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THE ROLE OF B-RAF MUTATION IN THE DIAGNOSIS AND PROGNOSIS OF CUTANEOUS MELANOMA
SUMMARY OF THE DOCTORAL THESIS

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TABLE OF CONTENTS

I. PART 1 – LITERATURE REVIEW

1. Melanocyte biology and development of melanocytic lesions

1.1 Melanocyte biology

Skin colour is the result of light reflected and absorbed into the skin layers and its complex reaction with the pigment present. The main molecules that make up to skin colour are melanin, haemoglobin and carotenoids, with melanin contributing the most to the final result. In contact with the skin, light can be reflected, scattered or absorbed in the skin in so-called chromophore molecules. In the epidermis, light dispersion occurs, and melanin subsequently absorbs the dispersed light, resulting in a black-brown or slightly reddish colour, depending on the type of melanin present. Thus, pigmentation is a feature of the epidermis, where the melanin produced by melanocytes is located. This can be of two types: constitutional or acquired. Constitutional pigmentation is an inherited genetic trait given by the amount, type and distribution of melanin. Acquired pigmentation is the result of the genetic basis interacting with environmental factors.

During intrauterine development, melanocytes develop from neural crests, an embryonic structure specific to vertebrates. Neural crests are located in the dorsal neural tube, between the primordium of the nervous system and the ectoderm. These neural crest cells eventually migrate into various embryonic structures and produce, in addition to melanocytes, neurons and glial cells of the peripheral nervous system, chondrocytes and osteocytes of the skull and face, endocardium, chromaffin cells of the adrenal medulla, and more. The development and differentiation of neural crest cells is a complex process that depends on numerous transcription factors and is regulated by complex intracellular networks. At the base of these mechanisms is a set of genes that regulate, in addition to the specialization of neural crest-derived cells and their migration from the neural tube level[1]. They encode beta-catenin-type regulatory proteins that control the expression of the transcription factor Mtif (microphthalmia-associated transcription factor). It is present specifically in immature melanoblasts and because it directly controls several genes involved in pigmentation, it plays a particularly important role in the development, specialization and survival of melanocytes[2].

Pigmented skin lesions, both benign and malignant, appear as a result of an increase in

either the number or the activity of melanocytes. Benign lesions – cutaneous naevi – can be congenital or, more frequently, acquired throughout life. They represent a local proliferation of melanocytes that develop in contact with each other, forming nests. They have various locations in relation to the layers of the skin and their development is triggered by mutations of oncogenes. A large number of changes are due to UV radiation [3]. The regulation of melanocyte proliferation is done through several pathways, one of the most important being the MAP (mitogen-activated protein)-kinase pathway. In normal cells, this occurs when binding of receptors for cytokines or tyrosine kinases activates "switch" proteins from the RAS (rat sarcoma virus) family, which, by activating a series of serine-threonine-kinases in turn, ultimately lead to the activation of MAPK. In the case of tumour cells, the MAPK pathway is permanently active[4]. B-RAF (v-raf murine sarcoma viral oncogene homolog B1) has an exclusive role in this signalling pathway and its most frequent mutation, V600E, the substitution between valine and glutamic acid in exon 5, codon 600, leads to the permanent activation of MAPK[5]. The theory of oncogenesis postulates that malignant cells originate from a normal adult cell, which, through repeated mutations, acquires functions typical of young or embryonic cells[6]. In the case of melanoma, this theory explains the aggressive mitotic activity and the significant potential for metastasis, melanocytic stem cells being characterized precisely by this increased ability to migrate to other regions of the fetal body.

1.2. Epidemiology. Risk factors

The incidence of skin melanoma (according to statistics from the United States) tripled between 1975 and 2005, so that one person in 55 is diagnosed with melanoma at some point in their life [7]. The risk factors for the development of melanoma can be classified into risk factors related to the environment and risk factors innate to the patient.

The most prevalent and well studied environmental risk factor for melanoma is ultraviolet radiation, and the increased incidence of skin cancers is explained on the one hand by the thinning of the ozone layer in the atmosphere, which leads to an increase in the amount of UV radiation that reaches the ground; and on the other hand, the increasing popularity of sunbeds, which emit an sigificant amount of UVB radiation [8]. Personal history of sunburns also increases the risk of developing both benign skin lesions (dysplastic naevi) and cutaneous melanoma, and the risk increases if the burns occurred in childhood. [9].

The use of tanning beds was demonstrated to increase the risk of developing melanoma. While using these devices, patients are exposed to amounts of UV radiation 10-15 times higher than after direct exposure to the sun for the same period of time. A study conducted by Lazovich et al., published in 2010, carried out in the United States between 2004 and 2007, on patients aged between 25 and 59 years, diagnosed with invasive melanoma, demonstrated that the use of tanning beds increases the risk of developing a melanoma, and this risk it increases the longer the use of these devices and does not vary with the type of device or the age at which the use began[10].

The patient-related risk factors were established by comparing groups of afflicted patients with control groups, thus resulting in relative risk tables [11]. Here, a significantly higher prevalence is observed in people with a small amount of melanic pigment, in the skin, hair and eyes. In contrast, individuals with intense skin pigmentation have a low prevalence of melanoma and in these cases, the tumours predominantly appear in atypical locations, such as acral or on mucous membranes.

Patient-specific risk factors for the occurrence of melanoma are associated with both the skin phenotype and the presence of pigmented naevi. Giant congenital naevi – over 9cm in diameter – although rare (Estimateded frequency at approximately 1:20,000 newborns [11]), bears a significantly higher risk of developing a melanoma (relative risk 5-15), and the treatment of these patients is quite problematic given the fact that these melanomas appear in childhood and often have unusual locations, such as the central nervous system. [12] The risk of melanoma associated with the presence of small congenital naevi is difficult to Estimatede given the difficulty in distinguishing them from acquired naevi.

Immunosuppression of any kind, including in the context of human immunodeficiency virus (HIV) infection, has also been correlated with the more frequent occurrence of melanoma and the increased aggressiveness of tumours for these patients [13].

The risk of developing melanoma is determined by a combination of environmental and genetic factors. Usually, the terms "sporadic" and "familial" are used to divide these cases. However, these terms can have different meanings depending on the geographical region analysed. Thus, in regions with a high incidence (e.g, Australia) the occurrence of a melanocytic tumour in two members of the same family is relatively frequent and does not involve a "familial" transmission, as is the case in a region with a low incidence of melanoma. The involvement of genetic risk factors is evident in truly familial cases, where

an increased incidence of cases is observed in genetically related individuals, who share a certain mutation transmitted to their descendants. In practice, most cases of melanoma (>90%) occur sporadically[7].

1.3. Classification of pigmented lesions

The factors that contribute to the differentiation of the various subtypes of melanocytic lesions and to their clinical appearance are their origin, wether epithelial or non-epithelial, and also the specific genetic changes present in the lesions. Most of the studies conducted indicate that the latter contribute most significantly to the phenotypic appearance of the injury. The typical mutations that induce proliferation are mainly activating mutations in the MAP-kinase (mitogen-activated protein kinase) pathway or receptor tyrosine kinase (RTK) translocations[4].

Acquired naevi (simple or dysplastic)

By definition, a naevus is a benign proliferation of melanocytes arranged in nests – meaning a group of three or more contiguous melanocytes. Unlike melanoma, naevi are organized, clearly delimited, relatively symmetrical and usually the melanocytes in their component appear in groups and not in individual cells.

The development of naevi depends, as for other pigmented lesions discussed, on genetic and environmental factors. The only known environmental factor is exposure to ultraviolet radiation, whereas the genetic factors can be several, out of which most notable are the skin phenotype and the MCR1 receptor type, which, together with other genes, encode skin and hair pigmentation and implicitly the individual's ability to protect themselves from the harmful effect of UV radiation through the production of melanin. Acquired naevi can be completely flat or slightly raised. Most of them are smaller than 6mm, symmetrical in shape and uniform in colour. Dermoscopically, they are reticular or globular, the reticular pattern is given by the distribution of melanin and melanocytes along the epidermal papillae, and the globular pattern is represented by the aggregation of melanocytes in distinct nests.

Congenital naevi

Congenital naevi are present at birth or appear in the first year of life and are present in approximately 1% of newborns. They are classified as small (< 1.5cm), moderate (1.5-20cm), large (>20cm) and sometimes giant (>40cm) [14]. Small naevi are more frequent,

and the large subtypes have an incidence of approximately 1:20,000 [15].

Most small and medium-sized congenital naevi can appear anywhere on the body, they are homogeneous lesions, clearly defined, often papillomatous and sometimes covered with hair. More clinically significant are large or giant naevi, especially those with a cranial distribution or at the level of the spine projection, since in these cases, the proliferation can extend to the meninges or the underlying nerve structures, thus speaking of leptomeningeal melanocytosis or neurocutaneous melanosis, entities that can evolves with complications such as hydrocephalus or meningeal melanoma. The risk is particularly high in the case of giant naevi with axial distribution and numerous satellite lesions [16], [17].

