“Effect of the Tumor Microenvironment Factor S100A4 on Malignant Melanoma Cells”

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MSc Pharmacy project

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ABSTRACT

Malignant melanoma is known as a highly aggressive cancer showing exceptional abilities to metastasize and resist existing therapies. Its incidence worldwide is increasing, having a prognosis of which is hardly to predict. Currently there are no effective treatments against malignant melanoma. Therefore, it is important to increase the understanding on how these aggressive properties of melanoma are regulated. Melanoma is composed of not only the malignant cells, but also the supporting stroma, including fibroblasts, endothelial cells, immune cells, soluble molecules and the extracellular matrix (ECM), which together is called the tumor microenvironment (TME).

Recently evidences implicating TME factors in cancer metastasis and modulation of drug-responses have been compelling. In the present work we investigated the influence of one of the TME factors S100A4 protein on the two melanoma cell lines Melmet 1 and Melmet 5, and how this affects the cell responses to the BRAF inhibitor vemurafenib. This study was based on in vitro cultures of melanoma cells stimulated with S100A4 and subsequently treated with vemurafenib before the cell proliferation and the cell viability were measured by the MTS assay. In addition, the levels of the tumorigenesis- and resistance-associated cytokine IL-8, secreted from melanoma cells in response to S100A4 and/or vemurafenib, was evaluated by the ELISA-method.

It was observed that extracellular S100A4 protein reduced the proliferation of melanoma cells in vitro. The melanoma cell sensitivity to the drug vemurafenib was also reduced in the presence of S100A4 leading to slightly higher cell survival. The S100A4-dependent effect on proliferation and the drug-sensitivity was observed in the proliferative phenotype cells Melmet 5, and not in the invasive phenotype cells Melmet 1. It was also observed that exposure to S100A4 stimulated the melanoma cells to secrete high levels of IL-8. Treatment with vemurafenib reduced the IL-8 secretion from both the non-stimulated controls and the S100A4-stimulated Melmet 1 and Melmet 5.
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1 INTRODUCTION

1.1 Malignant melanoma

Malignant melanoma is considered the most deadly form of skin cancer and one of the most devastating of all human cancers. The incidence and mortality of this disease continue to rise faster every year. According to Cancer Research UK almost 200,000 people worldwide were diagnosed and 46,000 people died of malignant melanoma in 2008 [3]. The Cancer Register in Norway recorded 1,413 new incidences and 296 deaths from malignant melanoma in Norway in 2009 [4]. The prognosis for melanoma patients with metastatic disease is really poor: the median survival time is 6-9 months and the 5-year overall survival rate is less than 20 % [5].

Figure 1: A) In the skin, melanocytes reside in the epidermis basal layer. Melanoma originates here when maliciously transformed melanocytes start to grow uncontrolled and invade the dermis (Illustrator ©2008 Terese Winslow) B) Illustrations of cutaneous melanoma. A bleeding mole or a growth on the skin lesions that changes color and keep changing appearance are signs of melanoma. The diameter of the spot is usually more than six millimeters and has irregular edges. The pictures are adapted from Hoek, 2011.

Melanoma arises from the pigment-producing cells melanocytes, which are predominantly found in the skin and eyes (Figure 1). Melanocytes absorb the ultraviolet radiation (UVR) to produce the dark pigment melanin protecting our skin from UV-damage. However, when the skin cells are exposed to intense UVR from e.g. sunshine and tanning beds, their DNA can be harmed leading to mutations. The danger of mutations increases in the cases of severe sunburns, and can allow the skin cells to multiply rapidly and eventually develop malignant
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tumors [6]. The fair-skinned population has a lack of skin pigmentation and has a higher risk of developing melanoma or other forms of skin cancer compared to the dark-skinned ones. There are many steps before normal melanocytes become metastatic melanoma. In Figure 2, a model for melanoma progression from benign nevus to metastatic melanoma is described.

![Figure 2: A model of melanoma development and progression. Melanomas evolve through several sequential steps. Step 1: common acquired and innate benign nevi with structurally normal melanocytes; step 2: dysplastic nevi with atypical structure and architecture; step 3: radial-growth phase, primary melanomas without metastatic competence; step 4: vertical-growth phase, primary melanomas with competence for metastasis; and step 5: metastatic melanoma. Each step is associated with certain biological events, as indicated at the bottom of the figure. Oncogenic BRAF mutations (see below) are found at various stages of melanoma, including very early stages like benign nevi [7, 8]. The picture is adapted from Miller and Mihm, 2006.](image)

Often melanoma progression is associated with the mutation in a gene called BRAF, encoding the proto-oncogene B-Raf, a member of the Raf kinase family of serine/threonine-specific protein kinases. This mutation is acquired relatively early, at the stage of benign nevus, and is found in approximately 50% of patients with malignant melanoma. B-Raf plays a role in regulating the mitogen-activated protein kinase (MAPK) signaling pathway (Figure 3) which transfers a signal from a receptor on the cell surface to the cell nucleus and modulates different gene expressions. This results in stimulation of cell proliferation and survival. Mutated BRAF assures constant activation of MAPK pathway, causing a cascade of
which signalizes the cancer cells to keep on proliferating. Therefore, inhibition of mutated B-Raf is a very important strategy to fight malignant melanoma [8].

Discovering melanoma in an early stage before it spreads to distant organs is important because it can be cured effectively by surgical resection. Even so, metastases of melanoma affect the skin, lymph nodes, lungs, brain and more, which all can be asymptomatic for years and difficult to discover. Once the disease has progressed to its late i.e. metastatic stage, it is incredibly difficult to treat. High metastatic potential and notoriously high resistance to therapy are two main hallmarks of malignant melanoma. Melanoma cells have also shown a lower capacity to undergo spontaneous apoptosis \textit{in vivo} compared to other tumor cell types. Furthermore, melanoma cells show high resistance to drug-inducible apoptosis \textit{in vitro}. Since most chemotherapeutic drugs work by inducing apoptosis in malignant cells, it is likely that resistance to apoptosis is one of the main reasons why melanoma is refractory to therapy [9].

\subsection{1.2 Therapy against malignant melanoma}

Until 2011, dacarbazine (\textit{DTIC}) and high dose interleukin 2 (\textit{HD IL-2}) were the only two single-agents approved by the US Food and Drug Administration (\textit{FDA}) for the treatment of malignant melanoma. However, these drugs did not show any improvement for median overall survival [10]. DTIC is an antineoplastic agent used in chemotherapy and was
approved in 1970 on the basis of overall response rate (10-20 %). It is believed that DTIC induces apoptosis of the malignant cells by acting like a purine analog and attaching an alkyl group to DNA, which affect the cell cycle progression by i.e. arresting in S and G2/M [11, 12]. However, DTIC is limited due to a low overall response rate and only eight months overall survival [10]. HD IL-2 was approved in 1998 based on complete response rate in a minority of patients (0-8 %) [10]. IL-2 is a cytokine produced endogenously by activated T cells and has both, immune-modulating and antitumor properties [13]. As a highly toxic agent it is associated with severe adverse effects like hypotension, supraventricular tachycardia and in the worst case, death. The effect of HD IL-2 is limited due to its toxicity and a low overall response rate (15 %) [14]. Various experimental drugs like the multi-target tyrosine kinase inhibitor Sorafenib® and various other MEK inhibitors, have been tested in clinical trials against melanoma without leading to big success. Generally there has not been much progress in melanoma therapy until recently. Increased understandings of melanoma biology, molecular characteristics and tumor immunology have led to the development of two novel promising drugs: the immune stimulatory agent ipilimumab (an antibody against the cytotoxic T-lymphocyte antigen 4, CTLA4) and the specific BRAFV600E inhibitor vemurafenib. The progression-free survival and overall survival associated with these drugs led to their FDA approval in 2011. Even though these agents are encouraging, these therapies also have their limitations. Vemurafenib has a relatively short duration of response (6-8 months), while ipilimumab has a low response rate (10-15 %) [10].

1.3 Vemurafenib and its mechanism of action

As mentioned before, the most common mutation found in cutaneous melanoma is in the gene encoding for serine/threonine kinase B-Raf. Substitution of glutamic acid for valine at codon 600 (i.e. V600E) accounts for about 85-90 % of all BRAF mutations in melanoma [15]. Mutated BRAF leads to constant stimulation of the MAPK pathway that is responsible for upregulation of a number of genes that stimulates cellular proliferation and survival (Figure 3). Therefore, it is “dangerous” to have constitutively active MAPK. The high incidence of mutant BRAF and its oncogenic potential encouraged the development of the selective BRAFV600E inhibitor vemurafenib (Figure 4).
Vemurafenib (V600E mutated B-Raf inhibitor) is made in tablets for peroral administration, developed by Plexxikon (now a part of Daiichi Sankyo group) and Hoffmann-La Roche for the treatment of late-stage melanoma. Six months after FDA approval, Health Canada and the European Commission approved this drug as a monotherapy for adult patients carrying the BRAF\textsuperscript{V600E} mutation with no way of resection and/or metastatic melanoma [1]. Vemurafenib is a small molecular kinase inhibitor that suppresses MAPK signaling by binding and blocking BRAF\textsuperscript{V600E} selectively, leading to suppression of uncontrolled cell proliferation as shown in Figure 5 [16]. Vemurafenib should not be used against the non-mutated BRAF (i.e. wild-type) which is found in half of all melanomas [17].

Figure 4: Molecular structure of vemurafenib, also known as Zelboraf, PLX4032, RG7204 or RO5185426. Its chemical name is propane-1-sulfonic acid [3-[5-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridine-3-carbonyl]-2,4-difluoro-phenyl]-amide and has the molecule formula $C_{23}H_{13}ClF_2N_2O_3S$ and a molecule weight of 489.9 g/mol.

