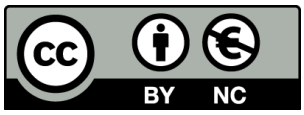
Universitat de Girona

CLINICAL EPIDEMIOLOGY AND MOLECULAR BIOLOGY OF CUTANEOUS AND MUCOSAL MELANOMA IN GIRONA 1994-2018

Anna Carbó Bagué



Universitat de Girona

DOCTORAL THESIS

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2022

Programa de Doctorat en Biologia Molecular, Biomedicina i Salut

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1. A. Carbó-Bagué, R. Fort-Culillas, H. Pla-Juher, J. Rubió-Casadevall. Nivolumab-Induced Autoimmune Haemolytic Anaemia and Safety of Subsequent Use of Ipilimumab: A Case Report. *Case Rep Oncol* 2021;14:1289–1294

2. J. Rubió-Casadevall, A. Carbó-Bagué, M. Puigdemont, G. Osca-Gelis, G. Oliveras, N. Vilar-Coromina, B. Ferrer-Fabrega, A. Urban, M. Llobet-Roma, F. Martín-Romero, F. Perez-Bueno, R. Marcos-Gragera. Population-based analysis of the prevalence of BRAF mutation in patients diagnosed with cutaneous melanoma and its significance as a prognostic facotr. *Eur J Dermatol 2021* Oct 1;31(5):616-622.

3. A. Carbó-Bagué, J. Rubió-Casadevall, M. Puigdemont, A. Sanvisens, G. Oliveras, M. Coll, B. Del Olmo, F. Perez-Bueno, R. Marcos-Gragera. Epidemiology and Molecular Profile of Mucosal Melanoma: A Population-Based Study in Southern Europe. *Cancers (Basel).* 2022 Feb; 14(3): 780.

- **List of abbreviations:**

AIHA. *Autoimmune Haemolitical Anemia*
AJCC. *Amercian Joint Comittee on Cancer*
ALM. *Acral Lentiginous Melanoma*
CI. *Confidence Interval*
CM. *Cutaneous Melanoma*
CNS. *Central Nervous System*
DFS. *Disease-free Survival*
DM. *Desmoplastic Melanoma*
GCR. *Girona Cancer Registry*
H&N. *Head and Neck*
HR. *Hazard Ratio*
ICI. *Immune Checkpoint Inhibitors*
LMM. *Lentigo Maligna Melanoma*
MAPK. *Mitogen-associated protein kinase*
MM. *Mucosal Melanomas*
NF1. *Neurofibromin 1*
NM. *Nodular Melanoma*
OS. *Overall Survival*
PFS. *Progression Free Survival*
RFS. *Recurrence-free Survival*
SBRT. *Stereotactic Body Radiation Therapy*
SLN. *Sentinel lymph node*
SRS. *Stereotactic RAdiosurgery*
SSM. *Superficial Spreading Melanoma*
TIL. *tumor infiltrating-lymphocytes*
TKI. *Tyrosine kinase inhibitors*
UVR. *Ultraviolet radiation*
WBRT. *Whole Brain Radiotherapy*

- **Dedicatòria; dedication:** aquesta tesi està dedicada en primera instància al Dr. Rubió i codirector de la meva tesi, per la seva paciència incansable, ajuda, perseverança i coneixement. Jordi sense tu no hagués sigut possible! També al director Dr. Marcos per facilitar-me el camí amb els seus coneixements epidemiològics i bagatge professional, per ajudar-me també en la organització i planificació de la tesi.
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1 INDEX

FIGURES AND TABLES INDEX

2 RESUM/RESUMEN/SUMMARY

CAT

En aquesta tesi doctoral s'analitza l'epidemiologia del melanoma cutani i de mucoses, així com el seu perfil molecular per aportar nous coneixements en base poblacional de la província de Girona. Com a font de casos s'ha utilitzat el registre de càncer de Girona. Es descriu la incidència i la supervivència global i relativa a 5 i 10 anys, a més a més, es fa un anàlisi molecular del gen BRAF en el melanoma cutani i un panell genètic en el melanoma de mucoses. A part, s'aporta evidència mèdica del tractament dels pacients a la pràctica clínica diària.

La supervivència global a 5 anys per tots els estadis en els melanomes cutanis és del 81% similar als altres estudis publicats amb una prevalença de mutacions en el gen BRAF del 50% i essent l'estadi l'únic factor pronòstic estadísticament significatiu per la supervivència.

La supervivència global a 5 anys per tots els estadis en els melanomes de mucoses és del 21% similar també als altres estudis publicats amb un perfil molecular on dominen les mutacions a NRAS i NF1.

Es confirma que les característiques epidemiològiques de Girona són similars a les de regions veïnes, que l'estadi segueix essent el factor pronòstic més important per supervivència així com no ho és l'estatus mutacional en el gen BRAF. I es demostra que els melanomes de mucoses a part de tenir un perfil mutacional diferent tenen un clar pitjor pronòstic.

CAST

En esta tesis doctoral se analiza la epidemiología del melanoma cutáneo y de mucosas, así como su perfil molecular para aportar nuevos conocimientos en base poblacional de la provincia de Girona. Como fuente de casos se ha utilizado el registro de cáncer de Girona. Se describe la incidencia y la supervivencia global y relativa a 5 y 10 años, además se realiza un análisis molecular del gen BRAF en el melanoma cutáneo y un panel genético en el melanoma de mucosas. Aparte, se aporta evidencia médica del tratamiento de los pacientes en la práctica clínica diaria.

La supervivencia global a 5 años por todos los estadios en los melanomas cutáneos es del 81% similar a los otros estudios publicados con una prevalencia de mutaciones en el gen BRAF del 50% y siendo el estadio el único factor pronóstico estadísticamente significativo para la supervivencia.

La supervivencia global a 5 años por todos los estadios en los melanomas de mucosas es del 21% similar también a los otros estudios publicados con un perfil molecular donde dominan las mutaciones en NRAS y NF1.

Se confirma que las características epidemiológicas de Girona son similares a las de regiones vecinas, que el estadio sigue siendo el factor pronóstico más importante para la supervivencia, así como no lo es el estatus mutacional en el gen BRAF. Y se demuestra que los melanomas de mucosas aparte de tener un perfil mutacional diferente tienen un claro peor pronóstico.

ENG

In this doctoral thesis, the epidemiology of cutaneous and mucosal melanoma is analyzed, as well as its molecular profile to provide new knowledge on a population basis in the province of Girona. The Girona cancer registry was used as a source of cases. The incidence and overall and relative survival at 5 and 10 years are described, as well as the molecular analysis of the BRAF gene in cutaneous melanoma and a genetic panel in mucosal melanoma. In addition, medical evidence of the treatment of patients in daily clinical practice is provided.

The 5-year overall survival for all stages in cutaneous melanomas is 81%, similar to other published studies with a prevalence of mutations in the BRAF gene of 50% and stage being the only statistically significant prognostic factor for survival.

The 5-year overall survival for all stages in mucosal melanomas is 21%, also similar to other published studies with a molecular profile where NRAS and NF1 mutations dominate.

It is confirmed that the epidemiological characteristics of Girona are like those of neighboring regions, that the stage continues to be the most important prognostic factor for survival, while the mutational status in the BRAF gene is not. And it has been shown that mucosal melanomas, apart from having a different mutational profile, have a poor prognosis.

3 INITIAL PAGE

This thesis is presented as a compendium of articles and opts for the international doctorate mention. The three articles are presented here under their full references together with the quality indices:**No se encuentran elementos de tabla de ilustraciones.**

1. J. Rubió-Casadevall, A. Carbó-Bagué, M. Puigdemont, G. Osca-Gelis, G. Oliveras, N. Vilar-Coromina, B. Ferrer-Fabrega, A. Urban, M. Llobet-Roma, F. Martín-Romero, F. Perez-Bueno, R. Marcos-Gragera. Population-based analysis of the prevalence of BRAF mutation in patients diagnosed with cutaneous melanoma and its significance as a prognostic factor. *Eur J Dermatol 2021* Oct 1;31(5):616-622. DOI: 10.1684/ejd.2021.4136

IF: 3.328

JCI 0.72

Q2 dermatologia

Citescore 2.1

2. A. Carbó-Bagué, J. Rubió-Casadevall, M. Puigdemont, A. Sanvisens, G. Oliveras, M. Coll, B. Del Olmo, F. Perez-Bueno, R. Marcos-Gragera. Epidemiology and Molecular Profile of Mucosal Melanoma: A Population-Based Study in Southern Europe. *Cancers (Basel). 2022* Feb; 14(3): 780. DOI: https://doi.org/10.3390/cancers14030780.

IF: 6.639

JCI 1.02

Q1 oncologia

Citescore 4.4

3. A. Carbó-Bagué, R. Fort-Culillas, H. Pla-Juher, J. Rubió-Casadevall. Nivolumab-Induced Autoimmune Haemolytic Anaemia and Safety of Subsequent Use of Ipilimumab: A Case Report. *Case Rep Oncol 2021*; 14:1289–1294. DOI: 10.1159/000518530

IF: 1.143

JCI 0.20

Q4 oncologia

Citescore 1.5

4 GENERAL INTRODUCTION

I would like to introduce the correlation between the three articles proposed in this thesis. The aim is to provide and expand the knowledge in the field of melanoma from a population-based view in both epidemiology and molecular biology and also a small sketch of routinary clinical practice. All of them are related to cutaneous and mucosal melanomas and its landscape. In this thesis uveal melanomas are not represented.

The database analyzed in the articles has been made thanks to the Girona Cancer Registry, a population-based registry of Girona's province, located in the north-east of Spain, which started registration in 1994. The patients were treated at the university Hospital Josep Trueta of Girona.

The incidence and survival of cutaneous melanoma in Girona was published before the registration of this thesis by our Girona Cancer Registry. (1)

In the first article (2) we did a population-based analysis of the prevalence of BRAF mutation in patients diagnosed with cutaneous melanoma and its significance as prognostic factor. The study was retrospective and included cases from 2009-2011. BRAF is mandatory in clinical practice guidelines The prevalence of BRAF mutation has been reported in between 38% and 48% of melanoma patients based on mainly stage III or metastatic disease however information on population-based analysis is lacking. BRAF mutation is a known predictive factor of response to iBRAF/iMEK treatments widely used in routine clinical practice. Results will be presented in the corresponding chapter.

In the second article (3) we did again a population-based analysis but with mucosal melanomas publishing the incidence and survival. The study was also retrospective and included cases from 1994-2018 as mucosal melanoma is a rare neoplasm. In this study we included a more extensive molecular profile with a panel of 44 genes. Molecular oncology is playing every day a more relevant role for understanding the biology of the tumors and finding target therapies.

In the third article (4) we explain a case report of real world data when treating melanoma patients in the routine clinical practice. Specifically, about the toxicity related to immune events. Immunotherapy is one of the pillars of melanoma cancer treatment in adjuvant and metastatic setting. Melanomas carry high tumor mutation burden typically responding well to immunotherapy.

Thanks to my experience in clinical practice as a medical oncologist and to all the patients we treat routinely, I have been able to perform these three articles which make up my thesis. Epidemiology and molecular oncology are essential to take care of melanoma patients.

5 INTRODUCTION OF THE THESIS

5.1 EPIDEMIOLOGY, CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF MELANOMA (RISK AND PROGNOSTIC FACTORS).

Melanoma arises from melanocytes which are specialized cells whose function is the production of melanin that serves as a shield to protect DNA from UV-radiation and primarily involves the skin. Melanomas can also arise on various mucosal surfaces, meninges, and the eye (uveal, conjunctiva and ciliary body) but the role of melanocytes in non-exposed areas remains unclear. Melanin is involved in antimicrobial defense. It is hypothesized that mucosal melanocytes have an immunogenic role, especially given their location in immunologically critical mucosal surfaces. (5)

In this thesis we will only talk about cutaneous and mucosal melanomas.

Melanoma is classified as in situ when confined within the epidermis, or invasive when invades into the dermis.

Melanoma accounts for only 3% of malignant neoplasms of the skin worldwide, but it accounts for the majority of deaths among skin tumors (6,7).

The considered risk factors for melanoma are a family history of melanoma, multiple benign or atypical nevi, a previous melanoma, sun sensitivity, and exposure to ultraviolet radiation (7).

Cutaneous melanoma (CM) is associated with sun exposure and UVR (Ultraviolet Radiation) molecular signature as a carcinogen. Incidence varies by regions (8) and phototypes described by Fitzpatrick scale in *Figure 1*. The fairer the skin, the easier it is for UV to cause inflammation (sunburn). Low Fitzpatrick phototype correlates with both low minimal erythematous dose and with higher risk of melanoma and other skin cancer risk. (9)

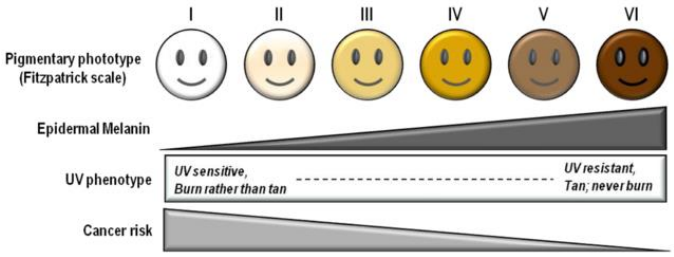

Figure 1. Schematic image of Fitzpatrick scale and UV risk. Orazio JD et al (9).

The main risk factor is intermittent exposure to ultraviolet radiation. Ambient UV exposure varies geographically according to intensity of sunlight in a particular location on Earth and UV

dose varies according to the amount of atmosphere it must pass through, making UV doses higher nearest the Equator (where sunlight strikes the Earth most directly) where we can find mor pigmented populations with lower incidence of skin melanoma (10,11).

Most melanomas arise out of pre-existing nevus, therefore having many nevi is another important risk factor for the disease (7).

Cutaneous melanoma incidence has increased steadily and significantly over the last several decades in white-skinned populations (European and USA populations) (12–15) while mucosal melanoma incidence has remained stable. This increase has been described especially in the initial stages ("in situ" o thin melanomas), which may reflect both the evolution of etiological factors and the efficacy of early diagnosis and awareness of the patients (1). It has been postulated that the use of regular protective sunscreen could modify this incidence trend (16) and there is one study by *Green and colleagues* that provides evidence to date that the regular use of sunscreen can prevent the development of melanoma for up to 10 years (17).

Unlike other cancers, the implementation of a national screening program is still under debate (18).

5-year-survival of CM of all stages is around 80% whereas for mucosal melanoma (MM) is much lower around 25%, results published in a large series of the CONCORD-3 study (19).

In *table 1* below clinical and pathological features of CM and MM are shown. (20)

Table 1. Clinical and pathological features of CM and MM in American population. Adapted from Carvajal et al (20)

		Cutaneous Melanoma	**Mucosal Melanoma**
Demographics	Proportion of all melanomas	90%	<2%
	Median age at diagnosis	55y	70y
	Male:Female ratio	60:40	35:65
	Race		
	white	94%	85%
	black	<1%	7%
Epidemiology	Incidence over time	Rising	Stable
	Risk factors	Ultraviolet radiation	Unknown
Pathology	Multifocality	<5%	20%
	Amelanotic	<10%	Up to 40%
Clinical Outcomes	Advanced stage at diagnosis	<30%	>50%
	5-year overall survival rate	80%	25%

Subtypes of **cutaneous melanoma (CM)** are distinguished by clinical and histopathological features into four major histological subtypes: superficial spreading melanoma **SSM** (41%),

followed by nodular melanoma **NM** (16%), lentigo maligna melanoma **LMM** (2.7%- 14%) and acral lentiginous melanoma **ALM** (1%-5% in non-Hispanic White population and higher rates in Asian or African American population). (21) There is also a very rare variant called desmoplastic melanoma **DM** (1-4%) that should be noted according to NCCN guidelines because it may impact on the decision about diagnostic staging and treatment.(22)

Clinical-pathologic subtypes are not included as prognostic factors in the current 8th edition AJCC staging system. (23)

In *table 2* below there is an enlightening summary of melanoma's classification including type of UVR exposure, subtype of melanoma, and genomic features.

Type of UVR exposure/CSD	Subtype of melanoma	Affected genes
Low-CSD melanoma	SSM	BRAF V600 E/K or NRAS CDKN2A TP53 SWI/SNF TERT
High-CSD melanoma	LMM Desmoplastic melanoma	NF1, NRAS, BRAF, KIT CDKN2A TP53 SWI/SNF TERT
Low to no UVR exposure (or variable/ incidental)	Spitz melanoma	HRAS, ROS1, NTRK1, NTRK3, ALK, RET, MET, BRAF, CDKN2A, TERT
	Acral melanoma Mucosal melanoma (genital, oral, sinonasal)	NRAS, KIT, NF1, SPRED1, BRAF, CCND1, ALK, ROS1, RET, NTRK1, CDKN2A, CDK4, TP53, SWI/SNF, TERT
	Uveal melanoma	GNAQ, GNA11, CYSLTR2, PLCB4, BAP1, SF3B1, ElF1AX
	Melanoma arising in congenital naevus	NRAS
	Melanoma arising in blue naevus	GNAQ, GNA11, CYSLTR2, BAP1, SF3B1, ElF1AX

CSD = Cumulative sun damage.

Table 2. Types of UVR exposure, subtype of melanoma and affected gens (21).

The AJCC (American Joint Committee on Cancer) analyzed 38.918 patients to determine factors significantly predictive of survival for patients with cutaneous melanomas. This and other studies have found that **Stage at diagnosis**, **Breslow tumor thickness**, **ulceration** (loss of epidermal matrix) and **mitotic rate** (number of mitoses/mm2) are the most important characteristics independently predictive of outcome by multivariate analysis (24).

Age is another independent predictive prognostic factor. There is an increase in mortality in melanoma patients 65 years of age or older (8). The features of primary melanoma among older patients are more locally advanced melanomas (thicker and more ulcerated) with a much greater proliferative activity as calibrated by the mitotic rate and higher incidence of lesions that are less likely to contain BRAF mutations due to sun-exposure areas (25). Alterations in the immunity system might explain why melanoma is more likely to be fatal in older patients.

Sex is a prognostic factor. In a large study of six European Latin countries, SUDCAN study (8), they found female sex associated with better survival, so did in a recent retrospective study made in Brasil (26) and in an Italian (27) study women had better survival than men at any time since diagnosis and reported that men were older that women, with a median age at diagnosis of 59.5 versus 55.8 years (P =0.0002). The results were independent of the effect of many other prognostic factors.

The reasons for this advantage for women are poorly understood. Some authors postulate that biological factors (hormonal influence, oxidative stress, and gene expression) and behavioral factors (exposure to UV and self-care/medical awareness) work together but they have not been confirmed and clinicians need to be aware of it (8,26,28).

In another retrospective study published in the USA (29) in models adjusted for age, melanoma subtype, and location, males were more likely to present with lesions with higher Breslow depths (OR: 1.261, 95% CI: 0.988-1.611, p=0.060) and also more likely to present with lesions with higher mitotic rates, after further adjustments for all other prognostic factors (OR: 1.244, 95% CI: 0.979-1.580, p=0.074). They conclude that differences in biological factors may contribute to the female prognosis advantage.

