RhoC in melanoma: possible target for statin treatment

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Co-promotor: Dr. Veronique Mathieu

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MASTER IN DE GENEESKUNDE
RhoC in melanoma: possible target for statin treatment

Thomas MALFAIT

Promotor: Prof. Dr. Lieve Brochez
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(Name student)

(handtekening promotor)
(signature promotor)

(Naam promotor)
(Name promotor)
Foreword

The combination of helping in an interesting research project, writing a thesis about it and following (almost all of) the lessons was quite a challenge. Nevertheless, these past two years have been a very enriching and inspiring period.

I definitely could not have brought this thesis to a good end without the support of many people. I would like to take the opportunity to thank all the persons who have been helpful and have encouraged me during this period.

First of all, I would like to express my gratitude to my promotor, Prof. Dr. Lieve Brochez (Dermatology, UZ Ghent), for all of her help. Without your aid, enthusiastic words and guidance, I probably would have been lost in the world of melanoma.

Many thanks also go to Dr. Veronique Mathieu (Toxicology, ULB), my copromotor. I appreciate your interest and support very much. You were always prepared for valuable advice and support when needed. Your comments have been of great value.

Other important persons, contributing to this thesis as well, deserve special mention; many appreciations to: PhD Dr. Barbara Boone (Dermatology, UZ Ghent), without her doctorate, there would not be a subject for me to write about; Dr. Tine De Backer (Cardiology, UZ Ghent), for her help in acquiring the statins used in this study and her interest in this matter; Prof. Robert Kiss (Toxicology, ULB) together with Dr. Veronique, Céline Bruyère and Gwendoline Van Goietsenoven (Toxicology, ULB) for being so supportive with the necessary hardware and software and their assistance in successfully completing this study.

I would also like to thank Koen Jacobs (Experimental Cancer Research, UZ Ghent), Mireille van Gele (Research Dermatology, UZ Ghent) and Dr. Reinhart Speeckaert (Research Dermatology, UZ Ghent) for their aid with the melanoma cell lines and technical preparations. In spite of your own busy schedules you still helped me for which I am very grateful.

I also wish to express my greatest thanks to my parents, my family and my friends for their support throughout these years. Last but not least, I wish to thank my girlfriend Fien of being so patient with me the last two (and more…) years. Fien, without your advice and unique support, your patience and understanding, those long nights working would have been much harder. Thank you for the many cups of coffee and inspiring conversations, for reading my thesis and commenting on my English (all remaining errors are to blame her and not me). Thank you!

Thomas Malfait, May 2010

(background picture: This is a rendered 3D image of a melanoma cell using ion abrasion scanning electron microscopy; Image courtesy of Donald Bliss and Sriram Subramaniam; National Library of Medicine, NIH)
### List of abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AJCC</td>
<td>American joint committee on cancer</td>
</tr>
<tr>
<td>ALM</td>
<td>Acral lentiginous melanoma</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-raf oncogene</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CM</td>
<td>Cutaneous melanoma</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxy-ribonucleic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
</tr>
<tr>
<td>GGR</td>
<td>Global growth ratio</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>HMG-CoA (RI)</td>
<td>3 - Hydroxy – 3 - methylglutary coenzyme A (reductase inhibitors)</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>MC1R</td>
<td>Melanocortin 1 receptor</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MMAC1</td>
<td>Mutated in multiple advanced cancers 1</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>NM</td>
<td>Nodular melanoma</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung cancer</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphate deleted on chromosome ten</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>RHC</td>
<td>Red hair color associated MC1R alleles</td>
</tr>
<tr>
<td>RhoC</td>
<td>Ras-homologous C</td>
</tr>
<tr>
<td>RhoGTPase</td>
<td>Ras-homologous guanine triphosphate</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SSM</td>
<td>Superficial spreading melanoma</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, Node and Metastasis</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet radiation</td>
</tr>
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</table>
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A. Summary (Dutch)
B. Videos of experiments
1 Abstract

**Background:** Although cutaneous melanoma (CM) comprises only ± 10% of all skin neoplasms, it is still responsible for three quarters of all deaths caused by skin cancer. Incidence rates are still increasing in many countries; mortality rates, however, seem to have leveled off, especially in the young cohorts. Management of early diagnosed lesions may be curative in as many as 97% of the cases. Unfortunately, due to its resistance to immuno-, chemo- and radiotherapy, treatment of advanced CM has been disappointing. Once CM has metastasized, 10-year survival drops below 20%. The future of melanoma therapy appears promising with emerging new molecular targeted therapies. Considering this aspect, we investigated whether RhoC could be a target for future therapy. RhoC is a member of the RhoGTPases, a distinct family of proteins within the superfamily of Ras-related small GTPases. Mounting evidence supports the knowledge that upregulation of RhoC is correlated with a worse prognosis, faster growth and metastatic tendency in many cancer cell lines and human cancers, including cutaneous melanoma.

The pleiotropic functions of Rho proteins include cytoskeletal reorganization during cellular motility, cell adhesion control and gene expression. In addition, Rho proteins are involved in transcriptional activation, growth processes, vesicle trafficking, enzyme regulation, cell cycle progression and apoptosis. Since transformation of a normal cell into a cancer phenotype is associated with changes in one or more of the above features, involvement of aberrant Rho signaling in tumor cells is not surprising.

RhoC protein is activated by isoprenylation, a necessary step for its correct localization, protein stability and downstream effects. Isoprenylation of RhoC requires geranylgeranyl pyrophosphate (GGPP), an intermediate in the HMG-CoA reductase pathway, also called the mevalonate or isoprenoid pathway. HMG-CoA reductase, the rate-limiting enzyme of this pathway, is inhibited by statins, resulting in a decrease of cholesterol synthesis. These widely prescribed anti-cholesterol drugs not only lead to a decrease in cardiovascular disease and mortality from coronary heart disease, but have also shown several other unintended beneficial effects. Epidemiological, in vitro and in vivo studies have suggested a protective effect towards cancer. A recent multiple linear regression study with 1318 melanoma cases and 6786 controls concluded that statin use is associated with a significantly reduced Breslow thickness, an important prognostic factor in cutaneous melanoma.

One of the proposed mechanisms by which statins could exert their protective effects towards tumors is the mevalonate pathway. By blocking this pathway, statin treatment leads to a depletion of GGPP thereby inhibiting the activation of isoprenylated proteins, such as RhoC.

**Purpose:** The purpose of this thesis was to study the effects of statins on melanoma cell lines through continuous phase-contrast cellular video microscopy. Second, correlation between the lipophilicity of different statins and their effectiveness of inhibiting cell proliferation in melanoma cell lines was
studied. We also investigated whether the anti-cholesterol properties of statins correlated with their effectiveness of inhibiting cell proliferation in different melanoma cell lines. As a last strategy, we tried to further assess the role of RhoC in melanoma proliferation.

**Methods:** First, in vitro effects of three different concentrations (50, 500 and 5000 nM) simvastatin treatment on melanoma BLM cell lines were studied through continuous phase-contrast microscopic cellular imaging during three days. The “Global Growth Ratio” (GGR), a measure of cell growth level, was calculated in each treated condition and compared to a control sample. Second, MTT assays of the five in Belgium available statins (atorvastatin, pravastatin, rosuvastatin, simvastatin and fluvastatin) were performed on four different human melanoma cell lines (G361, HT144, SKMEL-28 and C32) and one murine melanoma cell line (B16F10) in order to calculate the IC50, a measure of the effectiveness of reducing the global growth of cancer cells. Correlation was then studied between the IC50 and the anti-cholesterol properties of the statins. We also investigated whether there was a correlation between statin lipophilicity and their IC50.

Third, to further assess the role of RhoC deactivation in the effects of statins on melanoma cell lines, melanoma cell lines were electroporated with siRNA for RhoC and studied through continuous phase-contrast microscopic cellular imaging during six days. These pictures were then analyzed to calculate the GGR and compared to two different control samples. To confirm the results of these experiments, we studied the effects of SiRhoC and simvastatin on BLM melanoma cell lines by investigating the overall increase of cell area during a period of 72 hours.

**Results:** First, BLM cell lines treated with the highest concentration simvastatin (5000 nM) were associated with a significant decrease of GGR after incubation for 48 and 72 hours. Second, no correlation between the anti-cholesterol properties and the IC50 of the different statins was found. However, statin lipophilicity correlated with their strength of inhibiting melanoma cell lines. Pravastatin failed to show an inhibitory effect on each of the different cell lines. Last, RhoC silencing (SiRhoC) did not show anti-proliferative effects on the BLM melanoma cell lines. In a second experiment, SiRhoC showed a progressive increase of cell area over a period of 72 hours, also indicating that SiRhoC had no anti-proliferative effects.

**Conclusion:** This study confirms that simvastatin has anti-proliferative effects on BLM melanoma cell lines, but only in the highest concentration (5000 nM) and only after 48 hours of incubation. These effects probably occur at a concentration of 3 µM simvastatin. We found evidence that statin lipophilicity could play a role in their degree of inhibiting melanoma cell lines. The more lipophilic statins were correlated with a more efficient inhibition of the melanoma cell lines. RhoC silencing did not show the same remarkable effects as the statin treatment, meaning that we could not confirm that the anti-proliferative effects of statins are mediated by RhoC. However, RhoC could still play a role in invasion and/or migration of melanoma cell lines. Further studies will address the question whether adding statins to standard chemotherapy can increase the anti-proliferative effects on melanoma cell lines and whether RhoC might be a valuable target for molecular therapy.
2 Introduction

The first section of this introduction will describe some general aspects of cutaneous melanoma: epidemiology, risk factors, classification, diagnosis, staging and therapy.

The second section will highlight the importance of RhoC in cancer and, more specifically, in cutaneous melanoma.

The third and last section of this introduction focuses on the effects of statins on cancer and cutaneous melanoma in particular.

2.1 Cutaneous Melanoma

2.1.1 Epidemiology

Skin cancer can be broadly categorized into two groups: melanoma and non-melanoma. Non-melanoma skin cancer is the most common skin cancer and the most common cancer in the world (10), of which the vast majority is curable (2). Although cutaneous melanoma (CM) comprises only about 10% of all skin neoplasms, it is still responsible for approximately three quarters of all deaths caused by skin cancer (11).

Worldwide, an estimated 160,000 new cases of melanoma were diagnosed in 2002, representing 1.47% (12) of all newly diagnosed cancers. CM is responsible for 41,000 deaths each year (13).

The highest incidence rates are seen in Australia and New Zealand with 30 to 60 newly diagnosed cases per 100,000 inhabitants each year (11). In these countries CM represents the fourth most common cancer (14).

In the United States, it is predicted that 68,720 new cases will have been diagnosed in 2009, making CM the fifth and sixth most common cancer in men and women respectively. During the last decades American incidence rates have shown a three to fivefold increase, which makes CM the most rapidly increasing cancer in white populations (12).

Even though European incidence rates are still lower, they also follow these recent trend of increasing three to fivefold during the last decades (11). In Germany for instance, cancer registration has estimated a threefold increase during three decades (Figure 1) (15).

In Belgium, incidence rates of malignant melanoma have increased approximately by one-third in the period of 1999-2005. Cutaneous melanoma has become the second most important malignancy in males in the age group of 30-44 years. In the age group of 15-29 years, malignant melanoma is the most common cancer in women (16).