![Figure 4: Molecular structure of vemurafenib](image)

Figure 5: Vemurafenib and its mechanism of action. In the absence of vemurafenib, the MAPK signaling cascade transfers signals from mitogen-activated receptor tyrosine kinases (RTK), through the proteins RAS, B-Raf and MEK, to ERK leading to its phosphorylation i.e. activation of ERK, which further leads to modulation of gene expression that promotes cell proliferation and survival. Vemurafenib binds to mutated BRAF\textsuperscript{V600E} making BRAF inactive and therefore blocking ERK phosphorylation. As a result, downstream of signals and processes like abnormal cell proliferation are suppressed. The picture is adapted and modified from Medscape Education, 2011 [1, 2].

![Figure 5: Vemurafenib and its mechanism of action](image)
Introduction

In a phase 1 clinical study, vemurafenib showed a significant tumor regression in 81 % of the BRAF<sup>V600E</sup> metastatic melanoma patients. This led to a phase 2 follow up study (BRIM II) where treated patients in the phase 1 study demonstrated a 53 % response rate with a 6.8 months median duration of response. Phase 3 study was a randomized control trial (BRIM III) of previously untreated patients, where a comparison of vemurafenib to dacarbazine chemotherapy showed improvements in response rate (48 vs. 5 %), progression free survival (5.3 vs. 1.6 months), percentage of patients alive after six months (84 vs. 64 %) and a 75 % reduction in risk of dying [10]. Thus, although vemurafenib has shown to induce strong response in the patients, the effect has been short-lived leading to relapse in majority of the patients after 6-8 months, signifying the need for understanding how the vemurafenib-response is modulated [16].

1.4 Metastasis

Melanoma is one of the most metastatic human cancers. Metastasis is a spread of cancer from a place in the body where it arises (a primary tumor) to another place, the distant organs. This spreading process and a tumor that is formed by metastatic cells are both called a metastasis. The process of cancer metastasis consists of a long series of steps as shown in Figure 6. It starts with an in situ cancer progressively proliferating on an intact basement membrane. Destruction or disruption in cell adhesion molecules accompanies the invasiveness and the metastatic behavior of malignant cells. Cancer cells can then easier invade and move through the walls of nearby lymph vessels or blood vessels and circulate through the lymphatic system to, or directly to the bloodstream. Cancer cells that survive in the circulation will eventually be arrested in the capillary beds of a distant organ. Adherence and extravasation follow next, where the cancer cells invade the walls of the capillary and migrate into the surrounding tissue. Establishment of a cancer-supporting microenvironment at the distant site will further lead to proliferation of cancer cells forming small tumors called micrometastases. These micrometastases can stimulate growth of new blood vessels, known as angiogenesis, to obtain a blood supply with oxygen and nutrition necessary for following tumor growth and macrometastases. Not all cancer types need all these steps to metastasize, but they are general for most cancer forms [18, 19].
The most common sites of cancer metastasis are the lungs, brain, bones, and liver. Although most cancers have the ability to spread to many different parts of the body, often they tend to metastasize to some sites more often than to others. To explain this, in 1889 Stephen Paget introduced a theory known as the "seed and soil" hypothesis, where he proposed that metastasis depends on interaction between selected cancer cells (the “seeds”) and certain organ microenvironment (the “soil”). Since cancer cells have difficulty to survive outside their original area, they need a microenvironment that facilitates their survival and growth to establish metastases. This indicates that the microenvironment factors play an important role in the fate and behavior of cancer cells at the metastatic sites [21, 22].

### 1.5 “Phenotype switching” model

One of the recent hypothesis that attempts to explain how melanoma cells fulfill the metastasis cascade is a “phenotype switching” model proposed by Hoek et al. [23]. It suggests that during metastasis melanoma cells can switch back-and-forth between two different phenotypic states: the “proliferative phenotype” and the “invasive phenotype” (Figure 7). The cells that are in “invasive” state are more aggressive and show higher drug-resistant. Interestingly, Zipser et al. has shown that “invasive” cells are also less susceptible to MAPK pathway inhibition than the “proliferative” cells [24]. If the “phenotype switching” model proves to be true, it will open for new clinical aspects of melanomas. The phenotype
switching model may explain why treating patients with metastatic melanoma often give mixed-responses i.e. due to the presence of both, the proliferative and the invasive signature cells, where some cells respond stronger than the others. For example, chemotherapeutic drugs target fast proliferating cells, while invasive cells might remain unaffected. Interestingly, it has been proposed that phenotype switching is regulated by the signals from the microenvironment. However, specific microenvironment-factors that induce or stimulate such switching have not been identified [23].

**Figure 7:** Models of “phenotype switching” during melanoma metastasis. A) After transformation, melanoma cells initially have the “proliferative” phenotype which contributes to the formation of the primary tumor. Later, some melanoma cells are “pushed” to switch their phenotype to the “invasive” signature. The invasive phenotype cells can escape the primary lesion and lodge elsewhere in the body where they switch their phenotype back to the proliferative signature and starts to multiply, forming metastases. Each switch in phenotypic state is accompanied by the change in expression of a set of genes characteristic to proliferative and invasive signature (B). (Adapted from Hock et al., 2008) [23].

### 1.6 Tumor microenvironment and its role in cancer progression

Melanoma cells do not exist in isolation, but are rather surrounded by and interact with different factors from the tumor microenvironment (TME; **Figure 8**). The TME generally consists of extracellular matrix (ECM) and (non-malignant) stroma cells including fibroblasts, vascular endothelial cells, immune cells, inflammatory cells and various soluble factors like cytokines and growth factors. In melanoma cells numbers of mutations and/or deregulated expression of B-Raf, among others have been recognized, but in addition to genetic abnormalities, it has been shown that interactions between tumor cells and surrounding stromal environment are significant in facilitating tumor growth and metastasis [25].
These interactions occur via “cell-cell” or “cell-extracellular matrix” communications and usually involve soluble factors as mediators of these interactions. As illustrated in Figure 8, there are a number of mechanisms how TME factors can assist tumor cells leading to cancer progression. For example, fibroblasts can potentiate cancer cell growth and stimulate invasion, whereas inflammatory cells like macrophages can enhance survival and thereby contributing to drug-resistance. The fact that TME plays a role in cancer progression stimulated the interest in the involved TME factors and how they influence cancer cells. The present MSc project focuses on one of such factors, a pro-metastatic protein called S100A4.

1.7 S100A4 protein

In recent years, a number of S100-family proteins have been found to act as soluble factors mediating tumor-stroma interactions and facilitating metastasis [27]. These proteins are S100 superfamily calcium-binding proteins and have about 25 members. The first S100 proteins
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were discovered in 1965 and got the name based on the findings that they are soluble in 100% ammonium sulphate at neutral pH. Members of this family of low molecular weight acidic proteins (10-12 kDa) have no enzymatic activity, but act by interacting with and stimulating other “effector proteins” [28]. In this way, S100 proteins influence various processes like inflammatory responses, cell motility, growth, differentiation, cell cycle progression, transcription and secretion [29]. One family member, S100A4, is a well-known metastasis-associated protein found not only in cancer cells, but also in various stroma cells including fibroblasts, macrophages and granulocytes. S100A4 is as well observed as a secreted soluble factor in the extracellular space, suggesting that it might be an important player in the tumor microenvironment.

S100A4 protein is encoded by the S100A4 gene (also known as mts1, p9Ka, FSP1, CAPL, calvasculin, pEL98, metastasin, 18A2, and 42A). A number of studies have shown an upregulated expression of S100A4 in various cancers and that S100A4 correlates with bad prognosis in cancer patients [28]. Emerging preclinical and clinical evidences indicate that S100A4 has biological functions associated with cell invasion, motility, and angiogenesis, and take part in regulation of cell death of which all, but the latter, are involved in the metastasis process (Figure 9) [30, 31].

![Figure 9](image_url)  
*Figure 9:* A summary of S100A4s biological functions that are connected with cancer metastasis. (Adapted from Boye and Maelandsmo, 2010 [32])

S100A4 has been found to be localized in the cell nucleus, cytoplasm, and also in the extracellular space. Like other S100 proteins S100A4 has the ability to interact with various “effector proteins,” both intracellularly and extracellularly (Figure 10) [31]. The intracellular
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S100A4 protein and its mechanism of action have mostly been central. However, the focus of this MSc thesis is the extracellular S100A4. There has been suggested that this protein has to be in a certain oligomerization form, and not in a dimeric form, for the optimal activity as an extracellular factor [33]. The mechanism on how S100 family members are secreted to the extracellular space is still blurred [34]. In vitro studies have shown release of S100A4 from tumor cells, macrophages, and fibroblasts in culture [35, 36-38]. In vivo, secreted S100A4 has been detected in tumor interstitial fluid, and there has also been found an increased level of S100A4 in the serum of aging S100A4 transgenic mice [35].

![Figure 10: Binding partners and mechanisms of action of S100A4: A) intracellularly and B) extracellularly. (Adapted from Boye and Maelandsmo, 2010) [32].](image)

Furthermore, it has been shown that metastatic microenvironment has a higher level of S100A4-expressing stroma cells than the primary tumor [39]. How extracellular S100A4 influences the properties of the tumors cells is not fully elucidated. It has been shown in vitro that recombinant S100A4 protein, when added to non-melanoma cancer cells in culture, stimulated their motility, production of matrix metalloproteases (MMPs), activation of NF-κB, and MAPK signaling pathways, and secretion of metastasis-associated soluble factors like osteopontin [40, 32, 41]. It has been suggested that these biological effects of extracellular S100A4 come from induction through the cell surface receptor for advanced glycation end-products (RAGE), which is familiar for other S100 proteins. However, the RAGE-mediated
S100A4 signaling is controversially discussed when S100A4 signaling can also occur independent of RAGE [42].

