Lentigo maligna
Clinical management and histopathological diagnosis

Tio, D.C.K.S.

Publication date
2020
Document Version
Final published version
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D.C.K.S. Tio

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Darryl Christian Kim San Tio

Colofon

Lentigo Maligna: Clinical management and histopathological diagnosis – D.C.K.S. Tio

Lentigo Maligna

Clinical management and histopathological diagnosis

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Ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen

op vrijdag 17 april 2020, te 13.00 uur

door

Darryl Christian Kim San Tio

Geboren te Zaanstad
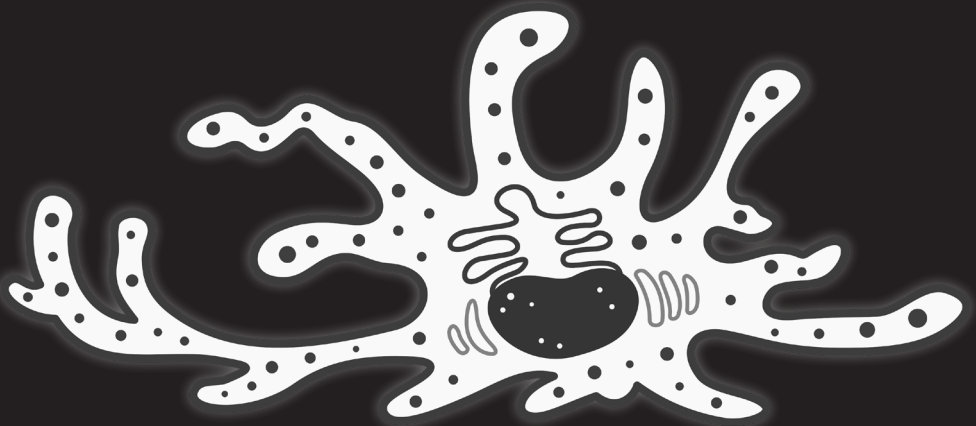
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General introduction and
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General introduction

Lentigo maligna (LM) was first described in a small case series by Hutchinson in 1894. Originally Hutchinson thought that the lesion was infectious in nature and he used the term "infective senile freckles" (1). Four years later, the term "Lentigo malin des viellards" was coined by Dubreuilh, which can be translated to "malignant lentigo of the elderly" (2). In 1912, it was Dubreuilh who classified LM as precancerous. He described LM as "De la mélanose circonscrite précancéreuse", which means "Circumscribed precancerous melanosis (3).

Clinical picture

A LM lesion visually looks like a brown and grey macule which can vary in size from several millimetres to several centimetres. Typically, LM occurs in UV-exposed areas like the head and neck area of elderly patients (Figure 1). These areas contain critical anatomical structures, and elderly patients often have multiple comorbidities. Consequently, this can make clinical management challenging (4).

Figure 1: Lentigo maligna on the right cheek of an elderly patient.

Epidemiology

LM is treated to prevent progression to lentigo maligna melanoma (LMM), which can metastasize. In 1985, Weinstock et al. showed that 4.7% of LM progresses to LMM when diagnosed at the age of 45, and 2.2% when diagnosed at the age of 65 (5). In a study from 2016 on the epidemiology of LM and LMM in Netherlands in the period 1989-2013, the cumulative chance of progression has been shown to be 2.0% for women and 2.6% for men during a period of 25 years (6). More recently an Australian study. reported a risk of progression of LM to LMM of 3.5% per year. Which equates to an average time to progression of 28.3 years (7).

The incidence of LM is on the rise. In Sweden an age adjusted incidence of 15/100,000 patient years was found (8). Studies from Denmark (0.55 LM/100,000 patient years to 1.05 LM/100,000 patient years between 2009-2011), Girona, Spain (0.36 LM/100,000 patient years to 1.1 LM/100,000 patient years between 1994-1996), the Netherlands (0.72 LM /100,000 patient years to 3.84 LM/100,000 patient years between 1989-2013) and the USA, Olmsted County (2.2 to 13.7/100,000 person years from 1970-2007) have all shown an increase of LM incidence (6, 9-11). A possible explanation for the rise in incidence is the increased life expectancy of patients. Another explanation is better awareness and recognition of the disease (Table 1).

Table 1: Increase of Incidence of Lentigo Maligna. LM = lentigo maligna.

Author and country	Journal	Period	Incidence increase
Greveling et al., the Netherlands (6)	J Invest Dermatol 2016	1989-2013	0.72/100,000 patient years to 3.84 LM/100,000 patient years
Toender et al., Denmark (9)	Melanoma Res 2014	1997-2011	0.55 LM/100,000 patients years to 1.05 LM/100,000 patient years
Hemminki et al., sweden (8)	Int J Cancer	2003	15/100,000 patient years
Vilar-Coromina et al., Spain (10)	Actas Dermosifiliogr 2010	1994-2005	0.36 LM/100,000 patient years to 1.1 LM/100,000 patient years
Mirzoyev et al., USA (11)	J Am Acad Dermatol 2014	1970-2007	2.2 LM/100,000 patiets years to 13.7 LM/100,000 person years

The diagnosis of LM usually based on a single 3 mm punch biopsy, which can potentially lead to a sampling error. LM is often larger than 10mm and situated on actinically damaged skin. If a large lesion is mapped with multiple punch biopsies, occult LMM might still be missed (12). Current guidelines therefore recommend excisional (for small lesions) or an incisional biopsy (for larger lesions) to obtain tissue for histopathology (13). Shave biopsies are not recommended because the tumour may be transected, thereby not allowing an accurate Breslow measurement if the lesion is an LMM. If direct excision is not possible, it is recommended to biopsy the darkest, most-palpable portion of the LM lesion (14). A recent study on LM diagnostics. has shown that 9% of histologically proven LM based on a biopsy are upstaged to LMM after histopathological examination of excision material (15).

Dermatoscopic examination of a lesion may aid diagnosis of LM. It can be used to differentiate lesions from lentigo senilis or seborrheic keratosis. In the past decade the usage of dermatoscopy has increased. A survey held in 2005 in Great Britain on the management of LM showed that 17.4% of 594 respondents used dermatoscopy for the diagnosis of LM (16). A European survey by our group in 2016 showed that the usage has risen to 83.4% of 415 respondents. Several sets of dermatoscopic criteria which characterize LM are available. Our survey showed that the most used set of criteria are the Stolz criteria, which 55.6% of 415 respondents use. The second most used criteria are the Schiffner criteria, used by 29.8% of respondents (17). The Stolz criteria include hyperpigmented follicular openings, Annular-granular pattern of pigmentation, dots aggregated around adnexal openings, short and polygonal lines around and between adnexal openings, pigmented rhomboidal structures and dark blotches and obliterated hair follicles (18, 19). The Schiffner criteria include asymmetric pigmented follicular openings, dark rhomboidal structures, slate-grey globules and slate-grey dots. Multivariate analysis showed that these criteria have a sensitivity of 89% and a specificity of 96% (20). In reality, clinicians might use a combination of these criteria.

Another useful tool which may aid the diagnosis of LM prior to biopsy or excision is reflectance confocal microscopy (RCM). It has been shown that RCM is at least as accurate as dermatoscopy in helping select a site for a biopsy. In fact, RCM selected biopsy sites often contain more histopathological criteria of LM compared to dermatoscopy (21). LM often has amelanotic parts which are easy to miss on macroscopic examination. To address this problem, RCM can be used to delineate the lesion prior to treatment. Several studies have underlined that scanning a

lesion with RCM prior to excision can improve the rates of radical excision (22-25). Dermatoscopy and RCM are useful assets for the management of LM in the diagnostic phase. However, the usage of RCM at the moment is limited. This could be due to the high cost of a confocal microscope (22). Another reason might be a lack of experience and the long learning curve using this microscope (26).

Histopathology

The histopathological diagnosis of LM is based on the presence of atypical melanocytes in the epidermal-dermal junction (Figure 2). As mentioned before LM is often found on sun-exposed and actinically damaged skin. Morphologically atypical melanocytes may simulate LM even though they are benign. From a histopathological viewpoint these melanocytes are indistinguishable, even with the use of immunohistochemical stains (MART1/melan-A, SOX10, MiTF and soluble adenylyl cyclase) (27, 28). What currently lacks, is a histopathological marker to discern these morphologically atypical melanocytes from LM.

LM is an entity surrounded by unsolved issues. Currently, the concept of LM encompasses what most likely are two separate entities. Flotte *et al*. proposed in 1999 that there are two histologic subtypes of LM with distinct biological behaviours. Under their definition, LM is defined as atypical melanocytic hyperplasia, whereas malignant melanoma in situ (MIS), LM type is characterized by confluence and nesting of atypical melanocytes at various layers of the epidermis (29).

Most therapeutic studies on LM do not make this distinction and describe a combination of both groups as defined above (30).

Treatment

In 2014, Tzellos *et al*. performed a Cochrane systematic review on the treatment of melanoma in situ, including LM. They concluded that there is a lack of high-quality evidence for both surgical and non-surgical treatments of LM (31). The European consensus guideline of 2016 and the American guideline of 2019 both recommend surgical excision as the first choice of treatment (13, 31). Excision with a 5 mm margin is preferred, either with a standard excision technique or staged techniques such as Mohs micrographical surgery. As alternative treatments, radiotherapy and topical imiquimod are mentioned, but there is no treatment algorithm. These recommendations however, are based

mainly on expert opinion and retrospective studies. Up until today no randomized controlled trials on the treatment of LM have been published (13, 32).

Conventional surgery has been the standard for quite some time. LM is usually excised with a 5 mm margin. The reported recurrence rates for this procedure vary from 6.8-30% (30, 33). In contrast, staged excision techniques have lower recurrence rates. Mohs micrographical utilizes frozen sections in which 100% of the margins can be immediately analysed. If the margins are not clear, additional tissue can be removed. For LM, an adapted technique called "slow Mohs" or "Breuninger Technique" is available where permanent sections are used instead. These techniques have reported recurrence rates of 0-5.9% (34-36). The "Spaghetti technique" is a technique where a narrow band of skin is excised just outside the clinical margin of a lesion. This is histopathologically analysed, if the margins are clear of LM the centre is excised and a reconstruction is performed. This technique has a reported recurrence rate of 4.6% (37). Surgical excision of large lesions however, may lead to potential aesthetic or functional impairment and often leading to large reconstructions.

Figure 2: H&E Stain of lentigo maligna. The black arrows indicate atypical melanocytes proliferating along the basal layer of the epidermis. Some of these melanocytes show ascension.
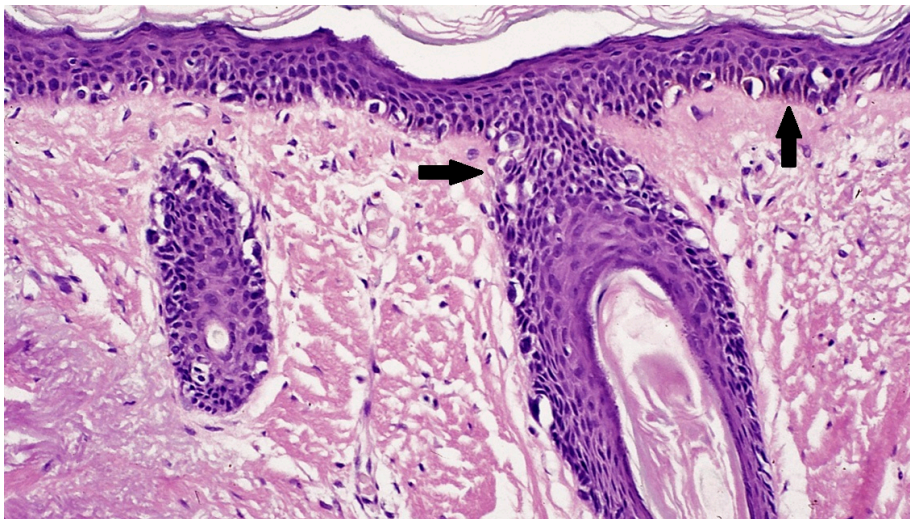
Radiotherapy (RT) is a non-surgical option which is superior in conserving tissue in comparison to surgical treatment. A review conducted by Fogarty *et al.* describes 9 studies including 537 patients treated with RT between 1941 and 2009. After a median follow-up of 3 years, 349 patients were assessed and 18 recurrences (5%) were found. The patients with a recurrence were retreated with RT,

surgery or other therapies. In this cohort op patients a progression to LMM was seen in 5 patients (1.4%) (38).

A second non-surgical treatment option is off-label topical imiquimod 5% cream (IMQ). Application of IMQ has an antitumoral effect by leading to direct apoptosis of tumor cells and by binding to toll-like receptor 7 and 8 on dendritic cells and macrophages. This leads to pro-inflammatory cytokine secretion and a cellular immune response to the tumor cells. The complete response rates range between 37–88% after this non-invasive procedure (39-42). Various treatment schedules are in use, these vary from 1 application 3-5 days per week to 1 or 2 applications per day for a period of 6-12 weeks. In a systematic review including 514 patients we showed that >60 applications in total has a 8 times greater odds of resulting in complete clearance (41). In this group we found 9 (1.7%) patients who showed progression to LMM after treatment with IMQ.

Cryotherapy or cryosurgery is another easily applicable, non-surgical option. However, there is a paucity of evidence regarding cryotherapy. A single study including 30 patients reported a recurrence rate of 6.6% after an average follow-up period of 3 years. Another problem is that cryotherapy potentially creates a scar under which occult LM or LMM may develop.

Various forms of laser therapy have been used to treat LM in the past. Studies using lasers with a wavelength ranging from 690 nm to 10.6 um including carbon dioxide, argon, Q-switched ruby, neodymium-doped yttrium aluminium garnet, alexandrite or a combination of the above have been used. The short term effects were promising, suggesting superior cosmetic outcomes, rapid treatment time and improved tolerability and reduced post-treatment care requirements. The long term follow-up was less positive where recurrence rates between 4.2-29.0% were found (43-50).

A single study reported on a combination treatment. The combination consisted of ablative laser treatment with either 2940-nm erbium-doped yttrium aluminium garnet laser or a 10,600-nm CO_2 laser followed by topical imiquimod 5% cream 5 days per week for 6 weeks. In total, 35 LM patients were treated and after a median follow-up of 14 months a cumulative recurrence rate of 23.5% (8 patients) was found. Out of these 8 patients, 5 had a LM on the nose (50).

Aim and outline of this thesis

The first goal of this thesis was to determine current practice regarding the management of LM across Europe. In **chapter 2** we held a survey among dermatologists who are members of the European Association of Dermatology and Venereology on the management of LM. The second goal was to investigate the effectiveness of non-surgical treatment using topical 5% imiquimod cream. In **Chapter 3** we performed a systematic review including all literature on the treatment of LM with topical 5% imiquimod cream. Our own results using this method are shown in **Chapter 4A** and **4b**. The third goal was to assess histopathological diagnosis. In **Chapter 5** we studied the correlation between a primary biopsy and subsequent excision. In **Chapter 6** we identified potential histopathological markers by analyzing the prevalence of Cancer/Testis antigen on cutanenous melanoma. With the findings from **Chapter 6** we selected several antigen of which we analyzed the prevalence on LM and LMM in **Chapter 7**. The fourth and final goal was to investigate the characteristics of LM and LMM in comparison to superficial spreading melanoma (SSM) and nodular melanoma (NM). To this end, we compared the epidemiological, clinical and genetic characteristics of metastatic LMM to metastatic NM and metastatic SSM in **Chapter 8**.

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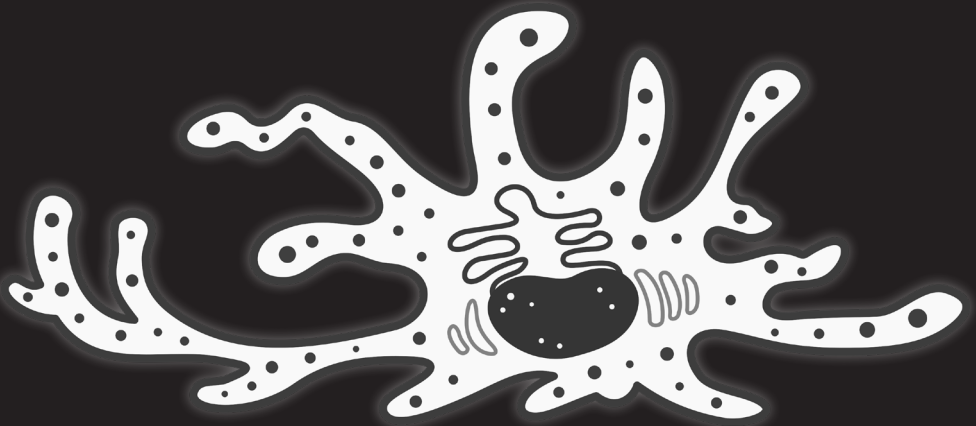
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2

Variation in the diagnosis and clinical management of Lentigo Maligna across Europe: a survey study among EADV members

D.C.K.S. Tio[1]; C.A.C. Prinsen[2], B. Dréno[3], R. Hoekzema[4], M. Augustin[5], C. van Montfrans[6]

1. Dermatology resident, Department of dermatology, Vrije Universeit Medical Center Amsterdam, The Netherlands
2. Clinical epidemiologist, Department of epidemiology and biostatistics, Amsterdam Public Health research institute, VU University Medical Center, Amsterdam, The Netherlands
3. Dermatologist. Department of Dermatology, Centre Hospitalier Universitaire, Nantes, France
4. Dermatologist, Department of dermatology, Vrije Universiteit Medical Center Amsterdam, The Netherlands
5. Dermatologist. Dermatologic Institute for Health Services Research in Dermatology and Nursing. Hamburg, Germany.
6. Dermatologist, Department of Dermatology, Erasmus Medical Center Rotterdam , The Netherlands.

Abstract

Introduction

Lentigo maligna (LM), a form of melanoma in situ, is treated to prevent progression to lentigo maligna melanoma (LMM). Surgical treatment is the gold standard. However, treatment guidelines are based on expert opinion and comparative studies are lacking.

The objective of this study is to assess the diagnostic methods and clinical management of LM patients among European dermatologists and residents.

Methods

A survey consisting of 29 questions about diagnostic methods and treatment options used for LM patients was sent to 3308 members of the European association of Dermatologists and Venereologists (EADV). Most questions were multiple choice, and multiple answers could be ticked per question.

Results

A total of N = 415 (12.5%) completed surveys were included into the analyses. A combination of clinical diagnosis 65.7%, dermatoscopy 83.4% and histopathology 88.2% is used by most respondents to diagnose LM. Tissue for histopathological evaluation was collected using most often by a single punch biopsy in 61.0%, The most common treatment for LM patients <60 years of age is surgery (97,6%). For LM patients >70 years of age, 66.8% of the respondents preferred surgical treatment. Non-surgical options such as radiotherapy (17.0%), topical imiquimod (30.6%), watchful waiting (19.6%) or cryotherapy (20.4%) were used in this elderly group. Sub-analysis showed that respondents who take into account patient preference, used topical imiquimod, radiotherapy and watchful waiting more often.

Conclusion

In conclusion, the results of this survey show that there is a variance in the diagnostic methods and treatment modalities used for LM across Europe. Surgery remains the most utilized option. However, non-surgical options, such as topical imiquimod and radiotherapy, are most often used for elderly patients. We recommend that future studies focus on patient preference and compare surgical to non-surgical therapy.

Introduction

Lentigo maligna (LM), a form of melanoma in situ, is treated to prevent progression to lentigo maligna melanoma (LMM). It typically progresses very slowly and can remain in a non-invasive form for years. A study from Sweden showed an age adjusted LM incidence of 15/100,000 patient years[1]. Recent studies showed an increasing incidence of LM in Denmark (0.55 LM /100,000 patient years to 1.05 LM /100,000 patients years between 2009-2011), Girona, Spain (0.36 LM/100,000 patient years to 1.1 LM/100.000 patient years between 1994-1996) and the Netherlands (0.72 LM /100,000 patient years to 3.84 LM/100,000 patient years between 1989-2013)[2-5]. The incidence of LM has been shown to rise with age[2-4]. The lifetime risk of developing a LMM within a LM has been reported by Greveling *et* al. to be as low as 2-2,6% over a course of 25 years[2].

LM typically occurs in UV-exposed areas like the face, which has critical anatomical structures. Elderly patients often have multiple comorbidities. As a consequence, clinical management may be challenging[6].

Several studies have reported a variance in the management of LM. A previous survey performed by Charles *et al.* showed that dermatoscopy was only used by 17.4% (N=597) of the respondents[7].

Another survey by Mahendran *et al*. showed that there was a variation in the management of LM among respondents in the United Kingdom (N=170). Of the total respondents, 94% used a biopsy to confirm the diagnosis LM. Of these respondents, 35.6% performed a single punch biopsy and 51.8% an incisional biopsy. The preferential treatment of respondents was surgery (89%), a minority used cryotherapy (6%), watchful waiting (3%) and radiotherapy (2%). For patients >70 years of age the preferential treatment was still surgery (50%), followed by watchful waiting (20%), cryotherapy (17%) and radiotherapy (13%)[8].

The most current consensus guideline advices complete excision of LM with at least a 5 mm margin[6]. Preferably, by using a staged excision technique like Mohs micrographical surgery. Non-surgical treatment options, such as radiotherapy or topical imiquimod can be considered[9]. However, comparative studies are lacking and treatment guidelines are based on expert opinion solely[10,11].

The primary objective of this study is to assess current practices of European dermatologists and residents regarding the diagnosis and treatment of LM.

Methods & Materials

Study population

The European association of Dermatologists and Venereologists (EADV) member database was consulted to identify all European dermatologists and dermatology resident members. All electronic contact information was retrieved manually from the EADV database and entered into a recipient database. We excluded physicians who had incomplete electronic address information. All remaining members (N = 3308) were invited to participate in an online survey between December 2014 and June 2015. Non-responders were sent two reminders after three and five months. If a survey was returned due to improper address information, a web based search was performed to update contact information.

Survey

The survey consisted of 29 questions and was designed to investigate the application of different diagnostic and therapeutic options among European dermatologists and residents. The survey consisted of three sections: 1. general information, 2. diagnostics and 3. treatment. The questions included queries about work setting, the number of LM patients seen by a respondent, what kind of diagnostic methods were used and if respondents use a multidisciplinary approach. The participants were asked whether they actively diagnosed or treated LM patients. If participants answered no, the survey finished after the diagnosis section. To assess treatment strategies, questions were included about preferences for non-surgical treatment such as topical imiquimod or radiotherapy. A sub section regarding "surgical treatment and treatment after non radical excision" was used to evaluate the type of surgical methods which are used. Questions for age specific groups were included for people <60 years of age, 60-70 years of age and >70 years of age to evaluate differences in treatment in relation to age of the patient. Questions were included to evaluate follow-up schedules. Lastly we included questions about decision making when opting for a certain treatment modality. Most questions were multiple choice, and multiple answers could be ticked per question. Therefore, the total of answers per question may exceed >100%. (see appendix 1 for the complete survey).

Surveys were included if the respondent was either a dermatologist or a dermatology resident. We excluded surveys if a respondent 1. Did not actively practice dermatology, 2. Was not a dermatologists or dermatology resident, 3. Did not fill in a complete survey.

Analysis

All data was extracted and analyzed using descriptive statistics. Data was expressed as means (%) or N (%) with a range where applicable. Sub analysis using X-square tests were performed to explore whether there was a relation between management of LM and respondents work setting, guideline usage, patient preference and geographical location.

Statistics

All data was analyzed using SPSS version 22.0 (IBM Corporation, Armonk NY, USA) statistics software. The survey responses and demographics were analyzed using descriptive frequencies. The total number of respondents who answered a section (general information, diagnostics or treatment) was set as 100% for that section.

Results

Participants/respondents

The survey was sent to 3308 EADV members. A total of 36 surveys was returned due to an incorrect email address. After an electronic internet search we identified 20 of the 36 incorrect email addresses, and the survey was successfully re-sent to these 20 potential respondents. The response rate was 12.8% (N=423). Of the returned surveys, six were returned blank and two were ineligible because the respondents were not active in the field of dermatology. A total of N = 415 (12.5%) completed surveys were included in the final analysis. The geographical spread of respondents per country are displayed in Figure 1.
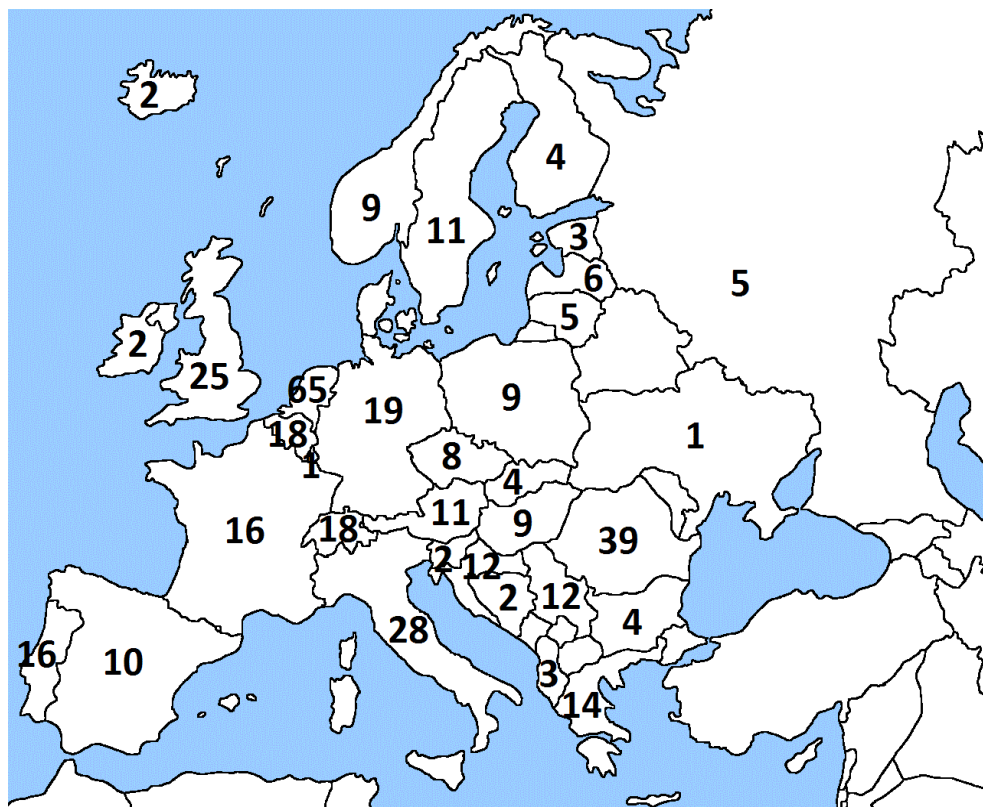
Figure 1: European respondents per country. The number of respondents is represented by the number indicated per country

The general demographics of the respondents are presented in Table 1. The majority of the respondents is certified as a dermatologist (91,5%) and a minority are residents (8,5%). Most respondents practiced either in an university hospital (40,1%) or in private practice (31,1%). For the diagnosis and treatment of LM patients more than half of the respondents use a guideline (58,6%), either a national guideline (58,0%) or the European guideline (42%). Most respondents see up to 10 new cases of LM per year (66,7**%**).

Table 1: Demographics of 415 respondents. All data is expressed as N(%) or mean(range)

Variable	N (%)
Gender	
Male	183 (43.1%)
Female	232 (55.9%)
Age	Mean 47.7 years
	(range: 26-80 years)
Profession	
Dermatologist	380 (91.5%)
Resident	35 (8.5%)
Practice setting	
University Hospital	170 (41.0%)
General Hospital	90 (21.7%)
Private Practice	129 (31.1%)
Independent center	6 (1.4%)
Other	20 (4.8%)
Guideline usage	
Yes	243 (58.6%)
National guideline	141 (58.0%)
European guideline	102 (42.0%)
No	172 (41.4)
Number of LM patients/year	
0-5	141 (34.0%)
5-10	136 (32.8%)
10-20	69 (16.6%)
20-50	50 (12.0%)
50+	19 (4.6%)

Diagnostic methods

A combination of clinical diagnosis (N=274; 65.7%), dermatoscopy (N=348; 83.4%) and histopathology (N=363; 88.2%) is used by most respondents to diagnose LM. As dermatoscopic criteria to diagnose a LM, half of the respondents use the Stolz criteria (N = 235; 55.6%), a third used the Shiffner (N = 126; 29.8%) criteria and a minority use the Pralong criteria (N=23, 5.4%).

Only a minority of the respondents use confocal microscopy (N = 23; 5,5%) or the Woods lamp (N= 8; 1,9%) in the diagnostic work-up. The confocal microscope is mostly used by dermatologists and residents working in a university hospital (N=15).

Respondents collect tissue for histopathological evaluation utilizing multiple techniques. A punch biopsy (N= 258; 61.0%) is used in most cases, sometimes an incisional biopsy (N = 117; 27.7%), an excisional biopsy (N =135; 31.9%) and/or skin mapping (N = 81; 19.1%).Most respondents select either the greyest area(s) of a lesion to locate the area of the biopsy (N = 311; 73.5%), or any palpable or elevated area(s) (N = 195; 46.1%).

Treatment

In most cases, the dermatologist or dermatology resident participate in choosing the treatment for their LM patients (N = 376; 90.6%). Less than half of these respondents (43.6%) sometimes discuss the treatment options for their LM patients in a multidisciplinary team (consisting of e.g. a plastic surgeon, a radiotherapist and a pathologist). Only some respondents often discuss their LM patients (21.3%) and a minority discuss all their LM cases in a multidisciplinary team (18.6%).

The choice of treatment is based on a multitude of criteria, among which were: anatomic localization of the lesion (N = 338; 89.9%); size of the lesion (N = 327; 87.0%); age of the patient (N = 304; 80.9%) and feasibility of the treatment (N = 248; 66.0%). The preference of the patient is only taken into account in half of the cases (N=220; 53.7%).

The most common treatment for LM patients is surgery (N = 367; 97,6%). The second most common option is topical imiquimod (N= 187; 49.7%), after which the respondents opt for radiotherapy (N = 101; 26,9%) or cryotherapy (N = 95; 25.2%) (Figure 2).
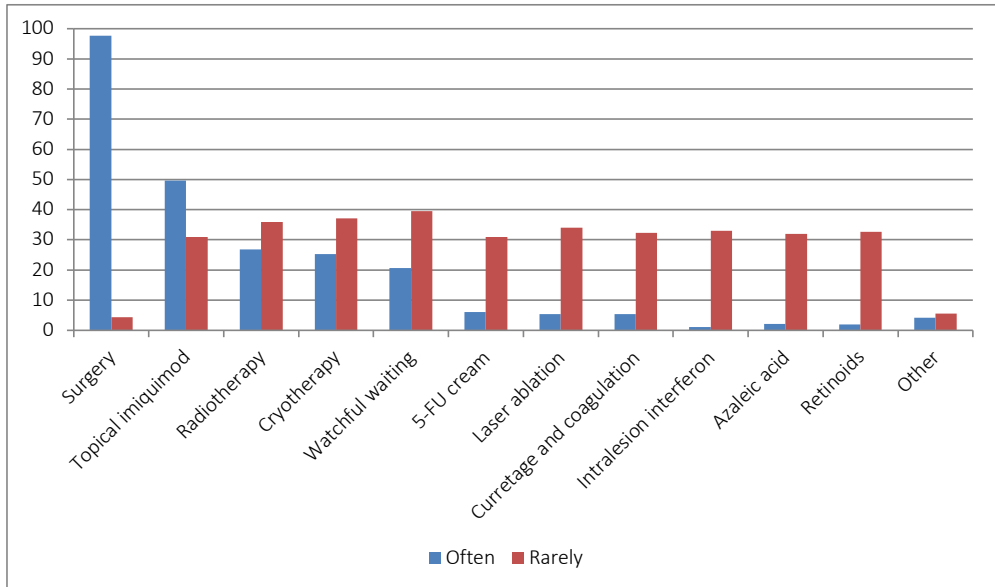
Figure 2: Variation in treatment modalities used for LM. Data is represented in % of 376 respondents who actively treat LM patients. Modalities used *often* are displayed in blue, modalities used *rarely* are displayed in red.

