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Mutational profiling of genes associated with melanoma progression: a single-center study of primary tumors and matched metastases

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"As to the remote and exciting causes of melanosis, we are quite in the dark, nor can more be said of the methodus medendi. We are hence forced to confess the incompetency of our knowledge of the disease under consideration, and to leave to future investigators the merit of revealing the laws which govern its origin and progress….and pointing out the means by which its ravages may be prevented or repressed" - Thomas Fawdington, The Manchester Royal Infirmary, 1826.

Abstract

Cutaneous melanoma is an aggressive cutaneous cancer originating from normal melanocytes through the accumulation and selection of somatic mutations in driver genes. Four molecular subtypes *BRAF, NRAS, NF1,* and triple wild type melanomas have been defined based on driver mutations in these genes. Additional mutations in other genes, such as *TP53*, *CDKN2A, RAC1, TERT promoter, DDX3X* and *PREX2*, occur in the advanced stages and contribute to progression of the disease and to the development of invasive phenotypes as well as metastatic potential. The study of molecular heterogeneity between primary tumors and paired metastases is essential for understanding tumor evolution and treatment with appropriate target therapies. Most published studies investigated primaries and metastases from different patients, making challenging to determine the discordance rates of their molecular profile and comprehend the natural progression accurately. In studies where primaries and metastases from the same patient were considered, the analysis was limited to *BRAF* and *NRAS* mutations, without exploring more comprehensive mutational profiles.

The primary aim of this study was to identify the molecular profiles of primary melanomas and matched metastases and estimate their clinical and prognostic significance. Moreover, we evaluated intra-patient and inter-patient heterogeneity of molecular profiles, that could be associated to mechanisms of progression.

We included both primary melanomas and paired metastatic tissues from 44 patients, for a total of 44 primary tumors and 63 cutaneous, lymph nodal, and visceral metastases. Sequencing analysis was performed through the IonTorrent methodology with a custom panel of 18 melanoma-related genes (*BRAF, NRAS, NF1, ARID2, TACC1, PPP6C, TP53, TERT promoter, RAC1, STK19, PREX2, EPHA3, GNA11, GRIN2A, DDX3X, WNT1, KIT, CTNNB1*). Healthy skin tissues from each patient were used to normalize the sequencing data. Descriptive statistics and multivariate regression models were used to study the impact of mutational status on clinical and prognostic features (histopathological subtype, Breslow thickness, ulceration, number of mitosis, stage). We evaluated differences in melanoma specific survival (MSS) among different molecular subgroups through Kaplan-Meier curves. Fisher's log statistics and Multiple Correspondence Analysis were performed to compare the mutational status of primary and metastatic tissues and to cluster patients according to the molecular profiles.

Overall, 43% of primary tumors exhibited *BRAF* driver mutations, 27% *NRAS*, 11% *NF1*, and 20% were triple wild type. Among metastases, the frequency of molecular subtypes was 30% with *BRAF* mutations, 14% *NRAS,* 14% *NF1* and 43% triple wild type. The wild type status resulted significantly increased in metastatic tissues compared to primary tumors (p=0.035). *BRAF*

were associated with the onset of tumors at a young age (median 57.6, range 25-73, p=0.036), with the presence of ulceration (p=0.003) and with higher number of mitosis (median 6 mitosis/mm^2, range 0-23, p=0.041) compared to *BRAF*-wild type melanomas. Moreover, we found that melanomas with *BRAF* driver mutations had a significantly increased risk to metastasize to the skin, independently of other prognostic factors, such as age at diagnosis, thickness, ulceration and mitosis (OR= 6.72, 95%CI 1.39-67.43, p= 0.028). Regarding *NRAS,* the presence of mutations was associated with later onset (median=72.5, range 45-84, p=0.004) and development of melanomas in chronic sun-damaged (CSD) areas, although this finding did not reach statistical significance (p=0.116). *NF1*-mutated melanomas were associated with more advanced stages at diagnoses (p=0.050). The triple wild type subgroup was strongly associated with the superficial spreading histotype (p<0.001) and with melanoma diagnosed at earlier stages (stage I/II, p=0.024).

A high frequency of mutations in *TACC1, PREX2, EPHA3*, and *TERT promoter* were found both in primary tumors (66%, 68%, 57% and 55%, respectively) and metastases (48%, 55%, 59%, and 45%, respectively). Mutations in *PREX2* and *RAC1* were significantly associated with higher Breslow thickness (p=0.002; p=0.006 respectively). *RAC1* variants were also associated with the nodular (p=0.012) and ulcerated (p=0.062) histologic features.

Intra-patient heterogeneity between primary melanomas and matched metastases was observed in 20% of patients for *BRAF* (p=0.223), and 15.9% for *NRAS* (p=0.127). When we considered the four main molecular subgroups, intra-patient discordance was observed in 53.3% of patients. Regarding the other genes, we observed a significant discordance of molecular status in *STK19* (p=0.007), *ARID2* (p=0.021), *PPP6C* (p=0.035), *TACC1* (p=0.046), *GRIN2A* (p=0.052).

Using Multiple Correspondence Analysis (MCA), we were able to identify two distinct clusters of patients based on the mutational status of the 18 genes analyzed. One cluster was characterized by primary lesions with *TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1*, and *DDX3X* mutations, while patients with triple wild type primary melanomas clustered in the second group. Moreover, MCA analysis also demonstrated that mutations in *PPP6C, GRIN2A, ARID2*, and *GNA11* tend to co-occur, both in primary and metastatic tissues, defining a unique molecular profile.

In conclusion, our findings identified interesting associations of clinical and prognostic features with the mutational status of melanoma-related genes and discovered a high rate of molecular heterogeneity between primary and metastatic tumors in the same patients. Finally, we defined two distinct clusters of melanoma lesions and identified specific molecular profiles of these clusters. Further investigations on large number of cases are required to confirm our data and fully comprehend the significance of these genetic patterns.

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Where the median time is not present it is because 50% of the group did not experience the event.

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M=metastasis.

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Introduction

1.EPIDEMIOLOGY

The term "Melanoma" derives from the Greek "*Melas"* (dark) and "*Oma"* (tumor). It was first described by Hippocrates in the 5th century BC and later reported by the Greek Physicist Rufus from Ephesus. Rene Laennec was the first, however, to realize that melanoma was a distinct disease and, in 1804, described it as "Melanose". In 1820, Dr. William Norris understood the possible heritable nature of some melanomas and the high propensity to metastasize (Rebecca et al., 2012).

Cutaneous melanoma is a skin cancer arising from genetically altered skin melanocytes. It is the third most common cancer of the skin, but account for 80% of all skin cancer deaths (Saginala et al., 2021). The incidence of melanoma is continuously increasing, with approximately 60 cases per 100 000 population per year in Australia and New Zealand, 30 cases per 100 000 population in the USA, and 25 new cases per 100 000 population in Europe (Long et al., 2023) (Figure 1A).

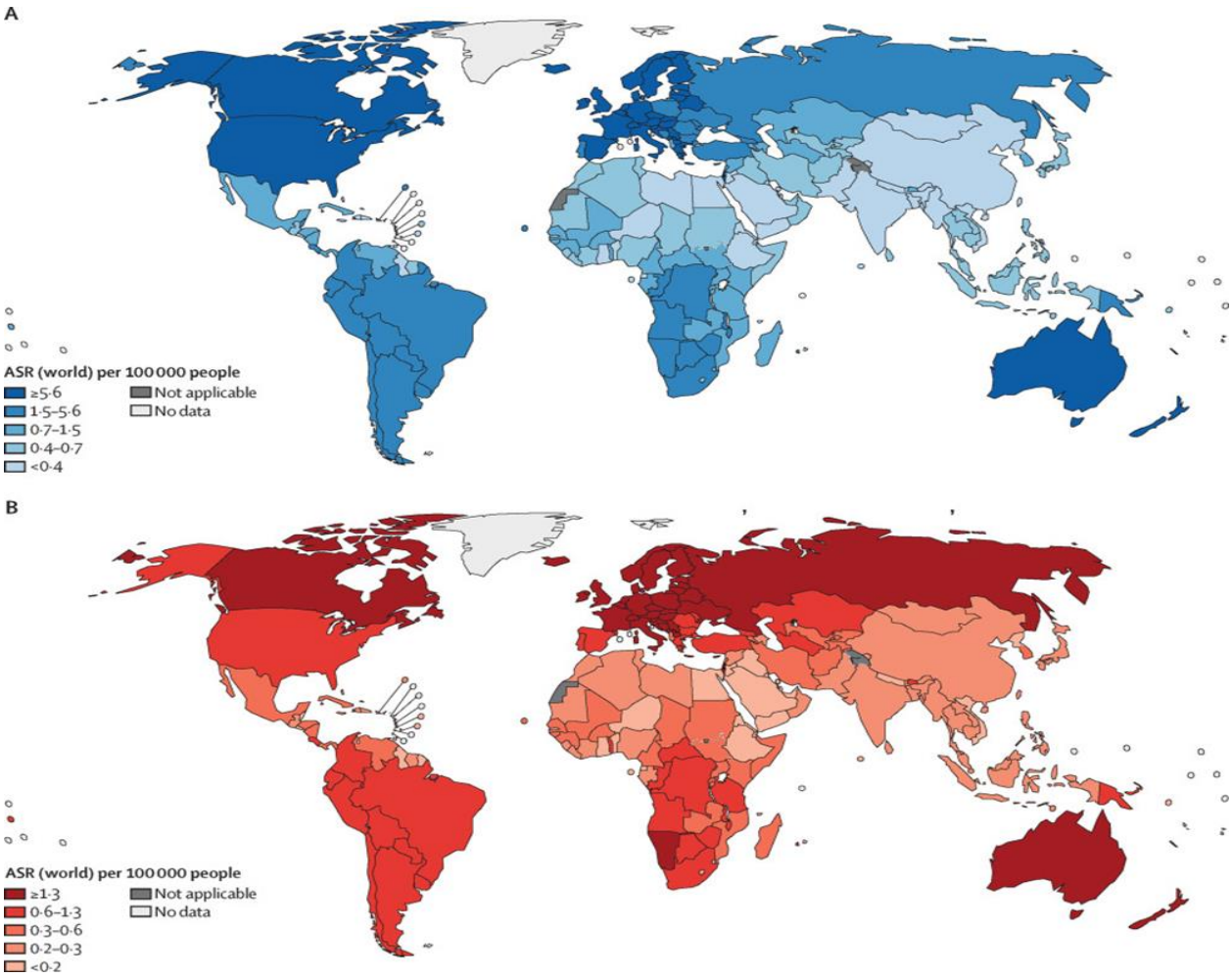


Figure 1. A) Incidence per 100 000 people per year. B) Mortality per 100 000 people per year (Long et al., 2023)

The mean age at melanoma diagnosis is 65 years, with 65.7% of diagnoses in patients aged 55 to 84 years. The global age-standardized incidence rate is 3.8/100,000 for males and 3.0/100,000 for females, with cumulative lifetime risks of 0.42% and 0.33%, respectively. Male and female incidence was 1.0 and 0.9 among Blacks and 5.0 for both sexes among Hispanics per 100.000 (Saginala et al., 2021). Overall, it is estimated that 97,610 new cases of cutaneous melanoma will be diagnosed in 2024 and 7,990 people will die of this disease (Siegel Mph et al., 2024). The 5-year survival rate is 99.4% for stage I–II disease, decreasing to 68.0% for stage III and 29.8% for stage IV (Saginala et al., 2021). Overall, mortality has declined as treatments have improved in recent years (Figure 1B) (Long et al., 2023). Females have a marginally higher survival rate than males for almost all subtypes; older patients have lower survival rates than younger patients, and urban locations have higher rates of survival than rural locations (Zheng et al., 2023).

2.RISK FACTORS

Genetic, individual and environmental risk factors contribute to the development of melanoma (Long et al., 2023).

An intense and intermittent UV exposure, mainly for individuals under 35 years of age, makes the greatest contribution to the development of melanoma (Conforti et al., 2021). In fact, intense UV can induce genetic alterations such as DNA damage and somatic mutations, accumulation of reactive oxygen species and oxidative stress, resulting in an inflammatory response related to malignant melanocyte change (Guo W. et al., 2021). Other risk factors supported by strong epidemiologic evidence include a high number of melanocytic nevi and a family or personal history of melanoma (Conforti et al., 2021). Moreover, fair skin, eye and hair phenotype indicate the insufficient ability of melanocytes to generate eumelanin, making individuals more vulnerable to UV damage and environmental stresses of the skin (Dzwierzynski et al., 2021). Finally, patients with an immunosuppressive status seem to have an increased risk of melanoma (Dzwierzynski et al., 2021).

Genetic susceptibility accounts for 5-10% of all melanoma cases and can be associated with an early onset and the occurrence of multiple primary melanomas. Germline variants are identified in about 40% of familial melanoma patients and involve high-penetrance genes such as *CDKN2A* (20-40% prevalence), *CDK4* (<1%), *POT1* (<1%), *ACD* (<1%), *TERF2IP* (<1%), *TERT* (<1%) and *BAP1* (<1%) and medium-penetrance genes as *MC1R* (70-90%) and *MITF* (1-5%). Variants in these genes favor the development of melanoma by altering normal cellular homeostasis, and thus, may also predispose to cancer at other sites (Zocchi et al., 2021).

3.MOLECULAR PATHOGENESIS OF MELANOMA

The molecular pathogenesis of melanoma is complex and several somatic mutational events in pathways critical for cell survival are required for tumor development (Teixido et al., 2021). Cutaneous melanoma has the highest mutational rate compared to all other cancers (excluding keratinocyte tumors), and these mutations tend to increase during its progression. At the early phase of pathogenesis, several mutated driver genes involved in key cellular pathways have been identified as being primarily responsible for carcinogenesis and leading melanoma onset (Guo W. et al., 2021). These pathways include the mitogen-activated protein kinase (MAPK), the protein kinase B (AKT), the cell cycle regulation, the p53 signaling and the pigmentation-related pathways. During tumor progression, additional pathways and mutated genes can be involved (Gosman et al., 2023). For example, *TERT promoter* mutations together with biallelic inactivation of *CDKN2A* and mutations of *PTEN, ARID2* and *TP53,* occur very frequently during melanoma progression, suggesting that selection mechanisms that preclude cellular senescence, epigenetic control and uncontrolled proliferation are pivotal events. Furthermore, the mutational load clearly increased from benign to intermediate and then to invasive melanoma lesions, with strong evidence of UV signature at all stages of tumor evolution (Testa et al., 2017).

