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Modeling mucosal melanomas: understanding the immune contexture and the molecular landscape for therapeutic targeting

Caratterizzazione dei melanomi mucosi: studio del microambiente immunologico e del profilo molecolare per sviluppare terapie mirate

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INTRODUCTION

Sinonasal Mucosal Melanoma: clinical aspects

Mucosal melanoma (MM) is a highly aggressive and rare form of melanoma arising from mucosal melanocytes with a pathogenesis unrelated to sun exposure (1). Head and neck, in particular the oral cavity and the sinonasal tract (SN), represent the most common MM sites (2). MM outcome is very poor (1, 2) and the reasons behind this aggressive clinical behavior are only partially understood.

Sinonasal mucosal melanoma (SN-MM) is a rare tumor, comprising about 1% of all melanomas and about 4–8% of sinonasal malignancies (3, 4). Tumors are equally distributed between men and women and the mean age of onset lies between 60-70 years old, although it can occur in any age group (5). SN-MM is one of most aggressive cancers of the head and neck region since it represents the histotype associated with the worst oncological outcomes among paranasal sinuses neoplasms, due to its high tendency to recur and metastasize at various distant sites, regardless of the stage of the disease at presentation (6). At present, survival rates are dismal, with 5-year overall survival less than 30% (3, 4). Despite technological developments in surgery, radiation therapy and systemic modalities, no increased survival is achieved (7).

SN-MM should be considered and managed as a separate disease from cutaneous melanoma (CM), including its staging system and treatment modalities (2, 3). Since the 7th edition of the AJCC staging system published in 2009, a specific classification for upper aereodigestive tract MM has been introduced where T1 and T2 have been omitted because of the aggressive behavior of the disease, and therefore all lesions should be classified as advanced disease, at least pT3 or pT4a-pT4b (8). The rarity of the neoplasm, the delay in diagnosis, its heterogeneity in growth pattern (superficial spreading vs deep infiltration), and different sites of origin (nasal fossae, paranasal sinuses, nasal septum, nasopharynx) have hampered identification of specific prognostic factors (7).

It is widely accepted that surgical resection of the tumor represents the standard of care for treatment of patients affected by SN-MM (3, 9). There is general agreement that achievement of negative surgical margins is a key element for the definitive outcome (10). However, it is worth remembering that radical excision with negative margins may be difficult to achieve due to the specific pattern of growth of the disease, featuring superficial spreading along the mucosal and submucosal planes and possibility of multiple satellite lesions around the primary along the whole sinonasal tract (3, 11). Moreover, the involvement of specific structures and areas (e.g. cavernous sinus, orbital apex, brain parenchyma, masticatory space, parapharyngeal spaces) may imply to leave residual disease unless to cause unacceptable morbidity to the patient (7). Endoscopic endonasal resection, whenever technically feasible, has outcomes similar to and possibly better than those seen with more mutilating and aggressive surgical approaches (3, 11). Some speculations may be offered to help explain outcomes in patients treated with a purely endoscopic resection: one of the most intriguing was advanced by Lund et al (12) who hypothesized that aggressive surgery might cause severe disturbances in the immune balance and, consequently, may promote dramatic recurrence and/or explain cases with rapid systemic dissemination.

Adjuvant protocols including different regimens of radiotherapy (RT) have shown promising loco-regional control benefits, but fail to provide a significant improvement in term of overall survival, since the incidence of systemic disease remains remarkably high (4, 7). Therefore, at present, adjuvant RT should be reserved for patients at increased risk for loco-regional recurrence, such as those with positive surgical margins, in case of involvement of critical structures (e.g. dura), and nodal metastasis (11). Emerging evidence supports the role of innovative irradiations modalities such as particle therapy, using protons and carbon ions, in order to provide a superior target dose and less collateral damage than intensity-modulated RT (IMRT) (13). Particle beam radiation therapy has been recently proposed also as definitive treatment for selected cases of sinonasal malignancies with excellent local-regional control and low serve toxicities (14). However, the role of RT as a primary treatment modality for SN-MM remains unclear. Further studies are required in order to fully

elucidate the potentials of particle therapies and its long-term toxicities in such rare and aggressive subset of cancer.

Chemotherapy, immunotherapy, or biochemotherapy (defined as systemic administration of a chemotherapeutic agent and at least 1 biologic agent, such as interferon-a or interleukin-2) have been used prevalently for the treatment of unresectable or metastatic lesions (7). It is worth mentioning, however, that the role of systemic therapy seems to gain relevance in recent years. Gore and Zanation (15) in a meta-analysis on sinonasal malignant melanoma, found that an increase in survival may be obtained by combining surgical resection of the primary lesion with systemic therapy, whereas Sun et al. (16) identified administration of biological agents as an independent positive prognostic factor. These articles reinforce the concept that only the introduction of agents able to prevent systemic spreading of the disease can significantly improve prognosis. At present, unfortunately, there is no consensus on the drugs or combination of drugs to be recommended for MM and systemic therapy is typically reserved for patients with unresectable or metastatic disease (17, 18).

This is in contrast to the significant advances obtained in CM, where a better understanding of the immune contexture (IC) and genomic landscape has significantly increased the array of available therapies (19). A recent molecular classification of CM identified four CM subgroups based on mutations targeting BRAF, NRAS and NF1, the fourth group being represented by triple negative CMs (20). CMs are also heterogeneous in term of tumor infiltrating immune cells, with variability associated to molecular types and disease stages (21). It is also clearly emerged that the IC in CMs not only can predict prognosis, but is highly relevant in various therapeutic settings (22). Target therapies (TT) with BRAF and MEK inhibitors (for BRAF-mutated CMs) and immunotherapies (IT) targeting immune check points (anti–CTLA-4 and anti–PD-1) have significantly changed the prognosis of metastatic CMs, with an improvement in the clinical responses and survival (23). Among tissues predictors of response to immune check points blockade, IFNγ-producing pre-existing T cells has been proposed (24). Of note, also the clinical response to BRAF inhibitors is partially mediated by CD8+ T cells, suggesting an immune cell contribution to TT (25). This remarkable amount of clinical success is, however, limited to CMs, with only marginal effect obtained on melanomas arising at the mucosal surfaces. Therefore, basic

research should be directed to better understand the IC and genomic landscape of SN-MMs leading to appropriate personalized therapies. The integration of such data will enable clinicians to propose new therapeutic targets and immune-based interventions for SN-MM founded on appropriate biomarkers for patient stratification.

Cancer immunoediting and melanoma: current evidences

Understanding how the immune system affects cancer development and progression has been one of the most challenging questions in oncology. The immune system plays a dual role in cancer: it can not only suppress tumor growth by destroying cancer cells or inhibiting their outgrowth but also promote tumor progression either by selecting for tumor cells that are more fit to survive in an immunocompetent host or by establishing conditions within the tumor microenvironment that facilitate tumor outgrowth. This phenomenon can be described by a unifying conceptual framework called "cancer immunoediting," which integrates the immune system's dual host-protective and tumor-promoting roles (26).

Cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immunity work together to destroy developing tumors long before they become clinically apparent (27). Many of the immune molecules and cells that participate in the elimination phase have been identified, but more work is needed to determine their exact sequence of action. If this phase goes to completion, then the host remains free of cancer, and elimination thus represents the full extent of the process. If, however, a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its outgrowth is prevented by immunologic mechanisms. T cells, IL-12, and IFN-g are required to maintain tumor cells in a state of functional dormancy, whereas NK cells and molecules that participate in the recognition or effector function of cells of innate immunity are not required; this indicates that equilibrium is a function of adaptive immunity only. Editing of tumor immunogenicity occurs in the equilibrium phase. Equilibrium may also represent an end stage of the cancer immunoediting process and may restrain outgrowth of occult cancers for the lifetime of the host. However, as a consequence of constant immune selection pressure placed on

genetically unstable tumor cells held in equilibrium, tumor cell variants may emerge that (i) are no longer recognized by adaptive immunity (antigen loss variants or tumors cells that develop defects in antigen processing or presentation), (ii) become insensitive to immune effector mechanisms, or (iii) induce an immunosuppressive state within the tumor microenvironment. These tumor cells may then enter the escape phase, in which their outgrowth is no longer blocked by immunity (28). These tumor cells emerge to cause clinically apparent disease. With this newfound knowledge of the immune system's capacity to not only recognize and destroy cancer but also to shape cancer immunogenicity, multiple forms of innovative therapies based on the specific IC analysis of each tumor are being explored.

The comprehension of the immunobiology of CM has radically changed treatment paradigms leading to an unprecedented improvement in patient survival. This can be attributed to the fact that CM is one of the most sensitive tumors to immune modulation (29). Several factors may explain melanoma cell susceptibility to immune system activation including high tumor mutational load due to ultraviolet light exposure, expression of cancer testis antigens and mimicry of melanocyte lineage proteins with pathogen-associated antigens (30). In this context T-cell response seems to play a central role to keep the melanoma at bay. Tumor infiltrated lymphocytes (TILs) are central to the development of an anti-tumor immune response and a subset of TILs demonstrate cytolytic activity against autologous tumors in melanoma patients (31). Their presence also correlates with increased survival and reduced risk of metastasis (31). In the past decades, several clinical trials aimed at eliciting T-cell response with local or systemic immunomodulatory drugs such as interferon (IFN)-α (32), interleukin IL-2 (33), cancer vaccines (34) and adoptive cell transfer (35). More recently, immune checkpoint inhibitors (ICIs) against cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) have dramatically changed the management of both unresectable and metastatic melanoma as well as those at high risk for recurrence after resection (36). In 2011, ipilimumab, a fully human monoclonal antibody (mAb) IgG1 that inhibits the interaction between CTLA-4 and its ligands, was the first ICI approved by the FDA (37). In a randomized phase III trial, the use of ipilimumab, showed a significant increase in median survival (10.1 months), compared to the administration of the glycoprotein 100 (gp100) peptide vaccine (6.4 months)

(37). In another phase III study, 502 patients with untreated metastatic melanoma were treated with a 1:1 combination of ipilimumab and dacarbazine, where outcomes were compared to dacarbazine monotherapy. The combination treatment showed a statistically significant increase in overall survival of 11.2 months, compared to 9.1 months obtained from dacarbazine monotherapy (38). Hence, multiple prospective and retrospective studies support the efficacy and safety of ipilimumab in the treatment of CM (39). The checkpoint inhibitor nivolumab operates by inhibiting interactions of ligands PD-L1 and PD-L2 with its receptor, programmed death-1 receptor (PD-1), thereby blocking T-cell activation. In a phase III trial of patients with ipilimumab-refractory metastatic melanoma, nivolumab showed a higher overall survival rate (72.9%) than dacarbazine (42.1%) (40). Furthermore, in a randomized phase III trial (CheckMate 037), patients who progressed after ipilimumab monotherapy or the combination of ipilimumab and BRAF inhibitor, reported a higher response to nivolumab compared to standard chemotherapy (41). In addition to nivolumab, other PD-1 checkpoint inhibitors, such as pembrolizumab, have shown more improvement in progression-free survival, toxicity, and overall survival than ipilimumab in the treatment of metastatic CM (42).

In order to increase the response rate to immunotherapy, especially for those patients who experienced primary or secondary resistance to single agent, a combination of anti-CTLA-4 mAb plus anti-PD-1 mAb have been evaluated in prospective clinical trials. The Checkmate-067, a phase 3 randomized clinical trial compared ipilimumab plus nivolumab to nivolumab alone and ipilimumab alone in unresectable/metastatic melanoma (43). Response rates were 57.6, 43.7 and 19%, respectively, and 5-year survival rates were 52% in the combination arm, 44% in the nivolumab group and 26% in the ipilimumab arm (43). However, the combination arm showed increased toxicity compared to each monotherapy treatment arm. In an attempt of reducing the toxicity burden of the combination, different dosing schedule by reducing ipilimumab dose and keep more standard dose anti-PD-1 single agents are currently under investigation (44). Brain metastases are a common cause of disabling neurologic complications and poor prognosis in patients with metastatic CM. The phase 2 clinical trial CheckMate-204 enrolled patients with small, untreated and

asymptomatic brain metastasis and showed that ipilimumab plus nivolumab have clinically meaningful intracranial efficacy (56% of intracranial response) (45).

Despite such advances obtained for CM, data about immunotherapy in MM are scarce and inconclusive (46). Data emerging from KEYNOTE 001, 002 and 006 clinical trials revealed that the objective response rate (ORR) in patients with MM treated with pembrolizumab was lower than what observed for CM (19% versus 33%) (47). In responders, the median durability of response was similar between mucosal and cutaneous primaries with 75% and 72% having an ongoing response, respectively. However, the median overall survival was significantly shorter for patients with mucosal melanoma (11.3 months) as compared to patients with a cutaneous primary (23.5 months) (47). D'Angelo et al. described a series of patients affected by MM treated with nivolumab alone, or in combination with ipilimumab (36): of the 35 patients with a mucosal primary who received combination therapy with ipilimumab and nivolumab, the ORR was 37%, as compared to 60% in patients with a cutaneous primary (36). To note, the response to immunotherapy was evaluated also by PD-L1 status, reporting similar ORR rates between MM and CM (53.3% versus 55%) when considering only the subset of PD-L1 positive patients (36). While PD-L1 is not typically used as a biomarker for treatment selection in CM, this difference in response rates may warrant further investigation in MM. The MM responses rates shown in this subset analysis are comparable to non-melanoma cancer response rates to immunotherapy such as bladder cancer (21%) (48) and lung cancer (18%) (49). While a "biomarker" predicting response to immunotherapy has yet to be found, tumor mutational burden is frequently cited as a predictor of response to immunotherapy. As compared to MM, CM has a high average tumor mutational burden of 13 mutations/ megabase (50). In contrast, MM has a tumor mutational burden of 2 mutations/megabase, similar to that of a less immunologically active cancer such as breast cancer (51).

Genetic and epigenetic alterations in melanoma

In contrast with the well-known molecular fingerprints of CM, the genetic and epigenetic profile of MM is poorly understood so far, probably due to the rarity of the

disease and the lack of recognized risk factor associated with its cancerogenesis. Studies using exome or whole-genome sequencing in small cohort of cases have shown that MMs are similar to acral melanomas (AM), having a lower tumor mutation burden and more frequent structural chromosomal variants than the cutaneous counterpart (52). Most frequent mutations in CM occur in BRAF (45%-66% of cases, classically the V600E mutation), NRAS (15%-20% of cases), NF1 (7%-14% of cases), MEK1/2 (2%-6% of cases), KIT (1%-2% of cases), and CDKN2A (0%-3% of cases) (53). Given their potential as a target for specific treatment, the Cancer Genome Atlas Network (TCGA) has proposed a classification of CM based on the mutational pattern in either BRAF, RAS or NF1, or none of these, the so-called triple-wild-type group (20). The presence of such mutations has been demonstrated to activate the Mitogen-Activated Protein Kinase (MAPK) pathway by extracellular binding of Receptor Tyrosine Kinases (RTK), leading to activation of the rat sarcoma (RAS) family protein, which subsequently activates intracellular serine-threonine protein kinases of the rapidly accelerated fibrosarcoma (RAF) family (ARAF, BRAF, CRAF). Activation of RAF leads to the phosphorylation of MAPK extracellular receptor kinase (MEK), which in turn phosphorylates extracellular signal-regulated kinase (ERK). ERK activation promotes cellular proliferation and activates mitochondrial proteins, which promote growth and inhibit apoptosis (54).