2. Melanoma

2.1. Melanoma subtypes

The current classification, proposed by Clark and his collaborators, classifies cutaneous melanoma into four subgroups, based on the clinical and histological characteristics, namely: superficial spreading melanoma, nodular melanoma, acral melanoma and lentigo malingna.

Superficial spreading melanoma

The most common tumour subtype, it reaches up to 60% of cases [18]. They are more common on the posterior chest and extremities, although they can appear anywhere on the body. They generally appear on intermittently sun-exposed skin, and in rarer cases on areas chronically exposed to the sun. It is the subtype most commonly associated with melanocytic naevi and develops more frequently from pre-existing naevi than other subtypes [18], [19]. The ABCDE rule is more relevant to this subtype than to others. Histologically, the lesions are characterized by a horizontal growth phase, with or without an associated vertical growth phase. The lesions are often asymmetrical and poorly defined peripherally, where an anarchic dispersion of single melanocytes is observed.

Nodular melanoma

This subtype of melanoma occurs on both intermittently and chronically sun-exposed areas. The ABCDE method is less useful in the diagnosis and follow-up of this subtype of melanoma, as the lesions are atypical in clinical presentation. They can be pigmented or non-pigmented, symmetric or asymmetric, flat or raised and can sometimes be confused

with basal cell or squamous cell carcinoma. Dermoscopically, some particular signs can sometimes be seen, such as heterochromia, polymorphic vascular points, a white veil or white striae at the level of the tumour [20].

Melanoma developed on lentigo maligna

This form appears specifically on skin chronically exposed to the sun, usually on the head and neck and sometimes on the distal portions of the extremities. Similar to superficial spreading subtypes, it is characterised by a horizontal growth phase and a vertical growth phase. The horizontal growth phase is long and less aggressive, before the transition to the vertical growth phase. Precursor lesions (lentigo solaris) are heterochromic pigmented spots appearing on an area of skin with chronic, sun-induced changes (poikiloderma, hypo- or hyperpigmentation, telangiectasias). Histologically, a specific aspect of this subtype of melanoma is the arrangement of melanocytes one by one, with varying degrees of cellular atypia, along the basement membrane.

Acral melanoma

This tumour subtype develops on the palms, soles and nail beds and is histologically similar to the malignant lentigo subtype, with a long period of horizontal evolution and histological arrangement in the form of single melanocytes along the basement membrane. A particular aspect of these tumours is the lack of mutations acquired under the influence of UV radiation, so that this causative factor does not seem to have a particularly significant role in the development of these lesions.

Desmoplastic melanoma

This subtype is the rarest, occuring in less than 4% of cases. It presents characteristics that differentiate it from other forms of melanoma, especially local aggressiveness. It afflicts elderly patients, usually men, usually in areas chronically exposed to the sun such as the scalp or face. The lesions do not show the clinical characteristics of melanoma, so the positive diagnosis is difficult to achieve and is often established late.

Genetic characteristics of melanoma

Although there is no general rule of genetic changes specific to each subtype of melanoma, some observations were made. Lesions appearing on naevi, in areas intermittently exposed to the sun [21], in young patients (<55 years old) [22], with melanocytes arranged in a relatively organized pattern, in groups, with intense pigmentation and epidermal extension, plead for a BRAF mutation. These features are also typical for the superficial spreading

subtype, which is also associated with a higher frequency of the BRAF mutation. Indeed, approximately 52% of superficial spreading melanomas show BRAF mutation and 20% NRAS mutation[21]. Since BRAF and NRAS mutations also occur in benign naevi, it can be hypothesized that they are not sufficient to induce malignancy. The accumulation of mutations over time, especially under the action of UV radiation, are what seems to trigger the neoplastic process [23]. In the case of acral melanoma, the most independent of UV radiation, structural aberrations of cell cycle regulatory and tumour suppressor genes may play the main role in oncogenesis.

2.2. Melanoma staging

Similar to other solid tumours, melanoma staging takes into account the primary tumour (T), the regional nodes (N), and the presence of distant metastases (M).

T: Primary tumour

The defining element in general to characterize the primary tumour is its size. In the case of melanoma, the Breslow index is used to determine tumour size, which measures the thickness of the tumour, more precisely the distance from the surface of the skin to the maximum depth of tumour invasion[24].

The presence of ulceration in the primary tumour is a separate and well-studied negative prognostic factor in cutaneous melanoma. It is defined as a full-thickness skin defect area, surrounded by a reactive area that presents changes at the level of the epidermis [25].

The mitotic index is another criterion that contributes to classifying patients into risk groups. It is defined as the number of mitoses per mm2 and is of interest in early stages. Mitotic rate influences prognosis and long-term survival and some authors considered it more important than ulceration in certain cases [26].

N: Regional lymph nodes

This takes into account the presence or absence of regional tumour dissemination at nodal or non-nodal level (satellite, microsatellite or transit nodes), in order to establish the appropriate treatment plan. Both the clinical evaluation methods and the surgical techniques indicated have been adapted over time, especially in recent years, to assess and stratify risk and to assist the clinician in choosing the optimal therapeutic approach for each individual patient. During the assessment of the disease, the patient is clinically examined and the presence or absence of regional adenopathies is noted. Depending on the

presence or absence of lymph node invasion, subsequent treatment is decided.

M: Distant metastases

In the international classification, category M indicates generalised disease, with M0 denoting absence, and M1 denoting presence of distant metastases. Subcategories indicate location as well as serum lactate dehydrogenase (LDH) values. In the case of patients with multiple metastases, category M is determined according to the most unfavourable location.

2.3. Therapeutic approach to melanoma

2.3.1. Excision of the primary tumour

Surgical excision of the primary tumour for melanoma – similar to most other skin cancers - remains the first-line approach, the gold standard, with curative intent and excellent results in early stages and which provides excellent information for establishing the prognosis both in early cases and in the advanced ones.

The goal of surgical excision of the primary tumour is to prevent recurrences and to control local and regional invasion of the disease. This aspect is particularly important for patients with small tumours without regional extension. Through wide excision, the appropriate removal of the primary tumour and any satellite nodes around it is ensured. Therefore, the macroscopic safety margin must be selected carefully, in order to minimise recurrence risk. The thickness of the tumour, expressed by the Breslow index, is the defining element in the case of choosing the safety margins. Thin melanomas, with a Breslow index < 1 mm, have a recurrence rate below 6% for a safety resection margin of 1 cm around the primary tumour [27]. Regarding melanomas with a Breslow index between 1 and 2 mm, many clinicians are reluctant to practice narrow excisions for these tumours, a fact justified by the slightly increased rate of recurrences. For tumours with a Breslow index greater than 2mm, a 2cm excision margin was considered indicated.

Once the positive diagnosis of melanoma is obtained, a wide resection of the lesion is often necessary to ensure adequate safety margins. In this sense, the correct orientation of the first incision is essential, since re-excision will lengthen the scar and possibly create a defect that must be covered. In rare cases, when the extension of the tumour is uncertain, it is necessary to graft the skin or leave the wound open and use various methods of temporary covering, such as a negative pressure system, until a definite diagnosis is

obtained. Once complete excision of the lesion is confirmed, the defect can be covered with a local flap if direct suturing is not possible.

2.3.2. Assessment of regional lymph nodes

Over time, several studies have shown that melanomas initially spread through the dermal lymphatic network, reaching regional lymph nodes and subsequently spread systemically. Sentinel lymph node technique, followed by complete lymph node dissection for patients with lymph node invasion has radically changed the therapeutic approach to the disease. The numerous studies carried out since then have demonstrated the benefits of this technique for the diagnosis, staging and development of a therapeutic plan for melanoma patients. The positive diagnosis of the sentinel node indicates the extension of the disease to the primary drainage lymphatic basin. The results of this invasive investigation are subsequently used to stage and Estimatede the prognosis of the disease as accurately as possible. It also indicates whether complete lymph node dissection or adjuvant therapy would benefit the patient. This investigation is indicated to all patients with melanomas with a Breslow index greater than 1 mm and for thinner tumours if they present unfavourable prognostic criteria, such as ulceration, frequent mitoses, or perivascular invasion. Regarding prognosis, patients who do not present tumour cells in the sentinel node have a 3-year survival rate of approximately 90%, whereas the rate drops to 60% if they are present[28]. With the evolution of targeted systemic therapies, the benefits of complete lymph node resection decreases on the one hand, and on the other hand, the role of the sentinel node biopsy shifts, from that of selecting patients who have a surgical indication to that of indicating an aggressive systemic therapy.