The matrix metalloproteases family (MMP family) is proteases that play an important role in tissue-remodeling and have shown capacity to degrade extracellular matrix components, which allow tumor cells to leave the primary tumor and enter the circulation [43]. A study where S100A4 proteins were downregulated in osteosacroma cells, showed a reduction of MMP-2 and MT1-MMP and also a lower capacity for the cells to migrate through Matrigel-coated filters [44].

S100A4 has also been shown to stimulate the invasive abilities in other human cancer cells than melanoma, like prostate cancer cells (MMP-9 was partly transcriptional activated in the cells), and like in colorectal cancer cells (effect of S100A4 on MMP was not investigated). [32]. Another investigation observed a correlation between S100A4 and angiogenesis in transgenic mice. S100A4 seemed to stimulate capillary-like growth in vitro when augmenting MMP-13 in endothelial cells, most likely through induction of NF-κB signaling [45]. Genes that are regulated by NF-κB are genes that control apoptosis, cell adhesion, proliferation, inflammation, and more [46].

As shown in Figure 10B, interaction between extracellular S100A4 and the annexin II receptor (A II) stimulates the cleavage of plasminogen to plasmin, which causes formation of capillary-like tube of endothelial cells. This S100A4-stimulated conversion of plasminogen also seems to activate MMP-2 and MMP-13, but seems unlikely to cause increased transcription of several MMPs. Even though there are good documentations about association of S100A4 and the regulation of MMPs, the mechanisms for this are not fully understood [32].

Furthermore, it has been shown that recruited S100A4-positive stroma cells stimulated survival and resistance of epithelial cancer cells [47]. The studies performed by others in our group at the Norwegian Radium Hospital revealed the influence of extracellular S100A4 protein also on melanoma cells, where an effect on cell motility and secretion of the soluble factor IL-8 has been demonstrated (chapter 1.8).
1.8 Interleukin-8 (IL-8)

IL-8 (also known as CXCL8) is a pro-inflammatory CXC chemokine, originally identified as a leukocyte and neutrophil chemoattractant. Interestingly, IL-8 is involved in melanoma progression and malignant melanoma patients often have elevated levels of IL-8. Tumor-associated IL-8 production has shown to correlate positively with tumor growth and metastatic abilities in i.e. melanoma cells (where their correlation first was discovered) [48]. It has also been confirmed in vivo using animal models, that downregulation of IL-8 levels in melanoma cells reduced metastatic potential of the cancer cells [49]. IL-8 can stimulate a large number of intracellular signaling pathways by binding to its receptors CXCR1 and CXCR2, as illustrated in Figure 11. Secretions of IL-8 have been found in cancer cells, endothelial cells, infiltrating neutrophils, and macrophages, suggesting an IL-8 role as an important regulatory factor within the TME.

**Figure 11**: A schematic diagram illustrating various IL-8 signaling pathways, including the MAPK-ERK which can be activated when IL-8 binds to its receptors CXCR1 and/or CXCR2. Majority of the activated pathways are linked to cell survival, proliferation, and invasion, suggesting that IL-8 might affect these processes. (Adapted from Waugh and Wilson, 2008) [50].
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IL-8 plays many roles in cancer biology, like controlling neutrophil recruitment and activation, stimulation of angiogenesis, stimulation of macrophages-derived growth factor release thus promoting cancer cell growth and invasion and enhancing survival of the cancer cells (Figure 12) [51-53].

Importantly, elevated levels of IL-8 have also been reported to be associated with drug-resistance. Thus, it has been shown that the chemotherapeutic agent DTIC stimulates the expression of IL-8 and the receptors CXCR1/CXCR2 in melanoma cells in vitro, and thereby can initiate autocrine survival signals in therapy-resistant tumor cells [54, 48]. Further, in a study in mice where IL-8 was “knocked out”, melanoma cells were more sensitive for DTIC [55], supporting the role of IL-8 in drug-resistance in melanoma cells.

Figure 12: Illustrative summary of the role of IL-8 in tumor microenvironment facilitating cancer aggressiveness. (Adapted from Waugh and Wilson, 2008) [50].
1.9 Aims of the study

1.9.1 Data produced in the group that motivated this MSc project

Previously, the PhD student Ingrid Bettum showed that addition of S100A4 protein to melanoma cells in culture in vitro resulted in:

I. enhanced motility (invasion and migration) of the melanoma cells. This effect was only observed in the proliferative cell lines like Melmet 5 (having low intrinsic invasiveness) (Figure 13A), and not in the invasive cell line Melmet 1 (having low intrinsic proliferation) (Figure 13B).

II. modulation of expression of some genes. For example, it was observed reduced expression of “proliferative genes” such as the microphthalmia-associated transcription factor (MITF) and protein melan-A (MLANA) (differentiation genes involved in the synthesis of the pigment melanin; Figure 13C).

![Figure 13: Effect of extracellular S100A4 on melanoma cell migration, invasion, and expression of “proliferative genes” (Bettum et al., manuscript in preparation). A) No difference on migration or invasion of Melmet 1 cells stimulated with 2μg/ml S100A4 (n=1). B) Enhanced migration and invasion of S100A4-stimulated Melmet 5 cells (n=2). C) S100A4-dependent reduced expression of MLANA and MITF genes in Melmet 5 associated with proliferative phenotype (n=2).](image)

Based on these results, it was hypothesized:

**Hypothesis 1**: Extracellular S100A4 contributes to “phenotype switching” where proliferative cells (Melmet 5) weaken their proliferative state and gain an “invasive state”. It can be speculated that such switching might influence drug-responses i.e. enhance drug-resistance. One of the aims in this MSc project was to address this possibility.
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Further, Ingrid Bettum has shown that under the influence of added S100A4 (extracellularly), melanoma cells started to secrete various soluble molecules, particularly the cytokine IL-8 \textit{(Figure 14)}. Knowing the role of IL-8 in cancer progression and drug-resistance as well as IL-8 association with the MAPK-ERK pathway, it was hypothesized:

\textbf{Hypothesis II:} Effect of vemurafenib on the MAPK pathway can modulate the IL-8 levels and S100A4-induced secretion of IL-8. Therefore it would be interesting to investigate an association between the response of vemurafenib and the IL-8 secretion with or without addition of S100A4 to melanoma cells

![IL-8 secretion](image)

\textit{Figure 14:} Increased secretion of IL-8 upon stimulation with S100A4 in different melanoma cell lines: Melmet 1, Melmet 5, Wm239, Wm983 and A375 \textit{(n=1)}.

\textbf{1.9.2 Main topics in this MSc project}

This MSc project is a part of a bigger project currently performed in Gunhild M. Maelandsmos group at the Department of Tumor Biology at the Radium Hospital, where the overall aim is to understand the influence of extracellular S100A4 on tumor cells and how this is related to their metastatic properties and drug-responses. The overall focus in this MSc project is to investigate these effects in two melanoma cell lines, Melmet 1 and Melmet 5, treated with S100A4 protein and/or the drug vemurafenib. The following specific aims are:

\textbf{I.} To explore how stimulation with S100A4 influences melanoma cell proliferation and their sensitivity to vemurafenib.

\textbf{II.} To study how IL-8 secretion is affected by melanoma cells stimulated with S100A4 alone and in combination with vemurafenib.
2 MATERIALS AND METHODS

2.1 Cell lines / General cell work

2.1.1 Melanoma cell lines: Melmet 1 and Melmet 5

Two different melanoma cell lines, named Melmet 1 and Melmet 5, were used in this study. Both Melmet cell lines have been established at the Radium Hospital Department of Tumor Biology where melanoma cells have been isolated from melanoma patient biopsies by using immunomagnetic beads conjugated with a melanoma-specific antibody, as described by Prasmickaite et al. [56]. The isolated melanoma cells were then cultivated as adherent monolayers in vitro.

Melmet 1: The human melanoma Melmet 1 cell line was established from subcutaneous metastases of a 36 years old female melanoma patient (Figure 15A). These are invasive, low-proliferative cells that show tumorigenic and metastatic abilities in nude mice, where the most common sites of distant metastasis are brain and bone.

Melmet 5: The human melanoma Melmet 5 cell line was established from lymph-node metastases of a 56 years old male melanoma patient (Figure 15B). These are proliferative, low-invasive cells that show tumorigenic and metastatic abilities in nude mice, where the most common sites of distant metastasis are brain, lung, liver and bone [57].

Figure 15: Morphology of A) Melmet 1 and B) Melmet 5. The pictures are adapted from Kotryna Vasiliauskaitė in the research group.
Materials and Methods

Both cell lines attach easily to the bottom of a culture flask and form adherent cell monolayers. Melmet 1 cells are bigger, rounder, and grow slower than Melmet 5 cells that are smaller and more elongated. In the experiments, Melmet 1 is seeded out with fewer cells in the wells than Melmet 5, due to fast confluence. Figure 15 shows the morphology of Melmet cells grown in vitro as cell monolayers.

2.1.2 Protein S100A4

Human recombinant S100A4 protein was produced by other members in the research group. Briefly, the protein was expressed in E.coli and purified by appropriate chromatographic techniques [58, 59]. In this study, three different batches of S100A4 - named T IV, G1, and G2 - were used (stock-concentrations were 0.74, 0.50, and 2.50 mg/ml, respectively). The stock solutions were stored at -80ºC, and were thawed on ice before use. Working solutions of S100A4 (usually 2 μg/ml) were prepared by adding the required amount of stock solutions to the cell growth medium (RPMI+, see 2.1.4).