3.1 Driver genes implicated in melanoma development and molecular classification

The MAPK pathway is the most important signaling pathway involved in the development of melanoma. It regulates numerous processes under physiological conditions, including cellular metabolism, survival, apoptosis, stress and differentiation, but its abnormal or uncontrolled activation can lead to tumor formation (Teixido et al., 2021). The pathway includes three different signaling in which the extracellular binding of growth factors to tyrosine kinases receptors (RTKs), produce a cascade of phosphorylation culminating in the activation of ERK, JNK, and p38, respectively (Carlino et al., 2015). Previous studies have shown that the JNK and p38/MAPK pathways are mainly related to cell stress and apoptosis. In contrast, the ERK/MAPK signaling is closely linked to cell division and development, through the sequential activation of Ras, Raf, MEK, and finally ERK (Guo Y.-J. et al., 2020) (Figure 2).

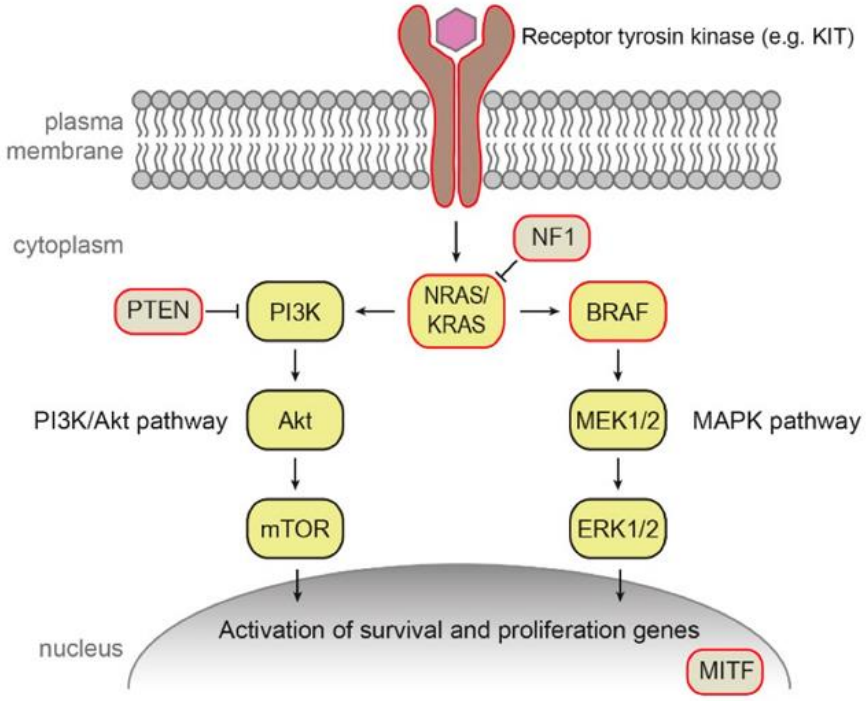


Figure 2. MAPK signaling (Horak et al., 2019)

The dysregulation of the ERK/MAPK signaling is the key event in melanomagenesis, occurring at the early phases of development. The presence of mutations in key components of this pathway, such as *BRAF, NRAS*, and *NF1* driver genes, defines the current molecular classification of melanoma in four subgroups (Figure 3) (*The Cancer Genome Atlas Program - TCGA*).

BRAF is a serine/threonine kinase belonging to the Raf family, implicated in signal transduction through the downstream MAPK pathway. In 2002, a genomic screening discovered a *BRAF* point mutation occurring more frequently in melanoma than in other types of solid tumors (Davies et al., 2002). The valine to glutamic acid substitution at codon 600 (V600E) of exon 15, can be detected in about 50% of melanomas (Guo W. et al., 2021). Biochemically, this substitution results in a negative charge in the activation loop, that destabilizes the inhibitory conformation and leads to the continuous activation of the BRAF protein as a monomer. Other variants, including V600K, V600D and V600R, account for 12%, 5% and 1% of *BRAF* mutations, respectively. Mutations in exon 14, while rare, have been also identified, but are insufficient for melanomagenesis (Alqathama, 2020).

NRAS is a small GTP-binding protein belonging to the Ras family that transduces RTK signaling Raf activation downstream. *NRAS* mutations usually occur at the G12, G13 and Q61 sites and are detected in about 25% of melanoma cases (Guo et al. 2021). In detail, *NRAS* Q61

mutants block the G protein in its active conformation, while *NRAS* G12 and *NRAS* G13 mutations activate the GTPase by decreasing NRAS sensitivity to GTPase-activating proteins (Savoia et al., 2019).

More recently, thanks to whole-exome sequencing approaches, *NF1*, has been identified as the third most frequently mutated gene in melanoma after *BRAF* and *NRAS* (Akbani et al., 2015). *NF1* encodes a negative regulator of RAS, therefore acting as tumor suppressor for the MAPK signaling. Loss of *NF1* or inactivating mutations of *NF1* are present in 46% of melanomas expressing BRAF and wild type RAS. NF1 suppression leads to Ras activation by reducing its intrinsic GTPase activity and induces hyperactivation of the MAPK pathway (Philpott et al., 2017). However, *NF1* mutant melanomas need additional changes for robust activation of the pathway, as a loss of NF1 does not result in a strong Ras activation (Mahalingam 2017). Mutations in *NF1* are frequent in melanoma showing UV-signature alterations and in those with a high tumor mutational burden (TMB).

The fourth molecular subtype of melanoma is defined triple wild type and includes a heterogeneous group of tumors lacking mutations in *BRAF,* N*RAS* and *NF1* (*The Cancer Genome Atlas Program - TCGA*) (Akbani et al., 2015). This subgroup is characterized by a low frequency of UV signature mutations but a high presence of copy number changes and complex structural genomic rearrangements. Usually, these tumors present pathogenic variants in the Ras pathway and mutations of *KIT, TP53, PTEN, TERT promoter, GNAQ, GNA11,* and *CTNNB1* (Pipek et al. 2023, Haugh et al. 2024). *KIT* mutations and amplification, which cause receptor dimerization, autophosphorylation of tyrosine residues and activation of downstream oncogenic pathways, are frequently found in this subgroup (9.5%) (Gong et al., 2018). *TERT promoter* mutations are present in only 6.7% of triple wild type melanomas. Moreover, these melanomas tend to have a lower TMB than other subtypes (Pipek et al., 2023).

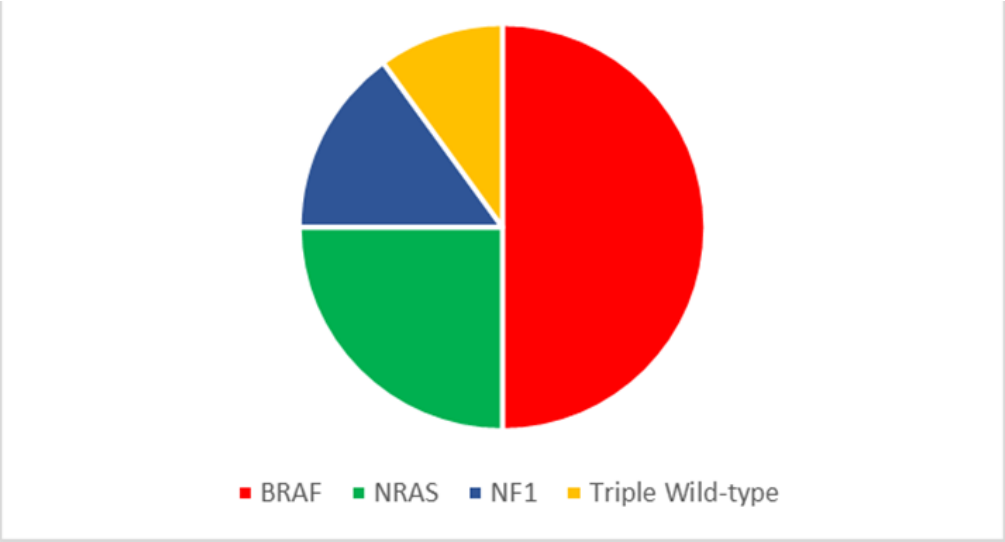


Figure 3. Frequency of molecular subtypes according to literature data: *BRAF* 50%; *NRAS* 25%, *NF1* 15%, Triple Wild type 10%

3.2 Clinical significance of the melanoma molecular classification

BRAF, *NRAS, NF1* and triple wild type melanoma subtypes appear to be associated with distinctive clinic-pathologic and prognostic features. Most studies have focused on characterizing the *BRAF* and *NRAS* subtypes, albeit with controversial results, while few reports have investigated the clinicopathological features of the other two molecular subtypes (Conway et al., 2020).

In the large meta-analysis by Kim et al. (2015), then confirmed in more recent studies, the *BRAF* mutations were associated with melanoma diagnosed at a young age (OR = 1.734; P < .001), trunk (OR = 2.272; P < .001), and superficial spreading melanoma (OR = 2.081; P < 0.001) and advanced stage (OR = 1.551; P = 0.003) (Kim et al., 2015). Different clinico-pathological aspects have been reported for different *BRAF* mutations. The V600E mutation is more common in melanomas of young patients and in those developed in anatomical areas not showing solar elastosis, suggesting the association with intermittent exposure to UV rather than chronic sun damage (Ottaviano et al., 2021). The *BRAF* V600E mutation appears to be associated with melanoma growth, increased Breslow thickness (p=0.030) and peritumoral lymphocytic infiltration (p=0.0008). (Zablocka et al., 2023). Contrariwise, the V600K substitution is more frequent in older male patients, in melanomas occurring on the head and neck region, and in those showing low histological regression (da Silva et al., 2019). Overall, the presence of *BRAF* mutations seems to predict an overall poor prognosis compared to *BRAF* wild type melanoma (Lovly et al. 2012), with a greater impact on stage IV patients (Castellani et al., 2023). Another

study confirmed that patients with *BRAF* V600E mutations tend to have shorter MSS when compared to patients wild type in *BRAF* (Wu et al., 2014). These findings could be biologically explained by the association of *BRAF* mutations with increased tissue invasion, cell migration, and immune response evasion (Wagstaff et al., 2022). In contrast, in some patient cohorts, *BRAF* mutated patients showed better overall survival (OS) than *NRAS* mutated patients (Kodali et al., 2024). However, it should be noted that different outcomes have been reported for *BRAF* variants. *BRAF* V600K appeared more aggressive in terms of cell proliferation than *BRAF* V600E (Nepote et al., 2022), and patients with V600K had shorter progression-free survival (PFS) than those with the V600E mutation (da Silva et al., 2019). Recently, Zengarini et al. (2022) found that melanoma with V600K has a greater tendency to metastasize than V600E lesions (Zengarini et al., 2022), despite the fact that a previous study reported a reduced time to progression for the V600E subgroup compared with the V600K subgroup ((Ponti et al., 2017). In addition, the V600K mutation is associated with higher TMB (Ottaviano et al., 2021) and better response to immunotherapy (Nepote et al., 2022).

Regarding the therapeutic approach for advanced cases, molecular BRAF inhibitors such as vemurafenib, dabrafenib, and encorafenib block the active form of the protein when the V600 mutations are present (Bollag et al., 2010). However, they are inactive on non-canonical mutations (Carlino et al., 2015). These treatments prolong median PFS and OS, but drug resistance usually develops within a year of starting treatment, either dependent or independent of MAPK signaling (Carlino et al. 2015, Rogala et al. 2022). The combined use of BRAF and MEK inhibitors is essential to achieve optimal therapeutic efficacy and overcome resistance (Subbiah et al., 2020). Three allosteric inhibitors of MEK1 and MEK2, Trametinib, Binimetinib, and Cobimetinib, are used in *BRAF*-mutated patients (Sullivan et al., 2015). In stage IV melanoma patients, current guidelines recommend an initial approach with anti-PD1 +/- anti-CTLA4 immunotherapy also for *BRAF*-mutated melanomas (Michielin et al. 2019, Garbe et al. 2022). The most successful immunotherapy option is the combination of nivolumab and ipilimumab, which results in 5-year survival rate of about 50% and is currently the gold standard for all metastatic melanomas, regardless of molecular status (Guhan et al., 2021). Recent trials have shown that combining immunotherapy with targeted treatment can improve effectiveness (Gutzmer et al. 2020, Ascierto et al. 2023). Notably, new studies are evaluating the possibility of employing machine learning-based approaches to differentiate the prognostic signature among patients with *BRAF* mutations who show a better response to dabrafenib or immunotherapy (Liu et al., 2023).

Regarding the clinico-pathological features of the *NRAS* subtype, mutations in this gene have been associated with elderly patients, nodular melanomas arising in chronically sun-exposed

anatomical areas, and thick ulcerated tumors (Savoia et al., 2019). There are discordant studies in the literature on survival and prognosis of *NRAS* mutated tumors. *NRAS* mutations do not seem to affect OS or increase it compared with tumors with *BRAF* mutations or wild type mutations, according to old reports (Muñoz-Couselo et al., 2017). In more recent papers, patients with *NRAS* mutant melanoma had significantly poorer PFS than wild NRAS melanoma (Zablocka et al., 2023) and was found to be a negative prognostic factor, with significantly reduced OS (Haugh et al., 2024). The first line of treatment for *RAS* mutated melanomas is immunotherapy, either as monotherapy or combination therapy (Garbe et al., 2022). However, *NRAS* mutational status did not influence PFS and OS in patients treated with anti-PD1 immunotherapy (Zaremba et al., 2023). Considering the nature of targetable hot-spot mutations in *NRAS,* many attempts have been made to design a specific NRAS inhibitor, all without success. Preclinical investigations have demonstrated effectiveness in *NRAS* mutated cases with MEK and cell cycle inhibitors (Randic et al., 2021). Dinter et al. (2024) recently reported that treating mutated *NRAS* melanomas with BRAF and MEK inhibitors showed significant benefits in an *in vitro* model (Dinter et al., 2024).