The male to female ratio varies in different countries. In countries with a high CM incidence, such as Australia and the United States, a higher incidence is observed in the male population. Countries with a lower incidence, such as Great Britain, Germany and Belgium, have a higher ratio of female patients with melanoma (11, 14, 16).
These mounting evidences of increasing incidence have made CM a cancer with growing medical importance (11).

In contrast to the incidence rates, mortality rates of melanoma have recently leveled off in many countries. Some studies have even described a decrease in mortality rates (11), especially in the younger cohorts (17). However, because CM especially affects young and middle-aged people, CM still represents a considerable health problem (18). The median age is about 55 years, which means that 50% of all CMs are already diagnosed before this age (11).

Figure 1: European age-standardized incidence and mortality rates in Germany, 1980 – 2004, cases/deaths per 100,000 (15).

The discrepancy in the rate of incidence increase and the favorable mortality trends has led to the hypothesis of “non-metastasizing melanoma”. One argument is that there is an over- or misdiagnosis of early not biologically significant lesions, leading to an inflation of the incidence. Another interpretation of this discrepancy is that, due to greater awareness of melanoma, malignant lesions are being found earlier. These lesions have a smaller Breslow depth, one of the most important prognostic factors in CM, resulting in decreased mortality (1).

2.1.2 Risk factors
Melanoma is considered as a genetically heterogenic disease and results from multiple interacting factors. A consistent body of evidence has identified important risk factors of which some contribute to the worldwide increase of melanoma incidence. These risk factors can be divided into three groups: environmental, host and genetic factors, summarized in Table 1 (1).
Environmental risk factors

Sun exposure Ultraviolet radiation (UVR) is the most investigated and also the most important environmental factor associated with melanoma. Different patterns of sun exposure have different effects on the development of melanoma. Chronic sun exposure (i.e. received during outdoor work on a daily basis) does not increase risk for melanoma and is in fact associated with a reduced risk (1, 17). Total sun exposure, however, is associated with a modest risk, whereas intermittent sun exposure (i.e. large amounts of UVR, received on weekends or holidays) confers the highest risk. The latter is the major pattern of UVR promoting melanoma development (1).

Different patterns of UVR exposure also have different effects on mutations found in melanoma: excessive sunlight exposure in youth increases the risk of melanoma associated with BRAF mutations. Among older individuals, cumulative sun exposure over a lifetime is associated with NRAS mutations (17). These findings support the idea of distinct genetic patterns in different primary melanoma subtypes, as discussed below (19).

Sun beds Increasing evidence points to the negative effects of tanning beds. A recent meta-analysis revealed that first exposure at young age is the most harmful. Ever and, surprisingly, never use of sun beds are both associated with increased melanoma risk (1, 17).

Sunburns A dose-response relationship with the number of sunburns and melanoma has been suggested. Timing of the sunburns appears important and confers an increased risk (1).

Host risk factors

Melanocytic nevi Nevi are the strongest risk factor for the development of melanoma. Studies have shown that people with a higher than average number of moles (40 or more on the back are considered high) or with dysplastic nevi, have a substantially increased risk for melanoma (17). The risk conferred by dysplastic nevi is much higher than those by common acquired nevi (1). The way in which the type and number of nevi may be involved has been considered in different ways. One theory is that nevi are on the causal pathway and some nevi develop into melanoma (17). Although it has been shown that nevi are not obligate precursor lesions, they can be used as risk markers.

Family history First-degree relatives of melanoma patients have a higher risk of developing the disease than individuals without positive family history, indicating the existence of a distinct hereditary component (17). CM is considered hereditary when two first-degree relatives or three relatives, irrespective of the degree of relationship, have been diagnosed with CM. Familial melanoma is seen in as many as 10% of melanoma cases. If a relative has a history of melanoma, the patient’s risk factor increases two to eight times (8).

Phenotypic characteristics Phenotype is an independent risk factor for the development of melanoma: light hair color, light eye color, poor tanning ability and light skin color, including skin that freckles easily. These phenotypic characteristics are highly correlated with melanoma (1, 17).
Genetic risk factors
The last decades, major advances have been made in identifying genetic factors contributing to melanoma susceptibility (17).

Pigmentation genes
The most important gene among the pigmentation genes known to be associated both with melanoma and phenotypic characteristics is the melanocortin 1 receptor (MC1R) gene (17). Some polymorphisms in the MC1R gene are associated with the red hair color phenotype (RHC) and others are not (rhc). Both polymorphisms result in increased melanoma risk (1, 17).

Individuals with multiple primary melanomas and a young age of onset are significantly more likely to carry multiple MC1R variants (1).

Other genes
Many other genes have been implicated in the development of melanoma, including CDKN2A (p16), CDK4, RB1, CDKN2A (p19), PTEN/MMAC1, ras and DNA repair genes (8, 17).

CDKN2A (p16), the familial melanoma gene, is rarely mutated among those with sporadic melanoma, although it accounts for approximately 30% of mutations in those with a hereditary form (17).

Because the increasing melanoma incidence is related to changing attitudes of leisure time behavior and of sun exposure, prevention and public health measures are essential to decrease the risk of melanoma (11). Within these three subgroups of risk factors, individual factors can be considered to be modifiable or fixed. By targeting modifiable risk factors, it may be possible to reduce the lifetime risk of melanoma or skin cancer in general (10).

2.1.3 Classification of cutaneous melanoma
Cutaneous melanomas are being classified into four major histogenetic subtypes: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM). The most important clinical and histological findings are summarized in Table 2. Besides these frequent subtypes, a wide variety of other uncommon subtypes of melanoma exists, which are not discussed in this thesis.
Superficial spreading melanoma (SSM)

SSM (Figure 2) is the most common form of CM in Caucasians (approximately 70%) (2, 8). It is usually found in patients in the fourth and fifth decade, more specific in “white-collar professions” (2). The lesion outline is usually sharp, yet irregular with variations in color, including pink, violaceous, black, brown, tan and rarely blue or white (7-8). It can arise on any body surface, but shows a preference on trunk in males and lower extremities in females (8).

After an initial radial growth phase, which can last from several months to a few years, the tumor becomes elevated and forms a prominent nodule or papule that can extend several millimeters above the skin surface (2, 7-8). This vertical growth phase is potentially more dangerous, because of its metastatic tendency and worse prognosis (2). High cure rates, however, can be achieved when these lesions are detected and treated in the early radial growth phase (8).

Nodular melanoma (NM)

NM (Figure 3) represents about 15% of melanomas and tends to develop in patients of middle age with a slight male preponderance. These lesions display, by definition, an immediate vertical growth phase (2, 7-8).

Clinically, these lesions usually present as a relatively uniform dark brown, black or blue-black nodular elevated tumor and are commonly found on both sun-exposed and non-sun-exposed body surfaces (2, 8).

Lentigo maligna melanoma (LMM)

Lentigo maligna (Figure 4) is a pre-invasive pigmented lesion, frequently found on head and neck. It typically arises on chronically sun damaged body surfaces. These lesions account for approximately 10% of cutaneous melanomas (2, 7-8). Only a small amount (± 5%) of LMs progress to the invasive LMM (8).

Clinically, LMMs are quite large and flat neoplasms, displacing a variety of coloration that includes tan, brown and black. They are characterized by a slow radial phase, sometimes taking up to 10 years before progressing towards vertical growth (2, 7-8).
Acral lentiginous melanoma (ALM)

ALM (Figure 5) represents about 5% of melanomas in Caucasians. In black population, however, it is the most common subtype (approximately 70%). ALMs are found on acral surfaces: soles, palms, ungleal and peri-ungeal surfaces. ALM is often diagnosed late, because it can be mistaken for other skin lesions, such as warts (2, 8).

Molecular classification of primary cutaneous melanoma

The above, traditional classification system for primary cutaneous melanoma has centered on clinical and pathological aspects. Although these melanoma subtypes are clinically and histopathologically distinct, such a classification is without independent prognostic value. Recent studies have shed new light on the molecular events associated with melanoma subtypes (20).

It is now clear that distinct patterns of genetic alterations exist in the four groups of primary melanomas. There are differences in both chromosomal aberrations and the frequency of specific gene mutations, suggesting the existence of different developmental pathways (19).

Newer tumor classification strategies, i.e. according to the degree of sun exposure and associated molecular defects, will advance our understanding of the heterogeneity of this disease and could be valuable in the design of targeted therapies (19-20).
<table>
<thead>
<tr>
<th>Clinical findings</th>
<th><strong>Superficial Spreading Melanoma</strong></th>
<th><strong>Lentigo maligna melanoma</strong></th>
<th><strong>Nodular Melanoma</strong></th>
<th><strong>Acral Lentiginous Melanoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Color</strong></td>
<td>Tan, brown, gray, black, violaceous, pink. Rarely blue or white</td>
<td>Tan, brown, black, flecks of pigment</td>
<td>Brown, black, blue-black</td>
<td>Similar to SSM. Striking black color, dusky blue or amelanotic region</td>
</tr>
<tr>
<td><strong>Outline</strong></td>
<td>Sharply margined Peninsula-like protrusions</td>
<td>Irregular outline</td>
<td>Plaque or nodule without surrounding flat pigmented lesion</td>
<td>Irregular borders. May extend onto the proximal or lateral nail fold (Hutchinson’s sign)</td>
</tr>
<tr>
<td><strong>Shape</strong></td>
<td>Palpable papule or nodule</td>
<td>Flat, with rare papule</td>
<td>Smoothly surfaced nodule, ulcerated polyp, or elevated plaque</td>
<td>Papular or nodular components, associated with dermal involvement. Advanced lesions often ulcerate and become hemorrhagic. Often cause nail dystrophy</td>
</tr>
<tr>
<td><strong>Anatomic site</strong></td>
<td>Trunk, extremities</td>
<td>Face and neck</td>
<td>Trunk, extremities</td>
<td>Acral surfaces, specifically the palms, soles and nails. It occurs most frequently on the plantar surface of the feet</td>
</tr>
<tr>
<td><strong>Sun exposure</strong></td>
<td>Intermittent</td>
<td>Chronic</td>
<td>Intermittent</td>
<td>?</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histological findings</th>
<th><strong>Superficial Spreading Melanoma</strong></th>
<th><strong>Lentigo maligna melanoma</strong></th>
<th><strong>Nodular Melanoma</strong></th>
<th><strong>Acral Lentiginous Melanoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-epidermal melanocytic proliferation</strong></td>
<td>Pagetoid and nested epithelial cells with amphophilic or finely pigmented cytoplasm, prominent nucleoli common</td>
<td>Increased density of individual melanocytes along the dermal-epidermal junction, large densely chromatic nuclei, multinucleated cells, extension down hair follicles</td>
<td>Minimal and only directly overlying the dermal tumor, no intra-epidermal nested melanocytic proliferation more than three rete lateral to the dermal tumor</td>
<td>Acanthosis, elongation of rete ridges, and lentiginous proliferation of atypical melanocytes</td>
</tr>
<tr>
<td><strong>Epidermis</strong></td>
<td>Hyperplasia</td>
<td>Atrophy</td>
<td>Atrophy or hyperplasia</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td><strong>Intradermal melanocytic proliferation</strong></td>
<td>Nests of variable sizes, expansile tumor nodule, cytology similar to epidermal component</td>
<td>Nested or infiltrative, epitheloid or spindle cells, may be similar to SSM and NMM</td>
<td>Small nests and aggregates of tumor cells that form an expansile nodule</td>
<td>Spindle cells, although epitheloid cells, small nevus-like cells or highly pleomorphic cell types could also be present</td>
</tr>
</tbody>
</table>

Table 2: Summary of the differences in clinical and histological findings in the four most common types of melanoma. 
1 adapted with permission from (7) 
2 adapted from (9)
2.1.4 Diagnosis

Key characteristics of clinical suspicious lesions can be memorized using the mnemotechnic “ABCDE”: Asymmetry, Border irregularities, Color heterogeneity, Diameter and Evolution or Elevation (Table 3) (8, 21).