The phosphatidylinositol 3-kinase pathway (PI3K) pathway has been found to be activated in 30%-60% of CM through functional loss of the tumor suppression protein PTEN, which is associated with BRAF V600E mutations (55). Also implicated in this line is the activation or amplification of serine/threonine protein kinase AKT3 in 40% to 60% of CM (55). This pathway follows the RTK-RAS-PI3K-(PTEN)-AKT3 signal cascade to the mitochondrial antiapoptotic protein BCL2 and cellular growth regulator mTOR (mammalian target of rapamycin). Upstream of RAS, amplifications or activating mutations in the gene encoding the RTK for stem cell factor, KIT, can also activate this pathway (54). This pathway has also been implicated in the development of melanoma brain metastases (56). The introduction of target therapy specifically addressing such pathways has completely revolutionized the range of treatments available for CM, especially in presence of unresectable and metastatic disease.

BRAF inhibitors (e.g. Vemurafenib, Dabrafenib, Encorafenib) selectively target the mutated BRAF kinase, thus decreasing signal transduction through the MAPK pathway (54). The BRIM-3 trial con- ducted in 675 patients with previously untreated advanced or unresectable BRAF V600 mutant melanoma demonstrated superior response with vemurafenib versus dacarbazine for the treatment of BRAF-mutant melanoma, with an overall response rate (ORR) of 48% versus 5% with dacarbazine, so that 84 of 338 patients on dacarbazine crossed over to vemurafenib during the conduction of this study (57). The addition of a MEK inhibitor, a downstream component of the MAPK pathway, delays the onset of resistance to BRAF inhibitors, which has been observed after a median time of 5 to 7 months of treatment (58). Combination BRAF/MEK inhibition has also demonstrated improved treatment response, PFS, and OS compared with BRAF-inhibitor monotherapy (58). The current standard of care is thus to use combination targeted therapy for eligible patients whose tumor harbors a mutation in BRAF V600 (59). The three approved combination therapies are dabrafenib/trametinib, vemurafenib/cobimetinib, and encorafenib/binimetinib on the basis of several large phase 3 trials affirming superiority of the combination (59).

Similarly, NRAS mutated cases can be treated using target therapy but with less successful results. Given the inherent difficulty in targeting NRAS directly and recognition of the downstream effects of RAS activation, MEK inhibition has been explored as an option to inhibit the MAPK pathway in NRAS mutant melanoma. Binimetinib, an inhibitor of MEK 1/2, demonstrated a modest 20% response rate in NRAS mutant melanoma, all partial responses (60). Therefore, it may represent an option for patients with progressive disease after front-line immunotherapy, especially in the absence of other targetable mutations or availability of clinical trial (61).

KIT mutations are less frequently observed in melanoma and, when present, can be targeted using specific RTK-inhibitors such as Imatinib mesylate, currently used for the treatment of gastrointestinal stromal tumors (GIST) and chronic myelogenous leukemia (CML). Hodi and colleagues performed a third phase 2 trial of imatinib with 24 evaluable target-enriched melanoma patients (62). The trial cohort included 8 patients with KIT mutations, 11 with KIT amplifications, and 5 with both. They reported a best ORR of 29%; responses were only seen in patients harboring KIT

mutation and not in those with KIT amplifications. Median time to progression was 3.7 months, and overall disease control rate (DCR) was 50% (62). Nilotinib is another selective bcr-abl tyrosine kinase inhibitor similar in structure to imatinib. In a phase 2 trial of nilotinib in 42 melanoma patients with KIT mutations, amplifications, or both, a response rate of 16.7% and DCR of 57.1% was reported (63). The median duration of response was 34 weeks. Most responses were again seen in patients with KIT mutations (6/7 patients) rather than KIT amplification (1/7 patient) (63). These trials suggest that durable responses may be seen in KIT mutant melanoma.

Conversely, the mutation profile of MM is far to be completely elucidated and involves genetic alterations not related to UV radiation or any other known carcinogen (52). At present, only a small proportion of MM patients harbored a recognized mutation profile while the large majority of cases is wild type, or, much more probably, driver mutations and genetic abnormalities involved in their pathogenesis are still unknown. Reports have indicated mutations in MM at an incidence of 5% in BRAF (range, 0-12%), 20% in NRAS (range, 5-41%), 15% in NF1 (range, 4-37%), 10% in KIT (range, 3-17%), while SF3B1, CTNNB1 and SPRED mutations have been described only in a few cases (52). The sequencing results of 67 MMs show that mutations of NRAS, BRAF, KIT, and SF3B1 are mutually exclusive, implying those mutations may converge on activating the MAPK pathways (64).

The differences in the mutation frequencies of BRAF are clinically important, as the majority of MM lack the target for the p.V600 based inhibitors that have revolutionized CM treatment. While these inhibitors have yet to be systematically investigated in a large cohort of MM, a small study (19 cases) on combined AM and MM showed an overall median progression-free survival (PFS) of 7.3 months (95% CI, 3.0–11.6), which was significantly lower than what observed for CM. (65) These data argue against clinically efficacy of targeted BRAF inhibitors for treatment of MM and strongly support the need for alternative treatments strategies.

Studies focusing on target therapies for NRAS-mutant cases of MM are sparse. MEK inhibitors have been the most developed targeted therapy approach. Although associated with benefits in terms of progression-free survival, MEK inhibitors do not provide any advantage in terms of overall survival (19). Combination strategies with PI3K-AKT-mTOR pathway and CDK4/6 inhibitors seem to increase MEK inhibitors'

benefit (19, 52). Nevertheless, results from clinical trials are still preliminary. A greater comprehension of the biology and intracellular interactions of NRAS-mutant MM is required in order to discover novel impactful strategies which could improve prognosis of these subgroup of patients. In details, the impact of an activating NRAS mutation on the immune composition of the melanoma tumor microenvironment is not well described and might be useful in future as a predictive marker in order to select patients who can benefit from diverse forms of immunotherapies such as a combination ICI treatment (anti-PD1±anti-CTLA4) versus anti-PD1 alone for patients with metastatic disease (66).

There are several ongoing studies of novel KIT inhibitors in KIT-mutant AM and MM, for which results are being awaited. The Pexidartinib (PLX3397) is tested in the PIANO trial (ClinicalTrials.gov Identifier, NCT02071940) in United Kingdom and in another phase 2 study in Asia (ClinicalTrials.gov Identifier, NCT02975700). Another novel agent under investigation is Regorafenib, which is already approved for the treatment of metastatic colorectal and hepatocellular carcinoma. Regorafenib is a multikinase inhibitor of VEGFR1-3, c-KIT, TIE-2, PDGFR-β, FGFR-1, RET, RAF-1, BRAF and p38 MAP kinase, and the investigators propose that this will provide better efficacy in KIT-mutated melanomas, including MM (NCT02501551).

It is worth mentioning that MM has a much higher level of structure variation and chromosomal instability compared to CM (2). As a result, specific attention should be paid to targeting the chromosomal rearrangements. Considerable variation also exists in epigenetic alterations within the subtypes of melanoma, such as cutaneous, mucosal, and uveal (19). Cytogenetic abnormalities have been largely described in uveal melanoma (UM) and used to stratify patients according to prognosis (69). The Cancer Genome Atlas (TCGA) uveal melanoma analysis has revealed four molecularly distinct UM subtypes. Two subclasses are associated with monosomy 3 and a poor prognosis, while the other two are associated with disomy 3 and a better prognosis (70). In addition to monosomy 3, amplification of 8q is associated with inferior prognosis. Frequently, these alterations will be present concurrently, and the presence of both corresponds with an even worse outcome (71). Other cytogenetic alterations associated with poor outcome include the loss of 8p, 6q, and 1p (72). The analysis of copy number alterations in MM are largely fragmentary and based on small

cohort of patients. In tumor samples obtained from SN-MM patients, 100%, 93%, and 57% gains have been reported in chromosome arms 1q, 6p, and 8q, respectively (73). Furthermore, ploidy analysis showed significant clear and high copy gains in 75% of triploid and tetraploid tumors (73, 74). Another aspect of SNMM is the occurrence of telomerase reverse transcriptase promoter mutations, which has been observed in 8% of SNMM patients (75). This results in higher transcriptional activity and an increased number of driver mutations (75). The current clinical significance of these alterations is unknown. However, they can facilitate the characterization of tumors and may eventually serve as therapeutic targets.

OBJECTIVES OF THE STUDY

1. To describe clinical presentation, treatment strategies, pattern of recurrences and survival outcomes from a retrospective series of consecutive patients affected by MM, treated uniformly in two tertiary-care Italian referral centers for skull base tumors, over a period of 18 years.
2. To define the immune contexture of MM by analyzing tumor-infiltrating immune cells (TIICs). The final aim is to identify the immunological interactions and the biological processes involved in the tumor progression and systemic metastasis development in such aggressive cancers, in order to explore the potentials of new cancer immunotherapies able to modify the immunological tumor microenvironment against tumor progression.
3. To obtain MM cell lines, displaying tumorigenic potential and containing discernable cancer stem cells (CSC), in order to define their phenotype, molecular landscape and transcriptional program and identify their vulnerable check points. This may represent a basis for further in vitro and in vivo experiments.
4. To investigate the molecular landscape of MM by exploring somatic genetic alterations, with particular attention to druggable genetic mutations or other genetic alterations useful to set up target therapies. In this regards, a secondary endpoint is to investigate the prognostic role of the identified genetic mutations in order to predict patient prognosis in terms of Overall Survival (OS) and the potential for early dissemination of disease assessed in terms of Disease-Free Survival (DFS).
5. To analyze cytogenetic abnormalities in MM, with particular focus on monosomy 3, amplification of 8q, and loss of 8p, 6q, and 1p, in order to describe their patterns in MM and investigate their potential role as prognosticator in such rare and aggressive cancer. In this setting, a secondary endpoint is to define copy number alteration (CNA) signatures that can be used to evaluate the risk of metastasization.

MATERIALS AND METHODS

Study design

A retrospective review of patients treated for sinonasal tract mucosal melanoma in two Italian tertiary care referral centres (University of Insubria, Varese, and University of Brescia) from 2000 to 2017 was performed. Retrieval of clinical information, therapies administered and survival outcomes data was performed from Institutional databases. Retrieval of paraffin-embedded tissue blocks and slides for pathological analysis was performed from the archives of the two Institutions involved. Only patients receiving surgical-based treatment with curative intent were included in the study. Exclusion criteria were as follows: (a) missing relevant materials and data (e.g. preoperative imaging, follow-up data, tissue blocks); (b) unresectable disease; (c) regional or systemic dissemination of disease at presentation; (d) less than 12 months of follow-up for surviving patients. Informed consent was obtained from all participants included in the study. The study was approved by the Institutional Review Board (Insubria Board of Ethics, approval number 0033025/2015). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Clinical management

Local extension of disease was assessed by means of multiplanar computed tomography (CT) scan and contrast-enhanced magnetic resonance imaging (MRI) in all cases. All patients underwent preoperative nasal endoscopy with multiple biopsies to define the tumor histology before treatment. Neck ultrasound and total body contrast-enhanced CT scan or positron emission tomography (PET) scan were obtained to rule out regional or systemic spread, respectively. Surgical resection was tailored on preoperative radiological findings with the goal to obtain a radical resection. The surgical procedures performed may include an exclusive endoscopic endonasal resection (EER); endoscopic endonasal resection with transnasal craniectomy (ERTC); combined cranio-endoscopic approach (CER); craniofacial

resection (CFR). Surgical procedures were performed according to a previously described technique (6). Final histology report was used to define the surgical margin status (infiltrated versus uninvolved). All cases were re-classified according to the 8th edition of the American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) classification for mucosal melanoma of the head and neck (76). In adjuvant setting, post-operative radiotherapy (PORT) was delivered in case of locally-advanced stage of disease (stage pT4a and pT4b) and in case of microscopically-involved surgical margins. The irradiation modality included 3D-conformal radiotherapy (3D-CRT) and intensity-modulated radiotherapy (IMRT) for patients treated before the 2012 and carbon ion radiotherapy (CIR) for patients treated since that date. Concurrent cisplatin-based chemoradiotherapy (CRT) was performed only in selected cases of macroscopic persistence of disease in sites not further amenable for surgery and in case of early systemic dissemination of disease during the post-operative period or during the adjuvant irradiation delivery. Recently, in selected cases who developed systemic metastasis during the follow-up period, immunotherapy using monoclonal antibodies anti-PD-1 (e.g. nivolumab, pembrolizumab) and/or anti-CTLA4 (e.g. ipilimumab) was delivered. All patients were followed according to a protocol that included monthly endoscopic examinations and MRI every 4 months during the first year; endoscopic examination and MRI every 2 and 6 months, respectively, during the second year; and both examinations at 6-month intervals thereafter, as previously described (3, 6). A total body PET-CT scan was prescribed once per year in order to rule out systemic dissemination of disease.

Immunohistochemistry analysis

Immunohistochemical evaluation of the immunological tumor microenvironment was performed by testing different antibodies, such as CD45, CD8, CD3, CD163, CD20, CD66b, BDCA2, CD56, CD274/PD-L1, HLA-DP, DQ, DR. The PD-L1, β-catenin, and PTEN immunostaining were also performed. For immunohistochemistry, a representative formalin-fixed paraffin-embedded tissue section of each case was immunolabeled using the standard avidin-biotin peroxidase method with specific antibodies, which have been summarized in **Table 1**.

Antibody		Clone	Diluition	Industry
CD 163	Mouse	10D6	1:50	Thermo Scientific
BDCA-2	Mouse	124B3.13	1:75	Dendritics
CD3	Rabbit	SP7	1:100	Thermo Scientific
CD20	Mouse	L26	1:250	Novocastra
CD56	Mouse	123C3.D5	1:30	Thermo Scientific
CD45	Mouse	X16/99	1:200	Leica
CD66b	Mouse	G10F5	1:180	Biolegend
CD274/PD-L1	Mouse	22C3	1:30	Dako
CD274/PD-L1	Rabbit	E1L3N	1:300	Cell Signaling
HLA-DP, DQ, DR	Mouse	CR3/43	1:500	Dako
β-catenina	Mouse	14	n.d.	Cell Marque
PTEN	Rabbit	138G6	1:100	Cell Signaling

Table 1. Antibodies used for immunohistochemistry

The histopathological slides have been analyzed using the software Aperio ImageScope (Leica Microsystems) and evaluated in a semi-quantitative way, producing a score system based on the total amount of CD45+ cells. The IHC Nuclear Image Analysis (Leica Microsystems) algorithm was used for the automatized cells count. The clinical and prognostic role of the different tumor-infiltrating immune cells patterns identified in sinonasal mucosal melanoma have been analyzed from statistical viewpoint.