NF1 gene is frequently altered in desmoplastic melanoma (Kiuru et al., 2017). *NF1*-mutated melanoma was significantly associated with male sex, advanced age at diagnosis, UV signature, and high TMB (Jour et al., 2023). In a study of 626 patients, *NF1* subtype showed a higher risk of death and poorer OS compared to *BRAF* (Cirenajwis et al., 2017). Increased MAPK pathway activity resulting from *NF1* mutations indicates that the use of MEK or RAF inhibitors may be an useful strategy as therapeutic approach for this subgroup of melanoma (Guhan et al., 2021), although the combination of these drugs with inhibitors of the mTOR/PI3K pathway seems to show improved effectiveness in clinical trials (Kiuru et al., 2017).

Finally, the clinical impact of the triple wild type status has yet to be clarified as there are only few studies focusing on this heterogeneous subgroup. Triple wild type usually occurs in old males with a low TMB. This subtype resulted clinically aggressive (Guhan et al., 2021), although some studies find no significant differences in the survival of these patients compared to other molecular subtypes.

3.3 Key molecular alterations in tumor progression and metastases

Besides the four subtypes described above, other molecular events have been differently involved in the pathogenesis, progression and metastatic spread of melanoma, although with discordant results (Cully et al. 2005, Stefansson et al. 2007, Balakrishnan et al. 2007, Hodis et al. 2012, Braeuer et al. 2014, Li Ding et al. 2014, Zhang et al 2020, Alkallas et al. 2020, Teixido et al. 2021, Liang Ding et al. 2022).

Mutations in genes controlling the cell cycle are very frequent (Hodis et al., 2012) both in the early phases and during progression. Variants in *CDKN2A* and *CDK4* are present at the somatic level in about 40% of cases and a high-level of copy number alterations containing the oncogenes *CDK4* and *CCND1* (Akbani et al., 2015) has been reported, especially in the triple wild type subgroup. The AKT/mTOR signal has been greatly engaged in melanoma metastasis with the loss of *PTEN* gene being the main reason for hyper-activation of this pathway. Several molecular and clinical analysis have revealed a strong association between AKT activation and metastasis development in specific areas such as the brain, lymph nodes, and lungs (Guo W. et al., 2021). When the *PTEN* gene is lost or downregulated, it prevents immunogenic cell death, which is essential to activate tumor-specific cytotoxic T lymphocytes. This immune evasion and escape hampers the effectiveness of T-cell-mediated immunotherapies, such as immune checkpoint inhibitors, making these patients more resistant to treatment (Sukniam et al., 2024).

More recently, genome sequencing studies identified many additional mutations in genes involved in cellular and molecular processes leading the melanoma progression, such as chromatin remodeling, epithelial-mesenchymal transition (EMT), and telomerase activity.

Alterations in Wnt/β-catenin signaling strictly affect EMT and lead to simultaneous activation of MAPK and AKT pathways. In addition, Wnt/β-catenin promotes neural crest migration of melanoma cells and induces an invasive phenotype (Sinnberg et al., 2018). In this pathway, mutated *SOX10, WNT1* and *CTNNB1* genes have been associated with metastatic spread, in particular with lymph node metastases (Braeuer et al. 2014, Guo W. et al. 2021). *ARID2* encodes for a protein that is part of the SWI/SNF chromatin remodeling complex. Mutations in this gene, identified in 5% of advanced melanoma cases, are associated with dysregulation of chromatin structure during pathogenesis (Hodis et al., 2012). In recent years, studies conducted on the chromatin remodeling complex observed that melanoma patients with *ARID2* mutations had better survival than wild type patients (HR: 1.22; 95% CI 1.0-1.5, p = 0.022), although no additional OS benefit was observed during treatment with anti-PD-1 inhibitors. Numerous mutations in the *TERT promoter* region have been identified in the *BRAF*, N*RAS*, and *NF1* mutated subtypes, suggesting a link to the MAPK pathway (Teixido et al., 2021). It has been shown that mutations in the *TERT* gene are involved in the progression of melanoma by conferring aggressiveness to the tumor, particularly in (superficial spreading melanoma) SSM and (nodular melanoma) NM histotypes (Loras et al., 2022). Mutations in the *TERT promoter* are indeed found predominantly in metastases as opposed to primary tumors (Sundararajan et al., 2024). In a retrospective study of 684 melanomas, the presence of -124C > T and -138_139CC > TT *TERT promoter* variants was associated with a Breslow thickness >2 mm and with a fast-growing phenotype. The tandem of

mutations showed a worse survival rate than the presence of only one variant (Manrique-Silva et al., 2024).

Other genes strongly associated to melanoma progression are *PPP6C, RAC1,* PREX2, *STK19* and *TP53.* In detail, the *PPP6C* gene negatively regulates the levels of the CCND1 oncogene during the G1 phase (Stefansson et al., 2007), and affects melanocyte differentiation through modulation of MITF activity. The R264C change is the most frequent *PPP6C* mutation identified in melanoma. Maskin et al. (2022) demonstrated that mutations in *PPP6C* together with *NRAS* Q61 accelerate melanomagenesis, while in the presence of *BRAF* V600E increase BRAF inhibitor resistance in a Zebrafish model (Maskin et al., 2022). *RAC1* is a RAS-associated GTPase of the Rho subfamily whose function is cytoskeletal reorganization, thus playing a crucial role in cell invasion. The *RAC1* P29S variant is an activating mutation, which preferentially switches RAC1 to a GTP-bound state. (Hodis et al., 2012). This mutation has been frequently observed in conjunction with mutations in the MAPK pathway, conferring to the tumor an alternative survival mechanism, and a significant increase of resistance to MAPK inhibitors (Lodde et al., 2023). Activating mutations of *RAC1,* loss of *PTEN*, or amplification of *PREX1/2* often occur alongside *BRAF* V600E and enhance migration by restoring activation of RAC1 pathway. Hyperactivation of ERK signalling by oncogenic *BRAF* mutants is sufficient to deregulate cell proliferation. However, the inhibition of RAC1 prevents both mesenchymal transition and migration (Gadal et al., 2023). Mutations in *STK19*, whose kinase function is still unknown, have been linked to melanoma through their impact on NRAS activation. Indeed, a recent study identified STK19 as a novel activator of NRAS, increasing its phosphorylation and binding to downstream effectors (Yin et al., 2019). Therefore, blocking STK19 kinase activity by targeting its hot-spot mutations such as D89N could be a promising strategy for the treatment of melanomas carrying *NRAS* mutations (Yin et al., 2019). *PREX2* and *GRIN2A* genes have also shown frequent somatic mutations in melanoma. Mutations in *PREX2* that generate a truncated protein appear to play an important role in melanomagenesis and in driving metastasis (Li Ding et al., 2014). *PREX2* mutant tumors are highly proliferative and show increased Ki67 staining, although the reasons for the biological alteration of cell cycle regulation by *PREX2* mutation remain unclear (Lissanu Deribe, 2016). The binding between PREX2 and RAC1 may explain the changes in cell cytoskeleton signaling, while together with the PTEN pathway, the *PREX2* gene is likely responsible for the increase in ribosome biogenesis. (Lissanu Deribe, 2016).

4.MOLECULAR HETEROGENEITY BETWEEN PRIMARY TUMOR AND METASTASIS

The prevailing theory of carcinogenesis suggests that most tumors originate from a single stem cell (Nowell, 1976). However, different cellular clones with distinct molecular, immunogenic, and proliferative behavior can emerge within the tumor during development and progression (Hunter et al., 2018). Metastatic tumors can arise from these dominant or minor cell subclones that consistently evolve into a more aggressive phenotype. Notably, melanoma has been shown to have the highest level of intra-tumor molecular heterogeneity compared with other cancers, suggesting significant variability of subclones that could progress differentially (Andor 2016). The acquisition of new mutations in evolving subclones during tumor progression underlies the genetic heterogeneity between primary and metastatic lesions and between multiple metastases (Hunter et al., 2018). These discrepancies constitute intra-patient tumor genetic heterogeneity and represent one of the critical aspects for molecular melanoma diagnosis and therapy (Grzywa et al., 2017).

The molecular and clinical significance of intra-patient heterogeneity was studied mainly by considering *BRAF* and *NRAS* driver genes, for which molecular discordance between primary and paired metastases could be a key concern for the proper administration of BRAF inhibitors. According to a large meta-analysis of 22 studies, the intra-patient heterogeneity rate of *BRAF* alterations was 13.4% considering primary melanoma and paired metastatic lesions and 7.3% considering two metastatic lesions (Valachis et al, 2017). Colombino et al. (2012) found high consistency in *BRAF/NRAS* status between the primary tumor and paired metastases (Colombino et al., 2012), while a single centre Japanese study reported high levels of discordance (47%) (Kaji et al., 2017). Our group found an intra-patient heterogeneity of 13.3% for both *BRAF* and *NRAS* genes by analyzing primary melanomas, related lymph nodes, and visceral metastases (Pellegrini et al., 2020). In the 41 Italian patients studied by Manca et al (2019), the discordance rate of *BRAF* mutations was 7.5% and of *NRAS* mutations was 14.5%. Interestingly, concordance rates were significantly lower when visceral lesions were analyzed compared with lymph node metastases (Manca et al., 2019). This reduced concordance of mutations from the primary tumor to regional and distant metastases could provide empirical support for the hypothesis of the accumulation of genetic alterations during the linear progression of melanoma. A recent meta-analysis revealed that the pooled concordance rate for *BRAF* was 89.4% [95% CI: 84.5; 93.5] considering 1220 patients and 97.8% [95% CI: 95.8; 99.4] for *NRAS* in 629 patients (Kerkour et al., 2023). In addition, the authors calculated the cumulative concordance of other known melanoma genes analyzed in a very small subgroup of patients, finding high concordance for mutations in the *TERT, CDKN2A, TP53, RPPIK3CA,* and *EPHB6* genes, and complete discordance for *CTNNB1, RAC1,* and *MAP2K1*, which were observed exclusively in primary or metastatic tumors (Kerkour et al.,

2023). In detail, the concordance rate of *TERT promoter* mutations was 64% in a total of 53 patients, 25% for *CDKN2A* in 4 patients, 44% for *TP53* in 9 patients and 20% for in 5 patients. (Kerkour et al., 2023). Harbst et al. (2016) sequenced the whole exome of two paired primary melanoma tumors and six metastases. Overall, 13% of all mutations identified were heterogeneous, meaning that they were not detected in all samples obtained from the same patient, and among them, 12% were driver mutations (Harbst et al., 2016).

Rationale and Aim

Extensive research has been conducted on the molecular genetics of melanoma at the somatic level, with particular emphasis on characterizing the *BRAF* and *NRAS* molecular subtypes. Deep molecular investigations have been performed mainly in metastatic tissues, besides the known issue of the discordance between the primary and metastatic samples. This could create a certain distortion in the analysis of the results. To better understand development and progression of melanoma, it is imperative to identify the molecular profiles of both primary tumor and metastases. In this context, the ideal cohort of study should include primary and matched metastases from the same patients. Some reports in the literature investigated molecular events in paired primary and metastases, but the majority lacked to estimate the overall mutational intra-patient discordance, only focusing on *BRAF* and *NRAS* genes. The few studies that described discordant mutational profile include very small number of patients. We believe that intra-patient molecular discordance analysis is necessary to truly understand metastatic progression, taking into account physiological inter-patient variability.

The main objective of our study was to characterize the molecular profiles of a panel of 18 melanoma-related genes and to identify their clinical and prognostic significance in primary and matched metastatic tissues. Moreover, the molecular profiling allowed us to evaluate the intra-patient and inter-patient mutational discordance that could be associated to mechanisms of progression.

Materials and Methods

1.RECRUITMENT OF PATIENTS AND SPECIMEN COLLECTION

Patients with metastatic melanoma were retrospectively collected from the Dermato-Oncology Unit of the University of L'Aquila from November 2020 to September 2023. The eligibility criteria included: age >18 years; the availability of formalin-fixed paraffin-embedded (FFPE) tissues of either primary melanoma, at least one metastasis and healthy skin; the consent for participating to the study. We collected information on the main epidemiological data and clinico-pathological parameters (Garbe et al., 2020) for primary melanoma and metastases. In detail, data on anatomical location, Breslow thickness, histotype, ulceration, tumor infiltrating lymphocytes (TIL), presence of regression, number of mitosis, elastosis, satellitosis were included. Five-year follow-up data, including therapy, progression and death were also registered, when available. We classified patients according to the AJCC Melanoma Staging System (8th edition) that currently represents the most widely accepted approach to staging and grading at initial diagnosis, essential for subsequent prognostic assessment (Table 1) (Gershenwald et al., 2018). Moreover, for each patient we collected complete anonymized medical records. Ethical approval of the study was obtained by Local Committee (30/CE2015), and the research was performed according to relevant guidelines and regulations stated in the Declaration of Helsinki.

Table 1: TNM Staging System according to American Joint Committee on Cancer, 8th edition (Gershenwald et al., 2017)

WHEN T IS…	AND N IS…	AND M IS…	THEN THE PATHOLOGICAL STAGE GROUP IS…
Tis	N0[b]	M0	0
T1a	N0	M0	IA
T1b	N0	M0	IA
T2a	N0	M0	IB
T2b	N0	M0	IIA
T3a	N0	M0	IIA
T3b	N0	M0	IIB
T4a	N0	M0	IIB
T4b	N0	M0	IIC
T0	N1b, N1c	M0	IIIB
T0	N2b, N2c, N3b or N3c	M0	IIIC
T1a/b–T2a	N1a or N2a	M0	IIIA
T1a/b–T2a	N1b/c or N2b	M0	IIIB
T2b/T3a	N1a–N2b	M0	IIIB
T1a–T3a	N2c or N3a/b/c	M0	IIIC
T3b/T4a	Any N ≥N1	M0	IIIC
T4b	N1a–N2c	M0	IIIC
T4b	N3a/b/c	M0	IIID

Any T, Tis	Any N	M1	IV

2.EXTRACTION AND SEQUENCING

For each sample, 7 sections were obtained from the FFPE tissues for DNA extraction. Only tumor section in which the percentage of cancer cells was greater than 40% were subjected to DNA extraction. DNA was obtained by macrodissection by using the QIAamp DNA Micro Kit (Qiagen). Target sequencing technology using NGS was chosen to study the molecular alterations of the genes involved in metastatic progression. After literature review and querying databases such as VARSOME and CLINVar, 18 genes were identified based on the distribution and frequency of mutations in metastatic melanoma (setting a mutational rate cut-off >10%), and included in the NGS panel (*BRAF, NRAS, NF1, ARID2, TACC1, PPP6C, TP53, TERT promoter, RAC1, STK19, PREX2, EPHA3, GNA11, GRIN2A, DDX3X, WNT1, KIT, CTNNB1*). Briefly, using the Ampliseq methodology, libraries were constructed using Ion Ampliseq™ Library Plus and subsequently quantified using Qubit. Twenty-six samples were first pooled and amplified using the OT2 emulsion PCR (Thermofisher), loaded onto chip540, and sequenced on the IonTorrentS5 platform (Thermofisher).