These criteria are most accurate when used in combination. Lesions exhibiting these features should be considered suspicious. Clinically distinguishing CM from severely atypical nevi may be difficult (8). Therefore, dermoscopy, carried out by an experienced examiner, can improve the diagnostic accuracy (21).

When a pigmented lesion is suspected for malignancy, it is important to perform a diagnostic excision biopsy, followed by a histopathological examination in an experienced pathology institute. Since Breslow thickness (maximum thickness of the lesion in millimeters) of a melanoma is one of the major prognostic factors in local disease and since it will also set the outlines for further management, it is important that a pathologist can evaluate a suspected lesion as a whole. (22)

Other factors that should be included in the histology report are level of invasion, presence of ulceration, presence and extent of regression and clearance of the surgical margins (21).

Once histologically confirmed, a therapeutic excision is the logical next step in the approach of CM (22). The objective of this wider therapeutic excision, after the initial diagnostic excision, is to reduce the risk of local recurrence. It is assumed that small aggregates of melanoma cells can be present in the immediate surroundings of the primary tumor, depending on the tumor thickness (22).
2.1.5 Clinical and pathologic staging

Staging of melanoma patients consists of a clinical examination and some para-clinical investigations. The clinical examination includes a total skin inspection, since the patient is at risk of developing a second primary melanoma. In about 40% of the cases such a second primary tumor presents synchronously with the first (22).

Palpation and inspection of the skin between the melanoma and the lymph node station is mandatory in order to detect satellite or in-transit metastases. A careful palpation of the regional lymph node stations and other stations (axilla, groin, neck) is performed. Further investigations are redundant in case of in situ melanoma. In case of thick invasive melanoma, a more elaborate investigation with CT scans of brain, chest and/or abdomen depending on the Breslow thickness is recommended in order to allow proper staging (21-22).

During the last 7 years, the 6th refined version of the American Joint Committee on Cancer (AJCC) staging and classification system for melanoma, has been the classification system of choice (22). Recently, begin 2010, the AJCC has approved and published the 7th version of the melanoma TNM staging system. These new recommendations, based on a multivariate analysis of 30,946 patients with Stage I, II and III melanoma and 7972 patients with Stage IV melanoma, contain some minor changes. For instance, after 40 years of being an integral component of melanoma staging, the Clark level is no longer recommended as a staging criterion. Since it is not an independent prognostic factor when mitotic rate is included in the analysis, Clark level can be replaced by the mitotic rate of the primary tumor. Increasing mitotic rate is associated with declining survival rates, especially within thin melanoma subgroups. In clinically localized melanoma, mitotic rate is the second most powerful predictor of survival after tumor thickness. The new proposed version of TNM categories along with the 5-year survival rates are summarized in Table 4 (23-24).

According to thickness, ulceration status, primary tumor mitotic rate, number of metastatic nodes and (site of) metastasis patients are subdivided into four broad stages (I-IV).

Patients with primary melanomas who have no evidence of regional or distant metastases are divided into two stages: Stage I for patients at low risk for metastases and melanoma-specific mortality or Stage II for those with intermediate risk for metastases and melanoma-specific mortality. The presence of melanoma ulceration “upstages” the prognosis compared to patients with non-ulcerated melanomas of equivalent thickness.

Stage III is characterized by the presence of regional metastases. There are five major determinants of outcome for pathological Stage III melanoma: (1) the number of tumor-bearing lymph nodes; (2)
tumor burden at the time of staging (i.e. microscopic or macroscopic); (3) the presence or absence of ulceration of the primary melanoma; (4) thickness of the primary melanoma and (5) the presence or absence of satellite or in-transit metastases (23).

Once melanoma has metastasized, the patient is immediately classified in Stage IV. Survival rates for patients with Stage IV melanoma are, unfortunately, more measured in months than in years. Only a minority of Stage IV patients survive beyond one year (25).

### TNM Staging categories for Cutaneous Melanoma

<table>
<thead>
<tr>
<th>T</th>
<th>Thickness (mm)</th>
<th>Ulceration Status/Mitoses</th>
<th>Stage</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>T1</td>
<td>≤ 1.00</td>
<td>a: Without ulceration and mitosis &lt; 1/mm²</td>
<td>IA</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: With ulceration or mitosis ≥ 1/mm²</td>
<td>IB</td>
<td>94%</td>
</tr>
<tr>
<td>T2</td>
<td>1.01 - 2.00</td>
<td>a: Without ulceration</td>
<td>IB</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: With ulceration</td>
<td>IIA</td>
<td>82%</td>
</tr>
<tr>
<td>T3</td>
<td>2.01 - 4.00</td>
<td>a: Without ulceration</td>
<td>IIA</td>
<td>79%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: With ulceration</td>
<td>IIB</td>
<td>68%</td>
</tr>
<tr>
<td>T4</td>
<td>&gt; 4.00</td>
<td>a: Without ulceration</td>
<td>IIB</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: With ulceration</td>
<td>IIC</td>
<td>53%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>No. of Metastatic Nodes</th>
<th>Nodal Metastatic Burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>N1</td>
<td>1</td>
<td>a: Micrometastasis¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: Macrometastasis²</td>
</tr>
<tr>
<td>N2</td>
<td>2 - 3</td>
<td>a: Micrometastasis¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b: Macrometastasis²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c: In-transit metastases/satellites without metastatic nodes</td>
</tr>
<tr>
<td>N3</td>
<td>4 + metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes</td>
<td>IIC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>Site</th>
<th>Serum LDH</th>
<th>1-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastases</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M1a</td>
<td>Distant skin, subcutaneous or nodal metastases</td>
<td>Normal</td>
<td>IV</td>
</tr>
<tr>
<td>M1b</td>
<td>Lung metastases</td>
<td>Normal</td>
<td>IV</td>
</tr>
<tr>
<td>M1c</td>
<td>All other visceral metastases</td>
<td>Normal</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Any distant metastases</td>
<td>Elevated</td>
<td>IV</td>
</tr>
</tbody>
</table>

Table 4. TNM Staging categories for Cutaneous Melanoma

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase; T, Tumor; N, Node; M, Metastasis; No., number

¹Micrometastases are diagnosed after sentinel lymph node biopsy

²Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically

³Depending on whether the primary tumor presents with/without ulceration

Adapted with permission from (23)
Individualized patient prognostic models and electronic prediction tools for localized and regional melanomas have been developed. These electronic prediction tools incorporate a recent AJCC database analysis, which, in contrast to the TNM-based AJCC melanoma staging system, includes key prognostic features, such as lesion site and patient age. They may therefore be more accurately in estimating survival than the recently validated 7th edition of the AJCC staging system. An initial version of such an individualized patient prognostic model is currently available on the Internet (http://www.melanomaprosnosis.org) (24, 26).

2.1.6 Therapy

Current guidelines for melanoma therapy subdivide the management according to the clinical stage: localized, locoregional metastatic or systemic metastatic disease (22, 27).

Management of local disease (Stage I-II)
The management of local disease starts with the wide excision of the primary tumor(s) depending on the Breslow thickness; 0.5 cm margin for in situ melanomas, 1 cm margin with a Breslow thickness up to 1 mm and 2 cm for thicker tumors. Routine elective lymphadenectomy or elective irradiation of the regional lymph nodes is not recommended, as the procedure does not afford any survival benefit and could in fact produce considerable morbidity (21, 27).

In this light, the technique of the sentinel node biopsy was developed. During this procedure the first draining lymph node of the cutaneous melanoma is excised and its histological status is used to predict the condition of the other lymph nodes in the station: in case of the absence of micrometastasis, the other draining lymph nodes are considered free of tumor (22). The risk of occult regional metastasis increases with increasing Breslow thickness. T1 to T4 tumors confer a risk of respectively 4%, 9%, 18% and 25% of occult regional metastasis (22, 27).

Although at this moment there are no indications for a survival benefit of patients undergoing this selective lymph node dissection, information obtained by this technique may be useful in planning subsequent treatments and follow-up regimens. Furthermore, staging with sentinel node technology should be required before entering clinical trials involving new surgical techniques or adjuvant therapy (22-23, 27).

Management of locoregional metastatic disease (Stage III)

If a palpable lymph node causes suspicions of melanoma metastasis, a lymph node biopsy can be performed to get histological confirmation (22). In the case of isolated locoregional lymph node metastases, a full dissection of the lymph node station is indicated, since removal of the tumor-bearing lymph node alone is insufficient (21-22, 27).
Management of systemic metastatic disease (Stage IV)

The classical chemotherapy in Stage IV patients is palliative and is only started in patients with clear metastatic disease in fairly good health. Patient selection is based on a multidisciplinary decision taking into account the number (solitary metastases are sometimes an indication for surgery) and location (subcutaneous, lymph node, brains, lung) of metastases, the health status of the patient and his disease-free interval (27).

The palliative therapy for advanced disease with several metastases in different anatomical regions should initially use well tolerated single-agent cytotoxic chemotherapy (i.e. dacarbazine) as any systemic therapy does not result in survival prolongation, but symptom palliation only.

Palliative radiotherapy should be considered especially for symptomatic brain or localized bone metastases (21).

2.1.7 Conclusion

Cutaneous melanoma is the deadliest form of skin cancer and has the fastest growing incidence rate compared to any other cancer. Melanoma results from multiple interacting risk factors. By targeting modifiable risk factors, it may be possible to reduce the lifetime risk of melanoma or skin cancer in general (10).

Traditionally, the classification system for primary cutaneous melanoma has centered on clinical and pathological aspects. However, such a classification is without independent prognostic value. Newer tumor classification strategies will advance our understanding of the heterogeneity of this disease and could be valuable in the design of targeted therapies (19-20).

Early diagnosed lesions may be curative in as many as 97% of the cases (23). Unfortunately, due to its resistance to immuno-, chemo- and radiotherapy treatment of advanced CM has been disappointing (11). Once CM has metastasized, 10-year survival drops below 20% (23). Since no systemic therapy has shown an impact on advanced melanoma, it is acceptable to include these patients in clinical trials, testing novel approaches. The follow-up of such patients should be conducted in specialized centers (22, 27).
2.2 RhoC in cancer and melanoma

2.2.1 RhoGTPases and RhoC

RhoC is a member of the Ras-homologous (Rho) guanosine triphosphate (GTP)-binding proteins, the RhoGTPases, a distinct family of small proteins (20 – 30 kDa) within the superfamily of Ras-related small GTPases (6, 28).

Rho proteins differ from other small GTPases because their sequences contain a Rho insert domain in the GTPase domain. This insert domain has been suggested to be involved in the activation of downstream effector proteins (6).