Surgery and treatment after non-radical excision

When respondents opt for surgical treatment, 57.5% excise the LM with a ≤ 5 mm margin (N = 217). A margin >5 mm is used by 38.6% of the respondents (N=145). Other surgical techniques include Mohs micrographical surgery (N=40; 10.6%), staged excision (N=56; 14.9%) or another strategy (N=26; 6.9%), such as the spaghetti technique (Figure 3). If there is uncertainty about the completeness of the excision, two third of the respondents recommended a re-excision (N = 266; 70,6%). When the histological margins are positive, 85% of the respondents recommended a re-excision (N = 321; 85,1%) and only a minority of the respondents (N = 40; 10,6%) would prescribe topical imiquimod after non-radical excision.
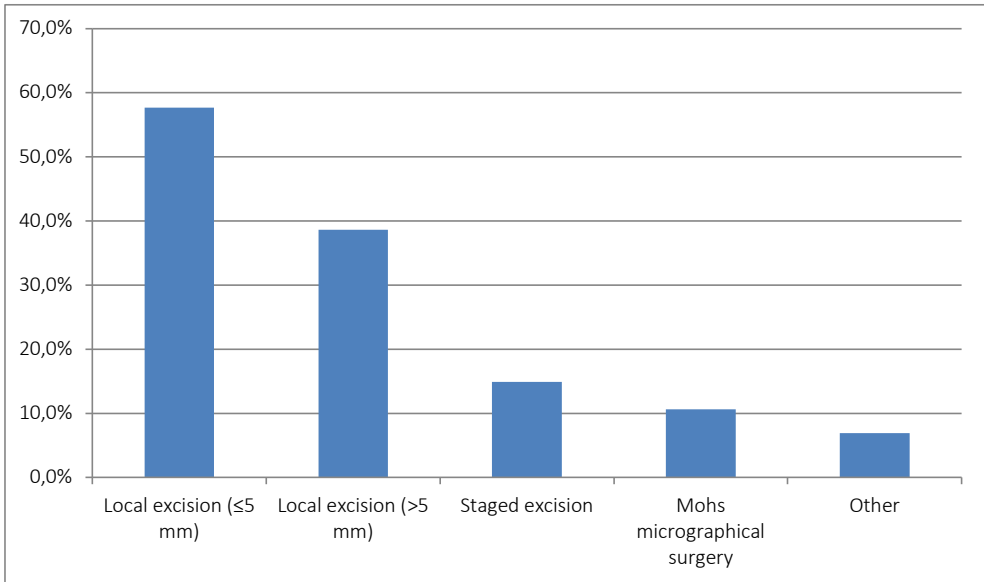
Figure 3: Types of surgical modalities used for LM. Data is presented as % of total number of respondents (N=376).

Age group specific treatment preference

We evaluated the preference of treatment for different age groups (i.e. <60 years, 60-70 years and >70 years of age. In the age group of <60 years surgery is the preferred treatment (N = 357; 94.9%). For LM patients between 60-70 years surgery is chosen in 86.7% (N = 327). For LM patients >70 years of age, 66.8% of the respondents prefer surgical treatment. However, non-surgical options such as radiotherapy (17.0%), topical imiquimod (30.6%) or watchful waiting (19.6%) are used more often in this group compared to patients <60 years of age. In addition, cryotherapy is considered by 20.4% of the respondents for LM patients >70 years of age. (Figure 4).
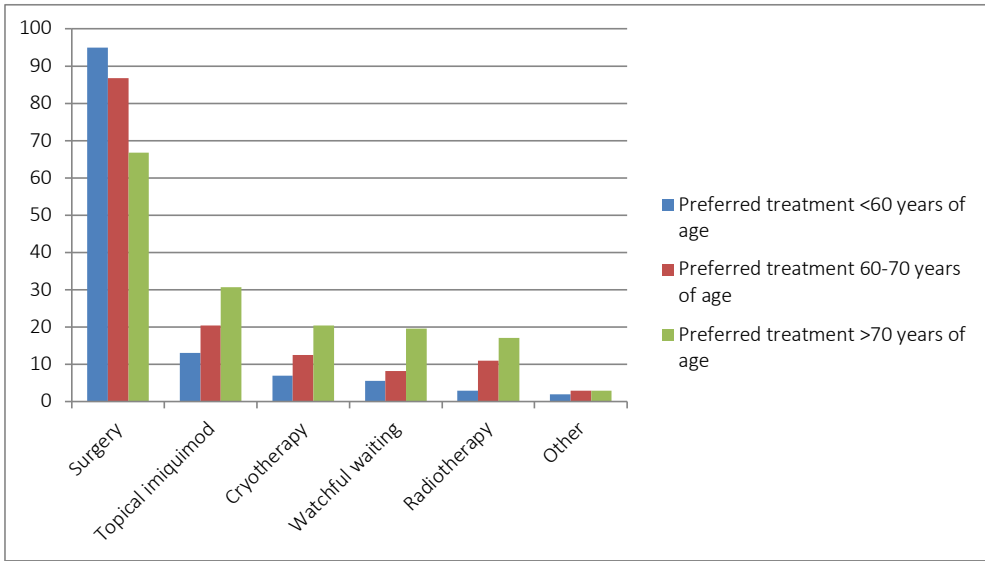
Figure 4: Treatment preference depending on the age of the patient. Data is presented as the % of respondents that actively treat LM patients (N=376). Respondents had the option of selecting multiple treatment modalities.

Follow-up of LM patients

After both macroscopic clearance and confirmed microscopic/histologic clearance, most respondents see their patients for a control visit within one year (N = 336; 89.4% and N = 320; 85.1%, respectively).

Sub analysis

Management of LM in relation to guideline usage, work setting or geographical location

Respondents who *do* use a guideline significantly used topical imiquimod, cryotherapy and watchful waiting less often, compared to respondents who *do not* use a guideline (Table 2).

Respondents in university hospitals used radiotherapy, and a multidisciplinary approach more often compared to respondents working in general hospitals, private practice or independent treatment centers (Table 2).

Respondents working with a multidisciplinary approach did not have a different treatment usage compared to respondents who do not have a multidisciplinary approach. Between respondents working in university hospitals, general hospitals, private practice or independent treatment centers there was no significant difference in 1. guideline usage 2. diagnostic methods used, 3. treatments rarely used, 4. type of biopsies performed, 5. types of surgery used or 6. treatment for different age groups (Data not shown).

Patient preference and treatment

Respondents *who take into account patient preference*, use topical imiquimod, radiotherapy and watchful waiting more often compared to people who *do not* take patient preference into account (Table 2).

Geographical location and management of LM

There was no significant difference in the usage of diagnostic tools and therapeutic options between individual countries (Data not shown).

Table 2. Sub analysis of management of LM depending on patient preference and work setting. All data is expressed as N respondents (% of total respondents). P values were considered significant <0.05 and are marked in bold text. IMQ = Topical imiquimod, RTx = radiotherapy, WW = Watchful waiting, CTx = Cryotherapy

N=376 Guideline usage vs treatment used	Surgery		IMQ		RTx		WW		CRx	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Yes	221 (58.8%)	146 (38.8%)	102 (27.1%)	85 (22.6%)	64 (17.0%)	41 (10.9%)	36 (9.6%)	42 (11.2%)	48 (12.8%)	47 (12.5%)
No	4 (1.1%)	5 (1.3%)	123 (32.7%)	66 (17.6%)	161 (34.8%)	110 (29.3%)	189 (50.3%)	109 (28.9%)	177 (47.1%)	104 (27.6%)
P		0.340		**0.037**		0.784		**0.006**		**0.032**
N=376 Treatment center versus	Surgery		IMQ		RTx		WW		CRx	

treatment used	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
University Hospital	166 (44.1%)	3 (0.8%)	76 (20.2%)	93 (24.7%)	65 (17.3%)	104 (27.7%)	33 (8.8%)	136 (36.2%)	36 (9.6%)	133 (35.4%)
General Hospital	88 (23.4%)	1 (0.3%)	53 (14.1%)	36 (9.6%)	18 (4.8%)	71 (18.9%)	20 (5.3%)	69 (18.4%)	25 (6.6%)	64 (17.0%)
Private practice	107 (28.5%)	5 (1.3%)	54 (14.4%)	58 (15.4%)	20 (5.3%)	92 (24.5%)	22 (5.9%)	90 (23.9%)	33 (8.8%)	79 (21.0%)
Independent treatment center	6 (1.6%)	0 (0.0%)	4 (1.1%)	2 (0.6%)	87 (23.1%)	4 (1.1%)	3 (0.8%)	3 (0.8%)	1 (0.3%)	5 (1.3%)
P		0.377		0.124		**0.001**		0.318		0.382
N=376 Patient preference vs treatment used	**Surgery**		**IMQ**		**RTx**		**WW**		**CRx**	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Yes	200 (53.2%)	167 (44.4%)	113 (30.0%)	74 (19.7%)	79 (21.0%)	26 (6.9%)	54 (14.4%)	24 (6.4%)	53 (14.1%)	42 (11.2%)
No	2 (0.5%)	7 (1.9%)	89 (23.7%)	100 (26.6%)	123 (32.7%)	149 (39.6%)	148 (39.4%)	150 (39.8%)	149 (39.6%)	132 (35.1%)
P		0,055		**0,01**		**0,0001**		**0.002**		0.640

Discussion

The results of this survey show that there is a variance among European dermatologists and residents in the diagnostic methods and treatment modalities used for LM. In general, a combination of clinical aspects, dermatoscopic morphology and histopathological examination is used to diagnose LM. Most often LM is diagnosed by a single punch biopsy. Skin mapping, an incisional or excisional biopsy is performed by a minority of the respondents. Currently, surgical treatment is the preferred choice for LM patients, although there is a shift towards non-surgical treatments in elderly patients.

The use of dermatoscopy has increased . This survey shows that 83.4% of the respondents use dermatoscopy for the diagnosis of LM compared to 17.4% reported by Charles *et al*[7].

Confocal microscopy is only used by a limited number of the respondents, this could be due to the high cost of a confocal microscope[12]. Another reason might be a lack of experience and the long learning curve using this microscope[13]. However, it could be a valuable tool to differentiate between lesions which may look like LM dermatoscopically, such as pigmented actinic keratosis, benign lichenoid keratosis, melasma and seborrhoic keratosis[14]. It could also be used as a tool to delineate LM for surgical excision or for follow-up[15-17].

The current European consensus guideline recommends an excisional or incisional biopsy to obtain specimens for histopathology[9]. It has been shown that a single punch biopsy could lead to sampling error, because LM are often larger than 10 mm[18]. Most respondents use a single punch biopsy (61.0%) to obtain material for histopathological examination. We hypothesize that single biopsies are taken by respondents to confirm the diagnosis of LM, and differentiate it from a benign lesion. It is possible that respondents do not actively perform further diagnostics if the primary biopsy shows LM, but this remains speculation.

For the treatment of LM, the current European consensus guideline recommends surgical excision with at least a margin of 5 mm, or preferably utilization of staged techniques such as Mohs micrographical surgery[9]. Standard excision may lead to aesthetic and functional impairment. Recurrence rates of standard excision with a 5 mm margin are reported to be 30% after 5.5 years[19]. Staged techniques , such as Mohs micrographical surgery or the spagetthi technique show a superior recurrence rate of 4-5.9%[20-22].

Recently, two reviews showed that imiquimod can be an effective treatment option. A complete clinical clearance rate of 74.3-76.2% and a histological clearance rate of 76.2-78.3% was found[23,24]. A trial by Marsden *et al*, in which 60 LM patients were treated with imiquimod applications 5 times per week, for a total of 12 weeks, showed a complete clearance rate in 37% of the treated patients[25].

Radiotherapy is superior to surgery in conserving normal tissue, it is also associated with an estimated recurrence rate of 5% after 3 years[26]. There is only a single study describing

cryotherapy for LM. In this study, 30 LM patients were treated by freezing the LM with liquid nitrogen delivered by an open spray. This study reported recurrence rates of 6.6% in 3 years

The current application of treatment for different age groups of patients seems similar to results of the survey by Mahendran *et al.* For LM patients <60 years of age respondents reported the use of mainly surgery (89.0%). For patients >70 years of age, half of the respondents used surgery (50.0%), only a minority used non-surgical options such as cryotherapy (17.0%), radiotherapy (13.0%) and watchful waiting (20.0%)[8]. Our survey showed that respondents used mainly surgery (93.4%) for patients <60 years of age. For patients >70 years of age respondents most often used surgery (66.8%). Non-surgical options such as topical imiquimod (30.6%), radiotherapy (17.0%), and watchful waiting (19.6%) are also used.

Respondents who do not use a guideline used topical imiquimod, watchful waiting and cryotherapy more often. This could be due the guidelines only making recommendations regarding surgical therapy. In the newest European consensus guideline non-surgical options like topical imiquimod, radiotherapy and watchful waiting are mentioned, but there is no advice on patient selection for the various therapies.

Respondents who take patient preference into account, also use more non-surgical options like topical imiquimod, radiotherapy and watchful waiting. Hypothetically respondents confer with their LM patients when opting for a treatment modality. The choice can be influenced by variables such as the size of the lesion, potential scarring, the age of the patient and comorbidity.

In a recent publication by Swetter *et* al, it was suggested that histopathological clearance should not necessarily be the gold standard for outcome measurement[27]. A study by Greveling *et al.* reports that the relative survival of LM patients (104%) and LMM (99%) patients does not differ significantly compared to the general population[2]. Arguably, when treating LM, complete histological clearance might not be the necessary goal. The fact that LM in daily practice is treated non-surgically shows that treatment outcomes, other than radical excision, might be considered of importance. Patients might express preference for non-surgical options. In this survey however, we did not ask respondents what they consider as the main outcome. Other outcomes could be clinical clearance of the LM, which additionally may be confirmed with histopathologic samples. Prevention of recurrence, or progression to LMM may also be a

clinical relevant outcome. The question remains whether LM should be considered as a malignant, pre-malignant or a benign lesion

Limitations of this survey are that the response rate of 12,5% induces a risk of reporting bias and limits the generalizability of our results. However, of all EADV members based around Europe, an average of 14% (range 1-80%) of the contacted EADV members per country responded. All invited countries were represented in the survey results except Belarus, Montenegro, Macedonia and Kosovo (Figure 1).

In conclusion, there is no standard procedure for diagnosing LM. Surgery remains the most utilized treatment option. In elderly patients, respondents more often advice non-surgical options such as topical imiquimod and radiotherapy. It is valuable for management of LM in general to study patient preference and. We also recommend that future studies compare non-surgical and surgical treatment options in a randomized controlled setting, and whether histopathological clearance should be the primary outcome measurement

Funding

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2

Appendix 1: Questionnaire on lentigo maligna: diagnosis & treatment

INTRODUCTION

Currently there are no specific guidelines regarding Lentigo Maligna (LM) that provide a clear evidence-based approach to the diagnostics and treatment of patients. The aim of this questionnaire is to investigate to what extent there is heterogeneity in diagnosis and treatment of LM patients among dermatology professionals. We here ask you about your current practice with LM patients.

This questionnaire is completely anonymous and consists of 26 questions. It will take approximately **5 minutes** to complete the questionnaire.

GENERAL INFORMATION

1. What is your gender?
    a. Male
    b. Female

2. What is your date of birth? (Day/Month/Year)

    ⬜⬜ - ⬜⬜ - ⬜⬜⬜⬜

3. What is your current profession?
    a. Dermatologist
    b. Dermatology resident
    c. Other (please specify) ……………………………….

4. Are you currently practicing?
    a. No
        i. If **NO**: the questionnaire stops here. Thank you for your time.
    b. Yes
        i. If **YES**: What kind of practice do you work in?
            1. University hospital
            2. General hospital
            3. Private practice

4. Independent treatment center

5. Other (Please specifiy) …………………………….

5. How many years of experience do you have in treating dermatology patients including residency?

    a. …… years

6. Do you follow a guideline for patients with a lentigo maligna?

    a. Yes/No

7. If yes, which guideline?

    a. National guideline

    b. European consensus basedguideline

DIAGNOSIS OF LENTIGO MALIGNA

8. How many new cases including referrals of lentigo maligna patients **per year** do you see in your practice on average? Please provide an estimate **over the last 5 years.**

    a. 0-5

    b. 5-10

    c. 10-20

    d. 20-50

    e. 50+

9. What method do you use to diagnose lentigo maligna? (*Please tick all that apply*)

    ( )Clinical diagnosis

    ( )Diagnosis based on dermatoscopy

    ( )Diagnosis based on Wood's Lamp

    ( )Diagnosis based on confocal microscopy

    ( )Diagnosis based on histopathology??

    ( ) Other

10. What dermoscopic criteria do you use in the diagnosis of lentigo maligna

    ( ) Classic Stoltz criteria: hyperpigmented follicular opening, annular-granular pattern, pigmented rhomboidal structures, obliterated hair follicles

( ) Shiffner criteria: asymmetric pigmented follicular openings, dark (brown or black) rhomboidal structures, slate-gray globules and slate-gray dots.

( ) Pralong criteria: darkening at dermoscopic examination, increased density of the vascular network, red rhomboidal structures, and target-like patterns.

( ) Other (Please specify): ....

11. If you use confocal microscopy, what type do you use?

    a. I do not use a confocal microscope

    b. VivaScope 1000/1500

    c. VivaScope 3000

    d. Both "a" and "b".

    e. Other (Please specify) ....

12. What is the reason you use confocal microscopy? (*Please tick all that apply*)

    ( ) As main diagnostic modality

    ( ) To guide the selection of biopsy site(s)

    ( ) For pre-surgical delineation

    ( ) To monitor treatment response

    ( ) Other (Please specify) ....

13. Do you take a biopsy if a lesion is suspect to be a lentigo maligna?

    a. Yes, all the time

    b. Yes, often

    c. Yes, sometimes

    d. No, never

14. How do you select the biopsy site? (*Please tick all that apply*)

    a. Center of the lesion

    b. Greyest area(s)

    c. Palpable/elevated area(s)

    d. Guided by confocal microscopy

    e. Other (Please specifiy) ....

15. What type of biopsy do you use?

a. Punch biopsy: ( ) <3mm ( ) 3mm ( ) >3mm

b. Skin mapping with punch biopsies

c. Incisional biopsy

d. Excisional biopsy

TREATMENT OF LENTIGO MALIGNA

16. Do you participate in choosing the treatment strategy for your patient?

a. Yes / No

If **NO NEVER** the questionnaire stops here

if **YES or YES OFTEN or YES SOMETIMES** please continue with question 10

17. Do you discuss the treatment strategy for your patient in a multidisciplinary team (with f.e. radiotherapist, plastic surgeon, pathologist)?

a. Yes, all the time

b. Yes, often

c. Yes, sometimes

d. No, never

18. On which criteria do you base your treatment strategy? *list from most important to least important with 1-8*

( ) Patient's age

( ) Anatomic localisation of the lesion

( ) Size of the lesion

( ) Feasibility of the therapy for the patient

( ) Preference of the patient

( ) Comorbidity

( ) experience with the proposed treatment

( ) guideline recommendations

19. Which treatment modalities do you use? *(Please tick all that apply)*

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

20. Which treatment treatment modalities do you **RARELY** use? *(Please tick all that apply)*

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

21. Which is your **MOST OFTEN APPLIED** therapy= List the therapies from **most often** applied to **rarely** applied from 1 to 6

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

22. What is your usual preferred treatment? *Please tick all that apply*.

a. In the age group of <60 years

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

b. In the age group of 60-70 years

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

c. In the age group of >70 years

( ) Cryotherapy

( ) Whatchful waiting

( ) Radiotherapy

( ) Surgery

( ) Topical Imiquimod

( ) Other, please specify…………………………………

23. Does your hospital facilitate access to Radiotherapy (either in your hospital or to a referral hospital)?

    a. Yes

    b. No

24. Which type of surgery do you use when performing surgical treatment of lentigo maligna? Tick all that apply

    a. Local excision with margin ≤ 0,5 cm

    b. Local excision with margin >0,5 cm

    c. Staged surgical excision (e.g. square excision, mapped serial excision)

    d. Mohs surgery with cryostat sections

    e. Other (Please specify) …………………………………

25. What is your definition of histological free margins?

26. What is your preferred treatment policy in cases of positive surgical margins?

    a. Adjuvant radiotherapy

    b. Adjuvant therapy with topical imiquimod

    c. Re-excision

d. Whatchful waiting policy

e. Other (Please specify) ……………………………………

FOLLOW UP

27. After confirmed microscopic clearance (i.e.histology on biopsy or excision), when do you schedule -in most cases- a control appointment for your patient?

    a. Never

    b. Within ≤ 1 year

    c. In the range of 1-3 years

    d. Within ≤ 5 years

    e. After ≥ 5 years

28. After **MACROSCOPIC** clearance, when do you schedule –in most cases- a control appointment for your patient?

    a. Never

    b. Within ≤ 1 year

    c. In the range of 1-3 years

    d. Within ≤ 5 years

    e. After ≥ 5 years

Where you feel that important aspects were not addressed in this questionnaire, please use the free text box.

Thank you very much for completing the questionnaire. Your input is very valuable and much appreciated. Please send your comments to C.vanMontfrans@vumc.nl

3

A systematic review on the role of imiquimod in lentigo maligna and lentigo maligna melanoma: need for standardization of treatment schedule and outcome measures

D.C.K.S Tio[1#]/ J. van der Woude [2#], CAC. Prinsen[3], EP. Jansma [4], R. Hoekzema[5], C. van Montfrans[6].
#Authors contributed equally

1. Dermatology resident, Department of Dermatology. VUmc Amsterdam, The Netherlands.
2. Dermatology resident, Flevoziekenhuis Almere, The Netherlands
3. Clinical epidemiologist, Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VUmc Amsterdam, The Netherlands.
4. Medical librarian, VU University, Amsterdam, The Netherlands
5. Dermatologist, Department of dermatology, VUmc Amsterdam, The Netherlands
6. Dermatologist, Department of dermatology, ErasmusMc Rotterdam, The Netherlands

Abstract

Introduction

Lentigo maligna (LM) is an in situ variant of melanoma. Our objective was to systematically review clinical and histological clearance and recurrence rates of imiquimod treatment of LM with emphasis on progression to lentigo maligna melanoma (LMM).

Methods

PubMed, EMBASE and the Cochrane library were searched from inception to May 2015. Articles were included if they described histologically proven LM treated with imiquimod 5% monotherapy or combined with another topical therapy. Analyzed outcomes were clinical and histological clearance, recurrence rates and number of LMM. The quality was assessed using the GRADE-like checklist and results reported according to the PRISMA Statement.

Results

Twenty-six case reports, 11 retrospective studies, 3 prospective studies and 1 randomized controlled trial were included. One case report of poor quality was excluded. Complete clinical clearance was seen in 369 of 471 patients (78.3%). Histological clearance was present in 285 of 370 (77%) patients. LMM was diagnosed in 9 (1.8%) patients 3.9 months (range 0-11 months) post treatment. Univariate multinominal logistic regression showed that 6-7 applications/week had a 6.47 greater odds (p=0.017) of resulting in complete *clinical* clearance compared to 1-4 applications/week. An intensity of 6-7 applications/week showed a 8.85 greater odds (p=0.003) of resulting in *histological* clearance compared to 1-4 applications. Applying imiquimod >60 times during a treatment period of 12 weeks (range 4-36) showed a 7.75 greater odds (p=0.001) of resulting in *histological* clearance compared to <60 total applications.

Conclusion

A treatment schedule using imiquimod 6-7 applications per week, with at least 60 applications shows the greatest odds of complete clinical and histological clearance of LM. Imiquimod is an option for patients unfit for not willing to undergo surgery or radiotherapy. Nine cases of LM progressed to LMM shortly after treatment. Our hypothesis is that these LMM may have been present before starting imiquimod.

Introduction

Lentigo maligna (LM) is an in situ variant of melanoma, which presents as a slowly enlarging brown to gray-black pigmented and sometimes amelanotic macule on chronically sun-exposed skin. Especially in patients older than 45 years, the incidence of LM and LM melanoma (LMM) is increasing[1]. By treating LM we aim to prevent progression to invasive LMM. An epidemiological study by Weinstock *et* al reports that a 45-year-old patient with LM, would - without treatment- have a lifetime risk of developing LMM of 4.7%[2]. If the diagnosis of LM is made at the age of 65, the lifetime risk of developing LMM would be 2.2% without treatment [2]. A recent Dutch study showed that the risk of progression of a LM to LMM is 2.0-2.6% lifetime risk[3].

Tzellos *et* al performed a Cochrane systematic review on the treatment of melanoma in situ, including LM. They concluded that there is a lack of high-quality evidence for both surgical and non-surgical treatments of LM[4]. In international guidelines, recommendations based on expert opinion state that surgical excision with at least a 5 mm margin is the therapy of first choice[5]. For various reasons, surgical management of LM can be challenging: the lesion may be located close to critical anatomical structures; the macroscopic margins are often unclear; in case of a large lesion reconstructive procedures may be needed after excision; histopathology often shows positive margins; most patients with LM are elderly and may be frail and suffer from comorbidity[1,3,6]. A review on the surgical treatment of LM showed that margin-controlled surgical techniques such as Mohs micrographical surgery, staged excision or the spaghetti technique are good alternatives to standard excision showing recurrences rates <5%[7].

Over the past 15 years, imiquimod cream has gained attention as an off-label, topical and non-invasive treatment modality for LM. Imiquimod targets atypical melanocytes both directly and by inducing an immune response against the atypical melanocytes[8]. This leads to secretion of pro-inflammatory cytokines and a cellular immune response to the tumor cells.

We aimed to systematically review all studies on imiquimod treatment of LM patients with emphasis on progression to LMM. Moreover, we assessed the clinical and histological clearance and recurrence rates after imiquimod treatment, and analyzed the optimal treatment schedule.

3

Methods

The results of our systematic review were obtained according to the guidelines for reporting systematic reviews as published in the PRISMA Statement (www.prisma-statement.org).

Eligibility criteria

Studies that were included in this review described patients of all ages with histologically proven LM, treatment with imiquimod 5% cream monotherapy or imiquimod combined with another topical therapy. Outcomes of the included studies were clinical and histological clearance, recurrence rates and the number of LMM. All lengths of follow-up were included and studies had to be published in English. Tzellos *et* al reported a paucity of high quality evidence regarding the treatment of LM[4]. Therefore we decided to include lower quality original studies as well such as case reports and cohort studies. Literature reviews, conference abstracts, animal studies, *in-vitro* studies, studies lacking full text, cases regarding melanoma in situ or LMM as the primary diagnosis were excluded .

Information sources and search

A systematic literature search was performed from inception up to and including May, 2015 in MEDLINE (PubMed) and EMBASE (www.embase.com), and The Cochrane Library (via Wiley) by a clinical librarian (EJ). Search terms included controlled MeSH terms in PubMed, EMtree in EMBASE.com as well as free text terms. We used free text terms only in The Cochrane library. Search terms expressing 'lentigo maligna', 'Dubreuilh melanosis' and 'Hutchinson's freckle' were used in combination with search terms identifying 'imiquimod', 'aldara' and 'zartra'. The full search strategy can be found in Appendix 1. References of included studies were checked for additional relevant reviews.

Study selection

Two reviewers (CvM and JvdW) independently screened all relevant titles and abstracts for eligibility. If necessary, full text articles were screened for eligibility. Differences in judgement were resolved with a third reviewer (CP) until consensus was reached (Figure 1).

Figure 1: Flowchart of the search and selection procedure of studies

Identification

Screening

Eligibility

Included

Records identified through database searching (n=285)

Records after duplicates removed (n=211)

Records screened (n=211)

Records excluded (n=137)

Reasons: reviews, animal studies non related subject

Full-text articles assessed for eligibility (n=74)

Full-text articles excluded:

-conference abstracts (n=8)

-no full text available (n=11)

- articles that were not published in English(n=10)

- Melanoma in situ/LMM primary diagnosis (n=1)

- commentary (n=2)

- duplicate (n=1)

Articles included in qualitative synthesis (n=41)

Data collection process

Two reviewers (JvdW and DT) extracted data from the included studies independently. Disagreements were resolved by consensus; if no agreement could be reached, a third author (CP) was consulted.

Data extraction

The following information was extracted from each study: age, gender, length of follow-up,

number of lesion(s), location of lesion(s), type of intervention, treatment schedule, treatment duration, total number of treatments, treatment intensity, inflammation, histological and/or clinical clearance, recurrence, number of LMM during or after imiquimod treatment and side effects.

We defined complete clinical clearance as no residual pigmentation and partial clinical clearance as residual pigmentation, based on clinical examination, dermatoscopy or confocal microscopy. Absence of clinical clearance was defined as lack of change in clinical appearance at any time point after finishing imiquimod treatment. Histological clearance was defined as absence of residual LM in a biopsy or excision specimen, obtained after finishing imiquimod treatment. Absence of histological clearance was defined as the presence of atypical melanocytes in a biopsy or excision specimen. We did not include partial histological response as an endpoint. This was only defined as an endpoint in a single study[9].

We classified the inflammatory response as 'no inflammation', 'mild inflammation' or 'severe inflammation'. When this classification was not applied, we registered the terms used to describe the inflammatory response. Recurrence was defined as clinical or histological presence of LM after previous complete clinical or histological clearance. Refractory lesions were defined as treated LM which did not show clinical and/or histological clearance. Patients were considered dropouts if they did not complete the treatment course due to excessive inflammation or other reasons.

Risk of bias in individual studies

Two reviewers (DT and JvdW) independently assessed the risk of bias using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach to grade quality of evidence and strength of recommendations (http://www.gradeworkinggroup.org) The GRADE system is a tool which can be used to distinguish articles of poor and excellent quality. The results of quality analysis can be used to formulate recommendations based on studies of good quality[10]. A 13-item checklist was used (one point for each criterion met): 0-4 points was defined as *poor* quality, 5-7 reflected a *fair* quality, 8-10 points as *good,* and 11-13 points as *excellent quality[11]*. Studies that scored "poor" quality were excluded. The studies, scoring "fair", "good" and "excellent" were considered as equals for the final analysis

Summary measures

Patient characteristics, lengths of follow-up and treatment regimens were analyzed using descriptive statistics. Data are reported as means or proportions, if appropriate with a 95% confidence interval. Clearance rates, recurrences, and development of LMM were expressed as means or proportions with a 95% confidence interval.

