Thesis

Chemokine Receptor Expression Profile in Malignant Melanoma and Brain-Specific Metastases

submitted by

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Graz, 06.02.2023

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis ist he 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.

Graz, 06.02.2023 Raphael Gassner eh.

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List of Abbreviations

ACKR	atypical chemokine receptor
C	Cystein
CKR	chemokine receptor
CCKR	conventional chemokine receptor
FFPE	formalin-fixed paraffin-embedded
HE	hematoxylin eosin
i.e.	id est
PCR	polymerase chain reaction
RGP	radial growth phase
RNA	ribonucleic acid
RT-qPCR	real-time quantitative PCR
UV	ultraviolet
VGP	vertical growth phase

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Zusammenfassung

Hintergrund

Das kutane Melanom zählt zu den häufigen malignen Tumoren in Industriestaaten und ist vielfach mit einer schlechten Prognose assoziiert. Dieser Umstand beruht auf der Entwicklung von Metastasen, welche gehäuft im Gehirn auftreten. Ein Mechanismus, auf dem die Metastasierung beruht, ist die Aktivität bestimmter Chemokine und deren Rezeptoren. Deren genau Rolle ist dabei jedoch noch nicht exakt geklärt. Aufgrund dieser Tatsache ist das Ziel dieser Arbeit, mögliche Unterschiede in der Expression von Chemokinrezeptoren in Melanomen und deren Hirnmetastasen herauszufinden. Ergebnisse daraus könnten die Basis für spezielle Therapien in diesem Bereich darstellen.

Methoden

Die Expression von Chemokinrezeptoren wurde auf neun primären malignen Melanomen sowie 25 Hirnmetastasen dieser Entität mittels RT-qPCR untersucht. Zusätzlich wurde jeweils umliegendes Normalgewebe mittels derselben Methode analysiert.

Ergebnisse

Valide Ergebnisse konnten für die Expression von fünf Chemokinrezeptoren (CCR4, CCR6, CCR9, CXCR4 und CXCR6) erzielt werden. Dabei zeigten CCR4, CCR6 sowie CCR9 eine erhöhte Expression in Hirnmetastasen gegenüber den Primärtumoren. Ebenso zeigte sich eine signifikant vermehrte Expression der CC Chemokinrezeptoren im umliegenden Normalgewebe gegenüber den Melanomproben ($p < 0,05$). CXC Rezeptoren (CXCR4 und CXCR6) zeigten ähnliche Ergebnisse wie die untersuchten CC Chemokinrezeptoren.

Schlussfolgerung

Kutane maligne Melanome unterscheiden sich in der Expression bestimmter Chemokinrezeptoren von deren Hirnmetastasen. Zusätzlich unterscheidet sich das Profil von Chemokinrezeptoren in Melanomen von dem im umliegenden Normalgewebe.

Abstract

Background

Cutaneous malignant melanoma is a common and potentially fatal malignancy in industrial countries, the developement of metastases is associated with poor prognosis. One of many mechanisms involved in its developing process is the expression of chemokine receptors. Since expression patterns of different chemokine receptors in the developement of brain metastases in primary malignant melanomas are still not known exactly, this thesis aimed on that issue. Furthermore, results in that field could be gate opening regarding the establishment of new therapeutical opportunities.

Methods

This thesis‘ target was to find out differences in expression patterns of chemokine receptors in primary malignant melanomas and their brain specific metastases. In total nine samples of primary melanomas and 25 samples of brain specific metastases as well as corresponding surrounding tissues were analysed regarding their chemokine expression patterns by using RT-qPCR.

Results

Five chemokine receptors (CCR4, CCR6, CCR9, CXCR4 and CXCR6) showed consistent expression patterns. All three CC chemokine receptors showed a decreased expression in melanomas in relation to control tissues as well as to metastases. Comparing CC chemokine receptor expression in melanomas and their surrounding skin tissue, significantly elevated levels of chemokine gene products were detected in melanoma‘s surrounding tissue ($p < 0.05$). Overall, CXCR4 was expressed in lower levels in all samples compared to controll tissue. These results resembled to those of CXCR6.

Conclusion

Chemokine expression patterns in melanomas are different to those in brain specific metastases. Furthermore, the chemokine profile is significantly different in primary melanomas compared to their surrounding skin tissue.

1 Introduction

1.1 Malignant Melanoma

Even in highly specialised and modern medicine cutaneous melanoma remains a potentially fatal malignancy (Saleem and Davar, 2022). It is caused by malignant transformation of melanocytes, which results in their potential occurance in even non-cutaneous areas like ocular, gastrointestinal or leptomeningeal (Ahmed et al., 2020). Nowadays the incidence of malignant melanoma is still increasing rapidly, although many other malignancies are decreasing. That circumastance is reasoned in its most important enviromental risk factor: exposure to ultraviolet (UV) rays because of their genotoxic effect (Rastrelli et al., 2014). It is responsible for about 80 % of all skin cancer related deaths, even though it is accountable for just about 5 % of cutaneous cancers (Zaremba et al., 2020). Once that cancer has metastasised, it rapidly deteriorates one patient’s prognosis and outcome (Schadendorf et al., 2018). Many studies have shown that melanoma‘s metastatic power is a result several genetic mutations that control central cellular processes such as proliferation (e.g. BRAF) or cell metabolism (PTEN) (Leonardi et al., 2018). Histopathological analysis combined with clinical characteristics are still the gold standard in diagnosis for melanoma, despite immunohistochemistry and genomic analysis. The most common subtype of that cutaneous cancer is the superficial spreading melanoma, followed by lentigo maligna melanoma (Elder et al., 2020).

1.1.1 General Classification of Melanoma

The gold standard for melanoma diagnosis is histopathological analysis supported by immunohistochemistry. The vast majority of cutaneous melanomas arise from epidermal melanocytes. Furthermore, most of them progress within two stages of growth. At first malignant melanocytes spread in a radial growth phase (RGP), followed by the vertical growth phase (VGP). Within the VGP the tumour is formed and is able to elevate the epidermis or infiltrate the dermis. In dependence of the RGP’s present, three main subtypes of melanoma can be distinguished: nodular melanoma, superficial spreading melanoma and lentigo maligna melanoma (WHO, 2021). Although these subtypes are the most common, many others can be described.

Table 1. Classification of melanoma, modified from 2018 WHO classification (adapted from Bobos, 2021)

Melanoma	Arising in cumulative sun damaged skin, low and high UV radiation	Lentigo maligna melanoma
		Superifical spreading melanoma
		Desmoplastic melanoma
	Not consistendly associated with cumulative sun damage, low or no UV radiation	Spitz melanoma
		Acral melanoma
		Mucosal melanoma
		Melanoma in congenital nevus
		Melanoma in blue nevus
		Uveal nevus
	Arising in variable UV radiation	Nodular melanoma
		Nevoid melanoma

1.1.1.1 Nodular melanoma

The nodular melanoma is defined by the abscense of RGP, which means it shows no intraepidermal proliferation of atypical melanocytes beyond three epidermal ridges on each tumour side. The grossing appearance is often a well circumscribed lesion with a small diameter in a symmetrical shape. Clinically the differentiaton to viral warts or basal cell carcinomas can be difficult. Histologically, the nodular melanoma consists of many different atypical cells that are epitheloid shaped with vesicular nuclei. The cell density within the nodule is high, mitosis and necrosis are abundant. Plasma cells are prominent in the microenviromental infiltrate. In terms of cytogenetic charakteristics nodular melanomas are associated with NRAS mutations, as well as BRAF mutations (WHO, 2021).