At this moment over twenty RhoGTPases have been identified (Figure 6). According to primary amino acid sequence, structural motifs and biological function, these proteins are divided into six groups (29): the Rho proteins (RhoA, RhoB, RhoC), the Rac proteins (Rac1, Rac2, Rac3, RhoG), the Cdc42-like proteins (Cdc42, TC10, TCL, Wrch1, Chp), the Rnd proteins (Rnd1, Rnd2, Rnd3/RhoE), the RhoBTB proteins (RhoBTB1, RhoBTB2, RhoBTB3) and the Miro proteins (Miro1, Miro2). Some Rho proteins do not belong to any of these subgroups (RhoD, Rif and RhoH/TTF) and some are classified as atypical, because they are not regulated as the other, classical GTPases (Rnd, Miro and RhoBTB subfamilies, RhoH, Wrch1 and Chp) (6).

These small Rho proteins, acting as molecular binary switches cycling between an active GTP-bound state and an inactive GDP-bound state, play an essential role in a broad range of signaling pathways, such as actin cytoskeletal reorganization during cellular motility, cell adhesion control and gene expression. In addition, Rho proteins are involved in transcriptional activation, growth processes, vesicle trafficking, enzyme regulation, cell cycle progression and apoptosis (28, 30).
Only in the active GTP-bound state, these proteins are able to bind effector proteins and transduce signals from a large variety of membrane receptors, including cytokine and growth factor receptors, adhesion receptors, ligand-stimulated G protein-coupled serpentine receptors, receptor tyrosine kinases and matrix-interacting integrins (Figure 7) (3, 6).

The activity of RhoGTPases in response to receptor stimulation is strictly controlled in order to stimulate, locally and temporally, specific downstream signaling pathways in cells (6).

An important example of this tight regulation is the post-translational prenylation of a conserved C-terminus of RhoA, B and C followed by methylation and proteolytic removal of the last three amino acids.

Prenylation, also called isoprenylation or lipidation, is the addition of hydrophobic molecules to a protein. This process appears to be an essential step for correct localization, stability and downstream effects of Rho proteins (Figure 8). The length of the prenyl group differs between the Rho proteins: RhoB can be prenylated either with a farnesyl or a geranylgeranyl group, whereas RhoA and RhoC are only geranylgeranylated. Inhibition of enzymes that synthesize prenyl groups have shown to induce a decrease in RhoA and B (31).

2.2.2 RhoC in cancer

It is well-known that Ras proteins are frequently mutated in (up to 30% of) human cancers of different origins. Being a distinct family of the Ras superfamily, it has been suggested that the same might hold true for the Rho family of small GTPases. Numerous in vitro and in vivo studies using tumor-derived cell lines, mouse-models and primary tumors clearly indicate that deregulated signaling of small RhoGTPases plays an important role in cancer development as well as the progression of (human) cancer (32).

Unexpectedly, to date, no mutations have been found in Rho proteins (6), except for one, RhoH, of which it is not clear if it plays a significant role in carcinogenesis (32).

Apparently, mutational activation or inactivation of Rho proteins is not favorable for the initiation or progression of tumors. However, deregulation of RhoGTPase signaling can still take place through the level of protein expression or activation of their regulators or downstream effectors.
Within the family of Rho proteins, higher expression of many members can be seen in different cancer-derived cell lines and human cancers. Rho proteins have shown to play a role in non-Hodgkin lymphomas, multiple myeloma, diffuse large cell lymphomas, breast, colon, lung and gastric cancers, as well as head and neck squamous cell carcinoma (HNSCC), bladder and testicular cancer. Furthermore, Rho overexpression in cultured cells has been shown to transform normal cells (32).

Our specific interest was focused on the role of RhoC in melanoma. As stated earlier, RhoC has not been found mutated in cancers, indicating that upregulated expression could be sufficient in its contribution to tumor growth and/or metastasis (31). Several in vitro and in vivo experiments have reported that RhoC overexpression promotes invasiveness, growth and/or metastatic behavior in many different cancer types (33). In breast cancer, RhoC expression has been correlated with tumor stage and lymph node metastasis. RhoC has been shown to be a specific molecular indicator for detecting invasive breast carcinoma with metastatic potential (34). Additionally, Van Golen et al. (1999) reported that the expression level of the RhoC gene is associated with rapid growth in inflammatory breast cancers (35). A recent study suggested that RhoC overexpression is a key genetic alteration in the development of inflammatory breast cancer (36).

Similar findings have been reported in epithelial ovarian tumors. RhoC expression was shown to be higher in serous carcinoma, which are more frequently associated with peritoneal dissemination. Moreover, expression levels of RhoC mRNA were significantly higher in ovarian tumors of Stages III and IV than those of Stages I and II (37). These results are concordant with a recent study by Yang Zhao et al. (2010). RhoC expression progressively increased in an array of ovarian cancer tissue samples ranging from well-differentiated to poorly differentiated. Transfection of ovarian cancer cells with siRNA-RhoC led to a suppression of RhoC gene expression and protein level, which correlated with a reduced ability to invade an artificial membrane or to migrate in vitro. These data strongly suggest a primary role for RhoC in the ability of ovarian tumors to become more invasive and to metastasize (38).

In renal cell carcinoma, high RhoC mRNA expression was associated with higher tumor grade, stage and shortened survival (39). High RhoC protein expression in bladder tumor has been correlated with poor differentiation, muscle invasion, lymph node metastasis and shortened survival (40).

In gastric cancer, expression levels of RhoC in metastatic tumors were significantly higher than in corresponding non-neoplastic mucosae. RhoC appeared to be a good genetic marker of metastatic potential. Conversely, it was observed that inhibition of RhoC expression by siRNA causes suppression of migration and invasion in gastric cell line (41).

RhoC mRNA levels in metastatic adenocarcinomas of the pancreas were shown to be significantly higher in the metastatic lesions than in the primary pancreatic carcinomas (42).
In a lung cancer model in mice, it was demonstrated that RhoC is largely involved in enhancing metastatic activity. RhoC overexpression enhanced migration as well as invasion. Interestingly, there were no differences among groups in macroscopic sizes, volumes or histological findings of the primary tumor, suggesting that RhoC was largely involved in the metastatic activity of lung cancer without modulating the growth or morphological change of the primary tumor. Overexpression of dominant negative Rho inhibited both migration and invasion, resulting in significantly smaller size and weights of mediastinal lymph nodes (43).

In colorectal carcinoma, it was found that the levels of RhoC mRNA transcripts in tumor tissue were significantly higher than in corresponding para-tumor and normal tissues. In addition, expression of RhoC in cancer with metastasized lymph nodes or liver was significantly higher compared to cancer without metastasis and was significantly correlated with the extent of local intestinal invasion. These results suggest the involvement of RhoC in the onset and development, as well as invasion and metastasis of colorectal carcinoma (44).

In non-small cell lung cancer (NSCLC), expression level of RhoC mRNA was positively correlated with the invasiveness of NSCLC, histologically detected as lymphatic and vascular permeation. Since lymphatic permeation and vascular permeation are independent prognostic factors in patients with Stage I NSCLC, overexpression of RhoC may be a significant determinant of the aggressiveness of the disease (45).

In prostate cancer, it was found that RhoC promotes tumor metastasis, but not tumor growth. Expression of RhoC served as a marker to predict metastatic status and survival of patients with prostate cancer. RhoC expression was inversely correlated with patient survival, suggesting that RhoC can serve as a prognostic marker as well as a potential therapeutic target for prostate cancer (46).

It has also been reported that RhoC gene expression in hepatocellular carcinoma was significantly higher in tumor tissue than in corresponding para-tumor normal liver tissues. Furthermore, expression level of RhoC gene was correlated with vein invasion, number of tumor nodes and metastatic lesions, suggesting that RhoC expression level could be an useful prognostic indicator (44).

RhoC promoted tumor growth, cell migration and metastatic capacity in human esophageal squamous cell cancer in nude mice (47). Expression of RhoC in oligodendroglioma was correlated with expression of miR-10b, which in turn, is correlated with glioma grading and malignancy (48). In squamous cell carcinoma of the head and neck, RhoC has been suggested to play an essential role in cell invasion and motility (49).

2.2.3 RhoC in melanoma

The involvement of RhoC has also been implicated in melanoma. In a variety of melanoma cell lines with low and high metastatic potential, a comparison of the gene expression profile showed that RhoC is expressed at higher levels in all metastases of human (A375) and mouse (B16) melanoma cell lines. Induced expression of RhoC, by infecting A375 melanoma cell line with retroviral particles containing
the full-length human RhoC gene, markedly enhanced their metastatic capacity, whereas dominant inhibitory Rho mutants suppressed motility as well as invasion (50).

In a recent study performed by Boone et al. (2009), two melanoma cell lines with different biological behavior were compared: DX3aza, a melanoma cell line with high metastatic capacity, and MeWO, a melanoma cell line with weak proliferative and metastatic capacity. RhoC mRNA expression levels in MeWO were comparable with that of pooled primary melanocytes, whereas they were upregulated in DX3aza. Analogously, RhoC protein expression appeared to be upregulated in the highly metastatic DX3aza compared to the low metastatic MeWo (33).

Although current insights in the role of RhoC in melanoma progression are mainly based on experiments in melanoma cell lines, a recent in vivo study confirmed these in vitro data in melanoma (33). Boone et al. (2009) were the first to investigate RhoC expression in primary melanoma tissue and clinicopathological follow-up data. Enhanced RhoC expression in primary cutaneous melanoma tissue was found to be strongly associated with Breslow thickness and ulceration, two major prognostic parameters correlating with growth of primary cutaneous melanoma. Lymphatic metastasis, disease relapse and mortality tended to be higher in patients with RhoC expression in their primary melanoma. Multivariate analysis revealed that these last associations were linked to the strong association of RhoC expression with tumor thickness and ulceration (33).

2.2.4 Conclusion

Overexpression of RhoC mRNA has been reported in different types of human cancers, such as inflammatory breast cancer (34-36), ovarian cancer (37-38), ductal adenocarcinoma of the pancreas (42), non-small cell lung cancer (45), bladder cancer (40) and lung cancer (43). In addition, studies investigating RhoC expression in gastric cancer (51-52), esophageal cancer (47), oligodendroglioma (48), squamous cell carcinoma of the head and neck (49), primary hepatocellular carcinoma (44), renal cell carcinoma (39) and colon carcinoma (53) showed that RhoC expression was associated with the presence of metastatic disease (33). Furthermore, RhoC was found to be a specific molecular prognostic marker in various tumors (33-34, 40, 44-46).

The involvement of RhoC has also been implicated in melanoma. Highly metastatic melanoma cell lines expressed RhoC at higher levels and enforced RhoC expression enhanced metastatic capacity. In contrast, Rho inhibition suppressed invasion and motility (50). In a recent in vivo study, RhoC expression in melanoma has been associated with thicker melanomas and tendency towards ulceration, two important prognostic factors in cutaneous melanoma (33).
2.3 Statins in cancer and melanoma

2.3.1 Statins

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG-CoA RI) or statins have become widely used cholesterol-lowering drugs. Their few side effects, effectiveness and long term safety make statins a frequently prescribed agent used in the treatment of lipid disorders (54).