Clinical and histological clearance rates were analyzed using multinomial- and binomial-logistic regression tests respectively. The effects of the following three variables on the clearance rates were analyzed: 1) treatment intensity, defined as number of applications of imiquimod per week, 2) the total number of applications during the entire treatment period and 3) the total treatment period defined as the number of weeks of imiquimod application. The results were expressed in odds ratios.

Clinical clearance, histological clearance, treatment intensity and treatment duration were analyzed using univariate logistic regression. These same variables were also analyzed using multivariate logistic regression. Multicollinearity between treatment intensity and treatment duration were examined by calculating a phi coefficient. All tests were performed using SPSS statistics software (version 22, IBM company).

Synthesis of results and risk of bias across studies

We pooled the data on clinical clearance, histological clearance, treatment intensity and treatment duration of each individual patient to create one large cohort[12]. In essence, we calculated the final results using individual patient data.

Results

Study selection

The literature search generated a total of 285 references. After removing duplicates of references, reviews, animal studies and non-related studies, 41 full text articles were included, comprising 26 case reports, 11 open label studies, 3 retrospective studies and a single prospective randomized trial (Figure 1).

Study characteristics

The characteristics of the included studies are summarized in Table 1.

Risk of bias within studies

According to the GRADE approach, the quality of 14 studies was rated as "excellent", 21 as "good", five as "fair" and one as "poor". In the studies graded as "excellent" the treatment rationale, treatment protocol and selection criteria for patients to have histological examination of the treated area during follow-up were described in more detail than in the other studies. The study that we scored as "poor" was excluded from the logistic regression analysis. We noticed a possible risk of publication bias regarding the case reports: of the 26 included case reports, only 1 reported negative outcomes. Therefore the combined Case reports and Cohorts were also analyzed separately.

Results of individual studies

A total of 509 patients with 514 LM lesions were included (mean age 69.5 years; range 33-95; SD 12). Information about localisation of the lesions was available in 380 patients/lesions .In 354 patients (93.1%) lesions were situated in the head and neck region, in 26 patients (6.9%) on the trunk or extremities.

Information about prior treatment was provided in 219 patients. Cryotherapy, surgery, laser ablation, radiotherapy or topical 5-fluorouracil was applied in 111 of these 219 patients, whereas 108 of these 219 patients did not receive any treatment before imiquimod. Of the 514 LM lesions, 280 were primary, 64 lesions were recurrent and in 170 cases it was unknown whether LM was primary, refractory or recurrent.

In several studies the imiquimod treatment regimen was only described in general terms , for example "on average 3-5 times a week". We calculated the average treatment frequency per individual patient. To achieve this, we multiplied the average number of weekly applications with the number of weeks of treatment duration.

The treatment protocols ranged from 15 to 440 applications during the total treatment period, and it was applied variably between once daily up to once a week over a period of 4 to 36 weeks. Even in individual cases the number of applications per week varied during the total treatment period (Table 1).

Table 1: Patient characteristics

Studies	41
Patients / Lesions	509/514
Male/Female/unknown	249/210/45
Mean age	69.5 year (33-95)
Location -Head/neck -Trunk/extremities	 354 26
Treatment (total number of applications)	71 (15-440)
Treatment (number of applications per week)	1-7
Treatment duration (weeks)	12.7 (4-36) weeks
Follow-up duration	21.9 (3-72) months

A uniform classification to describe the clinical inflammatory response induced by imiquimod was not applied in most studies. In 9 studies (22%) the inflammatory response was classified using the terminology 'mild', 'moderate', or 'severe'. In 17 studies (41%) the presence or absence of an inflammatory response was mentioned, but we were unable to classify the inflammation based on the reported data. In 15 studies (37%) there was no description of an inflammatory response.

Information about the presence of clinical clearance was available in 471 of 509 patients. Complete clinical clearance was seen in 369 of the 471 patients (78.3%). Absence of clinical clearance was reported in 23 of the 471 patients (4.9%). Partial clinical clearance and residual pigmentation was present in 79 of the 471 patients (16.8%). In 16 of these 79 patients with residual pigmentation biopsies were taken. No histological features of LM were observed in these patients. The other 63 patients were not histopathologically examined, and it is therefore unknown whether the residual pigmentation in these cases did or did not indicate incomplete clearance of LM (Table 2).

After imiquimod treatment, histopathological examination was performed on biopsies or excision specimens in 370 patients, including the 16 patients with clinical residual pigmentation. Histological clearance of LM was demonstrated in 285 of 370 patients (77%). Failure of histological clearance (LM still observed in H&E stained sections) was present in 85 out of 370 patients (23%). The time point at which either clinical or histological clearance was determined was not stated or varied between the described patients between 1 week and 15 months after finishing treatment (Table 2).

Side effects of imiquimod treatment were localized erythema, discomfort, swelling, erosions and severe inflammatory responses. Fourty-three patients dropped out during imiquimod treatment due to intolerable inflammation, unrelated causes or loss of follow-up. These patients were not included in the final statistical analysis.

Table 2: Clinical and histological clearance rates

Clinical response reported (N=471)	Clinical response reported (N=471)	Histology after treatment (N=370)
Complete clinical or histological clearance	369 (78.3%)	285 (77%)
Partial Clearance	79 (16.8%)	-
Clinical or histological non clearance	23 (4.9%)	85 (23%)

Recurrence and development of lentigo maligna melanoma during or after imiquimod treatment

The mean length of follow-up was 21.9 months (range 3-72 months). In 11 patients (2.2%) a recurrence was detected after a mean follow-up of 18.6 months (range 9-37 months). There is a concern about the risk of progression of LM to LMM during treatment with imiquimod (13;14). A LMM was detected in 9 patients (1.8%), on average 3.9 months (range 0-11 months) after completion of treatment (Table 3).

Table 3: Cases of lentigo maligna melanoma (LMM) after treatment of lentigo maligna with imiquimod.

Study	N LMM	Applications per week	Treatment duration (weeks)	Time to biopsy after treatment (weeks)	Breslow (mm)	Time to LMM after treatment (weeks)
Fisher et al[13]	1	3x	14	-	3,30	During treatment
Naylor et al[14]	1	7x	12	-	-	During treatment
Cotter et al[15]	1	5x	12	8	-	8
Powell et al[16]	1	3x	6	12	0,46	Non responder
Woodman see et al[17]	1	3x	8	44	0,78	44 (Intially a recurrence)
Hyde et al[18]	1	5x	12	8	0,32	8
Guitera et al[19]	2	5x	12	8	0,40	8
Swetter et al[20]	1	?	?	?	0,50	?

Additional analysis

Univariate, multinomial/binomial logistic regression of the effect of treatment intensity on clinical clearance rate showed that applying imiquimod 6-7 times per week has a 6.47 times greater odds of resulting in complete clinical clearance compared to 1-4 applications per week (odds ratio of 6.47; 95% CI, 1.40-30.03; p = 0.017). An intensity of 5 applications per week has a 3 times greater likelihood of producing a *partial* clinical clearance compared to 6-7

applications per week (odds ratio of 2.88; 95% CI 1.27-6.56; p=0.012). Applying imiquimod >60 times in total does not result in a significantly different clinical response (Table 4).

Table 4: The effect of treatment intensity (applications per week) and total number of applications on the odds ratio of achieving complete *clinical* clearance. Complete *clinical* clearance, >60 applications in total and 6-7 applications per week were used as reference categories. Data is presented as: odds ratio(range).

	Not clear	P	Partial clearance	P
<60 applications	2.0 (0.60-6.80)	0.285	1.5 (0.80-2.90)	0.219
>60 applications (ref)				
1-4 applications per week	6.47 (1.39-30.03)	**0.017**	0.46 (0.12-1.86)	0.278
5 applications per week	1.32 (0.28-6.23)	0.73	2.88 (1.27-6.56)	**0.012**
6-7 applications per week (ref)				

Applying imiquimod 6-7 times per week gives a 7.1 greater chance of histological clearance (odds ratio 7.10; 95% CI 4.02-10.30; p=0.01), compared to 1-4 applications per week. Applying imiquimod 5 times per week has a 8.85 times greater risk of *non*-histological clearance compared to 6-7 applications per week (odds ratio 8.85; 95% CI 5.33-11.15; p=0.003; Table 5). Multivariate multinomial/binomial logistic regression was not performed due to multicollinearity (Phi = 0.77; p<0.001) of the different independent variables.

Table 5: The effect of treatment intensity (applications per week) and total number of treatments on the odds ratio of achieving *Histological* clearance. *Histological* clearance, >60 applications in total and 6-7 applications per week were used as reference categories. Data is presented as odds ratios.

	Not clear	P
<60 applications	7.75 (4.02-10.30)	**0.001**
>60 applications (ref)		
1-4 applications per week	7.11 (4.52-10.45)	**0.001**
5 applications per week	8.85 (5.33-11.15)	**0.003**
6-7 applications per week (ref)		

Discussion

Overall, there is a lack of evidence regarding the effect of treatment with imiquimod cream for LM. No studies were found that compared imiquimod cream with either surgical treatment or radiotherapy. Based on 41 studies, evidence suggests complete clinical clearance rates of 78.3% and histological clearance rates of 77%. Nine cases of LMM were described that developed on average 3.9 months after the last application of imiquimod. The recurrence rate of LM was 2.2% after a mean follow-up of 18.6 months. The optimal treatment schedule to achieve clinical and/or histological clearance consisted of a cumulative dose of >60 applications and a treatment intensity of > 5 applications per week. These findings are in line with the results in 347 LM patients described in the review by Mora *et al*[21].

The results of our study are of relevance to dermatologists who seek an alternative treatment for patients with LM, who refuse to have or are not eligible for surgery or radiotherapy. These cases include elderly patients who cannot be operated without substantial risks or who are unable to make the frequent visits required for radiotherapy, patients who are reluctant to have a large facial scar, or patients with LM on functionally important areas such as the nose.

Strengths of this study are that we assessed the methodology of the included articles using the GRADE-like method. Using this method we were able to exclude studies with "poor" level evidence. Another strength is the use of individual patient data for our final analysis. This gives equal value to all cases included.

A limitation of this study is the finding that extrapolation of findings in the majority of studies was hampered by the lack of consistency in definitions of outcome, grading of inflammation and treatment schemes. There was no uniform time point defined when clinical or histological

clearance was evaluated.

There was a lack of consistency in the procedures to diagnose LM. Often, only a single biopsy was taken for the diagnosis. In a recent article, Kai *et* al suggested that this might lead to sampling error[22].

Secondly, the reported treatment schedules varied significantly (both frequency of application and treatment duration) and long term follow-up was lacking (mean follow-up 21.9 months). At review level we were only able to include 23 LM lesions from 7 studies reporting a treatment failure. Reporting bias may account for some of the beneficial effects we observed. No comparative RCT's were available to include in our analysis. In New-Zealand and Australia an RCT (ClinicalTrials.gov ID:NCT02394132) comparing radiotherapy with imiquimod started inclusion in 2015 for patients with LM, who are not eligible for surgery. Because of the lack of comparative studies our results are not generalizable to all patients with a LM.

Concern exists about the risk of progression of a LM to LMM induced by imiquimod treatment[23,24]. In this review we found 9 LM which progressed to LMM (1.8%) on average 3.9 months (range 0-11 months) after completion of treatment. This suggests that LMM may have been present before starting imiquimod therapy, but this remains a hypothesis. Another concern is incomplete clearance of a lesion after treatment with imiquimod. When using local imiquimod for LM it has been suggested that recurrence can be caused by incomplete clearance of atypical melanocytes extending deep into the pilosebaceous units[25]. In contrast, it has been suggested that the pilosebaceous units may act as a drug delivery route: potentially they can act as a low resistance shunt to viable skin strata[26,27]. However, this route of drug delivery is still poorly understood and needs further clarification[28]. A review by Ellis *et al* showed that cutaneous melanoma metastases have successfully been treated with topical imiquimod. Imiquimod has even been shown to be an effective treatment for dermal metastases. This suggests that imiquimod can penetrate the skin sufficiently to achieve farmacotherapeutic levels, even in the dermis[29,30]. It could be argued that topical imiquimod has the potential to clear the pilosebaceous units from atypical melanocytes. However, at this moment there is a lack of evidence supporting the efficacy of this potential drug delivery route for imiquimod. Therefore, we recommend excision of LM if it does not respond to topical imiquimod.

In a recent study 18 patients with a LM treated with imiquimod were followed for 5 years. The authors found no recurrences after analysis of patients with confocal microscopy[22]. Based on our own experience, we recommend a long term follow-up of at least 5 years after imiquimod treatment.

For future studies we recommend that experts reach consensus about the diagnostic procedures and outcome parameters. Quality of life and cosmetic results should be included as secondary outcomes. The common terminology criteria for adverse effects could be used to describe the clinical inflammatory response. The clinical and/or histological clearance after imiquimod treatment should be determined at a uniform time point.

In conclusion, based on the results of this review we recommend discussing the option of imiquimod treatment for LM with those patients who are not eligible or who are not willing to undergo surgery or radiotherapy. The treatment should involve an intensive schedule with >60 applications in total and a frequency of 6-7 applications per week. Future studies should use uniform outcome measurements including determination of clinical and histological clearance at uniform time points.

Funding

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Appendix 1: Summary of the results of the individual studies. CR = Case report; CS = Case series; OLS = open label study; RS = randomized controlled trial

Reference	Year	Type of study	Grade Score	n=lesions	complete response	partial response	no response	Histol clearance	Histol no clearance	Recurrence	LMM
Ahmed et al.[31]	2000	CR	10	1	1			1		No	
Alarcon et al.[32]	2014	PS	13	20	17		3	15	5	No	
Bratton et al.[33]	2015	CR	9	1	1			1		No	
Buetikker et al.[34]	2008	OLS	12	34	34		6	1			
Chapman et al.[35]	2003	CR	8	1	1			1		No	
Costa et al.[36]	2011	CR	6	1	1		n/a	n/a		no	
Cotter et al.[15]	2007	OLS	13	40	33	7		30	10		1
Demirci et al.[37]	2010	CR	7	5	3	2		n/a	n/a	No	
Ellis et al.[30]	2012	CR	8	1	1			1		no	
Epstein et al.[38]	2003	CR	8	1	1			n/a	n/a	No	

	Feldman et al.[39]	Fisher et al.[13]	Fleming et al.[40]	Guitera et al.[41]	Hyde et al.[9]	Kamin et al.[42]	Kirtschig et al.[43]	Kupfer-bessaguet	Lapresta et al.[45]	Ly et al.[46]	Mahoney et al.[47]	Martires et al.[48]
	2007	2003	2004	2014	2012	2005	2015	2004	2011	2011	2008	2010
	CR	CR	OLS	RS	PRT	CR	PS	CS	CR	OLS	OLS	CR
	7	8	10	13	11	10	12	9	9	12	11	4
	1	1	6	39	91	1	27	2	1	48	7	1
	1		2	19	56		20	2	1	20	6	
		1	3	9	23	1	4			18		1
			1								1	
	n/a	1	4	6	56	1	24	2	1	20	6	
	n/a	n/a	2	3	23	No	0	no	no	18	no	1
	1		n/a	3			1			n/a		n/a
		1		2	1							

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Study	Year	Type									
Powellet al.[58]	2004	OLS	13	11	6	3	2	9	2	no	
Powell et al.[16]	2008	RS	12	48	37	2	9	37	11		1
Powell et al.[57]	2004	CS	10	2	2			2		no	
Piazza et al.[56]	2009	CR	10	1	1			1		no	
O'Neill et al.[55]	2011	CR	5	1	1			n/a	n/a	no	
Noel et al.[54]	2005	CR	9	1	1			1		no	
Naylor et al.[14]	2003	OLS	12	30	26		2	26	2	no	1
Murchinson et al.[53]	2007	CR	5	1	1			n/a	n/a	no	
Munoz et al.[52]	2004	CR	10	1	1			1		no	
Misall et al.[51]	2009	CS	10	2	1	1		2		no	
Michalpoulos et al.[50]	2004	CR	10	1	1			1		no	
Micantonio et al.[49]	2006	CR	9	1	1			1		no	

Study	Year	Design									
Ramsdell et al.[59]	2009	CR	9	1	1			1		no	
Swetter et al.[20]	2015	RS	13	63	50	3	5	7	8		1
De Troya martin et al.[60]	2008	CR	10	2	2			2		no	
van Meurs et al.[61]	2007	CR	10	1	1			1		1	
van Meurs et al.[62]	2010	OLS	13	10	9	1		10		4	
Wolf et al.[63]	2005	OLS	11	6	6			6		no	
Woodmansee et al.[17]	2009	CR	8	1	1			1			1

Supplementary file 1: Search strategy in PubMed up to and including may 2015

"hutchinson's melanotic freckle"[MeSH Terms] OR "malignant lentigo"[tiab] OR "lentigo maligna"[tiab] OR "circumscribed precancerous melanosis"[tiab] OR "dubreuilh melanosis"[tiab] OR "malignant freckle"[tiab] OR "hutchinsons melanotic freckle"[tiab] OR "hutchinson freckle"[tiab] OR "hutchinson melanotic freckle"[tiab] OR "malignant freckle"[tiab] OR "melanosis circumscripta praecancerosa"[tiab] OR "melanosis circumscripta precancerosa"[tiab] OR "melanosis dubreuilh"[tiab] OR "melanosis dubreulh"[tiab] OR "melanosis hutchinson"[tiab] OR "melanotic freckle"[tiab]

"imiquimod" [Supplementary Concept] OR "aldara"[tiab] OR "zartra"[tiab] OR "zyclara"[tiab] OR resiquimod[tiab]

4A

Effectiveness of lentigo maligna treated with 5% topical imiquimod

D.C.K.S. Tio[1], C. van Montfrans[2], C.G.H. Ruijter[1], R. Hoekzema[1], M.W. Bekkenk[1]

1. Department of dermatology, Amsterdam University Medical Centers, Location Vrije Universiteit Medical Centre Amsterdam, the Netherlands
2. Department of dermatology, Erasmus Medical Centre Rotterdam, the Netherlands

Abstract

Introduction

Lentigo maligna (LM) is treated to prevent progression to lentigo maligna melanoma (LMM). Surgery is the gold standard, an alternative option is off-label topical imiquimod. The objective of this study was to evaluate the effectiveness of 5% topical imiquimod treatment for LM.

Methods

Between 2007-2017 patients with LM were treated with off-label topical imiquimod once daily for 12 weeks.

Results

57 LM patients were treated with topical imiquimod. Complete clinical clearance was observed in 48 patients (84.2%), partial clearance in three patients (5.3%). Three patients (5.3%) showed no response and another three patients (5.3%) stopped due to side effects. One patient developed a LMM 4.5 years during follow-up which was subsequently excised.

Conclusion

Treatment with topical imiquimod resulted in complete clearance of LM in 48 out of 57 patients (84.2%). Topical imiquimod is an acceptable treatment option for LM patients who prefer topical treatment over surgery or radiotherapy.

Introduction

Lentigo maligna (LM) is considered a type of melanoma in situ, which is prevalent in a predominantly elderly population with a fair skin type. It typically occurs on chronic sun-exposed skin such as the head and neck area, where critical anatomical structures are present. Elderly patients often suffer from multiple comorbidities, and as a consequence, clinical management may be challenging. Several studies showed an increased incidence of LM over the last decades (0.54 LM / 100,000 patient years, to 1.99 LM / 100,000 patient years)[1-4]. Treatment of LM is recommended in order to prevent progression to lentigo maligna melanoma (LMM), which can metastasize. The true progression rate is unknown, but a recent epidemiological study describing 10,545 LM and 124 LMM patients reported that the cumulative risk of LMM developing after a LM on any location after 25 years is 2.0-2.6%. Progression of individual lesions could not be evaluated in this study[1].

According to the current European consensus guideline, surgical excision is the gold standard for treatment of LM. Alternative options such as off-label topical imiquimod 5%, radiotherapy or watchful waiting are mentioned in the guideline, but there is no recommendation on application of these options[5]. Surgical excision of larger lesions can lead to disfiguring scars or functional impairment, and radiotherapy can potentially cause secondary malignancies or radiodermatitis[6,7]. Off-label topical imiquimod has the advantage of providing a good cosmetic outcome and it is easy to use for elderly patients[8].

The response rate of off-label topical imiquimod for LM has been reported to vary between 37.0-78.6%[9-13]. The wide range in the response rates could be due to different treatment regimens that were used.

A survey performed by our group among 415 dermatologists in Europe showed that non-surgical options are used quite often. Of the respondents, 17.0% indicated that they use radiotherapy, 30.6% topical imiquimod and 19.6% opt for watchful waiting when treating LM patients >70 years of age[14].

We treated LM patients with off-label topical imiquimod 5% since 2007. The patients recruited for this cohort between 2007 and 2012 were described in an earlier article by Kirtschig *et al.*, who treated 27 patients with topical imiquimod, of which 20 (74%) showed complete clinical

and histological clearance with a mean follow-up of 39 months[8]. We have expanded this cohort with 30 additional LM patients treated between 2012 and 2017.

The aim of this study was to analyse all LM patients prospectively treated at our centre between November 2007 and December 2017 with off-label topical imiquimod, in order to evaluate its effectiveness. Data was collected retrospectively by reviewing clinical records.

Methods

Being an academic referral centre, patients were usually referred to us when they were not eligible for surgical treatment or did not want surgical treatment. Often these patients were referred specifically for treatment with off-label topical imiquimod. All patients were informed about the advantages and disadvantages of excision, radiotherapy, off-label topical imiquimod, or watchful waiting. A shared decision for a treatment was made depending on the location of the lesion, comorbidity of the patient, feasibility of the treatment option and preference of the patient. If off-label topical imiquimod was chosen, informed consent was obtained prior to treatment. If watchful waiting was chosen, patients were offered check-up appointments for clinical revision every 3 months. When clinical or dermoscopic changes were seen during these check-up appointments, the treatment options were discussed again.

Patients were instructed to apply topical imiquimod to the lesion daily with a 1-2 cm margin for a total of 12 weeks. The aim was to achieve at least 10 weeks of inflammation. Every 4 weeks, patients were given a check-up appointment. Depending on the inflammatory reaction, the treatment schedule was adapted. If the inflammation was too intense, patients were instructed to apply imiquimod 3 times per week, and if the inflammatory response was too mild patients were instructed to apply imiquimod twice daily[8,15]. The treatment protocol of off-label topical imiquimod treatment for LM, was reviewed and consented by the ethics committee of the Vrije Universiteit Medical Center.

Some patients received treatment prior to treatment with topical imiquimod, by excision, cryotherapy, or radiotherapy. Such lesions were regarded as recurrent. Previous biopsies taken elsewhere, were sent to our pathology department for revision by an experienced dermatopathologist, confirming the diagnosis LM. All samples were examined using Hematoxylin and Eosin (H&E) stains, and MELAN-A (MART-1) stains. LM was histologically

defined as a proliferation of atypical melanocytes along the basal cell layer of the epidermis, with possible extension into hair follicles and ascension of melanocytes. Post-inflammatory hyperpigmentation (PIH) was defined by the presence of melanophages in the dermis without proliferation of atypical melanocytes[16].

After treatment, if no residual pigmentation was visible with the naked eye or by dermoscopy a lesion was deemed completely clinically clear. Lesions were classified as partially clear if pigmentation was less in comparison to pre-treatment photographs, but still visible macroscopically or by dermoscopy. When a lesion did not change at all, the patient was classified as a non-responder.

After completion of treatment, patients were invited for a check-up visit every 6 months. Clinical assessment included comparison to previous dermoscopic and photographic documentation. During follow-up, if a patient showed pigmentation at the treated site at any point in time, a three mm punch biopsy was performed to investigate whether the pigmentation was PIH or residual LM.

A sub-analysis was performed to determine whether there was a difference between a total of ≤60 applications or >60 applications in our cohort.

Statistics

Data was analysed using descriptive statistics with SPSS (version 22.0; IBM company). X-Square tests were used for the sub-analysis of the difference of ≤60 applications or >60 applications in total.

Results

In total, 57 patients with histologically proven LM were treated with topical imiquimod between 2007 and 2017.

Of the treated patients (N=57), 24 were men (42.1%) and 33 were women (57.9%), with a mean age of 76 years (SD +/- 10.6 years). There was a median follow-up of 36 months (IQR 24-60 months). Most lesions were located on the nose (N=23) or cheek (N=20), some on the forehead (N=8), the temple (N=3), the chin (N=1), the cutaneous upper lip (N=1) and the earlobe (N=1) (figure 1). The lesions had a median longest diameter of 15 mm (IQR 10-23 mm). Of the 57

patients, 46 patients had primary lesions (80.8%) and 11 patients had recurrent lesions (19.2%). The patients with recurrent LM were treated surgically (N=5), by cryotherapy (N=5) or by an unknown modality (N=1), prior to treatment with topical imiquimod.

Figure 1: Overview of LM patients treated with topical imiquimod 5%. LM = Lentigo maligna. LMM = Lentigo maligna melanoma.
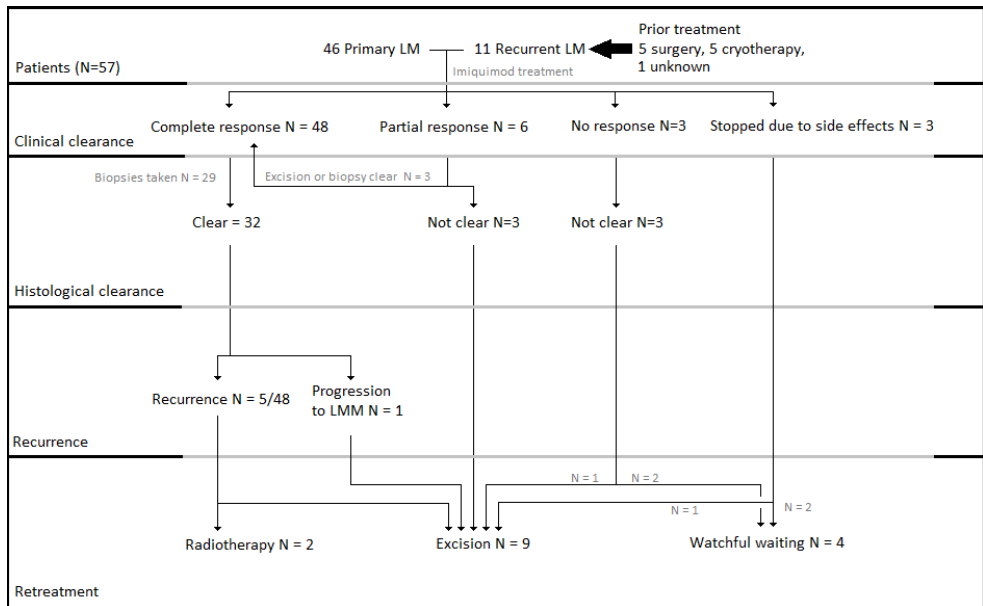
The median number of applications of topical imiquimod was 84 times in total (IQR 77-84 applications). Of the 57 patients, 10 applied imiquimod ≤60 times in total (17.5%). The remaining 47 patients applied imiquimod >60 times in total (82.5%). (Table 1).

Table 1: Demographics of LM patients treated with off-label 5% topical imiquimod. Data is shown as N (%), mean (Standard deviation) or mean (Interquartile range), where applicable. LM = Lentigo maligna. Mm = Millimetre. SD = Standard deviation. IQR = Interquartile range

LM patients treated with topical imiquimod	N = 57
Men/Women	24 (42.1%) /33 (57.9%)
Age	Mean 76 years (SD +/-10.6 years)
Follow-up	Median 36 months (IQR 24-60 months)
Primary/Recurrent	46 (80.8%) / 11 (19.8%)

Longest length of LM	Median 15 mm (IQR 10-23 mm)
Location	
Nose	N = 23 (40.4%)
Cheek	N = 20 (35.1%)
Forehead	N = 8 (14.0%)
Temporal	N = 3 (5.4%)
Chin	N = 1 (1.7%)
Cutaneous lip	N = 1 (1.7%)
Earlobe	N = 1 (1.7%)
Total applications of imiquimod	Median 84 applications (IQR 77-84 applications)
Patients who applied imiquimod <60 times	N = 10 (17.5%)
Patients who applied imiquimod > 60 times	N = 47 (82.5%)

Clinical clearance, histopathological clearance and retreatment

Complete clinical clearance was found in 48 patients (84.2%). Of these patients, 29 underwent a post-treatment three mm punch biopsy. All of these biopsies showed PIH and histological clearance of LM .

A partial clinical clearance was found in six patients (10.5%). One patient underwent re-excision of the LM lesion without a post-treatment biopsy. Histopathological examination confirmed the presence of residual LM. The remaining five patients had a three mm punch biopsy performed after treatment. Of these five biopsies, three showed PIH without residual LM, the two other biopsies showed residual LM. The three patients with a clear biopsy were added to the total of patients with a complete clinical clearance. The two patients with a biopsy showing residual LM underwent surgical excision.

Three patients (5.3%) did not respond to treatment. Of these, two had a biopsy performed which showed residual LM in both cases. Both patients declined surgical excision or radiotherapy and opted for watchful waiting. These patients were reviewed clinically every three months, so far they have not been re-treated. The third non-responder underwent surgical excision.

Another three (5.3%) patients ceased treatment early due to side effects. Side effects observed in this study included flu-like symptoms (n=11), lymphedema of the cheek (n=3), headache (n=7) and a sterile conjunctivitis (n=3). The three patients who discontinued treatment due to side effects did not have biopsies performed post-treatment. Residual pigmentation was still visible in these patients. One patient was retreated by excision and referred back to his original dermatologist. The 2 other patients were reviewed clinically every three months and have not been re-treated so far (Figure 1).

Recurrence after off-label 5% topical imiquimod

A total of 6 LM recurred (10.5%), after a mean follow-up period of 22.5 months (5-55 months). Recurrences after treatment with topical imiquimod were found on the chin (N=1), forehead (N=2), cutaneous upper lip (N=1), cheek (N=1) and the earlobe (N=1). In 2 of 6 patients recurrences were found after 5 months . Both patients had recurrent LM following surgery or cryotherapy, prior to treatment with topical imiquimod. In the other 4 patients recurrences were seen after 10, 29, 31 and 55 months. The patient who showed recurrence after 55 months, initially presented a histologically proven, primary LM on her left earlobe. After treatment a biopsy showed no residual LM and check-ups every 6 months were performed. No recurrence was seen, but after 4.5 years she reported repigmentation at the treated site. A biopsy showed LMM (Breslow thickness 0.4 mm, T1aN0M0), which was subsequently surgically excised. We have checked this patient regularly for 2 years after excision and so far she has not developed local recurrence or metastasis. All 6 patients with recurrent LM were offered alternative treatment, 4 patients opted for excision and 2 for radiotherapy (Table 2).

Table 2: Recurrence of LM after treatment with off-label 5% topical imiquimod. LMM = Lentigo maligna melanoma.