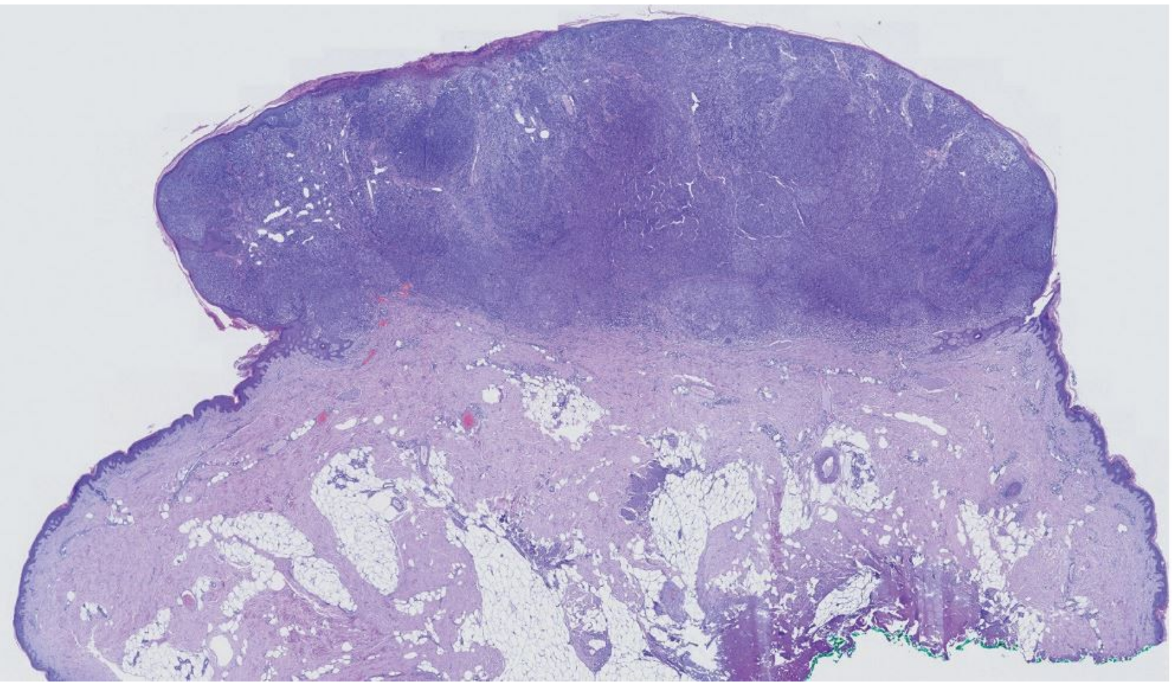

Figure 1. Nodular melanoma. No radial growth phase (RGP) can be seen on both sides of the tumour nodule. Adapted from Bobos, 2021

1.1.1.2 Superficial Spreading Melanoma

Superficial spreading melanoma is the most common subtype of cutaneous melanoma. Clinically they appear in a large variability in terms of colour, ranging from amelanotic to nearly black lesions and are usually bigger than ten mm in diameter and poorly defined. Arising as a melanoma in situ, it typically evolves through two phases of growth: RGP and VGP (Elder et al., 2018, WHO, 2021). The RPG consists of the melanoma in situ and early invasive phases, in which atypical melanocytes are located in the epidermis or in the superficial dermis as isolated cells or small sized nests. The VGP is characterised histopathologically by large nests of atypical melanocytes in the dermis, and solar elastosis is accompaning in the dermis as well (WHO, 2021). In superficial spreading melanomas BRAFp.V600E and NRAS mutations are often detected, although mutations of TP53 can occur additionally (Long et al., 2011).

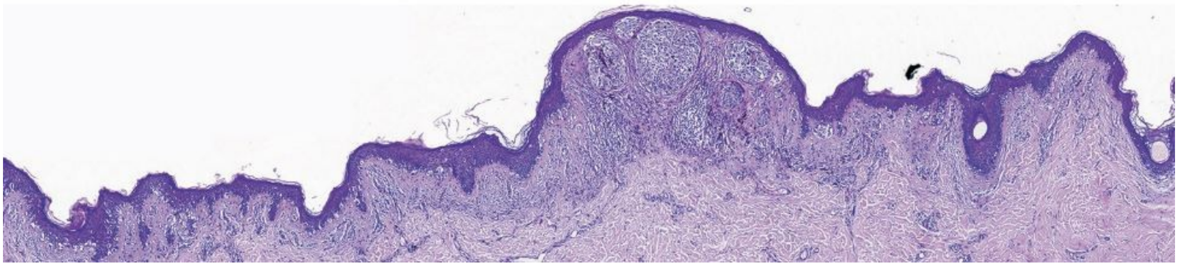

Figure 2. Superficial spreading melanoma. A wide radial growth phase (RGP) and only small vertical growth phase (VGP) is shown. Adapted from Bobos, 2021

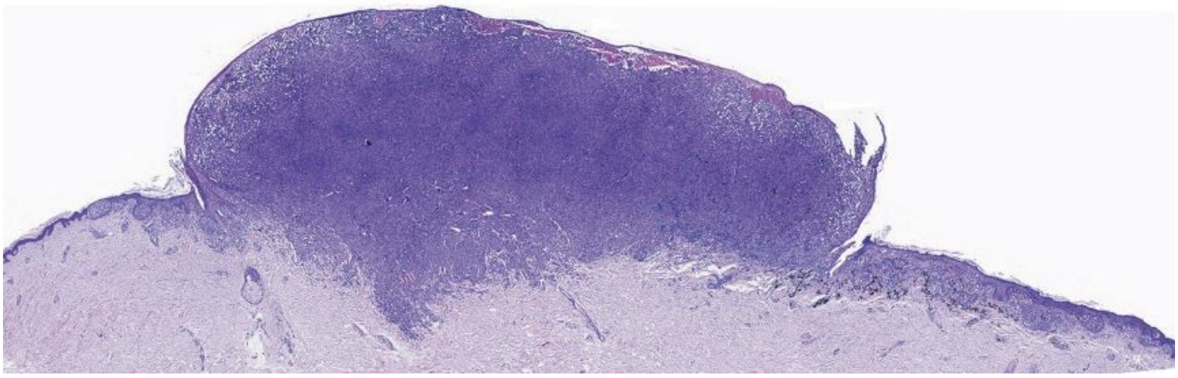

Figure 3. Superficial spreading melanoma with broad vertical growth phase (VGP). On both sides of the nodular growth a radial growth phase (RGP) can be detectet. Adapted from Bobos, 2021

1.1.1.3 Lentigo Maligna Melanoma

This type of melanoma mostly effects older patients in heavily sun exposed areas of the skin. Most times its clinically appearance is a brown coloured, irregular circumscribed lesion. This melanoma subtype can reach large diameters without infiltrating the dermis. As a melanoma in situ, atypical melanocytes proliferate in a continious row along the dermoepithelial and adnexal-stromal junction. In further progression nest formations are small in most cases and melanoma cells can be epithelioid, small and round with more than one nuclei. Furthermore, solar elastosis must be found in terms of diagnosis (WHO, 2021). Frequently detected mutations are BRAF V600K, NRAS and KIT (Bastian, 2014).