There are other factors less consistently independently predictive of outcome such us **microscopic satellites** in the initial biopsy defined as the presence of tumor nests greater than 0.5mm in diameter, in the reticular dermis, panniculus, or vessels beneath the principle invasive tumor but separated from it by at least 0.3mm of normal tissue (30). The presence of microscopic satellites correlates with decreased 5-year survival rate and increased frequency of occult regional lymph node metastases for clinical stage I melanoma patients. It is usually not possible to detect them with less than a complete excisional biopsy.

Other additional factors but recommended in the pathological report are **vertical growth phase**, tumor infiltrating-lymphocytes (**TIL**) and **regression**.

5.1.1 Epidemiology of Cutaneous Melanoma in Girona.

We had described the epidemiology of cutaneous melanoma in Girona in a previous article (1), not included in this thesis but we still find important to detail.

Using Girona's Cancer Registry database, we identified 1,482 melanoma cases of CM in the period from 1994 to 2013.

Of those patients, 55.3% were female with a median age at diagnosis of 60.2 years old, and 44.7% were male with a median age of 61.6 years old.

In both sexes, the most common histopathologic type of CM was superficial spreading melanoma (SSM), meaning 40.1%. Acral lentiginous melanoma (ALM) was the least common with 3.0%.

Incidence

For the entire period 1994-2013, CM incidence, including both invasive and "*in situ*", was 11.7 cases per 100,000 inhabitants/year in crude rate terms, and 9.7 and 7.2 cases in ASRe and ASRw.

We found a significant increase in incidence of melanoma cases in our area in the period 1994-2013, with an annual percentage of change (APC) using join-point analysis of 2.4%. However, the significance was restricted to men, "*in situ*" cases and thin melanomas with less than 1 mm in Breslow depth (pT1 tumours). No changes in incidence were observed in each histological type of melanoma or in the analysis by subsite.

Survival

Observed and relative survival at three, five and ten-years, depending on the melanoma's behavior (invasive or "*in situ*"), sex, site, subsite, histology, Breslow index, year of diagnosis and ulceration.

Five-year relative survival is, as expected, clearly better for "*in situ*" than for invasive melanoma (100% versus 81.9%), for confirmed skin melanoma than those without known primary (88.2% versus 26%), for SSM and LMM than NM and ALM (93.7% and 96.4% versus 58.8% and 56.6%), for those without ulceration than for those with confirmed ulceration (95.7 versus 60%) and for those with less than 1 mitosis per mm^2 than for those with more (100% versus 78.2%). To analyze these last two characteristics, only patients diagnosed between 2009 and 2012 were used, because previously these prognostic factors were not systematically registered in the pathologic reports.

Patients with melanoma with a Breslow thickness less than 1 mm, pT1, or between 1 and 2 mm, pT2, no statistically different relative survival at 5 and 10 years was observed, by contrast with those with pT3 or pT4 melanoma, who had a 10-year RS of 58% and 31.9%, respectively. In *table 3* there is a summary of survival and clinic-pathological characteristics.

Table 3. Observed and relative survival of melanoma according to clinicopathological characteristics in Girona, 1994-2013. (to see the whole table go to the publication (1))

Clinicopathological characteristics		Years of follow-up	OS (95% CI)	RS (95% CI)
Total		3y	83.1 (81.2; 85)	89.3 (87.3; 91.4)
		5y	76.1 (73.8; 78.3)	86 (83.5; 88.6)
		10y	64.1 (61.3; 67)	83.1 (79.5; 86.9)
Behavior	Invasive	5y	72 (69.3; 74.7)	81.9 (78.9; 85)
		10y	58.7 (55.5; 62.1)	76.7 (72.5; 81.1)
	In stiu	5y	90.3 (87; 93.7)	100 (96.4; 104)
		10y	83.4 (78.7; 88.4)	106 (99.8; 112)
Sex	Male	5y	70.9 (67.4; 74.6)	82.5 (78.4; 86.8)
		10y	57.4 (53.1; 62)	78.7 (72.8; 85.1)
	Female	5y	80.2 (77.4; 83.1)	88.7 (85.6; 91.9)
		10y	69.4 (65.9; 73.2)	86.3 (81.9; 90.9)
Histology	SMM	5y	87.2 (84.4; 90.1)	93.7 (90.7; 96.8)
		10y	76.6 (72.6; 80.8)	89.3 (84.7; 94.2)
	LMM	5y	74.4 (66.3; 83.4)	96.4 (86.1; 108)
		10y	53.4 (43.7; 65.4)	90.7 (74.3; 111)
	NM	5y	48.4 (41.4; 56.6)	58.8 (50.3; 68.7)
		10y	25.6 (18.7; 34.9)	38.1 (28; 51.9)
	ALM	5y	46.4 (33.6; 64.1)	56.6 (41.2; 77.7)
		10y	36.7 (24; 55.9)	51.6 (34.2; 77.8)
Breslow	≤ 1MM	5y	91.9 (89.4; 94.5)	99.2 (96.4; 102)
		10y	79.8 (75.4; 84.4)	94.2 (89; 99.7)
	1.01-2.0mm	5y	80.2 (74.7; 86.2)	90 (83.8; 96.6)
		10y	66.3 (59.2; 74.2)	85 (76; 95.2)
	2.01-4mm	5y	67.1 (59.5; 75.8)	79.4 (70.4; 89.5)
		10y	41.4 (32.4; 52.8)	58 (45.6; 73.8)
	4mm	5y	34.1 (27; 43)	42.8 (34; 53.9)
		10y	20.2 (13.7; 29.9)	31.9 (21.7; 46.7)
Ulceration	Present	5y	50.4 (38.9; 65.2)	60.1 (46.6; 77.6)
		5y	88.7 (83.8; 93.9)	95.7 (90.4; 101)
Mitotic count	≤1mm2	5y	94.5 (87.2; 100)	102.9 (95.1; 111)
	>1mm2	5y	68.8 (60.1; 78.8)	78.2 (68.4; 89.5)

SSM: superficial spread melanoma; LMM: lentigo malignant melanoma; NM: nodular melanoma ; ALM: acral lentiginous melanoma

OS: observed survival; RS: relative survival; NOS: not otherwise specified

5.1.2 Mucosal Melanomas

When talking about **mucosal melanomas (MM**) the value of pathological characteristics described above has not been consistently attested as validated prognostic factors. Although ultraviolet radiation exposure is an important risk factor for CM, it has not been associated with the development of MM.

MM account approximately 1.2% of all melanomas, despite in Asian populations where it has been reported up to 20% likely due to the lower prevalence of cutaneous melanoma (31). Although the absolute incidence of MM is greatest in whites, the proportion of MM in blacks, Asians, and Hispanics is greater than that observed in whites because as said before the incidence of CM in this populations is lower than Caucasians (20).

The head and neck (H&N) is the region most represented (31-55%), followed by the ano-rectum (17-24%), and the female genital tract (18-24%) (32). In rare occasions, primary mucosal melanoma has been observed in the urinary tract, esophagus, stomach, small and large intestine, and cervix (33).

MM is a highly lethal variant of melanoma that carries a very poor prognosis; 5-year survival reported is <25% (34). The low incidence and survival rates have led to few clinical trials and a lack of protocols and uniformed guidelines (35).

There is a European study published in 2012 from 76 population-based cancer registries that analyzed 8669 incident cases registered in the period 1995–2002. Five-year relative survival was 40.6% for MM. For mucosal melanomas, the most common sites were the head and neck (40.6%), the female genital tract (36.3%), and the anal canal/colo-rectal tract (18.5%) (36).

Vulvar melanoma has the better prognosis of all MM localizations with a 5-year survival around 40%. Recognition of abnormal pigmented vulvar lesions, often accompanied by pruritis and bleeding, allow early detection and prompt surgical approach leading to a curative attempt (37).

5.2 MOLECULAR CHARACTERISTICS OF MELANOMA.

The mutational landscape differs drastically from CM to MM. The genetic and molecular profile is believed to distinguish the biologic behavior between both types of melanomas.

In a large pan-tumor study melanoma was found to be the tumor with the highest somatic mutation frequency of all cancers analyzed (38).

Most CM have a well-known UVR (solar ultraviolet radiation) signature behind, in MM UVR is not de principal carcinogen involved except for conjunctival melanoma which is not part of this thesis and some from the nasal cavity. The evidence of UVR-related mutagenesis in the facial area implicates attenuated UVR exposure even in these relatively sun shielded sites (39).

-Mitogen-associated protein kinase **(MAPK) pathway** is an important intracellular and is commonly activated in melanoma. Its function is to promote tumorigenesis. The MAPK pathway responds to extracellular binding of growth factors to receptor tyrosine kinases. Then activation of downstream signaling starting with a GTPase (Ras) followed by phosphorylation of the following proteins: Ras/Raf/MEK/ERK. In CM, the MAPK pathway is commonly activated by mutations in BRAF, NRAS and NF1. A vast majority of cutaneous melanomas (94%) contain MAPK pathway activating mutations whereas only a 28% of mucosal melanomas harbor these mutations (33).

ESMO and NCCN clinical guidelines strongly recommend screening of BRAF, NRAS and KIT for all melanomas at diagnosis.

-**BRAF** is an oncogene part of the MAPKinasa-pathway. BRAF is highly mutated at codon V600 in many cancers including melanoma and it strongly activates MAPK pathway. The incidence of BRAF mutations in CM is 40-50% much lower in mucosal melanomas (3-15%) usually affecting different or non-activating regions. The most common mutation is V600E (80%) others are V600K (20%), V600R (5%) and V600M (1-2%). BRAF mutations are more frequent in lesions with superficial spreading and skin sites intermittently exposed to the sun. These mutations are important for being relevant clinical targets with iBRAF/iMEk therapies approved for CM. The combination of targeted inhibitors has had a very significant impact on survival with a median overall survival (OS) exceeding 2 years (40,41).

Interestingly, in mucosal melanoma, there is an increased number of non-canonical BRAF mutations (L505H, G469A, L597R, and T599I), which are known to lead to weaker MAPK activation as compared to BRAF-V600 and doubts whether they respond to iBRAF/iMEK (33).

-**NRAS** is an oncogene part of the MAPkinasa-pathway. NRAS mutations have been found in 8-15% of mucosal melanomas and in 20% of cutaneous ones. These mutations mainly involve exons 1 (codon G12 and G13) and 2 (codon Q60 and Q61) (42). Most frequently seen are NRAS Q61K 48% and Q61R 40%. Mutations in this gene confer aggressive clinical course and a

worse prognosis. They are more common in non-sun-exposed skin. To note, they can lead to acquired resistance to classic BRAF inhibitors. (40)

-**KIT** codes for a transmembrane receptor tyrosine kinase that, upon binding to its ligand, activates different intracellular signaling pathways some important in normal melanocyte development. It regulates the activation of several oncogenic downstream signaling pathways such as MAPK and AKT pathways (33). Activating mutations or amplifications in KIT are identified in 7 to 17% mucosal melanomas a higher value compared with CM (1-3%), and occasionally patients respond to the tyrosine kinase inhibitor imatinib or nilotinib though the mechanisms of respond should be better studied for further understanding. Predominantly, the mutations are found in exon 11 (L576P) or exon 13 (K642E) as in other KIT-dependent tumors (I.e GIST) but can also affect exons 9,12-21. KIT mutations in MM, are found particularly in the vulvovaginal and anorectal subtypes and often coexist with NF1 (43). Patients with KIT exon 11 or exon 13 mutations are shown to have a better response to KIT targeted therapy, suggesting that certain KIT alterations may be more sensitive to KIT inhibition.

-NF1 (Neurofibromin 1) is a negative regulator of Ras thus is considered to be a tumor suppressor. Loss of NF1 is associated with increased MAPK pathway activity. NF1 is significantly mutated at a same rate in CM than in MM, around 15%. (33,42). As said above, in MM mutations in NF1 and KIT coexist in 32% of cases. Theoretically, NF1 mutated tumors should respond to iBRAF/iMEK treatment but preclinical data show that these tumors are more resistant to iBRAF therapies but could be sensitive to the combination with MEK inhibition but has not been clinically tested. NF1 mutations appear to be correlated with the strongest UV signature and a high mutational burden and therefore there is rationale for using immunotherapeutic agents in this patient population (40).

Data published with The Cancer Genome Atlas (TCGA) found that melanomas could be classified into 4 genomic subtypes: mutant BRAF, mutant NRAS, mutant NF1, and triple-wild-type (42).

Melanomas from chronically sun-exposed skin tend to have the highest numbers of mutations, and often have NF1, NRAS, and occasionally BRAF V600K mutations present. BRAF V600E mutations, by contrast, are rare in these melanomas. Melanomas from intermittently sun-exposed skin frequently have intermediate numbers of mutations and have mutations in BRAF V600E (50%) or NRAS (15%-20%) (40).

It is interesting to note that melanoma precursors have been reported to progress through a stereotypical pattern of mutagenesis. Unequivocally benign lesions (nevi) acquire BRAF mutations in 70-80% of the cases. Although BRAF mutations clearly drive melanoma growth and

progression, they are insufficient by themselves to induce melanomas. Intermediate lesions acquired other mutations, such as telomerase reverse transcriptase (TERT) or NRAS mutations. Invasive melanomas often acquired cyclindependent kinase inhibitor 2A (CDKN2A) loss, PTEN loss, or TP53 mutations. Furthermore, point mutations increased at each stage of evolution (40). CDKN2A can be found in germinal linage linked to inherited familial melanoma. It has been hypothesized that mutations in this pathway may contribute to sensitivity to CDK4/6 inhibitors, but this has yet to be shown conclusively in clinical trials.

TP53 is the most famous tumor suppressor gene, is commonly mutated in many cancers and was found to be mutated in approximately 15% of CM melanomas, but also present in MM. Usually confers worse prognosis and aggressiveness (42). There are no target therapies available for TP53.

Whole-genome sequencing has shown that MM have a much lower burden of point mutations and a greater load of structural chromosomal variants compared to CM. These mutations bear no signatures of UVR or any other known carcinogen, and therefore their origin is unknown such as acral melanomas (39).

The largest study published in the literature (39) of whole-genome sequencing of 67 cases of MM showed the following significantly mutated genes. NRAS (12/67), BRAF (11/ 67), NF1 (11/67), KIT (10/67), SF3B1 (8/67), TP53 (6/67), SPRED1 (5/67), ATRX (4/67), HLA-A (4/67), and CHD8 (3/67). All BRAF mutations were in the protein tyrosine kinase domain and most targeted the 594–600 amino acids hotspot region similar to CM. NRAS mutations were targeted to hotspots on codon 61, which is the dominant hotspot in CM and codon 12, a hotspot less commonly mutated in cutaneous melanoma. NRAS mutations appear in approximately 10-15% of MM while in CM are 5%. SF3B1 and SPRED1 mutations were mostly present in primary samples and NRAS aberrations were predominantly in recurrent/metastatic tumors.

The authors conclude that all samples of MM had at least one well-established driver gene mutation i.e., MAPK pathway (NF1, NRAS, KIT, BRAF), SF3B1, TP53 and MDM2, SPRED1, TERT and ATRX, CDK4 and CCND1. These findings may help in the planification of oncological targeted therapies such as well-established iBRAF/iMEK, imatinib (c-Kit) or iCDK4/6 (CDK4, CCND1 mutations).

-SPRED1 (sprout-related, EVH1 domain containing protein 1) is a tumor suppressor gene that acts by transporting NF1 to the plasma membrane where it inhibits RAS-GTP signaling. Loss of SPRED1 function results in increased MAPK pathway signaling and can occur in approximately 26 to 37% of MM (33). Same procedure results in MAPK pathway activation when NF1 mutated

tumors. These findings provide a rationale for MAPK inhibition in SPRED1/NF1 deficient melanomas. NF1 and SPRED1 mutations doesn't coexist in MM suggesting that they may play similar roles in tumor progression in mucosal melanoma (44).

-**SF3B1** is a component of the spliceosome and plays a key role in initial stages of pre-mRNA splicing. SF3B1 is the most frequently mutated spliceosome gene in cancer also in hematological malignancies. SF3B1 mutations result in alternative splicing and aberrantly spliced mRNAs are subject to non-sense codons downregulating protein expression (33). SF3B1 mutations are rare in CM but nearly 20% of MM have a "hot-spot" mutation of SF3B1 at codon R625 (43). This data suggests that SF3B1 might be exploited as a novel prognostic and/or therapeutic target. Currently, there are phase I studies targeting the spliceosome.

Patient selection by molecular screening is essential to identify patients who might potentially benefit from targeted therapies. In *figure 2* there is a schematic graphic of molecular paths.

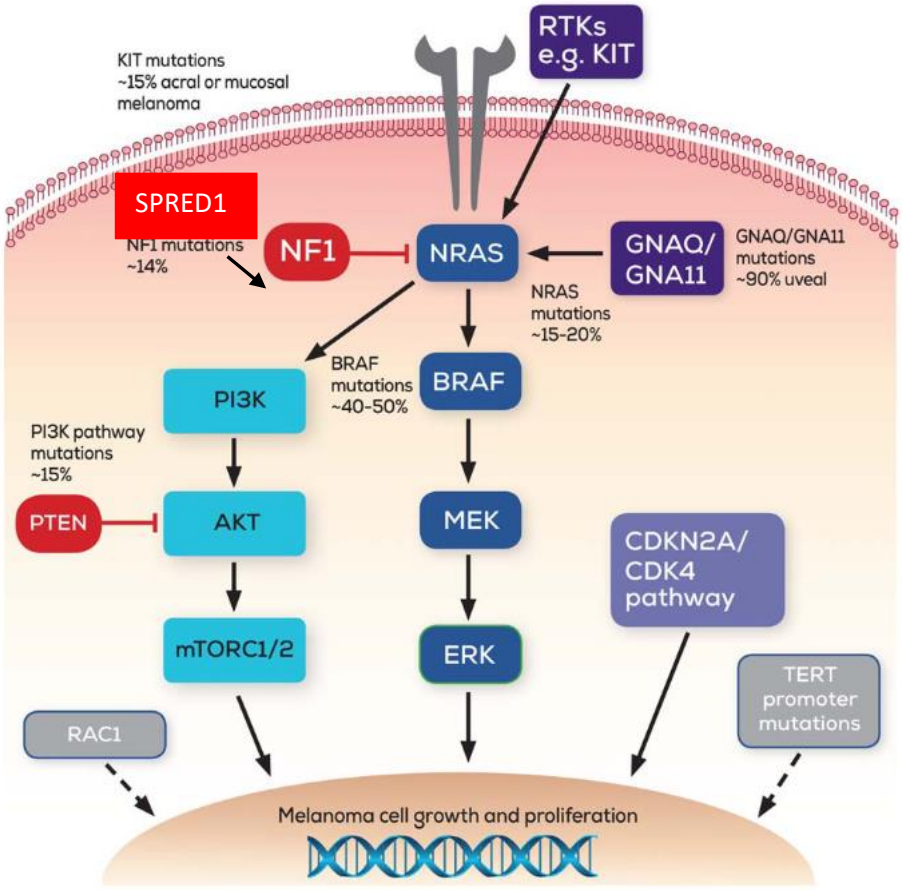

Figure 2. Schematic relevant cell signaling of melanoma. Alexandrov LB et al (29).