The resulting raw vcf files generated for each sample by the IonReporter software version 1.17 (Thermofisher) were subsequently analysed and filtered by the use of bcf tools (Danecek et al., 2021) by applying the following rules: "DP > 300 & AF > 0.05", where DP represents "total read depth at the genomic coordinates," while AF stands for "Allele Frequence frequency based on observation counts". Furthermore we considered a filtered coverage > 500X, Phred QUAL Score between 50 and 5000, pvalue <0.005, an Allele frequency between 0.05 and 1 and Homopolymer Length between 0 and 3. For each patient, the healthy skin specimen was used to normalize molecular alterations identified in the primary tumor and metastases. All variants shared by control tissue and primary tumor tissue (P) or metastatic tissue (M) were removed as considered patient-specific genetic background, not related to the studied phenotype. The remaining filtered variants were finally intersected between tumor specimens by the use of a custom bash script involving bedtools software (version 2.27.1) (Quinlan & Hall, 2010) and a following Python script. A final Excel file was created to store all variants found in each tissue alongside Excel sheets containing variants shared by any combinatorial intersection between specimens.

3.STATISTICAL ANALYSIS

For statistical analysis, we categorized the variables as follows: sex (Male, Female); location of the primary tumor (Head/ neck, Trunk, Extremities); CSD areas (head/neck areas,

extremities), histopathological subtype (SSM, NM); TIL (Absent, Brisk, Non-Brisk); associated nevus (presence, absence); ulceration (presence, absence); elastosis (presence, absence); satellitosis (presence, absence); metastatic site (lymph node, skin, visceral); therapy (therapy, no therapy), stage at diagnosis (Locoregional: I-II, Metastatic: III-IV). For patients with multiple metastases, we considered the chronological order of diagnoses to define the first, second or third. We separately analysed the molecular profile of the first metastases, the second and third. Regarding the four molecular subtypes, we grouped tissues as follows: *BRAF* (pathogenic variants, no-pathogenic variants, wild type), *NRAS* (pathogenic variants, no-pathogenic variants, wild type), *NF1* (all when *NF1* variants did not occur together with pathogenic *BRAF* or *NRAS*), and wild-type (when none of those above mutations were present). In detail, for *BRAF* we considered pathogenic the V600 variants while for *NRAS* we considered Q61, G12 and G13. Regarding other genes of the panel, we considered pathogenic all non-synonymous, missense, non-sense, splicing-related variants and we categorized samples on the base of the presence or the absence of this type of mutations.

We provided descriptive statistics both for categorical and continuous variables using frequency, means or median and standard deviations estimates, as appropriate. Univariate linear and logistic regression models were performed to analyze the impact of each gene on the sex, age at diagnosis, primary location, stage at diagnosis, histopathological subtype, Breslow thickness, mitosis, Brisk, associated nevus, ulceration, elastosis, satellitosis and metastatic site. The multivariate linear regression and logistic regression addressed the thickness and ulceration (principal AJCC prognostic factors) according to genes, adjusting for sex and age at diagnosis. Model fitting was assessed using the adjusted coefficient of Determination and tested using the $\chi 2$ statistics. Variables collinearity was addressed working out a VIF (variant inflation factor) analysis which enables us to exclude collinear variables, i.e. genes, negatively affecting model overall performance. According to the mutational status of the four main molecular subtypes, MSS was studied using the Kaplan-Meier curves, and differences were estimated with the log-rank test, when follow-up and treatment data were available. We excluded patients treated with target therapy from the MSS analysis due to the low number of cases.

To define the concordance of mutational status between the primary tumor and the group of first metastases, we analyzed the variation of each gene individually at the univariate analysis obtaining the individual level of discordance. The comparison was performed by the McNemar Test or Stuart-Maxwell test, as appropriate. The overall comparison of mutational status of the primary tumor *vs* the first metastasis was addressed using the Fisher's log statistics joining the p

values of the previous McNemar Tests (excluding *BRAF* and *NRAS)*. The p value associated to the corresponding $\chi 2$ distribution enabled us to test the presence of a global difference.

The definition of mutational profiles according to both the primary and metastatic groups were explored using the multiple correspondence analysis (MCA). The scree-plot was explored to identify a reasonable variability cut-off between dimensionality and data heterogeneity. A two-dimensional pattern of variability was accepted to describe the molecular heterogeneity among all 18 genes. Consequently, the biplot graph was generated.

The software STATA (version 17) and the statistical software R version 4.2.2 (Copyright (C) 2022 The R Foundation for Statistical Computing Platform) were used for the statistical analysis. The p-values are corrected for multiple hypothesis testing by using the Benjamini-Hochberg correction.

Results

1.POPULATION

Forty-four metastatic melanoma patients (29 males and 15 females), with a median age at first diagnosis of 61.60 years (range 25-84 years) were included in the study. Overall, we collected 44 primary tumors and 63 metastases. Demographic, clinical and histopathological characteristics of patients and melanomas are reported in Table 2. Melanomas were located mainly on the trunk (20/44, 45.4%) and on the extremities (19/44, 43.2%); only 5 (5/44, 11.4%) lesions were located on the head/neck region. Metastases were predominantly in the lymph nodes, accounting for 37/63 (58.7%) of the total occurrences. The remaining metastases were located in the skin (19/63, 30.2%), and in visceral organs (7/63, 11.1%). Patients and tumor characteristics are illustrated in Table 2.

Table 2: Demographic and clinical characteristics of melanoma patients and histopathological features of tumors and metastasis

Characteristics of patients		**No. of samples N = 44 (%)**
Sex	Males	29 (65.9%)
	Females	15 (34.1%)
Age	Median age (range)	61.6 (25-84)
No. of metastases	1	35 (79.5%)
	2	6 (13.6%)
	3	3 (6.9%)
Treatment type	Immunotherapy	15 (34.1%)
	Target therapy	4 (9.1%)
	No therapy	10 (22.7%)
Stage at Diagnosis	IA	2 (4.5%)
	IIA	4 (9%)
	IIB	2 (4.5%)
	IIC	5 (11.4%)
	IIIA	5 (11.4%)
	IIIB	6 (13.6%)
	IIIC	20 (45.6)
Progression	Yes	19 (43.2%)
	No	25 (56.8%)
Death	Yes	12 (27.3%)
	No	13 (29.5%)
	Unknown	19 (43.2%)
Characteristics of primary melanoma		**N=44 (%)**
Breslow thickness	Median value (range)	5.2 (0.6-20)
Histopathological subtype	SSM	13 (29.5%)
	NM	31 (70.5%)
Anatomical site	Head/neck	5 (11.4%)
	Trunk	20 (45.4%)
	Extremities	19 (43.2%)
TILs	Absent	17 (38.6%)
	Brisk	13 (29.5%)
	Non-Brisk	12 (27.2%)
Associated Nevus	Yes	14 (31.8)
	No	27 (61.4)
Mitosis	Median (range)	5.9 (0-24)
Ulceration	Yes	25 (56.8%)
	No	19 (43.2%)
Solar Elastosis	Yes	21 (47.7%)
	No	21 (47.7%)
Satellitosis	Yes	12 (27.2%)
	No	29 (65.9%)
Characteristics of metastases		**N=63 (%)**
Anatomical site	Lymph nodes	37 (58.7%)
	Skin	19 (30.2%)
	Brain	3 (4.8%)
	Visceral	4 (6.3%)

2.BRAF, NRAS, NF1 AND TRIPLE WILD TYPE SUBGROUPS

2.1 Frequency of mutations

We examined the frequency of driver mutations in primary tumors and metastases. We identified *BRAF* mutations in 43% of primary melanomas, *NRAS* in 27% and *NF1* in 11%; while 20% of tumors were triple wild type. Considering the group of first metastases, lesions with *BRAF* mutations were 30%, those with *NRAS* mutation 14% and those with *NF1* 14%. The 43% of first metastases were triple wild type. Even among the group of second metastases, the majority of lesions were represented by triple wild type subtype, which reached 60% of frequency (Figure 4).

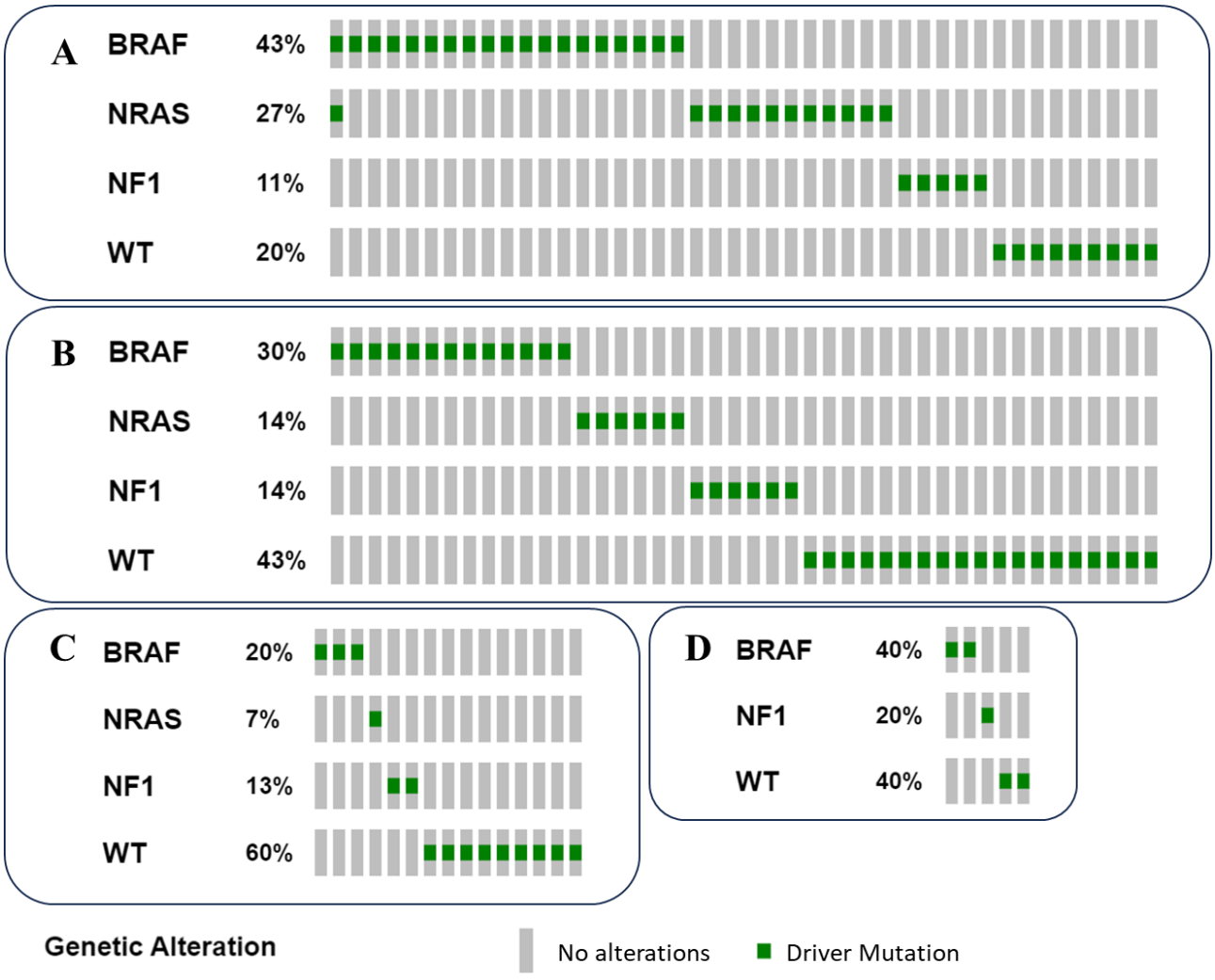


Figure 4 Frequency of driver mutations in A) Primary Tumor B) First Metastasis C) Second Metastasis D) Third Metastasis (*CBioPortal for Cancer Genomics:Oncoprinter*, n.d.)

Focusing on *BRAF* subtype, 36.4% of the primaries tumors harboured the *BRAF* V600E and 4.5% the *BRAF* V600K mutation while in metastases 23.8% carried the *BRAF* V600E, 6.3% *BRAF* V600K. For the *NRAS* subgroup, 9.1% of primaries were *NRAS* Q61L, 9.1% *NRAS* Q61R, and

6.8% *NRAS* Q61K. Among *NRAS* metastases, 4.8% were *NRAS* Q61L, and 1.6% *NRAS* Q61R. The variant *NRAS* Q61K was not detected in the metastases. Notably, the co-occurrence of *BRAF V600E* and *NRAS* Q61R driver mutations were present in one primary tumor (Allele Frequency = 0.312364 and Allele Frequency = 0.0813288, respectively). In the *NF1* subtype, the variants were distributed throughout the gene in both primary and metastases, without a specific hot-spot. The missense *NF1* variant V2086A was the most frequent, occurring in 30.7% of the *NF1*-mutated primary tumors. Additionally, the missense variant V2436M of uncertain significance was found four times (22.2%) in *NF1*-mutated metastases. Primary melanomas with the triple wild type status accounted for 20% of the cases; this percentage increased to 43% in metastases (p=0.034). Focusing on this fourth subgroup, we found that *PREX2, EPHA3, TERT promoter*, and *TACC1* were the most frequently mutated genes in both primary (78%, 33%, 56% and 67%, respectively), and metastatic cases (68%, 40%, 40% and 36% respectively). Mutations of the *KIT* gene were frequent in wild type metastases (32%) (Figure 5).