Although statins share a common mechanism of action, there are differences in their relative efficacy for improving the lipid profile, as well as in their chemistry and pharmacokinetics. An important example is lipophilicity (55). Statins can be divided into two groups: hydrophilic (pravastatin and rosuvastatin) and lipophilic (simvastatin, atorvastatin and fluvastatin) (4).

For lipophilic statins, passive diffusion through hepatocyte cell membranes is primarily responsible for efficient first-pass uptake, whereas for hydrophilic statins extensive carrier-mediated uptake is the major mechanism (55).

While lipophilicity results in efficient hepatic shunting, the same property results in ready passage through non-hepatic cell membranes and thus, as shown in Figure 9, lipophilic statins inhibit not only the cholesterol synthesis, but also the production of metabolic intermediates in many extra-hepatic tissues (4, 55). On the other hand, because the membrane of extra-hepatic cells consists of lipid bilayers, hydrophilic statins cannot penetrate it and are therefore not capable of reaching the intracellular enzyme of extra-hepatic cells (4). This contributes to the fact that hydrophilic statins exhibit greater hepatoselectivity and fewer side-effects, such as rhabdomyolysis, a common side effect of statins (4, 55).

Figure 9: Lipophilic, in contrast to hydrophilic, statins are capable of inhibiting HMG-CoA reductase in many extra-hepatic cells. This contributes to the fact that they are more likely to cause side-effects, such as rhabdomyolysis (4).
Statins, inhibitors of the rate-limiting enzyme of the cholesterol synthesis pathway, also called the mevalonate or isoprenoid pathway, have shown to decrease cardiovascular morbidity and mortality in primary and secondary prevention trials (56-58).

In addition to their cholesterol-lowering properties, statins seem to exert a number of protective effects against a number of conditions other than cardiovascular disease, including dementia (59-61), fractures (62-63), multiple sclerosis (64) and cancer (65).

These beneficial pleiotropic effects seem to be associated with HMG-CoA reductase inhibition. Inhibiting HMG-CoA reductase not only blocks the synthesis of cholesterol, but also decreases a number of isoprenoid intermediates, particularly farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The latter are important lipid attachments for the posttranslational modification (farnesylation or geranylgeranylation) of proteins including heterotrimeric G-proteins and small GTP-binding proteins. Isoprenylation converts small GTPase from a cytosolic (inactive) state to a membrane-bound (active) state (Figure 10). Hence, decreased levels of these isoprenoid intermediates can have far-reaching biologic consequences (66).

### 2.3.2 Statins in cancer

Although original reports had suggested potential pro-carcinogenic effects of statins, multiple lines of evidence now suggest that statins have anti-cancer properties in humans (67).

Several case-control studies have reported lower rates of cancer in persons using statins than in non-users (68). A statistical significant decreased risk of all cancers was associated with increasing statin use (69). A mini-review considering statins in their effects towards tumor suppression concluded that statins are capable of suppressing the growth of primary malignant cells from patients with chronic lymphocytic leukemia, hairy cell leukemia and immunoblastic cell lymphoma. Use of statins had a striking effect in diminishing the incidence of lung cancer and was associated with a risk reduction of 55% in patients that were using a statin for more than 6 months. In prostate cancer, there was an association between statin use and reduced risk of metastatic prostate cancer. Statins have also shown to suppress the growth of multiple myeloma cells. These results suggest that statins may have clinical potential as chemopreventive agents in at least some types of cancer (70).
In addition, the Air Force/Texas Coronary Atherosclerosis Prevention Study has shown that the lovastatin treated arm, compared to the placebo arm, had a significantly lower number of melanoma (71). A recent multiple linear regression study in 1318 cases and 6786 controls by Koomen et al. (2007) concluded that statin use was associated with a reduced Breslow thickness. This is an important finding since Breslow thickness at diagnosis is one of the strongest prognostic determinants (72).

In contrast to these exciting beneficial effects, recent meta-analyses have mainly concluded that the effect of statin on cancer incidence was close to null in randomized clinical trials (RCT), cohort studies and nested case-control studies (73-76). In the same study of Koomen et al. (2007), none of the statins used in this study consistently supported a risk reduction on the incidence of CM (72). A Cochrane Review published similar results; however, the authors stated that further exploration of statins in melanoma prevention is warranted to obtain sufficient power to exclude or confirm a clinically potential effect (77). These findings are in concordance with the most recent meta-analysis of 16 RCTs which concluded that statins do not offer any substantial increase or reduction in melanoma cancer (Figure 11) (5).

Despite massive amounts of epidemiological data, the topic remains inconclusive due to some limitations (78). A main issue in many epidemiological studies is cancer latency, the lag time between exposure to an agent and the effects on the disease; the majority of epidemiological studies published data of relatively short follow-up. Secondly, most of the epidemiological studies were designed to analyze the anti-cholesterol properties of statins, rather than the anti-cancerogenous. A third remark concerns the fact that, although synthesis of the existing randomized data could not support the hypothesis that low doses statins reduce the risk of melanoma, a risk reduction associated with higher doses has not been studied yet in full extent (5). A last remark is that some studies do not make a distinction between lipophilic and hydrophilic statins, which, as stated earlier, have other extra-hepatic effects (78).
The unsettled epidemiological data have been further investigated in various in vitro studies, yielding promising results regarding the chemoprevention of melanoma (5). Glynn et al. (2008) tested a range of melanoma cancer cell lines for their sensitivity to four statin drugs: simvastatin, lovastatin, mevastatin and pravastatin. The lipophilic statins (simvastatin, lovastatin and mevastatin) were shown to inhibit proliferation of all tested cell lines, whereas the hydrophilic pravastatin, did not show any inhibition. As stated earlier, a possible explanation of this difference may be the fact that, unlike the lipophilic statins, hydrophilic statins need a specific transporter to mediate their uptake. This transporter is absent on most of the extra-hepatic cells (54).

In a recent study by Saito et al. (2008), simvastatin was shown to induce apoptosis in A375M and G361 melanoma cell lines. It was elucidated that simvastatin treatment resulted in a G1 arrest of cell cycle progression, through upregulation of p21 and p27, two tumor suppressor proteins belonging to the cyclin-dependent kinase inhibitors. Expression of p21 has been reported to be inversely associated with high AJCC stage and recurrence free survival, whereas increased expression of p27 was significantly associated with decreasing tumor thickness and correlated with prolonged disease-free survival in primary nodular melanoma (79).

These results are in concordance with a study by Collisson and colleagues (2003). Atorvastatin has shown to inhibit invasion in A375M melanoma cells in a dose-dependent manner, without causing toxicity. RhoC expression was associated with increased expression levels of SRF, a downstream protein activated by different Rho proteins. Treatment of A375M cells with atorvastatin inhibited the RhoC-augmented transcription of SRF. A specific geranylgeranyl transferase inhibitor produced a similar effect, indicating that inhibition of geranylgeranylation is most likely responsible for the reduced transcriptional activity of treated cells. Similarly, atorvastatin treatment prevented invasion of melanoma cells in a Matrigel invasion assay (54). In contrast to these in vitro results, using in vivo imaging, Collisson et al. demonstrated that subcutaneous implanted tumors proliferated at similar rates in both treated and untreated animals. However, when treated with atorvastatin, the colonization of the pulmonary stroma with hematogenously seeded melanoma cells was prevented. These results suggest that atorvastatin treatment exerted an anti-metastatic effect due to inhibition of adherence, extravasation, seeding or colonization of the lung beds rather than through an anti-proliferative mechanism (54).
2.3.3 Conclusion

Statins have been widely investigated in epidemiological studies. Some have supported a protective effect towards cancer, others concluded that statins have no effect.

Epidemiological studies including higher doses of statins and/or discrimination in lipophilicity are therefore warranted. These studies need to be designed to specifically study the anti-cancerogenous properties of statins for a sufficiently long period.

In vitro studies are quite univocal in demonstrating a beneficial effect towards cancer. Since therapy for advanced melanoma is still disappointing and since statins have a good safety profile and are well tolerated, further in vitro and in vivo studies investigating statins in melanoma are recommended.

2.4 Thesis objectives

The primary objective of this thesis was to investigate the effect of statins on melanoma cell lines through continuous phase-contrast cellular video microscopy. To this end, the anti-proliferative effects of three different concentrations (50, 500 and 5000 nM) simvastatin on melanoma BLM cell lines were studied through continuous phase-contrast microscopic cellular imaging during three days. The GGR, a measure of cell growth level, was calculated in each condition and compared to a control sample.

Second, MTT assays of the five in Belgium available statins (atorvastatin, pravastatin, rosuvastatin, simvastatin and fluvastatin) were performed on four different human melanoma cell lines (G361, HT144, SKMEL-28 and C32) and one murine melanoma cell line (B16F10) to calculate the IC50, a measure of the effectiveness of reducing the global growth of cancer cells. Correlation was then studied between the IC50 and the anti-cholesterol properties of the statins. We also investigated whether there was a correlation between statin lipophilicity and their IC50.

Third, to further assess the role of RhoC deactivation in the effects of statins on melanoma cell lines, melanoma cell lines were electroporated with siRNA for RhoC and studied through continuous phase-contrast microscopic cellular imaging during six days. These pictures were then analyzed to calculate the GGR and compared to two different control samples.

To confirm the results of these experiments we studied the effects of SiRhoC and simvastatin on BLM melanoma cell lines by investigating the overall increase of cell area over a period of three days.
3 Materials and Methods

3.1 Literature search

Databases Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (http://www.isiknowledge.com) were consulted in acquiring relevant literature. Most important used search terms were: cutaneous, melanoma, epidemiology, classification, treatment, statins, RhoC, RhoGTPase(s), … Search results were limited to articles published in the last 10-15 years. Information about the anti-cholesterol effect of the five different statins used in the experiments were also obtained by literature search.

3.2 Cell Lines

The BLM melanoma cell line, kindly provided by Koen Jacobs (Experimental Cancer Research, University Ghent) was maintained in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (Greiner Bio-One, Belgium), 100 IU/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 2.5 µg/mL fungizone (Invitrogen). Cells were housed as monolayer cultures at 37°C, 99% humidity and 10% CO₂. Cells were passaged using 0.05% (w/v) trypsin and 0.02% (w/v) EDTA.

The four human melanoma cell lines (HT-144, G-361, C-32 and SK-Mel-28) and the murine melanoma cell line (B16F10), kindly provided by Dr. Veronique Mathieu and Prof. Robert Kiss (Institute of Pharmacy, ULB), were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultivated at 37 °C, at an humid atmosphere and 5% CO₂ in Roswell Park Memorial Institute medium (RPMI) (GibcoBRL/Life Technologies/Invitrogen, Merelbeke, Belgium), to which 10% fetal calf serum (FCS; GibcoBRL) was added, supplemented with a mixture of 0.6 mg/ml glutamine (GibcoBRL), 200 IU/ml penicillin (GibcoBRL), 200 IU/ml streptomycin (GibcoBRL) and 0.1 mg/ml gentamicin (GibcoBRL). The FCS was heat-inactivated for 1 hour at 56 °C. All cells were housed as monolayer. When cells were passaged, the medium was removed, cells were quickly rinsed with trypsin-EDTA and then incubated in trypsin-EDTA for two to five minutes at 37 °C. Trypsin-EDTA was inactivated by the serum of the culture medium. Cells were then subcultured at variable rates depending on the cell line.