2.3.3 Therapeutic approach to local relapses

Local recurrence is defined as the reappearance of the tumour either at the site of the initial scar, or between it and the regional lymphatic basin, the latter being defined as satellite metastases or in transit lesions. They are considered lymphogenic disseminations, their presence signifies stage III disease, since they represent nodal components (N) of the disease[29]. Once the relapses are detected, the optimal therapeutic option for the patient can be decided. Surgical excision or topical treatment can be performed locally, alternatively radiotherapy or systemic therapies can be selected. The therapeutic decision is

based on previous cancer treatments, age and co-morbidities, and also the number and location of the lesions identified. Patient history is essential, from the initial treatment, both surgical – such as the type of excision, or whether or not a biopsy of the sentinel lymph node or a lymph node dissection was performed; as well as non-surgical. Whether BRAF mutation testing and/or specific treatment was performed is also relevant, and all these factors determine the subsequent therapeutic approach.

2.3.4. Management of metastatic melanoma

Melanoma remains an aggressive oncological pathology, and sometimes the disease is diagnosed when metastatic disease is present. Currently, the multidisciplinary approach is essential in the management of these patients.

Surgical treatment remains indicated for patients with single metastases, or with less than 4 lesions usually limited to a certain segment (brain, liver, lung). Sometimes, in cases of bone or brain metastases, it is associated or replaced with radiotherapy, with good results in local disease control [30]. As for local recurrences, targeted therapies and immunotherapy play an important role in the therapy of metastatic melanoma. Several alternatives to traditional therapy have been developed and the pathophysiology of melanoma metastases is much better understood, so in the future there will be more alternatives to explore. Each modern therapy, separately, offers promising results, and their association is also of interest, as, for example, the combination of radiotherapy with immunotherapy shows promising results in the case of brain tumours [30].

2. PART 2 – STUDY AND RESULTS

1. Materials and method

In this paper, we present a retrospective, observational, longitudinal cross-sectional, non-randomized study on a sample of 30 patients diagnosed with malignant melanoma at different oncological stages. The patients may have had the BRAF mutation present or absent, and they were representative of a population diagnosed with malignant melanoma and treated at specialized centers for multidisciplinary oncological treatment.

The study cohort was formed using the following algorithm: 15 patients with malignant melanoma and the BRAF mutation were enrolled, while 15 patients without the mutation were identified through the same specific genetic testing. Complete and verifiable oncological data were retrieved from patient files for all participants.

The study group consisted of patients from the Bucharest Oncological Institute and the Bucharest Emergency University Hospital'

The inclusion criteria were as follows:

- Patients with histologically confirmed cutaneous melanoma.
- Patients who followed the specialized surgical and oncological treatment protocol, including targeted therapy for patients with the BRAF mutation.
- Patients who underwent genetic testing for the BRAF mutation, including those with and without the mutation.
- Patients aged 18 years or older.

The exclusion criteria were:

- Patients with acral, ocular, or mucosal melanoma.
- Patients who discontinued the treatment regimen.
- Patients with serious co-morbidities that prevented them from continuing the treatment.
- Patients who were not genetically tested for the BRAF mutation.

The patients underwent comprehensive treatment, including surgical procedures such as sentinel lymph node biopsy when indicated, as well as oncologic treatment. Patients who discontinued treatment due to adverse effects were excluded from the study. Patient follow-up for the current study was completed until September 1, 2021.

The main objective of the study was to investigate the influence of the BRAF mutation on the evolution and prognosis of melanomas. The secondary objective was to determine the impact of other oncological and clinical characteristics on disease prognosis and progression.

The primary endpoints of the study were overall survival (OS) and progression-free survival (PFS). OS was defined as the period from diagnosis/start of therapy of a malignant tumour until death, while PFS was defined as the period from diagnosis/beginning of therapy of a malignant tumour until the occurrence of specific events such as local recurrence, increase in tumour size based on imaging/clinical/in-vivo assessment, appearance of new metastasis, diagnosis of a new malignancy, or death. The study also considered survival at 5 years as a secondary endpoint, but due to the limited average follow-up period of approximately 3 years, its value was limited.

A classic descriptive statistical analysis was conducted for the variables included in the study. For continuous variables, measures of central tendency such as mean and median were estimated, along with measures of variability such as standard deviation (SD), minimum and maximum values, and the range distribution.

Inferential analysis employed methods from the "time to event" analysis category, specifically survival analysis. The methods used were based on the following estimators (statistics):

- The Kaplan-Meier non-parametric survival estimator, which graphically represented survival curves and calculated statistics such as median survival (the time period during which 50% of patients experienced the event). However, this statistic was not very useful in our study due to the relatively short follow-up period and a significant number of patients who did not experience the event. In some cases, a 95% confidence interval (95% CI) was attempted but not feasible for all situations. The analysis also calculated restricted mean survival time (RMST), which represented the average survival time if patients were followed for a limited period. Additionally, the average of years lost by patients (restricted mean time lost - RMTL) was calculated, representing the average period "lost" by patients over the limited follow-up period. The study compared survival curves for discrete predictors using two tests: the Martel-Haenszel log-rank test and the Gehan-Wilcoxon test with the Peto & Peto modification. RMST values were compared

using three indicators: the difference between RMST, the ratio between RMST, and the ratio between RMTL. This analysis was limited to categorical covariates with two categories, comparing the smaller of the two corresponding categories. RMST was also compared at several moments of the survival curves, such as 12, 24, and 60 months, whenever possible. In some cases, the permuted version of the RMST tests was also used considering the small sample size and the potential for type I errors in inferential analyses.

- The second estimator used was the hazard ratio (HR), calculated using Cox regression, a semi-parametric method that assumes proportional hazard rates throughout the entire evolution. This method could be applied to continuous predictors as well.

The level of significance (α) for the analysis in the study was set at 0.05, considering p-values lower than 0.05 as statistically significant. Marginally insignificant p-values (p between 0.05 and 0.10) were also considered due to the small sample size. It should be mentioned that a more advanced statistical methodology for survival analysis was used in the doctoral thesis, but the limited space of this summary does not allow for detailed explanation.

In this summary, we will present the results obtained only for the demographic, clinical, and paraclinical parameters that had a statistically significant or marginally insignificant influence on our population sample. We will also discuss the influence of the BRAF mutation on the evolution of malignant melanoma. However, the results presented will focus only on the OS analysis and not the PFS analysis.

2. Statistical analysis of the overall survival (OS)

Overall survival analysis for study lot

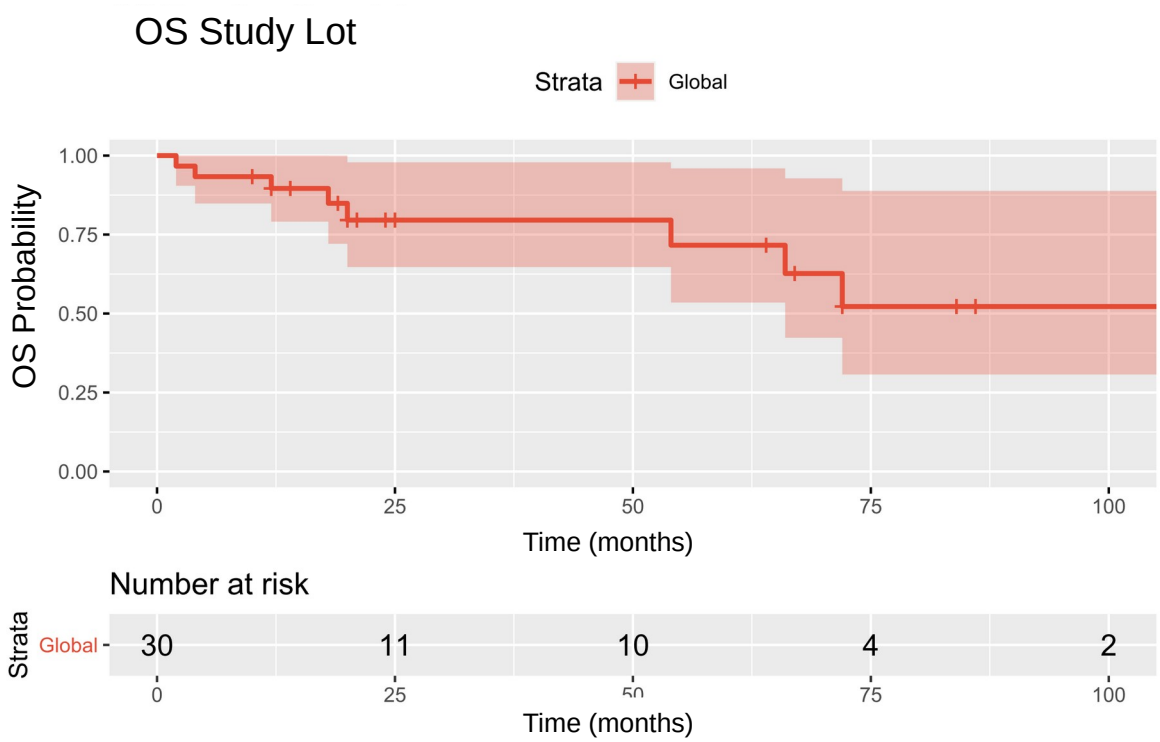
Table 2.1.