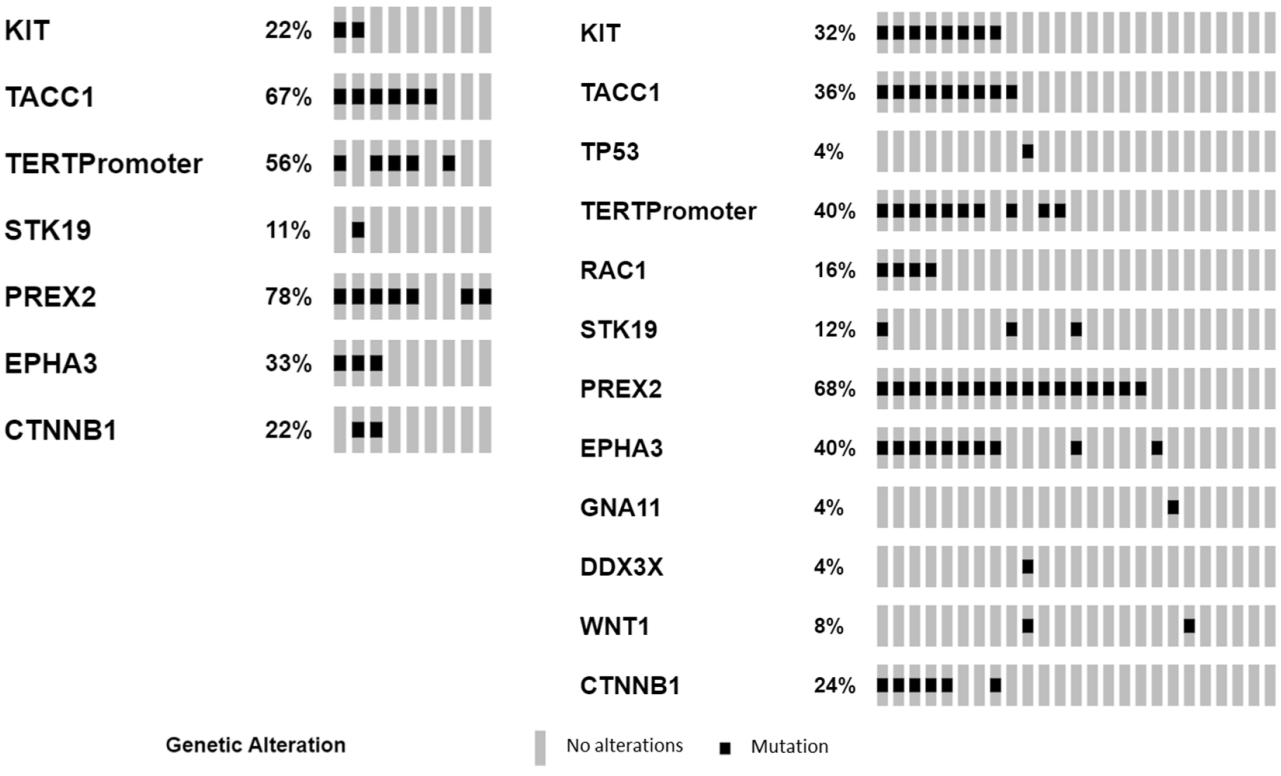


Figure 5 *Mutational profiles of A) triple wild type primary melanomas, B) triple wild type metastases* (*CBioPortal for Cancer Genomics:Oncoprinter*, n.d.)

2.2 Clinical associations with mutational status

Upon segregating our samples into the four main molecular subgroups, we investigated any potential link between molecular status and clinical data of patients and melanomas.

At the univariate analysis, we found that driver mutations in *BRAF* were associated with the onset of melanoma at a young age (median 57.6, range 25-73, p=0.036), with the presence of ulceration (p=0.003) and with higher number of mitosis (median 6 mitosis/mm^2, range 0-23, p=0.041) compared to *BRAF* wild type melanomas. Moreover, we found that melanomas with *BRAF* driver mutations had a significantly increased risk to metastasize to the skin, independently of other prognostic factors, such as age at diagnosis, thickness, ulceration and mitosis (OR= 6.72, 95%CI 1.39-67.43, p= 0.028). Regarding *NRAS,* the presence of mutations was associated with later onset (median=72.5, range 45-84, p=0.004) and development of melanomas in CSD areas, although this finding did not reach statistical significance (p=0.116). *NF1*-mutated melanomas were associated with more advanced stages at diagnoses (p=0.050), while triple wild type lesions were frequently identified as SSM (p=0.0001) and diagnosed at localized stages (I-II) (p=0.024).

Detailed follow-up and treatment data were available only for a subset of 25 patients who underwent molecular profiling. For these patients we conducted a Kaplan-Meier analysis to assess the MSS rate at 5-years of follow-up for the different molecular subgroups. We did not observe significant differences among subtypes (p=0.234). To take into account the impact of immunotherapy on the survival of the different molecular subtypes, we performed a subgroup analysis of patients treated and not treated. Regardless of whether therapy is administered or not, it appears that the *BRAF* subtype has a poorer, nonsignificant 5-year survival rate (p=0.481, p=0.756 respectively). Unfortunately, there were no *NF1* patients treated with immunotherapy in our cohort, thus we could not compare *NF1* cases with other groups regarding treatment with immunotherapy. In addition, it is worth noting that none of the patients with triple wild type died within 5 years and their survival rate remained unchanged, suggesting that their survival rate might be higher than that of other subgroups (Figure 6).

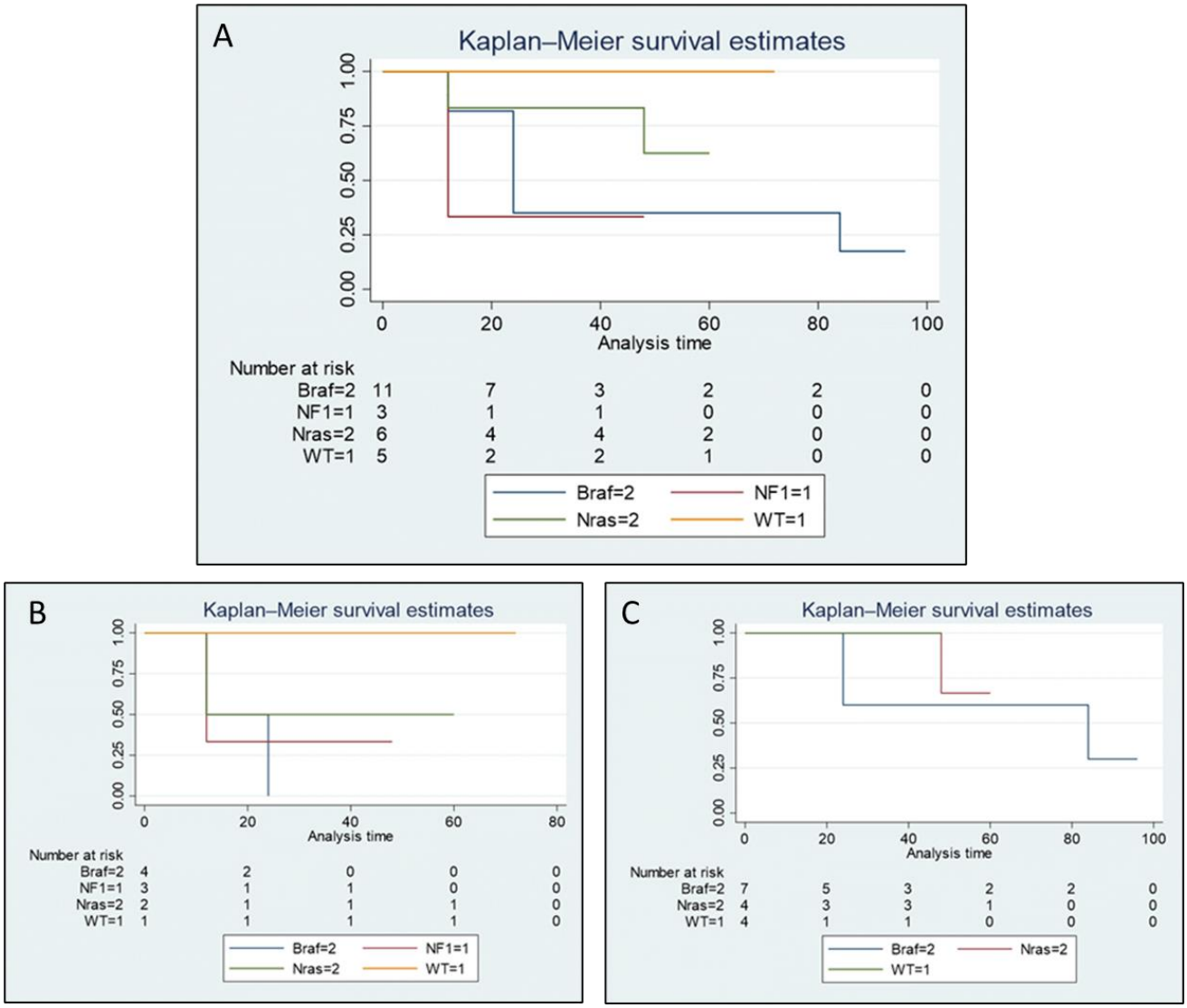


Figure 6 *Kaplan Meier melanoma specific survival curves* ***(A)*** *Survival curves of patients with BRAF primary melanomas (median time=24 months), NRAS primary melanomas, NF1 primary melanomas (median time=12 months) and triple wild type primary melanomas (p=0.234).* ***(B)*** *Survival curves of patients not treated with immunotherapy. BRAF (median time=12 months), NRAS (median time=12 months), NF1 (median time=12 months) and triple wild type (p=0.481).* ***(C)*** *Survival curves of patients treated with immunotherapy. BRAF (median time=84 months), NRAS, and triple wild type. No NF1 treated with immunotherapy were present in our cohort (p=0.756).*
We excluded patients treated with target therapy from the analysis.
Where the median time is not present it is because 50% of the group did not experience the event.

3.OTHERS MOLECULAR ALTERATIONS IN PRIMARY TUMORS AND METASTASES

3.1 Frequency of mutations

Sequencing analysis of other melanoma-related genes included in our NGS panel is reported in Figures 7-9. We identified a high frequency of mutations in *TACC1, PREX2, EPHA3*, and the *TERT promoter* both in primary melanomas (66%, 68%, 57% and 55%, respectively) and metastases (first metastasis: 48%, 57%, 48%, and 45% respectively; second metastasis: 47%, 40%, 47% and 40% respectively; third metastasis: 50%, 67%, 83% and 50%, respectively). Interestingly, 6 metastases had no mutations in the analysed genes.

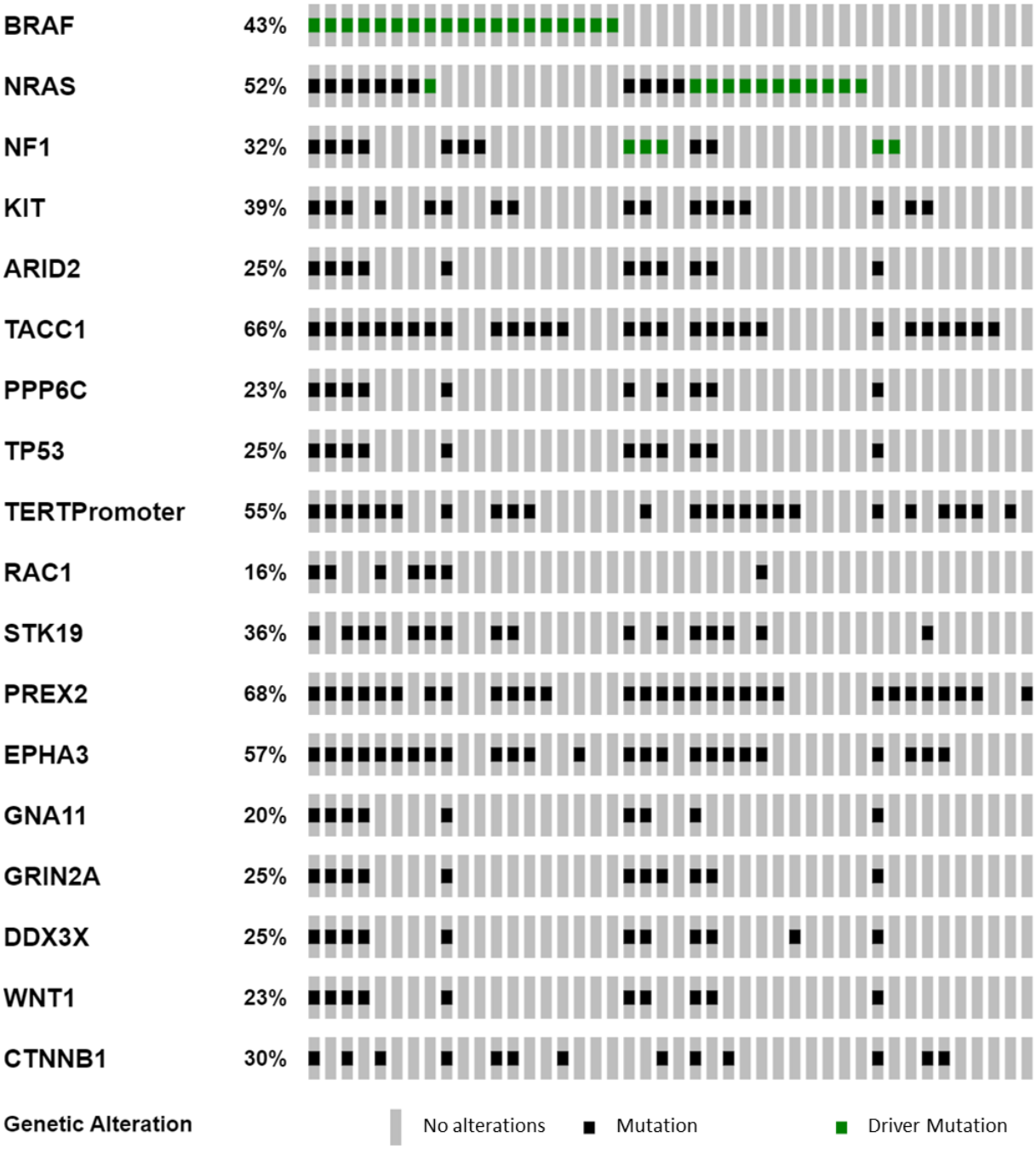


Figure 7 *Mutational status of additional genes included in our NGS panel in primary tumors* (*CBioPortal for Cancer Genomics:Oncoprinter*, n.d.).