Case #	Primary or recurrent	Previous treatment	Location	Time to recurrence (months)	Treatment after recurrence
Case 1	Recurrent	Cryotherapy	Cheek	5	Excision
Case 2	Recurrent	Excision	Upper lip	5	Excision
Case 3	Primary	-	Forehead	10	Radiotherapy

Case 4	Primary	-	Forehead	29	Excision
Case 5	Primary	-	Chin	31	Radiotherapy
Case 6	Primary	-	Earlobe	55 (Progression to LMM)	Excision

Subanalysis

A sub analysis showed no significant difference in complete clinical clearance rates between patients who applied imiquimod ≤60 or >60 times in total (p=0.24, data not shown).

Discussion

In our academic outpatient clinic we treated 57 LM patients with off-label topical imiquimod over a 10-year period. Imiquimod treatment (1 application daily, for 12 weeks) resulted in complete clinical clearance in 84.2% of patients, with a 10.5% recurrence rate during follow-up. One patient (1.8%) treated with topical imiquimod showed progression to LMM after 4.5 years of follow-up. The progression rate of LM to LMM in this study is 1,8%, which is similar to previous studies on topical imiquimod for LM. A systematic review of LM treated with topical imiquimod described 471 treated patients, with only 9 cases progressing to LMM following topical imiquimod (1.9%)[11].

Kai *et al.* reported a clearance rate of 62.5% (N=40). The patients in this study applied topical imiquimod 3 times per week for 6 weeks, followed by 5 times per week for 4 weeks, for a total of 38 applications[17]. Another study by Marsden *et al.* reported a 37% (N=27) histological clearance rate. These patients applied topical imiquimod 5 times per week during 12 weeks, for a total of 60 applications[9]. The more intense treatment regimen we used could explain the higher clearance rate observed in our study. This is concurrent with results of a systematic review, which has shown that the odds ratio of achieving complete clinical clearance is 8 times higher if topical imiquimod is applied >60 times in total[10,11].

Compared to staged surgical techniques or radiotherapy, topical imiquimod has a higher recurrence rate at 10,5%. Surgical excision with a 5 mm margin has a recurrence rate of 30% after 5.5 years[18] while staged excision techniques, such as Mohs micrographical surgery or the

"spaghetti technique" show a superior recurrence rate of 4-5.9%[1,19,20]. Radiotherapy has a reported recurrence rate of 5% after three years[21]. Topical imiquimod however, has the advantages of being non-invasive, providing a good cosmetic outcome and it is easy to use for elderly patients. To our knowledge, no comparative studies between treatments have been published so far.

To determine the position of topical imiquimod in a treatment algorithm it is necessary to define the primary goal of treatment. Currently, the main treatment goal for LM is to prevent progression to LMM. The true progression rate is unknown, although Greveling *et* al. reported that the cumulative risk of developing LMM after primary LM is 2-2.6% over a course of 25 years[1]. Patients with LM are mostly elderly patients and have been shown to have a relative survival rate of 104% compared to the general population, while LMM patients have a relative survival rate of 99% after treatment[1]. In contrast, studies on malignant melanoma (non-LMM) showed a relative 5-year survival of 76-83.4% after treatment[21,22]. In our study, we found no LM or LMM related deaths. A previous study on surgical treatment of LM and LMM by Gamblicher *et al.* reported similar findings. In a cohort of 270 patients (124 with LM and 146 with LMM) who were treated surgically they observed no LM- or LMM- related death after a mean follow-up of 55 months[23].

Swetter *et al*, have suggested that histological clearance should not necessarily be the gold standard to measure success of LM treatment[24]. In general, LM develops on actinically damaged skin. In sun damaged skin, morphologically atypical, but biologically non-malignant melanocytes may reside at the dermal-epidermal junction and may simulate LM. This makes the diagnosis difficult. Histologically these atypical but non-maligant melanocytes are indistinguishable from true malignant cells, even with the use of immunostains (MART1/melan-A, SOX10, MiTF and soluble adenylyl cyclase)[24,25]. Due to this problem it is difficult to prove radical excision and subsequently, striving for histological clearance could lead to large, perhaps unnecessary defects.

Our study has several limitations. Firstly, patients referred to us for LM usually are elderly patients, who often do not want to undergo surgical excision. Most of these patients did not want radiotherapy either, because it requires daily traveling to the hospital for several weeks. Therefore, this patient population is prone to selection bias, which may influence study results.

Secondly, 11 of our patients had been diagnosed with recurrent LM prior to treatment with topical imiquimod. This may have confounded the response to therapy. Lastly, the usage of single three mm punch biopsies for histopathological examination. In case of large LM this may have led to sampling error.

In conclusion, based on our results we consider off-label topical imiquimod an acceptable treatment option for patients with large LM lesions and for those who do not want surgical excision or radiotherapy. We recommend that future studies focus on comparing treatment options for LM, and whether histological clearance should be the most important outcome measurement or not.

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4A

4B

Lymphoedema in Lentigo Maligna patients treated with imiquimod, a long term adverse effect

D.C.K.S Tio[1], G. Kirtschig[2], R. Hoekzema[3], C. van Montfrans[4]

1. Dermatology resident, Vrije Universiteit Medical Center Amsterdam, the Netherlands
2. Dermatologist, University Hospital of Marburg, Marburg, Germany
3. Dermatologist, Vrije Universiteit Medical Center Amsterdam, the Netherlands
4. Dermatologist, Erasmus Universiteit Medical Center Rotterdam, the Netherlands

Lentigo Maligna (LM) is a potential precursor lesion of Lentigo Maligna Melanoma (LMM). It is treated to prevent progression to LMM. A recent epidemiological study reports a progression rate of 2.0-2.6% over a course of 25 years[1]. The gold standard of treatment is surgical excision with 5 mm margin[2]. Topical application of imiquimod cream is an off-label alternative[2,3].

Complete clinical response rates of LM treated with imiquimod vary from 37.1-100%[4-6].We report three patients with LM, who developed lymphoedema following application of topical imiquimod.

Three consecutive patients with LM were treated according to our protocol. Patients were instructed to apply imiquimod once daily to the lesion with a 1-2 cm margin, for 12 weeks. The goal was to achieve at least 10 weeks of inflammation. Depending on the inflammatory reaction, the treatment schedule was adapted. If it was too intense, patients were instructed to apply imiquimod 3 times per week, or if the inflammation was insufficient, patients were instructed to apply imiquimod 2-3 times daily[7].

The first patient is was a 66-year-old woman with a 9x10mm, pigmented brown macule on the left cheek. The diagnosis LM was confirmed by a punch biopsy. After 12 weeks of treatment with Imiquimod 5%, no residual pigmentation was visible macroscopically or by dermatoscopy. Within days after starting treatment, the patient developed erythema, soreness and oedema at the site of application. The erythema partially subsided, the soreness quickly disappeared, but a non-pitting swelling persisted. A punch biopsy obtained two years post-treatment demonstrated fibrosis, with increased numbers of fibroblasts and a mild lymphohistiocytic infiltrate which had replaced the normal subcutaneous tissue (figure 1a, b). D2-40 immunostaining showed several compressed lymphatic vessels within this fibrotic tissue. Four years post-treatment, the lymphoedema was still present.

The second case was a 68-year-old woman with a 14x14 mm irregularly pigmented macule on her right cheek. LM was confirmed histopathologically. She applied imiquimod once daily during the first 4 weeks of treatment. Due to intense inflammation she was instructed to apply the imiquimod 3 times weekly for the remaining 8 weeks, for a total of 12 weeks. One month post-treatment, a biopsy showed post-inflammatory hyperpigmentation, no LM was found. In the dermis oedema was observed. Histologically it was unclear if the oedema was

lymphoedema or residual oedema due to inflammation. The oedema persisted for three years, after which it disappeared.

The third case was a 69-year-old woman, who was referred following excision of a LMM on her right cheek. Histologic examination of the excised lesion showed radically excised LMM with a Breslow thickness of 0.6 mm. Several years later, pigmentation measuring 15x15 mm appeared around the scar. A biopsy showed LM, without evidence of LMM. The patient declined surgical treatment because she found the potential scarring unacceptable. She was treated with off-label imiquimod. During treatment, the patient developed an inflammatory reaction with erythema, swelling, soreness and crusting. After treatment, no residual pigmentation was present. The erythema and soreness disappeared but lymphoedema persisted. The lymphoedema disappeared gradually after a year.

Topical imiquimod is an off-label option for the treatment of LM patients, who do not qualify for, or do not opt for surgical treatment. Imiquimod is applied for a prolonged period of time to achieve a sufficient inflammatory response[5]. We hypothesize that lymphoedema may complicate treatment of LM patients with topical imiquimod. This adverse effect may be caused by the intense treatment regimen used in our patients, resulting in severe inflammation and significant dermal fibrosis, impairing normal tissue drainage by afferent lymphatic vessels.

In the two patients who were biopsied after imiquimod treatment (two years and one month post-treatment, respectively), fibrosis was clearly present in the reticular dermis histologically. We hypothesize that in our patients, similar to the sequence of events during cutaneous wound healing, a late phase of remodeling (maturation) may have followed previous phases of inflammation and proliferation in response to imiquimod. The remodeling phase involves degradation of excess collagen and organization of fibrotic connective tissue, which may take several years[8]. This may explain why lymphoedema persisted and only resolved in two of the three patients. Alternatively, the lymphoedema may have been related to other unknown/unrecognized factors

In conclusion topical imiquimod is an off-label alternative treatment option for the treatment of LM, for patients who are ineligible or do not opt for surgical treatment. When prescribing topical imiquimod for a lesion located on the cheek for a prolonged period of time, patients should be informed about the risk of secondary lymphoedema.

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5

Nine percent of biopsy proven lentigo maligna are reclassified as lentigo maligna melanoma after surgery

J. Zoutendijk, MD,[1] D.C.K.S Tio, MD,[2] S. Koljenovic, MD, PhD3,
R.R. van den Bos, MD, PhD[1]

1. Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
2. Department of Dermatology, VU Medical Center, Amsterdam, The Netherlands
3. Department of Pathology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Lentigo maligna (LM) is a melanoma in situ and the incidence is still rising in The Netherlands[1]. LM is mostly located in the face, therefore radical surgical therapy, which is the first choice treatment, can be challenging and staged excision is considered a useful option. The initial diagnosis is usually based on one or just a few biopsies which may be the cause that reclassification to LMM may occur after histopathological examination of a LM lesion. Due to patients age and LM localization non-surgical treatments such as topical imiquimod[2], combined with laser ablation[3], radiotherapy or careful clinical follow-up are sometimes considered. The histological clearance of these treatments is uncertain and many studies mention clinical clearance (with response rate until 74% with topical imiquimod)[2]. A recent study showed histological control after topical imiquimod with complete clearance in only 37%[4]. When alternative treatments are considered it is useful to have knowledge on the proportion of patients that are misclassified as LM during the initial biopsy. The aim of our study was to calculate the proportion of biopsy proven LM that turned out to be LMM after excision.

Data from our pathology department was requested; including all patients with LM or LMM in the pathology record during the period January 2010 until February 2017. We selected all patients that were diagnosed with LM(M) and were treated during this period at our center. Information on sex, age, size, anatomical location, diagnostics before treatment (punch or incisional biopsy), treatment method, number of excision rounds, diagnosis before (LM or LMM) and after treatment (LM or LMM) were retrieved from the database.

In addition 25, randomly chosen, LM cases that were treated with staged excisions were further investigated and Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks were selected based on corresponding Hematoxylin and Eosin (HE) sections. Each block containing LM was cut in 3 levels (of which 1 HE and 3 blancs were made) for evaluation of possible invasion.

SPSS statistics 24 were used for the statistical analyses.

In the studied period, 417 patients were diagnosed with LM or LMM and treated at the Erasmus Medical Center. In 284/417 (68.1%) patients the initial biopsy showed LM, 27/284 (9.5%) were not treated with a surgical procedure (topical treatment with imiquimod and laser) and 2 had a different definitive diagnaosis than LM or LMM/melanoma. Of the remaining patients 232/255 (91%) remained LM after complete excision, 23/255 (9%) of the LM were reclassified

to a LMM or melanoma. In the LM group 138/232 (59.5%) were female and in the LMM group 9/23 (39.1%). At the time of diagnosis the mean age in the LM and LMM group was 71 and 73 years respectively. The LM and LMM were mainly located in the head and neck region (86.6% and 78.3%) and had an average size of 1-2 cm (Table 1).

Table 1: Characteristics of patients diagnosed with LM(M) between 2010 until february 2017 in the Erasmus Medical Center. LM = lentigo maligna; LMM = lentigo maligna melanoma

Biopsy proven LM treated with surgery	LM after surgery	%	LMM/melanoma after surgery	%
n = 255	232	91	23	9
Male	94	40.5	14	60.9
Female	138	59.5	9	39.1
Age (years)				
Mean	71.1		73.4	
Median	72		73	
Size category				
1 (< 1 cm)	44	18.9	3	13
2 (1-2 cm)	83	35.8	12	52.2
3 (2-5 cm)	47	20.2	2	8.7
4 (> 5 cm)	6	2.6	1	4.3
Unknown	52	22.4	5	21.7
Anatomical location				
Head and neck	201	86.6	18	78.3
Extremities	17	7.3	2	8.7
Trunk	14	6.0	3	13

In the 25 cases of LM that we investigated with more and deeper sections, we did not found invasive melanoma.

This study shows that 9% of biopsy proven LM turned out to be LMM after complete excision. Previous epidemiological publication showed an cumulative risk of progression of 2-3% from LM (histologically confirmed) to LMM after 25 years follow up[1]. If there is a suspicion of LM current guidelines advise sampling with (punch or incisional) biopsy or in small lesions complete excision and surgical excision is the first choice of treatment[5,6]. This study adds that a biopsy alone may lead to an incorrect diagnosis of LM in lesions that are in fact LMM. A similar finding was reported before in a group of 46 patients in which an upgrade of 20% was found[7]. Also, invasion was shown in 33% of previously diagnosed melanoma in situ after deeper sections[8]. We could not confirm this in the 25 cases that we investigated with more and deeper sections.

In conclusion, the proportion of biopsy proven LM that turned out to be LMM or melanoma after complete staged excision or conventional excision is 9%. This should be taken in account when considering the treatment options for a patient with LM. Further and deeper histological investigation of the staged excision specimens does not contribute to higher detection rates of LMM and is therefore not of added value.

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6

Expression of Cancer/testis antigens in cutaneous melanoma: A systematic review

D.C.K.S Tio[1], F.R. Kasiem[2,3], M. Willemsen[2,3], R. van Doorn[4], N. van der Werf[5], R.Hoekzema[1,2], R.M. Luiten[2,3], M.W. Bekkenk[1,2]

1. Department of Dermatology, Amsterdam University Medical Centers, VU University.
2. Department of Dermatology and Netherlands Institute for Pigment Disorders, Amsterdam University Medical Centers, University of Amsterdam
3. Cancer Center Amsterdam and Amsterdam Infection & Immunity Institute
4. Department of dermatology, Leiden Universitair Medisch Centrum, Leiden, the Netherlands
5. Medical library, Leiden Universiteit Medisch Centrum, Leiden, the Netherlands

Abstract

Introduction

The Cancer/testis antigen (CTA) family, is a group of antigens that of which expression is restricted to male germ line cells of the testis and various malignancies. This expression pattern, makes this group of antigens potential *targets for immunotherapy.* The aim of this study was to create an overview of CTA expressed by melanoma cells at mRNA and protein level.

Methods

A systematic literature search was performed in MEDLINE (Pubmed) and EMBASE from inception, up to and including February 2018. Studies were screened for eligibility by two independent reviewers. A total of 65 full text articles were included in the final analysis.

Results

A total of 48 CTA have been studied in melanoma. Various CTA show different expression rates in primary and metastatic tumours. Of the 48 CTA the most studied were MAGE-A3, MAGE-A1, NY-ESO-1, MAGE-A4, SSX2, MAGE-A2, MAGE-C1/CT7, SSX1, MAGE-C2/CT10 and MAGE-A12. On average MAGE-A3 mRNA is present in 36% of primary tumours, while metastatic tumours have an expression rate of 55-81%. The same applies to the protein expression rate of MAGE-A3 in primary tumours, which is reported to be at 15-37%, while metastatic tumours have a higher expression rate of 25-70%. This trend of increased expression in metastases as compared to primary tumours is seen with MAGE-A1, MAGE-A2, MAGE-A4, MAGE-A12 and NY-ESO-1.

Conclusion

Many CTA are expressed on melanoma. This review provides an overview of the expression frequency of CTA antigens in melanoma and may aid in identifying CTA as therapeutic target for immunotherapy.

6

Introduction

The Cancer/testis antigen (CTA) family, is a group of antigens that is solely expressed in various malignancies and in germ cells of the testis [1] [2-4].

To date, more than 100 CTA gene families have been identified. The majority of these genes are located on the X chromosome and share a high sequence homology [5]. Of all CTA, the melanoma-associated antigen gene (MAGE) family has thus far been studied the most [3,5,6].

The MAGE family is subdivided into two categories, type I and II. Type I MAGE are located on the X-chromosome and consist of the MAGE-A, -B and -C subfamilies. Type II MAGE are not strictly X-chromosome bound and consist of the MAGE-D, -E, -F, -G, -H, -L and Necdin subfamilies. *In healthy tissue, type I MAGE expression is restricted to germ cells of the testis* [3]. *DNA methylation* of MAGE type I gene promotors, prevents protein expression in healthy somatic cells [7,8]. *Some* studies report expression in placental tissue and wound repair, but to a lesser degree compared to germ line cells of the testis [9,10]. One study reported MAGE-D1 expression in brain tissue of adult mice [2].

The normal function of CTA is largely unknown [11]. So far, it is known that MAGE possess a variety of cellular functions, such as complex formation with E3 RING ubiquitin ligases, involvement in substrate recognition, cellular localization and cell proliferation [3]. MAGE-A1 and MAGE-A4 are involved in the early spermatogenesis and MAGE-D2 plays a role in the embryonic development of mice [2,12]. In malignancies, several MAGE are known oncogenic drivers and play a role in malignant cell survival, tumour formation and metastasis [13].

MAGE-1, later renamed to MAGE-A1, was the first CTA to be identified. Studies showed that a short peptide fragment of MAGE-A1, named MZ2-E, could be presented on major histocompatibility complex (MHC) class I molecules. The association of MZ2-E and a MHC complex subsequently allowed specific cytotoxic T-cells to recognize and kill melanoma cells in a patient derived cell line [2,6,11,14,15]. The unique expression patterns of CTA, makes this group of antigens potential *candidate targets for immunotherapy* [1]. *MAGE-3.A1 peptide has been used as a vaccination, it could induce tumour regression in melanoma patients*[16].

Other possible functions are utilization of CTA as diagnostic or prognostic tumour markers. The prevalence of some CTA is often higher in more advanced malignancies and has been correlated with a poorer prognosis [17-21].

The expression of the various CTA is well evaluated in many malignancies, such as lung cancer, breast cancer, ovarian cancer, colon cancer, multiple myeloma and cutaneous melanoma [14,22-25].

Currently, there is no overview regarding the expression of CTA in melanoma. Reported expression of CTA differs widely between studies. The aim of this study was to create a comprehensive overview.

Methods and materials

Information sources and search

A systematic literature search was done in MEDLINE (Pubmed) and EMBASE (www.embase.com) by a clinical librarian from inception, up to and including February 2018. Search terms included controlled MeSH terms in PubMed, EMtree in EMBASE.com as well as free text terms. Search terms expressing 'cancer/testis antigen', ''melanoma antigen' and 'MAGE' were used in combination with search terms identifying 'malignant melanoma' and 'melanoma'. The full search strategy can be found in supplementary file 1. References of included studies were checked for additional relevant reviews.

Eligibility criteria

Articles were deemed eligible if they described mRNA or protein expression of CTA in tumor tissue (cutaneous melanoma or metastasis of cutaneous melanoma), melanoma cell lines, uncultured melanoma or short term cell cultures using immunohistochemistry, real time-polymerase chain reaction (RT-PCR), qRT-PCR, RNA sequencing V2 (RNAseqV2), Enzyme-Linked immune sorbent assay (ELISA), Flow cytometry (FACS), DNA methylation or immunofluorescent or immunohistochemical staining.

Non-English articles, review articles, inaccessible full text, animal studies and articles with non-cutaneous melanoma were excluded.

Study selection

Two reviewers (DT and FK) independently screened all relevant titles and abstracts for eligibility. If necessary, full text articles were screened for eligibility. Differences in judgement were resolved by a third reviewer (MW), until consensus was reached (Figure 1a and 1b).

Figure IA: Literature search in Embase
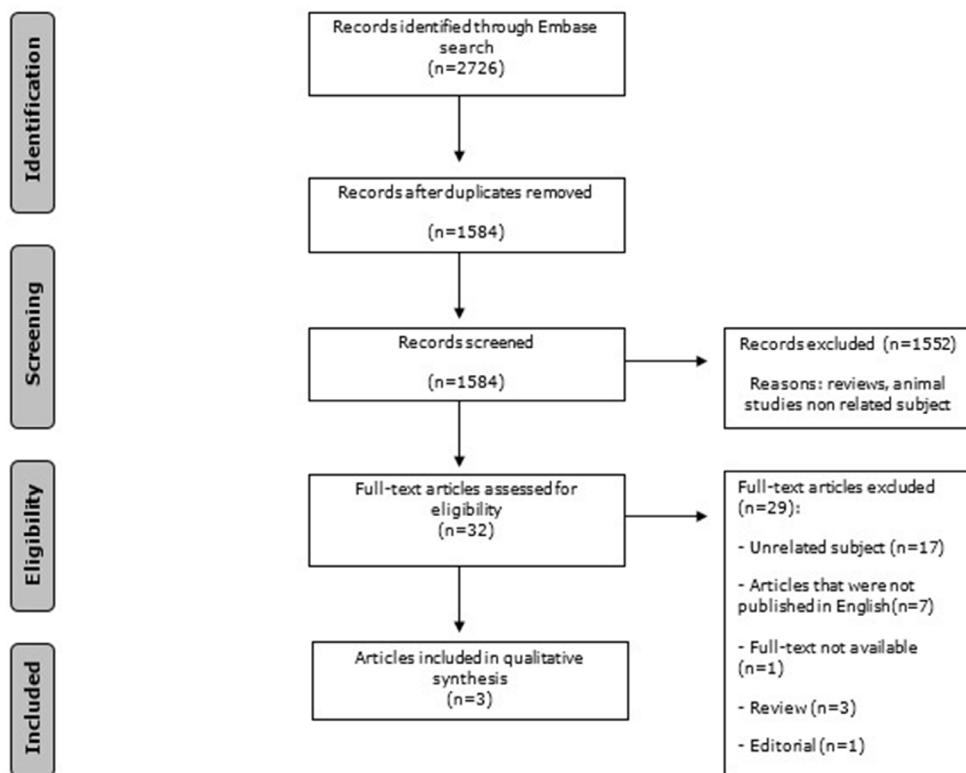
Data collection process

Two reviewers (DT and FK) extracted data from the included studies independently. Disagreements were resolved by consensus; if no agreement could be reached, a third author (MW) was consulted.

Data extraction

The following information was extracted from each study: CTA type described in the study, the type of tissue in which expression was evaluated, and the method of measuring the expression of CTA.

Figure IB: Literature search in Pubmed
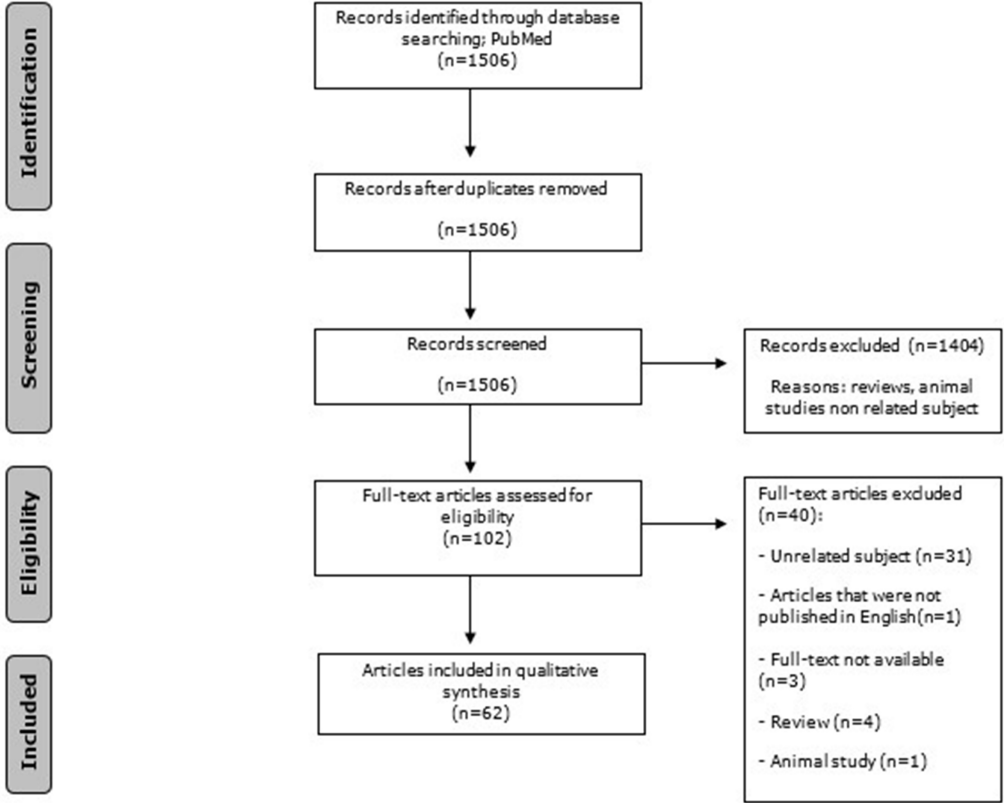
Results

A search in the Pubmed and Embase databases yielded 4232 articles. After a selection by 2 independent reviewers (DT, FK), based on title and abstracts, 134 studies were selected for full-text assessment. A total of 69 articles were *excluded* because: 1. Article was on an unrelated subject (N=48), 2. Article was not available in English (N=8), 3. Article was not unavailable in full-text (N=4), 4. Article was a review or an editorial (N=8). 5. Article was on an animal study (N=1). A total of 65 full-text articles were included into the final analysis.

Studies described expression of 48 different CTA found in melanoma. Studies evaluated expression based on either presence of CTA mRNA or presence of CTA protein. For the examination of mRNA, studies used either Real-time PCR (RT-PCR), qRT-PCR, RNA sequencing (RNAseqV2). Presence of protein was studied using mainly immunohistochemistry, but some

studies utilized ELISA, immunofluorescent or immunohistochemical staining or flowcytometry (FACS). All studies that described mRNA expression based on RNAseqV2 or DNA methylation only reported a single expression rate. To include these studies, we considered presence of DNA methylation to be equal to loss of RNA expression.

An overview of which studies described expression of a CTA based on mRNA or protein can be found in table 1. The most studied CTA were MAGE-A3, MAGE-A1, NY-ESO-1, MAGE-A4, SSX2, MAGE-A2, MAGE-C1/CT7, SSX1, MAGE-C2/CT10 and MAGE-A12 (Table 2, Figure 2).

Table 1: Overview of studies reporting on cancer testis (CT) antigen.

Data is presented as the range (median) of expression or a single expression rate where appropriate. The data shown is the expression of CTA in **all tumours** studied, this includes primary, metastatic tumours and tumours of unknown status. The reporting studies are displayed as references. mRNA = micro RNA, RT-PCR = Real Time-Polymerase Chain Reaction, ELISA = enzyme-linked immunosorbent assay, TMA = transcription-mediated amplification, FACS = fluorescence-activated cell sorting, IMF = immunofluorescent IHC = immunohistochemistry.