Strata	N Deaths (%)	RMST	Median Survival (95% CI)
Study lot	8 / 30 (26.66%)	77.73	N/A (66.00 for N/A)

Overall Survival for whole study lot

The overall survival rate on the entire lot was 73.34%. The median survival time (RMST) was nearly 78 months, with only the lower limit of the 95% confidence interval (CI) being determined, which was 66 months (5 and a half years).

Fig 2.1



Kaplan-Meier OS curve

Overall survival for presence/absence of B-RAF mutation:

table 2.2

BRAF Mutation	N Deaths (%)	Median Survival (95% CI)
Absent	2 / 15 (13.33)	54.00 (54.00 to N/A)
Present	6 / 15 (40.00)	72.00 (66.00 to N/A)

Overall survival for presence/absence of B-RAF mutation

Mortality was higher among patients with the BRAF mutation, but the median survival was 20 months longer in BRAF-positive patients. The results of the survival curve tests are displayed in the following tables:

table 2.3

BRAF	N	Deceased	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Absent	15	2	2.82	0.40 (1)	0.500
Present	15	6	5.18	-	-

Log-rank Martel-Haenszel test for OS for presence/absence of B-RAF mutation

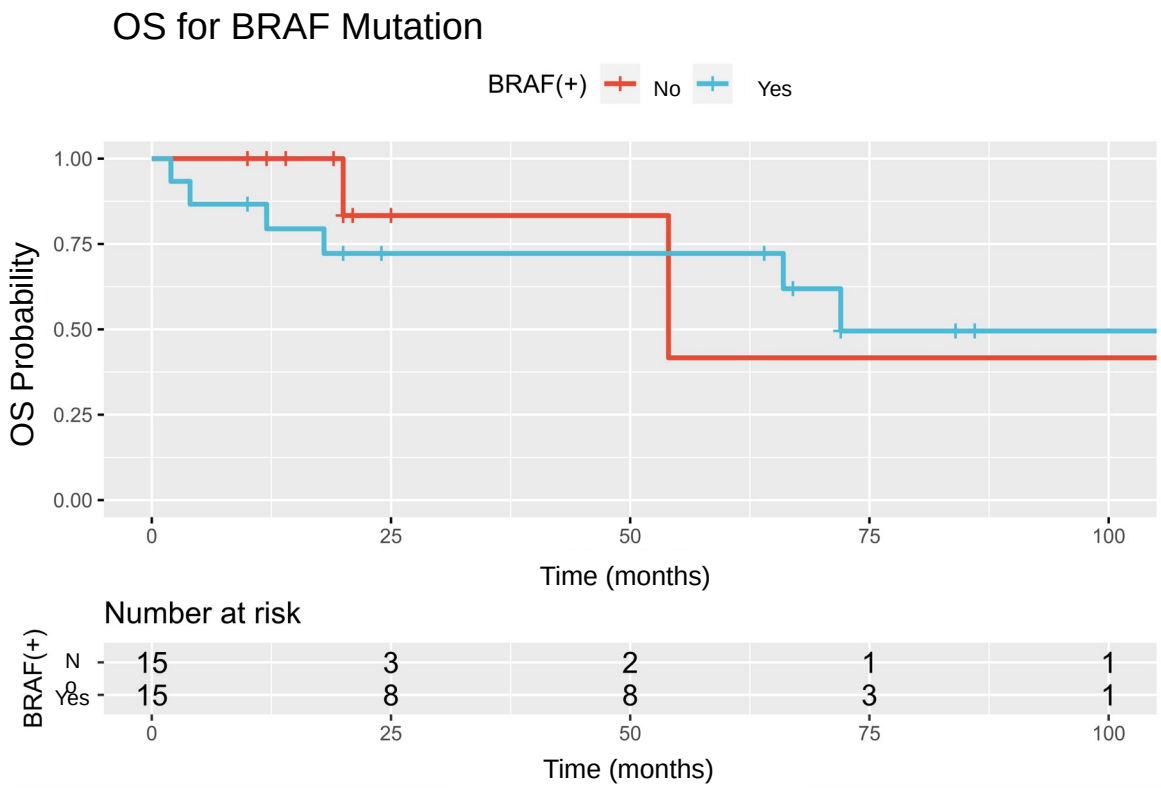
table 2.4

BRAF	N	Deceased	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Absent	15	1.64	2.53	0.60 (1)	0.400
Present	15	5.14	4.25	-	-

Gehan-Wilcoxon test for OS for presence/absence of B-RAF mutation

Both tests indicate that there are no statistically significant differences in terms of the survival curves.

Fig 2.2



Kaplan-Meier for OS for presence/absence of B-RAF mutation

table 2.5

Statistic	Estimated	95% CI
RMST BRAF Present	72.76	49.69 to 95.83
RMST BRAF Absent	71.66	34.41 to 108.91
RMTL BRAF Present	37.23	14.16 to 60.30
RMTL BRAF Absent	38.33	1.08 to 75.58

RMST and RMTL for OS for presence/absence of B-RAF mutation

For both RMST (Restricted Mean Survival Time) and RMTL (Restricted Mean Time Lost), the statistics showed similar values. Comparative tests were conducted to further analyze the data:

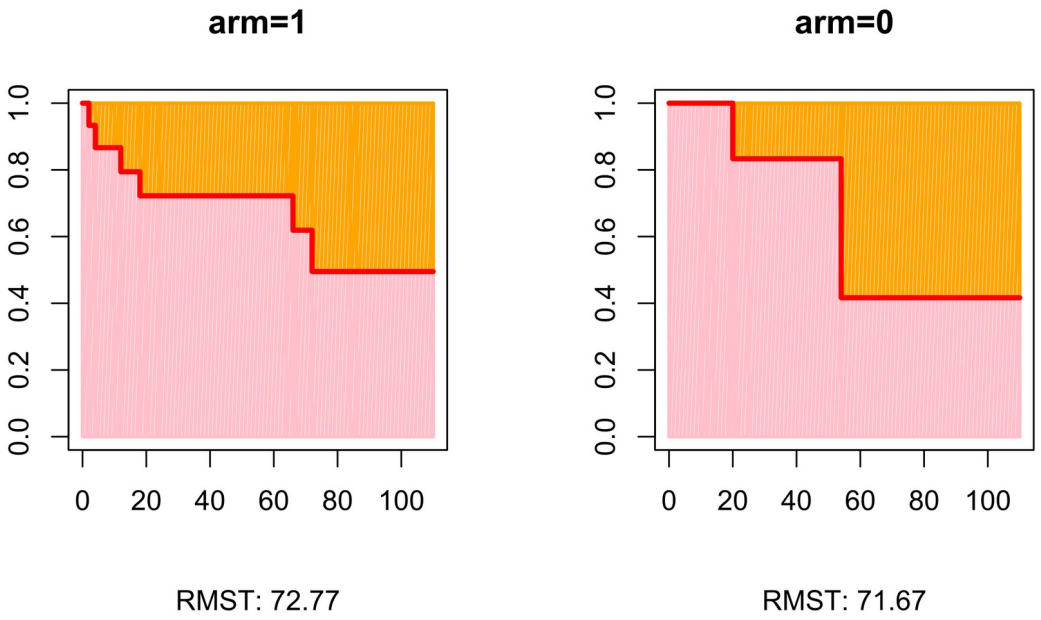
table 2.6

Test	Estimated (95% CI)	p value
Diferente RMST	-0.27 (-35.77 to 35.22)	0.961
Raport RMST	0.99 (0.63 to 1.57)	0.961
Raport RMLT	1.00 (0.33 to 3.01)	0.961

Comparative tests for OS for presence/absence of B-RAF mutation

No statistically significant differences were observed in any of the tests conducted, as indicated by the graph. The graph shows two arms: Arm 1 represents patients who were BRAF positive, Arm 0 represents patients who were BRAF negative.

fig 2.3



Comparative tests for OS for presence/absence of B-RAF mutation

Cox regression :

table 2.7

Predictor	Deaths N	HR (95% CI)[1]	p value
BRAF mutation			
Absent	2	—	

Predictor	Deaths N	HR (95% CI)[1]	p value
Present	6	1.72 (0.32 la 9.13)	0.523

[1] HR = Hazard Ratio

Cox regression for OS for presence/absence of B-RAF mutation

Although the hazard ratio (HR) was 1.72 in BRAF-positive patients, the effect did not reach statistical significance.