The CD274/PD-L1 expression was evaluated in a semi-quantitative way, according to the Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining (≥ 1+) relative to all viable tumor cells present in the sample (positive and negative), according to this formula:

$$TPS = \frac{\#\text{ PD-L1 positive tumor cells}}{\text{Total \# of PD-L1 positive + PD-L1 negative tumor cells}}$$

The PTEN expression was assessed according to the percentage of positive tumor cells, according to the following score system: score 0, absence of positive cells; score 1, 1% to 25% of positive cells; score 2, 25% to 50% of positive cells; score 3, 50% to 75% of positive cells; and score 4, >75% of positive cells. Digital images taken using the Olympus BX60 microscope were captured using a DP-70 Olympus digital camera and processed using Analysis Image Processing software (Olympus).

Cell lines generation and identification of cancer stem cells

Samples were collected from fresh tumors in operative room under a sterile setting. Tumor tissue was finely minced and, after extensively washing with culture medium, transferred into a 25-cm^2 culture flask at 37°C, with 8% CO_2. Cells were cultured in culture medium devoid of gentamicin, vancomycin, and fungizone. Controlled trypsinizations were done to preferentially remove the contaminating fibroblasts. The cells were routinely passed once a week, and the medium was changed twice in between. Stable cell lines were generated by the use of positive selection markers and selected through limiting dilution/colony-picking to provide a genetically homogenous and clonal population. Gene stability was verified for at least ten passages. Cells were assessed by fluorescence and phase microscopy and flow cytometry.

The identification of cancer steam cells of MM (CSCMM) was based on the evaluation of cell surface antigens by flow cytometry. CSCMM have been detected in culture in form of melanosphere. Confirmation of a CSCMM functional and molecular profile have been performed using classical CSC in vitro assays by generating melanospheres in non-adherent conditions. The clinical significance of the occurrence and density of CSC in MMs biopsies have been tested by immunohistochemistry using biomarkers identified on cell lines. We detected transcripts for many stem cell markers (CD44, NGFR, ABCB5, CD133, SNAI, TWIST, OCT3/4, NANOG, KIT) in RNA extracted from our SN-MM lines, suggesting the occurrence of a fraction of CSCMM.

As alternative approach to melanosphere assay for testing the stem cell features, we used the mouse transplantation system, using NOD-SCID mice (non-obese diabetic/severe combined immunodeficiency mice). A population of CSC-like cells was identified in MM xenografts (CSCMu) generated by injecting the human SN-

MM1 and SN-MM2 in NOD/SCID mice. In vitro and in vivo molecular targeting of CSCMu were performed as pre-clinical proof of concept.

DNA extraction

Tumor DNA was extracted from three representative 8µm-thick sections obtained from FFPE samples available for the molecular analyses and neoplastic areas were manually microdissected in order to have at least 50% of tumor cells. DNA was extracted using Maxwell® DNA FFPE Kit and Maxwell 16 system (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Each sample was quantified using Qubit dsDNA High Sensitivity Assay kit (Invitrogen, Thermo Fisher Scientific Inc, USA).

Gene mutation analysis with Sequenom MassArray system

Mucosal melanomas were analyzed for mutations in KRAS (NM_004985.4/LRG_344), NRAS (NM_002524.4/LRG_92), BRAF (NM_00433.4/LRG_299), and PIK3KA (NM_006218.2/LRG_310) genes using the Sequenom MassArray system (Diatech Pharmacogenetics, Jesi, Italy), based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This analysis was performed using the Myriapod Colon Status Kit (Diatech Pharmacogenetics) that includes a series of PCR assays designed to interrogate a total of 153 non-synonymous hotspot mutations in the four genes. This method is based on primer extension and offers two levels of specificity. First, a locus-specific PCR reaction, followed by a locus-specific primer extension reaction (iPLEX) in which an oligonucleotide primer anneals immediately upstream of the polymorphic site being genotyped. Through the use of MALDI-TOF MS, the mass of the extended primer is determined. The primer's mass indicates the sequence and, therefore, the alleles present at the polymorphic site of interest. Sequenom supplies software (SpectroTYPER) that automatically translates the mass of the observed primers into a genotype for each reaction. Briefly, sequenom analysis was done performing 5 µl PCR reaction mixture containing from 10 to 20 ng of DNA (**Table 2**). After PCR, terminal nucleotides were dephosphorylated by Shrimp Alkaline Phosphatase (SAP) reaction (**Table 3**) and this step was followed by the iPLEX single base extension reaction

(**Table 4**). The extension products (analytes) were desalted using clean resin and spotted in nanoliter volumes onto a matrix-arrayed silicon SpectroCHIP with 96 elements using the MassARRAY Nanodispencer (Diatech Pharmacogenetics). The chip is placed into the mass spectrometer and each spot is then shot with a laser under vacuum by the MALDI-TOF method. A laser beam serves as desorption and ionization source in MALDI mass spectrometry. Once the sample molecules are vaporized and ionized, they are transferred electrostatically into a time-of-flight mass spectrometer (TOF-MS), where they are separated from the matrix ions, individually detected based on their mass-to-charge (m/z) ratios, and analyzed. Detection of an ion at the end of the tube is based on its flight time, which is proportional to the square root of its m/z.

Reaction mix		95°C	120"	
Water	1,3 µl	95°C	30"	
10x PCR Buffer	0,5 µl	56°C	30"	X 45
MgCl2	0,4 µl	72°C	60"	
dNTP Mix	0,1 µl	72°C	300"	
Primer Mix	0,5 µl	4°C	300"	
PCR Enzyme	0,2 µl	10°C	Hold	
DNA	2 µl			
Total volume	5 µl			

Table 2. PCR reaction mix and thermic profile.

Reaction mix		37°C	2400"
Water	1,53 µl	85°C	300"
SAP buffer	0,17 µl	4°C	300"
SAP enzyme	0,3 µl	10°C	Hold
Total volume	2 µl		

Table 3. SAP reaction mix and thermic profile.

Reaction mix	
Water	0,56 µl
Buffer Plus	0,20 µl
Termination mix	0,20 µl
Thermosequenase	0,04 µl
Primer Mix	1 µl
Total volume	2 µl

94°C	30"	
94°C	5"	
52°C	5"	X 40
80°C	5"	
52°C	5"	
		X 5
80°C	5"	
72°C	180"	
4°C	300"	
10°C	Hold	

Table 4. IPLEX reaction mix and thermic profile.

Targeted next generation sequencing (NGS) analysis

A gene-targeted NGS analysis was performed on a subset of cases, for which a good quality DNA was available, using the Human Actionable Solid Tumor Mutations QIAseq DNA Panel (DHS-101Z, Qiagen, Hilden, Germany) that analyses 22 oncogenes (BRAF, PDGFRA, EGFR, KRAS, NRAS, KIT, AKT1, ALK, CTNNB1, ERBB3, ESR1, FOXL2, GNA11, GNAQ, IDH1, IDH2, MET, RAF1, RET, ERBB2, PIK3CA, TP53). A targeted amplicon-based library was constructed as described in a previous work of our group (77) according to the manufacturer protocol. Barcoded libraries were pooled together at 8pM and sequenced on an Ion S5 XL System (A27214, Thermo Fisher Scientific) using Ion 530 chip (Thermo Fisher Scientific). Unmapped BAM (uBAM) files were imported into the CLC Genomics Workbench (Qiagen Bioinformatics, Germany, version 12) and mapped on the Human hg19 genome. Sequencing data were analyzed using the Biomedical Genomics Analysis plugin and filtered ensuring a coverage of at least 100X and a variant allele frequency (VAF) higher than 5%.

MLPA Assay of chromosome abnormalities

Deletions/duplications analysis of regions in chromosomes 1p, 3, 6 and 8 was performed using SALSA MLPA (Multiplex Ligation-dependent Probe Amplification) probemix P027 Uveal Melanoma (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's protocol (www.mrc-holland.com). The probes used are detailed in **Table 5**.

Length (nt)	SALSA MLPA probe	Gene (exon[a])	Location (hg18)	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
Loss of 1p arm					
196	04888-L04272	*MFN2*	1p36.22	CTGGTGGACGAT-TACCAGATGGAC	7.9 **M**b
275 «	04148-L31286	*NBL1*	1p36.13	AGCTGCACAATT-TAATATATTCAA	8.5 **M**b
355	02267-L01425	*PTAFR*	1p35.3	CATCTTCATCGT-GTTCAGCTTCTT	6.7 **M**b
220	13671-L17892	*GJB3*	1p34.3	CCATGGGAGTGT-GTCAGGTGGAAG	10.6 **M**b
418	03964-L03351	*MUTYH*	1p34.1	CTCATACCATCT-ATTCAGAGACGT	23.1 **M**b
240	21549-L31285	*RPE65*	1p31.3	ATGCCCTTGTTA-ATGTCTACCCAG	51.6 **M**b
136	02867-L02334	*NOTCH2*	1p12	AAGCTGCAGACA-TCCGTAGGACAC	-
Loss of chromosome 3					
207	14147-L17987	*CHL1*	3p26.3	CCTAGGTGCTGT-AAACTGCAAACC	9.9 **M**b
283	15895-L18089	*BRK1*	3p25.3	AAAAGGTGAGAC-ACTCACCTAGAA	20.3 kb
391	13322-L14735	*VHL*, ex 2	3p25.3	CGTCAACATTGA-GAGATGGCACAA	3.3 kb
445	15899-L18091	*VHL*, ex 3	3p25.3	CCAAATGTGCAG-AAAGACCTGGAG	2.2 **M**b
260	06900-L06480	*PPARG*	3p25.2	ATACAACAAGGC-CATTTTCTCAAA	1.8 **M**b
474	06118-L05573	*XPC*	3p25.1	AGCAAGAGTGGT-GAGGCTTGGAGA	21.6 **M**b
190	15896-L17989	*MIR128-2*	3p22.3	GAGAGTGAGTAG-CAGGTCTCACAG	1.2 **M**b
141 Δ	15288-L20037	*MLH1*	3p22.2	TCTAACGCGCAA-GCGCATATCCTT	4.2 **M**b
454	16407-L18832	*CTNNB1*	3p22.1	GCTGACTATCCA-GTTGATGGGCTG	8.8 **M**b
364	15897-L18094	*RBM5*	3p21.31	ATATGATGACTA-CCGAGACTATGA	2.3 **M**b
483	16644-L19176	*BAP1*, ex 9	3p21.1	AACCTGATGGCA-GTGGTGCCCGAC	2.2 kb
226	16643-L20039	*BAP1*, ex 4	3p21.1	ATACGTCCGTGA-TTGATGATGATA	8.1 **M**b
178 ±	02292-L02212	*FHIT*, ex 5	3p14.2	GAGGACATGTCG-TTCAGATTTGGC	285.0 kb
337	02290-L01781	*FHIT*, ex 4	3p14.2	CCTGCCTGCTTA-GACCCTCTATAA	18.0 **M**b
409	21548-L31318	*ROBO1*	3p12.3	ATATGGATACGG-ATGCGCCAGAAG	16.4 **M**b
328 #	05297-L04685	*PROS1*	3**q**11.2	TGTGAATGCCCC-GAAGGCTACAGA	28.4 **M**b
247	05708-L19175	*CASR*	3**q**21.1	CTCCATCGTGTT-TAAGGAAGTCGG	32.9 **M**b
202	00487-L00069	*MME*	3**q**25.31	CGTTGACTGGTG-GACTCAACAGTC	38.5 **M**b
346	03271-L02708	*OPA1*	3**q**29	TGAAGATGGTGA-GAAGAAGATTAA	-
Gain of 6p and loss of 6q					
383	10252-L11363	*ECI2*	6p25.2	AAGGACTTGTTA-CTGAAGTTTTCC	20.3 **M**b
463	10253-L18092	*DCDC2*	6p22.2	GCAGAGAGGTCT-GAAACACGGGGG	12.4 **M**b
427	00585-L18090	*CDKN1A*	6p21.31	CGGCTGATCTTC-TCCAAGAGGAAG	8.7 **M**b
160 «	22020-L31270	*RUNX2*	6p12.3	GTTGTGATGCGT-ATTCCCGTAGAT	86.8 **M**b
266 +	04745-L19276	*CCN2*	6**q**23.2	ACCGAGCTAAAT-TCTGTGGAGTAT	28.0 **M**b
233	02798-L20038	*IGF2R*	6**q**25.3	TTCAACACAACA-GTGAGCTGTGAC	-
Loss of 8p and gain of 8q					
310	02552-L19178	*LZTS1*	8p21.3	GCTGCAGCGCAA-GAAGAACGAGGC	12.6 **M**b
318	04239-L03575	*NRG1*	8p12	CTGGGACAAGCC-ATCTTGTAAAAT	23.0 **M**b
184	16641-L19172	*RP1*	8**q**12.1	ATCCTGAGCTCT-GGAGCTGTGGTG	73.1 **M**b
166	15894-L16789	*MYC*, ex 1	8**q**24.21	CTGGAACTTACA-ACACCCGAGCAA	4.3 kb
154	20780-L27349	*MYC*, ex 3	8**q**24.21	GAACGAGCTAAA-ACGGAGCTTTTT	2.3 **M**b
299	16241-L18499	*ASAP1*	8**q**24.21	TTCCTTTCAGGC-TGTCCTTCGATG	-

Table 5. SALSA MLPA probes arranged according to chromosomal location.

Electrophoresis of the amplified products was performed with a SeqStudio Genetic Analyser and the electropherograms were checked with GeneMapper Software version 6 (Thermo Fisher Scientific). Output data were analyzed comparing the samples with three healthy controls using Coffalyser.net MLPA Analysis Software (MRC-Holland). The cut-off values used to evaluate gene/exon imbalances were 0.8 and 1.2 for loss and gain of signal, respectively.

Statistical analysis

The main endpoints analyzed were overall survival (OS) and disease free survival (DFS). OS was defined as time from surgical treatment to death for all causes. DFS was defined as the time from surgical treatment until the first event between relapse at any site or death for all causes. The Kaplan-Meier method was used to estimate the survival probability with Greenwood standard errors. Survival was estimated and compared based on log rank test. A multivariate proportional hazard Cox-regression model was implemented for the same endpoints (OS and DFS). Age at diagnosis, as a continuous variable, was analyzed using univariate Cox models. Results are shown in term of hazards ratios (HR), 95% confidence intervals (CIs), and p values. All statistical tests were two-tail and p values were considered significant when ≤ 0.05. All analyses were performed using IBM SPSS statistical software, version 25 (Chicago, IL, USA).

RESULTS

Clinical outcomes

Complete clinical history, current follow-up status and tissue blocks were fully available for 48 patients. **Table 6** summarizes the clinical features of the cohort.

