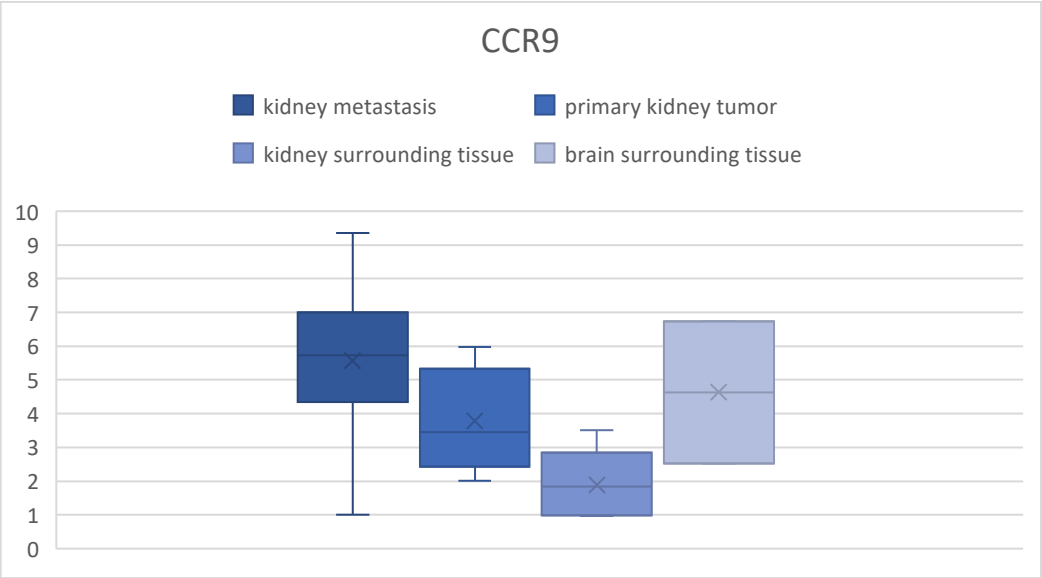


Figure 6: Relative expression of CCR9: each Box shows a different sample type

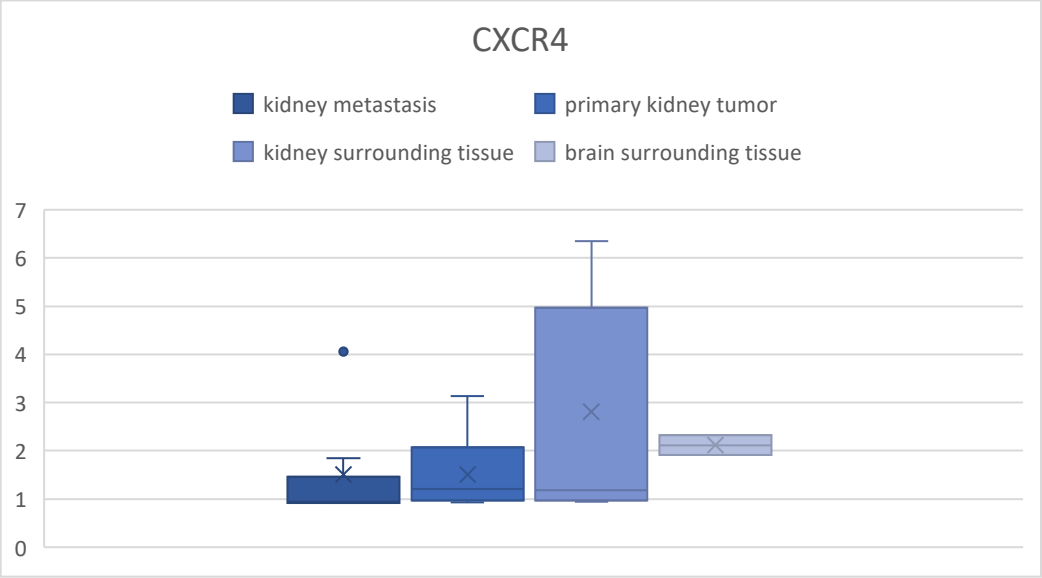


Figure 7: Relative expression of CXCR4: each Box shows a different sample type

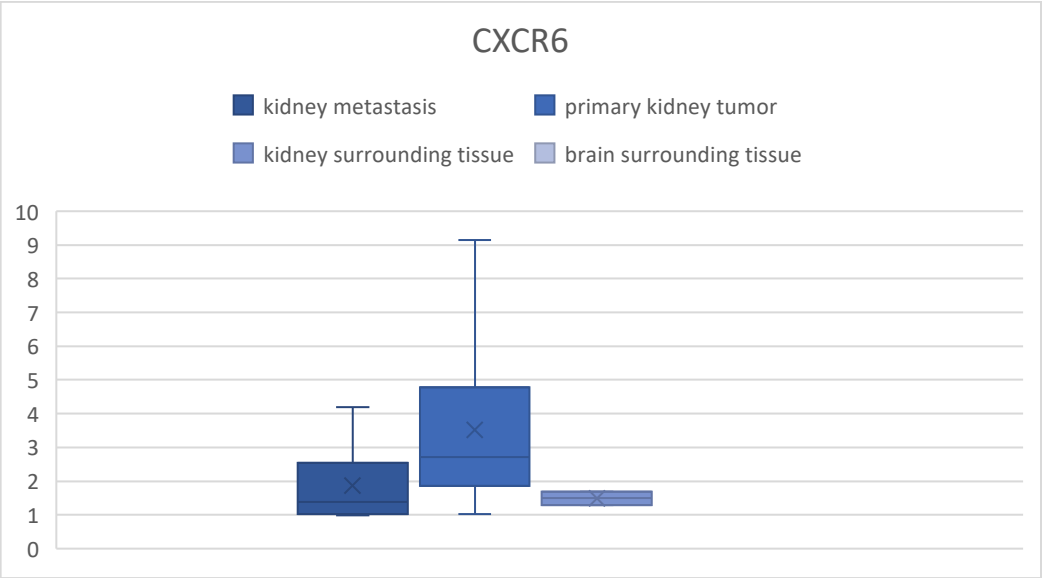


Figure 8: Relative expression of CXCR6: each Box shows a different sample type

5.2 Colorectal adenocarcinoma

The used samples were derived from 7 primary colorectal adenocarcinomas, 8 brain metastases and 6 surrounding colon tissues.

5.2.1 Chemokine expression pattern of colon carcinoma experiment

CCR6, CCR9 and CXCR6 were expressed in all samples of the metastases of the colon carcinoma. In comparison in the primary tumor samples these receptors were expressed to a lesser extent. The difference between the expression levels was the biggest with CCR6: just 3/7 primary tumor samples expressed this receptor. CXCR4 was expressed in all samples. CCR8 was expressed in all samples of the primary tumor, CCR4 expression was lower.

pos vs tested	CCR8	CCR4	CCR6	CCR9	CXCR4	CXCR6
Met.	6//8	6//8	8//8	8//8	8//8	8//8
prim Tumor	8//7	2//7	3//7	4//7	7//7	5//7
colon	6//6	4//7	2//7	4//7	7//7	2//7

Table 8: Expression pattern of chemokines in CRC

The CCR8 expression differed significantly between primary colon tumor samples and samples of the metastases (p=0,004). The CCR8 receptor gots lost in the metastases of colorectal adenocarcinoma. In all other tested receptors no significant differences between primary tumors and metastases were found.