2.1.3 Drug vemurafenib

Vemurafenib powder (Selleck Chemicals, LLC) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 20 mM stock solution. This stock solution was then divided into small tubes (10 μl/tube) and stored at -20ºC. Before the experiments, the vemurafenib stock solution was thawed on ice and diluted with cell growth medium to make the working-solutions of desired concentrations, usually ranged from 1-10 μM. Vemurafenib is sensitive to light, and therefore, working in dimmed light is beneficial. Thus, when handling the drug, the lights in the sterile cell bench were turned off and the tubes containing vemurafenib were covered with aluminum foil.

2.1.4 Cell culturing

Melanoma cells were routinely cultured in cell culture flasks in RPMI medium supplemented with Foetal Bovine Serum (FCS, 10 %) and L-glutamine (5 %) (further referred as
“RPMI+”) in an incubator with 5 % CO₂ and 95 % air atmosphere at 37°C. The cells were subcultured and the medium was changed 2-3 times a week depending on the cell confluence, which was controlled daily by an inverted microscope. Cells were subcultured when the confluence reached 60-80 %. All procedures were performed under sterile conditions. Materials and instruments used under cell culturing and general cell work are listed in Table 1.

2.1.5 Cell subculturing
To subculture the melanoma cells, their growth medium was first removed from the cell flask (size T75) and the cell monolayer was washed with 2 ml EDTA. Then a new 1 ml EDTA was added for 3-4 minutes, before the cell flask was roughly padded until the cells detached from the flask bottom. Next, few milliliters of RPMI+ was added to the flask, and the desired amount of the cell suspension was transferred to new flask (size T75). Usually, 1/5th of Melmet 1 suspension and 1/10th of Melmet 5 suspension were transferred to a new flask. Finally, the cell flasks where refilled with additional RPMI+ to a final volume of 12 ml.

2.1.6 Cell counting
To determine the melanoma cell concentration, first the cells were detached from the flask bottom (see Chapter 2.1.5) and the cell suspension was transferred to a tube. To determine the cell concentration, 10 μl of homogeneous cell suspension was mixed with 10 μl tryptan blue, a dye which stains only dead cells. Then, 10 μl of the mix was applied to a cell counting chamber slide and put into the automated cell counting instrument (Countess™). The Countess™ counts the amount of both viable and dead cells, but only the amount of viable cells was taken into consideration when preparing cells for the experiments. Knowing the cell concentrations (cells/ml) and the total volume of cell suspension in the tube, the total amount of viable cells was calculated. Total amount of viable cells = cell concentration (cell/ml) x total volume of cell suspension (ml). The cell suspension was further centrifuged at 1200 rpm for 5 minutes, and the cell pellet was resuspended in fresh RPMI+ to a final concentration of 1.0x10⁶ cells/ml.
Materials and Methods

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 Medium</td>
<td>BE12-167F</td>
<td>BioWhittaker®</td>
<td>Belgium</td>
</tr>
<tr>
<td>Foetal Bovine Serum Standard Quality</td>
<td>A15-101</td>
<td>PAA Laboratories</td>
<td>Austria</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>35050-038</td>
<td>Invitrogen™</td>
<td>USA</td>
</tr>
<tr>
<td>Versene, EDTA</td>
<td>BE17-711E</td>
<td>BioWhittaker®</td>
<td>Belgium</td>
</tr>
<tr>
<td>Sterile Cell Growth Flasks With Filter Caps (T75)</td>
<td>156499</td>
<td>NUNC™</td>
<td>Denmark</td>
</tr>
<tr>
<td>Sterile 2, 5, 10 and 25ml pipettes</td>
<td>13-675-3C</td>
<td>Fisherbrand®</td>
<td>UK</td>
</tr>
<tr>
<td>Sterile 15 ml tube</td>
<td>62.554.502</td>
<td>Sarstedt®</td>
<td>Germany</td>
</tr>
<tr>
<td>10, 100 and 1000 µl small pipettes</td>
<td>2279</td>
<td>ART®</td>
<td>UK</td>
</tr>
<tr>
<td>Tryptan Blue Stain 0,4 %</td>
<td>15250-061</td>
<td>Invitrogen™</td>
<td>USA</td>
</tr>
<tr>
<td>Countess™ - cell counting chamber slides</td>
<td>C10283</td>
<td>Invitrogen™</td>
<td>USA</td>
</tr>
<tr>
<td>Recombinant protein S100A4 (T IV, G1, and G2)</td>
<td>-</td>
<td>Produced in the research group</td>
<td>Norway</td>
</tr>
<tr>
<td>Vemurafenib (PLX4032)</td>
<td>S1267</td>
<td>Selleck Chemicals</td>
<td>USA</td>
</tr>
</tbody>
</table>

Table 1: List of the materials and instruments, used for general cell work.

<table>
<thead>
<tr>
<th>Instruments:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Countess™ - automated cell counter</td>
<td>C10281</td>
<td>Invitrogen™</td>
<td>Korea</td>
</tr>
</tbody>
</table>

2.2 Evaluation of cell proliferation and viability

2.2.1 MTS assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay)

The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (also called the MTS assay) is a colorimetric method used to evaluate cell proliferation/growth, and cell viability after treatment with a drug. When yellow MTS compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is added to the cells, it is reduced enzymatically by the viable cells into a formazan product that causes a color change and has an absorbance at maximum 490-500 nm wavelength (see Figure 16). The production of
formazan in the culture medium can be measured evaluating the absorbance which is directly proportional to the number of viable cells in culture [60]. The higher value of formazan indicates the higher amount of viable cells present in a well. The MTS assay is also used to evaluate cell sensitivity to drugs (i.e. drug-response). Lower absorbance values in drug-treated wells indicate fewer viable cells present in a well, which reflects higher effect of the drug.

In most of the experiments (except the experiment where the cell culture medium was collected for ELISA) 10 μl of MTS solution was added directly to the wells containing 80 μl culture medium above the growing adherent cells in a 96-well plate. The plate was incubated at 37°C for 0.5-2 hours until the yellow MTS-containing medium turned into darker/orange color. Further, the absorbance at 490 nm wavelength was measured directly in the 96-well plate by the Wallac 1420 Victor™ plate reader (see Table 2).

![Figure 16: Reduction reaction from MTS tetrazolium salt to its bioreduced product, formazan.](image)

In the experiments where the S100A4 effect on cell proliferation was evaluated, the absorbance values for the non-stimulated control cells were set to 100 % and the relative proliferation of S100A4-stimulated cells was calculated. In the experiments where the vemurafenib effect was evaluated, the absorbance values in the untreated cells (0 μM vemurafenib) were set to 100 % and the relative viability of the treated cells was calculated.
Materials and Methods

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nunclon™ 96-well plate</td>
<td>167008</td>
<td>Nunc™</td>
<td>Denmark</td>
</tr>
<tr>
<td>96® AQ aqueous Non-Radioactive Cell Proliferation Assay kit:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTS Solution  (G109A, 20 ml)</td>
<td>G5421</td>
<td>©Promega</td>
<td>USA</td>
</tr>
<tr>
<td>PMS Solution  (G110A, 1 ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Instruments:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country:</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Wallac 1420 Victor™ - multilabel, multitask plate reader</td>
<td>1420-018</td>
<td>PerkinElmer</td>
<td>USA</td>
</tr>
</tbody>
</table>

Table 2: List of the materials and instruments, used in MTS assay.

2.2.2 IncuCyte™ (kinetic proliferation assay)

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell seeding:</td>
<td>Adding S100A4 and measuring:</td>
<td>Collecting data:</td>
</tr>
</tbody>
</table>

Melmet 1:  
18 wells á 3800 cells in 100µl RPMI**

Removing 20µl cell culture medium from each well

Melmet 1:  
6 wells á 20µl G1 S100A4
6 wells á 20µl G2 S100A4
6 wells á 20µl RPMI** (control)

Melmet 5:  
18 wells á 4000 cells in 100µl RPMI**

Measuring the plate by the Incucyte™

Table 3: An overview of the performance of cell proliferation using IncuCyte™ (RPMI medium supplemented with 4% FCS and 5% L-glutamine is, and will also later be, referred as RPMI**).

Kinetic proliferation assay using IncuCyte™ is another method used to evaluate cell proliferation (and also cell sensitivity to drugs, which was not used in this study). In contrast to the MTS assay, IncuCyte™ can measure the same cells over time by imaging the wells (see Figure 17) and quantifying the cell confluence. In this way, temporal read outs of cell proliferation versus time is provided [61].
On the first day of the experiment, the Melmet cells were seeded in a 96-well microplate suited for the IncuCyte™ at a density of 3800 cells/well (Melmet 1) and 4000 cells/well (Melmet 5) in 100\(\mu\)l medium RPMI** as described in Table 3. The plate was incubated at 37ºC for 24 hours. The next day, 20 \(\mu\)l of the culture medium was removed from each well and was replaced with either 20 \(\mu\)l of G1 S100A4, 20 \(\mu\)l G2 S100A4, or 20 \(\mu\)l RPMI** in all the cell wells (the final concentration of S100A4 was 2\(\mu\)g/ml). The remaining empty wells were filled with 100\(\mu\)l PBS before the microplate was put into the IncuCyte and measured every second hour for 48 hours. Materials and instruments used in this study are listed in Table 4.