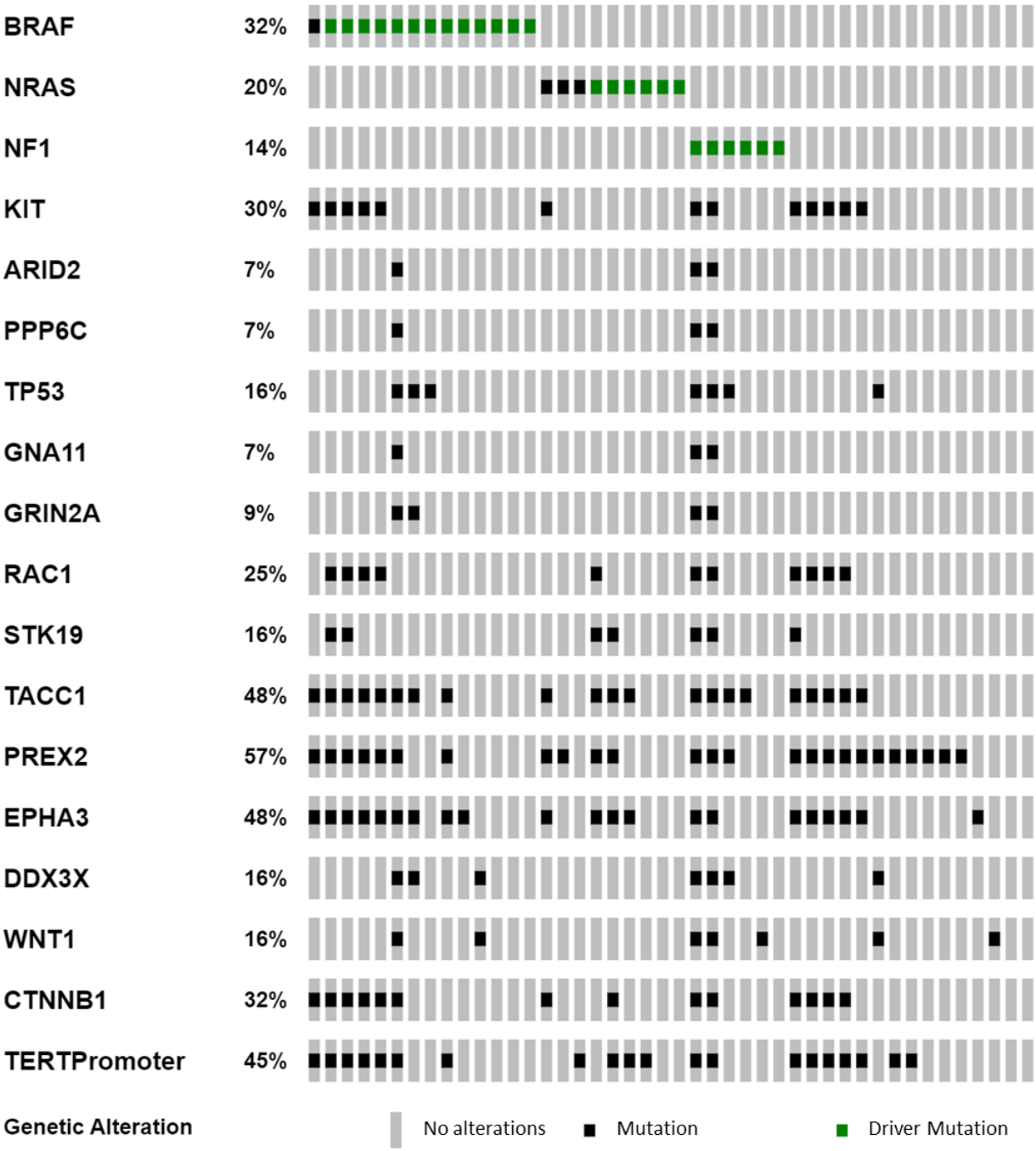


Figure 8 *Mutational status of additional genes included in our NGS panel in the first metastasis* (*CBioPortal for Cancer Genomics:Oncoprinter*, n.d.).

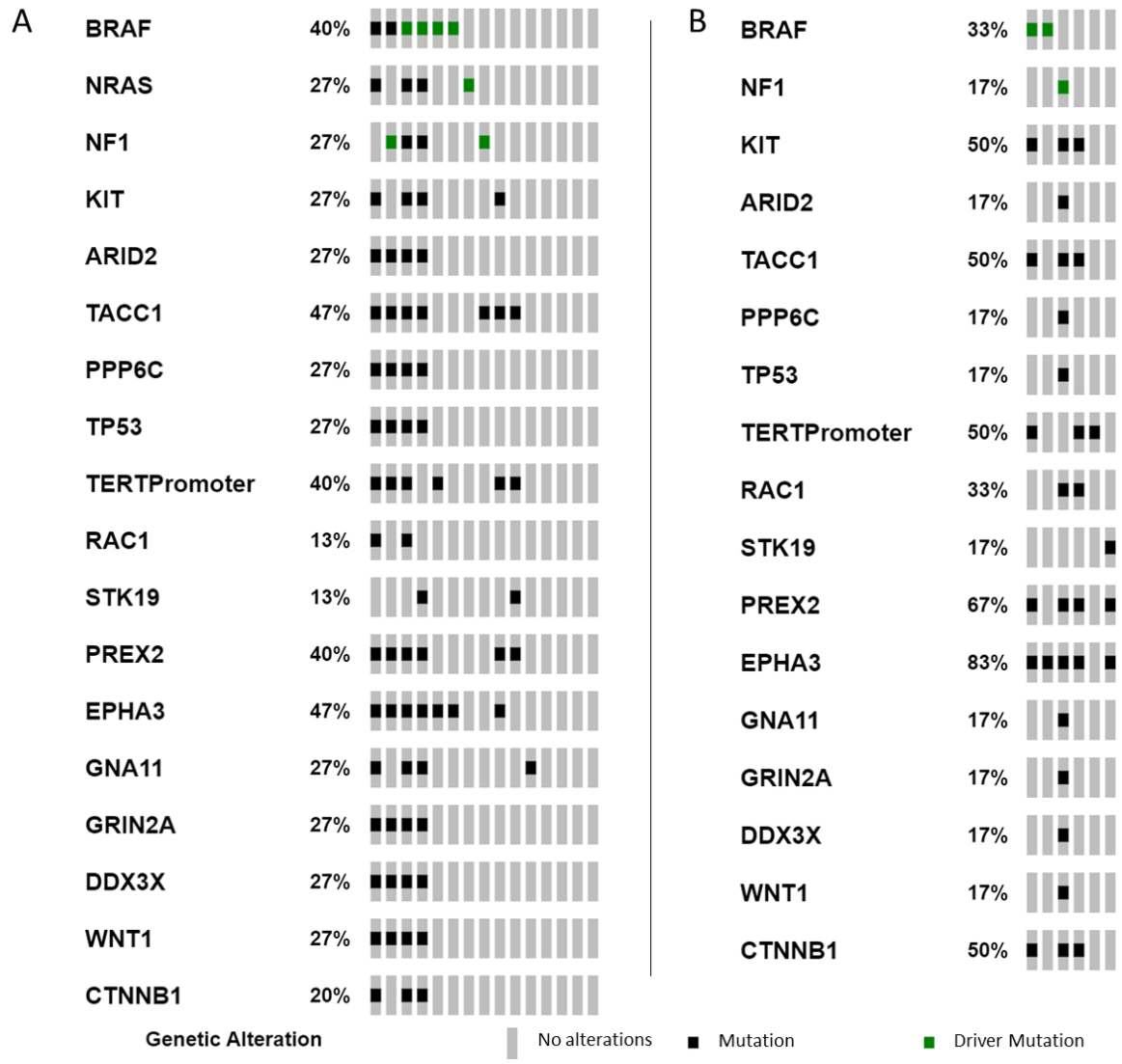


Figure 9 *Frequency of mutations in additional genes in the second (A) and third (B) metastasis* (*CBioPortal for Cancer Genomics:Oncoprinter*, n.d.)

3.2 Clinical associations with mutational status

We investigated whether mutations in the other 15 genes (*ARID2, TACC1, PPP6C, TP53, TERT promoter, RAC1, STK19, PREX2, EPHA3, GNA11, GRIN2A, DDX3X, WNT1, KIT, CTNNB1*) in our panel (except *BRAF, NRAS, NF1*) were associated with clinico-pathological characteristics using both univariate and multivariate statistical analyses.

The presence of variants in the *PREX2* and *RAC1* genes were significantly associated to thicker Breslow thickness (p=0.002 and p=0.001, respectively). In detail, at the multivariate analysis, adjusting for sex, age and mutations in other genes, the presence of these mutations remained significantly associated with thicker Breslow thickness (OR=6.02, 95%CI 2.36-9.81, p=0.002 for *PREX2* and OR=6.08, 95%CI 1.82-10.21, p=0.006 for *RAC1*). *RAC1* mutations were

also frequent in women (p=0.023) and in patients without dysplastic nevi (p=0.048). Moreover, we found *RAC1* mutations mainly in nodular (p=0.012) and ulcerated (p=0.062) lesions. *STK19* mutations were associated with satellitosis (p=0.050). Both *TP53* and *DDX3X* mutated melanomas progress more frequently to the lymph nodes (p=0.021 for both) compared than to other sites. No additional significant association of the mutational status of each gene with clinical parameters was observed.

Considering the impact of Breslow thickness on prognosis, we assessed whether *PREX* and *RAC1* mutational status was associated with survival. No significant differences in MSS were found between mutated and wild type melanomas for either genes (p=0.234, p=0.143, respectively).

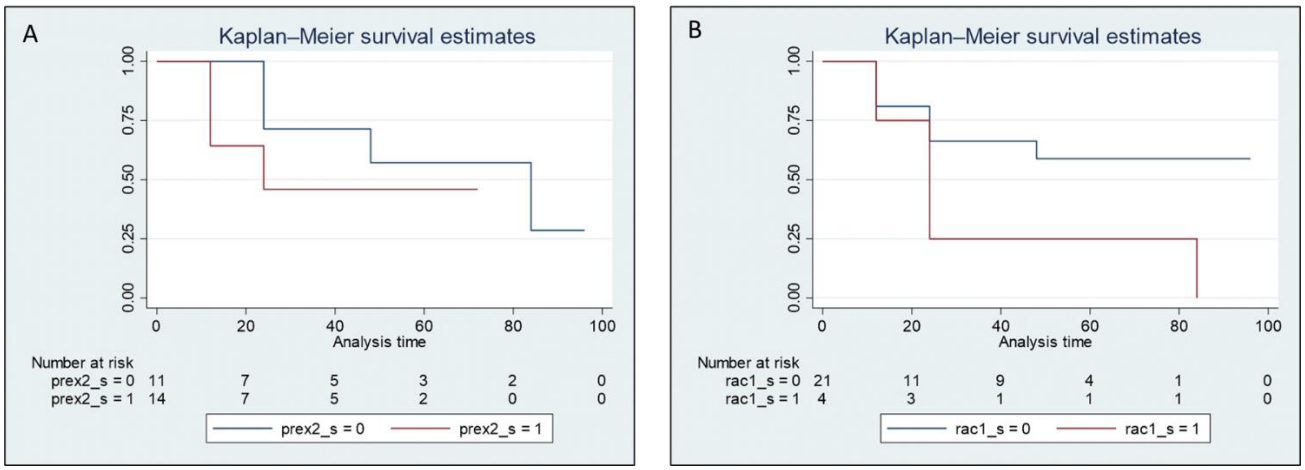


Figure 10 *Kaplan-Meier melanoma specific survival curves (A) PREX2 survival (median time=84 and 24 months respectively, p=0.234) (B) RAC1 survival (median time RAC1=1 is 24 months, p=0.143). Where the median time is not present it is because 50% of the group did not experience the event. 0 indicates absence of mutation while 1 indicates presence of mutation.*

4.INTRA-PATIENT HETEROGENEITY IN BRAF AND NRAS DRIVER GENES

Discordance of the 4 prevalent molecular subtypes (*BRAF, NRAS, NF1* and triple wild type) between primary melanoma and corresponding first metastasis was identified in 53.3% of patients. Considering *BRAF* and *NRAS* mutations, we observed a discordance of 20.0% and 15.9% respectively. There were only three patients who were concordant for all lesions including primary tumor and first, second and third metastases. Table 4 shows mutational changes between primary tumor and related metastases regarding the four main molecular subtypes of melanoma.

After conducting the marginal homogeneity test and the symmetry test to evaluate the statistical agreement of mutational status between primary and related metastases, we did not find

significant changes from *BRAF* and *NRAS* subtypes as compared to triple wild type (p=0.223, p=0.127 respectively). The wild type status resulted significantly increased in metastatic tissues compared to primary tumors (p=0.035). We compared MSS rates of patients with discordant driver mutations and those with concordant mutations between primary and first metastasis. Our analysis revealed that patients with discordant mutational status showed a nonsignificant lower survival rate regardless of immunotherapy treatment (median time of 24 and 84 months for concordant and discordant patients, respectively; p=0.32). The Figure 11 offers a visual representation of these findings.

Table 3: Molecular status changes between primary and related metastases, ndicating intra-patient heterogeneity.