3.3 Reagents

Atorvastatin (Lipitor, Pfizer), pravastatin (Prareduct, Sankyo), rosuvastatin (Crestor, AstraZeneca), simvastatin (Zocor, MSD) and fluvastatin (Lescol, Novartis), kindly provided by Dr. Tine De Backer (Cardiology, UZ Ghent), were obtained in tablet form (respectively 20 mg, 40 mg, 10 mg, 40 mg and 80 mg), dissolved in DMSO (dimethylsulfoxide) for all culture work.
3.4 Cellular imaging

The computer-assisted video microscopy, kindly provided by Prof. Robert Kiss and Dr. Veronique Mathieu, is a technique enabling cell observation in time, which allows the evaluation of the effect of a given product on the morphology, motility, death and proliferation of cells. This technique has been optimized by Dr. Olivier Debeir (80). The treated and control cells are maintained in closed flasks containing buffered medium at a controlled temperature of 37 ± 0.1 °C during the entire experiment. Cells are seeded at low density to allow cell proliferation over 72 hours (6,000 to 40,000 cells/ml) in 25 cm² flasks. They are monitored by means of a phase-contrast microscope coupled with a CCD camera (Olympus, Antwerp, Belgium). A control monitor placed between the camera and the computer allows visualization at any time. The program enables to take pictures every four minutes during a predetermined period. Movies are made by placing all these pictures/frames after each other accelerated in less than 1 min.

GGR

In each condition – control and treated – cell growth level was evaluated by the parameter “Global Growth Ratio” (GGR). The GGR is the ratio between the number of cells counted in a frame of a chosen timepoint and the number of cells counted on the first frames of the image sequences. Lower ratios indicate a decrease in proliferation, whereas higher ratios indicate an increase in proliferation. These experiments were performed in triplicate. BLM melanoma cells were treated with simvastatin at three different concentrations (50, 500 and 5000 nM).

3.5 MTT assay

To study the overall growth of the cell lines, we used the colorimetric MTT test. This test is based on the reduction of MTT (3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylTetrazolium Bromide, Sigma) into formazan crystals by the mitochondria of living cells. It allows to estimate the number of living cells in culture and therefore allows to analyze the effects of treatment on the overall growth in a considered cell population. The cells are seeded in a 96 well plate on day one; treatment is added on day two. The analysis is performed after a period of 72 hours of exposition to the treatment. The medium is then replaced by MTT dissolved in RPMI. This yellow product, used in excess (1 mg/ml), is incubated for 3 hours at 37 °C with these cells. During this time, the mitochondria of living cells transform MTT in formazan crystals. The formation of this new product is proportional to the number of living cells. The 96 well plates are then centrifuged for 10 minutes at 1000 rates per minute in order to pellet the formazan crystals. The excess of the MTT solution is removed by turning the plate on absorbent paper. The crystals are then dissolved in DMSO. The produced solution is purple; measurement of the color absorbance at 570 nm by spectrophotometry using a DIAS plate reader (Dynatech Laboratories, Guyancourt, France) reflects the number of remaining living cells. Each experimental condition is tested in sextuplicate on the plate.
The IC50 is defined as the concentration of the compound that reduces the global growth of the cancer cells by 50% after three days of drug exposure.

### 3.6 RhoC electroporation

SiRNA duplexes for human RhoC were purchased from Qiagen Benelux B.V. (Antwerp, Belgium). Silencing of Luciferase (siLuc) was used as a control, since Luciferase is not present in human melanoma cells. Transfection of siRNA was performed by electroporation using Amxa™ Nucleofector™ according to manufacturer's protocol. Briefly, cells were grown until 60% confluency, trypsinized and collected in an Nucleofector™ certified cuvette (Amaza GmBH, Germany) and mixed with 100µl Nucleofector™ solution “L”, containing 100 nM siRNA. Cells were electroporated in the Nucleofector™ electroporator with the X01 Nucleofector™ program. Functional assays were performed 96 hours post-transfection. These experiments were performed by Koen Jacobs and Mireille van Gele (Research Dermatology, UZ Ghent).

### 3.7 Statistical analysis

A p-value < 0.05 was considered significant; a p-value < 0.001 was deemed highly significant. On the figures, statistical significance (p < 0.05; p < 0.001) is represented respectively with one or three starts. All statistical analyses were performed using SPSS/PASW Statistics (version 17.0).
4 Results

Simvastatin decreases the GGR of BLM cell lines after 48 and 72 hours

The anti-proliferative effects of three different concentrations (50, 500 and 5000 nM) simvastatin were studied and compared to a control sample. These experiments were conducted in triplicate. The twelve conditions (3x controls, 3x 50 nM, 3x 500 nM and 3x 5000 nM) were monitored by continuous phase-contrast microscopy during 72 hours. The pictures were then analyzed to evaluate the GGR, a global measure for increase in cell number. This study indicates that after 24 hours none of the treated groups with 50 nM or 500 nM differed significantly from the control group (500 nM, p = 0.089; 50 nM, p = 0.061). BLM cell lines treated with 5000 nM simvastatin showed a tendency towards lower proliferation (5000 nM, p = 0.054 (borderline)). However, after 48 and 72 hours, 5000 nM simvastatin significantly decreased the degree of proliferation in BLM melanoma cell lines without causing toxicity (p < 0.001; p < 0.001). This was not the case for 50 nM (p = 0.431; p = 0.452) and 500 nM (p = 0.181; p = 0.113) (Figure 12 and Graph 1). The videos can be found in Appendix.

![Graph 1](image1.png)

Graph 1: Two different representations of the growth inhibitory effects of simvastatin on BLM melanoma cell lines. Cells were treated with 50, 500 or 5000 nM for 72 hours. Results are expressed as the mean Global Growth Ratio (GGR) ± SEM of triplicate determinations. *** P < 0.001 compared with control.
Figure 13: Pictures made by continuous phase-contrast microscopy. Only 5000 nM simvastatin significantly decreased the proliferation of the BLM melanoma cell lines.
Correlation between lipophilicity/anti-cholesterol effect of statins and mean IC50

Details about LDL-cholesterol reduction, a representation of statin strength, and lipophilicity of each statin were obtained by literature search. The IC50 of each statin on each cell line was obtained through MTT assays on four melanoma cell lines (G361, HT144, SKMEL-28 and C32) and one murine cell line (B16F10) (results not shown). The IC50 was defined as the concentration which inhibits overall cell growth by 50% after 72-hour incubation with statins. The “mean IC50” was then calculated as the mean of the IC50s of the five cell lines for each statin. Simvastatin showed the most efficient inhibition of the different melanoma cell lines. Pravastatin failed to shown an inhibition in all of the treated cell lines and was therefore excluded in the analysis. No correlation was found between the mean IC50 and the anti-cholesterol properties of the statins ($r = + 0.636; p = 0.364$) (Figure 14).

A correlation between statin lipophilicity and mean IC50 was found: the more lipophilic statins were correlated with a more efficient inhibition of the melanoma cell lines ($r = - 0.988; p = 0.012$) (Figure 15).

Table 4 shows the mean IC50, the LDL-cholesterol reduction with 40 mg/day and the lipophilicity of the five statins.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fluvastatin</th>
<th>Simvastatin</th>
<th>Atorvastatin</th>
<th>Rosuvastatin</th>
<th>Pravastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL reduction on 40 mg/day</td>
<td>27%</td>
<td>37%</td>
<td>49%</td>
<td>53%</td>
<td>29%</td>
</tr>
<tr>
<td>Lipophilicity (log D at pH 7.4)</td>
<td>1.27</td>
<td>1.6</td>
<td>1.11</td>
<td>0.33</td>
<td>0.84</td>
</tr>
<tr>
<td>Mean IC50</td>
<td>16.8</td>
<td>4.26</td>
<td>11.34</td>
<td>24.18</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 4: LDL reduction, lipophilicity and mean IC50 of the five statins.

1 adapted from (81)
2 adapted from (66)

Figure 14: Correlation between LDL reduction of 40 mg/day of each statin and mean IC50, calculated by MTT analysis of each statin on five different melanoma cell lines.
SiRhoC electroporation had no anti-proliferative effects on BLM melanoma cell lines

We studied the effects of SiRhoC, SiLuc and a control sample with or without 5 µM simvastatin. Since Luciferase is not present in human BLM melanoma cell lines, the silencing for Luciferase (SiLuc) was used as a second control sample. These experiments were conducted in duplicate. The twelve conditions (2x control ± 5 µM simvastatin, 2x SiRhoC ± 5 µM simvastatin, 2x SiLuc ± 5 µM simvastatin) were monitored by continuous phase-contrast microscopy during six days (144 hours). The pictures were then analyzed to evaluate the GGR. The degree of RhoC mRNA inhibition during the first four days was studied by functional assays (QT(mRNA)) and showed a downregulation of RhoC mRNA of 64% after 48 hours, 62% after 72 hours and 30% after 96 hours (Figure 16).

As suspected, SiLuc had no significant anti-proliferative effects on the BLM melanoma cell lines compared to the control sample over 144 hours (GGR24: p = 0.804; GGR48: p = 0.339; GGR72: p = 0.243; GGR96: p = 0.069; GGR120: p = 0.287; GGR144: p = 0.253). SiRhoC had also no significant anti-proliferative effects on the BLM melanoma cell lines over time (GGR24: p = 0.554; GGR48: p = 0.473; GGR72: p = 0.506; GGR96: p = 0.296; GGR120: p = 0.334; GGR144: p = 0.579). However,
when 5 µM simvastatin was administered, each group (Control; SiLuc and SiRhoC) showed a significant decrease proliferation after 48 hours and lasted for 144 hours (Control: GGR48: p = 0.047; GGR72: p = 0.046; GGR96: p = 0.029; GGR120: p = 0.009; GGR144: p = 0.003 / SiLuc: GGR48: p = 0.048; GGR72: p = 0.045; GGR96: p = 0.029; GGR120: p = 0.009; GGR144: p = 0.003 / SiRhoC: GGR48: p = 0.054 (borderline); GGR72: p = 0.047 (borderline); GGR96: p = 0.030; GGR120: p = 0.010; GGR144: p = 0.003) (Figure 17 and Graph 2).

Graph 2: Two different representations of the the growth inhibitory effects of simvastatin and SiRhoC on BLM melanoma cell lines. Results are expressed as the mean Global Growth Ratio (GGR) ± SEM of duplicate determinations. * P < 0.05 compared with control.
Figure 17: Pictures made by continuous phase-contrast microscopy over a period of 144 hours. Cells treated with 5 µM simvastatin showed a significant decrease of proliferation after 24 hours.
Simvastatin, but not SiRhoC, decreases the increase of cell surface area.

In order to verify our results of the SiRhoC experiment, a second experiment was performed, but now the increase of cell surface area (mm²) over 72 hours was investigated, instead of the GGR. Pictures were taken at starting point, after 8, 24, 32, 48, 56 and 72 hours and were analyzed by software developed to quantify the area filled by the cells over the period of the experiment. It was shown that SiRhoC had a significant lower area of cells compared to the control group after 24 hours (24h, 32h, 48h, 56h and 72h: p < 0.001). However, if we look at the graph, there is still a consistent increase of cell area in the SiRhoC group so we can conclude that this effect is probably due to the fact that the starting number of cells were lower in the SiRhoC group.