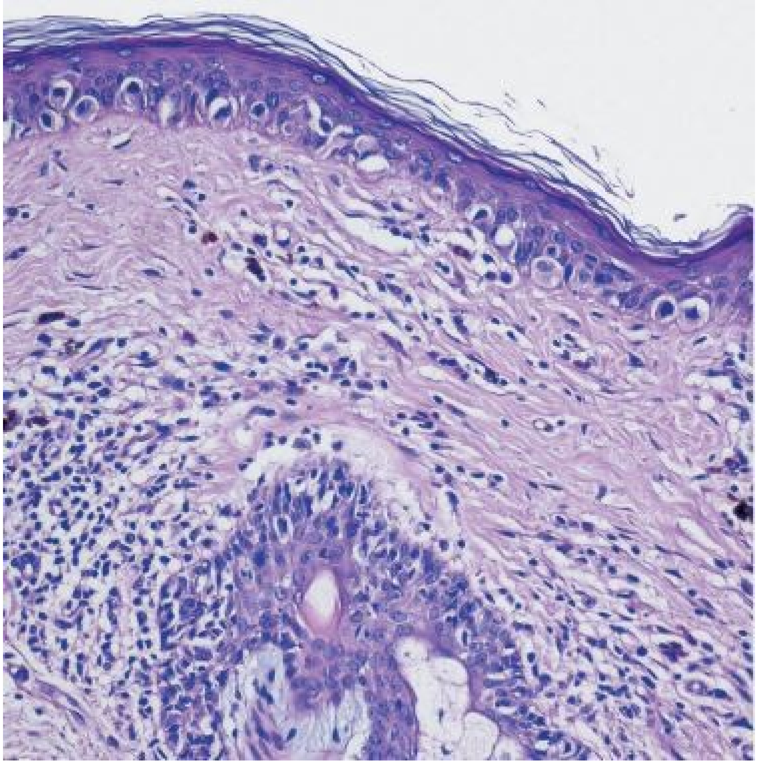

Figure 4. Lentigo maligna melanoma. Atypical melanocytes proliferate mainly along the epidermal basal layer. Adapted from Bobos, 2021

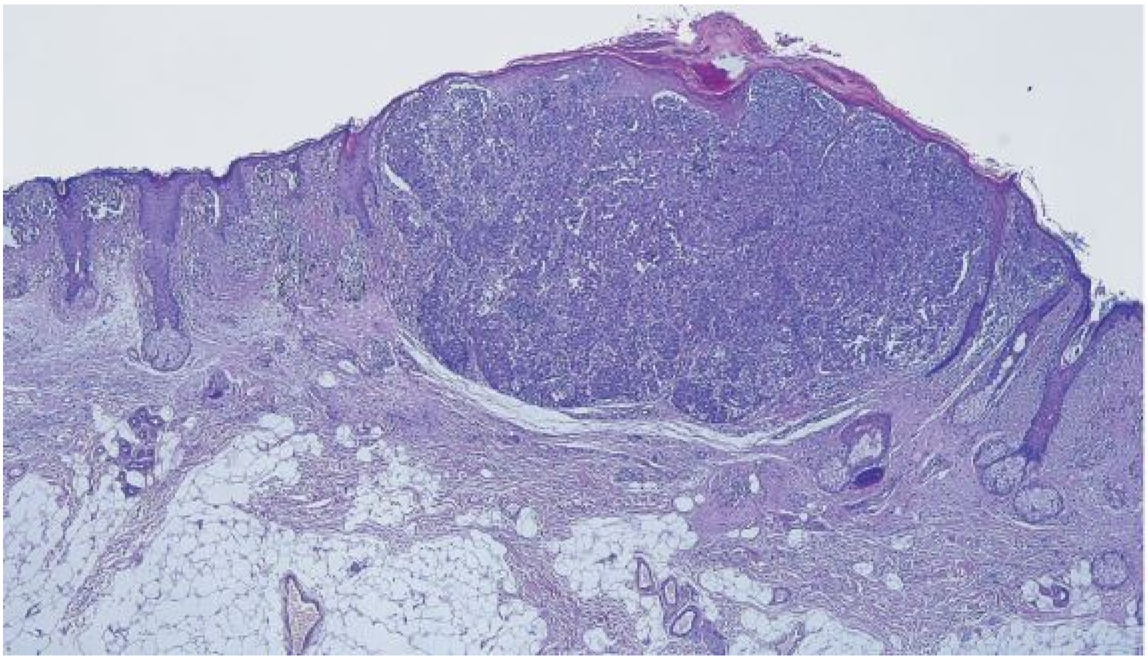

Figure 5. Lentigo maligna melanoma. A prominent vertical growth phase (VGP) can be seen, forming a tumour nodule. Adapted from Bobos, 2021

1.2 Chemokines

Chemokines are a subfamily of cytokines and small proteins, which play an essential role in stimulating cell migration - most notable leucocytes by signalling through G-protein-coupled chemokine receptors (CKRs). Cells that carry those receptors migrate to locations of interest, attracted by high chemokine concentration gradients (Morein et al., 2020). However, their activity is not limited to this function, as chemokines are also involved in cell proliferation, survival and differentiation. Consequently, chemokines are involved in all protective and destructive inflammatory responses (Hughes and Nibbs, 2018). Depending on the motif displayed by the arrangement of their structural important cysteins they can be divided into four subfamilies: CC, CXC, CX_3C and XC. In CC chemokines, two important cysteins are adjacent to each other, whereas in the CXC subfamily those cysteins are seperated by an amino acid. CX_3C chemokines are characterised by the placement of three amino acids in between those structural cysteins. In the XC class, the first and third cysteins are missing (Miller and Mayo, 2017). In matters of nomenclature the subfamily (CC, CXC, CX3C and XC) is followed by the letter “L“, denoting “ligand“ and a number according the time of the gene’s first isolation (Zlotnik and Yoshie, 2000).

Table 2. Chemokines in humans (adapted from Zlotnik, 2000)

Systematic nomenclature	Aliases
CCL1	I-309
CCL2	MCP-1
CCL3	MIP-1α
CCL4	MIP-1β
CCL5	RANTES
CCL7	MCP-3γ
CCL8	MCP-2
CCL11	Eotaxin
CCL13	MCP-4
CCL14	HCC-1
CCL15	HCC-2

Systematic nomenclature	Aliases
CCL16	HCC-4
CCL17	TARC
CCL18	PARC
CCL19	ELC
CCL20	LARC
CCL21	SLC
CCL22	MDC
CCL23	MPIF-1
CCL24	Eotaxin-2
CCL25	TECK
CCL26	Eotaxin-3
CCL27	CTACK
CCL28	MEC
CXCL1	GROα
CXCL2	GROβ
CXCL3	GROγ
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
XCL1	Lymphotactin alpha
XCL2	Lymphotactin beta
CX3CL1	Fractalkine