Overall Survival analysis to assess the impact of LDH (lactate dehydrogenase) levels at the time of diagnosis.

table 2.8

LDH	N Deaths (%)	Median Survival (95% CI)
Normal	1 / 10 (10.00)	N/A (N/A to N/A)
Elevated	7 / 20 (35.00)	72.00 (20.00 to N/A)

OS analysis for LDH level at the time of diagnosis

Mortality was higher among patients who had elevated LDH levels at the time of diagnosis. The results of the survival curve tests depicting this relationship are presented in the following tables:

table 2.9

LDH	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Normal	10	1	3.95	4.80 (1)	0.03
Elevated	20	7	4.05	-	-

log-rank Martel-Haenszel test for LDH level at the time of diagnosis

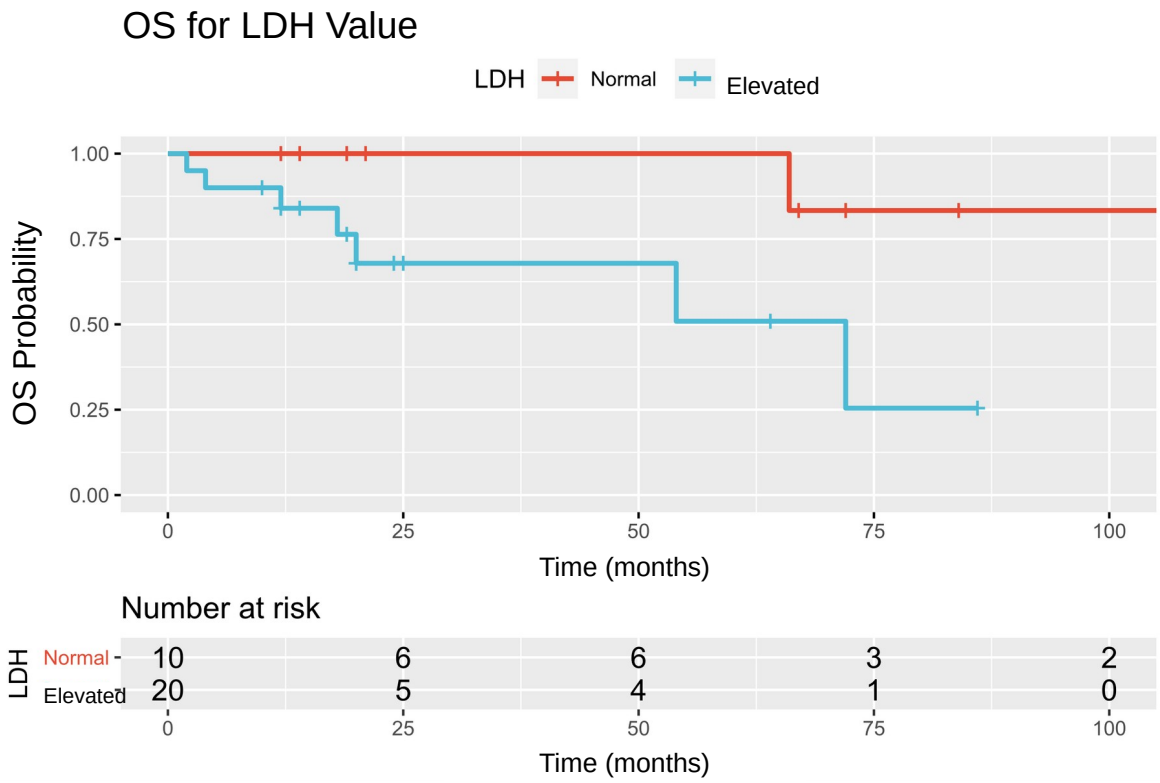
table 2.10

LDH	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Normal	10	0.71	3.22	4.60 (1)	0.03
Elevated	20	6.06	3.56	-	-

Gehan-Wilcoxon test for LDH level at the time of diagnosis

Both tests reported statistically significant p-values, indicating that there was a significant association between elevated LDH values at the time of diagnosis and higher mortality during the follow-up period.

Fig 2.4



Kaplan-Meier survival curves for LDH level at the time of diagnosis

table 2.11

Statistic	Estimated	95% CI
RMST – Elevated LDH	53.47	36.72 to 70.22
RMST – Normal LDH	82.66	76.70 to 88.63
RMTL – Elevated LDH	32.52	15.77 to 49.27
RMTL – Normal LDH	3.33	-2.63 to 9.29

RMST and RMTL for LDH level at the time of diagnosis

The RMST (Restricted Mean Survival Time) was nearly 30 months lower in patients with elevated LDH levels, indicating a shorter overall survival time compared to patients with normal LDH levels. On the other hand, the RMTL (Restricted Mean Time Lost) was approximately 30 months higher in patients with normal LDH levels, suggesting a longer duration of time lost due to mortality compared to patients with elevated LDH levels.

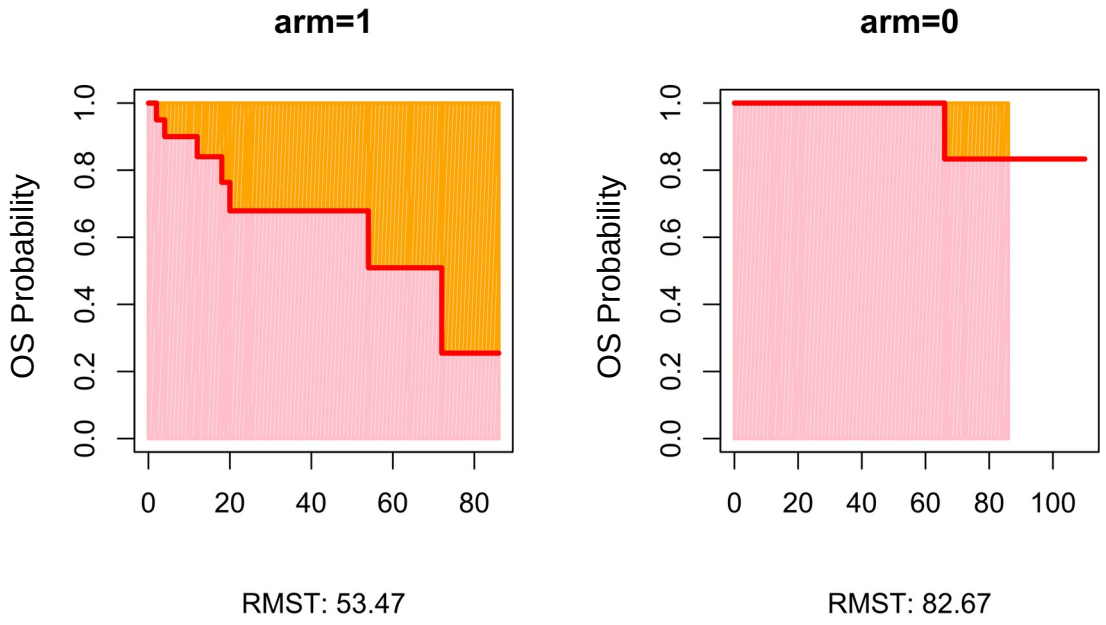
table 2.12

Test	Estimated (95% CI)	p value
Diferente RMST	-29.19 (-46.97 to -11.43)	0.001
Raport RMST	0.64 (0.46 to 0.89)	0.008
Raport RMLT	9.75 (1.51 to 62.79)	0.016

Comparative tests for LDH level at the time of diagnosis

All tests reported statistically significant differences, indicating that patients with elevated LDH levels at the time of diagnosis had an average of 30 months less survival and lost an average of 30 months more of their life compared to patients with LDH levels within normal range. This relationship is depicted in the graph where Arm 1 represents patients with elevated LDH levels and Arm 0 represents patients with normal LDH values.

Fig 2.5



Comparative tests for LDH level at the time of diagnosis

For a more comprehensive analysis, the RMST (Restricted Mean Survival Time) at 12, 24, and 60 months was compared using both the classic test and a permutation test with 100,000 resamplings without reintroduction from the population sample. The following table presents the RMST values for the analyzed period, the difference between RMST for patients with elevated LDH versus normal LDH, as well as the raw p-values and the results of the Permutation Test:

table 2.13

Time Point	RMST Elevated LDH	RMST Normal LDH	Difference	Raw p-value	Permutation Test
12 months	10.87	12.00	-1.12	0.133	0.209
24 months	20.38	24.00	-3.61	0.019	0.083
60 months	43.80	60.00	-16.19	0.004	0.039

Comparative analysis for LDH level at the time of diagnosis

Based on the provided information, we can observe the following findings:

At 12 months, there are no statistically significant differences in RMST between patients with elevated LDH and those with normal LDH. At 24 months, there is a statistically significant difference of 3.61 months in favor of patients with normal LDH. The p-value for the asymptotic test indicates statistical significance, while the p-value for the permutation test is marginally insignificant (0.08). At 60 months, there is a statistically significant difference of 16.19 months in favor of patients with normal LDH. Both the asymptotic test and the permutation test yield statistically significant p-values.

Considering these results, it can be concluded that starting from 24 months from the time of diagnosis, patients with elevated LDH have a worse prognosis compared to patients with normal LDH levels at the time of diagnosis. The data provides sufficient evidence to support this conclusion.

table 2.14

Predictor	Deces N	HR (95% CI)[1]	p value
Elevated LDH			
No	1	—	
Yes	7	8.00 (0.93 la 68.5)	0.058

[1] HR = Hazard Ratio

Cox regression for LDH level at the time of diagnosis

With a p-value that is on the borderline of statistical significance, it can be inferred that patients with elevated LDH levels at the time of diagnosis have approximately 8 times higher risk of death compared to patients who had normal LDH levels at diagnosis.