This experiment also confirms the previous experiments: 5 µM simvastatin treatment shows a significantly lower area of cells after 24 hours, compared to the control sample (24h, 32h, 48h, 56h and 72h: p < 0.001). When 5 µM or 3 µM simvastatin were added to the SiRhoC sample, we found a significantly lower area of cells in both groups.

We also investigated whether a lower concentration than 5 µM, but higher than 500 nM had an anti-proliferative effect on BLM melanoma cells. This experiment indicated that a concentration of 3 µM simvastatin led to a significant lower area of cells after 24 hours (24h, 32h, 48h, 56h and 72h: p < 0.001), suggesting that this lower concentration could have anti-proliferative effects on the cell lines.

It was found that the combination of SiRhoC and 3 µM simvastatin had a lower cell surface area increase than 3 µM simvastatin alone. Again, this could be explained by the fact that the starting number of cells were lower in the 3 µM simvastatin treated group compared to the other group.

Graph 3: Representation of the increase of cell surface (area, mm²) in BLM melanoma cells over 72 hours.
<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><img src="image1" alt="Control Start" /></td>
<td><img src="image2" alt="Control 24 hours" /></td>
<td><img src="image3" alt="Control 48 hours" /></td>
<td><img src="image4" alt="Control 72 hours" /></td>
</tr>
<tr>
<td><strong>3 µM Simvastatin</strong></td>
<td><img src="image5" alt="3 µM Simvastatin Start" /></td>
<td><img src="image6" alt="3 µM Simvastatin 24 hours" /></td>
<td><img src="image7" alt="3 µM Simvastatin 48 hours" /></td>
<td><img src="image8" alt="3 µM Simvastatin 72 hours" /></td>
</tr>
<tr>
<td><strong>5 µM Simvastatin</strong></td>
<td><img src="image9" alt="5 µM Simvastatin Start" /></td>
<td><img src="image10" alt="5 µM Simvastatin 24 hours" /></td>
<td><img src="image11" alt="5 µM Simvastatin 48 hours" /></td>
<td><img src="image12" alt="5 µM Simvastatin 72 hours" /></td>
</tr>
<tr>
<td><strong>SiRhoC</strong></td>
<td><img src="image13" alt="SiRhoC Start" /></td>
<td><img src="image14" alt="SiRhoC 24 hours" /></td>
<td><img src="image15" alt="SiRhoC 48 hours" /></td>
<td><img src="image16" alt="SiRhoC 72 hours" /></td>
</tr>
</tbody>
</table>

Figure 18: Selection of pictures made by phase-contrast microscopy. Cells treated with 3 and 5 µM simvastatin showed a significant decrease of cell surface area after 24 hours.
5 Discussion

Cutaneous melanoma is a disease characterized by many contrasts. Although it is the least common type of skin cancer, it is still the most deadly form, responsible for three quarters of all deaths caused by skin cancer (11). In the early stages, cutaneous melanoma is curative in the vast majority by surgical excision. However, the management of advanced melanoma is, due to its resistance to immuno-, chemo- and radiotherapy, disappointing with a five-year survival rate of less than 20% (23). Moreover, worldwide incidence rates of cutaneous melanoma are still increasing, reaching epidemic proportions in some countries. In contrast, mortality rates seem to have leveled off, especially in the young cohorts (11, 18). Fortunately, new therapies are on the horizon, using molecular targeted strategies to attack the tumor.

In this view, a recent thesis by Boone et al. (2009) has highlighted the importance of certain molecules and pathways involving melanoma progression. One of these molecules, RhoC, was found to be involved in growth and metastasis in melanoma patients (33).

RhoC belongs to the family of Rho proteins, a distinct family of proteins within the superfamily of Ras-related small GTPases (6, 28). These small proteins play an essential role in a broad range of signaling pathways, including cellular motility, cell adhesion control, gene expression, transcriptional activation, growth processes, vesicle trafficking, enzyme regulation, cell cycle progression and apoptosis. Since transformation of a normal cell into a cancer phenotype has been associated with different downstream effects of Rho proteins, it is not surprising that aberrant Rho signaling, and more specifically RhoC, is shown in many cancer cell lines and human cancers (28, 30). RhoC has not been found mutated in cancers, indicating that upregulated expression could be sufficient for it to contribute to metastasis (31). Several in vitro and in vivo experiments have reported that RhoC overexpression promotes invasiveness, growth and/or metastatic behavior in many different cancer types (33). Overexpression of RhoC mRNA has been reported in inflammatory breast cancer (34-36), ovarian cancer (37-38), ductal adenocarcinoma of the pancreas (42), non-small cell lung cancer (45), bladder cancer (40) and lung cancer (43). In addition, studies investigating RhoC expression in gastric cancer (51-52), esophageal cancer (47), oligodendroglioma (48), squamous cell carcinoma of the head and neck (49), primary hepatocellular carcinoma (44), renal cell carcinoma (39) and colon carcinoma (53) showed RhoC immunoreactivity to be associated with the presence of metastatic disease (33). Furthermore, RhoC was found to be a specific molecular prognostic marker in various tumors (34, 40, 44-46).
The involvement of RhoC has also been implicated in melanoma. Highly metastatic melanoma cell lines expressed RhoC at higher levels and enforced RhoC expression enhanced metastatic capacity. In contrast, Rho inhibition suppressed invasion as well as motility (50). RhoC expression in melanoma has been associated with thicker melanoma and tendency towards ulceration, two important prognostic factors in cutaneous melanoma (33). These data suggest that RhoC could be one of the future targets in (the prevention of) advanced melanoma.

RhoC protein is activated by isoprenylation, a necessary step for its correct localization, protein stability and downstream effects. Isoprenylation of RhoC requires geranylgeranyl pyrophosphate (GGPP), an intermediate in the HMG-CoA reductase pathway, also called the mevalonate or isoprenoid pathway (31, 66). HMG-CoA reductase, the rate-limiting enzyme of this pathway, is inhibited by statins, widely used cholesterol-lowering drugs.

These drugs have been shown to possess some other beneficial effects, besides decreasing cardiovascular morbidity and mortality. Many epidemiological studies have investigated the possible involvement of statins in cancer prevention. Some have supported a protective effect (68-72), others concluded that statins have no effect (5, 73-77).

However, these studies have some limitations. First, many epidemiological studies were designed to study the anti-cholesterol properties of statins rather than anti-cancerogenous effects. Second, a main issue in epidemiological studies is cancer latency, the lag time between exposure to an agent and the effects on the disease; the majority of epidemiological studies publish data of relatively short follow-up. A third remark concerns the fact that, although synthesis of the existing randomized data could not support the hypothesis that low doses statins reduce the risk of melanoma, a risk reduction associated with higher doses has not been studied yet in full extent (5). A last remark is that some studies do not make a distinction between lipophilic and hydrophilic statins, which, as stated earlier, have other extra-hepatic effects (78).

Epidemiological studies including higher doses of statins and/or discrimination in lipophilicity are therefore warranted. These studies need to be designed to specifically study the anti-cancerogenous properties of statins for a sufficiently long period.

One of the proposed mechanisms by which statin could exert their beneficial effects, is the inhibition of geranylgeranylation of RhoC (Figure 19). By inhibiting HMG-CoA, statin treatment leads to a depletion of GGPP, which in turn leads to a decrease of RhoC activation.

In this study, we examined the in vitro effects of three different concentrations (50, 500 and 5000 nM) of simvastatin on BLM melanoma cell lines through phase-contrast video microscopy. Only the highest concentration, 5000 nM, simvastatin led to a significant decrease of proliferation. In a second experiment, we could conclude that BLM melanoma cell lines treated with 3 µM simvastatin had a significantly lower area of cells, suggesting that this concentration could be sufficient to induce anti-
proliferative effects. These results are concordant with other studies examining statin effects on melanoma cell lines (54, 79, 82).

The concentrations obtained in this and other studies are higher than can be achieved in patients receiving a normal daily dose of simvastatin. The peak plasma concentrations of statins during a conventional therapy of hypercholesterinaemia are in the range of 10 – 100 nM (83). The peak plasma concentration of simvastatin achieved in patients receiving a 40 mg per day dose is approximately 3 ng/ml (7.2 nM) (54). Thus, it is therefore unlikely to obtain plasma concentrations of statins high enough to induce anti-proliferative effects (84). However, a recent dose study in patients with myeloma or lymphoma demonstrated that the maximum tolerated dose of simvastatin, given in combination with chemotherapy, was 15 mg/kg/day (54). Such high dose regimens result in peak plasma concentrations of approximately 4 mM, which is within the range that triggered apoptosis in our human melanoma cell lines (84).

![Figure 19: Through the inhibition of HMG-CoA reductase, statin treatment leads to a depletion of isoprenylated proteins, such as RhoC thereby preventing its downstream effects.](image)

In addition to being concentration dependent, the statin-induced apoptosis is also time dependent (84). The anti-proliferative effects of simvastatin were observed after exposure of 48 hours and 72 hours, not after 24 hours. This is in concordance with Saito et al. (2008), who demonstrated that exposure of 24 hours of statins did not reduce cell viability, whereas 48 hours of incubation dramatically reduced cell viability. The molecular mechanisms behind this phenomenon have not been identified yet.
In order to investigate whether the anti-proliferative effects of the different types of statins (atorvastatin, pravastatin, rosuvastatin, simvastatin and fluvastatin) is correlated with their lipophilicity, we calculated the IC50, defined as the concentration inhibiting overall cell growth by 50%, of each statin on four different human melanoma cell lines (HT-144, G-361, C-32 and SK-Mel-28) and one murine melanoma cell line (B16F10). The mean IC50 was then calculated for each statin separately. Simvastatin was the most efficient statin in inhibiting overall cell growth, whereas pravastatin failed to show any inhibition on each single cell line and was therefore excluded in our analysis. This finding is in agreement with a recent study by Glynn et al. (2008), where pravastatin failed to inhibit lung, breast and melanoma cancer cell lines. This could be due to the fact that pravastatin, a hydrophilic statin, is not capable to enter many extra-hepatic cells (Figure 19). This suspicion is strengthened by the fact that we found a correlation between the lipophilicity of the statins and their anti-proliferative effects. The more lipophilic statins showed a lower mean IC50, indicating that lower doses are needed to induce inhibition of overall cell growth on the different tested cell lines.

We also investigated whether the anti-cholesterol effect of the different types of statins correlated with their anti-proliferative effects. No correlation between their strength in LDL-cholesterol reduction in vivo and mean IC50 in vitro was found.

In ovarian cancer cells transfected with siRNA-RhoC, both RhoC gene expression and protein level were suppressed. This was correlated with a reduced ability to invade an artificial membrane or to migrate in vitro (38). Another in vitro study investigating the silencing of RhoC RNA reported an inhibition of proliferation in breast cancer cells (85). To further investigate the role of RhoC in melanoma, we conducted an experiment where RhoC mRNA was silenced by electroporation. However, in our study, no significant changes were observed in the SiRhoC electroporated group compared to control cells. In a second experiment investigating the increase of cell area over time, a consistent increase of cell area was seen in the SiRhoC electroporated sample. These results are supported by other experiments. In gastric cancer cell lines, no significant changes in the monolayer growth rate were observed for anti-RhoC siRNAs transfectants as compared to control cells (51). In agreement with these results, a RhoC knockout mouse model with mammary adenocarcinoma demonstrated that loss of RhoC did not affect tumorigenesis, but significantly decreased metastasis in this mouse. These data suggest that RhoC is involved in metastasis, but not in tumor cell proliferation (15).