**Figure 17**: Images of Melmet 5 cells taken at 0, 24 and 48 hours by IncuCyte™.

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country:</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 Well ImageLock Microplate</td>
<td>12011201</td>
<td>Essen BioScience</td>
<td>USA</td>
</tr>
<tr>
<td>PBS</td>
<td>BE17-516F</td>
<td>BioWhittaker®</td>
<td>Belgium</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Instruments:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country:</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Incucyte™ -kinetic imaging system</td>
<td>-</td>
<td>Essen BioScience</td>
<td>USA</td>
</tr>
</tbody>
</table>

**Table 4**: List of the materials and instruments used in the IncuCyte assay.
2.3 Treatment with S100A4 and vemurafenib

2.3.1 Effect on cell proliferation

Table 5: An overview over how the experiments on the cell proliferation using the MTS-assay were performed.

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 4 or 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell seeding:</strong></td>
<td><strong>Adding S100A4:</strong></td>
<td><strong>Measuring:</strong></td>
</tr>
<tr>
<td>Melmet 1: 6 wells á 4000 cells in 100µl RPMI**.</td>
<td>Removing 50µl cell culture medium from each well. Melmet 1: 3 wells á 20µl S100A4. 3 wells á 20µl RPMI** (control)</td>
<td>18 wells á 10µl MTS-solution.</td>
</tr>
<tr>
<td>Melmet 5: 6 wells á 4500 cells in 100µl RPMI**.</td>
<td>Melmet 5: 3 wells á 20µl S100A4. 3 wells á 20µl RPMI** (control)</td>
<td>Incubating at 37°C 0.5-2.0 hrs.</td>
</tr>
<tr>
<td>RPMI: 6 wells á 100µl RPMI**.</td>
<td>3 wells á 20µl S100A4. 3 wells á 20µl RPMI**</td>
<td>Measure the absorbance at 490 nm.</td>
</tr>
</tbody>
</table>

The Melmet cells were seeded in a 96-well plate at a density of 4000 cells/well (Melmet 1) and 4500 cells/well (Melmet 5) in 100µl medium RPMI** (Table 5). After incubation of the plate at 37°C until the next day, 50µl of cell culture medium was removed from each well and 20µl S100A4-suspension (giving a final concentration of 2µg/ml S100A4) was added to each well in half of the wells. The remaining wells were filled with 20µl RPMI** per well (control). After another 24-72 hours incubation, the MTS assay was performed as described above, in chapter 2.2.1.
### Materials and Methods

#### 2.3.2 Effect on cell sensitivity to vemurafenib

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
<th>DAY 5 or 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell seeding:</strong></td>
<td><strong>Adding S100A4:</strong></td>
<td><strong>Adding vemurafenib:</strong></td>
<td><strong>Measuring:</strong></td>
</tr>
<tr>
<td>Melmet 1: 30 wells á 3800 cells in 100µl RPMI**</td>
<td>Removing 50µl cell culture medium from each well</td>
<td>Melmet 1: 15 wells á 20µl S100A4 15 wells á 20µl RPMI** (control).</td>
<td>66 wells á 10µl of MTS-solution</td>
</tr>
<tr>
<td>Melmet 5: 30 wells á 4000 cells in 100µl RPMI**</td>
<td>Melmet 1: 15 wells á 20µl S100A4 15 wells á 20µl RPMI** (control).</td>
<td>Melmet 1: 6 wells á 10µl RPMI** 6 wells á 10µl 1µM VF 6 wells á 10µl 2.5µM VF 6 wells á 10µl 5µM VF 6 wells á 10µl 10µM VF</td>
<td>Incubating at 37°C for 0.5-2.0 hrs</td>
</tr>
<tr>
<td>RPMI: 6 wells á 100µl RPMI**</td>
<td>RPMI: 6 wells á 20µl RPMI**</td>
<td>Melmet 5: 6 wells á 10µl RPMI** 6 wells á 10µl 1µM VF 6 wells á 10µl 2.5µM VF 6 wells á 10µl 5µM VF 6 wells á 10µl 10µM VF</td>
<td>Measure the 96-well plate by the MTS assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melmet 5: 6 wells á 10µl RPMI** 6 wells á 10µl 1µM VF 6 wells á 10µl 2.5µM VF 6 wells á 10µl 5µM VF 6 wells á 10µl 10µM VF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPMI: 6 wells á 10µl RPMI**</td>
<td></td>
</tr>
</tbody>
</table>

*Table 6:* An overview over how the experiments on cell sensitivity to vemurafenib (VF) were performed.

The Melmet cells were seeded in a 96-well plate at a density of 3800 cells/well (Melmet 1) and 4000 cells/well (Melmet 5) in 100 µl medium RPMI** (*Table 6*), and incubated at 37°C. The next day, 50 µl of cell culture medium was removed from each well, and 20 µl S100A4-suspension (giving a final concentration of 2 µg/ml S100A4) was added to each well in half of the wells. The remaining wells were added 20 µl RPMI** per well (control) (*Figure 19*). Further, we incubated the plate for another 24 hours before 10 µl of vemurafenib-dilution was added directly to three of the wells with, and three of the wells.

*Figure 18:* An overview of how vemurafenib was diluted from the stock-solution of 20 mM to the desired concentrations of 1-10 µM in the wells.
Without S100A4 for each vemurafenib concentration. The same was done for both Melmet 1 and Melmet 5. The final drug concentration in each well was usually ranged between 1 and 10 μM. Figure 18 describes how vemurafenib stock solution was diluted to the desired concentration. After another 48-72 hours incubation, the MTS assay was performed as described above.

![Figure 18](image-url)

Figure 18: An overview of how vemurafenib stock solution was diluted to the desired concentration.

### 2.3.3 Effect of vemurafenib on S100A4-stimulated secretion of IL-8

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
<th>DAY X</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell seeding:</strong></td>
<td><strong>Adding S100A4 + vemurafenib:</strong></td>
<td><strong>Measuring MTS:</strong></td>
<td><strong>Measuring</strong></td>
</tr>
<tr>
<td>Melmet 1: 12 wells á 10000 cells in 100 μl RPMI**</td>
<td>Removing 30 μl of cell culture medium from each well</td>
<td>Collecting the cell growth supernatants and storing at -80°C</td>
<td>Performing ELISA assay</td>
</tr>
<tr>
<td>Melmet 5: 12 wells á 15000 cells in 100 μl RPMI**</td>
<td>Melmet 1: 6 wells á 20 μl S100A4 6 wells á 20 μl RPMI** ½ the plate á 10 μl 2 μM VF ½ the plate á 10 μl RPMI** (0μM VF)</td>
<td>30 wells á 80 μl of MTS-solution</td>
<td></td>
</tr>
<tr>
<td>RPMI: 6 wells á 100μl RPMI**</td>
<td>Melmet 5: 6 wells á 20 μl S100A4 6 wells á 2 0μl RPMI** (control) ½ the plate á 10 μl 1 μM VF ½ the plate á 10 μl RPMI** (0μM VF)</td>
<td>Incubating at 37°C for 0.5-2.0 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RPMI: 6 wells á 30μl RPMI**</td>
<td>Measure the 96-well plate by MTS assay</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: An overview over how the experiments on cell sensitivity to vemurafenib (VF) were performed.
The Melmet cells were seeded in a 96-well plate at a density of 10,000 cells/well (Melmet 1) and 15,000 cells/well (Melmet 5) in 100 μl medium RPMI** (*Table 7*). After incubation of the plate at 37°C until the next day, 30 μl of cell culture medium was removed from each well, and then 20 μl S100A4-suspension (giving a final concentration of 2 μg/ml S100A4) was added directly to each well in half of the wells; the wells in the remaining half of the plate were added 20 μl RPMI** (without S100A4). Subsequently, 10 μl of vemurafenib-dilution was added directly to three wells with and three of the wells without S100A4 for both Melmet 1 and Melmet 5. The final drug-concentration in each well was 2 μM in Melmet 1, and 1 μM in Melmet 5. The remaining six wells for each cell line were added 10 μl RPMI** (control). After 24 hours incubation, the cell growth medium was collected in small tubes and centrifuged at 13000 rpm for 5 minutes before the cell culture supernatants were collected in new small tubes and stored at -80°C for later analyzes (chapter 2.3.4). At last, 100 μl RPMI** containing the MTS solution was added to the remaining cell monolayer in each well, and the rest of the MTS assay was performed as described above.

2.3.4 ELISA assay for measuring secreted IL-8

In our experiments, we used the DuoSet® ELISA Development kit to measure human CXCL8/IL-8. The kit is designed for the analysis of conditioned cell media, and contains the basic components required to make the "sandwich ELISA" (illustrated in *Figure 20*). Capture antibodies was pre-coated in the bottom of the wells of modular microplates (containing 8 separately well strips). After the samples with the target protein (in our case; IL-8) was added to the wells and attached to the capture antibody, a second monoclonal antibody was added. This detector antibody attaches to a different epitope on the target protein, and are either directly labeled with biotin, or a biotin-labeled goat anti-mouse. Substrate plus HRP conjugated to the enzyme streptavidin were further added to the wells. These enzymes degrade the biotin and provide a colorimetric signal which can be measured at 450 nm wavelength by an ELISA plate reader. The stronger colorimetric signal (or the higher absorbance) indicates the higher levels of target protein (IL-8) present in the sample (well). Absorbance over 560 nm gives optical imperfections in the plate, and should be subtracted by setting wavelength correction to 540 or 570 nm.
Materials and Methods

**Figure 20:** The sandwich ELISA in a microplate. The picture is adapted from ©Abcam plc., 1998-2012 [62].

**General ELISA protocol:**

**Day 1:** First, we thawed the Capture Antibody on ice and diluted it 1:100 in PBS. Then, we immediately coated the 96-well microplate with 100 μl/well diluted Capture Antibody, sealed the plate with an adhesive strip, and incubated overnight at room temperature.