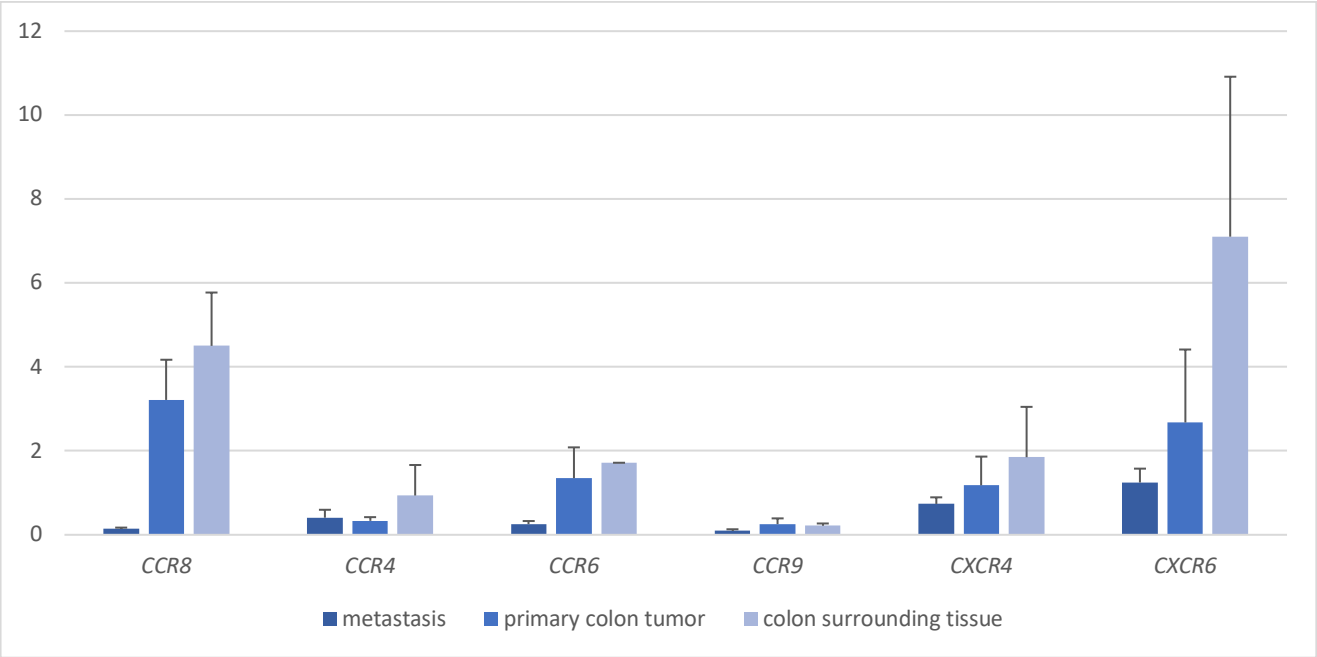


Figure 9: Relative expression of chemokines in CRC

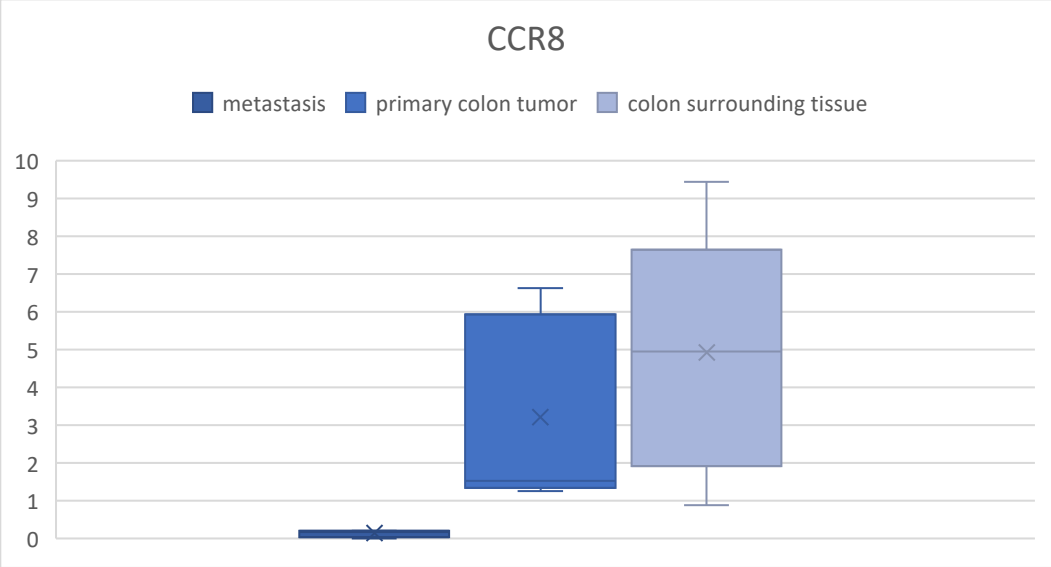


Figure 10: Relative expression of CCR8: each Box shows a different sample type

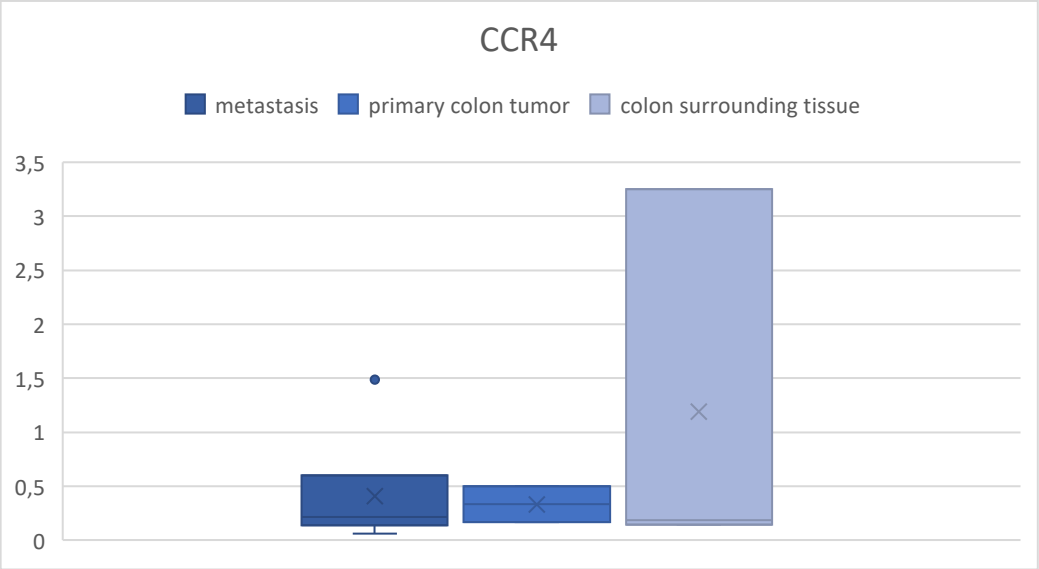


Figure 11: Relative expression of CCR4: each Box shows a different sample type

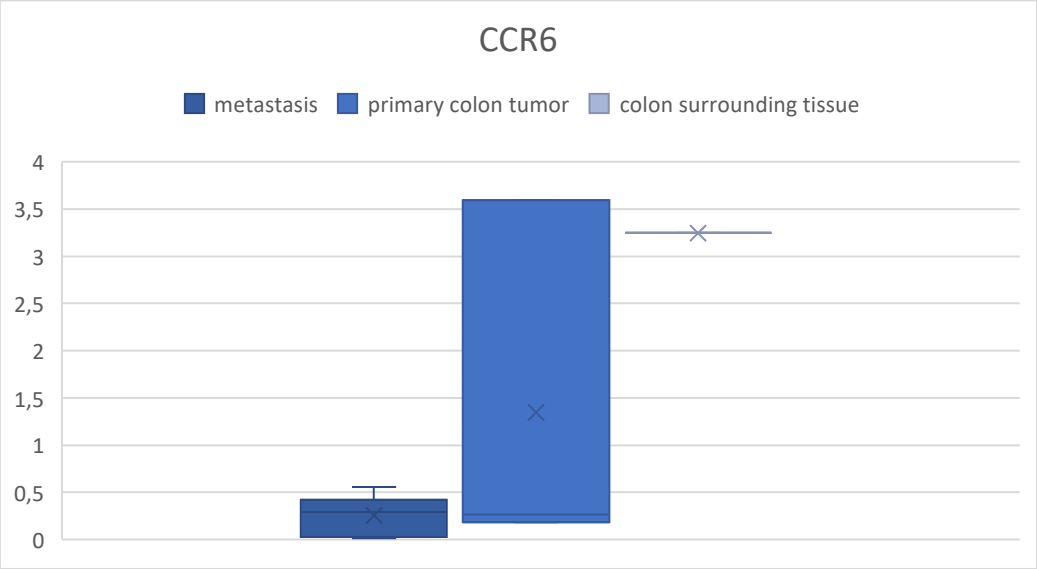


Figure 12: Relative expression of CCR6: each Box shows a different sample type