Status	First Metastases	Second Metastases	Third Metastases
Concordant	21 (47.7%)	3 (27.2%)	3 (60%)
BRAF->WT	5 (11.4%)	2 (18.2%)	1 (20%)
BRAF->NF1	3 (6.8%)	2 (18.2%)	1 (20%)
NRAS->BRAF	1 (2.3%)	-	-
NRAS->WT	4 (9.1%)	1 (9.1%)	-
NRAS->NF1	2 (4.5%)	1 (9.1%)	-
NF1->BRAF	2 (4.5%)	-	-
NF1->WT	2 (4.5%)	1 (9.1%)	-
NF1->NRAS	2 (4.5%)	-	-
WT->BRAF	1 (2.3%)	-	-
WT->NF1	1 (2.3%)	-	-

Triple wild type (WT)

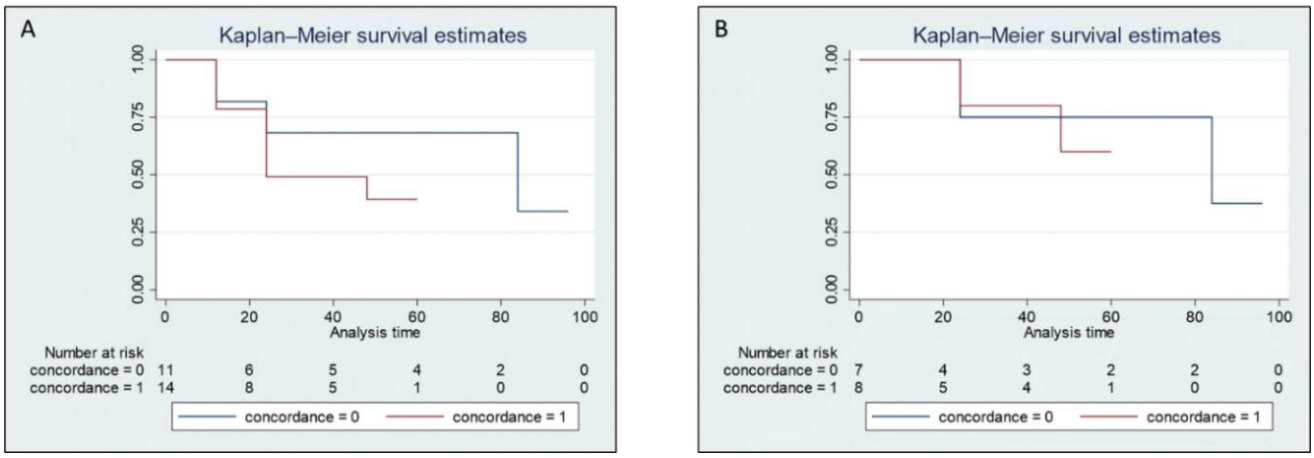


Figure 11. *Kaplan-Meier melanoma specific survival curves A) Cases treated with immunotherapy (median time = 84 months for concordant patients and 24 months for discordant, p=0.32) B) Cases not treated with immunotherapy median time=84 months for concordant patients (p=0.69).*
We excluded patients treated with target therapy from the analysis. Where the median time is not present it is because 50% of the group did not experience the event. (Concordance=0, intra-patient concordance; Concordance=1 intra-patient discordance).

To evaluate the discordance of the mutational status of additional genes included in our panel (excluding driver *BRAF* and *NRAS)*, between primary tumor and the group of first metastasis, we performed the McNemar test for each gene, and then we used the relative p-values to run the Fisher's log statistics. The p value associated to the corresponding $\chi 2$ distribution yielded a value of 8.594008e-05, that indicates an overall highly statistically significant difference between primary and matched metastatic tissues. In detail, the genes that mostly contributed to the discordance status were the following: *STK19* (p=0.007), *ARID2* (p=0.021), *PPP6C* (p=0.035) and *TACC1* (p=0.046), that were more frequently mutated in primary melanomas than in first metastases that were wild type for these genes. *GRIN2A* (p=0.052) and *GNA11* (p=0.057) showed the same trend.

5.INTER-PATIENT HETEROGENEITY

We performed a MCA encompassing all mutational status of primaries and first metastases to determine a specific molecular profile.

Firstly, we found two large and distinct molecular clusters (Cluster 1 and Cluster 2) of patients, and only three cases (no. 14, 17, 22) with a unique mutational pattern (Figure 12). The two main clusters did not share any clinical similarities. The explorative analysis showed that Cluster 1 was mainly characterised by mutations in the *TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1* and *DDX3X* genes in the primary lesion. Notably, all patients of this cluster but one are

patients with discordant *NF1* mutational status (presence in the primary and absence in the metastases) (Figure 12). In contrast, all triple wild type patients were present in the Cluster 2. The three patients that clustered separately (nr. 14, 17, and 22) were characterized by mutations in the *PPP6C, GRIN2A, ARID2*, and *GNA11* in the metastases (Figure 13, orange circle).

Moreover, the MCA analysis also demonstrated that *PPP6C, GRIN2A, ARID2*, and *GNA11* mutations tend to co-occur both in primaries and metastases (Figure 14).

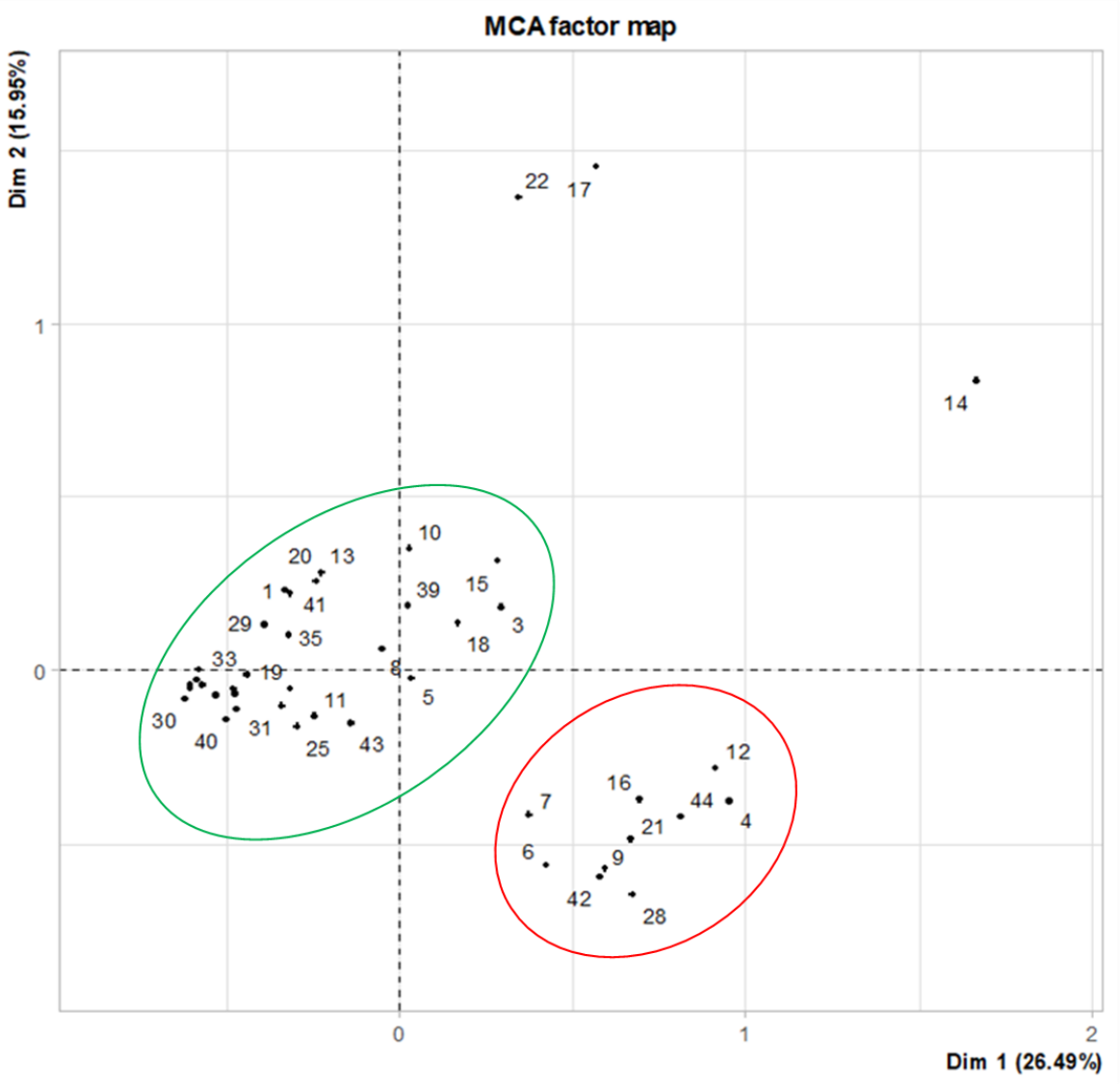


Figure 12 *Observations in MCA analysis.*
The observations correspond to patients who were divided based on their mutational status.
Cluster 1 has been circled in red (characterised by mutations in the TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1 and DDX3X genes in the primary lesion), Cluster 2 has been circled in green (characterised by all triple wild type).

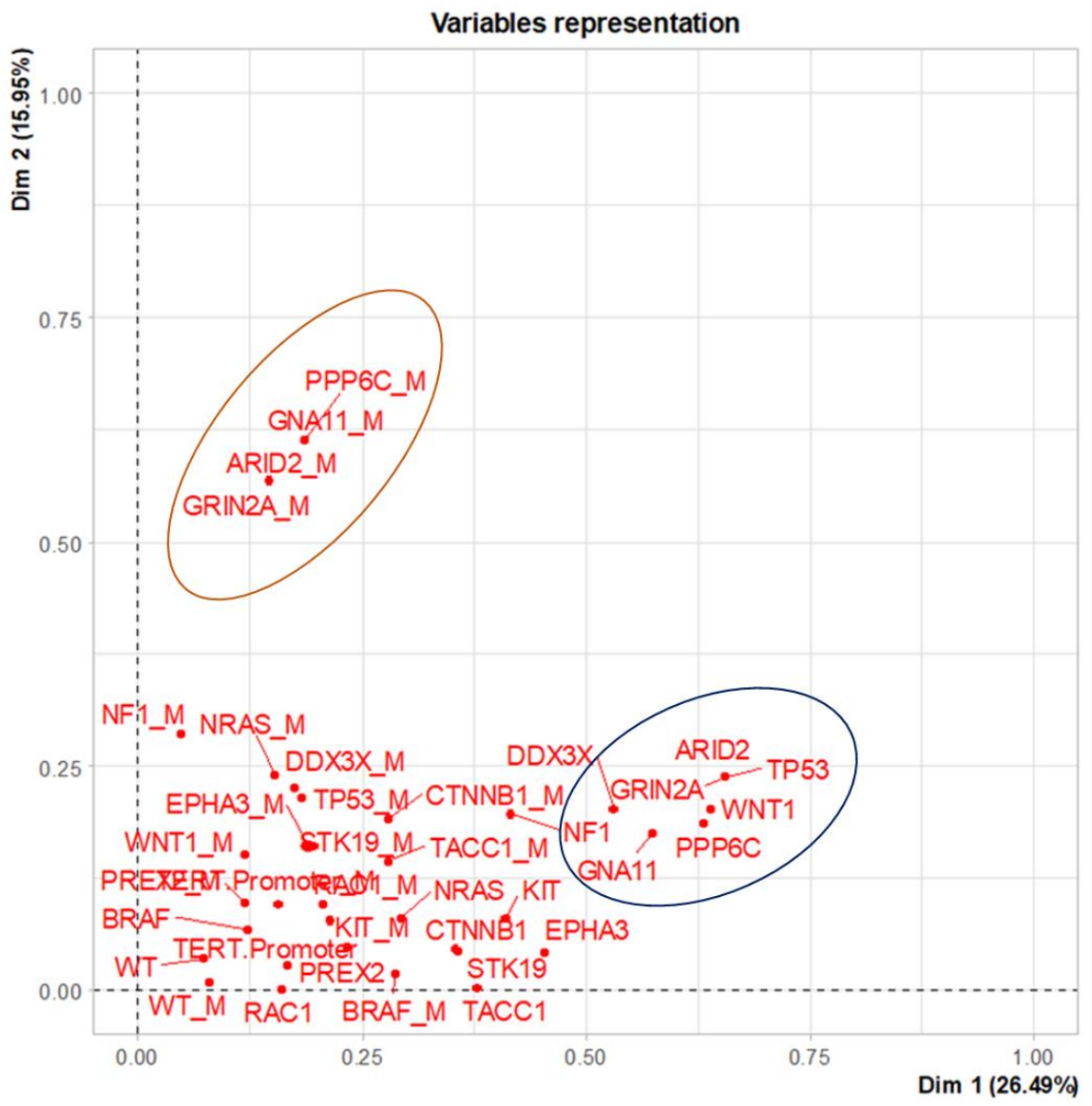


Figure 13 *Categories in MCA analysis.*
Two-dimension spatial distribution of genes on the base of their mutational status. The proximity of points indicates a molecular pattern. In blue are circled the genes that in the primary tumor tend to associate together while in orange are circled the genes that in metastasis tend to associate together.
M=metastasis

It should be mentioned that the clustering analysis through the MCA reported the distribution of the first two dimensions of complexity, explaining 42% of all the molecular variability in each tumor tissue regarding the 18 genes analyzed. Our sample cohort shows significant heterogeneity distributed across multiple dimensions, with ten dimensions explaining 83.1% of the significant heterogeneity as demonstrated by Fisher's test (Figure 14). However, following the "elbow rule," the initial four dimensions are adequate to explain all cases. Since representing four dimensions is not practical, we visualized the first two.