**Day 2:** We used a multi-pipette and washed each well with 400 μl Wash Buffer, three times. The liquid was removed from each well by inverting the plate and blotting it against clean paper towels. Further, we blocked the plate with 300 μl/well Block Buffer, sealed the plate, and incubated at room temperature for a minimum of 1 hour. Meanwhile, we thawed our 8 samples (cell culture supernatants) on ice. After 1 hour, we repeated washing the wells like described above. Then we diluted our samples (1:20 for Melmet 1 and 1:1 for Melmet 5) with Reagent Diluent, and added 100 μl diluted sample per well, sealed the plate, and incubated 2 hours at room temperature.

After 2 hours, we thawed the Detection Antibody on ice and repeated washing the wells exactly like earlier. The Detection Antibody was further diluted 1:100 in Reagent Diluent, and 100 μl diluted Detection Antibody was added per well before sealing the plate for 2 hours incubation at room temperature.
After 2 hours, we repeated washing the wells exactly like earlier. Then, we diluted Streptavidin-HRP 1:200 in Reagent Diluent, and added 100 μl diluted Streptavidin-HRP per well, covered the plate, and incubated for 20 minutes at room temperature. Streptavidin-HRP is sensitive for UV, and the plate should avoid direct light.

After 20 minutes, we repeated washing the wells exactly like earlier. Then, we prepared the Substrate Solution by mixing 1:1 of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine), and added 100 μl per well. The plate was further covered and incubated in room temperature for about 20 minutes (avoid direct light).

After the Substrate Solution in the wells turned slightly blue, we added 50 μl Stop Solution carefully to each well (caution with acid solutions) and tapped the plate gently to mix the solutions. Right after, we used a microplate reader at 450 nm to measure the optical density in each well. Materials and instruments used in ELISA assay are listed in Table 8.
# Materials and Methods

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Catalog no.:</th>
<th>Manufacturer:</th>
<th>Country:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear Microplate</td>
<td>DY990</td>
<td>R&amp;D Systems®</td>
<td>USA</td>
</tr>
<tr>
<td><strong>DuoSet® ELISA Development System Human CXCL8/IL-8:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capture Antibody</td>
<td>DY208</td>
<td>R&amp;D Systems®</td>
<td>USA</td>
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<td>Detection Antibody</td>
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</tr>
<tr>
<td>Streptavidin-HRP</td>
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<tr>
<td>Solutions required:</td>
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<td>Germany</td>
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<td>8.1 mM Na2HPO4</td>
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<td>Merck KGaA</td>
<td>Germany</td>
</tr>
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Table 8: List of the materials and instruments used in ELISA assay.
3 RESULTS

3.1 The influence of serum concentration on S100A4-induced effect on cell proliferation

To find the experimental conditions suitable for investigation of the effects of the added protein S100A4, first we tested the influence of different concentrations of fetal bovine serum (FCS) present. FCS was supplemented to the RPMI medium used to cultivate the Melmet 1 and Melmet 5 cells. To evaluate the effect of added S100A4 on melanoma cells it would be advantageous to exclude FCS that is rich in various proteins. However, the melanoma cells do not survive in the medium without serum. Therefore, FCS had to be added, but it would be beneficial to reduce the FCS concentration. In the initial experiments we used medium with 8 % FCS (traditional serum concentration) and 4 % FCS to evaluate whether the added S100A4 had an influence on melanoma cell proliferation after 24 hours stimulation. The amount of added S100A4 was 2 μg/ml. The choice of such S100A4 amount was based on previous experience in the group where it has been demonstrated that this amount induces the effects in various cancer cell lines, and is now used as a standard S100A4 concentration [63].

Figure 21: Effect of S100A4 on proliferation of A) Melmet 1 and B) Melmet 5 cells when cultured in medium containing 4 % and 8 % FCS concentrations. control columns represent Melmet cells without S100A4-stimulation. Each column indicates an average and each error bar indicates standard error of mean (SEM) from two independent biological experiments (n=2).
Results

Two independent biological experiments were performed for each FCS concentration. As can be seen in Figure 21, Melmet 1 did not respond to S100A4 at any FCS concentration. In contrast, Melmet 5 showed reduced proliferation upon S100A4 stimuli, particularly when 4 % FCS was used. Thus, Melmet 5 cell proliferation was reduced by 23 ±0.5 % and 10 ±7.9 % in the presence of 4 % and 8 % FCS, respectively. Based on the fact that the S100A4 effect was more pronounced at 4 % FCS, this concentration has been chosen for the subsequent experiments.

3.2 Time-dependency for the effect of S100A4 on cell proliferation

To follow the effect of S100A4 on cell proliferation over time, Melmet 1 and Melmet 5 cells were stimulated with 2μg/ml S100A4 for 24, 72, and 96 hours, before the cell proliferation was scored by the MTS assay. As can be seen in Figure 22A, S100A4 did not influence the proliferative abilities of Melmet 1 cells at any time point investigated. Since there was no observed difference in Melmet 1 proliferation at any time points tested (and no difference of S100A4 in the previous serum concentration test; Figure 21A), the experiment involving Melmet 1 was performed only once. On the contrary, Melmet 5 cells showed S100A4-induced reduction in cell proliferation at all time points (Figure 22B) in two biologically independent experiments, being in line with the data shown in Figure 21B.

However, there was no clear difference between the S100A4-induced effect at 24 hours versus the later time points (Figure 22B), indicating that the effect is not influenced by the longer stimulation with S100A4. Thus, Melmet 5 showed 24 ±0.4% reductions on the proliferation after 24 hours, 16 ±1.1% reductions after 72 hours, and 19 ±1.1% reductions after 96 hours treatment with S100A4. Altogether this indicates that S100A4 effect is induced already during first 24 hours of treatment, and also that S100A4 reduces cell proliferation only Melmet 5 cells, and not in Melmet 1 cell.
Results

Figure 22: Effect of S100A4 on cell proliferation in A) Melmet 1 and B) Melmet 5 cells after 24, 72, and 96 hours stimulation with (A4) or without S100A4 (control). A) Each column indicates an average and each error bar indicates standard deviation (SD) from three parallels in one biological experiment (n=1). B) Each column indicates an average and each error bar indicates SEM from two independent biological experiments (n=2).

3.3 Evaluation of Melmet cell sensitivity to vemurafenib

Both Melmet 1 and Melmet 5 cells have mutated BRAF, and were therefore suitable models to evaluate the effect of the BRAF inhibitor vemurafenib. To evaluate Melmet cell sensitivity to vemurafenib, the cells were treated with different drug-concentrations (ranging from 1-10 μM) for 48 and 72 hours in three biologically independent experiments before the cell viability was measured by the MTS assay.

Figure 23: Cell sensitivity to vemurafenib after 48 and 72 hours of treatment in A) Melmet 1 and B) Melmet 5. The data points indicate an average and the error bars indicate SEM from three independent biological experiments (n=3) where each experiment had three parallels.
Results

As can be seen in Figure 23, both cell lines showed a dose-dependent decrease in cell viability after treatment with vemurafenib. The median lethal dose (LD$_{50}$), or the vemurafenib dose that killed 50% of the cells after 48 hours treatment, was approximately 1 μM. In addition, the killing efficiency of Melmet 5 was more pronounced at 72 versus 48 hours treatment, where approximately 70% of the cells were killed by 1 μM vemurafenib (Figure 23B). However, Melmet 1 did not show any difference between the effects at 72 hours compared to 48 hours of the treatment (Figure 23A). The 72 hours treatment with the low doses of vemurafenib killed more cells in Melmet 5 than in Melmet 1 (70-80% vs. 60-70%), suggesting that Melmet 1 cells might be more resistant. However, the effect after the 48 hours treatment was the opposite. Majority of the cells were killed when treated with 10 μM vemurafenib: approximately 80-90% in both cells lines.

3.4 Effect of S100A4 on Melmet cell responses to vemurafenib

To explore how extracellular S100A4 influences Melmet cell sensitivities to vemurafenib, the cells were first stimulated with 2 μg/ml S100A4 (batch T IV) for 24 hours, and then treated with 5 and 10 μM vemurafenib for 48 and 72 hours, respectively, before cell viability was measured by the MTS assay.

As Figure 24A below shows, in Melmet 1 there was no difference between the viability of S100A4-stimulated versus the non-stimulated cells (control) at any vemurafenib-concentrations or time point tested. In Melmet 5 however, stimulation with S100A4 slightly enhanced the cell viability compared to the control cells without S100A4 (Figure 24B). Thus in Melmet 5, approximately 7% increase in the fraction of viable cells was registered at both vemurafenib-concentrations and both time points tested. Although the differences were not statistically significant, the same trend was observed in two independent biological experiments for each time point.
Figure 24: Effect of S100A4 (batch T IV) on the cell viability response (MTS assay) to vemurafenib after 48 hours (left panels) and 72 hours (right panels) treatment of A) Melmet 1 and B) Melmet 5. The control columns represent Melmet cells without S100A4-stimulation. A) The columns indicate an average and the error bars indicate SD from three parallels in one biological experiment (n=1). B) The columns indicate an average and the error bars indicate SEM from two independent biological experiments (n=2).

3.5 Effect of other S100A4-batches on Melmet cell proliferation and response to vemurafenib

After the S100A4 batch “T IV” used at the beginning of this MSc and for the Figures 21-24 was used up, the research group produced two new batches of recombinant S100A4, named “G1” and “G2”. By using the same experimental strategy as previously and adding the same amount of the new S100A4, we intended to evaluate whether the new batches induce the same effects in Melmet 5 as the previous batch (T IV). As mentioned earlier, 2 µg/ml S100A4 was used as a “standard concentration” in our group to stimulate various tumor cells. For the S100A4 batch G1 we accidently added less S100A4 i.e. 1.35 µg/ml (due to lower concentration in the G1 stock solution, which was observed only later).
Results

Figure 25: Effect of S100A4 (batch G1; 1.35 μg/ml) on the cell proliferation of A) Melmet 1 and B) Melmet 5. The relative proliferation was evaluated by the MTS assay after 72 and 96 hours stimulation with or without (control) S100A4. The columns indicate an average and the error bars indicate SEM from two independent biological experiments (n=2).