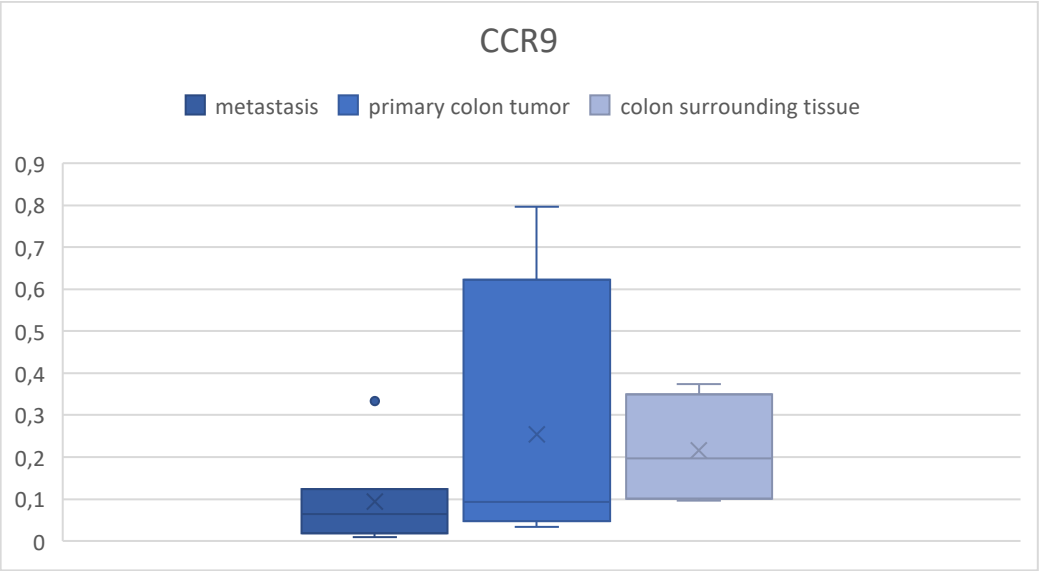


Figure 13: Relative expression of CCR9: each Box shows a different sample type

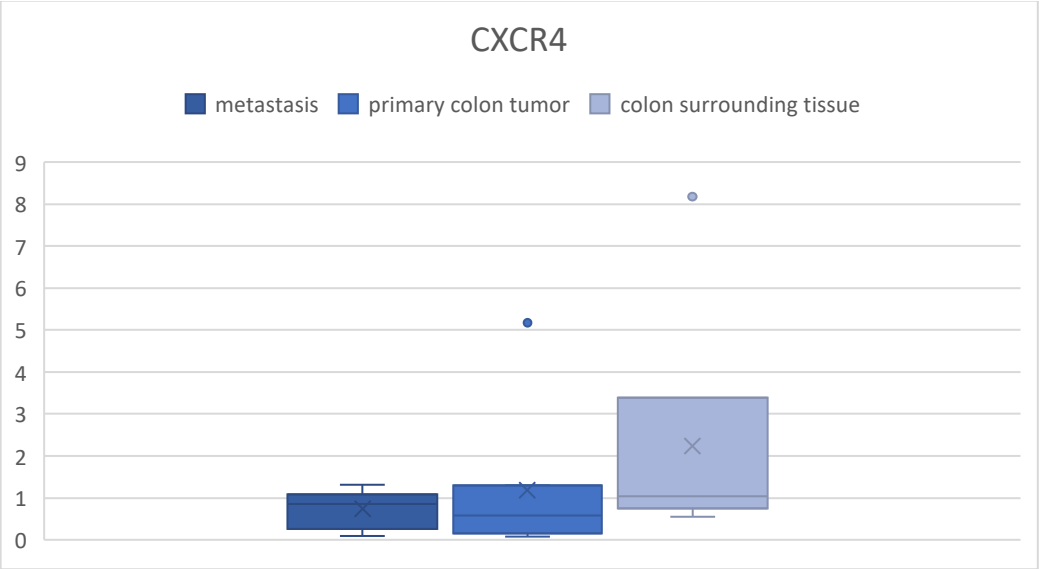


Figure 14: Relative expression of CXCR4 each Box shows a different sample type

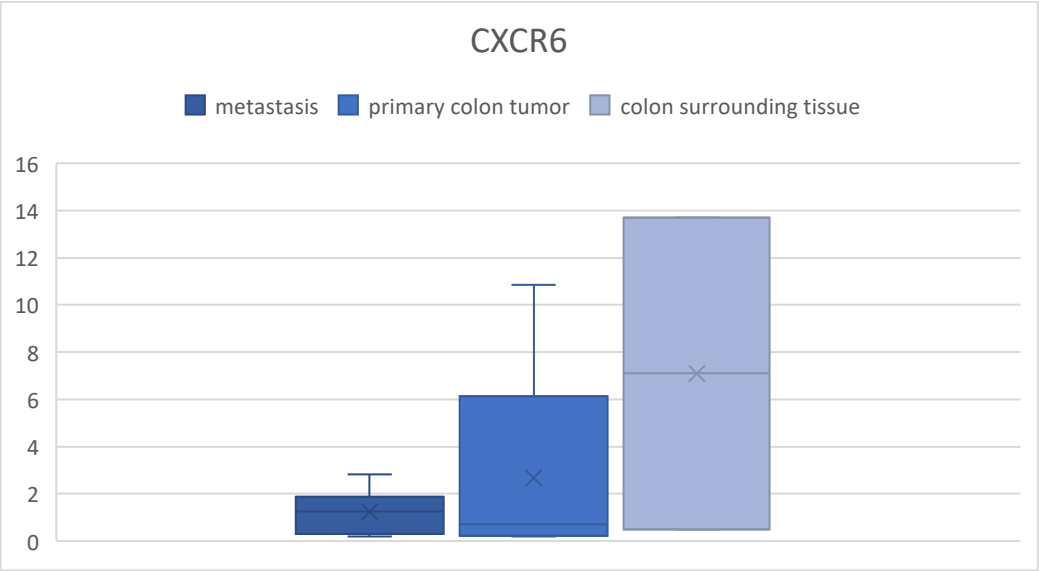


Figure 15: Relative expression of CXCR6: each Box shows a different sample type

6 Discussion

As described in this thesis, chemokines are mostly known for their role in the immune system causing not only cell migration but also having other effects. It has been known for a while, that they also have an impact in cancer progression and metastasis. Little is known about the role of this Cytokines in the progression into a brain-metastatic disease, the aim of this thesis was to get new data regarding clear cell renal cell carcinoma and colorectal adenocarcinoma.

CCR4 is expressed on T-regulatory cells who immigrate towards tumor cells and macropahges (following CCL22), promoting tumorgenesis (24). In ccRCC it is correlated with a poorer prognosis due a more aggressive course of the disease (85).
CCR4 was found in more samples of brain metastasis for both tumor types in this thesis than in the primary tumor. Limitation to that is the lack of significance, probaply due small sample size.