1.3 Chemokine Receptors

In general there are two types of surface molecules that chemokines can bind to: conventional chemokine receptors (cCKRs) and atypical chemokine receptors (ACKRs). cCKRs transduce signals through G-proteins and can induce cell migration or adhesion if stimulated. Nowadays 18 cCKRs are described in humans and named based on the predominant type of ligand they bind – the four subfamilies of chemokines, respectively. That is followed by the letter “R“, denoting “receptor“ and a number based on when it was first discovered (Hughes and Nibbs, 2018). So CC chemokines bind to CCR1 – CCR10, CXC chemokines bind to CXCR1 – CXCR6, XC chemokines bind to XCR1 and CX_3C chemokines bind to CX_3CR1, respectively (Murphy et al., 2000). Most of these receptors are not limited in one sole chemokine to recognise and bind, but in one subfamily. ACKRs can also be splitted into four subgroups (ACKR1 – ACKR4) and act as decoy receptors without signalling and have a nonredundant role in controlling inflammation. However, ACKRs are unable of activatiting cell migration. Both cCKRs and ACKRs have shown specific expression patterns especially on leucocytes as well as on different celltypes like endothelial cells or mutated cancer cells (Bachelerie et al., 2014).

Table 3. Chemokine receptors in humans (adapted from Zlotnik, 2000)

Systematic nomenclature	Aliases	Chemokine ligand
Conventional chemokine receptors		
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,

Systematic nomenclature	Aliases	Chemokine ligand
		CCL7, CCL14, CCL16
CCR6	-	CCL20
CCR7	EBI-1, BLR-2	CCL19, CCL21
CCR8	-	CCL1, CCL18
CCR9	-	CCL25
CCR10	-	CCL27, CCL28
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
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 Chemokine and Chemokine Receptor Network

Chemokines can be seperated into four subgroups, depending on the arrangement of their structural important cysteins. Furthermore they can be devided into two functional groups, based on the effect they are causing after binding to a chemokine receptor (Miller and Mayo, 2017). One group contains chemokines involved in keeping up homeostasis in the cellular immunsystem, meaning leucocyte migration into secondary lymphatic organs or lymphatic tissue developement without the presence of any inflammatory trigger. The other main group comprises to chemokines being involved in inflammatory processes, guiding leucocytes in damaged tissue. Based on the secreted chemokine pattern, different leucocytes as granulocytes, monocytes or eosinophils are attracted and migrate to their supposed location. For example, the signalling of both CXCR4 and CCR2 would only cause the homeostatic release of monocytes, but not granulocytes. That process of cytokine induced cell migration is also called chemotaxis (Griffith et al., 2014, Balkwill, 2004a). In addition, CKRs are not only present in leucocytes. They are involved in a wide spectrum of highly differentiated cells, i.e. epithelial cells, mesenchymal cells, neuronal cells as well as astrocytes. Chemokine related processes in those celltypes ranging from cell to cell adhesion, angiogenesis, embryonic developement to the production of even more cytokines (Gupta et al., 1998, Hughes and Nibbs, 2018).

1.5 Chemokine Network and Cancer

Malignant solid tumours do not exclusively consist of cancer cells, but rather a complex system of different celltypes, including many non malignant stromal cells and tumour associated leucocytes infiltrating the enviroment (Balkwill and Mantovani, 2001). As a result of that cellular tumour enviroment, tumour cells often show a complex chemokine and chemokine receptor network that influences the leucocyte infiltrate’s composition in terms of quantity and cellular variaty. Depending on that chemokine expression pattern a cellular microenviromental infiltrate can be predominantly consist of macrophages, lymphocytes, granulocytes or even fibroblasts and endothelial cells, as they also tend to express CKRs on their surface (Balkwill, 2004b). All these different cells with their complex chemokine and receptor network have distinct effects on cancer behaviour, i.e. tumour progression, angiogenesis, metastatic power and consequently the patients therapeutical outcome (Nagarsheth et al., 2017). Some chemokines are strongly and causatively linked to elevate pro-cancerious processes, while others are associated with anti-tumour activities (Morein et al., 2020).

1.5.1 Effects of Chemokines on Malignant Cells

Chemokines are mostly known for their guiding and attrackting leucocytes to tumorous microenviroment, where immune cells have a direkt impact on malignant cells. Because of CKRs presence on cancer cells themselves chemokines also interact with those cells directly. CCL2, CCL3 or CCL5 are capable of promoting tumour progression by inducing the secretion of matrix metalloproteinase 9 (Robinson et al., 2002). This collagenase is associated with angiogenesis and remodeling the extracellular matrix in malignant tumours (Nagarsheth et al., 2017). CCR9, the receptor for CCL25, is also a highly expressed and a well known receptor in many cancer types and responsible for an increased chemoresistance (Johnson et al., 2010). Moreover, one chemokine receptor is expressed on a majority of cancer types, independently of their tissue origin. CXCR4 can be found in more than 20 malignant entities, including cancers of epithelial, mesenchymal and haematopoetic origin. Those cancer cells also show high metastatic power as well as a reduced sensitivity to radiation (Balkwill, 2004b).

1.5.2 Effects of Chemokines on the Microenviroment

Chemokines can induce processes in terms of cancer progression in two directions: pro-cancerious and anti-cancerious. Those processes are mostly activated via leucocytes, which are part of the tumour's microenviroment. That indicates that the microenviroment is highly important in cancer progression (Morein et al., 2020). Lymphocytes as $CD8^+$ T cells, T_H1 cells and natural killer cells have potent anti-cancerious activity like inducing apoptosis and secreting cytotoxic granula. By expressing CXCR3 on their surface, they get recruitet by high tissue concentrations of CXCL9 and CXCL10, which can be found in many cancer tissues. Furthermore, T_H1 cells in an area of interest attract more T_H lymphocytes into the tumour's microenviroment (Morein et al., 2020, Kulbe et al., 2004). Another lymphocytic helper-cell, T_H22, expresses CCR6 and is attrackted by CCL20. That ligand can be found in many different cancer tissues. T_H22 is mostly known for secreting Interleukin 22, which is a pro-cancerious cytokine and leads to the upregulation of some protoonkogenes, resulting in increased cancer stemness (Zhuang et al., 2012).

1.6 Chemokines and Malignant Melanoma

A tumour's behaviour is based on many different factors. One of those is the amount of certain immune cells in its microinvoroment, which is orchestrated especially by chemokines. Although chemokines are mostly known for migrating leucocytes, they also have an influence on processes like angiogenesis, cell proliferation and with that progression of cancer. The same goes for malignant melanoma (Jacquelot et al., 2018).