Figure 26: Effect of S100A4 (batch G1) on the cell viability response (MTS assay) to vemurafenib after 48 hours treatment of A) Melmet 1 and B) Melmet 5. The control columns represent Melmet cells without S100A4-stimulation. The columns indicate an average and the error bars indicate SD from three parallels in one biological experiment (n=1).

As can be seen, 1.35 μg/ml G1 had no induced effect on Melmet 5 cell proliferation (Figure 25B) and cell response to vemurafenib (Figure 26B), which corresponded differently to the previous results where 2 μg/ml T IV batch was used (see Figure 22B and 24B). There was no clear effect of 1.35 μg/ml G1 in Melmet 1 cells either (Figures 25A and 26A), which corresponded to the previous results using 2μg/ml T IV S100A4 (Figures 22A and 24A). We speculated that the lack of effect of G1 S100A4 could be due to either too low concentration added to the cells or inactive S100A4 batch. Therefore, in the subsequent studies we switched to another batch “G2” and stimulated the cells with the standard
concentration (2μg/ml) of S100A4, exactly like the experiments performed with the T IV batch. The results on the Melmet cell proliferation and the sensitivity to vemurafenib in the presence or absence (control) of G2 S100A4 are shown in Figures 27 and 28, respectively.

**Figure 27:** Effect of S100A4 (batch G2) on the proliferation of A) Melmet 1 and B) Melmet 5 cells, 72 hours after S100A4 stimulation. The control columns represent Melmet cells without S100A4-stimulation. The columns indicate an average and the error bars indicate SD of three parallels in one biological experiment (n=1).

**Figure 28:** Effect of S100A4 (batch G2) on the cell viability response (MTS assay) to vemurafenib after 48 hours treatment of A) Melmet 1 and B) Melmet 5. The control columns represent Melmet cells without S100A4-stimulation. The columns indicate an average and the error bars indicate SD from three parallels in one biological experiment (n=1).

As can be seen, neither 2μg/ml G2 S100A4 notably affected Melmet cell proliferation (Figure 27) or sensitivity to the vemurafenib (Figure 28). Altogether, this indicated that G1 and G2 batches of S100A4 are less “effective” than the T IV batch in affecting melanoma cell proliferation and drug-sensitivity.
3.6 Evaluation of G1 and G2 S100A4 effect on cell proliferation using the IncuCyte system

Since G1 and G2 batches of S100A4 did not show the same effect on Melmet cell proliferation as seen for T IV S100A4 when measured by the MTS assay, we tested another method i.e. the IncyCyte™ for evaluation of cell proliferation. Previously, we measured cell proliferation using a “single” time point assays (MTS) which evaluates metabolic activity (i.e. mitochondrial activity) reflecting a number of viable cells. In contrast, IncuCyte system performs real-time imaging where the integrated software metrics can provide temporal readouts of cell proliferation at different time points, and thus provides growth curves.

![Growth curves for A) Melmet 1 and B) Melmet 5 in the presence and the absence (control) of G1 S100A4 (left panels) and G2 S100A4 (right panels), measured by IncuCyte. The plots show confluence in percents, reflecting cell proliferation from three parallels measured at two hours intervals for two days. Each data point is composited of several images at that time point (n=1).](image)

Figure 29: Growth curves for A) Melmet 1 and B) Melmet 5 in the presence and the absence (control) of G1 S100A4 (left panels) and G2 S100A4 (right panels), measured by IncuCyte. The plots show confluence in percents, reflecting cell proliferation from three parallels measured at two hours intervals for two days. Each data point is composited of several images at that time point (n=1).
Melmet 1 and Melmet 5 cells were seeded out in the presence (A4) and absence (control) of 2μg/ml G1 or G2 S100A4, and put into the IncuCyte. The cell proliferation was scored by measuring cell confluence every second hour for two days. **Figure 29** shows that for Melmet 1 cells, there was no effect on proliferation after stimulating with G1 or G2 S100A4. For Melmet 5, G1 did not have an effect, whereas G2 slightly reduced the proliferation after 24 hours, mimicking the effect seen for the T IV batch. It should be noted that the IncuCyte-experiment was performed only once.

### 3.7 Comparison of three different batches of S100A4 with respect to their ability to induce IL-8 secretion

Having observed that the cell response to the three batches of S100A4 (T IV, G1 and G2) is different, we decided to compare these batches in more detail. We studied their ability to stimulate the melanoma cells to secrete IL-8. Previously, it has been shown in our group that the effect of S100A4 on the induction of IL-8 is a very sensitive method for evaluating whether the added protein has an influence on cancer cells. Thus, the level of IL-8 secreted from the Melmet cells stimulated with 2 μg/ml S100A4 of the batches T IV, G1, or G2

\[\text{Relative level of IL-8} \]

\[\text{1 to 10} \quad \text{1 to 50} \]

**Figure 30**: Levels of IL-8 secreted into the conditioned cell media upon stimulation with 2 μg/ml S100A4 of batches T IV, G1, and G2 in **A** Melmet 1 and **B** Melmet 5. Control columns indicate Melmet cells with no S100A4 added. IL-8 was measured by ELISA after 24 hours S100A4-stimulation. Y-axis indicates the relative level of IL-8 (based on absorbance measurements, see Material and Methods chapter), and X-axis indicates the dilutions of the cell culture supernatants.
for 24 hours were measured. Specific IL-8 enzyme-linked immunosorbent assay (ELISA) was used to measure the level of IL-8 in the cell culture supernatants. The supernatants were diluted 1:10 and 1:50 for Melmet 1, and 1:2 and 1:5 for Melmet 5. Generally, Melmet 1 secreted substantially higher levels of IL-8 than Melmet 5 (see Figure 21 in chapter 1.9.1). Therefore, Melmet 1 cell culture supernatants had to be diluted more in order to avoid saturation of the signal during ELISA, and to achieve reliable measurements.

As can be seen in Figure 30, different batches of S100A4 (added at the same concentration) showed different ability to induce IL-8 secretion. This difference was pronounced more in Melmet 5 compared to Melmet 1 cells. The lack of difference in Melmet 1 at the lowest dilution (1:10) might be explained by too high levels of IL-8 present in the supernatant, leading to the saturation of the measured signal of absorbance. Thus, T IV batch induced the highest secretion of IL-8, while G2-mediated induction was the lowest (approximately 6% lower for Melmet 1 at dilution 1:50, and approximately 26% lower for Melmet 5 at dilution 1:10). This indicates that the three tested batches of S100A4 have different abilities to induce secretion of IL-8.

3.8 Effect of vemurafenib on S100A4-induced secretion of IL-8

To evaluate whether the treatment with vemurafenib influences S100A4-induced secretion of IL-8, we exposed the cells to both S100A4 and vemurafenib, and measured the IL-8 level in cell culture supernatant. We have chosen to use the drug dose 2 μM for Melmet 1 and 1 μM for Melmet 5, corresponding to approximately LD₅₀ (see Figure 23), and added the drug together with 2 μg/ml G2 S100A4. 24 hours later, the cell viability was evaluated by the MTS assay, and cell culture supernatants were collected and used for evaluation of IL-8 level by ELISA. The supernatants from three parallels were put together and diluted 1:20 in Melmet 1 and 1:1 in Melmet 5 before analysis by ELISA. In previous studies, we stimulated the Melmet cells with S100A4 the day before we added vemurafenib. In this study, S100A4 and vemurafenib were added at the same so that the effect of vemurafenib on S100A4-induced IL-8 secretion could be evaluated.
Results

**Figure 31:** Relative cell viability after 24 hours treatment with vemurafenib in A) Melmet 1 and B) Melmet 5 cells. The control represents Melmet cells without S100A4-stimulation. The columns indicate an average and the error bars indicate SD from three parallels in one biological experiment (n=1).

**Figure 31** shows the drug effect on cell viability i.e. the number of viable cells that could secrete IL-8. Thus, the viable cells were reduced by approximately 10 % in Melmet 1 and 25 % in Melmet 5 after 24 hours treatment with vemurafenib with or without S100A4. This indicates that the number of viable cells able to secrete IL-8 was 10 % and 25 % lower in the vemurafenib-treated cells versus the untreated control cells.