5.3 DIAGNOSIS AND STAGING.

To perform a correct diagnosis, the first step is to identify history of sun exposure and ask for family history of skin cancer.

The second step is to identify suspicious lesions for melanoma by physical examination usually made by a primary care practitioner or a dermatologist. If the first clinician is a primary care practitioner, a derivation to a dermatologist must be done with a dermoscopic study.

The ABCDE criteria represent a commonly used clinical guide for the diagnosis of early melanoma. A suspicious lesion is defined by the following criteria (45):

A: Asymmetry

B: Irregular Borders

C: Color – nevus with >1 color

D: Diameter of lesion > 6 mm

E: Evolution – nevus has gone through sudden changes in size/shape/color

Despite old and well-known ABCDE criteria, to assist in the detection of more subtle melanomas requires technology to augment a visual examination by an expert dermatologist.

The discipline of dermoscopy has improved the detection of melanoma and other skin cancers, has resulted in the detection of thinner melanomas, and has helped improve the ability to differentiate nevi (benign lesions) (46).

Patients with a suspicious pigmented lesion should undergo an excisional biopsy with 1 to 3mm negative margins, wider margins will interfere with accuracy of regional staging by SLN (Sentinel Lymph Node) biopsy (47). The orientation of the excisional biopsy should always be planned with definitive treatment in mind and must not interfere with sentinel node biopsy. Shave biopsy may compromise pathologic diagnosis and assessment of Breslow thickness, thus should not be performed (47).

The eighth edition of TNM AJCC staging system is the last being used worldwide for **cutaneous melanomas**. The seventh was implemented in 2009 and the eighth in 2018 (23).

Primary tumor thickness (Breslow) and ulceration continue to represent important prognostic factors for survival and define T-category strata. Clark's level is no longer part of the AJCC staging system nor does mitotic rate although it remains a major determinant of prognosis in melanomas and should be documented for all patients (7).

T Category	Thickness	Ulceration Status
TX: Primary tumor thickness cannot be assessed (eg, diagnosis by curettage)	Not applicable	Not applicable
T0: No evidence of primary tumor (eg, unknown primary or completely regressed melanoma)	Not applicable	Not applicable
Tis (melanoma *in situ*)	Not applicable	Not applicable
T1	≤1 mm	Unknown or unspecified
T1a	<0.8 mm	Without ulceration
T1b	<0.8 mm	With ulceration
	0.8–1.0 mm	With or without ulceration
T2	>1.0–2.0 mm	Unknown or unspecified
T2a	>1.0–2.0 mm	Without ulceration
T2b	>1.0–2.0 mm	With ulceration
T3	>2.0–4.0 mm	Unknown or unspecified
T3a	>2.0–4.0 mm	Without ulceration
T3b	>2.0–4.0 mm	With ulceration
T4	>4.0 mm	Unknown or unspecified
T4a	>4.0 mm	Without ulceration
T4b	>4.0 mm	With ulceration

Table 4. T category.

	Extent of Regional Lymph Node and/or Lymphatic Metastasis	
N Category	**Number of Tumor-Involved Regional Lymph Node**	**Presence of In-Transit, Satellite, and/or Microsatellite Metastases**
NX	Regional nodes not assessed (eg, SLN biopsy not performed, regional nodes previously removed for another reason) Exception: When there are no clinically detected regional metastases in a pT1 cM0 melanoma, assign cN0 instead of pNX	No
N0	No regional metastases detected	No
N1	One tumor-involved node or in-transit, satellite, and/or microsatellite metastases with no tumor-involved nodes	
N1a	One clinically occult (ie, detected by SLN biopsy)	No
N1b	One clinically detected	No
N1c	No regional lymph node disease	Yes
N2	Two or three tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with one tumor-involved node	
N2a	Two or three clinically occult (ie, detected by SLN biopsy)	No
N2b	Two or three, at least one of which was clinically detected	No
N2c	One clinically occult or clinically detected	Yes
N3	Four or more tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with two or more tumor-involved nodes, or any number of matted nodes without or with in-transit, satellite, and/or microsatellite metastases	
N3a	Four or more clinically occult (ie, detected by SLN biopsy)	No
N3b	Four or more, at least one of which was clinically detected, or presence of any number of matted nodes	No
N3c	Two or more clinically occult or clinically detected and/or presence of any number of matted nodes	Yes

Table 5. N category.

M Category	Anatomic Site	LDH Level
M0	No evidence of distant metastasis	Not applicable
M1	Evidence of distant metastasis	See below
M1a	Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph node	Not recorded or unspecified
M1a(0)		Not elevated
M1a(1)		Elevated
M1b	Distant metastasis to lung with or without M1a sites of disease	Not recorded or unspecified
M1b(0)		Not elevated
M1b(1)		Elevated
M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease	Not recorded or unspecified
M1c(0)		Not elevated
M1c(1)		Elevated
M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease	Not recorded or unspecified
M1d(0)		Normal
M1d(1)		Elevated

- Serum lactate dehydrogenase (LDH)
- Suffixes for M category: (0) LDH not elevated, (1) LDH elevated.
- No suffix is used if LDH is not recorded or is unspecified.

Table 6. M Category.

Clinical Staging (cTNM)*

	T	N	M
Stage 0	Tis	N0	M0
Stage IA	T1a	N0	M0
Stage IB	T1b	N0	M0
	T2a	N0	M0
Stage IIA	T2b	N0	M0
	T3a	N0	M0
Stage IIB	T3b	N0	M0
	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T, Tis	≥N1	M0
Stage IV	Any T	Any N	M1

*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic/biopsy evaluation for metastases. By convention, clinical staging should be used after biopsy of the primary melanoma, with clinical assessment for regional and distant metastases. Note that pathological assessment of the primary melanoma is used for both clinical and pathological classification. Diagnostic biopsies to evaluate possible regional and/or distant metastasis also are included. Note there is only one stage group for clinical Stage III melanoma.

Figure 3. Clinical staging

Pathological Staging (pTNM)**

	T	N	M
Stage 0†	Tis	N0	M0
Stage IA	T1a	N0	M0
	T1b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
	T3a	N0	M0
Stage IIB	T3b	N0	M0
	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage IIIA	T1a/b, T2a	N1a, N2a	M0
Stage IIIB	T0	N1b, N1c	M0
	T1a/b, T2a	N1b/c, N2b	M0
	T2b, T3a	N1a/b/c, N2a/b	M0
Stage IIIC	T0	N2b/c, N3b/c	M0
	T1a/b, T2a/b, T3a	N2c, N3a/b/c	M0
	T3b, T4a	Any N ≥ N1	M0
	T4b	N1a/b/c, N2a/b/c	M0
Stage IIID	T4b	N3a/b/c	M0
Stage IV	Any T, Tis	Any N	M1

**Pathological staging includes microstaging of the primary melanoma, including any additional staging information from the wide-excision (surgical) specimen that constitutes primary tumor surgical treatment and pathological information about the regional lymph nodes after SLN biopsy or therapeutic lymph node dissection for clinically evident regional lymph node disease.
†Pathological Stage 0 and pathological T1 without clinically detected regional or distant metastases (pTis/pT1 cN0 cM0) do not require pathological evaluation of lymph nodes to complete pathological staging; use cN0 to assign pathological stage.

Figure 4. Pathological staging.

Tables 4,5,6 and figures 3,4 show current TNM stage adapted from NCCN guidelines. https://www.nccn.org/professionals/physician_gls/pdf/cutaneous_melanoma.pdf

Regional lymph nodes represent the most common first site of metastasis in patients with primary melanoma. SLNB should be at the time of initial wide excision to not disturb the draining lymphatics.

Clinical occult nodal metastasis are those found at SLN biopsy but not detected in initial imaging or by clinical examination and generally have better survival than those with clinically evident disease (48). SLN biopsy detects clinically occult nodal metastases in 20% of patients at the time of diagnosis (49).

SLN biopsy should be performed from clinical stage IB (Breslow >0.8mm). 5% to 40% of patients will be upstaged to pathologic stage III (50).

When clinically detected lymph node involvement complete lymphadenectomy is the gold standard. Note that any positive lymph nodes are staged as stage III (49).

The number of tumor-involved lymph nodes is also an important predictor for survival. Primary tumor thickness is the single factor that most consistently predicts SLN positivity (51).

For patients with a positive SLN, complete nodal dissection is not anymore mandatory since 2 studies demonstrated no survival difference compared to close nodal observation by ultrasound

in specific populations. DeCOG-SLT phas III (52) and MSLT-II (53) trials have been practice-changing. Immediate complete lymph node dissection after a positive sentinel lymph node biopsy was associated with fewer initial nodal basin recurrences but similar melanoma-specific survival and distant metastases-free survival.

The German Dermatologic Cooperative Oncology Group–Sentinel Lymph node Trial (DeCOG-SLT was the first randomized clinical trial to assess the benefit of complete lymphadenectomy (CL) in melanoma patients with positive SLN biopsy. Enrolled in this trial were 483 patients with cutaneous melanoma on the trunk and limbs with a median follow-up of 35 months. No difference in metastasis-free survival was found between the groups with complete lymphadenectomy and the groups with spared nodal chain and followed by trimonthly ultrasound (66% of cases had micrometastases < 1.0 mm in the SLN). The authors concluded that CL should not be performed if the SLN presents micrometastasis less than 1.0 mm (50,52).

Patients in the MSLT-II study (Multicenter Selective Lymphadenectomy Trial II) had a positive SLN and were randomly assigned to receive CLND (Complete Lymph Node Dissection) immediately or observation with frequent clinical ultrasound evaluation. Melanoma specific survival was the main goal. The trial found no significant differences in melanoma-specific survival. However, the disease-free survival was slightly higher in the dissection arm. The authors concluded that complete lymphadenectomy, after SLN biopsy results were positive, could be waived as it had no effect on melanoma-specific survival, particularly in patients with little nodal deposit in the SLN and who were willing to undergo stringent ultrasound follow-up (50,53).

Complete lymph node dissection is associated with considerably greater morbidity than sentinel node biopsy alone and worsening of the quality of life specially for cronic lymphedema. In addition, patients with non-sentinel lymph nodes recurrences may still go on salvage lymphadenectomy (52,53).

8-10% of occult node metastases are found in complete lymph-node dissection after positive SLN biopsy (49).

Mucosal melanomas are not easily diagnosed because of their usually hidden location and because they are often amelanocytic. Therefore, they are diagnosed at later stages, with nodal involvement and with a worse prognosis. Even considering the stage at diagnosis, mucosal melanoma is associated with significantly worse survival outcomes compared to cutaneous and acral melanoma. In a recent retrospective study of a single center (Memorial Sloan Kettering

Cancer Center) of 3454 patients metastatic at diagnosis, they reported a median overall survival of 9.1 (MM), 13.4 (Uveal M), 11.4 (acral) and 11.7 (CM) months (54).

Surgical approach remains the only curative item for these patients. Wide local excision +/- SLN is the standard of care when initial stages. However, the anatomical surgical constraints and multifocal growth pattern significantly limit the ability for wide negative margins and impacts negatively in patient's quality of life. Unfortunately, 50–90% of patients exhibit postoperative local recurrence, even in the context of achieving negative margins (20).

Currently, there has been no reproducible staging system for MM. Information regarding Breslow and ulceration is often missing, making the staging system used for cutaneous melanomas futile (55). There are various systems proposed depending on location:

Various systems have been used depending on location, including the Ballantyne system which only considered three stages (Local, Regional and Disseminated disease) and the AJCC TNM 8th edition which has a specific staging system for H&N mucosal melanoma where assigns a minimum stage for the primary tumor as T3 (56). Other cases are extrapolated from same location staging system or cutaneous melanoma. NCCN guidelines include specific clinical guidelines for H&N and vulvar melanoma.

5.4 ONCOLOGICAL TREATMENT.

Surgical excision is the primary treatment for primary melanoma. Several prospective randomized trials have been conducted to define optimal surgical margins (57).

For in situ melanoma, a margin of 0.5 to 1 cm around the visible lesion should be obtained. For melanomas 1.0 mm or less, wide excision with a 1 cm margin is recommended. For melanomas measuring 1.01 to 2 mm in thickness wide excision with a 1 to 2cm margin is recommended. For melanomas measuring more than 2 mm in thickness, wide excision with 2 cm margins is recommended. Surgical margins may be modified to accommodate individual anatomic where 2cm margins would be difficult to achieve or for cosmetic considerations (58).

Role of SLN biopsy and complete lymphadenectomy has been discussed above at point 1.3.

Overall survival has historically been poor for patients with distant metastatic disease, and response to conventional chemotherapy is infrequent. The current oncological treatments will be discussed below, and all recommendations are made according to the current clinical guidelines.

5.4.1 Adjuvant radiotherapy may be considered for selected patients with clinically positive nodes or extracapsular involvement. Theres is one prospective phase III trial that showed statistical benefit in regional control versus placebo, but no benefit in PFS or OS. The dose range was 48-50Gy (59).

5.4.2 Adjuvant systemic therapy is not recommended for patients with stage I/II disease. Approved treatments are for stage III or stage IV completely resected disease.

High dose IFN alfa was used in the past with small but significant benefit (60). Nowadays, targeted therapies and immune checkpoint inhibitors have supplanted IFN alfa and is no longer recommended. There are several recent prospective trials supporting new therapies for adjuvant setting although none has been compared to IFN alfa. However, given the fact that they are more effective and less toxic.

There are four phase III representative trials, including patients with stage IIIA > 1mm or greater, that show statistically significant benefit in RFS/DFS. Note that the TNM system was 7th edition where stage IIIA disease is a higher risk group than in 8th edition.

These four trials are:

EORTC 1807: High dose Ipilimumab vs placebo. IIIA >1 mm, IIIB/C no IT (in transit) (61)

CheckMate 238: Nivolumab vs High dose Ipilimumab (10mg/kg). IIIB/C, **IV.** Results: adjuvant therapy with nivolumab resulted in significantly longer recurrence-free survival and a lower rate of grade 3 or 4 adverse events than adjuvant therapy with ipilimumab (62).

KEYNOTE-054: Pembrolizumab vs placebo**.** IIIA >1 mm, IIIB/C no IT (63).

COMBI-AD: Dabratenib/Trametinib vs placebo. IIIA >1 mm, IIIB/C**.** Patients were required to have **BRAF V600E or V600K mutation**. (64)

Entry criteria for all the trials mentioned above required primary tumor excision with adequate margins and complete lymphadenectomy in patients with nodal metastases detected by SLN biopsy. In patients with clinically occult nodal disease, it is reasonable to consider nodal basin ultrasound surveillance, although it is unclear whether the recommended adjuvant treatment options have similar efficacy.

Trials using immune checkpoint inhibitors were not stratified for BRAF status which make them an option for all commers, and the results are independent of PD-L1 expression.

Based on those studies pembrolizumab and nivolumab are the best options in adjuvant setting. If resected recurrence, and prior exposure to anti-PD1 agent, ipilimumab seems a reasonable option as an adjuvant option. Efficacy of ipilimumab was demonstrated using the high dose 10mg/kg (61) but the lower dose 3mg/kg is safer and may be equally effective.

For patients who harbor a BRAF V600-activating mutation and have a stage III resected disease, dabrafenib/trametinib is a feasible option for adjuvant treatment.

The duration of the adjuvant treatment is one year.

5.4.3 Neoadjuvant systemic therapy is not a recommended option outside of a clinical trial. There are few clinical trials with immunotherapy and tyrosine kinase inhibitors (TKIs) ongoing with promising results.

5.4.4 In-transit disease represents a distinct disease pattern whereby the disease recurs as dermal or subcutaneous nodules between the primary melanoma site and the regional lymph node basin. Excision to clear margins is the mainstay of treatment in patients with resectable in-transit metastasis with a small number of lesions. Another option is intralesional injection with IL-2 or T-VEC (Talimogene laherparepvec). There are several clinical studies that support IL-2 injection and complete response rates may be in >70% (65).

T-VEC is an agent that uses a modified herpes simplex virus to induce tumor cell lysis and to deliver localized expression of GM-CSF to injected lesions. In a phase III trial a 64% response rate is reported with bystander effect of other lesions (66).

Laser ablation, topical imiquimod, or RT are options that may help for palliation or to establish regional control for selected patients with unresectable in-transit disease.

5.4.5 Oncological treatments for advanced melanoma. Treatment for unresectable stage III or distant metastatic disease (stage IV).

-Immune Checkpoint Inhibitors are antibodies against T-cells/tumoral cells receptors (PD-1, PD-L1, CTLA4…) that trigger a signaling cascade that inhibits immune response. By preventing receptor-ligand interaction, immune response is released. In addition, melanomas carry a high mutation burden making them more sensitive to immune checkpoint inhibitors due to high number of “neoantigens” that are recognized as foreign to the adaptative immune system. For these reasons, immunotherapy is one of the backbones of oncological treatment in melanoma patients (67). Cytotoxic chemotherapy has very limited effectiveness and immunotherapy has completely changed the landscape of melanoma treatment.

The preferred regimens based on clinical trials are nivolumab, pembrolizumab and Ipilimumab/Nivolumab combination.

Single-agent ipilimumab monotherapy is no longer recommended first-line therapy option due to the results from the CheckMate 067 phase III trial (68) showing improved outcomes with nivolumab or nivolumab/ipilimumab combination therapy compared with ipilimumab monotherapy.

In the **CheckMate 067** study median progression-free survival was 11.5 months (95% confidence interval [CI], 8.9 to 16.7) for nivolumab plus ipilimumab as compared with 2.9 months (95% CI, 2.8 to 3.4) for ipilimumab alone (HR, 0.42; 95% CI, 0.31 to 0.57; p<0,0001) and was 6.9 months (95% CI, 4.3 to 9.5) for nivolumab alone (HR in the comparison with ipilimumab alone, 0.57; 95% CI, 0.43 to 0.76; P<0.00001). In PD-L1-positive patients, median PFS was 14.0 months in both the nivolumab plus ipilimumab and nivolumab alone groups, but in PDL1-negative patients, PFS was longer with the combination as compared with nivolumab alone (11.2 months [95% CI, 8.0 to not reached] versus 5.3 months [95% CI, 2.8 to 7.1]).

In this study the effectivity in BRAF-mutant patients is also reflected. The median PFS reported for the combination of nivolumab and ipilimumab in this study (11.7 months in BRAF-mutant patients) is similar to that recently reported for the combination of BRAF and MEK inhibition in BRAF-mutant metastatic melanoma (9.9 months for vemurafenib and cobimetinib; 9.3 to 11.4 months for dabrafenib and trametinib).

Selection between anti-PD-1 monotherapy and nivolumab/ipilimumab combination therapy should be informed by the consideration that, although combination therapy may improve PFS relative to nivolumab monotherapy, it is associated with a much higher risk of serious immunomediated toxicities compared with nivolumab monotherapy.

In the **Keynote-006** phase III trial (69) pembrolizumab was superior to ipilimumab in PFS and OS. Median PFS of pembrolizumab Q2W was 5.5 months (95% CI, 3.4 to 6.9), Q3W 4.1 months (95% CI, 2.9 to 6.9), and ipilumumab 2.8 months (95% CI, 2.8 to 2.9).