To date, the overall mechanisms in RhoC-dependent oncogenic effects have not yet been fully clarified and may perhaps differ according to cancer type (43). This could explain the differences seen in different cancer cell lines and human cancers. For instance, RhoC upregulation confers a metastatic nature in prostate cancer and in an orthotopic lung cancer model in mice, without affecting the growth
of primary tumors (43, 46). In contrast, RhoC overexpression in melanoma has been associated with metastasis as well as growth of primary tumor (33).

The apparent contradictory results by different groups may be due to the dependency of RhoC on cellular context. Therefore, it has been suggested by Hakem and colleagues (2005) to take a more systematic approach of testing the gene both in vitro and in vivo and to validate the outcome results in a clinical setting for each organ or tissue type in order to further clarify the role of RhoC in tumor progression (46, 86).

There are some limitations that may affect the interpretation of these results. First, SiRhoC was not suppressed 100% during the time of the experiments. It is possible that the inhibition was not sufficient to achieve an anti-proliferative effect. Secondly, we only investigated the anti-proliferative properties of silencing RhoC, we did not study anti-migratory effects, meaning we could not exclude that SiRhoC might lead to a decrease in invasion and/or metastasis.

In conclusion, our results demonstrate that simvastatin has an anti-proliferative effect on BLM melanoma cell lines. These findings are in concordance with the numerous in vitro studies on cancer cells in general and melanoma cells in particular. Although the tested concentration of 5 µM simvastatin is higher than attainable in human, these results support the idea that melanoma may be sensitive to statin treatment.

Freeman et al. (2006) suggested that given the in vitro evidence, together with the lower melanoma rates observed in some clinical trials and the disappointing treatment for advanced melanoma, it may be appropriate to perform clinical trials to combine statins with chemotherapy to test whether this combination is tolerable and, if it is, whether it is more effective than current chemotherapy alone (68).

Indeed, synergistic interactions have been reported between statins and several chemotherapeutic agents. In colon cancer cells, Agarwal et al. (1999) reported that lovastatin enhanced pro-apoptotic effects of cisplatin, 5-FU and sulindac through inhibition of geranylgeranylation (87). A recent study by Martirosyan et al. (2010) showed that, in a panel of ovarian cancer derived cell lines, lovastatin could synergize the apoptotic effects of doxorubicin (88). Fluvastatin was reported to synergistically potentiate the cytotoxic effects of gemcitabine on both human pancreatic cancer cells and human colon cancer cells (89). In myeloma cell lines and in bone marrow samples, lenalidomide significantly potentiated simvastatin-induced cytotoxicity through induction of apoptosis and inhibition of proliferation (90).

Consistent with these findings, a few studies have shown synergistic effects of statins with other treatments in melanoma. Pairing of lovastatin with d-γ- or d-δ-tocotrienol (three suppressors of HMG CoA reductase) yielded a significant synergy in suppressing the growth of the very resistant A549
cells and moderately sensitive B16 and DU145 melanoma cells. This effect was confirmed by in vivo experiments in mice (91).

Lovastatin has shown to potentiate the anti-tumor effects of doxorubicin in murine melanoma compared to the individual compound, resulting in an augmentation of apoptosis induced by doxorubicin (92).

Since statins have a good safety profile and are well tolerated and given the paucity of effective therapy for advanced melanoma, it is worthfull to address the question whether the combination of statins to standard chemotherapy is tolerable and if so, whether it is more effective than current chemotherapy alone (77). Further studies will also address the question whether RhoC might be a valuable target for molecular targeted therapy.
6 References


38. Zhao Y, Zong ZH, Xu HM. RhoC expression level is correlated with the clinicopathological characteristics of ovarian cancer and the expression levels of ROCK-I, VEGF, and MMP9. Gynecol Oncol. 2010;28(4):399-407.


7 Appendix

A. Summary (Dutch)

Achtergrond: Hoewel het cutane melanoom (CM) slechts ongeveer 10% van alle huidkankers vertegenwoordigt, blijft het toch verantwoordelijk voor 75% van alle doden door huidkanker. Incidentiecijfers tonen de laatste decennia in vele landen nog steeds een stijgende trend; sterftecijfers blijken evenwel hun top bereikt te hebben en vertonen soms zelfs een daling, voornamelijk in jongere cohorten. De behandeling van vroeg-gediagnosticeerde laesies is curatief in ongeveer 97% van de gevallen. Door zijn resistentie voor immuno-, chemo- en radiotherapie, blijft de behandeling voor het gemetastaseerd melanoom echter ontgoochelend. Eenmaal CM metastaseert, zakt de tienjaarsoverleving onder de 20%. De toekomst voor de behandeling is veelbelovend met de opkomst van nieuwe moleculaire therapieën.

In navolging van het doctoraatsonderzoek van Dr. Barbara Boone, waar het belang van RhoC in de groei en proliferatie van het CM aangetoond werd, werd onderzocht of RhoC één van deze toekomstige doelwitten kan zijn. RhoC behoort tot de RhoGTPases, een aparte familie van eiwitten binnen de superfamilie van Ras-gerelateerde kleine GTPases. Toenemende evidentie wijst erop dat opregulatie van RhoC gecorreleerd is met een slechtere prognose, een snellere groei en een grotere neiging tot metastasering in verscheidene kanker cellijnen en humane kankers, waaronder het cutane melanoom.

De pleiotrope functies van Rho activatie omvatten onder andere cytoskelet remodellering tijdens celmotilititeit, controle van celadhesie en gen expressie. Daarenboven zijn Rho proteïnes betrokken in transcriptie activatie, groeiprocessen, vesikeltransport, enzymregulatie, cyleclusprogressie en apoptosis. Aangezien transformatie van een normale cel naar een kankercel geassocieerd is met veranderingen in één of meer van deze eigenschappen, hoeft het niet te verwonderen dat afwijkingen in Rho expressie een belangrijke rol kan spelen bij tumorcellen.

RhoC wordt geactiveerd door isoprenylatie, een noodzakelijk stap voor zijn correcte lokalisatie, eiwitstabiliteit en downstream effecten. Isoprenylatie van RhoC vereist geranylgeranyl pyrofosfaat (GGPP), een intermediair in de HMG-CoA reductase pathway. Deze pathway, ook de mevalonate of isoprenoid pathway genoemd, is het therapeutisch aangrijpingspunt van statines. Deze frequent voorgeschreven anti-cholesterol medicatie leidt niet alleen tot een daling van mortaliteit door hart- en vaatziektes, maar vertonen ook enkele onbedoelde positieve effecten. Verschillende epidemiologische, in vitro en in vivo studies suggereren dat statines een protectief effect hebben tegen kanker. Een recente studie concludeerde dat inname van statines geassocieerd is met een verminderde Breslow dikte, een belangrijke prognostische factor in het CM. Eén van de vooropgestelde mechanismen waardoor statines dit gunstig effect zouden verwezenlijken, zou via de mevalonate pathway gebeuren. Door deze te blokkeren leiden statines tot een depletie van GGPP en daaropvolgend een depletie van geïsoprenyleerde eiwitten, waaronder RhoC.
**Doelstelling:** Het doel van deze masterproef was om de effecten van statines op melanoom celllijnen te bestuderen via continue fase-contrast videomicroscopie. Vervolgens werd onderzocht of er een correlatie was tussen de lipofiliciteit van de verschillende soorten statines en hun capaciteit om de proliferatie van melanoma cellen te inhiberen. Als een laatste strategie werd de rol van RhoC in melanoma proliferatie verder onderzocht.

**Methodologie:** Eerst werden de in vitro effecten van drie verschillende concentraties (50, 500 en 5000 nM) simvastatine behandeling op BLM melanoma celllijnen gedurende drie dagen met behulp van continue fase-contrast videomicroscopie onderzocht. De “Global Growth Ratio” (GGR), een globale maat voor celproliferatie, werd berekend in iedere conditie en vergeleken met een controle. Vervolgens werden MTT assays van de vijf in België beschikbare statines (atorvastatine, pravastatine, rosuvastatine, simvastatine en fluvastatine) uitgevoerd op vier verschillende melanoma celllijnen (G361, HT144, SKMEL-28 en C32), alsook op een melanoom cellijn van muizen (B10F16). Correlatie tussen de IC50, een maat voor de doeltreffendheid om de globale groei van een kankercel tegen te gaan, en de anti-cholesterol effecten van verschillende statines werd bestudeerd. Er werd ook onderzocht of er een correlatie was tussen de lipofiliciteit en de IC50 van de verschillende statines. Om de rol van RhoC desactivatie in melanoma celllijnen te bestuderen, werden melanoma celllijnen via elektroporatie voor RhoC mRNA uitgevoerd en bestudeerd via continue fase-contrast videomicroscopie. Deze afbeeldingen werden dan geanalyseerd om de GGR te berekenen en werden vergeleken met twee verschillende controlegroepen. Ter bevestiging van de resultaten van deze experimenten, werden de effecten van SiRhoC en simvastatine op de toename van celoppervlakte van BLM melanoma cellen over een periode van 72 uur bestudeerd.

**Resultaten:** Enkel BLM celllijnen behandeld met de hoogste concentratie simvastatine (5000 nM) vertoonden een significante daling in GGR na incubatie van 48 en 72 uur. Correlatie tussen de anti-cholesterol effecten en de IC50 van de verschillende statines kon niet aangetoond worden. Er werd wel een correlatie tussen lipofiliciteit en de IC50 van de verschillende statines gevonden. Hoe lipofieler een statine was, hoe lager de IC50, wat wijst op een sterkere inhibitie bij meer lipofiele statines. Pravastatine vertoonde geen enkel anti-proliferatief effect op de cellen en werd daarom niet in de correlatie analyse opgenomen. RhoC silencing vertoonde geen anti-proliferatieve effecten op de BLM melanoma celllijnen. In een tweede experiment waar de globale toename van celoppervlakte van BLM melanoma cellen werd bestudeerd, werd een consistente toename van celoppervlakte gezien.

**Besluit:** Deze studie bevestigt dat simvastatine een anti-proliferatief effect heeft op BLM melanoma celllijnen, maar enkel in de hoogste concentratie (5000 nM) en enkel na 48 uren. Vermoedelijk treedt dit effect al optreden vanaf 3 µM simvastatine.
Er werd aangetoond dat de lipofiliciteit van statines een rol kan spelen in hun doeltreffendheid om melanoom cellijnen te inhiberen. Hoe lipofieler de statine, hoe krachtiger de statine was in het inhiberen van de proliferatie.

RhoC vertoonde niet dezelfde effecten als simvastatine, wat betekent dat we niet konden bevestigen dat de anti-proliferatieve effecten van statines gemedieerd werden door RhoC.

Verdere studies zullen nagaan of het toevoegen van statines bij de standaard chemotherapie de anti-tumorale effecten van de chemotherapie op melanoom cellijnen kan versterken en of RhoC een waardevol doelwit kan zijn bij moleculaire doelgerichte therapie.
B. Videos

The CD-rom, containing the videos of the experiments using the continuous phase-contrast video microscopy, can be found on the back of the cover.