CCR6 is expressed on young dentritic cells who follow CCL20 which is expressed by tumor cells (26). This leads to the fact that a tumor is surrounded by young dentritic cells (31). This cells around the tumor strengthen the TH2-Immune response which means an increase of tumor progression because this immune response decreases anti-tumor effects (32). On the other hand CCR6 can be found on TH17 cells who mediate anti-tumor effects (20).
In clear cell renal cell carcinoma in this thesis this receptor was expressed in more metastases than in the primary. Although there was no significance for colorectal adenocarcinoma CCR6 was also found on more metastasis than on the primary tumors.

The data of the expression of CCR6 on colorectal cancer and their metastis in the liver are inconsistent. Jiao et. al says it is not elevanted (69), while Cheng et. al. and Frick et.al say the opposite. Our data on brain metastases supports the studies who found it is raised in metastases compared to the primum of colorectal cancers, although there is no significance.
This tendency we also found in clear cell renal cell carcinomas where CCR6 is higher expressed in the brain metastasis, this finding though is also not significant.

In conclusion, colorectal adenocarcinoma and clear renal cell carcinoma may upregulate their CCR6 receptors during the process of metastasizing.

CCR8 (CCL1)mediates the recruitment of Th2 and Treg cells in chronical inflammatory disease and there is a link in the tendency of clear cell renal cell carcinoma to spread into lymph nodes: a study says that expression leads to a reduction of recurrence (4).
We found CCR8 expressed on primums of ccRCC and on metastases, it was not significantly overexpressed in the brain metastases but we found a significance of that for colorectal cancer. We therefore claim that this receptor gets upregulated in the process of metastasizing to the brain in colorectal cancer.

CCR9 expressing tumor cells metastasize into tissue with a high level of CCL25, this is the case for melanoma that spread into the small bowel (42) .It also leads to chemoresistance and progression (43)(44). CCR9 was present in all brain metastasis but also in less tissues of tumors. It is not significant that ccRCC metastases have a higher expression than the primum. We also found CCR9 in a higher concentration on brain tissue, this might support the finding that CCR9 on tumors leads to a higher rate of metastasis. It could be evaluated if brain tissue has a higher CCL25 concentration in following studies.

CXCR4 can also be found on TH17 cells (20). Besides that also on B-lymphocytes, where it can show tumor promoting effects but in breast cancer the opposite is the case (20,29). It is overexpressed in NSCLC with brain metastases (62,63). This receptor was also found on primary colorectal cancer (79)(81). Data supports the fact, that CXCR4 expressing colorectal tumor cells are more likely to spread into the liver which means a poorer prognosis (80) (81). Regarding renal carcinomas there is a link between a higher tumor grade and the expression, so it can be used as a prognostic factor for this entity (92) (93). In this thesis the tumor grade was not included as a factor and there was no significance of the data. CXCR4 was found on all tissue samples we tested.

CXCR6 in high levels is linked to a worse survival rate (94). Other previous studies show a noverexpression of this receptor in tumors (primum and metastasis), furthermore it is linked to a poorer prognosis by upregulating the matrix metalloproteinasis (96,97). We found CXCR6 more on the metastases of ccRCC and colorectal cancer, in the ccRCC metastases even higher. This finding was not significant.

To summarise our data mainly support the known studies in this field.
There are some limitations to our data. There was no immunohistochemical analysis done, this would be a point of interest to get a better understanding of the expression of the chemokine receptors, tumor cells itself and/or the expression on the surrounding inflammatory reaction. More tissue samples would have been better for better statistical analysis. In this thesis there was a limiting factor for more sample acquisition that cancer of the kidneys and cancer of the colon in Austria are likely to be diagnosed before they spread into the brain.
An analysis including clinical data like sex, age, survival may be a point of interest in the future to do.
The used tissue was formalin fixated and in paraffin embodied, fresh tissue would have had higher RNA concentrations which would have made the laboratory work easier.
Our results support that there is more research to be done regarding cancer an chemokines and chemokine receptors especially to facilitate novel treatment options for metastases to the brain. Chemokines may have the potential for targeted therapy in the future in cancer that spread to the brain.

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