Studies have shown that different CKRs and their ligands are notably involved in human melanoma. Klein et al. associated high CCR4 expression levels with a higher risk of developing brain metastases. CCR4 and its ligand CCL22 are involved in regulating regulatory T cells and T_H2 cells. Furthermore, cutaneous melanoma which are resistant to Vemurafenib are associated with high expression levels of CCR4. That kinase inhibitor is used in patients with melanoma in palliative care setting (Klein et al., 2017). Upregulated CXCR4 is involved in melanoma processes that can even be seen macroscopically – the presence of ulceration and the general thickness of the melanoma lesion itself is among other things driven by high levels of that CKR (Koroknai et al., 2022, Mendt and Cardier, 2017, Mori et al., 2005). But CXCR4 expression has also shown different results, as Harlin et. al have suggested. Their results have shown that CXCR4 could be linked to a better outcome in patients suffering from melanoma because of it's power to costimulate T cells (Harlin et al., 2009, Mendt and Cardier, 2017). It is also known that this CKR is significally overexpressed in melanoma metastases as well (Monteagudo et al., 2012). Furthermore, the increased expression of CCR2 and its corresponding ligand CCL2 correlates with the invasive potential of malignant melanoma cells. That CCR2-CCL2 axis is mostly known for it's capability of introducing migration and invasion in many different cancers, including melanoma (Korbecki et al., 2020, Koroknai et al., 2022). Studies suggest that CCL19 and CCL21, as well as their CKR CCR7, play a role in the creation of lymph node metastases of primary melanoma. This mechanism is reasoned in the autocrine production and secretion of the ligands by melanoma cells themselves (Shields et al., 2010, Murakami et al., 2004). Moreover, CCR10 and CCL27 are correlated with melanoma’s progression. High expression pattern of CCR10 and its ligand are linked to increase the immune escape potential of tumour cells in malginant melanoma (Jaeger et al., 2007), but they are also downregulated significally in melanoma metastases (Monteagudo et al., 2012). Although it was only carried out in mouse models, Jacquelot et al. could demonstrate that a neutralised CCL25-CCR9 axis stimulated melanoma’s progression significantly (Jacquelot et al., 2016b).

1.7 Chemokines and Brain Metastases

Metastases are the leading cause of cancer mortality in almost all solid tumours. Their formation is based on a complex cascade of events, for example the so called epithelial to mesenchymal transition (EMT). Upon EMT, epithelial cells lose their adherence and polarity to each other and also change morphologically into a mesenchymal phenotype (Pietila et al., 2016). Another general mechanism in metastasing is signalling via CKRs and their specific ligands. CCR7 and its corresponding ligand, CCL21, are known for promoting the survival of metastasising cancer cells and modulating the inflammatory system to block the tumour‘s immune surveillance (Chitty et al., 2018). There is evidence that specific cancers have shown an overexpression of certain CKRs and corresponding ligands, i.e. CXCR4 and CXCL12 in non-small-cell-lung cancer with brain metastases (Chen et al., 2011) or CX3CR1 in breast cancer (Paratore et al., 2011). Furthermore, in cutaneous melanoma an overexpression of CCR4 is associated with an increased developement of brain metastases (Izraely et al., 2010). Lok et. al. were able to detect a generalised elevation of certain chemokine ligands in patients with cutaneous melanoma and present brain metastases, respectively CXCL10, CCL4 and CCL17 (Lok et al., 2014).

In summary, CKRs and chemokines are involved in many important developemental processes of cancer and most malign entities, as they can be found in every inflammatory event within the body. Furthermore, chemokines can be secreted by cancer cells themselves as well as CKRs can be found on malignant cells‘ surfaces. That reflects their importance in tumourgenesis and power of metastasising. Although there is already evidence for the effects of CCR4 and CCL22 in brain metastases of malignant melanoma, research on differences in expression patterns of other CKRs in brain metastases in relation to primary malignant melanoma is still lacking. Aim of this thesis is to find out significant differences in chemokine expression in primary malignant cutaneous melanomas compared to secondary brain metastases of cutaneous melanomas.

2 Material and Methods

2.1 Tissue Samples

Individual tissue samples of cutaneous melanomas as well as secondary brain metastases of cutaneous melanomas dating back to 2007 were obtained from the Medical University of Graz‘s Biobank. All samples have been verified by an experienced pathologist (Dr.^{in} med. univ. Marlene Leoni) in terms of correct diagnosis and availability of tumour tissue via microscopical analysis in hematoxylin eosin (HE) stained samples prior further analysis. Control tissues consisting of tonsil tissue and lymph node tissue were aquired from autopsies carried out at the Diagnostic and Research Institute of Pathology of the Medical University of Graz. All used samples and control tissues were formalin-fixed paraffin-embedded (FFPE) specimen.

In total 17 samples of primary cutaneous melanoma and 41 samples of secondary brain metastases from cutaneous melanomas were obtained from the Biobank Graz. Of all melanoma samples, eight were excluded from this experiment because there was no tumour tissue left on the FFPE blocks (because of prior diagnostic reasons). Furthermore, of all brain metastases samples 16 were excluded because of the same reason. Finally, nine samples of primary cutaneous melanoma and 25 samples of secondary brain metastases from primary cutaneous melanomas were included and analysed. Unfortunately, it was not possible to aquire matched samples from the same patients.

Furthermore, available surrounding non-neoplastic tissue of all samples was used in this experiment. In total surrounding tissue samples were nine for melanoma and six for brain tissue.

FFPE tissues (samples and controls) were cut using a microtome with a disposable blade. Serial sections were done in 10 µm slides, collecting approximately 50 µm in whole for further processing. The first slide was cut in 2 µm for HE staining in terms of ensuring that tumour tissue is still in the FFPE material as well as to distinguish between tumour tissue and surround normal tissue. Furthermore, the last slide was also cut in 2 µm thickness for HE staining, ensuring that all sections in between contained tumour tissue. Samples that contained normal surrounding tissue were macrodissected by hand using a single-use toothpick and a sterile, disposable blade. In the abscence of normal surrounding tissue the whole cut was used for RNA isolation without macrodissecting. All workspaces, the microtome and every used tool were constantly cleaned with Thermo Scientific^{TM} RNase AWAY^{TM} (Thermo Fisher Scientific Inc., Waltham, MA, USA). Moreover, within every new sample new gloves were used.

For this study the required ethical approval was obtained from the local ethics committee (31-144 ex 18/19).

2.2 RNA Isolation

Total RNA isolation was performed using Relia Prep^{TM} FFPE Total RNA Miniprep System (Promega, Madion, WI, UWA) using the manufacturer’s protocol (TM353 Revised 12/15). The isolated RNA was then diluted in 30 µl of nuclease free water. RNA quantity was measured by using the Thermo Scientific^{TM} NanoDrop^{TM} 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the corresponding NanoDrop^{TM} operating software (version 3.8.1). All isolated RNA samples were stored at – 80 °C until further processing.

2.3 cDNA Synthesis

cDNA synthesis was performed using the Thermo Scientific^{TM} RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the manufacturer’s protocol (revision 11). The total reaction volume was 20 µl including 1 µl random hexamer primer. Furthermore, the UNO96 thermocycler (VWR International GmbH, Darmstadt, Germany) was used for cDNA synthesis.

Cycling conditions were 25 °C for 5 minutes, 42 °C for 60 minutes and 70 °C for 5 minutes.

2.4 Real-Time PCR

Due to low isolated RNA quantity in some samples, a two-step approach was used for reverse transcription-quantitative real-time PCR. The first step included general testing of the chemokine receptor expression for all 19 receptors in one melanoma sample, one brain metastasis sample as well as in both control tissues (tonsillary and lymph node tissue, respectively). Melting curve results were evaluated and chemokine receptors with unspecific amplicons were subsequently excludet from further analysis. Those six receptors were CCR1, CCR5, CCR10, CXCR1, CXCR2 and CX_3CR1. All remaining chemokine receptors were tested on five melanoma samples, five brain metastasis samples and on both control tissues again. Samples, in which mRNA transpcripts of the receptors were detected were further tested on the whole sample size.