An analysis was conducted comparing tumour stages T1, T2, and T3 with stage T4:

table 2.15

T4 vs Other Stages	N Deaths (%)	Median Survival (95% CI)
T1&T2&T3	2 / 14 (14.28)	N/A (72.00 to N/A)
T4	6 / 16 (37.50)	54.00 (18.00 to N/A)

Overall Survival for T stage

Mortality was found to be higher in patients diagnosed with stage T4 tumors. The results of the survival curve tests, which demonstrate this relationship, are presented in the following tables:

table 2.16

T Stage	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
T1&T2&T3	14	2	4.67	3.80 (1)	0.05
T4	16	6	3.33	-	-

log-rank Martel-Haenszel test for T stage

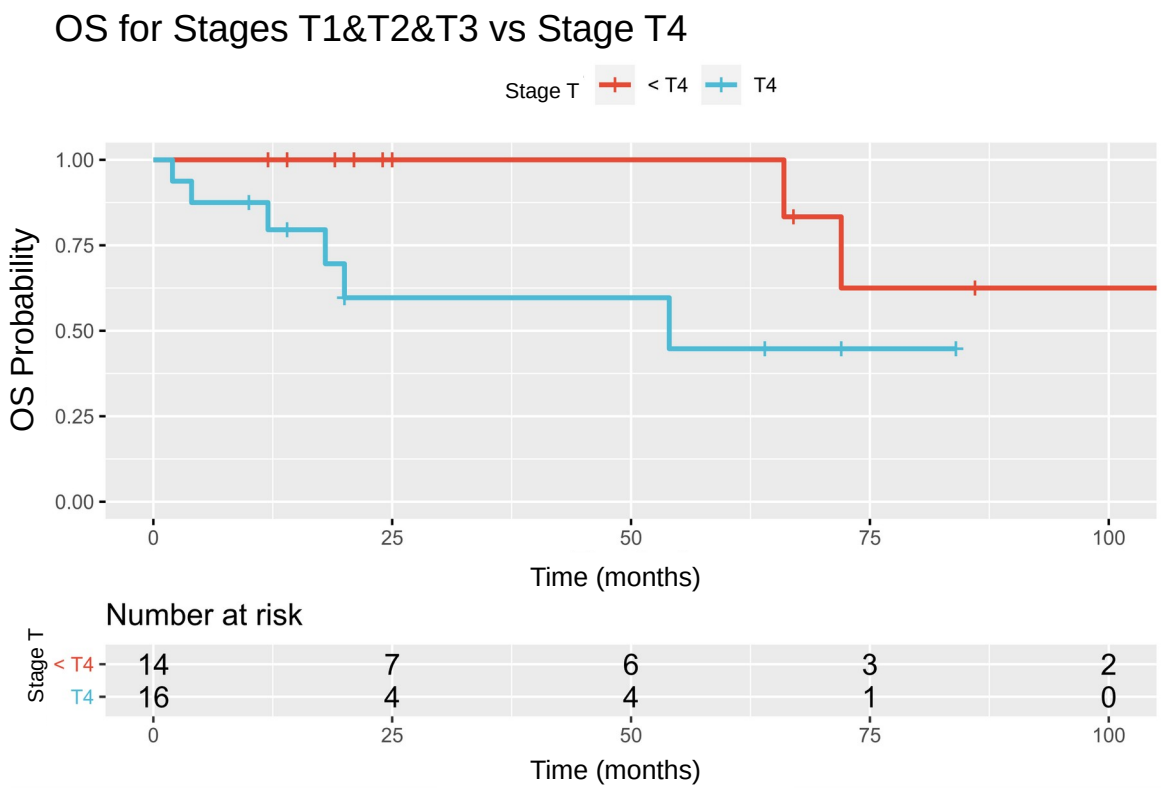
table 2.17

T Stage	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
T1&T2&T3	14	1.34	3.88	4.60 (1)	0.03
T4	16	5.44	2.90	-	-

Gehan-Wilcoxon test for T stage

The results of the tests provide evidence supporting the hypothesis that mortality is higher in patients in the T4 stage compared to the earlier stages. One of the tests yielded a p-value at the cut-off limit for statistical significance, suggesting a possible association, while the other test yielded a statistically significant p-value, providing stronger evidence of the relationship.

Fig 2.6



Kaplan-Meier survival curves for T stage

table 2.18

Statistic	Estimated	95% CI
RMST Stadiul T4	50.74	31.25 to 70.23
RMST Stadii T1&T2&T3	78.50	72.33 to 84.66
RMTL Stadiul T4	33.25	13.76 to 52.74
RMTL Stadii T1&T2&T3	5.50	-0.66 to 11.66

RMST and RMTL for T stage

The RMST was found to be nearly 28 months lower in patients with T4 stage tumours, indicating a shorter overall survival time compared to patients with lower T4 stages. Conversely, the RMTL was approximately 28 months higher in patients with lower T4 stages, suggesting a longer duration of time lost due to mortality compared to patients with T4 stage tumours.

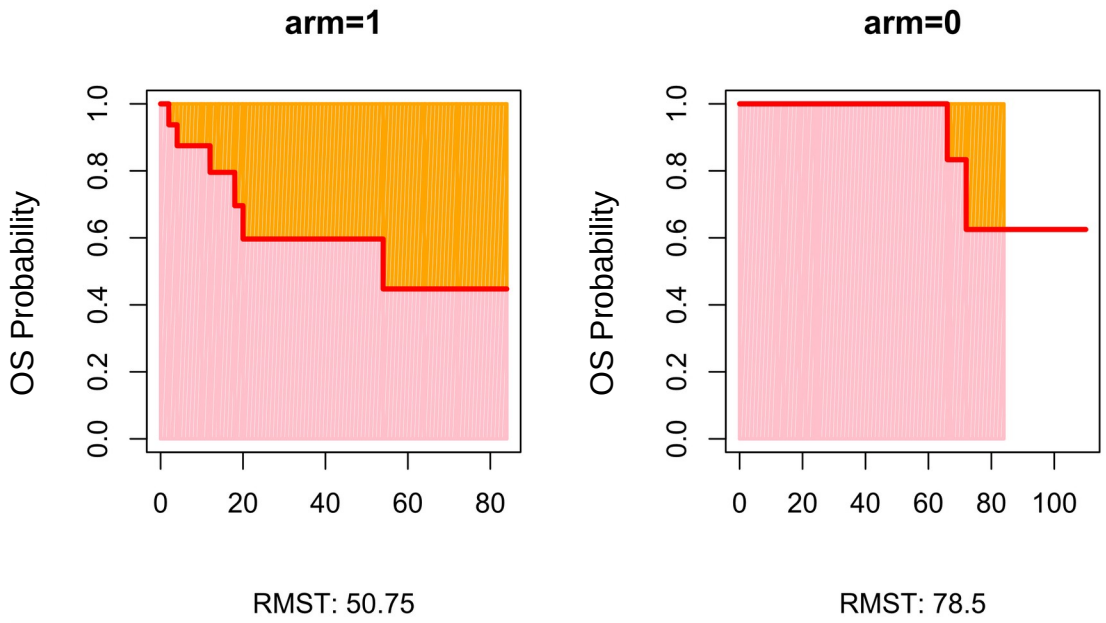
table 2.19

Test	Estimated (95% CI)	p value
Diferente RMST	-27.75 (-48.19 to -7.31)	0.008
Raport RMST	0.64 (0.43 to 0.95)	0.029
Raport RMLT	6.04 (1.70 to 21.42)	0.005

Comparative tests for T stage

All tests conducted reported statistically significant differences, indicating that patients in the T4 stage have an average survival of 28 months less and lose an average of 28 months more of their life compared to patients with earlier T stages. This relationship is depicted in the graph where Arm 1 represents patients in the T4 stage and Arm 0 represents patients in stages lower than T4. The findings emphasize the substantial impact of tumor stage on survival outcomes.

fig 2.7



Comparative tests for T stage

For a more comprehensive analysis, the RMST (Restricted Mean Survival Time) at 12, 24, and 60 months was compared using both the classical test and a permutation test with 100,000 resamplings without reintroduction from the population sample. The following table presents the RMST values for the analysed period, the difference between RMST for patients in stage T4 compared to stages T1/T2/T3, as well as the raw p-values and the results of the permutation test:

table 2.20

Time Point	RMST Stage T4	RMST Stages <T4	Difference	Raw p-value	Permutation Test
12 months	10.87	12.00	-1.12	0.133	0.209
24 months	19.42	24.00	-4.57	0.014	0.017
60 months	40.00	60.00	-20.00	0.002	0.009

Comparative analysis for T stage

Based on the provided information, the following observations can be made:
Within the first 12 months, there are no statistically significant differences in RMST between patients in the T4 stage and those in stages T1/T2/T3. At 24 months, there is a statistically significant difference of 4.5 months in favour of patients in stages T1/T2/T3. Both the asymptotic test and the permutation test indicate statistical significance. At 60 months, there is a statistically significant difference of 20 months in favour of patients in stages T1/T2/T3. Both the asymptotic test and the permutation test yield statistically significant p-values.
Considering these results, it can be concluded that starting from 24 months from the time of diagnosis, patients in the T4 stage have a more severe disease progression compared to patients in less advanced T stages. The data provides sufficient evidence to support this conclusion.

table 2.21

Predictor	**Deaths N**	**HR (95% CI)**[1]	**p value**
T4 Stage			
No	2	—	
Yes	6	4.40 (0.87 to 22.3)	0.073

[1] HR = Hazard Ratio

Cox regression for T stage

With a p-value that is marginally insignificant, it can be inferred that patients in stage T4 have approximately a 4.40 times higher risk of death compared to patients in stages lower than T4.