The most frequent clinical symptoms were epistaxis (39 cases, 81.2%), unilateral nasal obstruction (33 cases, 68.8%), headache (18 cases, 37.5%), facial pain (11 cases, 22.9%), and proptosis (6 cases, 12.5%).

According to definitive pathological examination, 10 (20.8%) patients were diagnosed with microscopically involved margins (orbital apex 4 cases, lateral wall of sphenoid sinus and cavernous sinus in 3 cases, masticatory space 2 cases, brain parenchyma in one case). In univariate analysis, surgical margins status (infiltrated versus cancer-free) was statistically associated with prognosis both in terms of OS (p=0.03) and DFS (p=0.01).

Most of the patients received adjuvant treatment (32 cases, 66.7%), in the form of PORT (60.4%) or concomitant CRT (6.3%). Irradiation was delivered using 3D-CRT in 9 cases (mean dose, 59.7 Gy; range, 55.3Gy-62Gy), IMRT in 12 cases (mean dose, 62 Gy; range, 57.7Gy-64Gy), and CIR in 11 cases (mean dose, 69Gy; range, 67Gy-72.2Gy). A total of 16 (33.3%) patients didn't receive adjuvant treatment, as it was not indicated after multidisciplinary discussion for several reasons: resection proved to be radical for an early-stage cancer in 9 cases; surgery performed as salvage treatment after previous RT or CRT in 4 cases; multiple comorbidities with compromised performance status in 2 cases; patient's refusal to adjuvant treatments in one case (**Table 6**). In univariate analysis, the delivery of adjuvant radiotherapy (32/48 cases) was associated with improved local control of the disease (p=0.02) but failed to be statistically significant associated with improved OS rates (p=0.37). In addition, when comparing survival outcomes between standard photon (3D-CRT and IMRT, 21 cases) versus heavy ion irradiation (CIR, 11 cases) in univariate analysis, improved rates in terms of DFS (p=0.03) but not in terms of OS (p=0.71) were observed in the group submitted to carbon ion irradiation.

After a mean follow-up of 60.8 months (range, 12-155 months; median, 57 months), 10 patients (20.8%) were alive without evidence of disease, 3 (6.3%) were alive with disease (AWD), 33 (68.8%) died of disease and 2 (4.1%) died from other causes (DOC) (**Table 6**).

Parameters		**N° (%)**
Gender	male	18 (37.5)
	female	30 (62.5)
Age	<69 years	20 (41.6)
	>69 years	28 (58.4)
Site	Naso-ethmoid	39 (81.2)
	maxillary	6 (12.5)
	nasopharynx	3 (6.3)
Stage	pT3	18 (37.5)
	pT4a	17 (35.4)
	pT4b	13 (27.1)
Treatment	Surgery	16 (33.3)
	Surgery + RT	29 (60.4)
	Surgery + RT-CHT	3 (6.3)
Status	alive	13 (27.1)
	dead	35 (72.9)
Overall Survival (OS)	1-year	76%±6.7%
	3-year	47%±7.4%
	5-year	38%±7.4%
	10-year	18%±7.1%
Disease Free Survival (DFS)	1-year	46%±7.3%
	3-year	30%±6.8%
	5-year	18%±6.5%
	10-year	14%±6.4%

Table 6. Clinical features of the patients included in the study. Abbreviations: RT, radiotherapy; CHT, chemotherapy.

Recurrences occurred in the large majority of patients (40/48 cases, 83.3%), after a mean period of 18 months (range, 3-139 months). Early recurrences within 36 months (range, 3-36 moths; median, 12 months) occurred in 28 (58.4%) cases while late recurrences after 36 months (range, 37-139 months; median, 89 months) were observed in 12 (25%) cases. Remarkably, the overall prognosis of patients who experienced an early recurrence was significantly lower than those who experienced late recurrences, both in terms of OS (p=0.02) and DFS (p=0.001). Early recurrences included systemic dissemination of disease (21/28 cases) without possibility to cure in most of the cases while late recurrences usually involved the site of primary tumor with possibility for salvage surgery (5/12 cases). A single recurrence was observed in 26 cases, 2 recurrences in 7 cases, 3 recurrences in four cases, and 4 recurrences in three cases. Local recurrences occurred in 19 cases, isolated (7 cases) or concomitant to systemic dissemination of disease (12 cases). Salvage surgery was performed only in 4 cases where there was possibility to remove the cancer with radical intent and possibility to cure. Sinonasal surgery was performed in other 3 cases as a palliative treatment in order to manage bleeding and nasal obstruction, which precluded daily clinical activities. Remarkably, three long-survivors experienced multiple local recurrences, which were amenable to complete surgical excision and/or irradiation. At present, two of them dead of disease for systemic metastases after 97 months and 112 months, respectively, while one patient is currently alive without evidence of disease after 131 months. Regional metastases in neck nodes were observed only in two cases, who were submitted to modified radical neck dissection as salvage treatment.

Finally, 36/48 (75%) cases experienced distant metastases during the follow-up involving the brain in 17 cases, lungs in 14 cases, bone in 12 cases, liver in 9 cases, kidney in 6 cases, adrenal gland in 3 cases, pancreas in one case. Patients who developed systemic metastasis dead of disease in 33 cases while the remaining three patients are currently alive with disease.

Cancer immune contexture

Immunohistochemical analysis of the immune infiltrate within MM tumoral cells revealed a mean density of CD45 positive cells of 301,6 (standard deviation ±42.4) while the mean density of CD8 positive cells was 132,1(standard deviation ±58.9), as

shown in **Table 7**. This is in contrast with the significant immune infiltration described in literature for cutaneous melanoma (22) and confirmed that MM is a *noninflammed tumor* with rare tumor-infiltrating immune cells.

	CD45			CD8			CD3	CD163	CD20	CD66b	BDCA2	CD56
ID	**Tot**	**Amm²**	**Densità**	**Tot**	**Amm²**	**Densità**	**%**	**%**	**%**	**%**	**%**	**%**
1	25515	070	**363,7**	10883	120	**90,9**	25	70	0	0	0	0
2	3191	72,31	**44,1**	2179	075	**29,2**	80	20	0	0	0	0
3	5631	090	**62,8**	1037	089	**11,7**	20	30	0	20	0	0
4	25644	135	**189,9**	8229	126	**65,2**	40	40	10	10	0	0
5	5606	025	**223,4**	3067	017	**185,9**	85	3	10	0	0	1
6	27318	088	**310,5**	10461	110	**95,0**	35	60	5	0	0	1
7	18987	36,82	**515,7**	12700	026	**487,7**	95	3	0	0	0	0
8	2509	016	**152,5**	1633	018	**93,2**	70	15	15	0	0	0
9	27371	093	**292,9**	7893	094	**83,6**	40	40	10	10	0	0
10	15479	24,4	**634,4**	3575	020	**182,6**	60	10	20	0	0	0
11	19714	058	**342,1**	13821	056	**248,6**	80	20	0	0	0	0
12	4857	055	**88,5**	1618	053	**30,6**	35	30	0	30	0	1
13	6572	022	**297,5**	1443	023	**62,6**	50	10	5	30	0	0
14	9388	044	**214,2**	10118	079	**127,7**	80	15	5	0	0	0
15	9627	060	**160,3**	785	067	**11,7**	25	10	0	65	0	0
16	2320	026	**88,0**	3325	070	**47,3**	70	20	5	5	0	0
17	4199	038	**111,8**	1097	039	**28,5**	45	50	0	0	0	0
18	24042	246	**97,6**	2812	209,37	**13,4**	45	50	2	2	0	0
19	5644	084	**67,3**	1027	079	**12,9**	20	45	0	35	0	0
20	27271	088	**310,4**	9460	089	**106,1**	35	50	10	1	0	1
21	5428	005	**1062,2**	541	006	**92,8**	15	15	0	30	0	1
22	83359	105	**790,7**	40393	103	**391,7**	60	35	5	0	0	0
23	2671	081	**32,9**	1854	082	**22,5**	80	10	5	1	0	1
24	16670	056	**295,4**	3940	087	**45,2**	25	35	0	40	0	0
25	22066	136	**162,8**	17005	134	**127,4**	85	10	5	0	0	0
26	2514	033	**76,6**	1072	031	**35,0**	60	35	0	2	0	0
27	8210	067	**121,9**	2594	068	**38,4**	40	50	1	1	0	0
28	403	005	**74,5**	214	006	**37,2**	50	45	0	1	0	1
29	44331	358	**123,8**	6013	358	**16,8**	20	70	0	5	0	1
30	61839	309	**199,9**	7451	291	**25,6**	15	80	1	1	0	0
31	007	000	**31,7**	019	001	**18,0**	60	40	0	1	0	1
32	2382	29,07	**81,9**	157	31,17	**5,0**	7	65	0	30	0	1

	CD45			CD8			CD3	CD163	CD20	CD66b	BDCA2	CD56
ID	**Tot**	**Amm²**	**Densità**	**Tot**	**Amm²**	**Densità**	**%**	**%**	**%**	**%**	**%**	**%**
33	2193	35,65	**61,5**	1047	35,76	**29,3**	55	40	1	0	0	1
34	4464	22,56	**197,9**	2462	26,3	**93,6**	80	20	0	0	0	0
35	5023	12,62	**398,0**	2013	11,71	**171,9**	70	25	1	0	0	0
36	9609	25,41	**378,2**	3209	21,28	**150,8**	70	30	0	0	0	0
37	16404	27,72	**591,8**	3811	26,86	**141,9**	55	30	10	0	0	0
38	5114	16,64	**307,3**	2043	15,84	**129,0**	60	20	0	20	0	0
39	34489	40,15	**859,0**	30881	42,44	**727,6**	90	10	1	0	0	0
40	1969	4,66	**422,5**	989	4,44	**222,7**	55	1	1	0	0	0
41	1626	29,39	**55,3**	804	25,28	**31,8**	60	1	0	0	0	1
42	23338	18,2	**1282,3**	13341	18,74	**711,9**	70	30	2	0	0	0
43	3793	17,18	**220,8**	625	17,81	**35,1**	30	65	0	1	0	0
44	1204	11,97	**100,6**	289	9,55	**30,3**	50	40	0	10	0	0
45	2568	10,18	**252,3**	980	9,95	**98,5**	55	40	0	5	0	0
46	4451	53,11	**83,8**	1204	57,66	**20,9**	35	60	2	0	0	0
47	17107	9,77	**1751,0**	7920	9,29	**852,5**	90	5	5	0	0	0
48	1643	24,99	**65,7**	498	20,22	**24,6**	80	10	0	5	0	1
mean	13662	60,75	**301,6** d.s. ±424	5428	62,8	**132,1** d.s. ±58,9	53,3	31,4	2,9	7,5	0	0,3

Table 7. Data on of the main leukocyte populations observed in the tumor samples of MM analyzed (two pages).

This results support the immune escape ability developed by the neoplasm during the immunoediting process, based on the selection of immune resistant tumor cells able to survive to the cytotoxic activities of T lymphocytes and NK cells. The first mechanism supporting this process is the T-cell exclusion, which is characterized by a very scarce presence of T lymphocytes, in particular CD8+, in the tumor microenvironment. Effective immunotherapy promotes the killing of cancer cells by cytotoxic T cells. This requires not only that cancer-specific T cells be generated, but also that these T cells physically contact cancer cells. The scarcity of T cells and the coexistence, in some patients, of cancer cells and T cells that recognize them indicates that tumors may exhibit the phenomenon of immune privilege, in which immunogenic tissue is protected from immune attack.

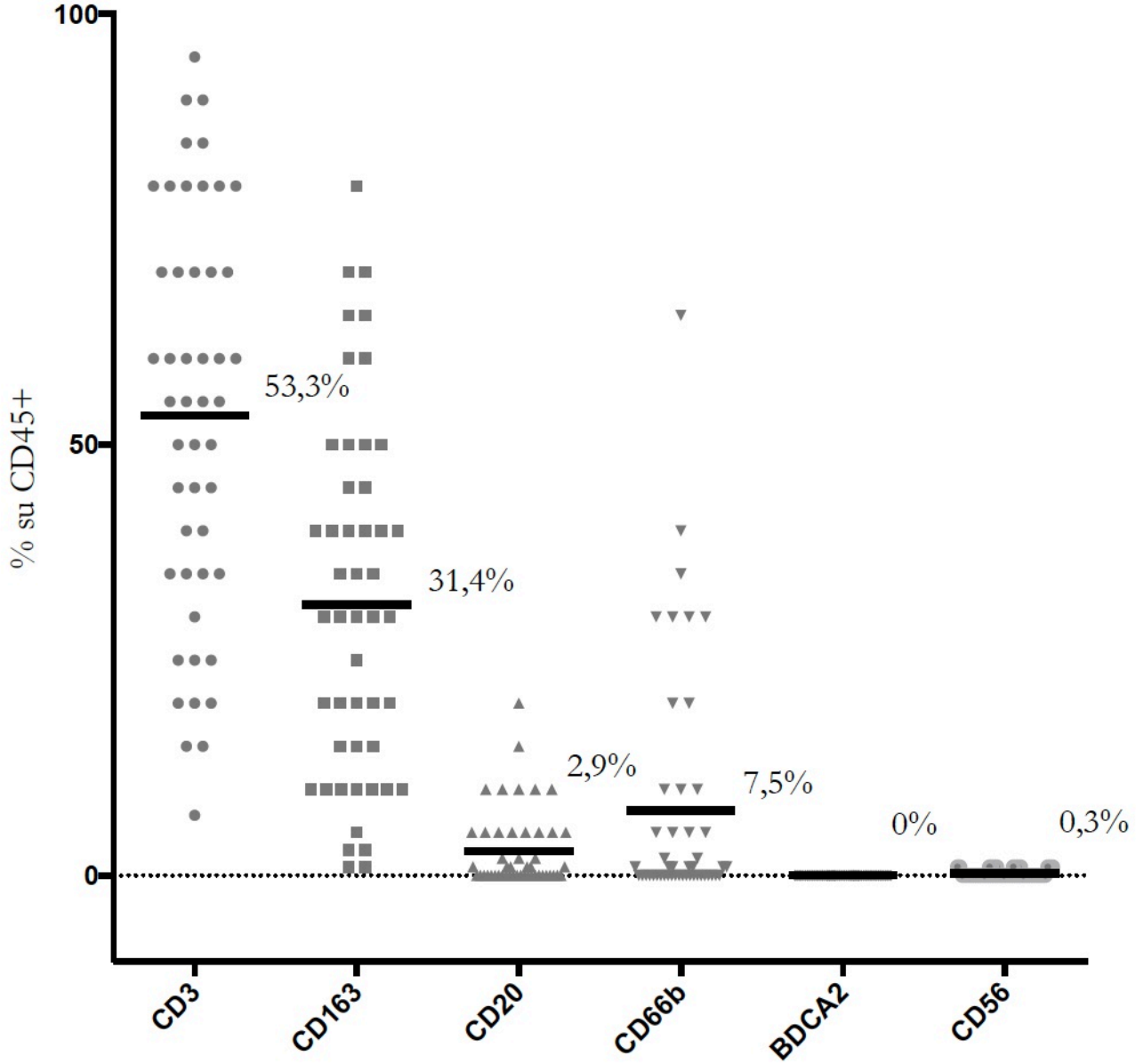

Figure 1. Distribution of immune cells as percentage, in relation to CD45+

The other markers analyzed (CD3, CD20, CD66b, CD163, CD56, BDCA2) have been evaluated using a semi-quantitative method in terms of percentage of positive cells compared to the total number of CD45+ cells (**Table 7**). The mean values of tumor-infiltrating immune cells were summarized as follows: CD163 positive cells, 31.4%; CD3 positive cells, 53.3%; CD66b positive cells, 7.5%; CD20 positive cells, 2.9%; CD56 positive cells, 0.3%; BDCA2 positive cells, 0%. Globally, the tumor microenvironment in MM included T cells, with preponderance of CD8+ rather than CD8-, macrophages and neutrophils. B lymphocytes and NK cells were extremely rare while plasmacytoid dendritic cells (pDCs) were absent. **Figure 1** shows the distribution of tumor-infiltrating immune cells observed in this cohort of MM.