The regimen's approved doses are:

-1 mg/kg nivo + 3 mg/kg ipi (same day), Q3W for 4 doses; then 3 mg/kg nivo monotherapy Q2W.

-Nivolumab 240 mg Q2W or 480 mg Q4W.

-Pembrolizumab 200 mg Q3W or 400mg Q6W.

The toxicity related to ICI is mainly derived from the autoimmune nature of the events. The most common adverse events are cutaneous toxicities (rash, pruritus, vitiligo…), gastrointestinal toxicities (diarrhea/colitis) and endocrinopathies (hypo- or hyperthyroidism, adrenal insufficiency, hypophysitis…) (70). Life-threatening immune-related toxicities but less common are nephritis, pneumonitis, and myocarditis. The treatment is based on glucocorticoids at a dose of mg/kg until the reversion of the autoimmune event.

-**BRAF targeted therapies.** Approximately half of patients with metastatic cutaneous melanoma harbor an activating mutation of BRAF. (71) Most BRAF-activating mutations are at residue V600 (usually V600E 90% but occasionally V600K 9% or other substitutions 1%) (72).

Co-administration of inhibitors of MEK, a signaling molecule downstream of BRAF, potentiates these effects and are recommended in front of single agent BRAF inhibitor monotherapy. The combinations improve the response rates, duration of response, PFS and OS.

Dabrafenib, encorafenib and vemurafenib are oral inhibitors of BRAF.

Trametinib, cobimetinib, and binimetinib are oral inhibitors of MEK1 and MEK2.

The proposed combinations are: dabrafenib/trametinib, encorafenib/binimetinib, vemurafenib/cobimetinib.

Hereby there is a summary of the most relevant phase III trials. In all the trials prior treatment with iBRAF was not allowed. In the COLUMBUS TRIAL IFN alfa or interleukins were permitted. Patients with brain metastasis were excluded from the trials unless treated and stable.

COMBI-d: Dab/Trame vs Dab/placebo. Median PFS 11m vs 8.8m, p=0.0004. OS 25.1m vs 18.7m, p=0,017 (73).

Co-BRIM: Vem/cobi vs Vem/placebo. Median PFS 12.3m vs 7.2m, p<0,0001. OS 22.3m vs 17,4m, p=0,005 (74).

COLUMBUS: Encor/bini vs Encor or Vem. PFS 14,9 vs 7.3 with vem, but no significant compared to encor 9.6m p=0,051 (75).

Most common toxicity related to the combos is: flu-like symptoms, diarrhea, hypertension, transaminitis, arthralgias and rash.

-**Imatinib** is a c-KIT, BRC-ABL and PDGFRA inhibitor. KIT mutations are associated with mucosal and acral melanomas. Several phase II studies testing imatinib have been performed, in patients with KIT-mutated or KIT-amplified metastatic melanomas. These studies demonstrated 20 to

30% ORR and 35% to 57% disease control rate (76). Two patients achieved durable complete responses and harbored a KIT-L576P mutation/amplification in exon 11 (77). This study suggests that some KIT mutations, such as L576P and K642E, may possess a greater oncogenic driver capacity, and thus increased sensitivity to KIT inhibition, hence the need for molecular oncology.

To summarize: For first-line therapy recommended treatment options include immune checkpoint inhibitors regardless of BRAF status, BRAF-targeted therapy for patients with BRAF mutation, or clinical trial if possible. The use of PD-L1 as a biomarker for selection of anti-PD-1 therapy alone or combination of nivolumab/ipilimumab is an emerging research issue with non-uniform application. The combination is far more toxic, and patients should be selected with caution. Symptomatic CNS disease must be controlled and stable before starting systemic therapies.

For patients with BRAF V600 mutations, selection between first-line immune checkpoint inhibitors or BRAF-targeted therapy remains controversial given the lack of comparative phase III clinical trials. Ideally, we would be able to determine molecular or clinical biomarkers to help guide these decisions. In routine clinical practice, decisions must be taken according to patient comorbidities, history of autoimmune disease, volume of the disease or cancer related symptoms. ICI's responses can take longer to develop, thus BRAF-targeted therapy may be preferred in cases where the disease is symptomatic or rapidly progressing.

Furthermore, overcoming resistance to BRAF/MEK inhibitors has been a challenging obstacle that could be targeted by ERK inhibitors or next-generation RAF inhibitors, but this is yet to come. Continuous research will bring improvements to take care of patients with this disease.

5.4.6 Radiotherapy.

-Radiation for Brain Metastases. Melanoma is the third most common source of brain metastases following lung and breast cancer, and more than 60% of patients with metastatic melanoma either present with or develop brain metastases during the course of their disease (78). New targeted systemic agents are improving outcomes but still have limited efficacy within the central nervous system and patients are underrepresented in clinical trials.

Stereotactic radiosurgery (SRS) is important in the management of CNS metastases from melanoma, and it is often being used in routine clinical practice. Retrospective studies have shown 1-year local tumor control rates from 72%-100% for patients with limited CNS disease, but lower rates for patients with multiple or large (>2 cm) tumors, bleeding after the procedure is the most common adverse event (79,80).

WBRT (Whole brain radiotherapy) formerly used for all patients is now being replaced by SRS/SBRT (Stereotactic Body Radiation Therapy). The trend of WBRT is to be reserved for patients with a high number or voluminous lesions. This is because some studies have demonstrated late adverse effects of WBRT on cognitive function and negative impact in quality of life (81).

-**Ablative treatment for extracranial metastases.** SBRT may offer more durable local control and freedom from regional or distant progression compared to systemic therapies when talking about oligometastatic patients. SBRT is a valid option to consider (82).

5.4.7 Mucosal Melanomas. Treatment is extrapolated from CM because their representation in clinical trials is very poor. There is a lack of validated clinical guidelines to follow. MM are challenging to treat. They are typically detected at a more advanced stage, lack dominant MAP kinase-activating mutations, and respond to immunotherapy less. Identifying actionable driver mutations such as c-KIT may change the course of this disease.

When localized disease at diagnosis, adjuvant RT may be considered to improve local control of the disease but does not change the overall survival (83,84).

A pooled analysis (85) shows a response rate of 23% in MM vs 41% in CM when using nivolumab alone. When using the combination (Nivo/Ipi) the response rate was 37% for MM vs 60% for CM.

In *figure 5* there is a summary of the disponible therapies and where they act.

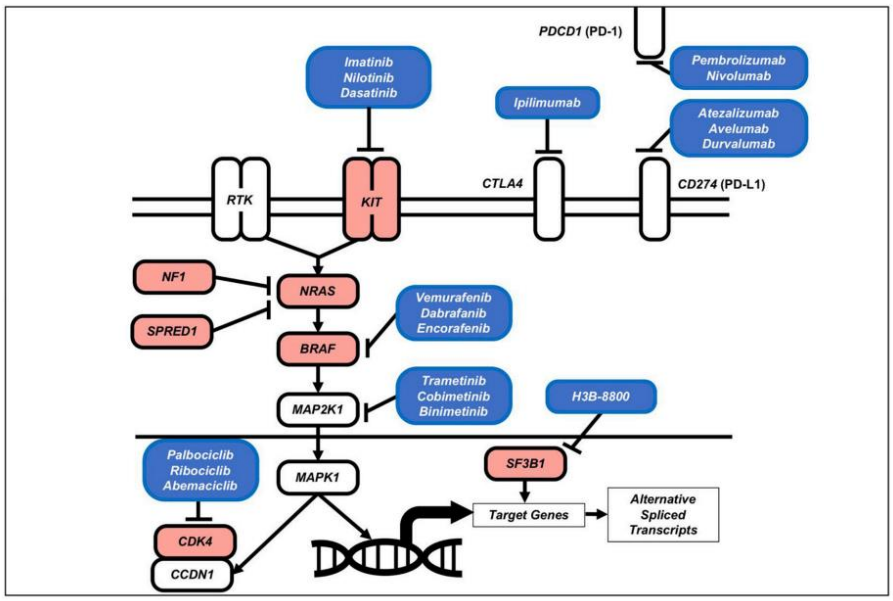

Figure 5 Oncogenic signaling and therapeutic targets in Mucosal Melanoma. Kelsey W et al. (33)

6 OBJECTIVES AND HYPOTHESES

HYPTOHESES

1-The epidemiological characteristics of skin and mucosal melanoma in Girona and province is comparable to that in neighboring areas.

2-The incidence of skin melanoma in Girona is increasing as it is in the rest of the world.

3-BRAF is a prognostic factor in skin melanoma.

4-The distribution of N-RAS, BRAF and c-KIT mutations in our population of mucosal melanoma is similar to the one published in other series.

5-Molecular findings in our mucosal melanoma cohort are in accordance with the other analysis published in the literature.

6-Toxicity of immune-checkpoint inhibitors differs from previous oncological treatments an needs clinicians awareness.

OBJECTIVES

1- To analyze the incidence, the trend of incidence, mortality, and survival of skin melanoma in Girona 2009-2011.

2- To analyze the incidence, the trend of incidence, mortality, and survival of mucosal melanoma in Girona 1994-2018.

3- To determinate the prevalence of BRAF mutation of cutaneous melanoma (2009-2011) and to correlate it with other prognostic factors and survival.

4- To perform a molecular analysis with NGS of mucosal melanoma of the Head and neck, vulvovaginal and rectal.

5-To exemplify routine clinical practice of management of melanoma patients.

7 MATERIAL AND METHODS

We analyzed the database belonging to the Girona Cancer Registry (GCR), a population-based cancer registry of Girona province in the north-east of Spain, which began keeping records in 1994 and covers a population of 761,627 inhabitants (census 2012 available at URL: www. idescat.cat). Cases are registered according to the International Agency for Research on Cancer (IARC) guidelines with a completeness of 96.3%. Regarding quality indicators from the period 2013-2017 of melanoma, the GCR has a 99.5% of histologic verification and a 0% of detection only by death certificate.

GCR participates actively through initiatives like the European Network of Cancer Registries (ENCR) in publishing data from population-based cancer registries throughout Europe and identifying epidemiological trends and associated survival.

Population-based data are essential for understanding cancer epidemiology and are subject to very low bias.

The region has seven community hospitals and a referral center, the University Hospital Josep Trueta. The seven community hospitals have their own dermatologists who could have made the first diagnosis and the surgical approach. These histological samples were referred to Hospital Josep Trueta for central pathologists to review the histological characteristics of each case, especially Breslow index, ulceration, and amount of mitosis per mm.

Incidence was analyzed in terms of crude rate (CR) and World and European age-standardized rate (ASRw, ASRe) in both articles, CM and MM. Trends were assessed using the estimated annual percentage of change (EAPC) of the ASRe. The joinpoint loglinear regression version 4.3.2.1.0 model was used to calculate EAPC.

In the first article about CM and BRAF gene (2) we analyzed observed (OS) and relative survival (RS) of the cohort as a whole and depending on prognostic factors, and performed a univariate and multivariate analysis of prognostic factors including sex, age above or below the median age at diagnosis of the cohort, sun-exposed area of primary tumor or not (skin of scalp, face and neck, or other areas), stage, histological subtype, number of mitosis (above or below and equal to 2 per mm2) and BRAF status (mutated versus wild-type).

We decided to perform a multivariate analysis including the variables that initially univariately approach significance to have more statistical power in our results. The model itself discriminates whether multivariately they continue to maintain significance or not and ends up

'choosing' which are more important for survival (86). If we had manually entered or excluded variables, we could have fallen into the error of entering or excluding variables that are really influencing in survival. Multivariate models determine the contribution of each variable in survival and provide a powerful test of significance compared to univariate techniques. On the contrary, they need a larger sample of cases to avoid high standard errors.

RS was analyzed to adjust survival relative to other causes of death in the same age groups in the cohort. Analyses of survival were computed using the Kaplan Meier method for OS and Pohar-Perme method for RS (87). Significance was determined at p = 0.05 to compare survival groups using the likelihood-ratio test. Vital status was updated on the 31st of October of 2019 by reviewing routine clinical practice reports and/or the Mortality Registry of Catalonia and the Spanish National Death Index. Multivariate Cox regression models were performed including variables with a p value at or below 0.2 based on the univariate analysis, we did not use relative excess risk models for competitive risks (88). Analyses were performed using R software v.3.

Relative survival (89) is a standard indicator for comparing cancer survival in population-based studies. Relative survival is the ratio of the observed survival of patients to the expected survival in a comparable group in the general population for the same region, age, sex and calendar year. It can be interpreted as the survival probability of cancer patients in the absence of other causes of death, which can vary widely between countries. Whereas overall survival is the probability to survive after a given time from diagnosis (5-year or 10-year), regardless from the cause of death, and can be less comparable between different geographic areas but is more commonly used in oncological clinical trials.

When studying population-based cancer survival, we are typically interested in estimating the probability that patients will die of their specific cancer. A common approach with competing-risks data is to classify the cause of death of each individual who dies and use this information to estimate what is commonly called cause-specific survival. Such an approach can be problematic with cancer registry data because information on cause of death is often unreliable or unavailable. As such, it is common in population-based cancer survival to instead estimate the chosen measure (crude or net probability) in a relative-survival (RS) framework, where cause-of-death information is not required (87).

In the second article about MM and molecular analysis (3) for the survival analysis, we calculated follow-up time from diagnosis to patients' last vital status recorded. To obtain these data, we reviewed hospital clinical reports and/or the Mortality Registry of Catalonia and the Spanish National Death Index. Vital status was updated on the 31st of August 2021. The observed

survival (OS) estimates were analyzed by Kaplan–Meier method using R software v.3.6.2 (90). Relative survival was not published due to the small sample, the results were inconclusive with overly wide confidence intervals.

See ANNEX 1 to view the complete form of the two articles cited below for detailed data of molecular analysis.

1. J. Rubió-Casadevall, A. Carbó-Bagué, M. Puigdemont, G. Osca-Gelis, G. Oliveras, N. Vilar-Coromina, B. Ferrer-Fabrega, A. Urban, M. Llobet-Roma, F. Martín-Romero, F. Perez-Bueno, R. Marcos-Gragera. Population-based analysis of the prevalence of BRAF mutation in patients diagnosed with cutaneous melanoma and its significance as a prognostic facotr. *Eur J Dermatol 2021* Oct 1;31(5):616-622.

2. A. Carbó-Bagué, J. Rubió-Casadevall, M. Puigdemont, A. Sanvisens, G. Oliveras, M. Coll, B. Del Olmo, F. Perez-Bueno, R. Marcos-Gragera. Epidemiology and Molecular Profile of Mucosal Melanoma: A Population-Based Study in Southern Europe. *Cancers (Basel).* 2022 Feb; 14(3): 780.

8 RESULTS

8.1 DESCRIPTIVE EPIDEMIOLOGY OF CUTANEOUS MELANOMA IN GIRONA (2009-2011).

We found 286 incident cases of CMM between 2009 and 2011; 149 women (52%) and 137 men (48%). Of these, 78 cases were in situ melanoma (27.3%) and 208 invasive melanoma (72.7%). Median age at diagnosis was 62 years. Ten cases (3.5%) were melanoma with an unknown primary. The distribution of the whole cohort according to stages was: 27.3% in Stage 0 or pTis (78 cases), 29.7% in Stage IA (85 cases), 11.9% in Stage IB (34 cases), 7.0% in Stage IIA (20 cases), 5.9% in Stage IIB (17 cases) and the same number of cases in Stage IIC, 2.1% in Stage IIIA (six cases), 1.4% in Stage IIIB (four cases), 5.6% in Stage IIIC (16 cases) and nine cases (3.1%) in Stage IV at the time of diagnosis. Therefore, 57.0% of all patients were diagnosed with early melanoma (in situ or Stage IA), which is also in concordance to the literature as melanoma is usually detected in early stages (23).

At the time of last follow-up visit, on 31st October 2019, 188 patients were still alive (65.7%), 76 were deceased (26.6%) and 22 were lost to follow-up (7.7%). The median time of follow-up was 8.5 years.

Table 7 shows overall survival and relative survival.

	n at risk	5-year OS (95% CI)	10-year OS (95% CI)	5-year RS (95% CI)	10-year RS (95% CI)
All cases	286	81.5 (77.1-86.2)	71.0 (65.3-77.2)	91.1 (85.9-96.7)	87.0 (76.8-98.5)
Gender					
Men	137	73.7 (66.5-81.6)	61.8 (52.5-72.6)	85.3 (76.5-95.1)	83.0 (68.2-101.0)
Women	149	87.9 (82.8-93.3)	79.0 (72.5-86.1)	95.2 (89.6-101.1)	90.0 (77.0-105.2)
***BRAF* mutation status**					
Negative	120	73.1 (65.5-81.5)	61.2 (52.0-72.0)	84.3 (75.3-94.8)	82.5 (64.5-105.5)
Positive	121	86.5 (80.6-92.9)	82.8 (76.2-90.0)	93.6 (87.1-100.5)	93.4 (83.0-105.0)
Stage					
Stage 0 (pTis)	78	93.6 (88.3-99.2)	84.7 (76.6-93.7)	104.7 (98.9-110.8)	101.4 (85.1-121.0)
Stage I	119	91.4 (86.4-96.6)	79.6 (70.0-90.4)	99.1 (93.4-105.1)	94.9 (82.8-108.7)
Stage II	54	69.3 (57.9-83.1)	61.4 (49.4-76.2)	86.9 (71.7-105.3)	85.0 (56.0-129.0)
Stage III	26	42.3 (27.0-66.3)	29.6 (16.2-54.2)	44.5 (28.5-69.5)	34.5 (19.4-61.5)
Stage IV	9	11.1 (1.7-70.5)	-	12.2 (2.7-55.6)	-
Ulceration					
Negative	142	88.5 (83.3-94.0)	77.8 (69.2-87.6)	96.1 (89.5-103.2)	90.6 (79.1-103.7)
Positive	56	52.0 (40.2-67.2)	39.6 (28.2-55.6)	65.8 (51.9-83.5)	63.5 (38.4-104.9)

n: number of cases; OS: observed survival; RS: relative survival; CI: confidence interval.

Table 7. Five- and ten-year observed and relative survival according to gender, stage, ulceration and BRAF mutation status.

We describe a 5-year OS of 81.5% for all stages which is strictly similar to other large studies and published to all clinical-guidelines (19).

Half of the cohort had a mutation in BRAF, and half doesn't. BRAF mutations have been described between 40-50% in CM, so we are in values, meaning that BRAF test was accurate (40).

Characteristics	Number of cases (%)
Gender	
Males	97 (48.5)
Females	103 (51.5)
Stage	
IA	81 (40.5)
IB	33 (16.5)
IIA	19 (9.5)
IIB	17 (8.5)
IIC	14 (7.0)
IIIA	6 (3.0)
IIIB	5 (2.5)
IIIC	16 (8.0)
IV	9 (4.5)
Presence of ulceration	
Positive	54 (27.0)
Negative	136 (68.0)
Missing	10* (5.0)
Histological subtype	
Nodular	35 (17.5)
Superficial spreading	114 (57.0)
Lentigo maligna	13 (6.5)
Acral	12 (6.0)
Other**	26 (13.0)
Mitotic count	
≤ 2 per mm^2	106 (53.0)
>2 per mm^2	83 (41.5)
missing	11 (5.5)
*Sun-exposed skin****	
Yes	33 (16.5)
No	167 (83.5)
BRAF mutation status	
Positive	98 (49.0)
Negative	84 (42.0)
Missing	18 (9)

We only performed the analysis of prognostic factors in cases with invasive CM, thus a total of 208 cases in the cohort. Eight cases were also discarded because we could not find a tissue sample (seven cases) or due to refusal to participate in the study (one case). *Table 8* shows the characteristics.