CT Antigen	mRNA (RT-PCR) *Range (median)*	mRNA (RNA SeqV2 DNA methylation) *Range*	mRNA (Other = ELISA, IMF or IHC, FACS)	Protein (IHC)	References *mRNA ; Protein*
Tumour Tissue					
MAGE-A1	16-90% (42%)	38.2%		7.5-57% (27%)	9,18,26-35,36 ; 22,32,34,37-42
MAGE-A2	41-100% (60%)	58.43%			9,18,27,29,31,33,36
MAGE-A3	36-90% (55%)	59.93%	45-51% (48%)	15-70% (31%)	9,18,26-31,43-45,36, 46,47; 22,43,45,48-50
MAGE-A4	0-22% (11%)			5-44% (18%)	9,18,27; 17,22,37,40,41

MAGE-A6	25-64% (44.5%)	61.42%			9,27, 36; -
MAGE-A10				38%	-; 51
MAGE-A12	34-75% (62%)	59.55%			9,27,52,36; -
MAGE-C1/CT7	57-70% (64%)	46.82%	28.3%	38-82% (60%)	30,53,54,36,55; 22,54
MAGE-C2/CT10	43-50% (46.5%)	48.81%	36.5%		56,57,36,55; -
NY-ESO1/LAGE-2	10-70.8% (40%)			0-61% (23.9%)	26,28-30,58-63; 22-24,37,38,40,41,60,61,64-67
SSX				34%	-; 68
SSX1	27-30% (28.5%)	49.06%			28,63,69,36
SSX2	0-50% (36%)			35%	28-30,69,70; 49
SSX4	26-27% (26.5%)				28,69; -
SSX5	5%				69; -
GAGE	47-49% (48%)			19-53% (36%)	30,31; 22,71
GAGE-1	20-31% (25.5%)				29,63,72; -
GAGE-2	20-24% (22%)				29,72; -
GAGE-3	30%				29; -
GAGE-6	30%				29; -
GAGE-7	20%				71; -
GAGE-8	30%				29; -
XAGE-1	38-51% (45.5%)				30,73; -

XAGE-1b				54.5%	- ; [64]
BAGE	15-22% (16%)				[30,31,74] ; -
PRAME	88-95% (91.5%)	95.88%			[63,75,76] ; -
KIF20A	72%			63%	[77] ; [77]
CTp11	59%				[78] ; -
LAGE-1	40%				[29] ; -
CTSP-1	59%				[79] ; -
CTSP-2	11%				[79] ; -
CTSP-4	0%				[79] ; -
SCP1	43%				[63] ; -
SEMG1	14%				[63] ; -
SPANXA	86%				[63] ; -
PASD	71%				[63] ; -
CSAG1		58.43%			[36] ; -
CDCA1				69.5%	- ; [80]
Uncultured melanoma					
TAG-1	59%				[81] ; -
TAG-2a	36%				[81] ; -
TAG-2b	23%				[81] ; -
TAG-2c	27%				[81] ; -
Cell lines					
MAGE-A1	53-100% (77%)				[9,29,63,82] ; -
MAGE-A2	50-100% (75%)				[9,29] ; -
MAGE-A3	25-100% (64%)		45%		[9,29,35,44,46,47,82,83,46] ; -
MAGE-A4	50%				[9] ; -
MAGE-A6	100%				[9] ; -

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MAGE-A12	100%				[9] ; -
MAGE-B	17%				[29] ; -
NY-ESO1	29%			25%	[29] ; [64]
SSX1	33-42% (27.5%)				[68,84] ; -
SSX2	4-42% (39%)				[29,68,84] ; -
SSX4	11-33% (22%)				[68,84] ; -
SSX5	6-8% (7%)				[68,84] ; -
GAGE				41%	- ; [71]
GAGE-1	29-71% (50%)				[29,71] ; -
GAGE-2	29-71% (50%)				[29,71] ; -
GAGE-3	42-76% (59%)				[29,71] ; -
GAGE-6	42-76% (59%)				[29,71] ; -
GAGE-7	29-76% (52.5%)				[29,71] ; -
GAGE-7b	76%				[71] ; -
GAGE-8	76%				[71] ; -
XAGE-1	43%				[73] ; -
XAGE-1a	9%				[85] ; -
XAGE-1b	61%			92%	[85] ; [64]
XAGE-1c	35%				[85] ; -
XAGE-1d	52%				[85] ; -
XAGE-2	16.5%				[85] ; -
XAGE-3	0%				[85] ; -
KIF20A	100%				[77] ; -
LAGE-1	42%				[29] ; -

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CTp11	26%				[78] ; -
CDCA1	100%				[80] ; -
Short term cell cultures					
MAGE-A3			51%		[47] ; -
Tumour tissue cell lines					
MAGE-C1/CT7			28.3%		[55] ; -
MAGE-C2/CT10			36.5%		[55] ; -

Figure 2: Mean expression of CTA by primary and metastatic melanoma. Data is presented as a percentage. mRNA = micro RNA
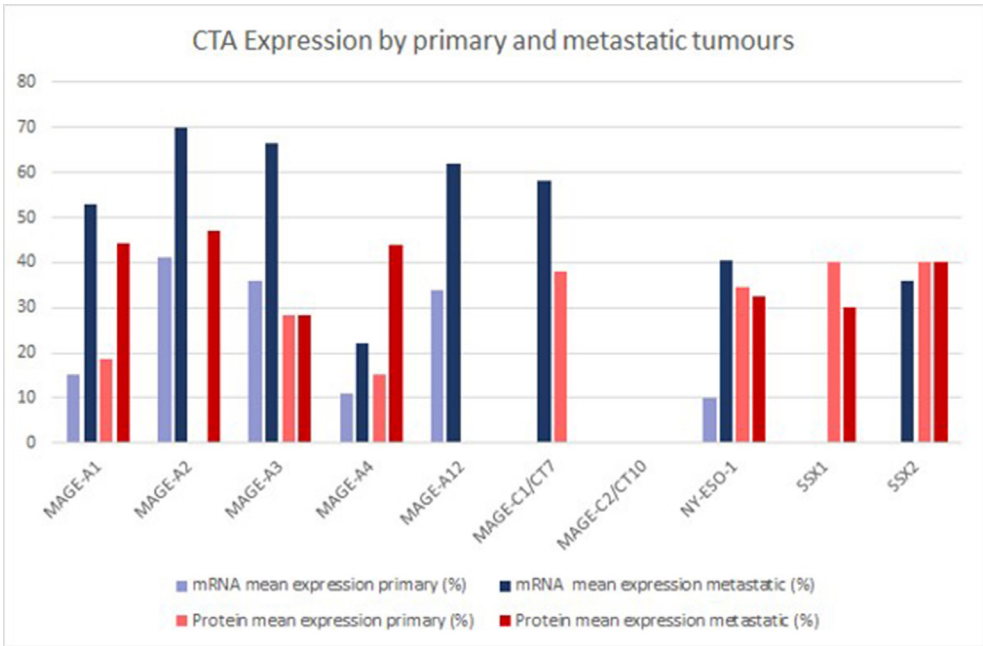
Table 2: Results of studies which specify expression of the 10 top studied CTA in primary and metastatic melanoma. Data is expressed as N tumours/ Percentage expression

CTA		Primary N Tumour / %	Metastatic N Tumour / %	Reference
MAGE-A1	mRNA	100 / 16% - 4 / 0%	145 / 48% 47 / 70% 3 / 33%	[18] [30,35]
	Protein	251 / 20% 40 / 7,5% 38 / 21%	335 / 51% 264 / 36%	[37] [38] [22]
MAGE-A2	mRNA	100 / 41%	145 / 70%	[18]
	Protein	-	64 / 47%	[31]
MAGE-A3	mRNA	100 / 36% - - -	145 / 76% 47 / 81% 316 / 62% 64 / 55 %	[18] [30] [43] [31]
	Protein	38 / 15% 91 / 37% 85 / 25% -	- - 120 / 25% 10 / 70%	[22] [48] [49] [50]
MAGE-A4	mRNA	100 / 11%	145 / 22%	[18]
	Protein	251 / 9% 321 / 18% 38 / 29 %	335 / 44% - -	[37] [40] [22]
MAGE-A12	mRNA	83 / 34%	243 / 62%	[52]
	Protein	-	-	-
MAGE-C1/CT7	mRNA	- -	47 / 57% 11 / 64%	[30] [54]
	Protein	38 / 38%	-	[22]
MAGE-C2/CT10	mRNA	-	-	-
	Protein	-	-	-
NY-ESO-1	mRNA	20 / 10%	32 / 47%	[58]

		-	64 / 31,3%	61
		-	47 / 49%	30
	Protein	251 / 45%	335 / 45%	37
		75 / 29%	38 / 50-61.5%	64
		40 / 5%	264 / 14%	38
		16 / 0%	206 / 28.2%	23
		321 / 37%	-	40
		61 /13%	63 /32%	24
		-	60 /46.6%	61
		-	11 / 36%	66
SSX1	mRNA	-	-	-
	Protein	35 / 40%	66 / 30%	68
SSX2	mRNA	-	47 / 36%	30
	Protein	35 / 40%	66 / 40%	49

Discussion

This study has shown that many CTA are present on cutaneous melanoma. Various CTA show different expression rates in primary and metastatic tumours. We found that MAGE-A3 mRNA is present in 36% of primary tumours, while metastatic tumours have an expression rate of 55-81%. The same applies to the protein expression rate of MAGE-A3 in primary tumours, which is reported to be at 15-37% while metastatic tumours have an expression frequency of 25-70%. This trend of increased expression in metastases as compared to primary tumours is seen with MAGE-A1, MAGE-A2, MAGE-A4, MAGE-A12 and NY-ESO-1. In contrast, primary tumours express more SSX1 protein (40%), compared to metastatic tumours which only express SSX1 protein in 30% of the cases. SSX2 protein expression seems to be the same in both primary and metastatic tumours, at a rate of 40%.

Expression of MAGE-A3 antigen is associated with promotion of cell proliferation and primary tumour size. In addition it is linked to the number and size of metastatic lung foci[86]. MAGE and other CTA have been shown to facilitate the malignant phenotype, by conferring resistance to chemotherapeutic agents, such as paclitaxel and doxorubicin[87-89]. It could be argued that

presence of certain MAGE antigen subtypes such as MAGE-A3 on melanoma, could increase the oncogenic potential of a tumour.

Silencing of SSX in vivo can significantly impair growth of melanoma tumour xenografts[90]. Expression of SSX2 by has been linked to DNA damage induction and immediate promotion of genomic instability. In the long-term SSX2 has been shown to support tumour cell growth. SSX has been shown to promote growth and survival properties of melanoma cells trough modulation of the MAPK/Erk and Wnt signalling pathways[91].

We hypothesize that demethylation of MAGE and NY-ESO-1 gene promoters occurs in a later stage of the oncogenic process, which might explain the expression rate differences between primary and metastatic tumours. This correlates with increasing epigenetic instability and global DNA hypomethylation occurring during tumour progression. It is possible that SSX is demethylated at an earlier stage, to promote primary tumour growth.

Though the expression of CTA might heighten the oncogenic potential of a tumour, it also offers a unique target for therapy. Normally CTA antigen are expressed exclusively on healthy germ line cells of the testis, and malignancies such as melanoma. Since MHC is not expressed on germ line cells, these cells will not be recognized by CTA-reactive T cell responses. This makes this group of antigens a potential target for immunotherapy of cancer.

In a previous study, vaccinations with MAGE-3.A1 peptide has been administered to melanoma patients, which successfully induced tumour regression in 7 of 25 patients [16]. Other studies reported tumour regression in vivo and in-vitro tumour cell killing, using with MAGE-3 tumour-specific antigen[92,93].

Cancer testis antigen have been studied in other tumour types as well. In multiple myeloma, it has been shown that autoantibodies against SSX-2 and NY-ESO-1 are capable of activating complement and increasing CTA uptake by antigen-presenting cells [94]. In theory, CTA protein or specific antibodies could be an adjuvant therapy alongside immune-checkpoint inhibitors.

Adjuvant MAGE-A3 therapy has been utilized for the treatment of non-small cell lung carcinoma (NSCLC). The results, were disappointing. After primary resection of the tumour, a total of 1515 patients with NSCLC were treated with adjuvant recombinant MAGE-A3 protein with AS15 immunostimulant. This group of patients did not have a better survival after resection

compared to patients who 784 patients who received placebo, after a mean follow-up of 38.1 months (27.9-48.4 months). However, this study did not, combine immune checkpoint inhibitors with adjuvant MAGE-A3 protein or antibodies. It could be possible that patients who express MAGE-A3 mRNA do not respond to adjuvant MAGE-A3 antibodies because the tumour does not present the actual protein. [95].

A limitation to this study is the highly heterogenic data. Due to this fact it was impossible to perform a meta-analysis.

In conclusion, many CTA are expressed by melanoma. This review provides an overview of the expression frequency of CTA antigens in melanoma and may aid in choices of CTA as therapeutic target for immunotherapy.

6

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("Melanoma"[Mesh] OR "Melanoma"[tw] OR "Melanoma"[tw] OR "lentigo maligna"[tw] OR "melanotic"[tw] OR "melanomas"[tw]) AND ("MAGEA6 protein, human" [Supplementary Concept] OR "MAGEA3 protein, human" [Supplementary Concept] OR "MAGEA1 protein, human" [Supplementary Concept] OR "Mage-a2 antigen" [Supplementary Concept] OR "MAGE-A1 protein (278-286), human" [Supplementary Concept] OR "MAGEC1 protein, human" [Supplementary Concept] OR "MAGEC2 protein, human" [Supplementary Concept] OR "MAGEA4 protein, human" [Supplementary Concept] OR "MAGE-A10 antigen" [Supplementary Concept] OR "MAGEA12 protein, human" [Supplementary Concept] OR "MAGEA11 protein, human" [Supplementary Concept] OR "CSAG1 protein, human" [Supplementary Concept] OR "MAGEB2 protein, human" [Supplementary Concept] OR "MAGEB1 protein, human" [Supplementary Concept] OR MAGE*[tw] OR "CSAG1"[tw] OR "SSX-2 peptide (41-49)" [Supplementary Concept] OR "SSX2"[tw] OR "SSX 2"[tw] OR "PRAME protein, human" [Supplementary Concept] OR "PRAME"[tw] OR "MAPE"[tw] OR "NY-ESO-1 protein, human (91-110)" [Supplementary Concept] OR "peptide NY-ESO-1 157-170" [Supplementary Concept] OR "peptide NY-ESO-1 157-165" [Supplementary Concept] OR "NY-ESO-1:161-180 peptide, human" [Supplementary Concept] OR "NY ESO 1"[tw]OR "NY ESO1"[tw]OR "NY-ESO-1"[tw]OR "NY-ESO1"[tw]OR "NY ESO-1"[tw]OR "NY-ESO 1"[tw]OR "NYESO1"[tw]OR "NYESO-1"[tw]OR "NYESO 1"[tw] OR (("Antigens"[Mesh:NoExp] OR "antigen"[tw] OR "antigens"[tw] OR "antigenic"[tw] OR "antigenicity"[tw]) AND ("cancer/testis"[tw] OR "CT"[ti] OR "cancer testis"[tw])))

Embase

(exp melanoma/ OR "Melanoma".ti,ab. OR "Melanoma".ti,ab. AND (melanoma antigen/ OR melanoma antigen 1/ OR melanoma antigen 2/ OR melanoma antigen 3/ OR melanoma antigen 4/ OR melanoma antigen 10/ OR MAGE*.ti,ab. OR "CSAG1".ti,ab. OR "SSX2".ti,ab. OR "SSX 2".ti,ab. OR "PRAME".ti,ab. OR "MAPE".ti,ab. OR NY ESO 1 antigen/ OR CTAG1*.ti,ab. OR "NY ESO 1".ti,ab. OR "NY ESO1".ti,ab. OR "NY-ESO-1".ti,ab. OR "NY-ESO1".ti,ab. OR "NY ESO-1".ti,ab. OR "NY-ESO 1".ti,ab. OR "NYESO1".ti,ab. OR "NYESO-1".ti,ab. OR "NYESO 1".ti,ab. OR cancer testis antigen/ OR ((antigen/ OR "antigen".ti,ab. OR "antigens".ti,ab. OR "antigenic".ti,ab. OR

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"antigenicity".ti,ab.) AND ("cancer/testis".ti,ab. OR "CT".ti. OR "cancer testis".ti,ab.))) NOT (exp animal/ NOT human/) NOT "conference abstract".pt.

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Supplementary table II: Cancer/testis mRNA expression in melanoma

Technique	Tissue origin	Cancer/testis antigen	N$_{Total}$	N$_{Primary}$	N$_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
RT-PCR	Tumor tissue	MAGE-A1	245	100	145	-	16	48	18
			23[1]	-	-	35	-	-	28
			189/202 [2]	8	194; In.tr:37, LN:48, Di:109	41	-	-	26
			10	-	-	90	-	-	29
			47	0	47	-	-	70	30
			64[3]	0	64	52	-	-	31

[1] Mostly metastatic
[2] Not all probes were available for biopsies during the time the sample was taken.
[3] Obtained from a smaller amount of patients

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Technique	Tissue origin	Cancer/testis antigen	N_Total	N_Primary	N_Metastatic	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			7	-	-	57	-	-	32
			19	-	-	42	-	-	33
			4	-	-	25	-	-	9
			47[4]	6	34; LR:24, Di:10	32	-	-	27
			6	-	-	67	-	-	34
			10; Mel.is: 3	4	3	-	0	33	35
		MAGE-A2	245	100	145	-	41	70	18
			10	-	-	60	-	-	29
			64[3]	0	64	-	-	47	31
			19	-	-	84	-	-	33

[4] 7 lesions of unknown origin

Technique	Tissue origin	Cancer/testis antigen	N[Total]	N[Primary]	N[Metastatic]	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			4	-	-	100	-	-	9
			47[4]	6	34 LR:24, Di:10	45	-	-	27
		MAGE-A3	245	100	145	-	36	76	18
			23[1]	-	-	52	-	-	28
			316	0	316; Cut:230, LN:54, IO:32	-	-	62	43
			105	-	-	69	-	-	44
			202	8	194; In.tr:37, LN:48, Di:109	55	-	-	26
			10	-	-	50	-	-	29
			47	0	47	-	-	81	30

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Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			10/61	-	-	90	-	-	[45]
			64[3]	0	64	-	-	55	[31]
			4	-	-	50	-	-	[9]
			47[4]	6	34 LR:24, Di:10	45	-	-	[27]
		MAGE-A4	245	100	145	-	11	22	[18]
			4	-	-	0	-	-	[9]
			47[4]	6	34 LR:24, Di:10	17	-	-	[27]
		MAGE-A6	4	-	-	25	-	-	[9]
			47[4]	6	34 LR:24, Di:10	64	-	-	[27]
		MAGE-A12	4	-	-	75	-	-	[9]

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			47[4]	6	34 LR:24, Di:10	74	-	-	27
			326	83	243	55	34	62	52
		MAGE-B	10	-	-	50	-	-	29
		MAGE-C1/CT7	10	-	-	70	-	-	53
			47	0	47	-	-	57	30
			11	0	11	-	-	64	54
		MAGE-C2/CT10	70	-	-	43	-	-	56
			10	-	-	50	-	-	57
		BAGE	47	0	47	-	-	15	30
			64[3]	0	64	-	-	16	31
			178	38	140	22	8	26	74

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		GAGE	47	0	47	-	-	49	30
			64^3	0	64	-	-	47	31
		GAGE-1	169	39	130	31	13	36	72
		GAGE-2	168	39	129	24	13	28	72
		GAGE-1,-2,-7	10	-	-	20	-	-	29
		GAGE-3-6,-8	10	-	-	30	-	-	29
		XAGE-1	47	0	47	-	-	51	30
			69	8	61	-	0	38	73
		SSX1	23^1	-	-	30	-	-	28
			37	-	-	27	-	-	69
		SSX2	23^1	-	-	44	-	-	28
			10	-	-	0	-	-	29

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			47	0	47	-	-	36	[30]
			37	-	-	35	-	-	[69]
			16	-	-	50^5	-	-	[70]
		SSX4	23^1	-	-	26	-	-	[28]
			37	-	-	27	-	-	[69]
		SSX5	37	-	-	5	-	-	[69]
		PRAME	201	49	152	-	88	95	[75]
		LAGE-1	10	-	-	40	-	-	[29]
		CTSP-1	17	-	-	59	-	-	[79]
		CTSP-2	9	-	-	11	-	-	[79]
		CTSP-4	9	-	-	0	-	-	[79]

[5] Expression analysis was performed with oligonucleotides specific for HOM-MEL40; an antigen coded for by SSX2 gene.

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Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		CTp11	10	-	-	70	-	-	[78]
		KIF20A	38	28	10	-	64	80	[77]
		NY-ESO-1/ LAGE-2	52	20	32; LR:22, Di:10	32.7	10	47 (LR:45, Di: 50)	[58]
			23^1	-	-	44	-	-	[28]
			67	-	-	34.3	-	-	[59]
			202^2	8	194; In.tr:37, LN:48, Di:109	70.83	-	-	[26]
			120	-	-	40	-	-	[60]
			64	0	64	-	-	31.3^{16}	[61]
			10	-	-	20	-	-	[29]
			47	0	47	-	-	49	[30]

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			12	1	11	42	-	-	62
	Uncultured melanoma	TAG-1	22	-	-	59	-	-	81
		TAG-2a	22	-	-	36	-	-	81
		TAG-2b	22	-	-	23	-	-	81
		TAG-2c	22	-	-	27	-	-	81
RT-PCR	Cell lines	MAGE-A1	24	1	23	83	-	-	29
			2	-	-	100	-	-	9
			17	-	-	53	-	-	82
			7	0	7	-	-	71	63
		MAGE-A2	24	1	23	50	-	-	29
			2	-	-	100	-	-	9
		MAGE-A3	81	-	-	81	-	-	44

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Technique	Tissue origin	Cancer/testis antigen	N$_{Total}$	N$_{Primary}$	N$_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			28	-	-	64	-	-	46
			24	1	23	38	-	-	29
			41	-	-	49	-	-	83
			2	-	-	100	-	-	9
			17	-	-	88	-	-	82
			69	19	50; LN:25, Di:25	26	5	LN:40, Di:28	47
			10 Mel.is:3	4	3	-	25	67	35
		MAGE-A4	2	-	-	50	-	-	9
		MAGE-A6	2	-	-	100	-	-	9
		MAGE-A12	2	-	-	100	-	-	9

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		MAGE-B	24	1	23	17	-	-	29
		GAGE-1,-2,-7	24	1	23	29	-	-	29
		GAGE-1, -2, -8	17	-	-	71	-	-	71
		GAGE-3-6,-8	24	1	23	42	-	-	29
		GAGE 3-7b	17	-	-	76	-	-	71
		XAGE-1	14	-	-	43	-	-	73
		XAGE-1a	28	5	23	-	0	9[6]	85
		XAGE-1b	28	5	23	-	0	61[6]	85
		XAGE-1c	28	5	23	-	0	35[6]	85
		XAGE-1d	28	5	23	-	0	52[6]	85
		XAGE-2	28	5	23	-	20	13[6]	85

[6] For primary and metastatic: results from 60 PCR cycles included

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Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		XAGE-3	28	5	23	-	0	0[6]	85
		SSX1	12	-	-	42	-	-	84
			18	-	-	33	-	-	68
		SSX2	24	1	23	4	-	-	29
			12	-	-	42	-	-	84
			18	-	-	39	-	-	68
		SSX4	12	-	-	33	-	-	84
			18	-	-	11	-	-	68
		SSX5	12	-	-	8	-	-	84
			18	-	-	6	-	-	68
		LAGE-1	24	1	23	42	-	-	29
		CDCA1	9	-	-	100	-	-	80

Technique	Tissue origin	Cancer/testis antigen	N_Total	N_Primary	N_Metastatic	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		CTp11	23	-	-	26	-	-	78
		KIF20A	10	-	-	100	-	-	77
		NY-ESO-1/ LAGE-2	24	1	23	29	-	-	29
qRT-PCR	Tumor tissue	SCP1	7	0	7	-	-	43	63
		SEMG1	7	0	7	-	-	14	63
		SPANXA	7	0	7	-	-	86	63
		PASD	7	0	7	-	-	71	63
		SSX1	7	0	7	-	-	100	63
		PRAME	7	0	7	-	-	100	63
		GAGE-1	7	0	7	-	-	100	63

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Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
		NY-ESO-1/ LAGE-2	7	0	7	-	-	43	63
RNAseqV2, DNA methylation	Tumor tissue	MAGE-A1	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	38.2	-	-	36
		MAGE-A2	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	58.43	-	-	36
		MAGE-A3	$N_{RNAseqV2}$: 267; N_{DNA}	-	-	59.93	-	-	36

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			N_{DNA} methylation: 255						
		MAGE-A6	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	61.42	-	-	36
		MAGE-A12	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	59.55	-	-	36
		MAGE-C1/CT7	$N_{RNAseqV2}$: 267; N_{DNA}	-	-	46.82	-	-	36

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			$_{methylation}$: 255						
		MAGE-C2/CT10	$N_{RNAseqV2}$: 267; N_{DNA} $_{methylation}$: 255	-	-	49.81	-	-	36
		SSX1	$N_{RNAseqV2}$: 267; N_{DNA} $_{methylation}$: 255	-	-	49.06	-	-	36
		SSX2	$N_{RNAseqV2}$: 267; N_{DNA}	-	-	20.97	-	-	36

Technique	Tissue origin	Cancer/testis antigen	N_{Total}	$N_{Primary}$	$N_{Metastatic}$	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
			methylation: 255						
		PRAME	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	95.88	-	-	36
		CSAG1	$N_{RNAseqV2}$: 267; N_{DNA} methylation: 255	-	-	58.43	-	-	36
ELISA	Short term cell cultures	MAGE-A3	69	19	50; LN:25, Di:25	51	37	56 (LN:52, Di:60)	47
TMA	Tumor tissue Cell lines	MAGE-C1/CT7	222 50	59	163	-	20.3-24	40.5	55

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Technique	Tissue origin	Cancer/testis antigen	N_Total	N_Primary	N_Metastatic	mRNA expression total(%)	mRNA expression Primary (%)	mRNA expression Metastatic (%)	Ref.
	Tumor tissue Cell lines	MAGE-C2/CT10	206 68	51	155	-	33	40	55
FACS	Cell lines	MAGE-A3	28	-	-	45	-	-	46

SSM: superficial spreading melanoma; Nod: nodular; ACL: acrolentiginous melanoma; US: unspecified; Mel.is: melanoma in situ; SC: spindle cell component; D: desmoplastic; Epi: epithelioid component; Junc: junctional component; Cut: cutaneous melanoma; In.tr: in transit melanoma; Mel.inv: melanoma invasive; LN: Lymph nodes; Visc: Visceral; LR: Locoregional; Di:Distant; IO: internal organs; S.c.: subcutaneous;

Supplementary file III: Cancer/testis protein expression in melanoma

Tissue origin	Cancer/testis antigen	N[Total]	N[primary]	N[metastatic]	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
Tumor tissue	MAGE-A1	586[3]	251	335; LN:174, S.c.:71, Di:90	-	20	Di: 51	MA454	37
		56[3]	9	47	27[7]	-	-		39
		314[3]	40	274	-	7.5	36		38
		321	321	0	-	30	-	MA454	40
		32[8]			SC: 12.5 (4/32) Epi: 9.7 (3/31) Junc: 20 (4/20)	-	-		41
		38	38; SSM: 20;	0	-	21	-	MA454	22

[7] From HMB-45 negative tumors 35% (6/17) stained strongly positive (>33% intratumoral staining) and from HMB-45 positive tumors 23% (9/39) stained positive (> 5% intratumoral staining)

[8] All tumors were desmoplastic melanoma based on accepted histological criteria, including spindle cell proliferation separated by abundant collagen, cytologic atypia of the spindle cells, atypical melanocytic proliferation at the dermal–epidermal junction and neurotropism.

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Tissue origin	Cancer/testis antigen	N_Total	N_primary	N_metastatic	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
			Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.IS: 1						
		7	-	-	57	-	-	77B	[32]
					57			34B	
		30 -HMB-45 neg: 22 (SC: 8, D: 8, Epi:6) - HMB-45 pos: 8	-	-	57 -HMB-45 neg: 64 (14/22) - HMB-45 pos: 38 (3/8)	-	-	MA454	[42]
		6	-	-	50	-	-	34B	[34]

Tissue origin	Cancer/testis antigen	N_{Total}	$N_{primary}$	$N_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
	MAGE-A3	52	-	-	90.4[9]	-	-	57B	[43]
		38;	38 SSM: 20; Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.is: 1	-	-	15[13]	-	M3H67	[22]
		91	91	0	-	37[10]	-	57B[11]	[48]
		205;	85; Mel.is:20, Mel.inv: 65	120; Cut:33, LN:66, IO:21	-	25	25 (Cut:30, LN:23, IO:24)	57B[11]	[49]

[9] The antibody used detects MAGE-A1, -6, -12 and –A4 more strongly than MAGE-A3. Of these 52 metastases, 17 were negative for MAGE-A3 (by RT-PCR) and 35 (67%) showed the presence of MAGE-A3 mRNA.
[10] Immunoreactivity defined as positive when more than 15% of tumor cells stain positive.
[11] Antibody also recognizes other members of the MAGE family, including MAGE-1, -4, -6, and -12. Crossreactivity can not be fully excluded.

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Tissue origin	Cancer/testis antigen	N_{Total}	$N_{primary}$	$N_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
		61	40	21	44	-	-	57B[11]	[45]
		10	0	10	-	-	70	57B[11]	[50]
	MAGE-A4	586[3]	251	335; LN:174, S.c.:71, Di:90	-	9	Di: 44	supernatant 57B	[37]
		321	321	0	-	18	-	57B	[40]
		32[8]	-	-	SC: 12.5 Epi: 19.4 Junc: 5	-	-	supernatant 57B	[41]
		60	-	-	28	-	-	57B	[17]
		38	38;	0	-	29	-	57B[12]	[22]

[12] mAb 57B was initially generated to MAGE-A3, but later considered to be a poly MAGE reagent and in this study regarded as reactive to MAGE-A4.

Tissue origin	Cancer/testis antigen	N_{Total}	$N_{primary}$	$N_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
			SSM: 20; Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.is: 1						
	MAGE-A10	50	0	50	-	-	38	GA11.1	51
	MAGE-C1/CT7	38	38; SSM: 20; Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.is: 1	0	-	38[13]	-	CT7-33	22
		11	0	11	-	-	82	CT7-33	54

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Tissue origin	Cancer/testis antigen	N_{Total}	$N_{primary}$	$N_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
	GAGE	38	38; SSM: 20; Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.is: 1	0	-	19[13]	-	GAGE	22
		40	-	-	53	-	-	pGAb	71
	XAGE-1b	113	75	38; Cut:12, LN:26	-	30.7	Cut: 75.0, LN: 57.7	Mouse US09-13	64
	SSX	101	35	66	34	40	30	E3AS	68
	SSX2	101	35	66; LR:17; LN:38; Di:11	-	40	LR: 24; LN: 32; Di: 36	E3AS[14]	49

[13] A non-specified subset was evaluated for expression.
[14] Generated mouse E3AS Anti-SSX mAb found to recognize SSX2, SSX3, and SSX4 proteins expressed in formaldehyde-fixed and paraffin-embedded tissue.

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Tissue origin	Cancer/testis antigen	N$_{Total}$	N$_{primary}$	N$_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
	CDCA1	70	56	14	73	75	64	Polyclonal anti-CDCA1	80
	KIF20A	60	51	9	-	59	67	Polyclonal rabbit	77
	NY-ESO-1/ LAGE-2	586[3]	251	335; LN:174, S.c.:71, Di:90	-	45	Distant: 45	E978	37
		113[3]	75	38; Cut:12, LN:26	-	29.3	LN: 61.5; Cut: 50	Mouse E978	64
		314[3]	40	274	-	5	14	-	38
		79; SSM:31; Nod:26;	-	-	SSM:6; Nod:23;	-	-	E978	23

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Tissue origin	Cancer/testis antigen	N_Total	N_primary	N_metastatic	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
		ACL:8; LMM:7; US: 7			ACL:37.5; LMM:43; US: 29				
		222[3]	16	206	-	0	28.2		[65]
		321	321	0	-	37	-	E978	[40]
		124[3]	61; SSM:27, Nod:23, ACL:6, LMM:3, D:2	63	-	13	32	E978	[24]
		120	-	-	45	-	-	Synthesized E978[15]	[60]

[15] Two antibodies used in this study: ES121 and E978. Both antibodies had very similar immunohistochemistry reactivities. With peptide ELISA was determined that both antibodies were specific for NY-ESO-1 because neither bound to homologous LAGE-1 peptides despite 84% overall amino acid homology.

Tissue origin	Cancer/testis antigen	N_{Total}	$N_{primary}$	$N_{metastatic}$	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
		32[8]			SC: 6.25; Epi: 6.5; Junc: 15			E978, ES121	41
		60	0	60	-	-	46.6[16]	E978	61
		38	38 SSM: 20; Nod: 8; LMM: 2; ACL: 2; US: 5; Mel.is: 1	0	24	-	-	ES121	22
		11	0	11	-	-	36	ES121	66
		38	-	-	32	-	-	D8.38	67
Cell lines	GAGE	17	-	-	41	-	-	pGAb	71

[16] Both RT-PCR and IHC combined gave a total NY-ESO-1 expression of 38.7% (48/124)

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Tissue origin	Cancer/testis antigen	N_Total	N_primary	N_metastatic	Total protein expression (%)	Protein expression in primary tissue (%)	Protein expression in metastatic tissue (%)	Monoclonal antibody	Ref.
	XAGE-1b	12	5	7	92	100	86	Mouse US09-13	64
	NY-ESO-1/ LAGE-2	12	5	7	25	20	29	Mouse E978	64

SSM: superficial spreading melanoma; Nod: nodular; LMM: Lentigo maligna melanoma; ACL: acrolentiginous melanoma; US: unspecified; Mel.is: melanoma in situ; SC: spindle cell component; D: desmoplastic; Epi: epithelioid component; Junc: junctional component; Cut: cutaneous melanoma; In.tr: in transit melanoma; Mel.inv: melanoma invasive; LN: Lymph nodes; Visc: Visceral; LR: Locoregional; Di:Distant; IO: internal organs; S.c.: subcutaneous

[i] Mostly metastatic

[ii] Not all probes were available for biopsies during the time the sample was taken

[iii] Obtained from a smaller amount of patients

[iv] 7 lesions of unknown origin

[v] Expression analysis was performed with oligonucleotides specific for HOM-MEL40; an antigen coded for by SSX2 gene.