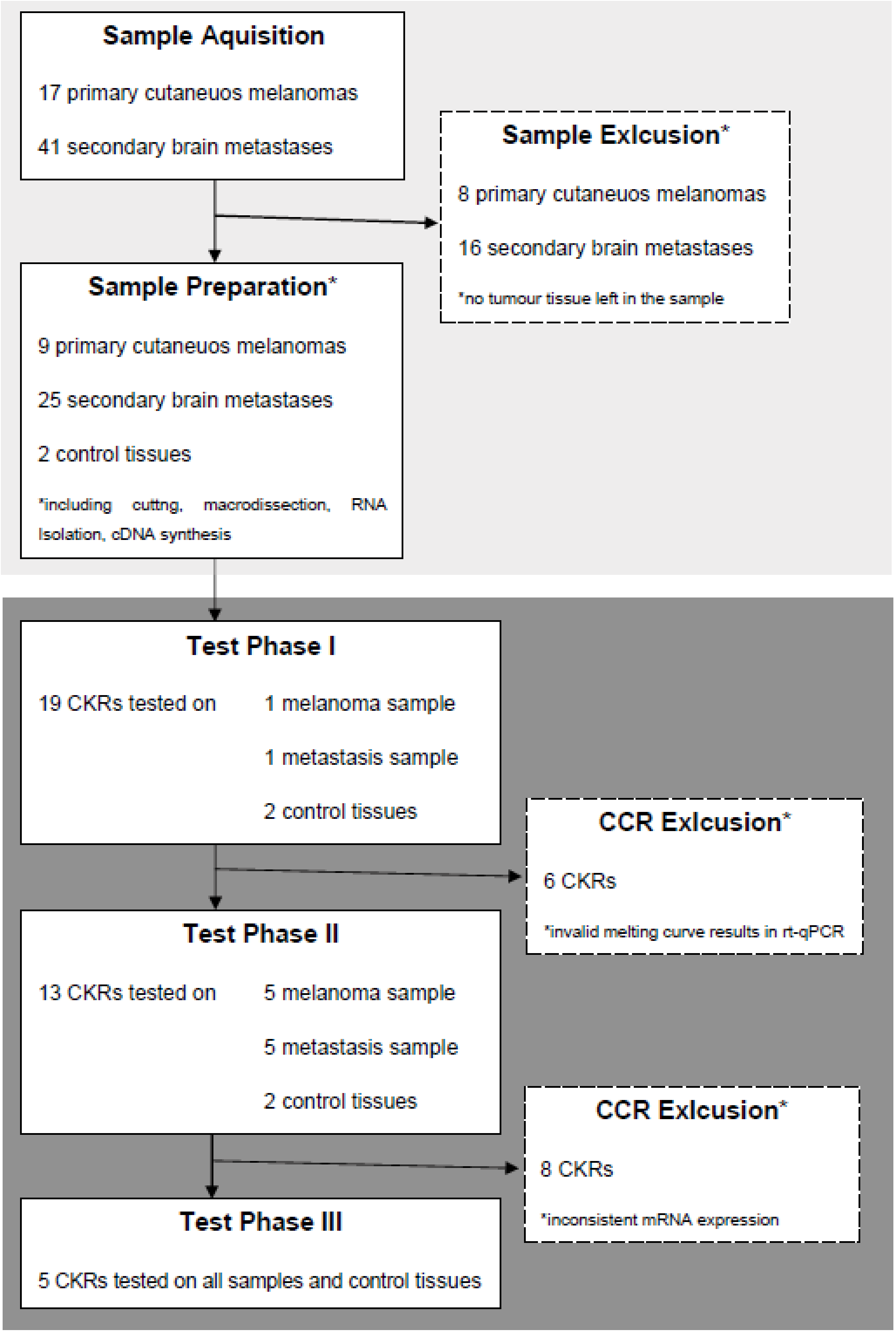

Figure 6. Illustration of sample aquisition, sample preparation and ensuing test phases (I-III). (CKR = chemokine receptor)

Primers were ordered from Ingenetix GmbH in Vienna, Austria – except for CCR1, CCR6, CCR8, CCR10, CXCR4 and CX_3CR1. Those six primers were ordered from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Table 4 shows the chemokine receptor primers and their individual sequence. In terms of preparing the complimentary DNA it was deluted 1:20 with nuclease-free water. The PCR was run using the Luna® Universal qPCR Master Mix M3003 (Applied Biosystems, Foster City, CA, USA) with a modified protocol. The total reaction volume was 10 µl, including 5 µl of Master Mix, 4 µl cDNA and varying concentration of forward and reverse primer. The primers concentration was evaluated prior on tonsil and lymph node tissue. In Table 5 all evaluated and applied concentrations for the reverse and forward primers are shown.

Table 4. Primer sequences used in rt-qPCR

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. Primer's concentration (nM) used within the experiment

Chemokine Receptor	Primer concentration (forward and revers) in nM
CCR1	100
CCR2	250
CCR3	250
CCR4	500
CCR5	125
CCR6	250
CCR7	500
CCR8	125
CCR9	250
CCR10	125
CXCR1	250
CXCR2	250
CXCR3	125
CXCR4	100
CXCR5	500
CXCR6	500
CXCR7	500
CX3CR1	250
XCR1	500
GAPD	500
ACTB	500

Thermocycling conditions were 50 °C for 2 minutes and 95 °C for 10 minutes for polymerase activation, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 30 seconds. Upon RT-qPCR, a melt curve stage, i.e. fluorescence data capturing during gradual temperature increase from 60 °C to 95 °C, was conducted to differentiate specific amplicons from nonspecific products.

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

ACTB and GAPD were used as reference genes to compare the cycle number in relation to the genes of interest. The geometric mean value of Ct values of ACTB and GAPD was used for calculation. Samples with bigger Ct values than 32 in the reference genes were exluded in terms of inaccuracy. As calibrators, FFPE tissues of tonsil and lymph nodes derived from post-mortem examinations were used (processed identically to the samples as mentioned). Likewise, the geometric mean value of Ct values was used for calculation.

2.5 Statistical Analysis

SPSS (Version 27) was used for statistical analysis. To detect differences between chemokine receptor expression levels among melanoma tissue and metastatic tissue the nonparametric Mann-Whitney U test was performed. P values less than 0.05 were considered significant.

3 Results

3.1 Chemokine Receptor Expression Pattern

In whole, 19 CKRs were analysed in three phases (Figure 6). Within the first phase, all CKRs were tested on one melanoma sample, one brain metastasis sample and both control tissues. Because of invalid melting curve results in PCR analysis, six CKRs were excluded from further testing (CCR1, CCR5, CCR10, CXCR1, CXCR2 and CX_3CR1, respectively). The remaining CKRs were further tested on five samples of each entity and both control tissues as well. Inconsistent mRNA expression of eight CKRs led to their exclusion, which resulted in testing five CKRs (CCR4, CCR6, CCR9, CXCR4 and CXCR6) on the whole sample size. Table 6 contains the mRNA expression patterns in all samples, including the surrounding tissues.