The immune contexture that characterizes the density, the location, the organization and the functional orientation of tumor-infiltrating immune cells in cancers was tested to investigate its clinical impact on patient's outcome.
The Kaplan-Meier analysis showed a statistically significant correlation between the density (High versus Low levels) of CD45 (p=0,018), CD8 (p=0,019), CD3 (p=0,047), CD163 (p=0,038) and CD66b (CD66b+ vs CD66-; p=0,027) in terms of Overall Survival. Specifically, high density levels of CD45, CD8, CD3 and CD66b- were associated with better OS rates, while high density levels of CD163 were associated with worst prognosis in terms of OS. Regarding the analysis of CD56+, no statistically significant values have been obtained, but a significant trend (p=0.061) between the presence/absence of this type of immune cell was recognizable (**Figure 2**).

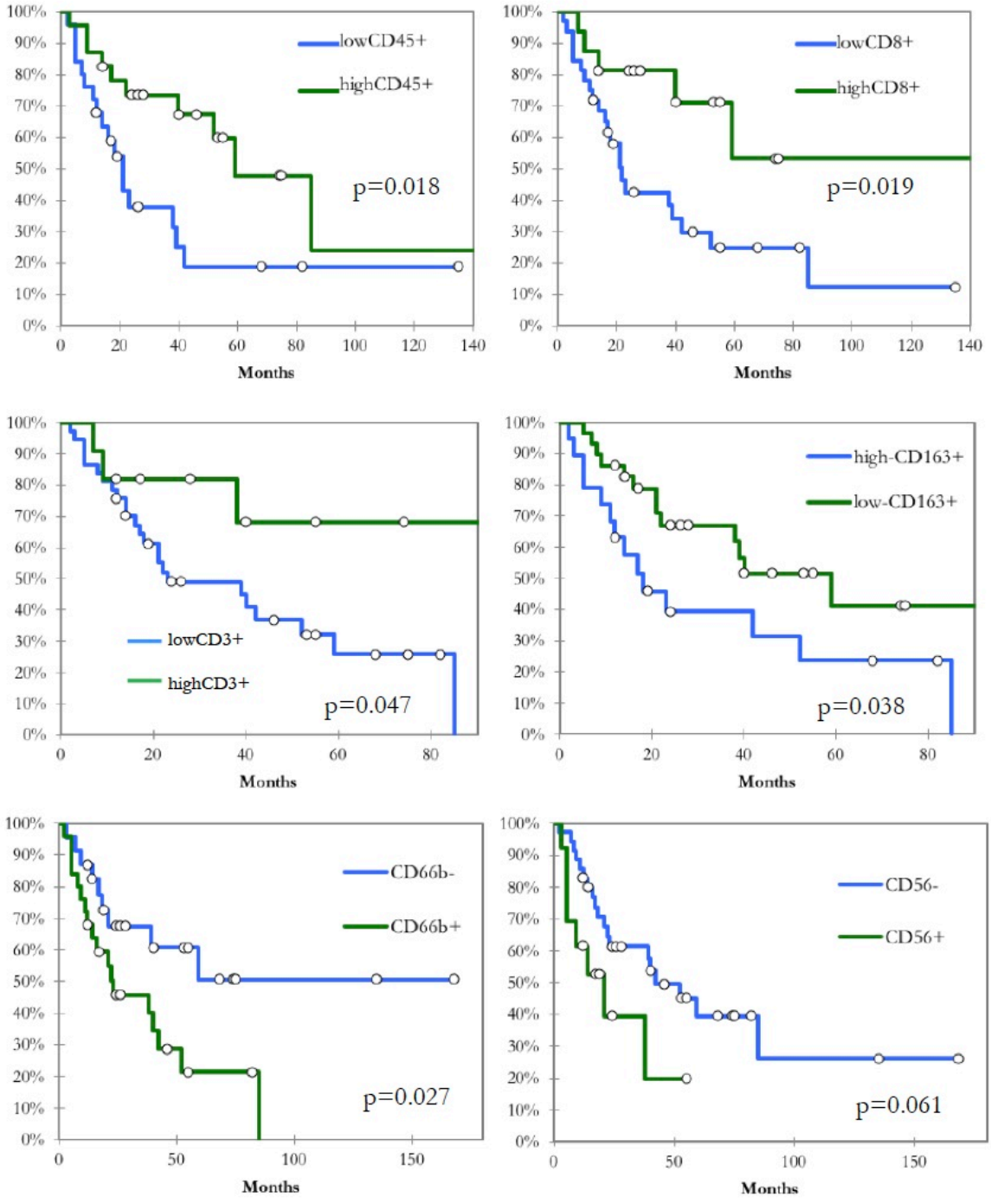

Figure 2. Kaplan-Meier univariate analysis in terms of Overall Survival based on density levels of CD45, CD8, CD3, CD163, CD66b- and CD56 positive cells.

Merging the results obtained from different markers was very useful in order to obtain patterns of immune microenvironment statistically significant associated with different prognosis. In details, the cohort was stratified according to the levels of CD163+, CD66b- and CD56- obtaining that the pattern characterized by low levels of CD163 positive cells, CD66b negative cells and CD56 negative cells was associated with statistically significant better OS (p=0.002, **Figure 3**).

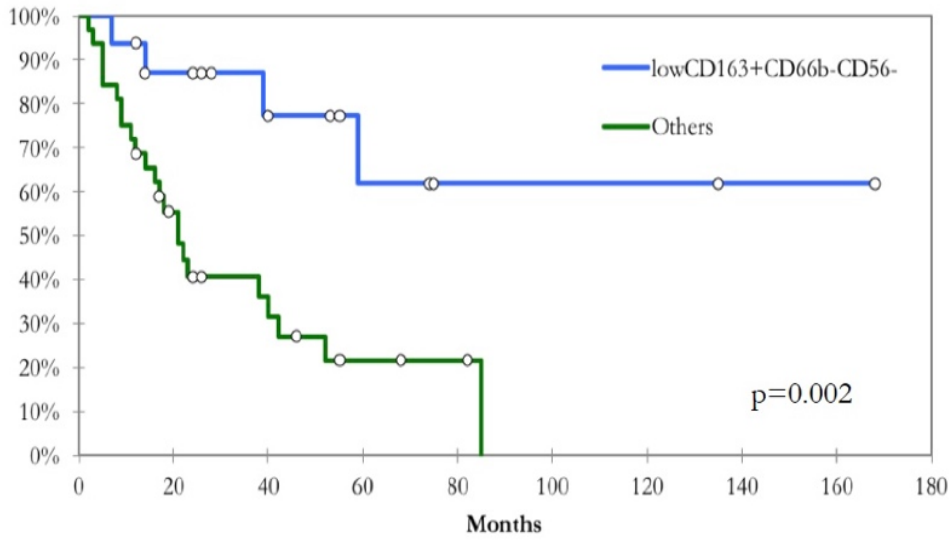

Figure 3. Kaplan-Meier univariate analysis in terms of Overall Survival based on low levels of CD163+/CD66b-/CD56-

When stratifying the patients' cohort according to another combination of immune markers (low level of CD163+ and CD66b- associated with high level of CD8+), we obtained statistically significant differences in terms of OS between the two groups (p=0.026, **Figure 4**). These findings proved that the presence of immune infiltration within the tumor microenvironment is relevant to be analyzed and, in details, in our cohort of MM, the presence of CD3 and CD8 positive cells was associated with better prognosis while the presence of CD163 and CD66b positive cells was associated with worst prognosis in terms of OS. The prognostic significance of the different patterns of immune infiltration observed has been summarized in **Figure 5.**

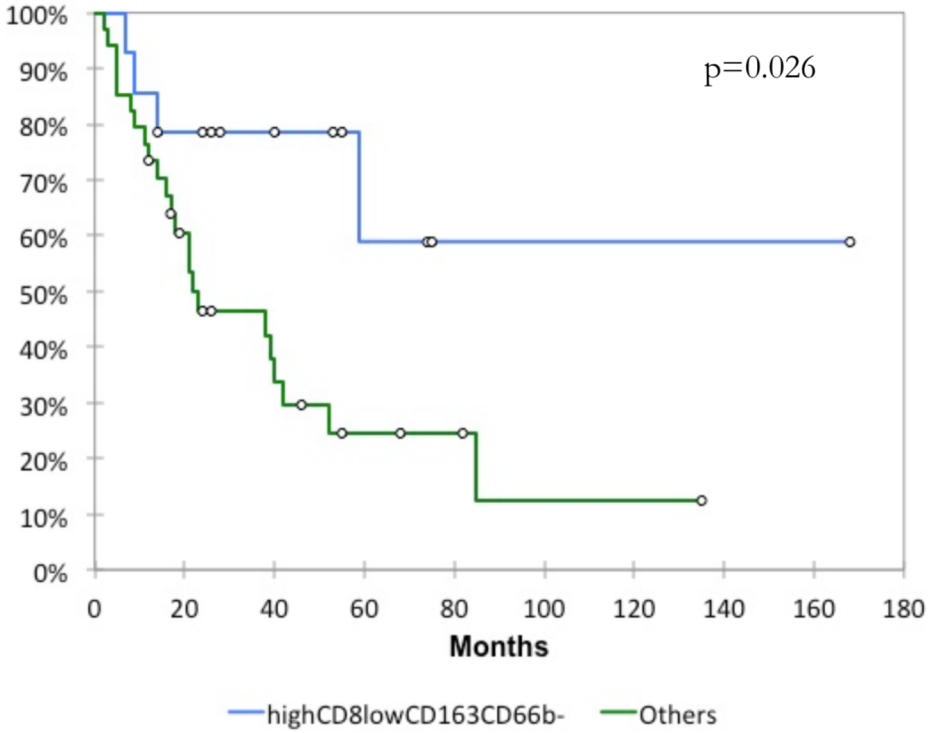

Figure 4. Kaplan-Meier univariate analysis in terms of Overall Survival based on high level of CD8+/low levels of CD163+ and CD66b-

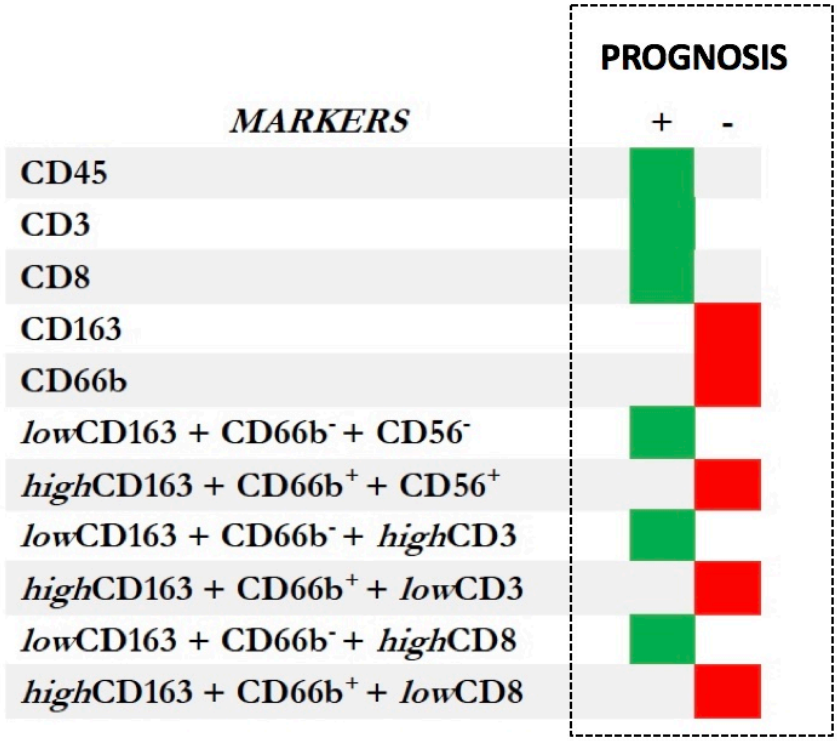

Figure 5. Summary of the prognostic impact of the immune markers found within the tumor microenvironment of MM samples.

According to the results obtained in the profiling of the immune contexture of this cancer, the biological mechanisms and the potential efficacy of specific forms of immunotherapy have been analyzed.

PDL-1 expression by immunohistochemistry was found in a small percentage of cases. In details, 79,3% (38/48) of cases were negative, showing an internal positive control in the immune infiltrate cells in 13/48 cases; 18,7% (9/48) cases displayed only a focal positivity (<5% tumor cells); while 2% (1/48) of cases stained with a mild positivity. However, even in the case showing mild positivity for PDL-1 in immunohistochemistry, the associated absence of CD8+ cells may support the possibility of immunoescape of this marker (**Figure 6**).

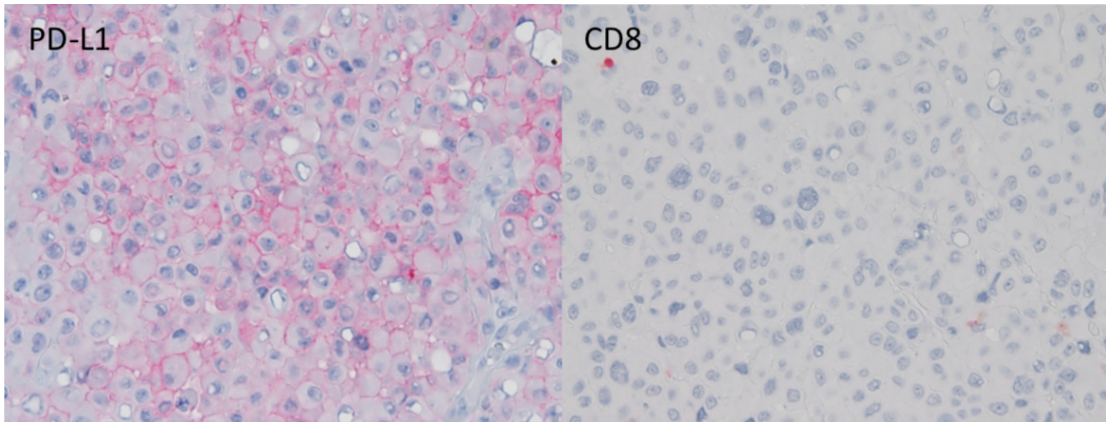

Figure 6. Expression of PDL-1 associated with absence of immune infiltrate, as displayed by the negative CD8 staining.