[vi] For primary and metastatic: results from 60 PCR cycles included

[vii] From HMB-45 negative tumors 35% (6/17) stained strongly positive (>33% intratumoral staining) and from HMB-45 positive tumors 23% (9/39) stained positive (> 5% intratumoral staining)

[viii] All tumors were desmoplastic melanoma based on accepted histological criteria, including spindle cell proliferation separated by abundant collagen, cytologic atypia of the spindle cells, atypical melanocytic proliferation at the dermal-epidermal junction and neurotropism.

[ix] The antibody used detects MAGE-A1, -6, -12 and –A4 more strongly than MAGE-A3. Of these 52 metastases, 17 were negative for MAGE-A3 (by RT-PCR) and 35 (67%) showed the presence of MAGE-A3 mRNA.

[x] Immunoreactivity defined as positive when more than 15% of tumor cells stain positive.

[xi] Antibody also recognizes other members of the MAGE family, including MAGE-1, -4, -6, and -12. Crossreactivity cannot be fully excluded.

[xii] mAb 57B was initially generated to MAGE-A3, but later considered to be a poly MAGE reagent and in this study regarded as reactive to MAGE-A4.

[xiii] A non-specified subset was evaluated for expression.

[xiv] Generated mouse E3AS Anti-SSX mAb found to recognize SSX2, SSX3, and SSX4 proteins expressed in formaldehyde-fixed and paraffin-embedded tissue.

[xv] Two antibodies used in this study: ES121 and E978. Both antibodies had very similar immunohistochemistry reactivities. With peptide ELISA was determined that both antibodies were specific for NY-ESO-1 because neither bound to homologous LAGE-1 peptides despite 84% overall amino acid homology.

[xvi] Both RT-PCR and IHC combined gave a total NY-ESO-1 expression of 38.7% (48/124)

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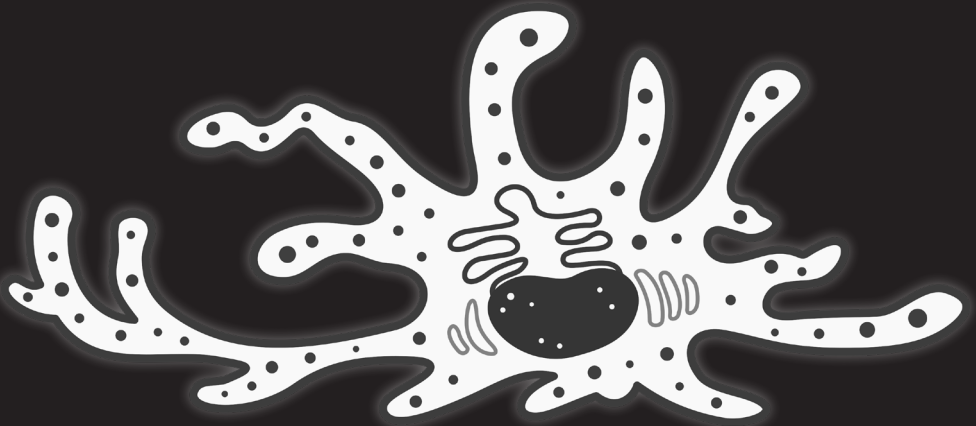
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Lower expression of Cancer/Testis antigen in Lentigo Maligna Melanoma as compared to other types of primary cutaneous melanoma

D.C.K.S. Tio[1,3], M.Willemsen[2,3], G. Krebbers[2,3], F.R. Kasiem[2,3,] R. Hoekzema[1,2,]
R. van Doorn[4], M.W. Bekkenk[1,2], R.M. Luiten[2,3]

1. Department of Dermatology, Amsterdam University Medical Centers, VU University.
2. Department of Dermatology and Netherlands Institute for Pigment Disorders, Amsterdam University Medical Centers, University of Amsterdam
3. Cancer Center Amsterdam and Amsterdam Infection & Immunity Institute
4. Department of Dermatology, Leiden University Medical Center

Abstract

The cancer/testis antigens (CTA) are a group of antigens expressed on germ cells of healthy testis and malignant tumors. We studied whether CTA are present on lentigo maligna (LM) and lentigo maligna melanoma (LMM) samples. Immunohistochemical expression of a panel of CTA (MAGE-A1, A2- A3, NY-ESO-1, PRAME, SSX-2 and a MAGE-A antibody reactive with -A1, -A2, -A3, -A4, -A6, -A10 and -A12) was investigated in formalin-fixed paraffin-embedded samples from LMM (n=20), LM (n=8), chronically sun-exposed skin (n=7) and healthy skin (n=7). In 4 LMM lesions the MAGE-A marker was positive. Another 3 LMM lesions were positive for MAGE-A1, MAGE-A2 and MAGE-A3. PRAME was positive in 18/20 LMM and 6/8 LM. We did not find expression of MAGE, NY-ESO-1 or SSX-2 in LM, thereby excluding these CTA as diagnostic marker to discern malignant melanocytes in LM from normal melanocytes. LMM did express MAGE, NY-ESO-1 and SSX-2. If a biopsy from a lesion suspect for LM shows positivity for MAGE, NY-ESO-1 and SSX-2, the lesion may actually be LMM. In contrast, PRAME expression was found in LM at low levels and in LMM at much higher levels, and absent in normal melanocytes. PRAME can potentially be used to discern normal melanocytes from malignant melanocytes.

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Introduction

Cancer/testis antigens (CTA) are a group of antigens expressed by germ cells of healthy testis and by malignant tumors of different histological origin, including cutaneous melanoma. This selective expression pattern qualifies CTA as diagnostic and prognostic tumor markers and make CTA key candidate targets for immunotherapy (1)*.* Lentigo maligna (LM) is considered to be a variant of melanoma in situ and a precursor of lentigo maligna melanoma (LMM) (2). Classically, 4 types of cutaneous melanoma are discriminated, namely superficial spreading melanoma, nodular melanoma, LMM and acrolentiginous melanoma (3). The "divergent pathways" model, postulated in 2003, differentiates melanoma associated with chronic sun damage from melanoma arising in intermittently sun-exposed skin (4). It has been shown by Stadelmeyer *et al*. that LM more often have BRAFV600K mutations, which are associated to chronic sun damage, than superficial spreading melanoma (SSM) and nodular melanoma (NM), which are more associated to intermittent sun damage (5). Based on these facts, LM might warrant a different management approach. Therefore this study aims to identify phenotypic differences between LM, LMM and normal melanocytes, that could aid in the diagnosis of LM and LMM.

Currently, the diagnosis of LM is based on the histological presence of melanocytes proliferating along the basal layer of the epidermis. However, a specific marker to discriminate malignant melanocytes from normal melanocytes is lacking (6).

This study aims to investigate whether CTA are present on LM and lentigo maligna melanoma (LMM), and to evaluate if CTA can be used to discern malignant melanocytes in LM from normal melanocytes.

Materials & Methods

Formalin-fixed paraffin-embedded tissue sections of LMM (n=20), LM (n=8), chronically sun-exposed skin (n=7) and healthy skin (n=7) were kindly provided by the department of Pathology at the Amsterdam University Medical Centers, VU Medical Center, Amsterdam, the Netherlands. This study was approved by the biobank ethical committee of the VU Medical Center. LM was defined as atypical melanocytes, singly and in nests, usually confined to the basal layer with little pagetoid invasion of the epidermis. LMM was defined as LM with an

invasive component composed of spindled melanocytes or epitheloid melanocytes with variable cytological atypia, nuclear pleomorphism and tumor giant cells, as described by Patterson *et al.* (7). Tissue sections were deparaffinized in xylene and rehydrated by serial passage through graded ethanols. Heat-induced antigen retrieval was performed for 20 min. at 98°C in TrisEDTA pH9.0 buffer. Antibodies used included anti-MAGE-A mAb 6C1, reactive with MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12 proteins, anti-MAGE-A1 mAb MA454, polyclonal anti-MAGE-A2 Ab, polyclonal anti-MAGE-A3 Ab, anti-SSX2 mAb CL3202 (all obtained from Thermo Fisher Scientific), anti-NY-ESO-1 mAb E978; (Santa Cruz Biotechnology), and anti-PRAME mAb EPR20330 (Abcam). Next, tissue sections were incubated with either Poly-AP anti-mouse or Poly-AP anti-rabbit (Immunologic) and visualized with Perma Red/AP (Diagnostic Biosystems). Tissue sections were counterstained with hematoxylin (Sigma Aldrich). Coverslips were mounted using Pertex (VWR International). Images were taken with Olympus Cell Sens software (Olympus).

Results

MAGE-A showed a nuclear and cytoplasmic staining pattern with no background staining. Four of 20 (20%) LMM stained positive with the anti-MAGE-A antibody. MAGE-A1 was expressed by 50% of tumor cells in 1 LMM sample, while in 3 LMM samples MAGE-A1 expression was limited to 5% of tumor cells. Three different LMM lesions expressed MAGE-A1, MAGE-A2 and MAGE-A3 in less than 5% of the tumor tissue. PRAME showed a nuclear and membranous staining pattern and no background staining. PRAME expression was seen in 18 of 20 (90%) LMM. Of these 18 positive samples, 14 showed expression in 90-100% of the tumor cells while the other 4 showed positive expression in 1-50% of the tumor cells. We did not find expression of NY-ESO-1 or SSX-2 in LMM. In the LM group, 6 of 8 (75%) LM showed expression of PRAME at low levels. In one of these LM tissues, PRAME was expressed in 20-30% of the tumor cells, while five other LM showed PRAME expression in <1% of tumor cells. No expression of MAGE-A, MAGE-A1, -A2, -A3, NY-ESO-1 or SSX-2 was seen in LM. Sun-exposed skin did not show any positive staining for CTAs (MAGE, NY-ESO-1, SSX-2 or PRAME). One out of 7 normal skin tissues showed positive expression of PRAME in <1% of melanocytes with a similar staining pattern as in LM and LMM. The results are summarized in table 1.

Discussion

LM is considered a melanoma in situ and CTA expression can be a feature of malignant tumors. We did not observe any MAGE-A1, -A2, -A3, -A4, -A10, -A12, NY-ESO-1 or SSX-2 expression, but we did observe PRAME expression in LM, albeit at lower levels compared to LMM. This is concurrent with the hypothesis that LM is the precursor of LMM, and perhaps the pre-malignant stage prior to LMM.

We demonstrated expression of MAGE-A1, MAGE-A2 and MAGE-A3 antigen on LMM. In our systematic review cutaneous melanoma was described to express MAGE-A1 protein in 7.5-30% of 659 primary tumors and MAGE-A3 protein was expressed in 15-37% of 254 primary tumors. In comparison, LMM seems to have a lower expression rate of MAGE-A1 and MAGE-A2 at 1/20 (5%) tumors. In this same review, we found that PRAME expression is reported in 88% of 49 primary cutaneous melanoma and in 95% of 152 metastatic cutaneous melanoma. This review also revealed that primary tumors express CTA at lower rates compared to metastatic tumors (8). Although the expression of PRAME (90% in LMM) in this study does not differ in comparison to cutaneous melanoma, the pattern of lower MAGE-A expression levels supports the notion that LMM is a distinct entity compared to SSM and NM.

In our samples, we did not find any positive staining of MAGE-A1, -A2, -A3, -A4, -A10, -A12, NY-ESO-1 or SSX2 in LM tissue. Therefore, these specific CTA cannot be used to discern malignant melanocytes from normal melanocytes to confirm the diagnosis of LM. However, it is possible that if a biopsy from a lesion suspect for LM shows positivity for MAGE, NY-ESO-1 or SSX-2, the lesion may actually be LMM. A recent study showed that 9% of biopsy proven LM are reclassified as LMM after surgical excision (9).

Interestingly enough we found positive PRAME expression in LM at low levels but not in sun-exposed skin. In a clinical setting, it is difficult to accurately distinguish LM from solar lentigines. Usually a biopsy is taken to confirm or disprove the diagnosis LM. Another uncertain situation is when a sample might not show all the classical characteristics of LM on histopathological examination. PRAME specifically could potentially be used to differentiate normal melanocytes in sun-exposed or chronic sun-damaged skin from malignant melanocytes, indicating LM. Our

findings are similar to a recent study by Lezcano *et al*, in which they found positive PRAME expression in 24 of 27 (88%) LM and 15 of 17 (88%) LMM. They also found rare isolated cases of junctional melanocytes with immunoreactivity for PRAME in benign non-lesional skin (10).

A single normal skin sample showed positive expression of PRAME. Expression of PRAME has been described in normal skin in an earlier study by Ikeda *et al* and the aforementioned study by Lezcano *et al.* (10, 11). A potential pitfall is that PRAME seems to be less specific for testis and malignancies in comparison to other CTA. If a normal skin sample is false positive it could lead to the incorrect diagnosis of LM and consequently to overtreatment. Because the staining pattern is the same, it is important to correlate clinical information with the histopathological findings to prevent incorrect diagnosis.

Limitations to this study are a small sample size and the lack of LM samples which consecutively progressed to LMM. We recommend future studies to investigate the prevalence of PRAME in larger cohorts of LM and LMM to analyze whether PRAME can be used as a discerning marker between normal and atypical melanocytes.

In conclusion, we did not find expression of MAGE-A, NY-ESO-1 or SSX-2 in LM, thereby excluding these CTA as diagnostic marker to discern malignant melanocytes in LM from normal melanocytes. LMM does express MAGE, NY-ESO-1 and SSX-2 but at lower levels compared to cutaneous melanoma. If a biopsy from a lesion suspect for LM shows positivity for MAGE, NY-ESO-1 and SSX-2, the lesion may actually be LMM. In contrast, PRAME expression was found in LM at low levels and in LMM at much higher levels. This specific CTA can potentially be used to discern normal melanocytes from malignant melanocytes in LM.

Figure 1: MAGE1 and PRAME expression in skin sections. FFPE sections were immunohistochemically stained for MAGE1 (mAb 6C1) and PRAME (mAbERP20330) and visualized using Perma Red/AP chromogen. Images are taken at 400x magnification.
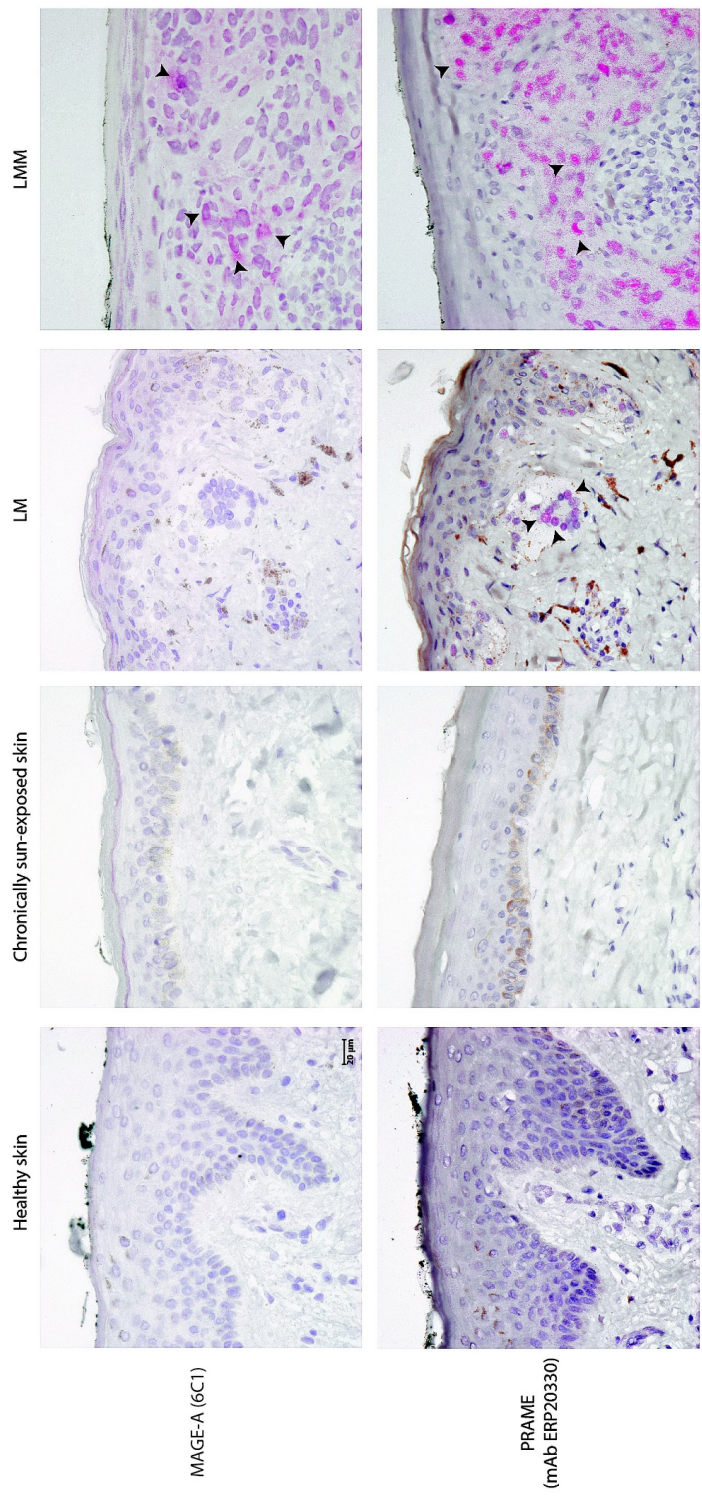
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Table 1: Summary of expression patterns of Cancer Testis Antigens in lentigo maligna and lentigo maligna melanoma.

	MAGE-A (mAb 6C1)	MAGE-A1 (MA454)	MAGE-A2 (polyclonal)	MAGE-A3 (polyclonal)	NY-ESO-1 (mAb E978)	PRAME (mAb EPR20330)	SSX2 (mAb CL3202)
Staining pattern	Nuclear and Cytoplasmic	Cytoplasmic	Cytoplasmic	Cytoplasmic	-	Nuclear and membranous	-
Healthy skin (N=7)	Negative	Negative	Negative	Negative	Negative	1/7 samples positive expression in <1% of cells	Negative
Sun-exposed skin (N=7)	Negative	Negative	Negative	Negative	Negative	Negative	Negative
LM (N=8)	Negative	Negative	Negative	Negative	Negative	6/8 samples positive expression in <1-30% of cells	Negative
LMM (N=20)	4/20 samples positive expression in 5-50% of cells	1/20 samples positive expression in <5% of cells	1/20 samples positive expression in <5% of cells	1/20 samples positive expression in <5% of cells	Negative	18/20 samples positive expression in 50-100% of cells	Negative

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8

Patient characteristics and oncogenic mutations of metastasized lentigo maligna melanoma: Results from the Dutch Melanoma Treatment Registry.

D.C.K.S. Tio[1], M.W Bekkenk[1], R. Hoekzema[1], A.J.M van den Eertwegh[2], W.J. Louwman[3] A.J. ten Tije[4], J.W.B. de Groot[5], E. Kapiteijn[6], G. Vreugdenhil[7], M.J Boers[8],
Suijkerbuijk K.P.M[9], A.A.M van der Veldt[10], R.S. van Rijn[11], D. Piersma[12], M.J.B. Aarts[13], J.B.A.G Haanen[14,15], G.A.P. Hospers[16], F.W.P.J. van den Berkmortel[17], R.M. Luiten[1],
W. Ouwerkerk[1,18]

1. Department of Dermatology, Netherlands Institute for Pigment Disorders, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands; Amsterdam Infection & Immunity Institute; Cancer Center Amsterdam.
2. Department of Medical Oncology Amsterdam UMC, VU University Amsterdam, Amsterdam, the Netherlands; Cancer Center Amsterdam
3. Department of Research and Development, Netherlands Comprehensive Cancer Organisation (IKNL), Utrecht, The Netherlands
4. Department of Internal Medicine/Oncology, Amphia Hospital, Breda, the Netherlands
5. Department of Medical Oncology, Isala Oncological Center, Zwolle, the Netherlands
6. Department of Medical Oncology, Leiden University Medical Center, Leiden, the Netherlands
7. Department of Internal Medicine/Oncology, Maxima Medical Center, Eindhoven, the Netherlands
8. Department of Medical Oncology, Radboud University Medical Center, Nijmegen, the Netherlands
9. Department of Medical Oncology, University Medical Center Utrecht, University of Utrecht, Utrecht, the Netherlands
10. Department of Medical Oncology, ErasmusMC Cancer Institute, Rotterdam, the Netherlands
11. Department of Internal Medicine/ Oncology, Medical Center Leeuwarden, Leeuwarden, the Netherlands
12. Department of Internal Medicine/Oncology, Medisch Spectrum Twente, Enschede, the Netherlands
13. Department of Medical Oncology, Maastricht University Medical Center, Maastricht, the Netherlands
14. Dutch Institute for Clinical Auditing
15. Division of Medical Oncology, Antoni van Leeuwenhoek-Netherlands Cancer Institute, Amsterdam, the Netherlands –
16. Department of Medical Oncology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
17. Department of Internal Medicine, Zuyderland Medical Center Geleen-Heerlen, Sittard-Geleen, the Netherlands
18. National Heart Centre Singapore, Singapore

Abstract

Introduction

Patients with metastatic Lentigo Maligna Melanoma (mLMM) represent a subgroup of cutaneous melanoma patients. Using data from the Dutch Melanoma Treatment Registry (DMTR), we studied the epidemiological, clinical and genetic characteristics, treatment efficacy and survival of mLMM patients in comparison to patients with metastatic Nodulair Melanoma (mNM) and patients with metastatic Superficial Spreading Melanoma (mSSM), respectively.

Methods

In the Netherlands, all patients with metastatic melanoma are registered in the DMTR. Data from 2013 to 2018 were extracted. Clinical, histological and genetic aspects, efficacy of treatment and survival were analyzed. In addition, the proportion of patients with mLMM in comparison to patients with primary LMM was analyzed using national incidence data.

Results

Data were extracted concerning 3,959 patients with advanced melanoma including 59 (1.5%) mLMM patients mLMM and 2,313 (58.4%) mNM/mSSM patients. The proportion of patients with LMM developing metastases was lower (59/1,840; 3.2%) than in cases of NM/SSM (2,313/35,055; 6.6%). In general, BRAFV600 mutations were less frequently encountered in patients with mLMM than in patients with mNM or mSSM. In contrast, the proportion of V600K mutations was higher in mLMM. KIT mutations were demonstrated more often in patients with mLMM. Although overall survival between patient groups was similar, melanoma-related death occurred less common in patients with mLMM.

Conclusion

Based on our analysis of the DMTR data, LMM seems to have a lower metastatic potential than SSM and NM . The genetic profile of mLMM also appears different, having more BRAFV600K mutations. Interestingly, patients with mLMM patients die less often from metastases than patients with SSM or NM, despite similar overall supervival. These findings suggest that mLMM shows less aggressive behavior than mNM and mSSM.

8

Introduction

The development of immunotherapy and targeted therapy has markedly improved the survival of patients with metastatic cutaneous melanoma . Current treatment guidelines however, make no distinction between the different subtypes of the primary melanocytic malignancy(1-3).

Primary cutaneous melanoma is mostly caused by the interplay between sun damage or exposure to UV radiation and host factors. This combination results in characteristic patterns of driver mutations in BRAF, NRAS and other oncogenic genes (4). In 2003 the "divergent pathway" model was introduced. This hypothesis differentiates between melanoma associated with chronic sun damage and melanoma arising on intermittently sun-exposed skin (5, 6). Lentigo maligna (LM) is considered a variant of melanoma in situ, which can progress to LMM. In general, LM is most prevalent in elderly patients with fair skin, often with a history of chronic sun damage (7, 8). Previous studies have shown that LM and LMM are associated with chronic sun exposure and a history of non-melanoma skin cancer, whereas nodular melanoma (NM) or superficial spreading melanoma (SSM) are associated with intermittent sun exposure (9). Consequently, intermittent sun exposure can cause other types of mutations than chronic sun exposure. Stadelmeyer *et al.* showed that in LM and LMM lesions oncogenic mutations in BRAFV600K are more common than BRAFV600E mutations (10). Furthermore, BRAFV600K specific mutations were predominantly found in chronic sun-damaged skin in elderly people (11). This may explain why LM and LMM occur mainly in chronically sun-exposed skin, such as the head and neck area (7, 8).

Based on the aforementioned clinical and genetic differences, LMM could be considered as a different type of melanoma with   a different prognosis, possibly justifying a different therapeutic policy . Further knowledge about LMM- specific characteristics, including molecular markers predictive of homing preferences, may help to understand and manage this heterogeneous disease in terms of prognosis and follow-up procedures (12). In the Netherlands, care for patients with advanced melanoma is centralized in 14 hospitals across the country. Since   July 2013 all patients diagnosed with metastatic melanoma in The Netherlands have been registered in the Dutch Melanoma Treatment Registry (DMTR), irrespective of treatment modality (13). The aim of this study is to identify the differences

between mLMM and mNM and mSSM with respect to epidemiology, clinical characteristics, tumor characteristics, survival, drug efficacy and driver mutations, using DMTR data.

Methods

Patient data

The DMTR is a registry of all patients with stage IV or irresectable stage IIIC melanoma (including uveal melanoma), referred to or discussed with one of the melanoma centers. Data were recorded at the time of primary diagnosis of advanced melanoma and during follow-up, irrespective of treatment modality. Limited data were collected for patients in poor condition from referring hospitals, for whom a melanoma center was only consulted and who were not eligible for systemic treatment, as previously described (13). For all patients referred to a melanoma center, the DMTR contains detailed clinical information on baseline characteristics, type of treatment and regime, dosages, immune-related adverse events, time period until the next treatment, survival, and healthcare resource use. The DMTR was approved by the medical ethical committee and was not subject to the Medical Research Involving Human Subjects Act. Patients without cytological or histological proof of melanoma were not included

Data from the DMTR was provided from 2013-2018. From this data we extracted 216 parameters in total, describing patient and melanoma characteristics, location of melanomas, histological data on ulceration, dermal mitosis, satellites, in transit metastasis, anatomical locations of metastasis and tumor mutational data in BRAF, NRAS, KIT and GNAQ genes. In addition, treatment and survival data was retrieved. All parameters retrieved are presented in Supplementary Table S1.

Treatment results of patients are recorded in the DMTR as "treatment episodes" per patient for each type of treatment received. For each patient, the first treatment received, for example ipilimumab, was assigned "episode one". The episode was closed upon termination of that particular treatment regimen. A new episode was opened when the same patient subsequently received a different type of treatment, for the duration of that treatment regimen. The clinical

result of each episode was recorded on a 5-point categorical scale defined as complete response, partial response, stable disease, progressive disease or death.

To compare the incidence of primary melanoma with the incidence of metastatic melanoma, data on the incidence of primary melanoma from 2013 to2018 were retrieved from The`Netherlands Cancer Registry (NCR), hosted by the Dutch Association of Comprehensive Cancer Centers (IKNL). The NCR is a nationwide, population-based cancer registry, which has recorded all primary diagnoses of melanoma in the Netherlands from 1989 onwards.

Statistics

Registered cases of mLMM were compared to cases of mNM and mSSM taken together, using standard descriptive statistics including, as appropriate, mean ± standard deviation (SD) and median plus 25th and 75th interquartiles (IQR) or numbers and percentages. Differences between groups were tested using the t-test, Kruskal-Wallis test (for continuous variables) or the χ^2 test (for categorical variables). Differences were tested using χ^2 tests, t-tests, Wilcoxon signed-rank test where appropriate.

To find the parameters that best distinguished mLMM from mNM and mSSM, a penalized (LASSO) logistic regression analysis was performed, using a comprehensive list of 216 clinical variables (Supplementary file 1). Missing values were imputed 5 times using multi-chain Monte Carlo methods Gibbs sampling (14). Five imputed datasets were created. A 10-fold cross validation was performed to ensure optimal penalty parameters and used all analyses for each imputed dataset (15, 16). Variables were included that were selected in all 5 imputations and were averaged over all 5 imputation sets.

Cox proportional hazards models were used to calculate the hazards for survival of mLMM and other diagnoses (mNM and mSSM), corrected for confounding factors such as age. The proportional hazards assumption of variables in the Cox proportional hazards model was checked using Grambsch and Therneau's test implemented in the cox.zph function of the "R" statistical program and none of the variables violated these assumptions (17). Survival time was calculated as the time from the start of treatment to mortality or latest date of follow-up. Treatment differences were analyzed by using an ANOVA test.

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In order to evaluate differences in genetic mutation between mLMM and the other diagnoses, we first investigated if there were any mutations known for BRAF, NRAS, KIT, GNAQ or GNA11. If there were differences in these known mutations, we compared the numbers of specific mutations according to melanoma subtype using univariate χ^2 tests. All analyses were performed using SPSS (version 22.0; IBM) and "R" version 3.5.

Results

Primary characteristics and incidence of metastatic melanoma

The DMTR registered 3,959 unique patients from 2013 to 2018. This included 59 (1.5%) patients with mLMM, 800 (20.2%) with mNM, 1,513 (38.2%) with mSSM, 77 (1.9%) with acrolentiginous melanoma, 32 (0.8%) with desmoplastic melanoma and 1,478 (37.3%) with other or unknown melanoma subtypes. All subsequent analyses were performed on the patients with mLMM and the patients with mNM or mSSM.

We compared the characteristics of mLMM patients to the combined group of patients with mNM or mSSM. Patients with mLMM were significantly older at age of diagnosis than other melanoma patients, while gender distribution was comparable. The primary tumor location of patients with mLMM was more often in the head and neck area. Satellite metastases were found more often in the mLMM group. Lactate dehydrogenase levels and number of tumor-positive sentinel lymph nodes were similar in both groups (Table 1).

The cumulative incidence of primary cutaneous melanoma in the Netherlands between 2013 and 2018 was 37,126 resulting in an average incidence of 7,425 primary cutaneous melanomas per year (18). Of all registered melanomas 1,840 (4.9%) were of LMM subtype and 35,055 (94.4%) were SSM or NM subtype (Figure 1). Looking at metastatic melanomas registered in the DMTR 3.2% (59/1,840) were mLMM, whereas 6.6% (2,313/35,055) were either mNM or mSSM.The proportion of total mLMM in the Netherlands, registered in DMTR,was 3.2% (N=59/1,840) and the proportion of mNM/mSSM was 6.6% (N=2,313/35,055). Interestingly, the proportion of LMM (3.2) that became metastatic is lower in comparison to the SSM/NM (6.6%) group. This observation that advanced mLMM patients were relatively less frequently found in the DMTR than the national incidence at diagnosis, might imply a less aggressive disease course.

Genetic analysis

DMTR patient data was analyzed to compare the mutation profiles of mLMM and mNM and mSSM. Less BRAF mutations were found in mLMM than in mNM or mSSM (32.2% versus 59.6%; p<0.001). In contrast, more KIT mutations were present in the mLMM group, as compared to the mNM and mSSM group (5.1% versus 0.65%; p=0.002). GNAQ and GNA11 mutation frequency did not differ between groups (Table 1).

In table 2 the percentages of encountered specific BRAF, KIT, GNAQ and GNA11 mutations are presented. BRAFV600E mutations were less frequently found in mLMM than in mNM and mSSM (35.0% versus 79.6%; p<0.001). Interestingly, most BRAF mutations in mLMM were BRAF V600K, while this mutation was only present in a minority of mNM/mSSM (45.0% versus 10.6%; p<0,001). No statistically significant difference was found in the distribution of NRAS mutations.