Our "missings" did not exceed 10% which is a reasonable number compared to other population-based studies.

40% of the cases were stage IA, non-ulcerated, superficial spreading subtype and in non-sun-exposed skin. Sun-exposed-skin refers to the scalp, face, and neck.

Table 8 Characteristics of the 200 cases of infiltrative melanoma.

8.2 BRAF AND PROGNOSTIC FACTORS

Analysis of BRAF status was performed in 271 cases (94,7% of the total cohort). We only performed the analysis of prognostic factors in cases with invasive CMM, thus a total of 200 cases as said before 8 were not available.

Excluding missing cases and those in which the DNA was insufficient, BRAF-mutated cases constituted 38.9% of "in situ" melanoma cases (23 of 59) and 53.8% of invasive melanoma cases (98 of 182).

The univariate and multivariate analysis of prognostic factors including BRAF mutation status are summarized in *table 9*. Only those variables with $p < 0.2$ significance based on the univariate

analysis were included in the multivariate analysis. Stage, BRAF status and mitotic count were significant based on the univariate analysis, but only stage maintained its significance for survival in multivariate analysis.

Although the number of mitoses has not been included as a prognostic factor in the TNM 8th edition and its prognostic value is currently under discussion as it is affected by inter-observer variation, we decided to include it as a prognostic factor because we believe it still has its role. We used more than two mitoses per mm2 as the cut-off point. No difference in distribution of BRAF mutation was identified based on mitotic count.

Variable (number of cases)	5-year relative survival	10-year relative survival	Univariant significance	Multivariant significance
Age				
<62 ($n=96$)	91.4 (85.5-97.7)	90.7 (84.2-97.8)	$p=0.974$	
≥62 ($n=104$)	78.6 (67.3-92.0)	69.8 (50.0-97.4)		
Gender				
Men ($n=97$)	75.9 (64.9-88.7)	74.8 (59.3-94.3)	$p=0.239$	
Women ($n=103$)	91.4 (83.7-99.7)	82.7 (65.9-103.7)		
***BRAF* mutation status**				
Negative ($n=84$)	76.3 (64.6-90.2)	73.0 (52.1-102.2)	$p=0.10$*	$p=0.907$
Positive ($n=98$)	90.0 (82.1-98.5)	90.5 (78.8-103.9)		
Stage				
I ($n=114$)	99.0 (93.1-105.2)	94.3 (81.7-108.8)		
II ($n=51$)	86.0 (70.1-105.5)	83.8 (54.0-129.8)	$p<0.001$*	$p<0.001$
III ($n=26$)	44.5 (28.5-69.5)	34.5 (19.4-61.5)		
IV ($n=9$)	12.15 (2.7-55.6)	12.15 (2.7-55.6)		
Mitotic count				
≤2 ($n=106$)	99.6 (93.5-106.0)	95.3 (82.2-110.4)	$p<0.001$*	$p=0.505$
>2 ($n=83$)	70.4 (58.4-84.9)	66.4 (47.0-93.7)		
Sun-exposed skin				
No ($n=167$)	85.3 (78.5-92.6)	82.0 (69.4-96.8)	$p=0.690$	
Yes ($n=33$)	76.5 (56.7-103.2)	69.1 (40.6-117.4)		
Histological type				
Nodular ($n=35$)	81.7 (67.6-98.8)	86.5 (67.6-110.7)		
Superficial spreading ($n=114$)	89.0 (81.8-96.9)	87.9 (57.8-100.0)	$p=0.240$	
Acral ($n=12$)	75.7 (47.1-121.6)	60.0 (16.5-218.8)		
Lentigo maligna ($n=13$)	95.4 (75.3-120.9)	-		
Other ($n=26$)	53.9 (34.0-85.2)	63.1 (39.9-99.9)		

**only those variables with $p<0.2$ were included in multivariate analysis.*

Table 9. Five- and ten-year relative survival and results of univariate and multivariate analysis of prognostic factors. (

8.3 EPIDEMIOLOGY OF MUCOSAL MELANOMA 1994-2018.

Forty-two patients were identified: 14 (33%) had vulvar-vaginal melanoma, 15 (35.7%) had rectal melanoma, 12 (28.6%) had melanoma located in the head and neck sphere and 1 male patient had a urethral melanoma.

The ASIRw of MM in Girona between 1994 and 2018 for both sexes was 0.14. It was 0.10 for males and 0.16 for females, which was higher because of the number of vulvar-vaginal melanomas according to other articles in the literature.

The ASIRe for vulvar-vaginal, rectal, and head and neck melanoma were 0.09, 0.1 and 0.09 cases/100,000 inhabitant-years, respectively.

Results are summarized in *table 10.*

Characteristics				CR (per 100.000) (95% CI)			ASIRe (per 100.000) (95% CI)			ASIRw (per 100.000) (95% CI)		
Site	*n* (%)	M/F (%)	Med Age [IQR]	M	F	Total	M	F	Total	M	F	Total
Head & Neck	12 (28.6)	6/6	72.5 [57.0–91.2]	0.07 (0.01–0.13)	0.07 (0.01–0.13)	0.07 (0.03–0.11)	0.09 (0.03–0.22)	0.08 (0.03–0.17)	0.09 (0.05–0.16)	0.05 (0.02–0.15)	0.025 (0.01–0.13)	0.04 (0.02–0.09)
Rectal	15 (35.7)	6/9	69.9 [65.9–82.3]	0.07 (0.01–0.13)	0.11 (0.04–0.18)	0.09 (0.05–0.14)	0.09 (0.03–0.23)	0.10 (0.05–0.20)	0.10 (0.06–0.17)	0.05 (0.02–0.15)	0.036 (0.01–0.14)	0.04 (0.02–0.10)
Vulvar-vaginal	14 (33.3)	0/14	64.4 [57.8–75.4]	–	0.17 (0.08–0.26)	0.09 (0.04–0.13)	–	0.18 (0.10–0.30)	0.09 (0.05–0.16)	–	0.10 (0.05–0.22)	0.05 (0.03–0.11)
Urethral	1 (2.4)	1/0	69.8	0.01 (0–0.04)	–	0.01 (0–0.02)	0.01 (0–0.13)	–	0.01 (0–0.04)	0.008 (0–0.106)	–	0.004 (0–0.054)
All	42 (100)	13/29	68.4 [59.9–84.0]	0.16 (0.07–0.24)	0.36 (0.23–0.48)	0.26 (0.18–0.33)	0.19 (0.10–0.36)	0.36 (0.24–0.52)	0.29 (0.21–0.40)	0.10 (0.05–0.22)	0.16 (0.10–0.29)	0.14 (0.09–0.21)

N: Number of cases; M: males; F: females; CI: confidence interval; CR: crude rate; ASIRe: European age-adjusted standard incidence rate ASIRw; world age-adjusted standard incidence rate and IQR: interquartile range.

Table 10. Gender distribution and incidence rates of mucosal melanoma in Girona 1994–2018.

OS at 5 years was 7.7% in men, 34.5% in women and 26% for both sexes. Survival rates were 35.7% (Vulvo-vaginal) ,20% (rectal, only females were alive at 5-years) and 25% (H&N).

In *table 11* results for OS are shown.

Site	5y OS (%) (95% CI)		
	Males	Females	Total
Head and Neck	16.7 (2.8–99.7)	33.3 (10.8–100)	25.0 (9.4–66.6)
Rectal	0	33.3 (13.2–84.0)	20.0 (7.3–55.0)
Vulvovaginal	NA	35.7 (17.7–72.1)	NA
All	7.7 (1.2–50.6)	34.5 (20.9–56.9)	26.2 (15.8–43.5)

Table 11. Five-year observed survival for all stages of mucosal melanoma in Girona 1994–2018.

Women survived longer, and vulvar-vaginal melanoma was the subgroup with better survival. This has algo been published in other MM studies (37).

8.4 MUTATIONAL ANALYSIS OF MUCOSAL MELANOMA.

From the 42 cases of the cohort, 24 cases (60%) were suitable for NGS analysis: nine rectal melanomas, eight head and neck (five nasal and three pharyngeal) melanomas and seven in the vulvar-vaginal area.

Detailed results can be found in the original article exposed in material and methods chapter.

Pathogenic somatic mutations of the studied genes were identified in 18 cases (75%). Eight cases had only one mutation, three cases had two mutations, five cases had three mutations and two had four mutations.

Only one patient had a BRAF G596R mutation, which is more typically seen in lung cancer and is present in 0.02% of all malignant solid tumors (91).There are open clinical trials for this type of mutation, also in melanoma (www.mycancergenome.org Accessed on 28 November 2021). MM do not usually harbor mutations in V600 as normally CM do.

NRAS mutations were found in three cases (12.5%), and one well-known KRAS G12C mutation, for which there are clinically tested drugs, was found.

NF1 mutations dominated, with seven cases (29%) predominantly in the rectum.

KIT mutation L576P exon 11 was found in one case of vulvar-vaginal melanoma. This type of mutation is the typical and most frequent found in MM. It is true, that more mutations in KIT were expected.

TP53 mutations, widespread in solid tumors, are present in MM too: in the present cohort, four cases were found (16.6%).

Amplifications of MYC were the most frequent copy number variations (CNV) with an average number of five copies found in eight patients.

Several other mutations were found that are very uncommon in melanomas and are of uncertain meaning, such as POLE mutations, which are well-characterized in endometrial tumors.

One patient with vulvar MM had a frameshift mutation in the BRCA1 gene with a variant allele frequency (VAF) of 80%. Another patient with rectal MM had a CDKN2A mutation with VAF of 45%. For this reason, an underlying germline mutation was suspected, and genetic counseling was recommended.

9 DISCUSSION

9.1 EPIDEMIOLOGY OF CUTANEOUS MELANOMA.

The Age Standardized Incidence-Rate of skin melanoma in our area, formerly published from 1994-2013 in an article out of this thesis, was ASRe 9.7 and ASRw 7.2 per 100.000inhab/year. For invasive melanoma ASRe 7.52, ASRw 5.6 and ASRe 2.15, ASRw 1.59 for “in situ” melanoma. We have also observed in our region a significant increase in incidence of “in situ” melanomas

or melanomas of less than 1 mm based on Breslow index in concordance with the trend in Europe and USA (1).

In a study published in 2010 of our area (Catalonia) (13) they confirmed the rising trends of the incidence. The predicted number of CMM patients increased markedly for females, with a more than doubling of cases for the period 2015– 2019 compared with the most recent available observed period (2000–2002). A large proportion of the increase in observed CM incidence rates, mainly among women, is probably comprised of thin melanomas as has been observed in other European populations. It has been observed that in Australia, Northern Europe and among Caucasian populations of the USA, CM incidence has increased in the older age groups but has remained more stable among the Young (92). These observations among the elderly people are consistent with the hypothesis that the increased incidence of CM is real and not just because of a higher detection associated with the implementation of screening programs, increased coverage of cancer registries or changes in diagnostic criteria implemented in recent decades. They conlcude that although improvements on diagnostic practices applied, the most probable reason of the increase of CMM incidence is changes on exposure to ultraviolet radiation.

Annual incidence has risen as rapidly as 4–6% in many fair-skinned populations that predominate regions like North America, Northern Europe, Australia, and New Zealand (6). This increase in the incidence can be caused by evolution of etiological factors and the efficacy of early diagnosis and awareness of the patients (8).

The last GLOBOCAN 2020 (results from IARC available from: http://globocan.iarc.fr) published the incidence of countries worldwide. Spain has a ASRw per 100.000 for both sexes and all ages of 6.8. Near to our region, Italy has a ASRw 12.2, France 15.2, Portugal 5.6, and Greece 7.2. In the northern Europe, in higher latitudes, Germany 20.5, UK 16, Sweden 23.3 and Norway 26.4. Incidence of melanoma varies by geographic location among people of the same ethnicity (6).

Melanoma survival trends are variable and, as it happens with incidence, are influenced by geography, ethnicity, age, and sex (6). Worldwide, females have greater survival rates than males. In our cohort 5-year-RS of women was 95.2% (CI 89.6-101.1) vs men 85.3% (CI 75.6-95.1). Survival is the greatest among individuals beyond their seventh decade worldwide, in our cohort we stratified by <62 or ≥62. For the subgroup of <62 5-year-RS was 91.4% (CI 85.5-97.7) and for ≥ 62 was 78.6% (CI 67.3-92).

Trends in survival were published from the SUDCAN population-based study (8). The SUDCAN study aims to compare cancer net survivals between Belgium, France, Italy, Portugal, Spain, and Switzerland. The survival from SMM has increased constantly in Europe since the 1980s (93).

Switzerland and France had the highest net survivals, Italy and Spain had intermediate net survivals, whereas Belgium and Portugal had the lowest net survivals. Overall, the 5-year age-standardized net survivals ranged from 79 to 90%. Between 1992 and 2004, in long study period countries, a moderate absolute increase in the 5-year net survival was observed in Switzerland (2%) and France (4%), whereas a 9% increase was observed in Spain or Italy. Unfortunately, the study included no data on cancer subsite, histology, stage at diagnosis, comorbidities, or treatment; this makes the results difficult to interpret/understand.

In our study, only stage maintained its significance as independent prognostic factor for survival in the multivariate analysis (*see figure 12*). Stage is a clear prognostic factor and well established, it also is, the number of lymph nodes affected in stage III disease but was not the subject of the present study. Other classical prognostic factors are now being under discussion.

It must be considered that all cases analyzed in the articles that compose the thesis had been staged with AJCC TNM 7th edition. Since 2018 the 8th edition is on use, accuracy of different TNM subgroups according to survival could be modified.

9.2 BRAF MUTATION AS A PROGNOSTIC FACTOR IN SKIN MELANOMA.

Although there is a benefit for OS in BRAF-mutated patients in the whole cohort, this is lost when RS is analyzed. Since the main part of the cohort represents patients with thin melanoma and the number of recurrences was not very high, we do not consider the effect of target treatments to be relevant in the subgroup of positive BRAF.

BRAF is not a significant prognostic factor, but it is a significant predictive factor for response to target therapies as it well explained in the general introduction. By contrast, NRAS which has not been analyzed in our cohort, is a prognostic factor of bad prognosis (94).

We found significative differences in the distribution of BRAF mutation according to the presence of ulceration or sub-location, with a higher percentage of BRAF wildtype in ulcerated melanoma and in skin with more exposure to the sun. In addition, although the number of cases was low, wildtype cases were greater for acral and malignant lentigo melanoma. The distribution of BRAF mutation regarding stages might have been influenced by the number of cases corresponding to each stage as well as a lack of information, but it can be affirmed that thin and thick melanomas exhibit a similar distribution of BRAF mutation and mutation status does not influence the aggressiveness of melanoma at diagnosis although in our multivariate analysis we found significant differences. Other studies with similar multivariate analysis found also no differences between stages (94).

In primary melanomas, clinicopathologic features consistently reported to be associated with BRAF mutation include younger age, fewer markers of chronic sun damage in surrounding skin and higher total body nevus counts. However, the presence of the mutation in a primary melanoma has no apparent impact on disease-free interval or overall survival (41). This is consistent with our publication that BRAF is not a prognostic factor but is associated with some clinicopathologic features. The higher frequency of BRAF mutations in superficial spread melanoma and in those arising in skin with less exposure to the sun that we found is consistent with other publications (94,95).

In addition, our data are consistent with a recent review carried out by Gutierrez-Castaneda et al. (96) of 32 studies, very few of them population-based, in which BRAF mutation correlated mainly with superficial spread located in the trunk. In their analysis, NRAS was correlated to nodular melanoma and KIT to mucosal melanomas as was expected.

Thomas et al. (97) published a large population-based study including 912 patients diagnosed from 1998 to 2003 in the United States, Canada, Italy and Australia. The authors did not find a significant difference in melanoma specific survival between melanoma harboring mutations in NRAS or BRAF compared to non-mutated melanoma adjusted for other prognostic factors including stage, however, focusing on high-risk tumors, survival was worse for BRAF-mutated cases compared to non-mutated cases.

Si et al. (98) found a correlation between BRAF and NRAS mutation and poorer survival in a cohort of 432 Chinese patients diagnosed with thick CM.

In a population-based cohort of 437 cases, Meckbach et al. (99) were unable to identify a prognostic impact of BRAF V600 mutations in survival in patients with Stage I or II melanoma.

So, to conclude there's is a debate about mutational status and prognosis but the tendence of most studies is that mutational status has no relevance as a prognostic factor, but it does for treatment decision that at the end may impact in patients' survival.

In our population-based series, thin melanoma predominated as it does in the other series. For this reason, the statistical power for BRAF mutation as a prognostic factor in more advanced stages was decreased. Target therapies didn't influence much on our study, they were implemented upon 2011. Our results show differences in OS between patients with BRAF mutation and wild-type BRAF. However, this statistical significance is lost when analyzing relative survival.

We also found significant differences in the median age between the two groups. The median age for mutated BRAF cases was 56.5 years and for BRAF wild-type cases was 62.7 (p = 0.032). This data is consistent with other studies which revealed a young median age for BRAF-mutated patients (94).

We want to highlight the fact that we obtained a 94.7% of paraffin-tissue samples of the total cohort, which makes our study robust.

9.3 EPIDEMIOLOGY AND MOLECULAR ANALYSIS IN MUCOSAL MELANOMA.

MMs are a very rare type of cancer, with a much lower incidence and worse prognosis compared to cutaneous melanomas and account for approximately 1.2% of all melanomas. Five-year survival of MM patients is around 25% even less depending on the anatomical site (100).

The head and neck (H&N) is cited as the region most heavily represented (~50%), followed by the ano-rectum, and the female genital tract.

Deborah Kuk et al. (54) conducted a single-center, retrospective analysis of 3,454 patients with melanoma diagnosed with distant metastases from 2000 to 2013. The median overall survival for those with mucosal, uveal, acral, nonacral cutaneous, and unknown primary melanoma was 9.1, 13.4, 11.4, 11.7, and 10.4 months, respectively. Patients with uveal melanoma, cutaneous melanoma (acral and nonacral), and unknown primary melanoma had similar survival, but patients with mucosal melanoma had worse survival.

In our study, women survived longer, and vulvar-vaginal melanoma was the subgroup with better survival. This subgroup was the one in which we observed a more standardized diagnostic and treatment approach, probably due to the feasibility of applying the known evidence extrapolated from the management of CM (37). By contrast, H&N are the subgroup with worse survival rendering it even more unfeasible to carry out effective surgical outcomes.

MMs are underreported, which makes it difficult to develop large studies about epidemiology and treatment.