Given the profile of *noninflammed* tumor of MM and its limited positivity for PDL-1 expression, alternative strategies for immunotherapy should be investigated and possibile mechanisms for T-cell exclusion should be investigated.

Therefore, four sinonasal MM cell lines (SN-MM1, SN-MM2, SN-MM3, SN-MM4) were generated and validated, with a proved melanocytic identity and showing tumorigenic potential in vitro and in vivo (**Figure 7**). CSCMM were identified and fully characterized in order to define their phenotype. The immunoexpression of hematoxylin eosin, SOX10 and MRT was tested in order to confirm their nature, as shown in **Figure 8**.

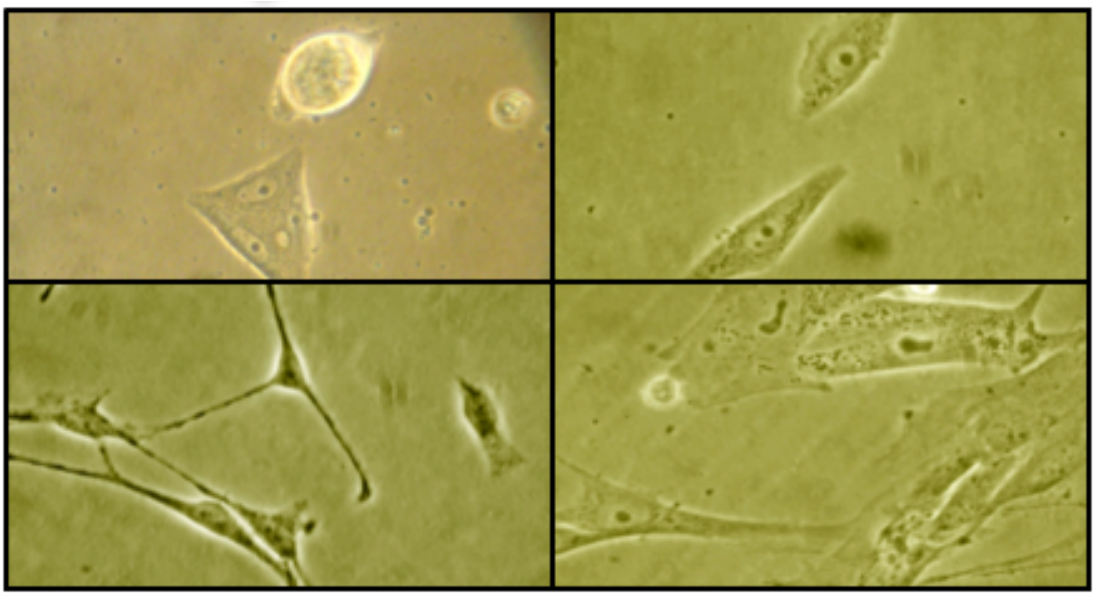

Figure 7. Mucosal Melanoma cell lines generation

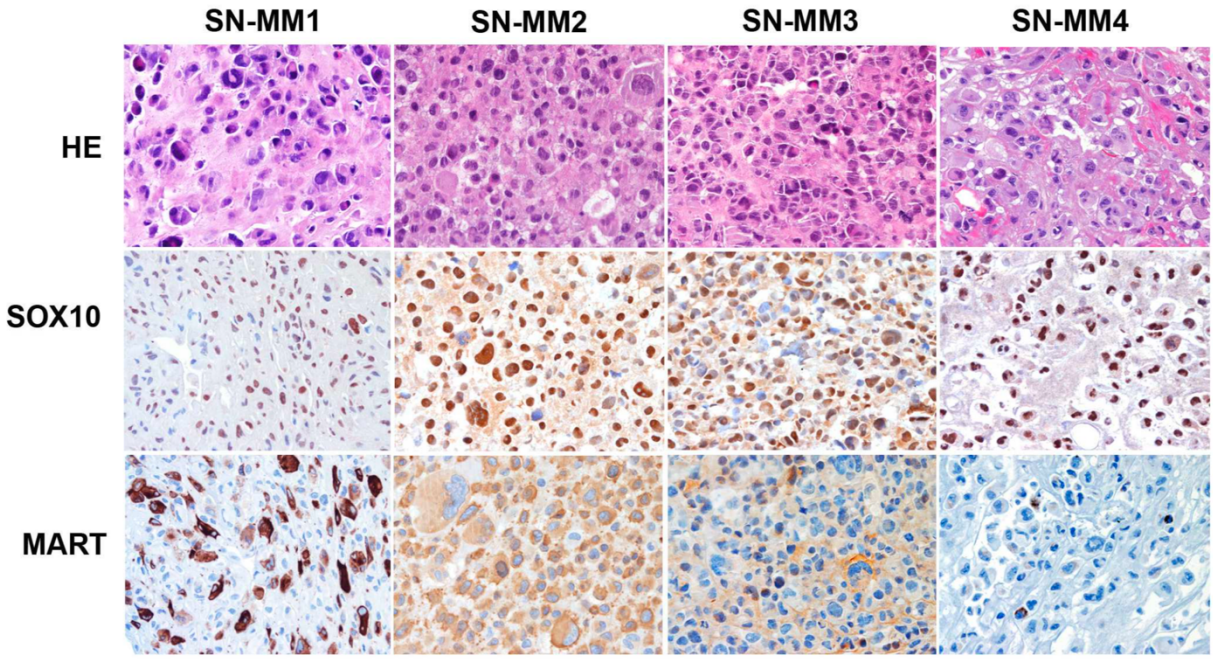

Figure 8. Morphology and melanocytic phenotype of human SN-MM cell lines. Sections are from cell blocks and stained as labeled (Magnification 200X).

A population of CSC-like cells in MM xenografts generated by injecting the human SN-MM1 and SN-MM2 in NOD/SCID mice was detected (CSCMu). Compared to surrounding atypical and large MITF+ZEB1- melanocytes, CSCMu cells are found in cluster and resulted smaller showing a MITF- ZEB1+ phenotype. According to their CSC phenotype, they also lack ZEB2 and CDH1 but strongly express membrane CD44 and CD271, as shown in **Figure 9**.

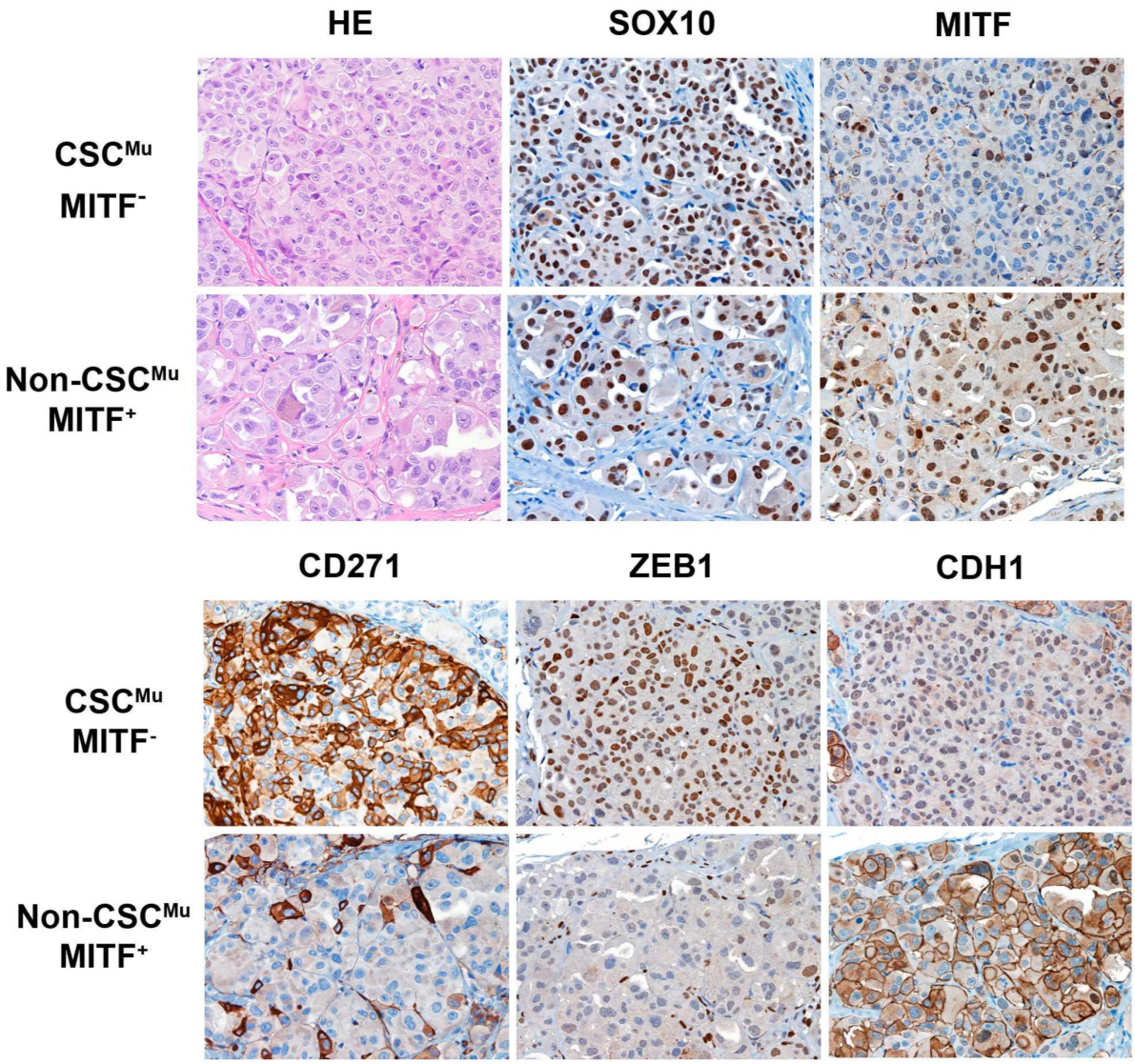

Figure 9. Identification of CSCMu in SN-MM. Sections are from the mouse xenograft obtained by subcutaneous injection of SN-MM1 in NOD/SCID mice and stained as labeled (Magnification 200X)

When directly stimulated, SN-MM cell lines can efficiently respond to IFN-γ by producing significant levels of chemokines CXCL9, CXCL10 and CXCL11, as shown in **Figure 10**. Similarly, high levels of response to IFN-γ were observed in CSCMu derived from the xenograft previously arranged.

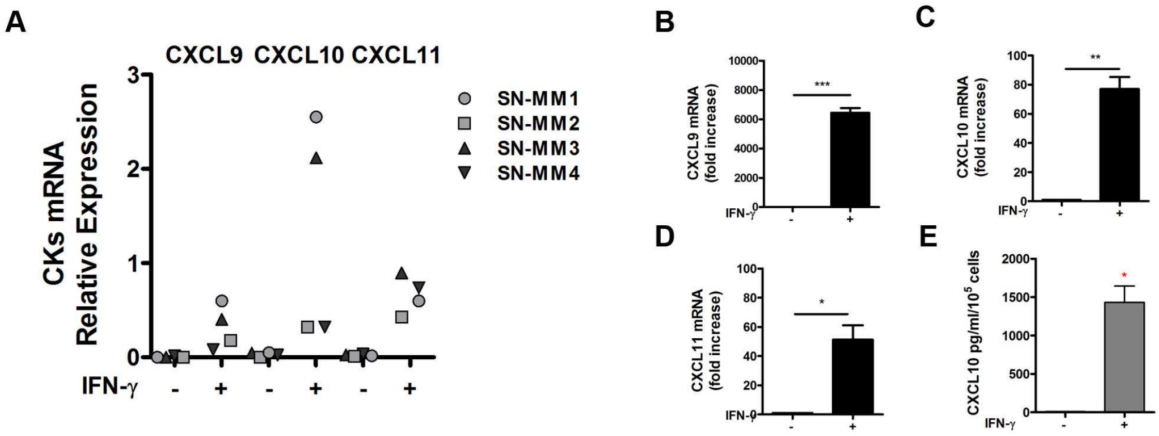

Figure 10. Chemokines (CKs) production in SN-MM cell lines. The graphs illustrate the Cks mRNA relative expression in the four SN-MM cell lines (A). In B, C, D we can observe that a triplication of CXCL9, CXCL10 and CXCL11 was observed in the SN-MM 1 cell line. In E, the graph reports CXCL10 secretion by SN-MM2 cells, unstimulated and after INF-gamma stimulation (48 hours).

Therefore, complex cellular and molecular interactions within the human tumour microenvironment might explain the "cold" phenotype observed in vivo. Cold cancers resist to the attack of the immune system through a set T cells exclusion (TCE) mechanisms (78). Cancer cell-intrinsic molecular abnormalities plays a key role in driving the TCE, including gain of function (WNT-β catenin, MYC) and loss of function (LKB1, PTEN, p53) genetic variants. These abnormalities ultimately result in the modulation of T cells-attracting chemokines or in the recruitment and differentiation of cells that establish an immunosuppressive environment promoting TCE (78). In CMs, a WNT-β catenin-dependent TCE is mediated by reduced accumulation of antigen-presenting cells (79); on the other hand, PTEN loss leads to inhibition of the lipidation of the autophagosome protein LC3 and autophagy in tumour cells, a process that can diminish T cell priming (80). Furthermore, PTEN loss is also associated with immunosuppressive chemokines and cytokines (80).

Therefore, we tried to identify cellular and molecular mechanisms of TCE in our cohort of MM by analyzing β-catenin (CTNNB1) and PTEN expression.

In our cohort, by using immunohistochemistry, β-catenin (CTNNB1) nuclear expression was negative in 100% of cases (**Figure 11**). The expression of this marker was regularly limited to the cytoplasm and cell membrane of MM cells, indicating lack of WNT-β catenin activation. This finding is completely in contrast to what is emerging from cutaneous melanoma, where the WNT/β-catenin pathway seems to be one of the main mechanism associated with the TCE from the immune contexture of the cancer. The lack of WNT- β-catenin pathway activation in MM, associated with the poor T cell immune infiltrate, seems to suggest that probably other cellular and molecular mechanisms of TCE might be present in this cancer.