Overall survival and treatment effectiveness

As described in the method section, the DMTR registers treatment of patients with metastatic melanoma in episodes. Patients were treated with various systemic therapies including BRAF inhibitors, combined BRAF/MEK inhibitors, anti-PD1 antibodies, chemotherapy, ipilimumab, combined ipilimumab/nivolumab and/or talimogene laherparepvec (T-VEC). The 59 patients with mLMM, 800 with mNM and 1,513 with mSSM received 86, 1,377 and 2,787 treatment episodes, respectively.

In contrast to clinical trials where patients are treated according to treatment arms and protocol, physicians in clinical practice change therapeutic regimens when treatment appears to be ineffective or if complications occur. Patients with metastatic melanoma who survive the first episode, but are treated with a different agent in a consecutive episode, most likely suffer from progressive or refractory disease. In this context, a surprising finding was that patients with mLMM underwent less treatment episodes than patients with mNM/mSSM . While patients with mNM/mSSM underwent up to 10 treatment episodes , patients with mLMM underwent up to 5 episodes.

Unfortunately, due to the small size of treatment groups in mLMM (N=1 to N=11), no reliable comparison could be made with the mNM/mSSM treatment groups. In the mNM/mSSM

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treatment groups, the best results were seen with anti-PD1 antibodies followed by ipilimumab and nivolumab combination therapy (Table 3). Figure-S1 summarizes the results of every individual treatment type. The result per treatment episode (complete response, partial response, stable disease, progressive disease and death) are displayed in percentages.

The overall survival of the mLMM and mNM/mSSM groups was similar , also when adjusted for age at presentation (HR 1.22, 95% CI 0.79-1.88; p=0.37). However, regardless of treatment, patients with mLMM suffered significantly (p=0.02) less melanoma- related death (figure 2).

Discussion

In this study we observed several differences between mLMM and mNM/mSSM. Firstly, mLMM was diagnosed at a significantly higher age than mNM/mSSM and more often localized in the head and neck area.. Secondly, the proportion of primary LMM progressing to mLMM was lower than in patients with NM/SSM. Thirdly, genetic analysis showed that patients with mLMM had less BRAF mutations in general, but relatively more BRAFV600K mutations, and more KIT mutations. And lastly, the overall survival of patients with mLMM and mNM/mSSM was similar, although death in the mLMM group was significantly less often melanoma-related.

The prevalence of primary LMM in the Netherlands appears to be lower than in Sweden, Southern California USA and Spain. Epidemiological studies have shown that primary LMM represents 7-14.3% of all primary cutaneous melanomas in these countries (19-21). Possibly, the different geographical locations and differences in chronic sun exposure (outdoor activities) contribute to the higher prevalence of LMM in certain countries. Studies have shown that the incidence of melanoma was positively associated with living closer to the equator (22, 23).

Another intriguing finding is the mean age at diagnosis of mLMM in the DMTR patient group, which was 65 (±14.6) years. This is relatively young, considering a previous epidemiological study of LM and LMM in the Netherlands, which indicated an average age at primary diagnosis of 71 and 72 years respectively in 2013 (24). This suggests that the group analyzed in our study may represent a subpopulation of LMM patients with a higher progression risk than other LM patients or non-metastatic LMM patients.

Currently, no distinction is made between cases of metastatic melanoma with regard to the primary tumor. Based on recent genomic analyses, the Cancer Genome Atlas Network (CGAN) categorizes cutaneous melanoma into 4 genomic subtypes: mutant BRAF, mutant NRAS, mutant NF1 and triple wild-type (25). This classification is mainly used for clinical stratification prior to therapeutic decision-making. .

In this study we found less BRAF mutations overall in patients with mLMM, but more BRAF V600K mutations, which is concurrent with earlier studies (10). Several studies showed that Kit mutations occur in <2% of all melanomas, but are found in 25-28% of LM(M) (26-28). In this study we found KIT mutations in 5.1% of patients with mLMM. Although this is lower than the reported expression rates, it is considerably higher than the 0.6% KIT mutations we found in patients with mNM/mSSM, which is also lower than reported in literature. Genetic screening of KIT is not standard of care in the Netherlands, which may explain the low percentage of KIT mutations in this study compared to what is reported in the literature. Although LMM, SSM and NM all primarily arise from melanocytes, the different underlying driver mutations may result in different biological behavior.

Because patients with mLMM frequently carry KIT mutations, they may benefit from therapeutic approaches that target mutated KIT by cKIT tyrosine-kinase inhibitors, such as imatinib. This therapeutic target has been suggested before in 2010 by Garrido *et al.* (29). Imatinib has already been successfully used for the treatment of metastatic melanoma. A retrospective study by Wei *et al.* analyzed 78 patients, who received imatinib 400mg/day continuously. They found an overall survival of 13.1 months and progression-free survival of 4.2 months (30).

In this study we were unable to investigate possible differences in therapeutic efficacy per treatment modality between the mLMM and mNM/mSSM groups, due to the limited number of patients in the mLMM group. In the mNM/mSSM group however, overall survival rates after treatment were comparable to previous studies *(31-33)*. Overall survival of patients with mLMM and mNM/mSSM did not differ, but patients with mLMM died significantly less frequently from melanoma-related causes. It is unlikely that this was solely due to age-related death by other causes in the group with mLMM, as the difference in mean age at diagnosis of

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metastatic melanoma was only 8 years (65 years in mLMM versus 57 years in mNM/mSSM). This finding suggests that mLMM may have a less aggressive behavior than mNM and mSSM.

A strength of this study is that it is based on nationwide real-life data. However, even though DTMR records all metastatic melanomas in The Netherlands regardless of the type of primary melanoma, only 59 mLMM could be identified. Due to this small number it was not possible to relate differences in survival to different treatment regimens and compare those to mNM/mSSM. A possible confounder in this study, and inherent of a national registry, is the introduction of selection- and reporting bias. Despite the compulsory registration of all patients with metastatic disease and the efforts of DMTR to record all data, certain relevant data may have been missed in daily clinical practice.

In conclusion, our results suggest that the biological behavior of mLMM differs from mNM/mSSM. Patients with primary LMM develop metastatic disease less frequently than patients with NM/SMM. . Furthermore, mLMM carries different oncogenic mutations, with a higher frequency of BRAFV600K and KIT mutations. Lastly, despite similar survival rates, patients with mLMM suffered less melanoma-related death. Taken together, these data indicate that it may be worthwhile to discriminate mLMM from mNM or mSSM in treatment decision-making. We recommend that future research should focus on predictive markers for overall survival based on melanoma subtype and that management in patients with metastatic disease is adapted based on these melanoma subtypes.

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Table 1. Patient demographics. Univariate analysis and multivariate logistic regression between metastatic lentigo maligna melanoma (mLMM) and metastatic nodular melanoma (mNM) or metastatic superficial spreading melanoma.(mSSM)

	mLMM	mNM/mSSM	p-value	OR		multivariate p
N	59	2313				
Sex (male)	36 (62.1%)	1392 (60.2%)	0.77	1.711	(0.98-3.00)	0.06
Year of Birth	1946 (12.7)	1953 (13.5)	<0.001	0.96	(0.94-0.99)	<0.001
Mean age of diagnosis metastatic melanoma	65 (SD 14.6)	57 (SD 13.7)	<0.001			
Location primary tumor			<0.001			
Head and Neck	47 (79.7%)	315 (13.6%)				
Trunk	4 (6.8%)	1134 (49%)				
Extremities	8 (13.6%)	832 (36%)				
Acral	0 (0.0%)	32 (1.4%)				
Histological characteristics						
Ulceration	6 (10.2%)	844 (36.5%)	<0.001			
Dermal mitosis	22 (59.5%)	1359 (91.1%)	<0.001	0.01	(0.00-0.04)	<0.001
Satellites or in transits			0.31			
Satellites	5 (9.8%)	179 (8.8%)		1.55	(0.72-3.35)	0.26
In transits	1 (2.0%)	39 (1.9%)		0.57	(0.07-4.49)	0.60
Both	2 (3.9%)	22 (1.1%)		6.67	(2.02-22.00)	<0.001
Macroscopic characteristics						
Number of tumor positive SNs			0.59	0.17	(0.02-1.70)	0.13
No	5 (62.5%)	519 (54.3%)				
1	1 (12.5%)	324 (33.9%)		0.86	(0.46-1.60)	0.63
>1	2 (25.0%)	106 (11.1%)		1.13	(0.36-3.51)	0.84
Number of lymph nodes removed	5 (3.5-21.5)	4 (2.0-21.5)	0.15	0.99	(0.96-1.03)	0.85
Node metastases (macroscopic) recurrence	10 (16.9%)	599 (25.9%)	0.12	3.97	(1.78-8.89)	<0.001
LDH (units/liter)	5.78 (5.67-6.26)	5.98 (5.70-6.26)	0.14	1.13	(0.61-2.09)	0.70
Genetic mutations						
BRAF	19 (32.2%)	1378 (59.6%)	<0.001	0.39	(0.24-0.63)	<0.001
KIT	3 (5.1%)	15 (0.6%)	<0.001	3.19	(0.82-12.39)	0.09
GNAQ	0 (0.0%)	8 (0.3%)	0.65	3.23	(0.34-30.84)	0.31
GNA11	0 (0.0%)	8 (0.3%)	0.65			

OR = odd's ratio, The odds indicate variables predictive of indicating mLMM CI = confidence interval. SN, sentinel node;

Table 2. Frequency of oncogenic mutations (percentages).

BRAF	mLMM	mNM/mSSM	p
c.1799T.A.p.Val600Glu. (V600E)	35.00	79.60	**<0.001**
c.1798_1799delinsAA.p.Val600Lys (V600K)	45.00	10.62	**<0.001**
c.1798_1799delinsAG.p.Val600Arg. (V600R)	0.00	1.77	1.00
c.1799_1800delinsAT.p.Val600Asp. (V600D)	0.00	0.28	1.00
c.1799_1800delinsAA.p.Val600Glu (E2.variant)	0.00	1.20	1.00
c.1781A.G.p.Asp594Gly.	0.00	0.07	1.00
c.1794_1796dup.p.Thr599dup.	0.00	0.07	1.00
c.1795_1797dup.p.Thr599dup.	0.00	0.07	1.00
c.1799_1802delinsAAAT.p. (Val600_Lys601delinsGluIle)	0.00	0.35	1.00
Other	20	4.89	0.01
NRAS			
c.181C.A.p.Gln61Lys.	0.00	33.10	0.08
c.182A.G.p.Gln61Arg.	33.33	41.83	0.86
c.182A.T.p.Gln61Leu.	33.33	9.40	0.07
c.183A.T.p.Gln61His.	11.11	2.91	0.66
c.180_181delinsTA.p.Gln61Lys.	11.11	0.67	0.13
c.34G.T.p.Gly12Cys.	0.00	0.45	1.00
c.35G.A.p.Gly12Asp.	0.00	0.22	1.00

The NRAS mutations c.35G.C.p.Gly12Ala.; c.37G.T.p.Gly13Cys; c.37G.A.p.Gly13Ser.; c.38G.A.p.Gly13Asp.; c.44G.A.p.Gly15Glu. were not found. P: Chi-square

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Table 3: Survival based on treatment of the first episode.

Treatment given in first episode	First treatment episode Median PFS	First treatment episode Median DFS	OS rate after all treatment	Median OS after all treatment
mLMM				
BRAF-inhibitor (N=3)	3.1 (2.4-4.7)	3.7 (3.5-4.5)	66.7%	9.6 (7.5-11.6)
BRAF + MEK (N=5)	5.5 (4.4-5.9)	3.2 (3.0-4.2)	25.0%	11.6 (11.6-11.6)
Anti PD1 (N=11)	5.8 (5.2-8.0)	3.5 (2.9-3.9)	45.5%	25.3 (4.6-29.4)
Ipilimumab (N=11)	4.7 (3.7-12.6)	3.9 (3.2-4.1)	18.2%	23.3 (15.3-30.1)
Ipilimumab+ Nivolumab (N=1)	9.8	3.1	100.0%	30.2
Chemotherapy (N=1)	3.0	3.0	0%	-
mNM/mSSM				
BRAF (N=536)	4.9 (3.0-7.8)	3.3 (2.7-4.0)	31.5%	11.0 (6.0-21.2)
BRAF + MEK (N=262)	5.8 (3.8-9.5)	4.3 (2.7-3.9)	47.0%	11.8 (6.5-19.5)
Anti-PD1 (N=378)	6.9 (3.9-14.7)	3.7 (3.2-4.2)	52.6%	16.1 (9.4-25.0)
Ipilimumab (N=303)	4.6 (3.3-8.5)	3.6 (3.0-4.2)	43.9%	12.5 (7.0-29.7)
Ipilimumab+Nivolumab (N=54)	5.8 (3.2-12.0)	3.6 (2.8-4.2)	48.1%	15.8 (9.8-27.8)
Chemotherapy (N=93)	3.2 (2.4-4.7)	3.1 (2.3-3.8)	36.6%	8.7 (5.4-18.5)

Median overall (OS), disease free (DFS) and progression free (PFS) survival durations are displayed as months (IQR).

Figure 1. Incidence of primary lentigo maligna melanoma (LMM), nodular melanoma (NM), superficial spreading melanoma (SSM) and metastatic LMM, NM/SSM.

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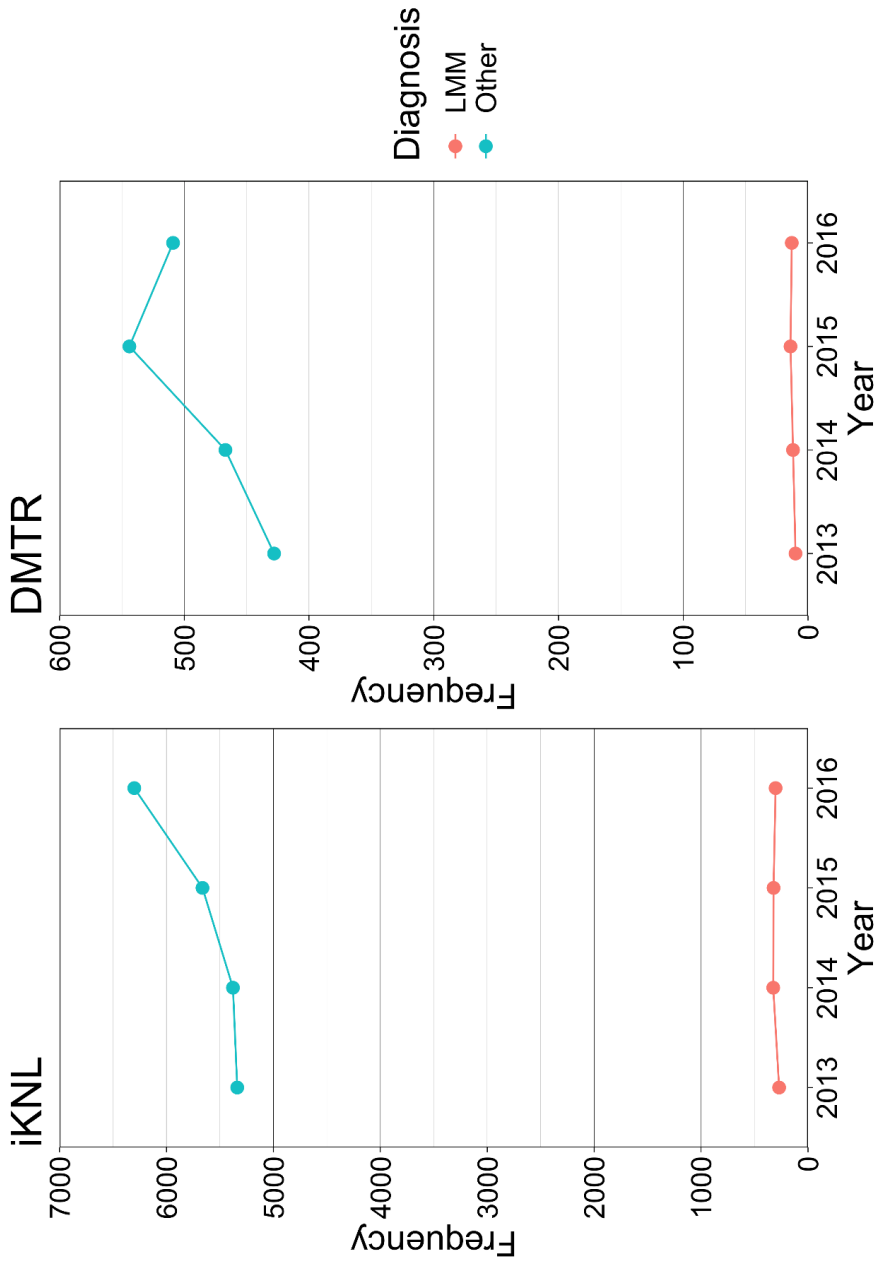
Figure 2. A: Overall survival corrected for age at presentation. C: Causes of death. Metastatic lentigo maligna melanoma versus metastatic nodular melanoma and metastatic superficial spreading melanoma
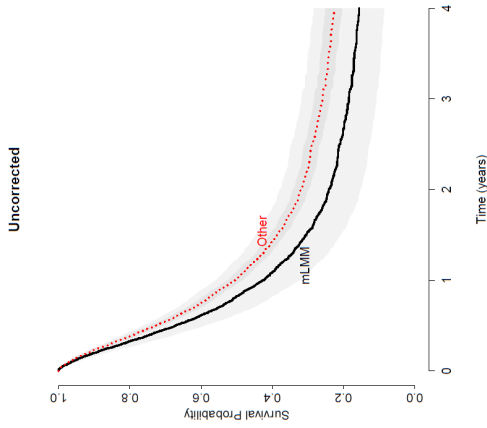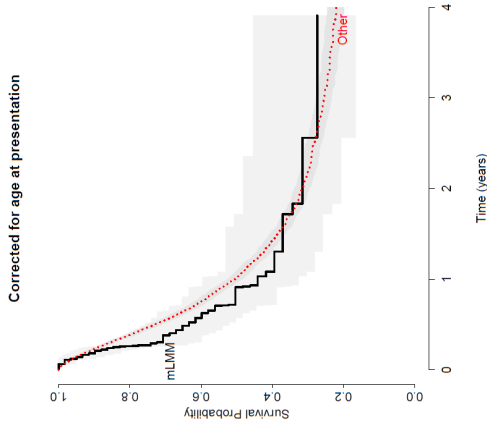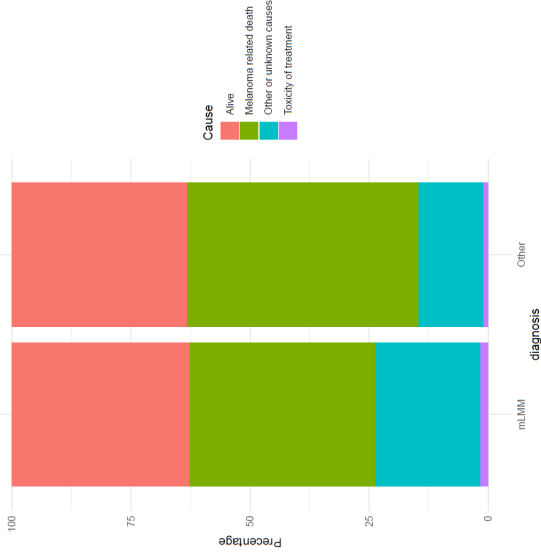
Supplementary Table S1: Clinical variable list of DMTR data set

Variable

Gender

Year of birth

Limited or expansive registration

Date first registration

Tumor expansion

(Sub)cutaneous

Date of primary tumor diagnosis

Location primary tumor

Subtype melanoma

Breslow Thickness

Histological ulceration

Histological dermal mitosis

Histological Sattelite metastasis

Lymph node metastasis

Distant metastasis

Subcutaneous metastasasis

Nodal metastasis

Pulmonary metastasis

Hepatologic metastasis

Brain metastasis

Gastro-intestinal metastasis

Bone metastasis

Other metastasis

Did the patient receive treatment for the primary tumor

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Primary traetment

Local treatment for primary tumor

Treatment in-transit metastasis

Positive sentinel nodes

Lymph node dissection

Number of removed lymph nodes

Number of positive lymph nodues

Immunotherapy

Vemurafenib

Dabrafenib

Ipilimumab

Dabrafenib and Rametinib

Vermurafenib en Cobimetinib

Nivolumab

Pembrolizumab

Ipilimumab plus Nivolumab

Number of recurrence

Date first recurrence

Local recurrence

In transit metastasis

Lymph node station

Distant metastasis

Systemic

Systemic therapy

Sub-cutaneous metastasis

Nodal metastasis

Pulmonary metastasis

Hepatologic metastasis

Brain metastasis

Gastro-intestinal metastasis

Bone metastasis

Other metastasis

Systemische therapie

Immunotherapie

Vemurafenib

Dabrafenib

Ipilimumab

Dabrafenib en trametinib

Vermurafenib en cobimetinib

Nivolumab

Pembrolizumab

Ipilimumab plus nivolumab

Treatment episode number

Date of diagnosis

Date of first presentation at clinic

Are there proven metastsis

Histological classification primary tumor

Revision of histological classification of primary tumor

Histology and/or cytological specimen of primary tumour present?

BRAF mutation?

NRAS mutation?

KIT mutation?

GNAQ mutation?

GNA11 mutation?

Other mutations?

Sanger sequencing used?

Next generation sequencing used?

Sequenom analysis used?

Real-time PCR used?

Cobas-BRAF test used?

Is there a BRAF mutation?

c.1799T>A (p.(Val600Glu))

c.1798_1799delinsAA (p.(Val600Lys))

c.1798_1799delinsAG (p.(Val600Arg))

c.1799_1800delinsAT (p.(Val600Asp))

c.1799_1800delinsAA (p.(Val600Glu)), (=E2 variant)

c.1781A>G (p.(Asp594Gly)

c.1794_1796dup (p.(Thr599dup))

c.1795_1797dup (p.(Thr599dup))

c.1799_1802delinsAAAT (p.(Val600_Lys601delinsGluIle))

Other

Is there a NRAS mutation?

c.181C>A (p.(Gln61Lys))

c.182A>G (p.(Gln61Arg))

c.182A>T (p.(Gln61Leu))

c.183A>T (p.(Gln61His))

c.180_181delinsTA (p.(Gln61Lys))

c.34G>T (p.(Gly12Cys))

c.35G>A (p.(Gly12Asp))

c.35G>C (p.(Gly12Ala))

c.37G>T (p.(Gly13Cys)

c.37G>A (p.(Gly13Ser))

c.38G>A (p.(Gly13Asp))

c.44G>A (p.(Gly15Glu))

Other

Is there a KIT mutation?

c.1671G>C (p.(Trp557Cys))

c.1672A>G (p.(Lys558Glu))

c.1676T>A (p.(Val559Asp))

c.1679T>A (p.(Val560Asp))

c.1727T>C (p.(Leu576Pro))

c.1922T>A (p.(Leu641His))

c.1924A>G (p.Lys642Glu))

c.2591C>T (p.(Ser864Phe))

Other

Is there a GNAQ mutation?

c.626A>T (p.(Gln209Leu))

c.548G>A (p.(Arg183Gln))

Other

Is therea GNA11 mutation?

c.626A>T (p.(Gln209Leu))

c.547C>T (p.(Arg183Gln))

Other

Type of systemic treatment given

Date start of BRAF inhibitor

Name BRAF inhibitor

Dosage of BRAF inhibitor

Stop date BRAF inhibitor

Date start of MEK inhibitor

Name MEK inhibitor

Dosage of MEK inhibitor

Stop date MEK inhibitor

Date start of ipilimumab

Dosage of ipilimumab

Number of ipilimumab treatments

Was a PD-L1 test performed?

Start PD1-antibodies

Name PD1-antibodies

Dosage PD1-antibodies

Number of PD1-antibodies treatments

treatment episode

Result treatment episode

Cause of death treatment episode

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Supplementary Figure S1: Results of individual treatment. All treatment is reported in episodes. An episode is defined as the time period of treatment with a single modality such as a BRAF inhibitor. If a second treatment is given to a patient this is reported as the second episode. All response is classified on a 5-class scale of "complete response, partial response, stable disease, progressive disease and death". Data is displayed in percentages.
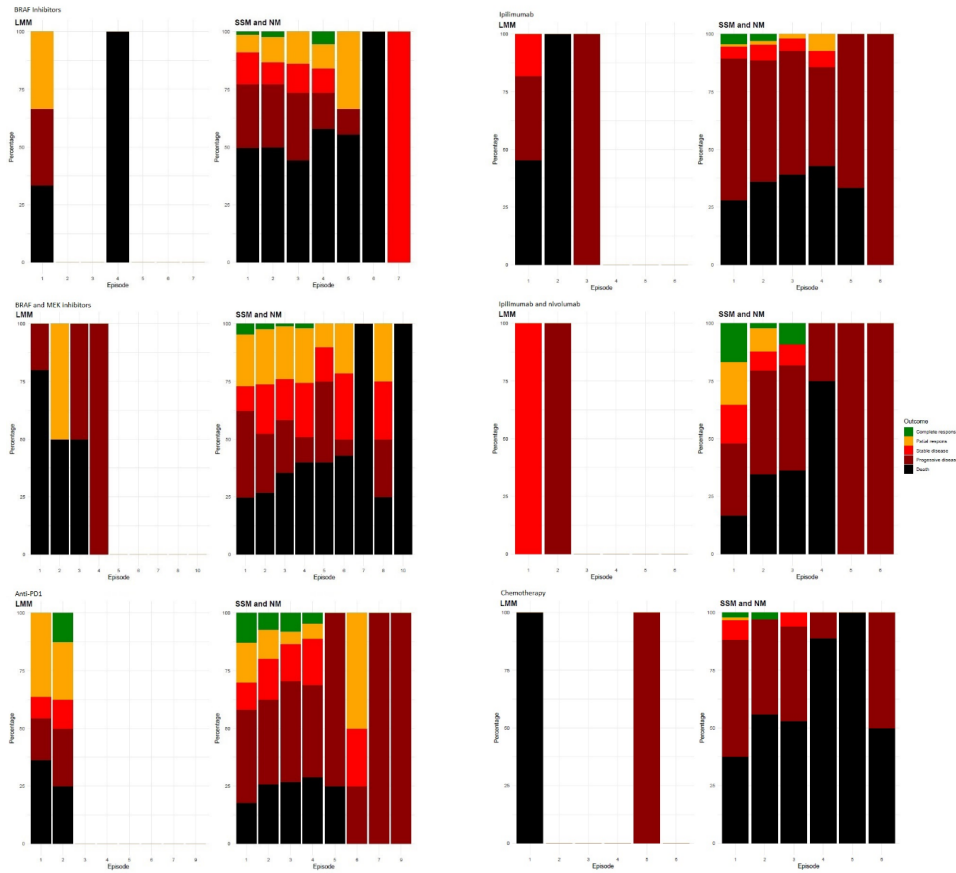
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General discussion

General discussion

Currently there are several questions regarding management of Lentigo Maligna (LM). The first question is: "How do we properly diagnose LM?". A second question is "How should we treat LM?". While it is possible to diagnose and treat LM the third question that remains is "Should we treat LM?".

Histopathological diagnosis of LM or LMM is difficult. The diagnosis of LM is based on the presence a lentiginous proliferation of atypical melanocytes along the basal layer (1). The first problem that pathologists face on examination of a biopsy or excision of LM is the differentiation between normal melanocytes and atypical melanocytes of LM. In early LM lesions it is especially difficult to differentiate melanocytic hyperplasia in chronically sun-exposed skin from atypical melanocytes representing LM using standard Haematoxylin and eosin (H&E) sections. A very sensitive immunohistochemical marker is S-100 protein (2). It has a relatively low specificity, dermal dendritic cells are invariably positive for S-100, making specific identification of individual melanocytes in the dermis difficult (2). Another marker that can be used to identify melanocytes is Melan-A/MART1 (3). Melan-A is a component of the premelanosomal membrane. Therefore, Melan-A is a marker for melanosomes and not exclusive for melanomas (4, 5). It can also be present in perivascular epithelioid cell tumours. Otherwise it has a high specificity for melanocytes (6).

A second problem is the differentiation between LM and LMM. In many biopsies, dermal cells staining positive for Melan-A are found. These cells represents a conundrum, because they may represent LMM, a superficially invasive melanoma, dermal naevi or non-specific staining of dermal melanophages (7). Consequently, using the current routine markers it is difficult to differentiate normal melanocytes from atypical melanocytes for the diagnosis of LM in early lesions. And it if there are Melan-A positive dermal cells present in a biopsy from a lesion clinically suspect for LM it could be misinterpreted as LMM and vice versa.

To solve these problems, we sought to find new markers to help in the histopathological diagnosis of LM. The Cancer/testis antigen (CTA) family, is a group of antigens that is solely expressed in various malignancies and in germ cells of the testis (8-10). Currently over 100 CTA families have been identified. One of the most studied CTA is the melanoma-associated antigen gene (MAGE) family (10-12). The expression of the various CTA is well evaluated in many

malignancies, such as lung cancer, breast cancer, ovarian cancer, colon cancer, multiple myeloma and cutaneous melanoma (13-17). It has been shown that the prevalence of some CTA is often higher in more advanced malignancies and has been correlated with a poorer prognosis (18, 19). The prevalence of CTA on LM and LMM has only been reported in a single study by Brasseur et al. in which 4 samples of LM were examined for the expression of MAGE-A1, -A2, -A3 and -A4. No expression was found in these samples (20).The exact function of CTA is largely unknown. So far it has been shown that MAGE possess a variety of cellular functions, such as complex formation with E3 RING ubiquitin ligases, involvement in substrate recognition, cellular localization and cell proliferation (10). In malignancies, several MAGE are known oncogenic drivers and play a role in malignant cell survival, tumour formation and metastasis (21). As mentioned above, the function of many CTA is unknown. MAGE-A specifically plays a role in tumour promotion. P53 is a tumour suppressor gene which is a target for genetic alternations in cancer (22). MAGE-A proteins interact with p53 proteins and may block the association of p53 with its cognate sites in chromatin, thus impairing the function of p53 (23). PRAME is a dominant repressor of the retinoic acid signalling pathway, thereby inhibiting retinoic acid-induced differentiation, cell cycle arrest and apoptosis (24). It has also been shown to induce cell proliferation, inhibit apoptosis and reduce cytotoxic drug sensitivity (25-28). SSX protein has been shown to contain repressor domains which repress DNA transcription (29). It has been implicated that it plays a role in the regulation of cell differentiation (30). Hypothetically, SSX represses normal cell differentiation. The exact function of NY-ESO-1 is still unknown. It is believed that it is involved in cell cycle regulation progression, growth and apoptosis (31).

We investigated the prevalence of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A10, MAGE-A12, PRAME, NY-ESO-1 and SSX2 on LM and LMM (Chapter 6 and 7). The results of these studies show that MAGE-A and PRAME are useful for the diagnosis of LM. We found the presence of MAGE-A on LMM but not on LM. This implies that if a lesion suspect for LM expresses MAGE, it could indicate that the LM is in actuality LMM. Additionally, PRAME can potentially be used to distinguish atypical, but benign melanocytes from malignant melanocytes of LM. Staining with MAGE-A and PRAME antibodies should be considered if there is doubt about the histopathological diagnosis of lesions clinically suspect for LM, in addition to the currently used MELAN-A/MART1 stain. With these potential new markers, lesions can be

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stratified and treated accordingly. However, it is imperative that further studies investigate the sensitivity and specificity of these markers.