An analysis was performed for tumour thickness at the time of diagnosis. Being a continuous variable, the Cox model was exclusively used for this analysis.

table 2.22

Predictor	Deces N	HR (95% CI)[1]	p value
Tumour Thickness	8	1.13 (0.99 to 1.27)	0.064

[1] HR = Hazard Ratio

Cox regression for OS for tumour thickness

With a p-value that is marginally insignificant, it can be observed that a 1mm increase in tumour thickness is associated with a 13% increase in the probability of death. Although the statistical significance of the p-value may be slightly uncertain, the findings suggest a positive association between tumour thickness and the risk of mortality.

An analysis was conducted to investigate the presence of visceral metastases, excluding cutaneous and lymph node metastases.

table 2.23

Metastases	N Deaths (%)	Median Survival (95% CI)
Absent	2 / 19 (10.52)	N/A [66.00 to N/A]
Present	6 / 11 (54.55)	54.00 (12.00 to N/A)

Overall Survival analysis for presence/absence of metastases

Mortality was found to be 5 times higher in patients with visceral metastases. The results of the survival curve tests, which demonstrate this relationship, are presented in the following tables:

table 2.24

Metastases	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Absent	19	2	4.97	4.80 (1)	0.03
Present	11	6	3.03	-	-

log-rank Martel-Haenszel test for presence/absence of metastases

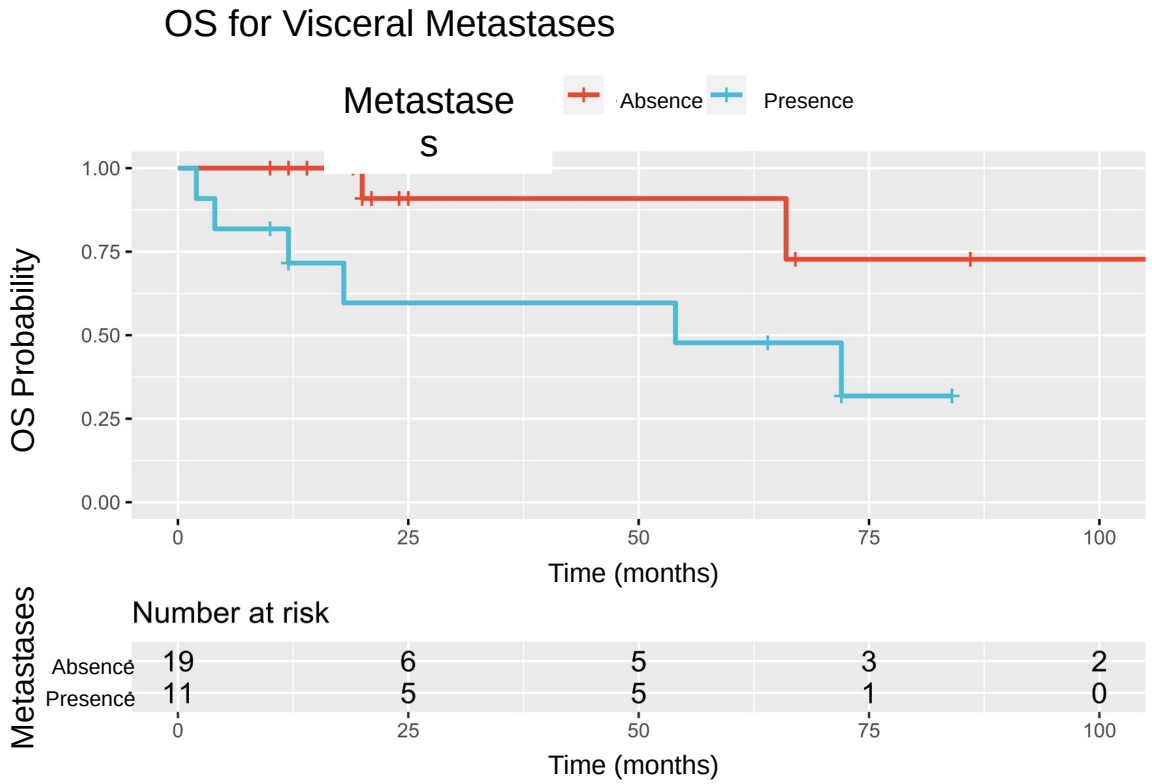
table 2.25

Metastases	N	Deaths Observed	Deaths under H0	χ2 statistic (degrees of freedom)	p value
Absent	19	1.57	4.26	5.40 (1)	0.03
Present	11	5.22	2.53	-	-

Gehan-Wilcoxon test for presence/absence of metastases

The results of the tests indicate that the approximately 5 times higher mortality in patients with visceral metastases is statistically significant, both tests performed yielding a p-value of less than 0.05.

Fig 2.8



Kaplan-Meier survival curves for presence/absence of metastases

RMST and RMTL for presence/absence of metastases

table 2.26

Statistic	Estimated	95% CI
RMST Metastases +	48.54	27.40 to 69.68
RMST Metastases -	74.90	63.15 to 86.66
RMTL Metastases +	35.45	14.31 to 56.59
RMTL Metastases -	9.09	-2.66 to 20.84

RMST and RMTL for presence/absence of metastases

Based on the provided information, it can be observed that:

The RMST (Restricted Mean Survival Time) was approximately 26 months lower in patients with present visceral metastases compared to those without metastases. This indicates a shorter overall survival time in patients with metastases. Conversely, the RMTL (Restricted Mean Time Lost) was approximately 26 months higher in patients without visceral metastases compared to those with metastases. This suggests a longer duration of time lost due to mortality in patients without metastases.

Comparative tests:

table 2.27

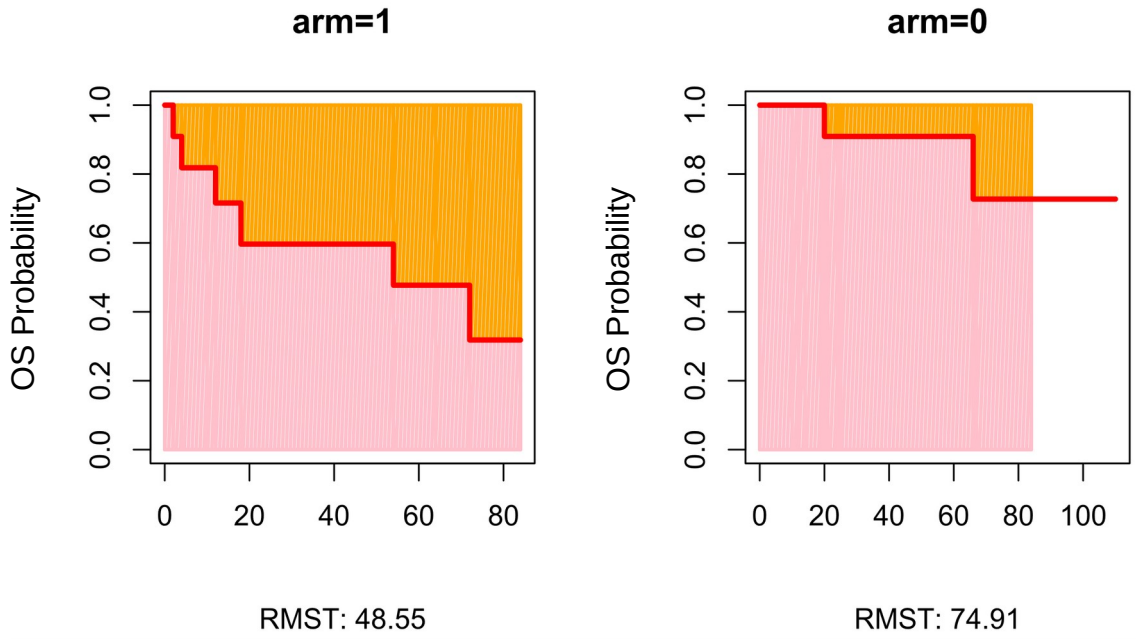
Test	Estimated (95% CI)	p value
Diferente RMST	-26.36 (-50.55 to -2.17)	0.033
RMST Raport	0.64 (0.40 to 1.03)	0.066
RMLT Raport	3.90 (0.93 to 16.20)	0.061

Comparative tests for presence/absence of metastases

One of the tests reported a statistically significant p-value, indicating a significant difference in survival between patients with and without visceral metastases. However, the other two tests yielded marginally insignificant p-values, suggesting a less clear-cut association.