Beaudoux et al. (55) published the epidemiology of mucosal melanoma in the region of Champagne-Ardenne in France in the period of 2001–2014. They identified 39 cases of MM, including those arising in the eye. Their incidence of 0.18/100,000 inhabitants-year in ASIRw is similar to ours of 0.14/100,000 inhabitants-year. The five-year survival for all stages in the French study was 31.8%, slightly higher than that of our study, 26.2%

However, Beaudoux et al.'s study has an important difference compared to ours: they included conjunctival melanoma. We excluded conjunctival melanoma (C69.0) from our analysis mainly for two reasons: first, it is subject to the bias of a non-specific registration, since it belongs to the same location in ICD-O-3 as uveal or choroidal melanoma (C69.3 and C69.4), which we cannot consider mucosal melanoma. Second, conjunctival melanoma is genetically and biologically different, while head and neck, rectal and vulgo-vaginal melanomas are molecularly similar, and it is the only mucosal melanoma exposed to the sun, as all other mucosal melanomas are found in non-exposed sites. This could explain the higher frequency of BRAF-V600E mutations found in conjunctival melanoma published in other studies, closer to the cutaneous one (101).

Bishop et al. (102) published results from the Surveillance Epidemiology and End Results Program (SEER) in the period of 1988–2010. They identified 2755 cases of MM and reported an incidence of 0.23/100,000 inhabitants-year and a 5-year survival for all stages of 34%. The authors also described better survival in vulvar melanomas than in other subgroups, with 40% of patients alive at 5 years.

The California Cancer Registry, which analyzed 1824 mucosal melanomas diagnosed between 1994 and 2015 (103) published a 5-year survival of 27.6%. Stage and anatomic site determine prognosis of MM. Mucosal melanomas from less common anatomic sites (e.g., spine/CNS, lung and pleura, liver, and pancreas) conferred the worst prognosis.

The North American Association of Central Cancer Registries, in a study where 1806 cases of melanoma diagnosed in the period of 1996–2000 were analyzed. They were stratified by cutaneous, ocular, and mucosal. MM were more frequent in women. They reported an incidence of MM of 0.28 for women and 0.15 for men (104).

In the Netherlands they published an epidemiologic study of extracutaneous melanoma. Mucosal melanomas were the second most frequent subsite following uveal melanomas and reported European ASR of 1.8 cases per million among men and 2.8 cases per million in women, in the period 1989– 2006 (105).

Differences in the incidence between the population studies mentioned above and ours may be due to the inclusion of conjunctival melanomas or geographical variability. Our study is the first population-based in southern Europe, to our knowledge.

There is one European study (36) inside The Surveillance of Rare Cancers in Europe project (RARECARE). The RARECARE is a large collaboration project of population-based cancer registries

across Europe funded to deal with the issue of rare cancers. This work provides descriptive epidemiological data of malignant mucosal and uveal melanomas and adnexal skin carcinomas in Europe. Thet analyzed 8669 incident cases registered in the period 1995–2002 by 76 population-based cancer registries.

Considering the geographical variation in ASR, malignant melanomas of uvea was the most common site (4.4 per million) ranging from 3.1 in Southern Europe to 5.8 in Northern Europe. For mucosal melanomas, the ASR ranged from 0.9 per million in Eastern Europe to 2.7 in Northern Europe. They found a 5-years RS after the diagnosis for mucosal melanomas of female genital tract (43.9%) compared to the ones of head and neck (25.5%) and anal canal/colo-rectal tract (19.0%). The highest 5-year relative survival for mucosal melanomas was observed in Northern Europe (44.8%) and the lowest in Southern (36.2%) and Eastern Europe (37.1%).

Their 5-year OS survival for all cases is 32.1% a little bit better than ours but still consistently that in Southern Europe 5-year survival is worse.

In our study, some information about clinical and pathological characteristics was missing in a considerable percentage of cases, not allowing us to perform a multivariate analysis for prognostic factors. The reason for the lack of information is mainly that the patients were often diagnosed in advanced disease and a surgical approach was not used, and because Breslow and ulceration are not easily reproducible in MM. This limitation has also been observed in all other studies explained above, also with considerable missing data in pathological characteristics. In contrast to primary CM, the value of pathological characteristics such as Breslow or ulceration as prognostic parameters has not been consistently attested in MM. The prognostic or predicting factors of MM that cause unfavorable outcomes are not certain, although LDH level and performance status were found to be significant in a recently published survival meta-analysis (35).

MM differ from CMs in molecular profile, as the primary risk factor of CM, sun exposure, does not play a role in the development of mucosal melanomas (see introduction chapter for deep information).

BRAF mutations, frequently seen in CM, are not associated with MM. Therefore, there is the necessity to explore molecular pathways altered in MM. In addition, molecular profiling will help in the development of specific treatments for MM. Current clinical practice guidelines do not include a specific section for MM. BRAF status is the only validated predictive biomarker at the moment and it is not very useful in MM. Most conclusions are based on case reports. Melanoma patients routinely receive either a combination of BRAF/MEK inhibitors or immunotherapy with

antiPD1/DPL1 alone or in combination with antiCTLA4; the best sequence is still under discussion. Personalized systemic therapies targeting, for example, KIT mutations are only possible under clinical trials.

Analyzing results from the literature the basic biology of MM still remains unclear, but improvements have been made in molecular oncology the recent years.

Newell et al. (39) performed the largest study published so far with WGS-analysis of 67 mucosal melanomas from Europe, Australia and China. They confirmed that mucosal melanomas show low contribution from the UVR-signature. Interestingly, patients with somatic mutation in BRCA genes had no germline translation, which has yet to be analyzed in our cohort. They identified a total of 10 significantly mutated genes: NRAS (12/67), BRAF (11/ 67), NF1 (11/67), KIT (10/67), SF3B1 (8/67), TP53 (6/67), SPRED1 (5/67), ATRX (4/67), HLA-A (4/67) and CHD8 (3/67). NRAS mutations were targeted on hotspots on codon 61, which is also seen in our cohort and in what Mikkelsen et al. (101) published afterwards.

Alterations in KIT and NF1 are more frequent than in CM, whereas the MAPK-pathway typically including Ras/Raf/MEK/ERK is less dominant. The mutations observed in the BRAF gene in MM affect regions other than codon 600, which are known to lead to weaker MAPK-pathway activation and therefore are not predicted to respond to BRAF inhibition therapies, which are by the way the standard of care in CM. In our study, we confirmed the relevance of NF1 mutations, but more KIT mutations were expected, it could be a problem of the custom panel technique. We found one L576P exon 11 on KIT mutation, which was first described in GIST, suggesting that the molecular profile may indicate target therapies such as imatinib for selected patients.

A limitation of our study is the small size of cases that were suitable for gene sequencing, for this reason solid conclusions could not to be drawn. We assume that old FFPE tissues harbor high levels of degradation of DNA, and that produces artifacts that complicate the interpretation of NGS results. Part of these artifacts could be eliminated before preparing the libraries, using uracil-DNA glycosylase or nuclease S1 (106).

There were six cases for which we could not find any pathogenic mutation. Since MMs carry at least one well-established driver mutation, there is a possibility that we did not detect them because our panel was limited to 44 genes. For example, SF3B1 or SPRED1, described in other articles, were not covered in our panel (see introduction chapter to learn more about these genes). However, there are several copy number variations in mucosal melanoma, not all

detected in our study, which can also explain the negative cases, and some detected that do not have a proper interpretation yet.

9.4 IMMUNE-CHECKPOINT INHIBITORS.

Immunotherapy has been an emerging treatment since 2015, and it is currently being used to treat many types of cancer with particular side effects different from those of chemotherapy. Tumors can evade normal immune surveillance by several mechanisms including upregulation of immune checkpoint molecules such as PD1 and PD ligand 1 or CTLA4. Immunotherapy has been an emerging treatment since 2015, and it is currently being used to treat many types of cancer with particular side effects different from those of chemotherapy. Tumors can evade normal immune surveillance by several mechanisms including upregulation of immune checkpoint molecules such as PD1 and PD ligand 1 or CTLA4. The use of monoclonal antibodies (nivolumab, pembrolizumab, etc.) that block co-inhibitory immune checkpoint molecules, such as CTLA-4 and PD-1, may serve to increase a baseline T-cell-specific immune response that turns the immune system against the tumor (107). However, a disruption in the functioning of immune checkpoint molecules can lead to imbalances in immunologic tolerance that result in an uncontrolled immune response. This may clinically manifest with autoimmune/inflammatory side-effects, which cause collateral damage to normal organ systems and tissues (70). Such adverse effects have been the subject of much clinical interest and mechanistic research. We published an interesting case report of our clinics to contribute in this knowledge (4).

As explained in the introduction, CIs are the backbone of melanoma's oncological treatment and metastatic melanoma was one of the first cancers to be treated with ICIs.

Immune-related adverse events (irAEs) can involve all organs and although usually are low grade and manageable, sometimes can be life-threatening. Knowledge of toxicities associated with PD-1/PD-L1 blockade, as well as effective management algorithms for these toxicities, is pivotal in order to optimize clinical efficacy and safety. Standard treatment algorithms include corticosteroids as the treatment of choice, antihistamines, antitumor necrosis factors, immunoglobulins and rituximab.

In general, toxicities with anti-PD-1/PD-L1 appear to be less common and less severe when compared with antiCTLA-4 (70).

The frequency of irAEs is dependent on the agents used, exposure time and the administered dose but also on the patient's intrinsic risk factors (infectious disease, autoimmune disease, chronic disorders…) ; conversely, the timing of appearance is often dictated by the affected organ systems as it is represented in *figure 6* (108).

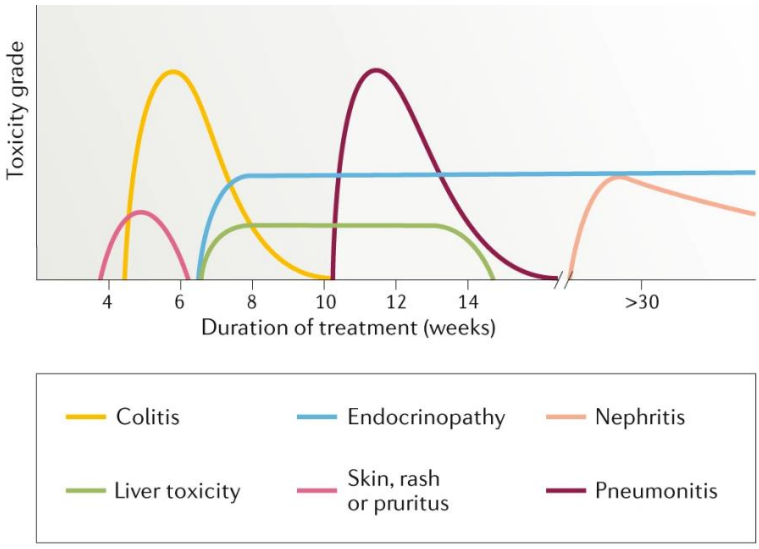

Figure 6. Kinetics of main irAEs. Martins F et al (108).

Hematological irAEs are rare and not extensively described in the literature. In the largest review published, a total of 68 cases were identified in the database of the Food and Drug Administration (FDA), 43 were associated with nivolumab, 13 with pembrolizumab, 7 with ipilimumab, and 5 with atezolizumab. Cases were included until March 2018, but only a few of them have been published (109).

Our case report describes grade 3 autoimmune haemolitical anemia (AIHA) induced by nivolumab in a melanoma patient of our clinical practice. Although the precise frequency is unclear, the estimated incidence of AIHA induced by ICI is <0.1%. That is why this case report has special interest.

The diagnosis of AIHA is made through laboratory findings. In our case, a decrease in hemoglobin, indirect hyperbilirubinemia, elevated LDH, and reduced haptoglobin were found. Coomb's test was positive for complement 3d but negative for IgG, according to most of the cases reported in the literature (110). Clinical guidelines recommend corticosteroids as the treatment of choice in AIHA. In refractory cases, rituximab with or without intravenous immunoglobulins may be useful. Our patient did well with corticosteroids at mg/kg and fully recovered.

AIHA can occur at any time with ICI, so clinicians should be aware of it even if the patient has not suffered any hematologic events previously. The median time between initiation of anti-PD1 or anti-PDL1 and occurrence of haem-irAE is 10 weeks, with a range between 2 and 78 weeks.

Our patient developed AIHA 7 weeks after the immunotherapy initiation, according to previous cases reported.

After the remission of the AIHA with steroids, we started treatment with ipilimumab, an anti-CTLA4, based on the proven efficacy in metastatic melanoma and due to its different mechanism of action compared to nivolumab. AIHA did not recur, and these data may provide some support for the safety of changing strategy in patients who have had haematological toxicities after anti-PD1/PDL1. Although there are unclear recommendations of the management due to the rarity of this toxicity.

10 GENERAL CONCLUSIONS

We confirm that the epidemiological characteristics of melanoma in Girona and province is comparable to that in neighboring areas.

The incidence of skin melanoma in Girona is increasing as it is in the rest of the world at expenses of thin melanomas (<1mm).

In our population-based study, BRAF mutation was not shown to constitute an independent prognostic factor for survival in melanoma patients. Stage is a consistent prognostic factor for survival. Since our results are population-based, the study is free of biases normally associated with hospital series.

Our second study confirms the steady incidence and low survival of mucosal melanomas in a region of southern Europe. We could not establish a comprehensive distribution of NRAS, BRAF and c-KIT mutations due to low cases of the sample and technical problems. However, we amplified the already published knowledge of NRAS and NF1 playing a role in the molecular landscape of mucosal melanoma and hypothesized MEK inhibitors may be useful in these patients. Population-based data are essential for the understanding of biological behavior in MM and for improving the lack of clinical evidence in treating patients. Treatments for mucosal melanomas are extrapolated from data based on therapies for metastatic cutaneous melanoma but understanding its mutational profile will allow medical oncologists to design better treatment strategies in the context of more precise medicine. We believe target therapies might be a very good option to a not-underestimated group of patients with certain actionable mutations.

Given the bad prognosis of MM and the delay of its diagnosis, dermatologists should incorporate examination of the oropharynx and genitalia in the full body skin exam to promote early detection.

The oncological treatment for Melanoma is based on immune-checkpoint inhibitors. They play the principal role, but they are not exempt of toxicities as we can exemplify with our third publication. Clinicians must suspect and be prepared for early detection of adverse events and proceed with caution and security. Human immune system is complex and needs further investigation to better understand its mechanisms of action.

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12 ANNEX 1

J. Rubió-Casadevall, A. Carbó-Bagué, M. Puigdemont, G. Osca-Gelis, G. Oliveras, N. Vilar-Coromina, B. Ferrer-Fabrega, A. Urban, M. Llobet-Roma, F. Martín-Romero, F. Perez-Bueno, R. Marcos-Gragera. Population-based analysis of the prevalence of BRAF mutation in patients diagnosed with cutaneous melanoma and its significance as a prognostic factor. Eur J Dermatol 2021 Oct 1;31(5):616-622.

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Background

The prevalence of *BRAF* mutation has been reported in between 38% and 48% of melanoma patients, based on mainly Stage III or metastatic melanoma, however, information based on population-based studies is scarce.

Objectives

We performed a population-based retrospective cohort study to determine the prevalence of the *BRAF* mutation in patients diagnosed with *in situ* and infiltrating cutaneous malignant melanoma in the province of Girona between 2009 and 2011.

Materials & Methods

Using the database of the Girona Cancer Registry, we performed *BRAF* mutation analysis based on paraffin-embedded tissue. This data was then correlated with other known clinical and histological prognostic factors for survival.

Results

We found 286 incident cases of cutaneous melanoma in the Girona Cancer Registry database. Excluding missing cases, *BRAF*-mutated patients constituted 38.9% of "*in situ*" melanoma cases and 53.8% of invasive melanoma cases. Five-year relative survival was not statistically different between *BRAF*-mutated patients (93.6%; 95% CI: 87.1-100.5) and non-mutated patients (84.3%, 95% CI: 75.3-94.8). Only stage was significant as a prognostic factor for survival based on multivariate analysis.

Conclusion

From our population-based study, we conclude that *BRAF* mutation is not an independent prognostic factor for melanoma survival.

cancers

MDPI

Article

Epidemiology and Molecular Profile of Mucosal Melanoma: A Population-Based Study in Southern Europe

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Simple Summary: There are few population-based studies focused on the epidemiology of mucosal melanoma, a rare neoplasm. Its poor prognosis, the different etiology from cutaneous melanoma and the lack of effective treatment beyond corrective surgery, make the knowledge of the mutational profile of this type of cancer a useful tool in understanding its natural history and also for the investigation of new target therapies. The aim of our population-based study is to analyze the incidence and survival of mucosal melanoma, which mainly arises from the head and neck sphere, genitourinary tract and rectal area, and to carry out the mutational analysis of selected cases. We used the Girona Cancer Registry database, which registered all cancer cases in Girona, a province of Spain in southern Europe, during the period of 1994–2018.

Abstract: Background: Mucosal melanoma is a rare neoplasm on which few epidemiological population-based studies have been published. A good surgical approach is the standard treatment, but the prognosis is worse than that of skin melanoma. The analysis of mucosal melanoma's mutational profile can help to develop target therapies in advanced disease or adjuvant settings. Methods: We analyzed the database of the Cancer Registry of Girona, a region located in the northeast of Spain, in the period of 1994–2018. We selected cases of primary invasive melanoma, excluding those located in the skin, eye, central nervous system and an unknown primary site. Epidemiological analysis included incidence and survival. Mutational profile analysis was performed with a custom gene panel. Results: Forty-two patients were identified: 14 (33%) had vulvar-vaginal melanoma, 15 (35.7%) had rectal melanoma, 12 (28.6%) had melanoma located in the head and neck sphere and 1 male patient had a urethral melanoma. European age-standardized incidence rates for vulvar-vaginal, rectal and head and neck melanoma were 0.09, 0.1 and 0.09 cases/100,000 inhabitant-years, respectively. Five-year observed survival rates were 37.5%, 20% and 25% for these types of cancers. NRAS Q61 was the most frequent mutation found. Conclusion: Our study confirms the steady incidence and low survival of mucosal melanomas in a region of southern Europe. NRAS and NF1 play a role in the molecular landscape of mucosal melanoma. MEK and PI3K/mTOR inhibitors could be reasonable treatment options and are being studied in clinical trials.

Keywords: mucosal melanoma; epidemiology; incidence; survival; DNA mutational analysis; NRAS

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1. Introduction

Melanomas of the mucosa (MMs) are neoplasms that arise from melanocytes of the epithelium of the otorhinolaryngological sphere (oral and nasal cavities), conjunctiva, genitourinary tract (especially in the vulvovaginal area) and anorectal area.

MMs are a very rare type of cancer, with a much lower incidence and worse prognosis compared to cutaneous melanomas (CMs) and account for approximately 1.2% of all melanomas. Five-year survival of MM patients is less than 25%, and 23% of patients are diagnosed with metastasis [1]. MM patients have a median survival of 9 months and the worst prognosis compared with other melanoma subgroups such as uveal, acral, non-acral cutaneous and unknown primary melanoma [2].

Clinical guidelines strongly recommend testing BRAF, NRAS and KIT in all melanomas. The BRAF mutation in MM has been reported in 3–15% of cases, while it has been reported in nearly 50% of CM cases. Mutations of KIT can be identified in 7–17% of cases, a much higher value than in cutaneous melanoma, especially in vulvovaginal melanomas, where they may be identified in 30% of cases [1]. Mutations in NRAS have been found in 15–20% of mucosal melanomas, such as cutaneous ones.