The current paradigm states that LM is considered a melanoma in situ and it is treated to prevent progression to lentigo maligna melanoma (LMM). Consequently the goal of treatment is complete removal of LM which is usually achieved by surgical excision. Alternative options are topical imiquimod, radiotherapy and watchful waiting. When choosing and option clinicians are confronted by the question: "How should we treat LM?". It is difficult to determine which of these options is the best, regardless of the surgical or non-surgical character. One reason why this is difficult is because globally there is no proper management algorithm. In the newest European consensus guideline (2016) and the newest American Association of Dermatology (AAD) guideline on the management of primary cutaneous melanoma (2019) for diagnosis it is advised to perform an excisional biopsy, or if that is not possible an incision biopsy of lesions for histopathological analysis (32, 33). If a lesion is diagnosed as LM, surgical treatment is recommended. Preferably staged excision or excision with margin control. As alternatives, topical imiquimod and radiotherapy are mentioned, but there is no recommendation on when to use surgical or non-surgical options. A second reasons why it is difficult to determine the best treatment option is because there is a paucity of knowledge. Currently there are no randomized controlled trials which compare surgery, topical imiquimod and radiotherapy. Globally there is a only a single randomized controlled trial in progress comparing radiotherapy and topical imiquimod for patients who cannot undergo surgical excision (RADICAL trial: NCT02394132).

Evidence on non-surgical treatment using topical imiquimod is limited but there are studies reporting varying effectiveness. The LIMIT-1 study by Marsden et al. studied the effect of imiquimod for LM using a treatment schedule of 5 applications during 12 weeks (total 60 applications). Out of the 27 treated patients, 10 (37%; 95% CI 19-58%) showed a pathological complete remission (34). A study by Kai et al. reported on 40 LM patients treated with topical imiquimod 3 times per week during a period of 6 weeks. After treatment the LM were excised, 11/40 patients (27.5%) had residual LM. Total histological clearance was found in 29/40 patients (72.5%). They found no recurrence after a 5 year follow-up (35). In comparison, our systematic review including 471 patients found a response rate of 78.3% and a recurrence rate of 2.2.%. We also showed that <60 applications in total has a 8 times higher odds of achieving

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complete clearance. In total 9 patients (1.8%) showed progression to LMM (**Chapter 3**). In our own prospective trial including 57 patients we found a success rate of 84.2% and a recurrence rate of 10.5%. A single patient (1.8%) showed progression to LMM (**Chapter 4A**). In comparison, conventional excision of LM with a 5 mm margin has recurrences rates varying between 6.8-30% (36, 37). Staged excision techniques such as Mohs micrographical surgery or " Slow Mohs" have a lower reported recurrence rate of 0-5.9% (38-40). One of the questions critics of topical treatment always ask is whether topical imiquimod is sufficient to treat LM in order to decrease the risk of death due to LMM. Currently there are no reported LMM related deaths after treatment with imiquimod. There is a single study on the long term survival of LM patients after surgical excision. This study by Gambichler et al. included 270 patients of which 124 had a LM and 146 LMM. In the LM group a 5-year local recurrence rate of 3% (N=4/124 patients) was found and in the LMM group 3% (N=5/146 patients). They reported a single LMM related death (N=1/146 patients; 0.7%) (41). The lack of evidence shows that very little is known about the actual mortality rates of LMM patients in general. The prospective studies and systematic review together show that topical imiquimod 5% is a serious alternative option to surgical therapy. This option should definitely be considered and discussed with patients who do not want surgical treatment or cannot undergo surgical treatment.

The term "Lentigo maligna" suggests something that should be removed immediately. In reality, LM shows behavior comparable to a pre-malignant lesion like actinic keratosis and in daily practice actinic keratosis is not always treated, even though it can progress to squamous cell carcinoma. An epidemiological study from 2016 describing LM the Netherlands showed that LM progresses slowly at a 2.0-2.6 cumulative progression rate over the course of 25 years (42). A more recent study by Menzies et al. showed a risk of progression of LM to LMM of 3.5% per year. Which equates to an average time to progression of 28.3 years (43). Taking into account that the average age of diagnosis of LM and LMM are 73 and 72 years of age respectively (42), it does seem unlikely that large groups of LM patients have the time to progress to LMM. In our study on Dutch Melanoma Treatment Registry study (Chapter 8) we found that patients with metastatic LMM have an average age of diagnosis of 65.3 years. These patients progressed from LM to LMM and metastatic LMM comparatively fast. This would suggest that there is a sub-group among LM patients who have a higher chance of progression. To determine whether a LM patient should be treated or not it is essential to determine

whether patients belong to this group of "high risk" LM patients. These "high risk" LM patients can be treated more aggressively using surgical techniques. If patients do not belong to this group alternatives such as topical imiquimod, radiotherapy or watchful waiting can be discussed alongside surgical excision. It is important that future studies aim to identify markers for "high risk" LM. Perhaps in the future we can discern "low-risk" LM and "high risk" or LM, subsequently these patients can be treated accordingly.

Based on current knowledge on LM we believe that LM should be treated, because currently it is not possible to identify the subgroup of "high risk" LM. However, we do not believe it should be treated as aggressively as is suggested in current guidelines. A putative treatment algorithm that can be used is that if patients are younger, surgical excision for lesions <1.0 cm diameter can be considered. If a patient is older (>75 years), the LM is larger or patients do not want surgical excision, treatment with topical imiquimod or radiotherapy should be considered. Depending on the overall condition and the age of the patient watchful waiting is an option.

Conclusions

Management of LM remains difficult. Diagnosis of LM can be difficult but the usage of MAGE-A and PRAME can aid the process. The Presence of MAGE-A on a lesion clinically suspect for LM can indicate that it is in actuality LMM. Furthermore PRAME can be used to differentiate atypical melanocytes from malignant melanocytes of LM. The paradigm states that LM is a melanoma in situ and should be treated to prevent progression to LMM. Whether the best treatment should be surgical or non-surgical is unknown because there are no trials comparing the various options. Studies do show that topical imiquimod is a good option for patients who cannot undergo or do not want surgical excision. Based on current knowledge on LM we believe that LM should be treated, but not as aggressively as is suggested in current guidelines. If in the future stratification of "low risk" and "high risk" LM is possible, patients should be treated according to their risk profile.
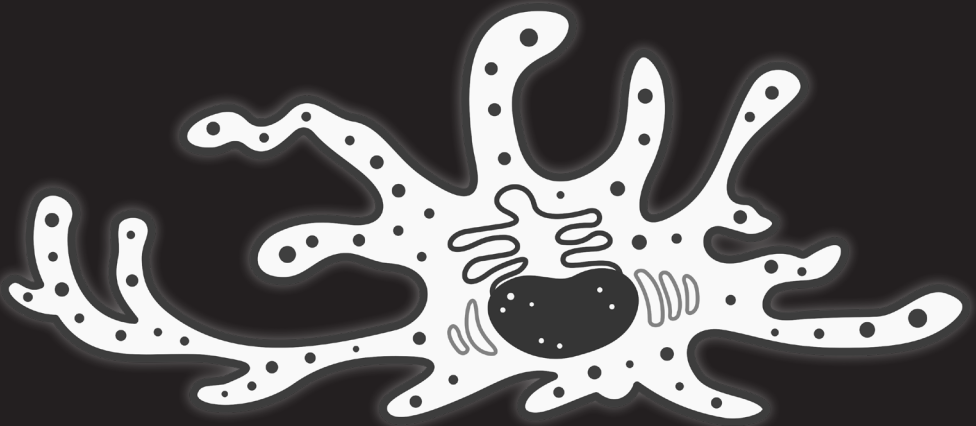
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General Summary
Nederlandse samenvatting
Dankwoord
PhD Portfolio
Curriculum Vitae
List of publications

General Summary

Lentigo maligna (LM) is considered a melanoma in situ, predominantly found in the head and neck area of elderly patients. It is treated to prevent progression to lentigo maligna melanoma (LMM), which can potentially metastasize. This thesis covers several topics on the management of LM. The first part describes current management tactics among clinicians across Europe and the treatment of LM using topical imiquimod. In the second part a difficulty in the diagnostic process is discussed and a potential solution to this problem. In the last part a register study on metastatic LMM is discussed.

In **chapter 2** we performed a survey among dermatologists who are members of the European Association of Dermatology and Venereology. The purpose of this survey was to assess how LM is managed in daily practice. We found that LM is often biopsied with a 3 mm punch biopsy for primary histopathological diagnosis (N=258/415 respondents; 61%). This is in contrast with current guidelines, which advise excisional biopsies. Another discrepancy which we found is that treatment of LM is usually surgical, but only for patients <60 years of age (357/376 respondents; 94.9%). However, for older patients who are >70 years of age, non-surgical treatment becomes more frequent. Many respondents use topical imiquimod (N=115/376 respondents; 30.6%) or radiotherapy (N=64/376 respondents; 17.0%) instead of surgery. These data show that that non-surgical treatment is used more often if the patient is older.

In **chapter 3** we systematically reviewed the effectiveness of topical imiquimod treatment for LM. In this study we included 26 case-reports, 11 retrospective studies, 3 prospective studies and 1 randomized controlled trial including 471 patients. Complete clinical clearance was found in 369/471 (78.3%) of the cases. In this same study we found that a more intense treatment regimens with >60 applications in total has a 6.47 greater odds (p=0.017) of achieving clinical clearance and a 8.85 greater odds (p=0.003) of histological clearance. After a mean follow-up of 18.6 months (range 9-37 months) a recurrence rate of 2.2% (11/471 patients) was found in this group. Progression to lentigo maligna melanoma was seen in 1.8% (9/471 patients). In **chapter 4A** we assessed the effectiveness of topical imiquimod in a prospective trial. A total of 57 patients were treated with topical imiquimod. The patients applied imiquimod once daily during a period of 12 weeks. A complete clinical clearance was found in 84.2% of the patients with a recurrence rate of 10.5% (6/57 patients) after a mean follow-up of 22.5 months. A single

case showed progression to LMM (1/57 patients; 1.8%). In chapter 4B a case series is described of patients who were treated with topical imiquimod for LM. These patients all had LM on the cheek and after treatment they developed persistent lymphedema. This was a novel adverse advent not previously described in the literature.

As stated before, in chapter 2 we found that LM is often diagnosed based on a 3 mm punch biopsy. In chapter 5 we studied whether the diagnosis on initial punch biopsy is the same as the diagnosis after excision of an LM. This study included 255 patients with a lesion diagnosed as LM on the initial biopsy, which were subsequently treated surgically. Of these patients, the diagnosis of 232/255 patients (91%) remained LM after excision. However, in 23/255 patients (9%) the final diagnosis was LMM. This was an indication that the initial biopsy represented a sampling error. However, one of the questions that remains, is whether this has clinical consequences. We did not assess the long-term follow-up of these patients.

On histopathological examination it is difficult to distinguish atypical melanocytes from malignant melanocytes of LM. In Chapter 6 we performed a systematic review on the prevalence of Cancer Testis Antigen (CTA) in cutaneous melanoma. In this chapter we included a total of 65 articles describing 48 different CTA found in cutaneous melanoma (Superficial spreading melanoma or nodular melanoma). We found that cutaneous melanoma shows high rates of expression of several CTA. The CTA with high expression rates were MAGE-A3, MAGE-A2, MAGE-A1, MAGE-A4, MAGE-A6, Preferentially Antigen expressed in Melanoma (PRAME), NY-ESO-1, and SSX2. The expression of CTA in general are higher in metastatic melanoma, compared to primary melanoma.

In Chapter 7 we studied the prevalence of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, PRAME, NY-ESO-1 and SSX2 to determine whether they can be used to aid in the diagnosis of LM. We examined 7 samples of normal skin, 7 of chronically sun-exposed skin, 6 of LM and 20 of LMM. We used specific antibodies for the different targets mentioned above and a cross-reactive MAGE-A antibody. The cross-reactive MAGE-A antibody detects expression of MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12. In our samples we found positive expression of MAGE-A in 4 LMM samples, and expression of MAGE-A1, MAGE-A2, MAGE-A3 in 3 different LMM samples. We also found expression of PRAME in 18/20 (90%) LMM samples and 6/8 (75%) LM samples. With regard to the problem of misdiagnosis on initial biopsy, immunostaining for

MAGE-A may be useful to detect LMM. Furthermore PRAME can potentially be used as a marker to differentiate normal melanocytes from LM and LMM.

In Chapter 8 we studied the epidemiological, clinical, histopathological and genetic characteristics of patients with metastatic LMM (mLMM), metastatic nodular melanoma (mNM) and metastatic superficial spreading melanoma (mSSM). Since July 2013 all patients with metastatic melanoma are prospectively registered in the Dutch Melanoma Treatment Registry (DMTR) database. From this database we extracted information on 3959 unique patients, including 59 with LMM, 800 with nodular melanoma and 1513 with superficial spreading melanoma. After analysis of the DMTR data, we found that mLMM was diagnosed at a significantly higher age than mNM/ mSSM, and was more often localized in the head and neck area. mLMM showed less dermal mitosis but more satellite metastasis. Genetic analysis showed that in patients with mLMM the tumors carried less BRAF mutations in general (35.0% versus 79.6%; p<0,001%), but relatively more BRAFV600**K** mutations (45.0% versus 10.6%; p<0,001), and more KIT mutations (5.10% versus 0.65%; p=0,002). From the Dutch Intergrated Cancer Institute (iKNL) we extracted data on the incidence of cutaneous melanoma between 2013-2018. The proportions of LMM and NM/SSM that metastasized were compared. We found that a less patients with primary LMM developed metastases (59/1,840; 3.2%) in comparison to patients with primary NM/SSM (2,313/35,055; 6.6%).

The efficacy of treatment could not be compared between mLMM and mSSM/mNM due to small numbers of mLMM. In the DMTR treatment is registered in episodes. For every new treatment regimen a new episode is opened. In the mSSM/mNM group the best treatment results were obtained with the combination of ipilimumab/nivolumab followed by treatment with anti-PD1 antibodies. The overall survival between mLMM and mSSM/mNM groups was similar. In conclusion patients , mLMM patients do not seem to have a worse overall survival and death was significantly less often melanoma-related even though mLMM patients are older, suffer from more comorbidities and receive less treatment episodes,

In Chapter 9 current problems concerning management of LM are discussed. We conclude that there is a paucity of evidence on the question which treatment option for LM is the best, as there are no published randomized controlled trials. Diagnosis of LM can be aided by the usage of the cancer testis antigen MAGE-A and PRAME. Expression of MAGE-A in a lesion clinically

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and histologically suspect for LM may be an indication that the lesion actually represents LMM. Immunostaining for PRAME can be helpful to discern atypical melanocytes from malignant melanocytes of LM. Lastly, we discuss that data from a national registry of metastatic melanoma including metastatic LMM indicates that there is a "high risk" group of LM patients with a higher progression risk.

Nederlandse samenvatting

Lentigo maligna (LM) wordt beschouwd als een melanoom in situ. Meestal komt het voor in het hoofd/hals gebied van oudere patiënten. De behandeling van LM is erop gericht om progressie naar een lentigo maligna melanoom (LMM) te voorkomen. Een LMM kan metastaseren en dit risico wordt ingeperkt door behandeling van de precursor afwijking (het LM). In het eerste deel van deze thesis wordt besproken hoe Europese dermatologen op dit moment LM behandelen. Tevens wordt ingegaan op het gebruik van imiquimod crème en de effectiviteit hiervan. Het tweede deel van de thesis omvat diagnostische problemen en een mogelijke oplossing hiervoor. In het laatste deel van dit proefschrift wordt een register studie over gemetastaseerd LMM beschreven.

In **hoofdstuk 2** beschrijven we de resultaten van een vragenlijst die we hebben uitgezet onder dermatologen die lid zijn van de Europese Associatie voor Dermatologie en Venereologie. Het doel van deze vragenlijst was om te onderzoeken hoe LM in de dagelijkse praktijk wordt behandeld. Uit de antwoorden bleek dat LM meestal wordt gediagnosticeerd met behulp van een 3 mm stansbiopt (N=258/415 respondenten; 61%). Dit is niet in overeenstemming met de huidige Nederlandse richtlijn melanoom, die een primair excisiebiopt adviseren. In de Nederlandse en internationale richtlijnen wordt geadviseerd dat LM chirurgisch behandeld wordt ongeacht de leeftijd. We vonden dat er voor behandeling van LM vaker wordt gekozen voor chirurgisch behandeling bij patiënten <60 jaar oud (357/376 respondenten; 94.9%). Echter, als patiënten >70 jaar oud zijn dan wordt niet chirurgische behandeling vaker toegepast. De meest toegepaste niet-chirurgische behandeling is imiquimod crème (N=115/376 respondenten; 30.6%) en daarna radiotherapie (N=64/376 respondenten; 17.0%). Concluderend blijkt uit de enquête dat bij oudere patiënten (>70jr) vaker wordt gekozen voor niet-chirurgische behandeling.

In **hoofdstuk 3** hebben we een systematische wijze de effectiviteit van behandeling van LM met imiquimod crème bestudeerd. In deze studie hebben we 26 *case reports*, 11 retrospectieve studies, 3 prospectieve studies en 1 *randomized controlled trial* geïncludeerd, die samen 471 patiënten beschreven. Na behandeling met imiquimod crème werd in 369/471 (78.3%) patiënten een complete klinische respons gezien. Een andere bevinding uit deze review was dat een behandelregime met >60 applicaties in totaal een 6.47 (p=0.017) keer grotere kans op

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complete klinische respons en een 8.85 (p=0.003) keer grotere kans op een volledige histologische respons gaf. Na een gemiddelde follow-up van 18.6 maanden (bereik 9-37 maanden) werd een recidief gezien in 2.2% van de patiënten (11/471 patiënten). **Hoofdstuk 4A** beschrijft een prospectieve studie over de effectiviteit van lokale behandeling van LM met imiquimod crème. In totaal zijn 57 patiënten gedurende 12 weken eenmaal per dag behandeld met imiquimod crème. Een complete klinische respons werd gezien bij 84.2% (48/57 patiënten) waarbij er bij 10.5% (6/57) een recidief werd gezien na gemiddeld 22.5 maanden follow-up. Een enkele patiënt toonde hierbij progressie van LM naar LMM (1/57 patiënten; 1.8%). In **hoofdstuk 4B** wordt een *case-series* beschreven. Drie LM patiënten ontwikkelden persisterend lymfoedeem na behandeling met imiquimod crème. Deze bijwerking was nog niet eerder beschreven.

Zoals beschreven in **hoofdstuk 2** wordt LM meestal gediagnosticeerd op basis van een 3 mm stansbiopt. In **hoofdstuk 5** hebben we gekeken in hoeverre de diagnose LM na een primair stansbiopt ongewijzigd blijft na excisie van de gehele afwijking. In deze studie zijn 255 patiënten geïncludeerd waarbij de LM, zoals gediagnosticeerd in het initiële biopt, aansluitend is geëxcideerd. Bij het gros van deze patiënten (232/255 patiënten; 91%) bleef de diagnose LM ongewijzigd. Echter, in een klein deel (23/255 patiënten; 9%) bleek sprake van LMM. Dit suggereert dat er bij het initiële biopt sprake was van een *Sampling error.* Over de (lange termijn) klinische consequenties hiervan kunnen wij geen uitspraak doen, omdat deze niet waren opgenomen in de studie.

Histopathologisch onderzoek van LM is niet eenvoudig. Het onderscheiden van atypische melanocyten en melanocyten die duiden op LM blijft een lastig probleem. De zogeheten *Cancer Testis Antigens* (CTA) zijn antigenen die exclusief voorkomen gezonde testis en op verscheidene maligniteiten, waaronder melanoom. In **hoofdstuk 6** hebben we systematisch gekeken naar de prevalentie van de CTA op cutaan melanoom. In deze studie hebben we 65 artikelen geïncludeerd die expressie beschrijven van 48 verschillende CTA op cutaan melanoom (superficieel spreidend melanoom en nodulair melanoom). Uit de analyse bleek dat cutaan melanoom hoge expressie heeft van de volgende CTA: *Melanoma associated antigen-*A3 (MAGE), MAGE-A2, MAGE-A1, MAGE-A4, MAGE-A6, *Preferentially Antigen expressed in Melanoma* (PRAME), NY-ESO-1 en SSX2. De expressie van CTA is tevens hoger in gemetastaseerd melanoom in vergelijking met primair melanoom.

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In **hoofdstuk 7** hebben we de prevalentie onderzocht van MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, PRAME, NY-ESO-1 en SSX2 in LM en LMM om te evalueren of deze CTA gebruikt kunnen worden voor de diagnostiek van LM en LMM. Er zijn 7 monsters van normale huid, 7 van zon-beschenen huid, 6 van LM en 20 van LMM bekeken met behulp van specifieke antilichamen voor de verschillende antigenen en een MAGE-A kruis-reactief antilichaam. Hierbij vonden wij expressie van MAGE-A in 4 LMM en van MAGE-A1, MAGE-A2 en MAGE-A3 in 3 andere LMM monsters. Daarnaast werd PRAME gezien in 18/20 (90%) LMM en 6/8 (75%) LM. Onze conclusie is dat door bepalen van expressie van MAGE in het initiële biopt de kans op het missen van LMM kleiner is. Daarnaast kan PRAME gebruikt worden om een onderscheid te maken tussen normale melanocyten en maligne melanocyten van LM en LMM.

In **hoofdstuk 8** hebben we de epidemiologische, histopathologische, klinische en genetische karakteristieken van gemetastaseerd LMM (mLMM), gemetastaseerd nodulair melanoom (mNM) en gemetastaseerd superficieel spreidend melanoom (mSSM) bestudeerd. Het doel was om de karakteristieken van mLMM te vergelijken met die van mNM/mSSM. In de *Dutch Melanoma Treatment Registry* (DMTR) worden sinds juli 2013 alle patiënten met gemetastaseerd melanoom geregistreerd. Uit deze database hebben wij data geëxtraheerd van 3959 unieke patiënten. In deze groep hadden 59 patiënten mLMM, 800 mNM en 1513 mSSM. Wij hebben aangetoond dat mLMM vaker werd gediagnosticeerd op een hogere leeftijd dan mNM/mSSM. Tevens kwam mLMM vaker voor in het hoofd/hals gebied. Uit de database van het geïntegreerde kanker instituut Nederland (iKNL) hebben wij vervolgens data geëxtraheerd over het vóórkomen van primair cutaan melanoom in Nederland in de periode 2013-2018 en vergeleken met het aantal gemetastaseerde melanomen. Hieruit bleek dat het aantal primair LMM (N=59/1840; 3.2%) dat uiteindelijk metastaseerde relatief lager was dan het aantal primaire NM/SSM (N=2.313/35.055; 6.6%) dat metastaseerde. Genetische analyse liet zien dat mLMM patiënten minder vaak BRAF mutaties hadden (35,0% versus 79,6%; p<0,001%) maar relatief vaker het subtype BRAFV600**K** (45,0% versus 10,6%; p<0,001) en meer KIT mutaties (5,10% versus 0,65%; p=0,002). De effectiviteit van behandeling kon niet worden vergeleken tussen de mLMM en de mNM/mSSM groep vanwege te kleine aantallen mLMM. Wel bleek dat binnen de groep mNM/mSSM de overleving het beste was bij patiënten die als eerste behandeling de combinatie ipilimumab/nivolumab of anti-PD1 antilichamen kregen. De

overleving tussen mLMM en mNM/mSSM ongeacht behandeling was vergelijkbaar, maar opvallend was dat de melanoom-gerelateerde overleving bij mLMM significant beter was. Samenvattend lijken mLMM patiënten een vergelijkbare overleving te hebben met de mNM/mSSM groep. Daarnaast overlijden mLMM patiënten significant minder vaak aan melanoom, ondanks het feit dat deze patiënten gemiddeld ouder zijn en ze in totaal minder behandelingen krijgen.

In **hoofdstuk 9** worden de huidige dilemma's rondom de behandeling van LM besproken. Onze conclusie is dat er een tekort is aan bewijs voor de beste behandelmodaliteit doordat er tot op heden geen *randomized controlled trials* zijn uitgevoerd (of gepubliceerd). Diagnostiek van LM kan worden verbeterd met behulp van de *Cancer Testis* antigenen MAGE-A en PRAME. Als een huidafwijking suspect voor LM expressie toont van MAGE-A, dan bestaat het risico dat er mogelijk sprake is van een LMM. PRAME kan worden gebruikt om atypische melanocyten van maligne melanocyten te onderscheiden en kan op die manier bijdragen tot het onderscheid tussen LM en LMM. Tot slot worden data uit de *Dutch Melanoma Treatment Registry* bediscussieerd en tonen wij aan dat er een hoog risico LM groep lijkt te bestaan die een groter risico heeft op progressie tot LMM.

Dankwoord

Bedankt iedereen, voor een fantastische tijd.

PhD Portfolio

Name PhD student: Darryl C.K.S. Tio

PhD Period: 2015-2020

Courses

Basis Cursus Regelgeving en Organisatie Klinisch Wetenschappelijk Onderzoek (BROK) AMC	2019	0.9 ECTS
Hugh Greenway's 35th Annual Superficial Anatomy & Cutaneous Surgery Course. San Diego California, United States of America	2018	1.0 ECTS
ESDR Future Leaders Academy .	2019	1.0 ECTS

Presentations

Imiquimod usage for Lentigo Maligna. The Pros and Cons. The annual national science meeting of the Dutch Society for Dermatology and Venerology (NVDV) in Amsterdam, the Netherlands. (Oral presentation)	2015	0.5 ECTS
Lymphoedema, a novel side effect of LM treated with imiquimod. The European Association of Dermatology and Venereology annual conference (EADV) in Copenhagen, Denmark. (Oral presentation)	2015	0.5 ECTS
Imiquimod treatment of lentigo maligna. A systematic review. Annual Science exchange day the Vrije Universiteit Medical Center (VUMC) in Amsterdam, the Netherlands. (Poster presentation)	2015	0.5 ECTS
Imiquimod for lentigo maligna. A systematic review. The Annual Dutch Society of Experimental Dermatology scientific meeting (NVED) in Lunteren, the Netherlands. (Oral presentation)	2016	0.5 ECTS
Variance in the management of Lentigo Maligna among European dermatologists. The European Association of Dermatology and Venereology annual conference (EADV) in Vienna, Austria. (Oral Presentation)	2016	0.5 ECTS

Variance in the management of Lentigo Maligna among European Dermatologists. Annual retreat of the Cancer Centre Amsterdam (CCA).	2017	0.5 ECTS
Long term Follow-up of lentigo maligna patients treated with topical imiquimod. The Annual Dutch Society of Experimental Dermatology scientific meeting (NVED) in Lunteren, the Netherlands. (Oral presentation)	2017	0.5 ECTS
Vascular Dermatoses. Annual European association of Dermatology and Venereology Review (EADV Review) in Utrecht, the Netherlands. (Oral presentation)	2018	0.5 ECTS
Long term follow-up of lentigo maligna treated with imiquimod. The European Association of Dermatology and Venereology annual conference (EADV) in Paris, France. (Poster presentation)	2019	0.5 ECTS
Prevalence of Cancer Testis Antigens on Lentigo maligna and Lentigo Maligna Melanoma. The Annual Dutch Society of Experimental Dermatology scientific meeting (NVED) in Lunteren, the Netherlands. (Poster presentation)	2019	0.5 ECTS
Patient characteristics and oncogenic mutations of metastasized lentigo maligna melanoma: Results from the Dutch Melanoma Treatment Registry. The Annual Dutch Society of Experimental Dermatology scientific meeting (NVED) in Lunteren, the Netherlands. (Oral presentation)	2020	0.5 ECTS

Conferences

Dutch Society of Experimental Dermatology (NVED)	2015, 2016, 2018, 2019; 2020	1.25 ECTS
European Association of Dermatology and Venereology	2015, 2016, 2018, 2019	4.0 ECTS
American Academy of Dermatology	2018	1.0 ECTS

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European Society of Dermatological Research (ESDR)	2019	0.25 ECTS
Breaking down walls in cutaneous oncology	2019	0.25 ECTS

Teaching

F.R. Kasiem, scientific internship AMC/UvA and LUMC	2018	3.0 ECTS
C.G.H. Ruijter, scientific internship AMC/UvA	2019	1.5 ECTS

Grants

Grant from the European Association of Dermatology and Venereology to perform a survey on the clinical management of Lentigo Maligna among European Dermatologists. (€40.000)	2015

Curriculum Vitae

Darryl Christian Kim San Tio werd geboren op 27 december 1987 in Zaandam en is daar opgegroeid. In 2006 begon hij aan de studie Biomedische Wetenschappen aan de Vrije Universiteit van Amsterdam waarvoor hij in juni 2009 een Bachelor diploma behaalde. Aansluitend aan deze studie is hij in september 2009 gestart met de studie geneeskunde waarvoor hij zijn artsendiploma in december 2014 heeft behaald. Na een korte pauze is hij in april 2015 gestart met promotieonderzoek onder begeleiding van Prof. dr. R. Hoekzema en dr. C. van Montfrans. In 2016 is dit team versterkt door Prof. dr. R. Luiten en dr. M.W. Bekkenk. Naast het promotietraject is hij in oktober 2015 met de opleiding dermatologie (opleider Dr. M. Wintzen) gestart aan het VU Medisch Centrum te Amsterdam. De opleiding dermatologie heeft hij afgerond in december 2019. Sinds januari 2020 is hij deels werkzaam in het Amsterdam Universitaire Medische Centra locatie AMC te Amsterdam als staflid op de afdeling Dermatologie en deels in Centrum Oosterwal te Alkmaar.

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1. Tio D, Willemsen M, Krebbers G, Kasiem FR, Hoekzema R, van Doorn R, Bekkenk MW, Luiten RM. Differential Expression of the Cancer Testis Antigens on Lentigo Maligna and Lentigo Maligna Melanoma. Am J Dermatopathol. 2020 Jan 14. doi: 10.1097/DAD.0000000000001607.

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7. Tio D, Kasiem FR, Willemsen M, van Doorn R, van der Werf N, Hoekzema R, Luiten RM, Bekkenk MW. Expression of cancer/testis antigens in cutaneous melanoma: a systematic review. Melanoma Res. 2019 Jan 4. Doi: 10.1097/CMR.0000000000000569

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10. Tio D, J van der Woude, CAC Prinsen, EP Jansma, R Hoekzema, C van montfrans. A systematic Review on the Role of Imiquimod in Lentigo Maligna and Lentigo Maligna Melanoma: Need for standardization of Treatment Schedule and Outcome Measures. J Eur Acad Dermatol Venereol 2017 apr;31(4)616-624. doi: 10.1111/jdv.14085

11. Tio D, van Doorn R, Mooi WM, van Montfrans C. Gepigmenteerde huidafwijkingen in het gelaat. Huisarts en Wetenschap 2016 aug; 59. 356-368.

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