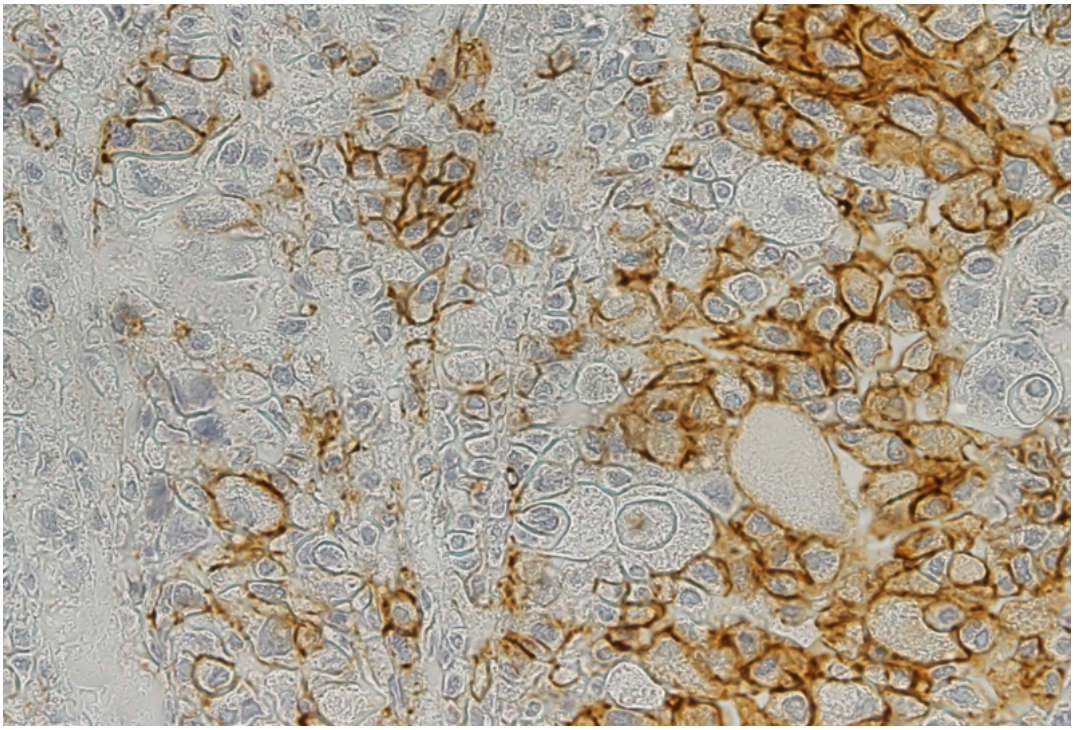

Figure 11. Negative β-catenin nuclear expression in MM.

On the contrary, when analyzing the immunoexpression of PTEN, we found that PTEN loss was recurrent in our series of sinonasal MM. PTEN loss was found in 48% of MM samples with very low immunoreactivity scores also in cases which were positives, as shown in **Figure 12**.

Recent evidences had shown that loss in phosphatase and tensin homolog deleted on chromosome 10 was associated with immunotherapy resistance in different cancers, which may be attributed to the non-T-cell-inflamed tumor microenvironment (81).

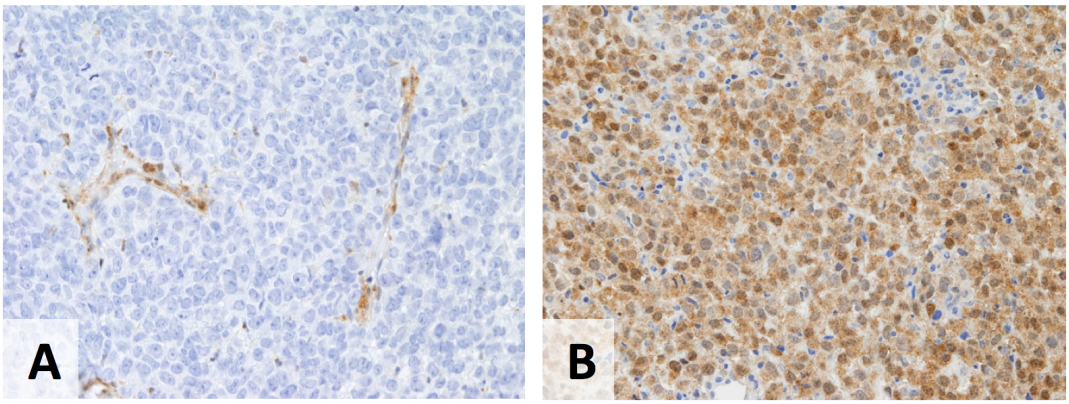

Figure 12. PTEN expression in sinonasal Mucosal Melanoma. Negative immunoreactivity (score 0) in A; diffuse positive staining (score 4) in B.

In this regards, it has been described in literature that deletion or inactivating PTEN mutations have been associated with a non-T cell inflamed infiltrate in cutaneous melanoma and it has been shown that, in tumors characterized by genetic heterogeneity, only areas that maintain PTEN expression show partial T cell immune infiltrate. Therefore, the PTEN loss may represent an escape mechanism also in MM. Remarkably, in our series, PTEN loss was frequently observed in cases with a low T cell infiltration, supporting such speculation.

Moreover, this hypothesis is supported also by the observation that our patients affected by metastatic MM treated with anti-PD1 agents showed a better clinical response if PTEN expression was preserved. These data may bring insight into the role of PTEN loss in T cell exclusion and immunotherapy resistance, and may inspire further research on immune modulating strategy to augment immunotherapy.

Mutational status

By combining data obtained from Sequenom MassArray system and gene-targeted NGS analysis, 31 cases provided suitable results.

Somatic mutations were observed only in a relatively small fraction of primary sinonasal MM, as summarized in **Table 8**. The most frequently observed mutations involved NRAS gene (10/31, 32.2%), which represents a relevant finding compared to previous report where NRAS mutations have been described in proportion of cases ranging from 9% to 21% (82). Paradigmatic cases harboring NRAS mutations are depicted in **Figure 13** and **Figure 14**. To note, in one patient (case #30), who experienced multiple local recurrences over a period of 15 years, different types of NRAS mutations were observed in different samples of recurrence. During the time span analyzed, the patient underwent several therapies including surgery, intensity-modulated radiotherapy, carbon ion therapy, cisplatin-based chemotherapy, and immunotherapy with dual checkpoint inhibition based on a combination of ipilimumab (targeting CTLA-4) and nivolumab (targeting PD1). These treatments might have impacted in the molecular profile of the cancer. Tumor heterogeneity in melanoma is well known, and a switch of the driver mutation or a selection of a tumor clone for a specific driver mutation in response to radiochemotherapy and/or immunotherapy has been reported in few cases of MM (83). Another possible theory that would perhaps explain the presence of different oncogenic drivers in different tumor samples of a same patient might be the occurrence of a second primary tumor, which, in the specific case, should have happened four times. However, this hypothesis is less likely since the cancer was spatially located always in the same region (right nasal fossa) and the patient was found negative for hereditary cancer syndromes. Globally, this result is relevant and provide an important rationale for longitudinal molecular testing, based on evidence for an unforeseen recurrent event of molecular driver switch in progressing sinonasal MM. This finding also provides the basis for further studies on a potential causal relation of emerging NRAS mutant clones and immunotherapy.

KIT mutations were found in 2/31 (6.5%) cases, suggesting a marginal role for the therapeutic use of tyrosine kinase inhibitors (TKIs) in MM. Similarly, KRAS mutations were found in 2/31 (6.5%) cases. To note, NRAS, KRAS and KIT mutations were mutually exclusive events.

Conversely, in three cases harboring NRAS mutation, additional variants were found in one case each: PIK3CA, RET and CTNNB1 mutations. Given the rarity of this finding, further studies should be performed in order to understand if such mutations might be involved in the pathogenesis of MM or can be considered only as passenger mutations.

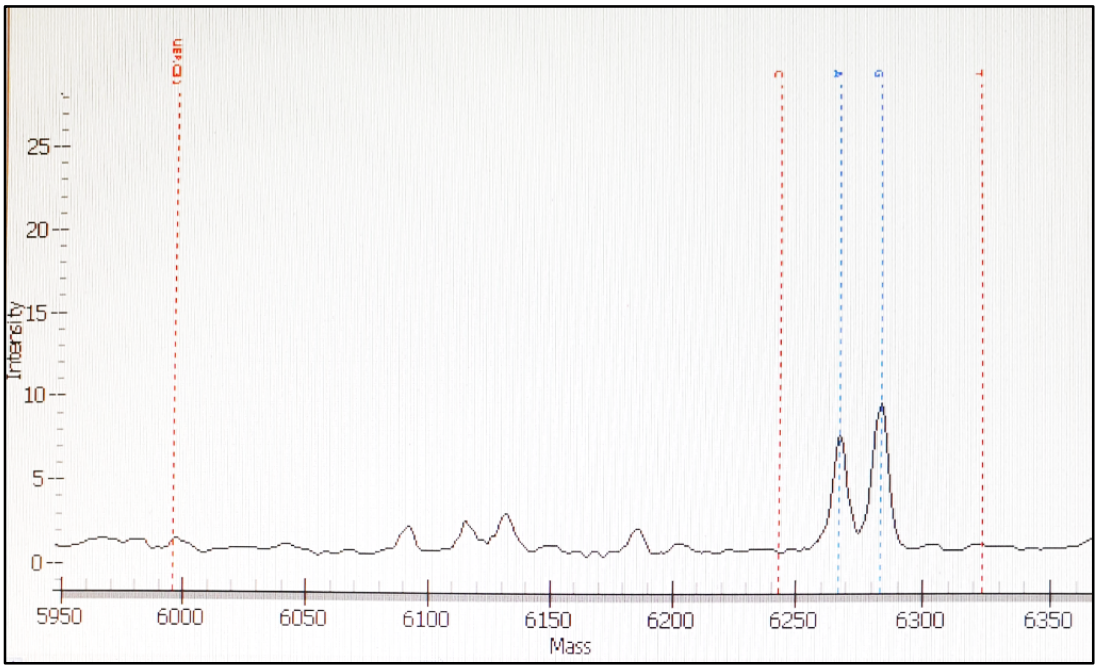

Figure 13. Case #29, gene mutation analysis with Sequenom MassArray system. The NRAS c.38G>A p.G13D mutation has been found.

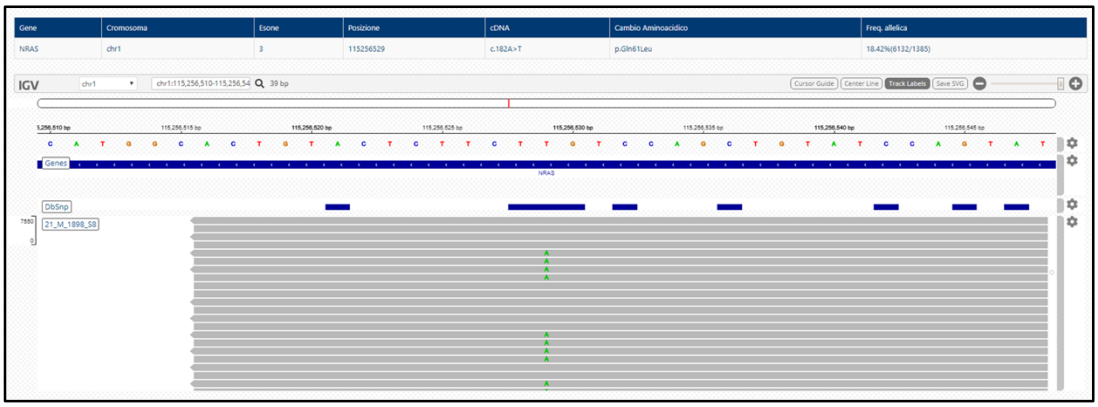

Figure 14. Case #19, gene-targeted NGS analysis revealed the presence of NRAS c.182A>T p.Q61L mutation.

Remarkably, no BRAF mutations were observed in any case. This emphasizes the molecular differences between MM and CM, where the classical V600E BRAF mutation was observed in more than 60% of cases (53). Moreover, this finding strongly argues against the clinical efficacy of the recently introduced RAF inhibitors in MM. Globally, it appears clear that the set of genetic alterations found, although of potential relevance, is still insufficient to open windows of opportunity for MM treatment with specific target therapies (82). Additional, unknown abnormalities might drive or cooperate in sunlight-independent melanoma genesis.

Patient ID	NRAS	KIT	KRAS	PIK3CA	RET	CTNNB1
Case #2	wt	wt	wt	wt	wt	wt
Case #3	wt	wt	wt	wt	wt	wt
Case #4	**p.Gly12Ala**	wt	wt	**p.Arg524Lys**	wt	wt
Case #5	wt	wt	wt	wt	wt	wt
Case #7	wt	wt	wt	wt	wt	wt
Case #10	wt	wt	**p.Gly12Ala**	wt	wt	wt
Case #11	wt	wt	wt	wt	wt	wt
Case #12	wt	wt	**p.Gly12Ala**	wt	wt	wt
Case #14	wt	wt	wt	wt	wt	wt
Case #15	wt	wt	wt	wt	wt	wt
Case #16	wt	wt	wt	wt	wt	wt
Case #17	wt	wt	wt	wt	wt	wt
Case #18	**p.Gly12Asp**	wt	wt	wt	wt	wt
Case #19	**p.Gln61Lys**	wt	wt	wt	wt	wt
Case #21	wt	wt	wt	wt	wt	wt
Case #22	**p.Gln61Arg**	wt	wt	wt	wt	**p.Thr41Ala**
Case #24	**p.Gln61His**	wt	wt	wt	wt	wt
Case #27	wt	**p.Asn822Tyr**	wt	wt	wt	wt
Case #29	**p.Gly12Asp**	wt	wt	wt	wt	wt
Case #30	**p.Gly12Arg** **p.Gly12Arg** **p.Gln61His** **p.Gly12Asp**	wt wt wt wt	wt wt wt wt	wt wt wt wt	wt wt wt wt	wt wt wt wt
Case #32	**p.Gln61Hys**	wt	wt	wt	wt	wt
Case #33	wt	wt	wt	wt	wt	wt
Case #34	wt	wt	wt	wt	wt	wt
Case #35	wt	wt	wt	wt	wt	wt
Case #38	wt	wt	wt	wt	wt	wt
Case #39	**p.Gly12Ala**	wt	wt	wt	**p.Arg886Trp**	wt
Case #40	wt	wt	wt	wt	wt	wt
Case #43	wt	wt	wt	wt	wt	wt
Case #45	**p.Gly12Asp**	wt	wt	wt	wt	wt
Case #46	wt	wt	wt	wt	wt	wt
Case #48	wt	**p.Leu576Pro**	wt	wt	wt	wt

Table 8. Genetic mutations found in the cohort of samples analyzed.

The prognostic impact of the gene mutations found in this case-series of 31 cases was analyzed in univariate analysis. No statistically significant values were found both in terms of OS and DFS when analyzing the study population according to the mutational status of KIT (p=0.7; p=0.09), PIK3CA (p=0.1; p=0.9), RET (p=0.8; p=0.8), and CTNNB1 (p=0.3; p=0.4).

Conversely, NRAS and KRAS mutations were found to be statistically associated with prognosis (**Figure 15**): NRAS mutated cases had statistically significant increased OS rates (p=0.05) compared to wild type cases but this difference was not found in terms of DFS (p=0.3); KRAS mutations were associated with worst prognosis both in terms of OS (p=0.01) and DFS (p=0.01).