Table 6. mRNA expression pattern of analysed chemokine receptors in distinct sample groups

	CCR4	**CCR6**	**CCR9**	**CXCR4**	**CXCR6**
Cutaneous melanoma	9/9	7/9	7/9	9/9	9/9
Brain metastasis	25/25	24/25	24/25	25/25	25/25
Surrounding melanoma tissue	7/9	8/9	5/9	8/9	8/9
Surrounding metastasis tissue	6/6	5/6	6/6	6/6	6/6

3.2 Relative Expression of CC Chemokine Receptors

All tested CC chemokine receptors showed a homogenious decreased expression within the cutaneous melanomas in relation to the control tissues. There were inconsistend results in the expression in brain metastases. Within a wide range, CCR4 and CCR9 were similar highly expressed as in control tissues. The expression of mRNA encoding CCR6 showed high levels in relation to control tissues. Comparing expression levels between primary tumour and metastases, all CC chemokine receptors showed a decreased expression in the primary tumour (CCR4 $p = 0.711$, CCR6 $p = 0.887$, CCR9 $p = 0.925$, respectively). Measurement of expression levels in both surrounding tissues showed slighty lower levels in CCR4 and CCR6, but higher level in CCR9. No results could reach a significant p-value < 0.05.

Comparing the expression patterns of CCR4, CCR6 and CCR9 in melanomas and their surrounding skin tissue, significantly elevated levels of chemokine gene products could be detected in surrounding skin tissues (CCR4 $p = 0.05$, CCR6 $p = 0.011$, CCR9 $p = 0,019$, respectively).

Expression levels of surrounding brain tissue in comparison to brain metastases, the expression of CCR4 and CCR9 showed consistent levels (CCR4 $p = 0.881$, CCR9 $p = 0.917$) and expression level of CCR6 was decreased in surrounding brain tissue ($p = 0.18$).

3.3 Relative Expression of CXC Chemokine Receptors

Both CXC receptors (CXCR4 and CXCR6) showed a decreased expression level in the primary tumour compared to the control tissues. Furthermore, CXCR4 was consistently lower expressed in metastases and both the surrounding tissues in comparison to control tissues. Although it was significantly lower expressed in melanomas than in their surrounding tissue ($p = 0.043$). Similar results were detected in the chemokine receptor CXCR6. That receptor's expression pattern was overall lower in primary melanoma, brain metastases and their corresponding surrounding tissues. Expression levels of mRNA of CXCR6 was also decreased in the primary tumour compared to brain metastases ($p = 0,334$). Both CXC receptors were expressed without any difference in metastases and their surround brain tissues, respectively.