Our aim is to conduct a population-based study of the incidence and survival of mucosal melanomas in Girona's province, a region of southern Europe, from 1994 to 2018 and to perform a genetic analysis to determine the molecular landscape of these neoplasms.

2. Materials and Methods

2.1. Study Cohort

This is a retrospective cohort population-based study. We analyzed the database from the Girona Cancer Registry (GCR), a population-based cancer registry in Girona's province, located in the north-east of Spain, which started case registration in 1994. The population covered is 749,656 inhabitants according to the 2018 census. GCR cases are registered according to the International Agency of Research on Cancer (IARC) guidelines with a completeness of 95.0% (http://ico.gencat.cat/web/.content/minisite/ico/professionals/documents/registre_cancer_girona/arxius/CanGir-2013-17.pdf, accessed on 28 November 2021). The International Classification for Diseases-Oncology, Third Edition (ICD-O-3), was used to register cases [3].

We restricted our analysis to cases of primary invasive melanoma (ICD-O-3, histological codes: 8720-8723, 8730, 8740-8746 and 8761-8774) and excluded those located on the skin (code C44), in an unknown primary site (C80.9) and in the eye and central nervous system (C69-C72). Patients were eligible if diagnosed from the 1 January 1994, to 30 December 2018.

We obtained paraffin-embedded tissue samples from all the hospitals in our province by previous contact and agreement with the collaborating pathology labs. Samples with the highest proportion of tumor cells were selected by pathologists from the University Hospital Josep Trueta.

2.2. Descriptive Epidemiology

Descriptive statistics were expressed as median and interquartile ranges (IQR) for quantitative variables and as absolute frequencies and percentages for qualitative variables. Crude (CR) and age-standardized incidence rates using the 2013 European standard population ($ASIR_e$) and world standard population (ASIRw) were calculated and expressed per 100,000 person-years. For the survival analysis, we calculated follow-up time from diagnosis to patients' last vital status recorded. To obtain these data, we reviewed hospital clinical reports and/or the Mortality Registry of Catalonia and the Spanish National Death Index. Vital status was updated on the 31st of August 2021. The observed survival (OS) estimates were analyzed by Kaplan–Meier method using R software v.3.6.2.

2.3. DNA Extraction, Library Preparation and Sequencing

DNA extraction was performed with cobas sample kits. We excluded almost half of the cases either for not having enough archived tissue or due to bad quality of DNA. DNA quality and quantity were assessed using a genomic DNA ScreenTape on a TapeStation 2100 instrument from Agilent Technologies. We found 24 out of 42 cases with proper DNA quality for NGS analysis (60% of the cohort): n = 9 rectal melanoma, n = 8 head and neck (5 nasal and 3 pharyngeal) melanoma, n = 7 vulvar-vaginal.

Old paraffin tissue samples have high degradation levels of DNA; therefore, a screen test that served as quality control for DNA or RNA samples was used.

We used a custom gene panel, designed and validated internally at Gencardio Diagnostics (University of Girona-IDIBGI). This panel covers the entire exonic regions from the following 44 genes: AKT1, AKT3, ALK, APC, BRAF, BRCA1, BRCA2, CDKN2A, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ERBB4, FGFR1, FGFR2, FGFR3, NF1, HRAS, JAK1, JAK2, KIT, NRAS, MAP2K1, MAP2K2, MET, MYC, NRAS, NTRK1, NTRK2, NTRK3, PDGFRA, PIK3CA, POLD1, PLE, PTEN, RB1, RET, ROS1, SLC34A2, SOX2, STK11, TERT and TP53. In addition, the panel covers hotspots for 8 known fusion genes— ALK, BRAF, EGFR, FGFR1, FGFR2, FGFR2, RET and ROS1—and a selection of common SNVs to create a backbone for Copy Number Alteration (CNA) detection.

Sample library preparation was performed following the Sureselect XT HS Target Enrichment System (Agilent Technologies). Upon enzymatic fragmentation and adapter and index ligation, DNA fragment size and concentration were assessed using a TapeStation instrument. DNA fragments were hybridized using biotinylated RNA probes (Agilent Technologies) corresponding to the regions of interest of the panel design. The capture was performed using streptavidin-coated beads, and the captured DNA was PCR-amplified. To improve the on-target capture, a second hybridization and capture were performed. Finally, the specific molarity of each library was checked in the TapeStation instrument in order to multiplex the samples. Libraries were sequenced on a MiSeq instrument using 2 $\times$ 76 base pairs read length (Illumina, San Diego, CA, USA).

2.4. NGS-Analysis

NGS analysis was performed using a custom bioinformatics pipeline available at https://github.com/GENCARDIO/GC_NGS_PIPELINE (accessed on 28 November 2021). Raw FASTQ files were preprocessed to remove low-quality bases and adapters using fastp (v0.21.0). Read alignment to the human reference genome (GRCh37/hg19) was performed using the Burrows-Wheeler Aligner (BWA-MEM; v0.7.17). Sequencing and optical duplicates were removed with Picard (v2.18.9). SNV detection was performed using Mutect2 (v4.2.2.0) in combination with Lancet (v1.1.0). INDELs (<50 bp) were detected using Lancet. Structural Variants (SVs) were detected using Manta (v1.6.0). Copy Number Alteration (CNA) detection was performed using CNVkit (v0.9.8). Variants displaying significant strand bias due to FFPE artifacts were removed using GATK FilterByOrientationBias tool. Only variants with a Variant Allele Frequency (VAF) higher than 10% were kept for downstream annotation. Variant annotation was performed using Variant Effect Predictor (Ensembl release 101), with the selection of MANE isoforms. General population frequencies were annotated with gnomAD v2.1.1, ExAC and 1000Genomes. Clinical annotation levels of evidence were extracted from CIViC. Gene fusions were annotated with chimerKB v4.

All significant variants were manually checked with the Integrative Genomics Viewer (https://www.broadinstitute.org/software/igv/home, accessed on 28 November 2021).

3. Results

3.1. Descriptive Epidemiology

Forty-two patients with MM were identified in the cohort: 14 female patients with vulvar-vaginal melanoma, 15 rectal melanoma cases, 12 patients with head and neck melanoma (eight nasal and four pharyngeal) and 1 male patient with a penile urethral melanoma.

The ASIRw of MM in Girona between 1994 and 2018 for both sexes was 0.14. It was 0.10 for males and 0.16 for females, which was higher because of the number of vulvar-vaginal melanomas according to other articles in the literature.

Table 1 shows sex and site distribution with incidence rates of the whole cohort and of each subgroup of patients in CR, ASIRw and ASIRe, for men, women and both sexes.

Table 1. Gender distribution and incidence rates of mucosal melanoma in Girona 1994–2018.

Characteristics				CR (per 100.000) (95% CI)			ASIRe (per 100.000) (95% CI)			ASIRw (per 100.000) (95% CI)		
Site	***n* (%)**	**M/F (%)**	**Med Age [IQR]**	**M**	**F**	**Total**	**M**	**F**	**Total**	**M**	**F**	**Total**
Head & Neck	12 (28.6)	6/6	72.5 [57.0–91.2]	0.07 (0.01–0.13)	0.07 (0.01–0.13)	0.07 (0.03–0.11)	0.09 (0.03–0.22)	0.08 (0.03–0.17)	0.09 (0.05–0.16)	0.05 (0.02–0.15)	0.025 (0.01–0.13)	0.04 (0.02–0.09)
Rectal	15 (35.7)	6/9	69.9 [65.9–82.3]	0.07 (0.01–0.13)	0.11 (0.04–0.18)	0.09 (0.05–0.14)	0.09 (0.03–0.23)	0.10 (0.05–0.20)	0.10 (0.06–0.17)	0.05 (0.02–0.15)	0.036 (0.01–0.14)	0.04 (0.02–0.10)
Vulvar-vaginal	14 (33.3)	0/14	64.4 [57.8–75.4]	–	0.17 (0.08–0.26)	0.09 (0.04–0.13)	–	0.18 (0.10–0.30)	0.09 (0.05–0.16)	–	0.10 (0.05–0.22)	0.05 (0.03–0.11)
Urethral	1 (2.4)	1/0	69.8	0.01 (0–0.04)	–	0.01 (0–0.02)	0.01 (0–0.13)	–	0.01 (0–0.04)	0.008 (0–0.106)	–	0.004 (0–0.054)
All	42 (100)	13/29	68.4 [59.9–84.0]	0.16 (0.07–0.24)	0.36 (0.23–0.48)	0.26 (0.18–0.33)	0.19 (0.10–0.36)	0.36 (0.24–0.52)	0.29 (0.21–0.40)	0.10 (0.05–0.22)	0.16 (0.10–0.29)	0.14 (0.09–0.21)

N: Number of cases; M: males; F: females; CI: confidence interval; CR: crude rate; ASIRe: European age-adjusted standard incidence rate ASIRw; world age-adjusted standard incidence rate and IQR: interquartile range.

Table 2 shows the OS results. We summarize the 5-year OS for all stages for the whole cohort and each subgroup. OS at 5 years was 7.7% in men, 34.5% in women and 26% for both sexes.

Table 2. Five-year observed survival for all stages of mucosal melanoma in Girona 1994–2018. OS: observed survival; NA: not applicable.

Site	**5y OS (%) (95% CI)**		
	Males	**Females**	**Total**
Head and Neck	16.7 (2.8–99.7)	33.3 (10.8–100)	25.0 (9.4–66.6)
Rectal	0	33.3 (13.2–84.0)	20.0 (7.3–55.0)
Vulvovaginal	NA	35.7 (17.7–72.1)	NA
All	7.7 (1.2–50.6)	34.5 (20.9–56.9)	26.2 (15.8–43.5)

Women survived longer, and vulvar-vaginal melanoma was the subgroup with better survival. This subgroup was the one in which we observed a more standardized diagnostic and treatment approach, probably due to the feasibility of applying the known evidence extrapolated from the management of CM [4]. Table 3 summarizes the characteristics of the patients with vulvovaginal melanoma. Staging has been extrapolated from the cutaneous melanoma TNM, 7th Edition from the American Joint Committee on Cancer system since there is no specific staging for MM.

3.2. Genetics

From the 42 cases of the cohort, 24 cases (60%) were suitable for NGS analysis: nine rectal melanomas, eight head and neck (five nasal and three pharyngeal) melanomas and seven in the vulvar-vaginal area. In Table 4, results from the genetic profiling are shown. All pathogenic somatic mutations found in the assay are represented.

Table 3. Clinical characteristics of patients with vulvar-vaginal mucosal melanoma.

Characteristics	N = 14 (33%)
Pathology	
Breslow	
<1 mm	1 (7.1)
1–2 mm	0
2–4 mm	3 (21.4)
>4 mm	10 (71.5)
Ulceration	
Positive	8 (57.2)
Negative	5 (35.7)
Missing	1 (7.1)
Initial treatment	
Local surgery only	7 (50)
Local surgery + lymphadenectomy	3 (21.4)
Local surgery + lymphadenectomy + adjuvant radiotherapy	2 (14.3)
Local surgery + lymphadenectomy + adjuvant interferon	1 (7.1)
Radical radiotherapy	0
Systemic treatment only	1 (7.1)
Palliative treatment only	0
Sentinel node biopsy	5 (35.7)
Stage information (TNM 7th Edition)	
Stage IB	2 (14.3)
Stage IIA	2 (14.3)
Stage IIB	1 (7.1)
Stage IIC	6 (42.9)
Stage IIIB	2 (14.3)
Stage IIIC	1 (7.1)
Vital status (at 31 of August 2021)	
Alive without disease	3 (21.4)
Alive with disease	0
Deceased for specific disease	10 (71.5)
Deceased for all causes	1 (7.1)

Table 4. Results from the genetic profiling of the 24 mucosal melanoma samples analyzed.

Mutation/Site	Rectum									Nasal				Pharynx			Vulvovaginal					
	1	4	6	7	9	16	17	21	23	2	3	10	19	20	8	12	18	5	11	13	14	15	22	24
BRAF G596R																								
KRAS G12C																								
NRAS Q61 H/K/R																								
KIT L576P																								
NF1 L1611T																								
NF1 Y1401 *																								
NF1 A1660 *																								
NF1splice acceptor																								
NF1 I766 *																								
NF1splice donor																								
NF1 F624V																								
TP53 S241C																								
TP53 F134L																								
TP 53 R273L																								
TP 53 K139E																								
CDKN2A E10 *																								
MYC F7L																								

Table 4. *Cont.*

Mutation/Site	Rectum									Nasal			Pharynx				Vulvovaginal					
	1	4	6	7	9	16	17	21	23	2	3	10	19	20	8	12	18	5	11	13	14	15	22	24
BRCA2 E1734K																								
BRCA2 D935N																								
BRCA1 G1777P																								
APC E892G																								
AKT3 A21L *																								
PTEN V166Sfs * 14																								
FGFR3 R158Q																								
MAP2K2 R74S																								
POLE R2165H																								
POLE M1748L																								
POLE R1294C																								
POLD1splice donor																								
ERBB2 V153L																								
ERBB3 R1077W																								
ROS1 M710T																								
MET R359Q																								
JAK2 N1108S																								
STK11 V66G *																								
Wildtype in assay																								

Missense | Nonsense → Frameshift | splice site * Stop codon; Blank cell = no mutation found.

Pathogenic somatic mutations of the studied genes were identified in 18 cases (75%). Eight cases had only one mutation, three cases had two mutations, five cases had three mutations and two had four mutations.

Only one patient had a BRAF G596R mutation, which is more typically seen in lung cancer and is present in 0.02% of all malignant solid tumors [5]. There are open clinical trials for this type of mutation, also in melanoma (www.mycancergenome.org Accessed on 28 November 2021).

NRAS mutations were found in three cases (12.5%), and one KRAS G12C mutation, for which there are clinically tested drugs, was found. NF1 mutations dominated, with seven cases (29%) predominantly in the rectum. KIT mutation L576P exon 11 was found in one case of vulvar-vaginal melanoma. TP53 mutations, widespread in solid tumors, are present in MM too: in the present cohort, four cases were found (16.6%).

Amplifications of MYC were the most frequent copy number variations (CNV) with an average number of five copies found in eight patients.

Several other mutations were found that are very uncommon in melanomas and are of uncertain meaning, such as POLE mutations, which are well-characterized in other solid tumors. One patient with vulvar MM had a frameshift mutation in the BRCA1 gene with a variant allele frequency (VAF) of 80%. Another patient with rectal MM had a CDKN2A mutation with VAF of 45%. For this reason, an underlying germline mutation was suspected, and genetic counseling was recommended.

4. Discussion

There are few population-based studies published in the literature focused on mucosal melanoma, meaning that its epidemiology remains sparsely analyzed. MMs are underreported, which makes it difficult to develop large studies. Treatment is also not well-standardized, although this is not the purpose of this study.

Beaudoux et al. published the epidemiology of mucosal melanoma in the region of Champagne-Ardenne in France in the period of 2001–2014. They identified 39 cases of MM, including those arising in the eye. Their incidence of 0.18/100,000 inhabitants-year in ASIRw is similar to ours of 0.14/100,000 inhabitants-year. The five-year survival for all stages in the French study was 31.8%, slightly higher than that of our study, 26.2% [6].

However, Beaudoux et al.'s study has an important difference compared to ours: they included conjunctival melanoma. We excluded conjunctival melanoma (C69.0) from our analysis mainly for two reasons: first, it is subject to the bias of a non-specific registration, since it belongs to the same location in ICD-O-3 as uveal or choroidal melanoma (C69.3 and C69.4), which we cannot consider mucosal melanoma. In addition, a significant number of diagnoses used to complete the GCR database in this site are coded as C69.9 without further specification of sublocation. Second, conjunctival melanoma is genetically and biologically different, while head and neck, rectal and vulgo-vaginal melanomas are molecularly similar, and it is the only mucosal melanoma exposed to the sun, as all other mucosal melanomas are found in non-exposed sites. Therefore, there is no known modifiable risk factor. This could explain the higher frequency of BRAF-V600E mutations found in conjunctival melanoma published in other studies, closer to the cutaneous one [7].

Bishop et al. published results from the Surveillance Epidemiology and End Results Program (SEER) in the period of 1988–2010. They identified 2755 cases of MM and reported an incidence of 0.23/100,000 inhabitants-year and a 5-year survival for all stages of 34%. The authors also described better survival in vulvar melanomas than in other subgroups, with 40% of patients alive at 5 years [8].

Similar results to those of SEER were obtained in the California Cancer Registry, which analyzed 1824 mucosal melanomas diagnosed between 1994 and 2015 [9], and the North American Association of Central Cancer Registries, in a study where 1806 cases diagnosed in the period of 1996–2000 were analyzed [10].

Differences in the incidence between the population studies mentioned above and ours may be due to the inclusion of conjunctival melanomas or geographical variability, this study being the first population-based one in southern Europe, to our knowledge.

In our study, some information about clinical and pathological characteristics was missing in a considerable percentage of cases, not allowing us to perform a multivariate analysis for prognostic factors. The reason for the lack of information is mainly that the patients were often diagnosed in advanced disease and a surgical approach was not used. This limitation has also been observed in other studies, also with considerable missing data in pathological characteristics [5,10]. In contrast to primary CM, the value of pathological characteristics such as Breslow or ulceration as prognostic parameters has not been consistently attested in MM [5,10]. The prognostic or predicting factors of MM that cause unfavorable outcomes are not certain, although LDH level and performance status were found to be significant in a recently published survival meta-analysis [11,12].

We were able to describe characteristics of vulvar-vaginal melanoma cases, where the treatment approach is similar to CM. Our results are not far from those published by Aliteri et al. [9] in terms of survival but are lower than those obtained by Sanchez et al. [13] and Wolhmut et al. [14], who reported a 5-year OS of up to 50% for vulvar-vaginal melanoma. Indeed, vaginal melanoma has worse prognosis than vulvar melanomas [15]. Initial staging, Breslow index and complete surgery with lymphadenectomy are the main independent variables for survival that have been reported [14–16].

It is believed that MMs differ from CMs in molecular profile, as the primary risk factor of CM, sun exposure, does not play a role in the development of mucosal melanomas. BRAF mutations, frequently seen in CM, are not associated with MM. Therefore, there is the necessity to explore molecular pathways altered in MM. In addition, molecular profiling will help in the development of specific treatments for MM.

Current clinical practice guidelines do not include a specific section for MM. BRAF status is the only validated predictive biomarker at the moment. Most conclusions are based on case reports. Melanoma patients routinely receive either a combination of BRAF/MEK inhibitors or immunotherapy with antiPD1/DPL1 alone or in combination with antiCTLA4; the best sequence is still under discussion. Personalized systemic therapies targeting, for example, KIT mutations are only possible under clinical trials.