Nevertheless, considering the average survival rates, it can be observed that patients without visceral metastases tend to have higher survival rates compared to those with metastases. This relationship is depicted in the graph, where Arm 1 represents patients with metastases present and Arm 0 represents patients with metastases absent.

fig 2.9



Comparative tests for presence/absence of metastases

table 2.28

Predictor	Event N	HR (95% CI)[1]	p value
Visceral Metastases			
No	2	—	
Yes	6	5.07 (1.01 to 25.4)	0.048

[1] HR = Hazard Ratio,

Cox regression for presence/absence of metastases

The risk of death was 5 times higher in patients with metastases, the effect being statistically significant.

In the discussion section, we addressed the similarity between the data obtained in our study and the current literature on malignant melanoma. We observed a significant variability of results in the literature, which can be attributed to several factors. These include variations in the medical systems of the authors, differences in the characteristics of the population samples, variations in study designs (prospective, retrospective, controlled trials), as well as variances in diagnostic and treatment protocols.

While there were differences between our results and those reported in the literature, the majority of these differences fell within the expected range of errors inherent in clinical studies. To account for this, we reported a confidence interval (95% CI), which reflects the standard error of the statistics calculated in our study, such as RMST, survival median, and HR, among others. By providing this confidence interval, we aimed to express the level of uncertainty associated with our findings and to acknowledge the inherent variability that can arise in clinical research.

3. Conclusions

3.1. Results

Following the statistical analysis of the group of patients in the study, the following results were obtained:

- *Characteristics of the study group:*

• The average age of diagnosis with malignant melanoma was 57 years

• There were no demographically significant differences regarding the gender of patients diagnosed with melanoma.

• BRAF mutation was found in half of the patients in the study. At a population level, the proportion of BRAF mutations identified varies greatly, and the proportion of BRAF-positive melanomas in the total number of melanomas cannot be accurately estimated at the present time.

• In 1/3 of cases the primary tumour was located peripherally (i.e. on arms or legs) and in the remaining 2/3 of cases the tumour was located axially.

• 2/3 of the patients had an elevated LDH level at the time of admission to the study.

• In almost 75% of patients, ulcerations were observed at the level of the primary tumour.

• More than half of the patients were diagnosed with tumour stage T4.

• At the time of diagnosis, 70% of patients the disease had extended beyond the localised stage.

• 1/3 of the patients had distant metastases at the time of diagnosis.

• In 70% of the patients who benefited from surgical treatment, the procedure used was that of extensive resection of the tumour (wide excision) and sentinel node biopsy.

• Approximately half of the patients received adjuvant chemotherapy.

• Approximately 15% of patients underwent radiotherapy.

• About ½ of the patients benefited from immunotherapy.

- *For the role of the BRAF mutation*

• In the patients in the sample, the BRAF mutation had no prognostic role, both overall survival and progression-free survival not being influenced by this parameter; however, considering the fact that targeted therapy was applied to all patients with BRAF mutation, the hypothesis can be supported that patients with BRAF mutation who received targeted therapy have practically the same prognosis as patients without BRAF mutation.

- *For the objective – patient survival:*

• The median survival of patients in the study was almost 78 months, an underestimated value because it was estimated using RMST, the study having censored patients.

• We were unable to estimate a median survival for patients in the study, but we obtained a calculated lower threshold of 66 months.

• Mortality in our study was 26.66%; 5-year survival was 60%, but this was estimated on a smaller sample of patients than the baseline.

• Survival at 5 years in our group was lower than in similar studies conducted in other countries. In the patients in the present study group, the initial diagnosis was made mainly in more advanced stages of the disease, which can explain the unfavourable prognosis.

• An influence of age on disease progression was observed for the studied sample. Elderly patients showed a decrease in median survival time.

- *For the objective – survival without progression:*

• Patients with an elevated LDH level at the time of diagnosis have a worse prognosis than patients with normal LDH levels, both in terms of survival and disease progression; a possible explanation for this effect is that elevated LDH is associated with the presence of metastases and/or more advanced disease.

• Patients with T4 melanomas have a more unfavourable prognosis compared to patients with melanomas in lower T stages, both in terms of progression and survival.

• In patients with organ metastases, the prognosis is worse compared to patients without organ metastases, both in terms of survival and progression.

• Patients with regionally extensive disease have a more severe prognosis than patients with limited disease, but only in terms of disease progression, our study showing no difference in survival between patients with limited disease and those with regional disease.

• Surgical treatment did not influence the prognosis, in our study the surgical procedure used was selected according to the stage of the melanoma, considering the advanced stage in which the primary tumour was detected in most patients.

• In the patients in our study, chemotherapy did not influence the evolution of the disease; it being reserved for patients with an already unfavourable prognosis, when the rest of the therapeutic resources have been exhausted.

• We cannot issue a conclusion related to the role of radiotherapy in the patients in the

analysed sample, only 4 patients had an indication for this therapeutic modality reserved for certain categories of metastases.

• Immunotherapy influenced the prognosis of patients in the sample, by increasing the average survival period, but there were no differences in terms of mortality for patients who benefited from this therapy vs patients who did not.

3.2. Personal contributions

Melanoma is a malignant disease that has garnered significant attention in recent decades. This increased focus has led to a better understanding of its underlying mechanisms and the development of numerous innovative treatment approaches. Consequently, melanoma, which was once associated with a particularly poor prognosis despite adjuvant therapies, can now be effectively managed for extended periods of time. The introduction of targeted therapy and immunotherapy has revolutionized the approach to treating this condition.

The objective of the present study is to enhance our comprehension of melanoma within the specific contexts of the participating centres. We aim to analyse the survival, progression, and prognosis of melanoma patients. Unlike studies conducted in other regions or countries, our results are specific to the patient profile treated by clinicians in these centres. Notably, this profile includes characteristics such as a high incidence of advanced-stage melanomas at the time of diagnosis, large tumor thickness, and increased ulceration rates, as discussed in *Chapter 3.1 - Results.*

Furthermore, the study aims to identify patients at risk of developing aggressive forms of the disease, as well as those prone to recurrence and the development of local, nodal, or distant metastases. For instance, we have observed that patients with primary tumours exhibiting ulceration or elevated levels of lactate dehydrogenase (LDH) at the time of enrolment, as well as elderly patients, are more susceptible to adverse outcomes. This information can assist clinicians in determining the most appropriate therapeutic approach, as highlighted in *Chapter 3.1 - Results*.

Simultaneously, the study examines the outcomes of patients undergoing current treatments. The implementation of oncological therapies requires careful consideration of various medical, ethical, economic, and availability-related factors, as well as the meticulous selection of patients. Studies involving cancer patients must account for the inherent risk of disease progression in the placebo group, as well as the potential adverse

effects associated with experimental treatments. Although our study is retrospective, its aim is to provide valuable insights without subjecting patients to additional risks.

Surgical intervention plays a pivotal role in melanoma treatment, particularly in the early stages, as corroborated by our study findings. Patients who receive appropriate surgical treatment, employing the correct techniques, demonstrate improved overall survival and progression-free survival rates. Adequate surgical procedures with clear margins serve to prevent regional disease progression and facilitate accurate staging. In our study, all patients received proper and comprehensive surgical treatment, supplemented with sentinel node biopsy in select cases. Analyzing the outcomes under this treatment paradigm contributes to our understanding of how the disease evolves within the current standard of care.

3.3. Limitations and future perspectives

As a retrospective study, the present work has certain limitations that need to be acknowledged. Firstly, the study's reliability depends on the accuracy and completeness of the data obtained from patient files. Any inconsistencies or missing information in the records could potentially impact the validity of the findings. Additionally, due to the retrospective nature of the study, there are inherent limitations in the ability to adjust for confounding variables or include new variables that may have been of interest.

To address these limitations and improve future research, several suggestions can be made. One proposal is the establishment of a national database specifically designed for cancer patients, including those with melanoma. Such a database would greatly facilitate a multidisciplinary approach to treatment, ensuring that each patient receives standardized care regardless of the treatment centre. It would also enable equitable access to resources and treatments in accordance with current guidelines. By doing so, the quality of care could be enhanced, the risk of complications reduced, and unnecessary interventions minimized, ultimately leading to cost savings. Moreover, developing an infrastructure suitable for comprehensive data collection purposes would enable more robust statistical analyses on larger study cohorts. The results obtained from these analyses could then inform and further improve the therapeutic approaches to various cancers, including melanoma.

In conclusion, our study aims to provide valuable data on the progression and survival of

patients with BRAF+ melanoma. This information can be utilized independently or in conjunction with other studies to evaluate and enhance the therapeutic approaches for these patients. However, it is important to acknowledge the limitations inherent in retrospective studies and consider the potential improvements that can be made in data management systems and infrastructure to support future research endeavours in the field of oncology.

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