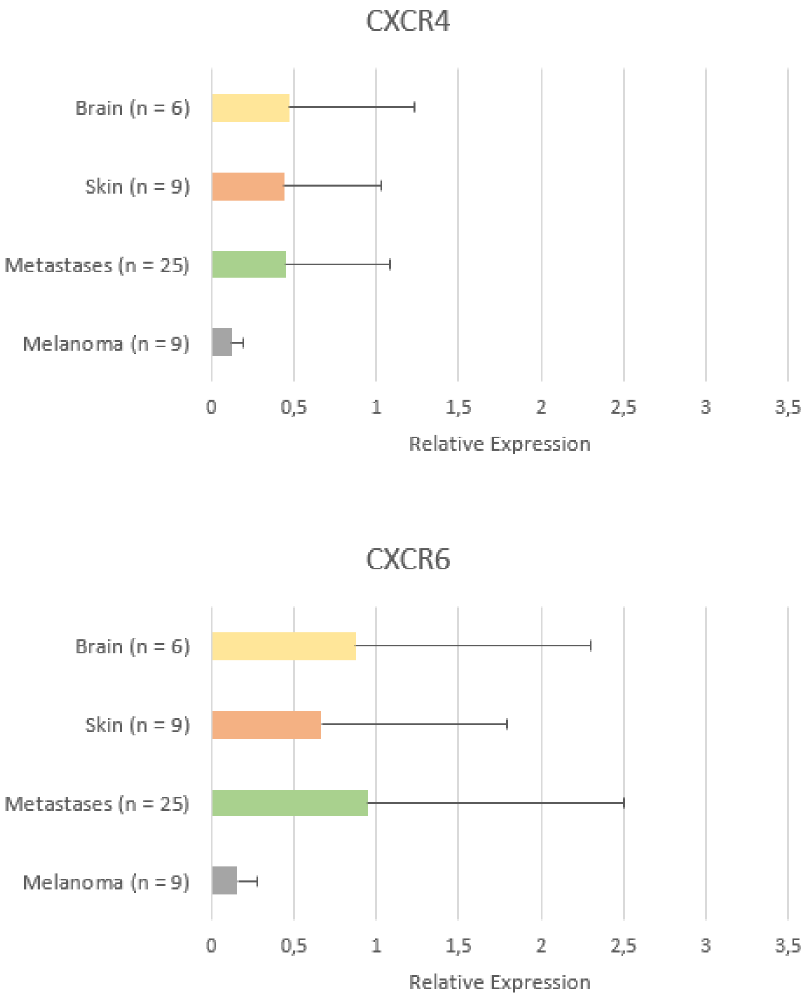

Figure 7. Relative expression of CXCR4 and CXCR6 of analysed samples

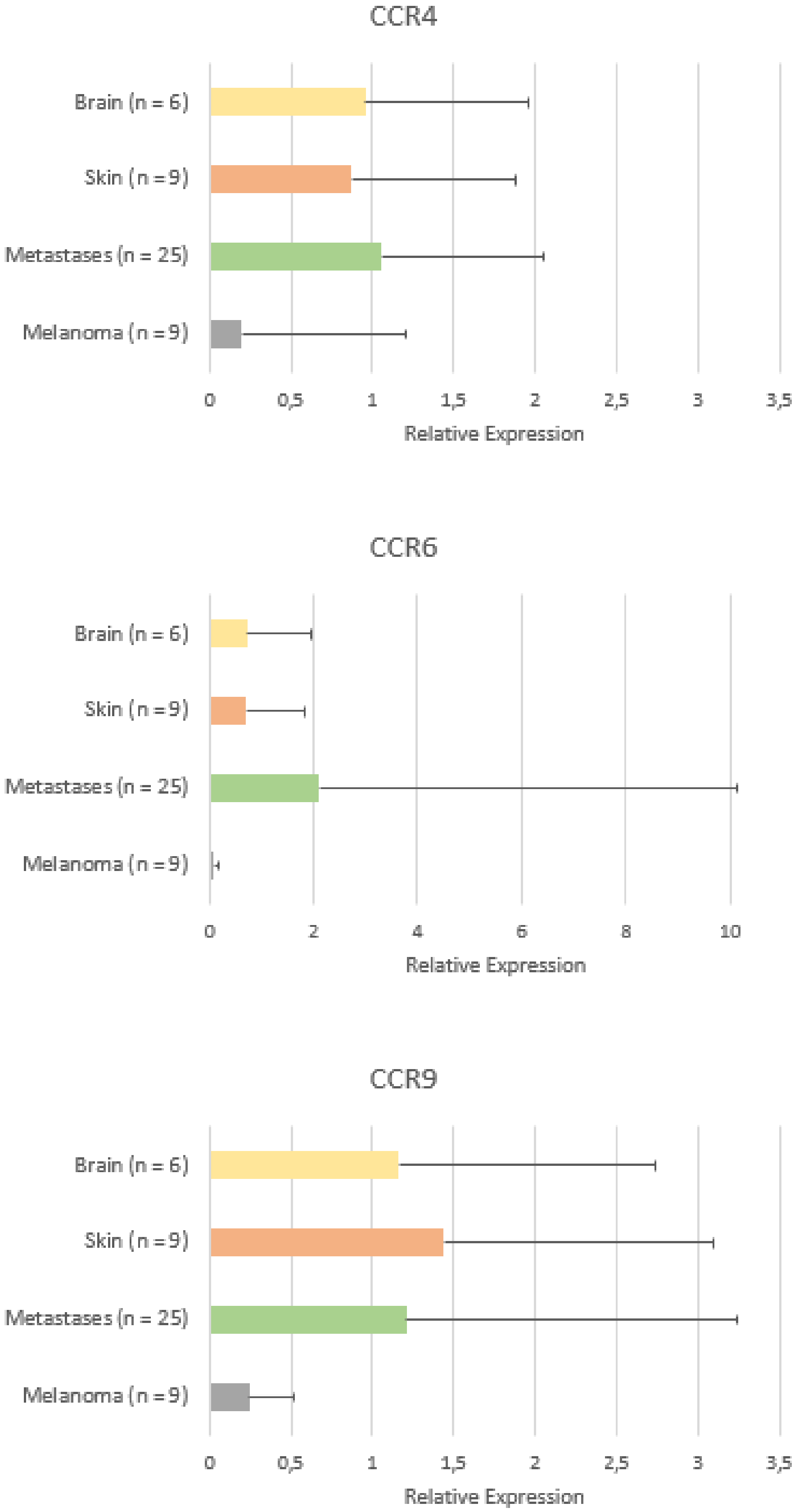

Figure 8. Relative expression of CCR4, CCR6 and CCR9 of analysed samples

4 Discussion

Nowadays there is strong evidence that chemokines and their receptors play an essential role in guiding inflammatory processes (Morein et al., 2020). Due to the fact that malignant diseases are associated with inflammatory reactions in the tumours surrounding tissue as well as in malignant cells themselves, chemokines are an important factor in tumour progression (Kakinuma and Hwang, 2006, Kulbe et al., 2004, Nagarsheth et al., 2017). As a common skin cancer, malignant melanoma‘s progression and ability in metastasising is also subject to that chemokine network (Klein et al., 2017, Weber et al., 2020, Murakami et al., 2004).

This thesis‘ aim was to analyse the chemokine receptor expression pattern in malignant melanomas and their specific brain metastases. Changes in chemokine expression patterns could be potential targets in pharmacotherapeutical issues (Shields et al., 2010).

Klein et al. associated elevated levels of CCR4 in melanoma with a increased resistance to certain therapies using kinase inhibitors. Furthermore, that research group and others as well presented evidence that melanomas with high CCR4 expression levels are more likely in developing brain metastases (Klein et al., 2017, Korbecki et al., 2020). Regarding high expression levels of CCR4, Izraeli et al. were also able to find significant evidence (Izraely et al., 2010). The results regarding the existing evidence of the expression of CCR4 could not be replicated in this thesis. CCR4 showed identical expression levels in brain metastases, normal skin tissue as well as in normal brain tissue compared to the control samples. Contrary to this the expression of this chemokine receptor was highly decreased in primary melanoma samples ($p = 0.711$). This difference could be explained by the brain’s microenviroment, which could be involved in downregulating the expression of that chemokine receptor.

CCR6 is already known to be highly expressed in malignant melanomas, but moreover in circulating tumour cells of that entity. This indicates a higher rate in metastasising of malignant melanomas, as Jacquelot et al. could show in 2018 (Jacquelot et al., 2018). Very low expression levels of CCR6 were observed in the analysed cutaneous melanoma samples compared to the control tissues. Furthermore, increased levels in brain metastases were obtained. That does not support the evidence of prior studies because of the lack of high expression levels in the primary tumour. Nevertheless, that chemokine profile also indicates the presumption of higher rates in metastasising because of increased levels of mRNA transcripts of CCR6 in metastases. The metastases‘ microenviroment could therefore facilitate expression of CCR6.

The presence of CCR9 increases T cell migration and is also known to be found on circulating tumour cells. This phenomenon can also be seen in CCR6 (Jacquelot et al., 2018, Jacquelot et al., 2016a). High expression levels of CCR9 can be found on melanoma cells in fact, but are not associated with patients prognosis (Jacquelot et al., 2018). As well as in the other tested CC chemokine receptors, CCR9 gene product levels are decreased in cutaneous melanoma, but slightly increased in both surrounding tissues and brain metastases. The same constellation was detected in CCR6. Tumours may lose this receptor after metastasising because it is not beneficial anymore in the microenviroment of the brain.

This thesis could also show that the expression of CXCR4 was decreased in primary melanomas as well as in their brain metastases and all surrounding tissues. Interestingly, that chemokine receptor is well known for its high expression levels in malignant melanomas and the developement of metastases in liver and lung (Jacquelot et al., 2018, Mendt and Cardier, 2017, Monteagudo et al., 2012). That could imply, that melanomas with low levels of CXCR4 favour metastasising into the brain instead of lung and liver. This fact could be an interesting starting point for further studies referring tumour progression of malignant melanomas into the central nervous system.

Chemokine receptor CXCR6 expression was decreased slightly in surrounding tissues (brain and skin, respectively), but very low in melanomas and metastases. There is only limited evidence that CXCR6 is involved in the developement of malignant melanomas or their metastases (Adamski et al., 2017, Di Pilato et al., 2021) , which indicates its overall secondary importance. Nevertheless, the decreased expression may imply that it is beneficial for the primary tumour.

An overall decrease of all analysed CKRs was seen in primary cutaneous melanomas. A possible explanation may be that melanomas do not depend on chemokines in terms of survival or progression, which leads to a reduced receptor expression. Otherwise that entity could maybe lose its ability to express CKRs based on occuring mutations as it progresses.

One secondary observation was, that CCR4, CCR6, CCR9 and CXCR4 showed a significantly decreased expression in melanomas compared to their surrounding skin tissues. That could be explained by the high amount of leucocytes within the surrounding tissue as an inflammatory response to the tumour.

The data collected in this thesis is unfortunately limited in a few aspects, which strongly indicates its interpretation along with other studies on that specific topic. One major problem working on melanoma samples is often a high amount of melanin, which is known to hamper RNA isolation as well as PCR reactions. That can lead to imprecise and invalid results (Dorrie et al., 2006). Furthermore, the small sample size is one major criterium regarding the exclusion of specific CKRs. Because of the small sample size and the used testing approach (Figure 6) CKRs were excluded from further processing and testing. Moreover, there was no chance to obtain matched samples from the same patient. Comparing samples of malignant melanomas with brain metastasis samples from the same patient could have lead to more precise and coherent results.

As chemokines and their receptors are involved in tumour developement mechanisms, they are a potential pharmakotherapeutical target in cancer therapies. There is evidence, that CKR profiles are different in various entities and stages of cancer progression. Given that fact, further research on CKR expression patterns in cutaneous melanomas and their specific brain metastases including bigger cohorts could generate interesting results.

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