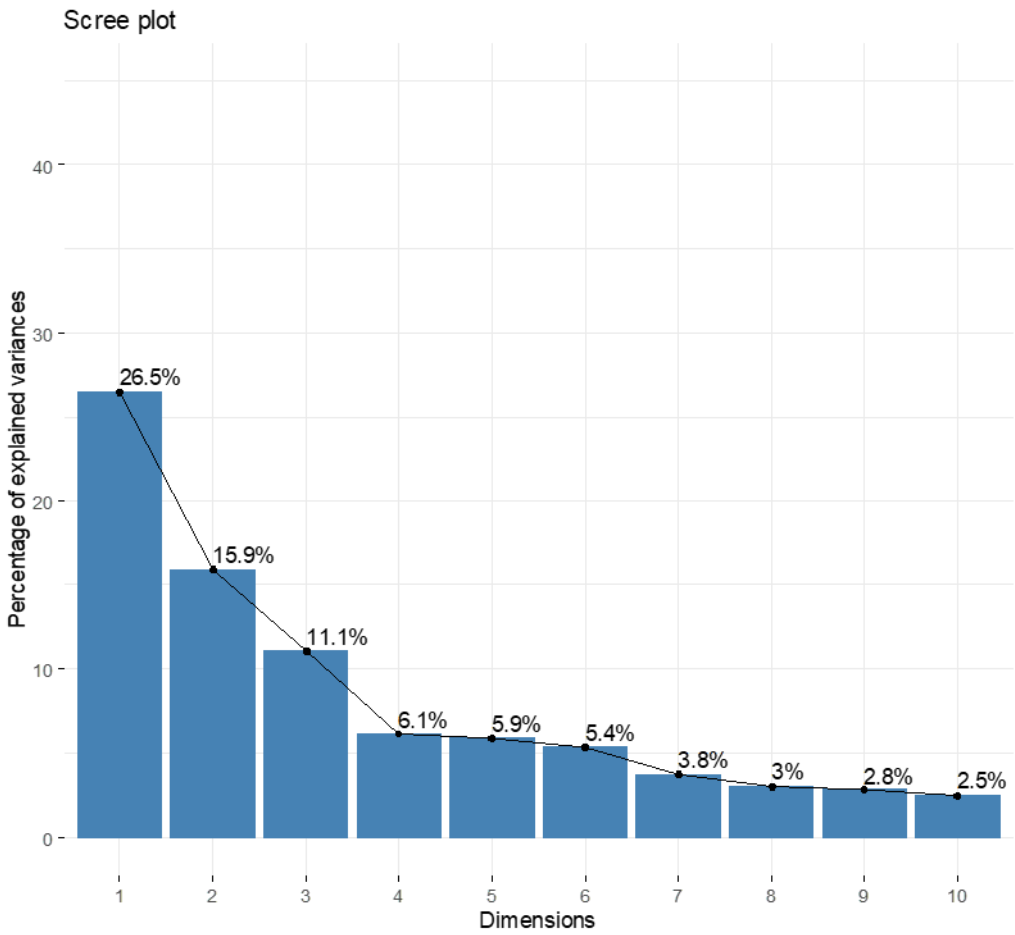


Figure 14 *Scree plot with 10 dimensions that explain the 83.06% of variability of samples Distribution of events in the different dimensions explaining the high heterogeneity of the sample.*

Discussion

We analyzed the molecular profiles of 18 melanoma-related genes in primary melanomas and paired metastases in order to identify their clinical and prognostic significance and to assess the intra-patient and inter-patient mutational discordance that might be associated with mechanisms of progression. Our results consolidate the known evidence on clinical and molecular features of *BRAF, NRAS, NF1* and triple wild type melanoma subgroups. We found a high frequency of mutations in *TACC1, PREX2, EPHA3* and *TERT promoter* in both primary triple wild type tumors and metastases, although no clinically significant association was observed for these genes. In particular, mutations in *RAC1* and *PREX2* were associated with worse prognostic factors. A high rate of intrapatient heterogeneity between primary and metastatic tissues, potentially affecting prognosis, was identified, associated with differences in *STK19, ARID2, PPP6C, TACC1*, and *GRIN2A* genes. Finally, we identified two distinct clusters of patients with different molecular profiles, characterized by the presence of mutations in *TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1* and *DDX3X* genes in primary lesions.

The molecular pathogenesis of melanoma is complex and several mutational events accumulate in critical pathways during the development and progression of the disease (Teixido et al. 2021). The most important pathways altered in melanoma include MAPK, AKT, cell cycle, pigmentation, and p53. Mutations in the key components of the MAPK pathway, including *BRAF, NRAS* and *NF1* are responsible for the hyper-activation of the signalling and drive melanoma development. Melanoma without mutations in these genes are considered triple wild type and comprise 10% of cases (Akbani et al. 2015). Consistent with literature data, we found *BRAF* mutations in 43% of cases, with V600E as the most common change, followed by the V600K variant. *NRAS* mutations were observed in 27% of melanomas, with changes at the codon Q61 more frequently identified in our cases. *NF1* mutations were detected in 11% of cases, while the triple wild type accounted for 20% of primary tumors in our group. Notably, we observed a co-occurrence of *BRAF* V600E and *NRAS* Q61R mutations in one primary lesion. Although, *BRAF* and *NRAS* mutations are considered mutually exclusives in melanoma (Reddy et al. 2017), the co-occurrence can be identified, with a recent estimated rate of 1% in primary lesions and 5% in metastases, suggesting a higher frequency than previous hypothesised (del Regno et al. 2020).

BRAF, NRAS, NF1 and triple wild type subtypes exhibited distinctive clinico-pathological and prognostic characteristics. We confirmed that *BRAF* mutations are linked to the early onset of melanoma and are found with a high frequency in ulcerated lesions, those with a high number of mitoses and a tendency to metastasize to the skin. In contrast, *NRAS* mutations resulted associated

with a late onset of melanoma and with tumors developed on CSD areas. Our results are in line with the clinical characteristics of the *BRAF* and *NRAS* molecular subgroups widely reported in the literature (Ellerhorst et al. 2011, Yaman et al. 2015, Pellegrini et al. 2020). In our patients, *NF1* mutations correlated with advanced stages and nodular histologic subtype, while the triple wild type subgroup was associated with SSM histotype and lower stage at diagnosis. In a large NGS study, *NF1* mutations in melanoma were associated with high Breslow thickness, ulceration, and fast-growing phenotype (De Unamuno Bustos et al. 2017), indicating a potential more aggressive behavior, as observed in our cases. Regarding triple wild type melanoma, no previous studies investigated the clinical characteristics associated with this subgroup of melanomas. It is only known that this subtype has been reported more frequently in elderly males (Cirenajwis et al. (2017). Our study represents the first attempt to clinically characterized the triple wild type subgroup.

There are discordant data in the literature on survival rates associated with the four molecular subtypes, with some studies identifying *BRAF* as a predictor of worse prognosis, others *NRAS*, and still others reporting no differences between subgroups (Muñoz-Couselo et al., 2017). In our exploratory analysis of a small group of patients, we observed no significant differences in 5-year melanoma survival between subtypes, although melanomas mutated in *NF1* tend to have a worse outcome, followed by lesions mutated in *BRAF*. This is in agreement with the results described by Cirenajwis et al. (2017), who reported an increased risk of melanoma death for *NF1* subtype versus *BRAF* mutated subtype in three different cohorts of 626 patients.

In addition to the four molecular subtypes, our analysis of additional melanoma genes involved in melanoma progression revealed a high frequency of mutations in *PREX2*, *TERT promoter*, *EPHA3*, and *TACC1*, occurring mainly in the triple wild type subtype. However, no clinical characteristics resulted associated with mutations in these genes. Published data reported *PREX2* mutations in 27% of TGCA cases, which have been shown to initiate the development of tumors ("The Cancer Genome Atlas Program [TCGA]). In a Spanish study of 100 melanoma samples, *PREX2* mutations were associated with high number of mitoses (De Unamuno Bustos et al. 2017). *TERT promoter* mutations increase TERT activity and have been observed in 50% of melanoma cases. Notably, mutations in the *TERT promoter* are present in greater numbers in metastases and appear less frequently in primaries, confirming their importance in tumor progression (Kerkour et al. 2023). Mutations in *TERT promoter* have been significantly associated with female sex, increased Breslow thickness, presence of ulceration, presence of mitosis, absence of regression, and fast-growing melanomas (De Unamuno Bustos et al. 2017). Activating mutations of the *EPHA3* receptor tyrosine kinase are involved in tumor growth and survival, and have been

identified in several tumors types, including melanomas. An *in vivo* assay demonstrated indeed that the inhibition of Epha3 is able to suppress melanoma cell proliferation and migration, by decreasing expression of p38-MAPK and p-ERK1/2 (Balakrishnan et al. 2007, Li Ding et al. 2014, Reddy et al. 2017, Hilke et al. 2020, Luo et al. 2022, Ming and Ma 2022). The somatic mutations found in melanoma in the *EPHA3* gene are able to eliminate the tumor-suppressive activity of the encoded protein and drugs such as Indolium appear to have excellent efficacy by delaying the development of metastases in melanoma Vemurafenib-resistant (Radi et al., 2022). Finally, although *TACC1* mutations are less common, when they are present they appear to activate Ras and PI3K pathways and their activity appears to influence that of *PPP6C*.

We identified for the first time a significant strong association of *PREX2* and *RAC1* mutations with Breslow thickness. Moreover, *RAC1* variants resulted also associated with ulceration and nodular histotype, suggesting a worse prognostic behaviour for melanomas harbouring these mutations. *RAC1* encodes for a protein critical in cytoskeleton organization and motility, making it a potential key player in metastasis formation and tumor progression (Ma et al. 2023). Recent studies have shown that *RAC1* mutations could increase the aggressiveness of melanoma and lead to resistance either to BRAF and MEK inhibitors and to immunotherapy (Cannon et al. 2020). Upregulation of RAC1 expression, in particular via the Scar/Wave complex, can promote lamellipodia formation and drive the migration of melanoma cells, facilitating metastasis (Ma et al. 2023). Therefore, targeting this gene could improve patients' life expectancy (Krauthammer et al. 2012, Barrows et al. 2015, Reddy et al. 2017). A recent in vitro and in vivo study tested that taxifolin successfully suppresses RAC1 expression through inhibition of deubiquitinase USP18, thereby blocking downstream target cascades including ERK/c-Myc, p38/IL-6, and the JNK/β-catenin/MITF pathway (Xu et al., 2024).

Discrepancies in the molecular profiles of primary tumors and paired metastases are commonly recognized (Chang et al. 2020). This molecular heterogeneity can arise from point mutations, copy number changes, deletions and duplications, whole chromosomal rearrangements, gene expression, epimutations, or reversible phenotypic changes. Most studies focused exclusively on the discordance of *BRAF* and *NRAS* mutational status. In a comprehensive meta-analysis and recent articles, intra-patient heterogeneity between primary and paired metastases was reported to vary from 4.8% to 15.5% of cases for *BRAF* and from 2.2% to 14.3% for *NRAS* (Valachis et al. 2017, Manca et al. 2019, Kerkour et al. 2023). Differences in heterogeneity rates between studies were attributed both to physiological variability between case cohorts and to the technical approaches used for analysis, i.e., molecular-based versus IHC-based methods (Grzywa et al. 2017). We found an intra-patient heterogeneity of 20.0% for *BRAF* and 15.9% for *NRAS*, and an

overall discordance of 53.3% when considering all four molecular subtypes. Notably, we found that triple wild type profile was significantly increased in metastases compared to the primary tumors consistent with the meta-analysis of Kerkour et al. (Kerkour et al. 2023). We further investigated how the primary tumor molecularly differs from metastases by considering the additional 18 genes related to melanoma progression. A significant variability between mutations present in the primary and those present in the metastases, especially in *STK19, ARID2, PPP6C, TACC1* and *GRIN2A,* was observed. Manca et al. (2019) analysed a panel of 25 genes to investigate heterogeneity in cutaneous melanoma between primary and matched metastases in 41 patients, reporting a 76% of consistency in mutational profiles between primary and metastatic melanomas. The concordance was statistically higher between primary lesions and lymph node metastasis compared to visceral metastasis (Manca et al. 2019). In addition, in the recent study by Bezrookove (2024) that analysed the primary melanoma and lymph node metastasis of 17stage IV patients, a considerable discordance between primary and related metastasis was observed, thus suggesting that distant metastasis might not arise from the lymph node. In fact, authors presented a patient with a wild type primary lesions, a *BRAF*-mutated lymph node metastasis and a *NRAS* mutated the distant metastasis (Bezrookove et al., 2024). In this context, there is an emerging perception that a single biopsy is inadequate to determine the molecular status of a tumor, and that alternative diagnostic approaches, such as liquid biopsy analysis that accounts for molecular heterogeneity, are needed (Ito et al. 2021). We observed a tendency for discordant patients to have a worse prognosis despite treatment. Therefore, in addition to contributing to a better understanding of tumor biology, the study of molecular profiles of primary melanoma and corresponding metastases could be considered for patient stratification and realistic implementation of a personalized treatment strategy (Geiger et al. 2024).

Finally, we conducted MCA analysis to investigate specific molecular profiles characterizing the primary tumors and related metastases. We identified two molecular clusters distinguished by a mutational pattern including variants in *TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1* and *DDX3X* genes in the primary tumor. Moreover, our analysis revealed that mutations in *PPP6C, GNA11, ARID2*, and *GRIN2A* tend to co-occur both in primary and metastatic tissues. Previous studies have shown that mutations in *PPP6C* and *GNA11* are involved in pathways promoting cell survival, while *ARID2* and *GRIN2A* in chromatin remodelling and cell metabolism signalling, respectively (Wei et al. 2011, Krauthammer et al. 2012,W. Guo, Hodis et al. 2012, Xia et al. 2014, J. J. Lee et al. 2015, Wang, and Li 2021). The co-occurrence of these nongenetically related determinants has not been reported in the literature, so further evidence is needed to shed light on the underlying association between these genes (Betancourt et al. 2021).

One limitation of our study is that the cohort was small and collected from a single center. The small sample size did not allow us to perform some deep analysis of data. Indeed, it was not possible to explore the 5-years MSS for specific categories, such as the triple wild type and *NF1* patients treated with immunotherapy. In addition, considering the potential variability in the evaluation of broad molecular profiles, as with our panel of 18 genes, more cases would have been useful. Our patients were diagnosed over a long period of time, so although every effort was made to ensure the accuracy of prognostic factors over time, some histopathological features may have been reported differently over the years. A centralized pathology review would have been necessary but was not feasible for this project. The DNA extraction was perfomed using macrodissection, which can be problematic when evaluating tumour heterogeneity, although the tumor-rich areas to be macrodissected were selected by an experienced pathologist. In addition, it might be interesting to evaluate in real time, during tumor progression, the results in liquid biopsy to complete the study and single cell could resolve the problem of detection <1%. Finally, detailed follow-up and treatment data were available only for a subset of 25 patients who underwent molecular profiling.

A strength of our work was that we were able to study the molecular profiles of primary and metastatic melanoma in a group of rare and valuable tissues from the same patients, representing the ideal cohort to study molecular variability during progression.

In conclusion, we characterized the molecular profiles of 18 melanoma-related genes in primary melanomas and matched metastases, identifying their clinical and prognostic significance and demonstrating a significant intra-patient heterogeneity mainly sustained by *STK19, ARID2, PPP6C, TACC1* and *GRIN2A* genes. We identified two large and distinct molecular clusters of patients distinguished by mutations in *TP53, PPP6C, ARID2, GRIN2A, GNA11, WNT1* and *DDX3X* and *GRIN2A*, suggesting potential interactions and shared pathways in melanoma development and progression. Further investigations on large number of cases will help to confirm this data and fully comprehend the significance of these molecular patterns.

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