Analyzing results from the literature in the molecular landscape [17–20], the basic biology of MM still remains unclear, but improvements have been made in recent

years. Newell et al. performed the largest study published so far with WGS-analysis of 67 mucosal melanomas from Europe, Australia and China [17]. They confirmed that mucosal melanomas show low contribution from the UVR-signature. Interestingly, patients with somatic mutation in BRCA genes had no germline translation, which has yet to be analyzed in our cohort. They identified a total of 10 significantly mutated genes: NRAS (12/67), BRAF (11/ 67), NF1 (11/67), KIT (10/67), SF3B1 (8/67), TP53 (6/67), SPRED1 (5/67), ATRX (4/67), HLA-A (4/67) and CHD8 (3/67). NRAS mutations were targeted on hotspots of codon 61, which is also seen in our cohort and in what Mikkelsen et al. published afterwards [7].

Alterations in KIT and NF1 are more frequent than in CM, whereas the MAPK-pathway typically including Ras/Raf/MEK/ERK is less dominant. The mutations observed in the BRAF gene in MM affect regions other than codon 600, which are known to lead to weaker MAPK-pathway activation and therefore are not predicted to respond to BRAF inhibition therapies, which are by the way the standard of care in CM [20].

KIT is already an established therapeutic target agent in other cancers, specifically in gastrointestinal stromal tumors (GIST). Identification of these known mutations in patients with MM may take into consideration KIT inhibitor treatments [21]. In a trial in which imatinib was used to treat 24 patients with either KIT-mutated or KIT-amplified tumors in mucosal, acral or chronically sun-damaged melanoma, the authors concluded that it was effective in KIT-mutated tumors but not in those where the gene was amplified only [22]. In our study, we found L576P exon 11 on KIT mutation, which is frequent in GIST, suggesting that the molecular profile may indicate target therapies such as imatinib for selected patients.

NRAS Q61 mutations are typically seen in MM, as we confirmed in our study, and are associated with a poor prognosis and a potential cause of BRAF inhibitor resistance. MEK inhibitors may be effective in these patients. The MEK inhibitor binimetinib has shown activity in this setting, with a response rate of 20% in a Phase II trial of 30 patients with NRAS-mutated melanoma [23].

NF1 is a tumor suppression gene. Loss of NF1 is associated with increased MAPK activity and is significantly mutated both in CM and MM. Similarly to NRAS mutations, alterations in NF1 result in poor response to BRAF inhibitors and may be targeted by MEK inhibitors [20].

A limitation of our study is the small size of cases that were suitable for NGS that did not allow solid conclusions to be drawn. We assume that old FFPE tissues harbor high levels of degradation of DNA, and that produces artifacts that complicate the interpretation of NGS results. Part of these artifacts could be eliminated before preparing the libraries, using uracil-DNA glycosylase or nuclease S1 [24,25]. Nevertheless, with this study we have contributed to the understanding of molecular pathways in MM, but more genetic research needs to be done.

There were six cases for which we could not find any pathogenic mutation. Since MMs carry at least one well-established driver mutation, there is a possibility that we did not detect them because our panel was limited to 44 genes. For example, SF3B1 or SPRED1, described in other articles, were not covered in our panel [7,17,18]. However, there are several copy number variations in mucosal melanoma, not all detected in our study, which can also explain the negative cases, and some detected that do not have a proper interpretation yet [7,17].

Today, immune checkpoint inhibitors are the standard of care for many cancer types, including CMs, and have incredibly improved patients' chance of survival. Three mucosal melanomas respond less to immunotherapy [7,26] than CMs. A postulated reason for this is that the mutation burden is much lower in mucosal melanoma as compared to cutaneous melanoma [20]. Angelo et al. evaluated the efficacy of ipilimumab and nivolumab alone or in combination. The study included data from several clinical studies with 889 melanoma patients, 10% of which had mucosal melanoma; the response rate was 37.5% and the progression-free survival was 5.9 months [27]. In our cohort, two patients with vulvar

melanoma received anti-PD1/PDL1 treatment and one patient with nasal melanoma received anti-CTLA4; none of them responded. All other patients who needed systemic treatments received conventional chemotherapy due to the antiquity of our cohort.

5. Conclusions

Population-based data are essential for the understanding of biological behavior in MM and the lack of clinical evidence in treating patients. Treatments for mucosal melanomas are often extrapolated from data based on therapies for metastatic cutaneous melanoma, but knowing and understanding its mutational profile will allow us to design better treatment strategies in the context of more precise medicine.

Our study supports the steady incidence and poor patient survival of mucosal melanoma in a region of southern Europe. NRAS and NF1 are confirmed to play a role in mucosal melanoma. We believe target therapies may be a very good option to a not-underestimated group of patients with certain actionable mutations.

Author Contributions: A.C.-B. and J.R.-C. conceived and coordinated the study, analyzed data and results, and wrote the manuscript. M.P. was responsible for the coding of cases and formal analysis of the database in the Girona Cancer Registry (GCR). A.S. performed the statistical analysis. G.O. and F.P.-B. selected paraffin embedded tissue and made histological diagnoses. B.d.O. and M.C. performed the mutational analysis. R.M.-G. is the coordinator of GCR and also conceived and reviwed the study. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Josep Trueta of Girona on 19 January 2021.

Informed Consent Statement: Written informed consent was obtained from subjects involved in the study following Ethics Committee indications.

Data Availability Statement: Data are available at Genetic Descriptive, Genetic and Prevention Epidemiology Group, Biomedical Research Institute of Girona (IDIBGI). This study was carried out using anonymized data from the Girona Cancer Registry (GCR), which complies with the legal regulations in Law for Data Protection and management of clinical data in force in Spain. The GCR also belongs to and complies with the regulations and rules of the International Association of Cancer Registries and the International Association for Research in Cancer (IARC) Cancer Registry (IACR) and the International Association for Cancer.

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Case Report

Nivolumab-Induced Autoimmune Haemolytic Anaemia and Safety of Subsequent Use of Ipilimumab: A Case Report

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Keywords
Immune checkpoint inhibitors · Immune-related adverse event · Autoimmune haemolytic anaemia · Nivolumab · Ipilimumab

Abstract
Autoimmune haemolytic anaemia (AIHA) is a rare immune-related adverse event and appears to be more common with anti-PD1/PDL1 than anti-CTLA4. Little is known about the safety of re-treating with anti-PD1/PDL1 or changing to anti-CTLA4. We present a case of grade 4 AIHA due to nivolumab (PD1-inhibitor) treatment in a patient with melanoma for adjuvant setting after surgery and the safeness of subsequent treatment with ipilimumab (anti-CTLA4). After the remission of AIHA with steroids, ipilimumab was started with the rationale of its different mechanism of action. Fortunately, AIHA did not recur. The mechanism by which checkpoint inhibitors cause AIHA is likely by augmenting or redirecting immune surveillance, especially by activating pre-existing red blood cell autoantibodies, but further studies must be done. To our knowledge, this is the first case published in the literature with the change of immunotherapy treatment to anti-CTLA4.

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Introduction/Background

Immunotherapy has been an emerging treatment since 2015, and it is currently being used to treat many types of cancer with particular side effects different from those of chemotherapy. Tumours can evade normal immune surveillance by several mechanisms including upregulation of immune checkpoint molecules such as PD1 and PD ligand 1 or CTLA4.

Nivolumab is a fully human IgG4 monoclonal antibody which binds to and blocks the activation of PD1 as a checkpoint inhibitor (CPI). This release of check on the immune system can also trigger a reaction against the body's own tissues leading to autoimmune adverse effects such as pneumonitis, hepatitis, colitis, hypophysitis, arthritis, or nephritis, which are the most known side effects that appear in between 20 and 30% of the patients [1].

Autoimmune haemolytic anaemia (AIHA) has been described as a very uncommon immune-related adverse effect. We present a case of AIHA in a patient treated with nivolumab for adjuvant setting after melanoma surgery and treated later with ipilimumab, a fully human IgG1k against CTLA4, without reproducing this type of toxicity.

Case Report

A 62-year-old male was diagnosed with BRAF-negative stage IVa completely excised acral melanoma in February 2019. He was considered for adjuvant nivolumab 3 mg/kg every 2 weeks [2]. In June 2019, after the third cycle/dose, he presented to the emergency room with severe asthenia and fatigue. He claimed not to have shortness of breath, thoracic pain, fever, or bleeding episodes. Physical examination showed mild conjunctival jaundice. The rest of the clinical examination was unremarkable. Routine laboratory tests showed 5.8 g/dL haemoglobin levels, 1,200 absolute neutrophil count, indirect hyperbilirubinaemia 2.4 mg/dL, high lactate dehydrogenase (LDH) 912 U/L, and low haptoglobin <10 (Fig. 1–3). The direct antiglobulin test was positive for complement 3d but negative for IgM and IgG.

Our diagnosis was AIHA. Considering that the patient was currently on immunotherapy treatment and given the timing association between nivolumab and anaemia, we could establish the immune-related adverse event grade 4. The patient had not started other concomitant medications associated with AIHA, so we concluded it could be reasonably related to nivolumab.

We started treatment with a high dose of methylprednisolone (1 mg/kg) and 3 red blood cell transfusions. After 4 days, the haemoglobin levels raised to 9.5 g/dL, the bilirubin levels became normal, and LDH levels took a bit longer to normalize (Fig. 1–3). The patient was feeling well, so he was discharged from the hospital with a slow descending dose of cortisone. We decided to stop adjuvant treatment and start controls.

The first recurrence was detected after 1 year. He came with skin metastases, and CT scans showed one unique 6-mm temporal cerebral lesion. He underwent radiosurgery with complete response. Despite having suffered a CTCAE grade 4 immuno-related event, we thought to give a chance with ipilimumab (anti-CTLA4) as it was another mechanism of action. The first cycle was given at 1 mg/kg and next at 3 mg/kg. No immune-related adverse events were found during the treatment. Four weeks after the fourth cycle of ipilimumab, abdominal adenopathies and new skin metastases appeared.

In August 2020, he started second line with fotemustine 80 mg/m^2 every 2 weeks. After 7 cycles, a PET scan was performed with partial response only, persisting one skin

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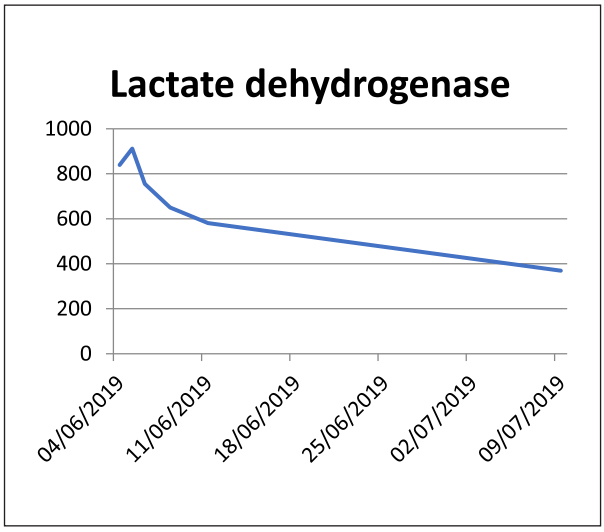

Fig. 1. Evolution of lactate dehydrogenase levels.

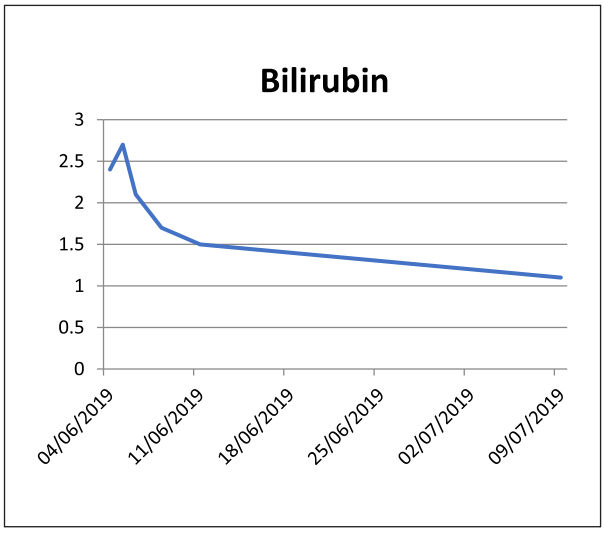

Fig. 2. Evolution of bilirubin levels.

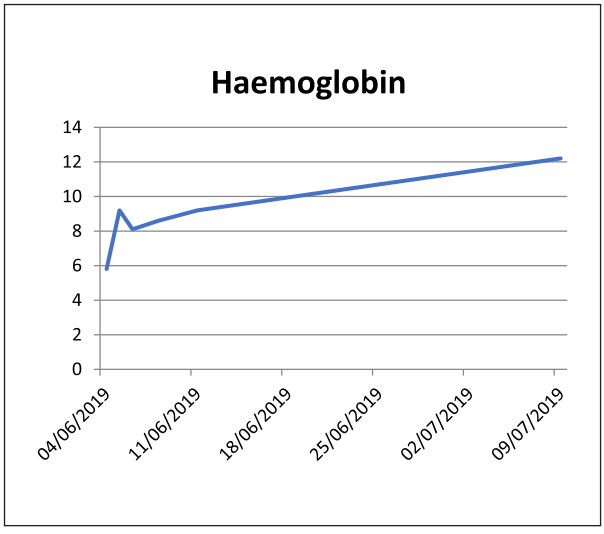

Fig. 3. Evolution of haemoglobin levels.

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metastasis in the pretibial zone. In December 2020, he received surgery of the unique active focus on the skin with free margins. He continued with fotemustine every 3 weeks for better tolerance. In the last follow-up in April 2021, PET scan showed complete metabolic response.

Discussion

Novel immunotherapies such as anti-PD1 and anti-PDL1 can induce immune-related adverse events (irAEs) that can involve all organs. Haematological irAEs are rare and not extensively described in the literature.

A recent observational study by Delanoy et al. [3] found that the frequency of haematological irAEs associated with anti-PD1 and anti-PDL1 is low, mostly appearing as neutropenia, haemolytic anaemia, and thrombocytopaenia. Although grade 2 or worse have been described in <1%, those were serious and/or life threatening.

There are few case reports in the literature, 10 with nivolumab and 3 with pembrolizumab. We have performed a review of the literature and compared our case report to others published since the moment about solid tumours.

In the largest review published, a total of 68 cases were identified in the database of the Food and Drug Administration (FDA), 43 were associated with nivolumab, 13 with pembrolizumab, 7 with ipilimumab, and 5 with atezolizumab. Cases were included until March 2018, but a few of them have been published. Thus, AIHA is a rare but potentially serious adverse effect. Although the precise frequency is unclear, the estimated incidence of AIHA induced by CPI is <0.1%. It seems to be more common with anti-PD1 and anti-PDL1 than with anti-CTLA4, and apparently its frequency is not related to the underlying malignancy [4].

Drug-induced AIHA may be either "warm" or "cold" depending on the temperature at which the autoantibodies become active. Warm AIHA is generally mediated via IgG or via C3, and the autoantibodies are active at temperatures >37°C. Cold AIHA is mediated via IgM activation, and the autoantibodies are active at temperatures of 0–4°C. AIHA after nivolumab therapy appears to be warm and is commonly mediated through IgG or C3 which was our case, but there are others published with IgG positive alone or both [5].

In addition, Michot et al. [6] found bone marrow failure in 3 cases of nivolumab therapy for melanoma, suggesting a central origin for immune-related cytopaenia as well. We do not think that this is our case, but physicians should suspect bone marrow suppression in some cases when pancytopaenia appears because AIHA has been described together with bone marrow aplasia [7]. Then, a bone marrow biopsy needs to be done.

The diagnosis of AIHA is made through laboratory findings. In our case, a decrease in haemoglobin, indirect hyperbilirubinemia, elevated LDH, and reduced haptoglobin were found. Coomb's test was positive for complement 3d but negative for IgG, according to most of the cases reported in the literature [3, 4].

Clinical guidelines recommend corticosteroids as the treatment of choice in AIHA. In refractory cases, rituximab with or without intravenous immunoglobulins may be useful [3, 4].

First-line treatment choice was high-dose prednisolone, generally 1 mg/kg, which required to be increased up to 1.5–2 mg/kg if the response is insufficient. Starting with high-dose pulse methylprednisolone (1,000 mg/24 h per 3 days) has no evidence of better outcomes [3]. Treatment with rituximab associated with corticosteroids has also been reported [3]. Generally, anaemia responded fairly well to steroids, but it could be fatal, as in the cases described by Palla et al. [8], where despite increasing the dose of steroids, the

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patient expired due to respiratory failure, and Tanios et al. [4], where the patient developed bradycardia and cardiac arrest [9].

AIHA can occur at any time with nivolumab, so clinicians should be aware of it even if the patient has not suffered any haematologic events previously [8]. The median time between initiation of anti-PD1 or anti-PDL1 and occurrence of haem-irAE is 10 weeks, with a range between 2 and 78 weeks [3, 4]. Our patient developed AIHA 7 weeks after the immunotherapy initiation, according to previous cases reported.

For patients with a history of mild AIHA diagnosis or a positive Coombs test without haemolysis, CPIs could probably be considered under special precautions and monitoring [4] assuming that in some cases, oral corticosteroids may be needed for recurrence especially if detected early by haemoglobin decrease in the routine blood tests as these patients should be monitored as a precaution [7].

After the remission of the AIHA with steroids, we started treatment with ipilimumab, an anti-CTLA4, based on the proven efficacy of this CPI in metastatic melanoma and due to its different mechanism of action compared to nivolumab. AIHA did not recur, and these data may provide some support for the safety of CPI in patients who have had haematological toxicities after anti-PD1/PDL1.

Schwab et al. [10] re-challenged a patient with ipilimumab and nivolumab, and the patient redeveloped AIHA. Conversely, Tardy et al. [11] re-challenged the patient with nivolumab without recurrence of AIHA.

The mechanism by which CPIs cause AIHA is likely by augmenting or redirecting immune surveillance, especially by activating pre-existing red blood cell autoantibodies. This mechanism of action is different from other drug-induced AIHA. It is speculated that CPIs cause a random activation of the immune system resulting in the formation of auto-antibodies, activation of T-cell clones, and diminishing the function of regulatory T cells [12].

Conclusions

Haematological irAEs induced by anti-PD1 or anti-PDL1 are rare and appear to be more common with anti-PD1/PDL1 than anti-CTLA4. The estimated frequency of AIHA is <1% of all immune-related side effects, but physicians should be aware because it often appears in a severe form.

Indications of re-challenge with immunotherapy remain unclear. It could be reasonable using anti-CTLA4 such as ipilimumab after a haematological irAE associated with anti-PD1/PDL1 in metastatic melanoma, and our case report supports its safety. Ipilimumab is not free from AIHA risk but it is lower.

Statement of Ethics

Written informed consent was obtained from the patient for publication of this case report. The study is exempt from ethics committee approval because there is no experimental intervention, and we only describe a case report about routine and standard clinical practice. Patient's identity has been protected and treated confidentially.

Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors helped to draft the manuscript, read, and approved the final manuscript. A.C. and R.F. are co-authors and were involved in collecting the data, performing the analysis, and writing the manuscript. H.P. participated in the analysis of the data. J.R. coordinated the drafting of the manuscript and its preparation for publication.

Data Availability Statement

All data generated or analysed during this study are included in this article and/or its online suppl. material files (for all online suppl. material, see www.karger.com/doi/10.1159/000518530). Further enquiries can be directed to the corresponding author.

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