**Figure 32:** Effect of vemurafenib on the levels of IL-8 secreted from A) Melmet 1 and B) Melmet 5 without (control) and with (A4) G2 S100A4. The level of IL-8 was measured in cell culture supernatants by ELISA. The numbers in red next to the symbol “↑” indicate elevation of IL-8-level (in percentage) in cells with S100A4 compared to controls without S100A4.
Results

*Figure 32* shows that secretion of IL-8 was reduced by vemurafenib in both, S100A4-stimulated and non-stimulated (control) cells. In Melmet 1, vemurafenib-induced reduction of IL-8 levels was much higher than the reduction of cell viability shown in Fig. 31A. In Melmet 5, however, the levels of vemurafenib-induced reduction of IL-8 and cell viability were very similar. In Melmet 1 without vemurafenib, S100A4 enhanced the level of IL-8 by 45 % compared to the control cells without S100A4. In the presence of vemurafenib, S100A4-induced enhancement of IL-8 was approximately 21 % i.e. 2-fold lower. In Melmet 5, without vemurafenib, S100A4 enhanced the level of IL-8 by 85 %, while in the presence of vemurafenib the induction was 90 % i.e. almost non-affected. Altogether, treatments with vemurafenib seem to lower the levels of IL-8 and S100A4-stimulated IL-8 induction in Melmet1. The effect in Melmet 5 was unclear and remains to be proved.
4 DISCUSSION

In the present work we have investigated the influence of the extracellular S100A4 protein on the two melanoma cell lines, Melmet 1 and Melmet 5, and how this affects cell response to the drug vemurafenib. We have demonstrated that upon stimulation with S100A4, melanoma cells can reduce their proliferation. This is in line with the data of PhD student Ingrid Bettum who has shown that the expressions of several genes like MITF and MLANA, which are associated with a proliferative phenotype, are also reduced when the cells are stimulated with S100A4. It should be noted that reduced proliferation was observed only in Melmet 5, and not in Melmet 1 cells. Bettum et al. have also observed that only Melmet 5 cells respond to S100A4 by increasing their invasiveness. Furthermore, other studies has also revealed that S100A4 stimulate cell invasion in various human cancer cell lines [32]. Based on the fact that the influence of S100A4 leads to reduced cell proliferation and increased invasion, and having in mind that Melmet 5 represents proliferative phenotypic cells with low intrinsic invasiveness, it can be speculated that S100A4 might stimulate the cells to switch from a proliferative towards an invasive phenotype. Occurrence of such phenotype switching was suggested by Hoek et al. to explain the progression of melanoma metastasis [23]. Since S100A4 is a metastasis promoting protein it could be conceivable that S100A4 might play a role in phenotype switching.

When studying S100A4 influence on vemurafenib-induced cell death, it has been observed that the protein slightly reduced the cell sensitivity to the drug leading to 7 % higher cell viability after the treatment. This was observed only in Melmet 5, and not in Melmet 1, which corresponds to the data discussed above and the phenotype switch hypothesis. If S100A4 “pushes” the cells into the invasive phenotype, these cells might become more resistant to treatments. According to Zipser et al. [24] invasive cells represent a cell subpopulation with enhanced resistance to MAPK inhibitors. This strengthens the hypothesis that the sensitivity of melanoma cells to drugs like vemurafenib might be depended on their phenotypic state. It should be pointed out that the effect of S100A4 on the cells response to vemurafenib was quite low and not statistically significant, although it was observed consistently in several experiments and for several drug concentrations. The low effect of S100A4 might be due to
the fact that only a small fraction of cells responded to S100A4 and therefore the effects measured in the whole cell population might be small. Thus the data of Bettum et al. indicates that only a small cell subpopulation (a few percent) responded to S100A4 by enhancing their invasiveness. This suggests that cell response to S100A4 might be heterogeneous: some cells might show strong response, while other cells show weak or no response to the protein. This phenomenon could not be investigated in our study, where we measured the proliferation and drug-response in the whole cell population. For that reason, it cannot be excluded that the induced effects of S100A4 on the proliferation and/or the drug response in some cells might be much higher than the effect registered by our method in the total cell population.

In this study we used the MTS assay for the evaluation of cell proliferation and viability after treatment with the drug. The advantage of such assay is the ability to evaluate the amount of viable cells directly in the cell wells i.e. without having to “disturb” the cells or running additional analysis. However, Huang et al. have studied inaccuracies of the MTS assay and observed some disadvantages. One of them was that conduction of the assay in culture medium in the present of 10 % serum albumin, led to reduction in the absorbance that the assay is based on [64]. By decreasing the serum-concentration in our experiments (using “only” 4 % FCS) we think we limited this inaccuracy. Overall, the MTS assay is a well-known and recognized method for evaluating proliferation or viability. In this study we also used the IncuCyte™ to measure cell proliferation. Although the IncuCyte™ do not have the same limitation as the MTS assay, the weakness of this method is that the IncuCyte™ only takes images of the cells in one part of the well, and not the whole well. In this way, this method is depended on how evenly the cells are seeded in the wells.

An important observation of this study is that the S100A4 effect was depended on the recombinant protein batch used to stimulate the cells with. The two newest batches, G1 and G2, had no or lower effect than the first batch, T IV, despite that the same S100A4-concentration (2μg/ml) was used. This might be explained by different grade of protein oligomerization in these three batches. Previously, it has been shown that for the optimal activity as an extracellular factor, the protein has to be in a certain oligomeric, and not a
dimeric conformation [33]. It could be that the batch T IV contained more of this active form than the two other bathes, G1 and G2. However, the effective concentration of S100A4 oligomers in our three batches has not been investigated in the present study, but is taken into consideration in the group.

Another clear effect of S100A4 was the induction of enhanced secretion of the cytokine IL-8, which is also one of the genes belonging to the invasive phenotype signature. IL-8 plays an important role in regulating tumor cell behavior and drug-resistance (see Figure 12) [54, 48]. Therefore, the observed ability of S100A4 to stimulate IL-8 secretion leading to the elevated level of IL-8 in the conditioned media of the tumor cells might have an impact for tumor progression and therapy. It should be mentioned, that in this study we used the ELISA assay (R&D System) for the detection of IL-8 levels. Siawaya et al. have compared different fluorescent bead-based immunoassays (commercial Luminex kits) to ELISA assays for the measurement of cytokines in whole blood or culture supernatants, and reported high accuracy and reliability of ELISA [65].

We observed higher effect of S100A4 on the IL-8 induction in Melmet 5 cells (85 % elevation) compared to Melmet 1 cells (45 % elevation). Besides, we observed that the degree of IL-8 induction varied depending on the S100A4-batches. Altogether, this is in line with the previous results on proliferation and drug-response. Interestingly, such drastic increase of IL-8 in Melmet 5 might be associated to the lowered Melmet 5 cell sensitivity to vemurafenib observed in the presence of S100A4. One of the pathways associated with the IL-8 is the MAPK-ERK, the same pathway that the drug vemurafenib is targeting suggesting that IL-8 and vemurafenib can have an influence on each other. Of curiosity we investigated how the treatment with vemurafenib affected the IL-8 secretion. We observed that vemurafenib reduced the levels of IL-8 in both the non-stimulated controls and the S100A4-stimulated cells. It should be mentioned that such reduction in IL-8 levels could be reasoned by fewer viable cells present in the vemurafenib treated wells compared to the untreated once. However, the fact that Melmet 1 cell viability was reduced by approximately 10 % while IL-8 level was reduced by almost 50 % (comparing Figures 24A and 25A), suggests that other
biological mechanism than reduced cell numbers might contribute to the reduction of the IL-8 levels. This possibility is however questioned since in Melmet 5 the percentage reflecting the reduced cell viability corresponded to the percentage reflecting the reduced IL-8 level (comparing Figures 24B and 25B). Whether vemurafenib affected the S100A4-mediated induction of IL-8, remains also to be proved. Our data so far show that such induction was reduced 2-fold in Melmet 1, but not in Melmet 5. However, this experiment was performed only once and more studies are needed to conclude about the influence of vemurafenib on S100A4-stimulated secretion of IL-8.

In summary, we have shown that when adding S100A4 protein to melanoma cells their proliferation is affected, and their sensitivity to the drug vemurafenib is slightly modulated. These effects were more pronounced in Melmet 5 cells than in Melmet 1 cells, suggesting that S100A4 has the highest influence on melanoma cells of the proliferative phenotype. Besides, S100A4 could significantly induce secretion of the pro-tumorigenic cytokine IL-8, which is expected to mediate a variety of processes associated with melanoma cell aggressiveness.
5 CONCLUSIONS

- When melanoma cells are exposed to S100A4 protein in vitro, the cells reduce both their proliferation and sensitivity to the drug vemurafenib leading to slightly higher cell survival.

- The S100A4-dependent effect on proliferation and the vemurafenib-sensitivity was observed in the proliferative phenotype cells Melmet 5, and not in the invasive phenotype cells Melmet 1.

- Exposure to S100A4 stimulates the melanoma cells to secrete high levels of the pro-tumorigenic cytokine IL-8. Treatment with vemurafenib reduces the IL-8 secretion in both the non-stimulated controls and the S100A4-stimulated cells of Melmet 1 and Melmet 5.

- Three different batches of the recombinant S100A4 protein demonstrate different abilities to induce the above-discussed effects. The batch T IV is the most “powerful.”
The evidence implicating S100A4 in cancer metastasis is compelling, but a more comprehensive understanding of the molecular mechanisms of how S100A4 exerts its functions to influence tumor metastasis, is needed. This should be a central focus in future research efforts, along with the investigation of the MAPK-ERK pathway on the molecular level to see how the microenvironment factors (i.e. S100A4) might influence the effect of vemurafenib (or other drugs targeting this pathway) on cell death. Due to time-limitation, our study involving the effect of S100A4 on the Melmet cell lines responses to vemurafenib was performed only once or twice. It would be interesting to see whether more repetitions of the experiments could achieve statistically significant differences of the cell treatments and effects. The effect of vemurafenib on IL-8 secretion as well as the effect of IL-8 on cell response to vemurafenib is an interesting issue for further investigation.
## ABBREVIATIONS

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<td>LD$_{50}$</td>
<td>Median lethal dose, 50 %</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase (MAP2K)</td>
</tr>
<tr>
<td>Melmet 1</td>
<td>Metastatic melanoma 1 cell line</td>
</tr>
<tr>
<td>Melmet 5</td>
<td>Metastatic melanoma 5 cell line</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase (originally called ERK)</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MLANA</td>
<td>Protein melan-A (also known as melanoma antigen recognized by T-cells 1 or MART-1)</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NF-$\kappa$B</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B-cells</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma (small GTPase)</td>
</tr>
<tr>
<td>RPM</td>
<td>Round per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophage</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plaminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet radiation</td>
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</table>
REFERENCES