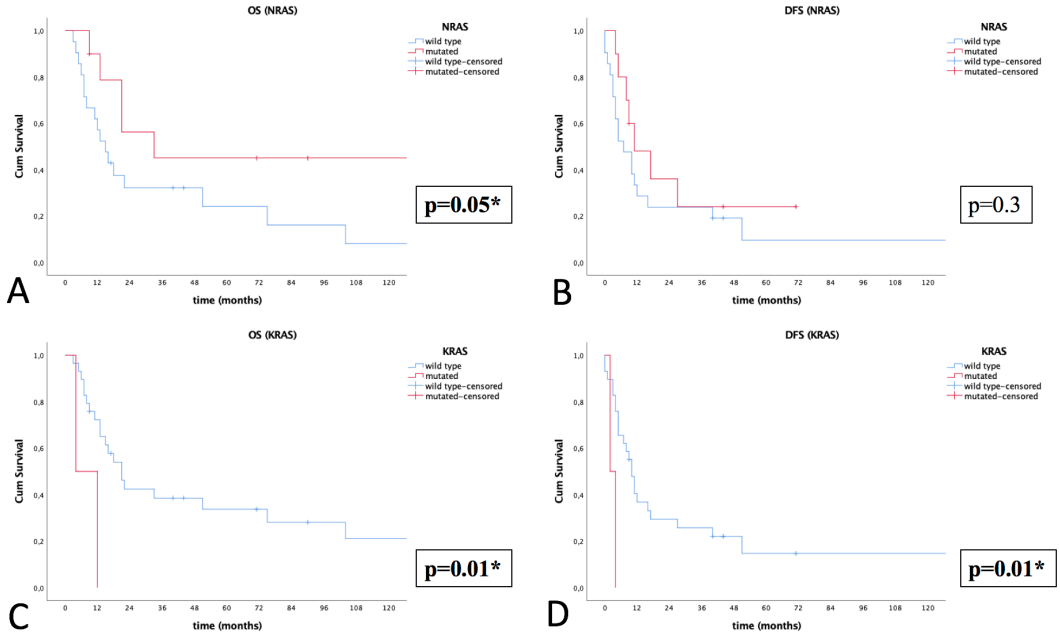

Figure 15. Kaplan-Meier survival analysis according to the mutational status of NRAS (A, OS; B, DFS) and KRAS (C, OS; D, DFS).

Copy number alterations

MLPA assay for the detection of deletions or duplications in chromosomes 1p, 3, 6 and 8 obtained suitable results in 26 cases, with a specific pattern of chromosome abnormalities as depicted in **Figure 16**. In details, the most frequently observed alterations were the gain of 6p (25/26 cases, 96.1%) and the gain of 8q (26/26 cases, 100%), which could be considered as a distinctive signature of MM (**Figure 17**).

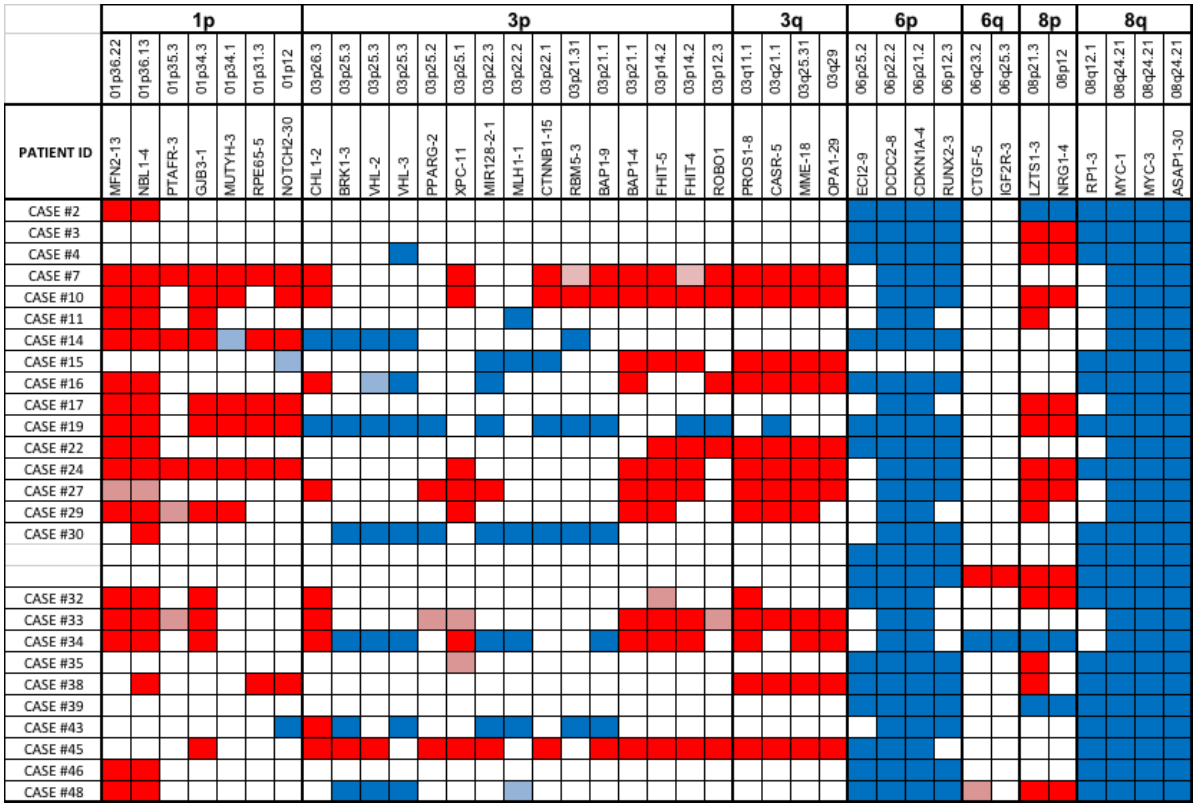

Figure 16. Results obtained from MLPA analysis. Red box, loss; Blue box, gain.

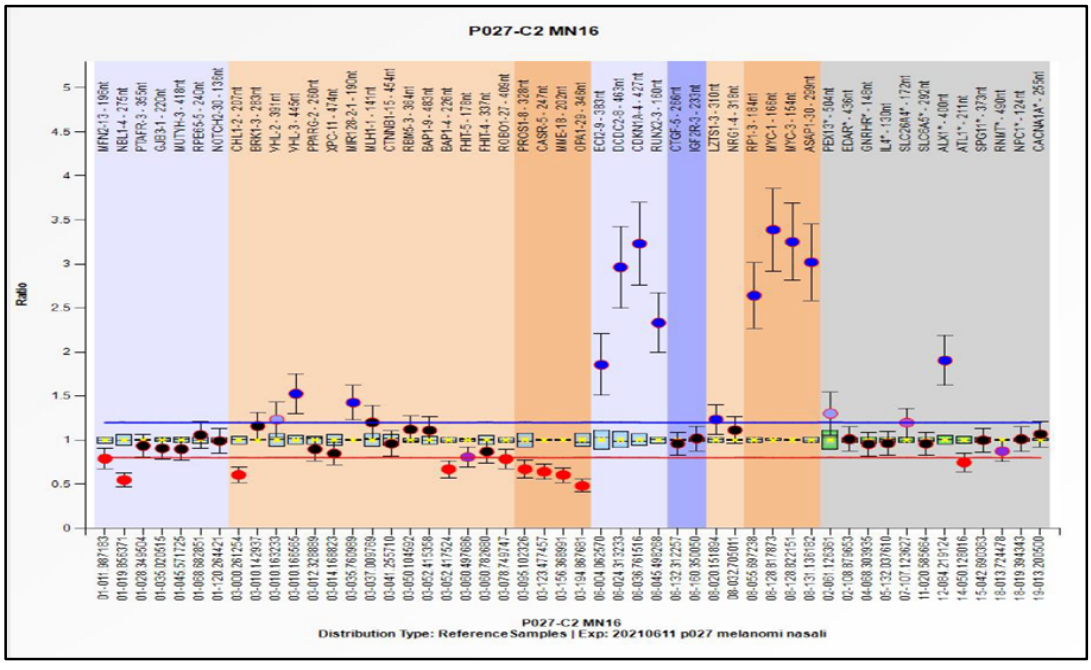

Figure 17. Case #16, MLPA amplification analysis that showed loss of 3q and gain of 6p and 8q.

Loss of 1p was observed in 18/26 (69.2%) cases and it was associated with poor prognosis, with statistically significant values both in terms of OS (p=0.006) and DFS (p=0.01). Loss of 3p and/or 3q was observed in 11/26 (42.3%) cases and it was associated with poor prognosis, with statistically significant values both in terms of OS (p=0.004) and DFS (p=0.009). Loss of 8p was observed in 10 (38.4%) cases and it was associated with poor prognosis, with a statistically significant value only in terms of OS (p=0.003) but not for DFS (p=0.14). Gain of 3p and/or 3q was observed in 7/26 (26.9%) cases but no statistically significant associations with prognosis were found in terms of OS and DFS. Globally, the MLPA CNA pattern characterized by 1p loss, 3p/3q loss and 8p loss (5/26 cases, 19.2%) was found to be a strong negative prognosticator, with statistically significant worse outcomes both in terms of OS (p=0.02) and DFS (p=0.05), as shown in **Figure 18**.

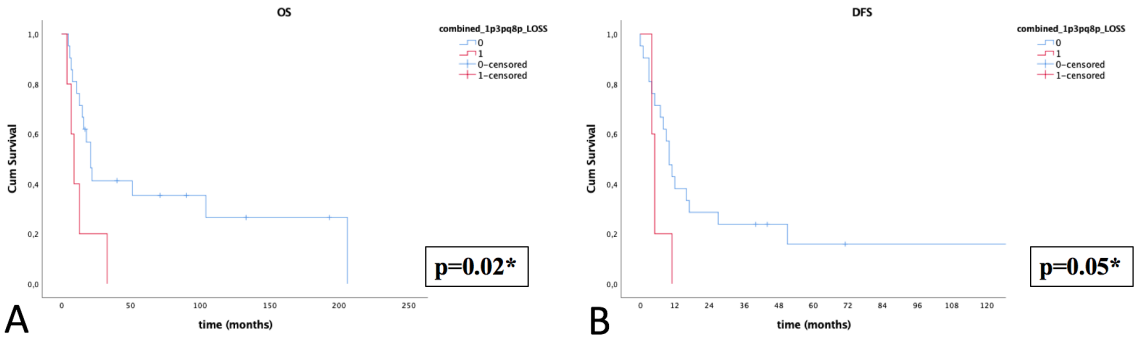

Figure 18. Kaplan-Meier survival analysis according to the MLPA amplification pattern characterized by 1p loss, 3p/3q loss and 8p loss (A, OS; B, DFS)

Finally, a Cox regression model was implemented in order to identify independent prognostic factors in MM (**Table 9**). To note, wild-type NRAS status was associated with worse prognosis and increased risk of overall death (HR=6.40, p=0.01) while the combined loss of 1p, 3p/3q and 8p was associated with and increased risk of overall death (HR=9.12, p=0.002) and increased of recurrence (HR=3.58, p=0.003).

	OS			**DFS**		
Variable	**HR**	**HR 95%**	**P-value**	**HR**	**HR 95%**	**P-value**
Age (continue variable)	1.01	0.96 – 1.05	0.65	1.02	0.97 – 1.06	0.37
NRAS (wt vs mutated)	6.40	1.45 – 28.17	0.01*	2.55	0.82 – 7.92	0.10
1p-3p/3q-8p LOSS (yes vs no)	9.12	2.19 – 37.97	0.002*	3.58	1.08 – 11.87	0.03*

Table 9. Cox regression model for OS and DFS.

DISCUSSION

Both basic researches and drug discoveries in CM have achieved enormous progresses, whereas little is known about either the genetic alterations which initiate MM cancerogenesis and the immune contexture of such rare cancer. As a result, patients affected by MM are suffering from limited treatment options and undesirable response rates that lead to extremely poor prognoses. Data currently available in literature on clinical trials both on immunotherapy and target-therapy for MM have produced sparse and conflicting results, which seem to support a limited efficacy of therapies currently in use for CM when applied for MM patients. Therefore, extensive analysis of the immune contexture and molecular landscape of MM are urgently required in order to find out biomarkers able to predict prognosis and select patents who can benefit from specific protocols of immunotherapy and/or target therapy.

In light of these considerations, we have analyzed retrospectively a cohort of 48 patients affected by SN-MM in order to describe their clinical course and investigate their genetic, molecular and immunologic profile. We found that the global prognosis of the patients included in this study is dismal, with 5-year OS rates of 38% and median time to recurrence of 18 months, in line with previous findings available in literature (3-5). Complete surgical resection, with negative margins, was a critical prognostic factor associated with improved prognosis, as described in literature (11). However, it should be emphasized that, even in patients presenting with localized disease, definitive surgical management of the primary is more difficult than for patients with cutaneous primaries. Patients with MM often suffer from a "field effect" characterized by frequent development of multiple primaries in nearby tissues, precluding clear margins. Adjuvant irradiation, although failed to improved overall survival as described in literature (11-14), in our series, was delivered in case of infiltrated surgical margins and was associated with improved local control of the disease. To note, carbon ion radiotherapy, introduced in our experience since 2012, proved to be a highly effective method of adjuvant irradiation with improved DFS rates compared to standard photon irradiation modalities, especially for those cases with persistence of disease after surgery in critical areas (e.g. cavernous sinus, orbital

apex, masticatory space) not amenable for further resection. Although the extremely high recurrence rates observed in our cohort (83.3%) and the significant risk of systemic metastasis, which occurred in 75% of patients, were able to recognize two groups of SN-MM patients characterized by a divergence in the clinical course: a group of 28/48 (58.3%) cases who experienced early recurrence and rapid development of systemic metastasis after a median time of 12 months (range, 3-36 months) without significant possibilities to cure, and a smaller group of 12/48 (25%) cases who can be defined as "long-survivors" since they developed late recurrences after a median time of 89 months (range, 37-139 months), mainly involving the site of primary tumor with possibility for multiple salvage surgeries. The profound discrepancy in OS (p=0.02) and DFS (p=0.001) observed between these two groups, regardless the uniform treatment protocol adopted, represents one more reason for deciphering comprehensively the immune microenvironment and the genetic profile of such rare and aggressive cancer.

The most relevant immunological, genetic and genomic factors analyzed in our study, which proved to have a prognostic role or might be relevant for their therapeutic implications, have been summarized in **Table 10**.

The immune contexture analysis of 48 cases of SN-MM performed in our study revealed that this is a *noninflammed* tumor, also referred as "cold" cancer, given the scarce immune infiltration by CD45, CD8 and CD3 positive cells. However, when directly stimulated "in vitro", SN-MM cell lines can efficiently respond to IFN-γ by producing CXCL9, CXCL10 and CXCL11. Complex cellular and molecular interactions within the tumour microenvironment might thus explain the "cold" phenotype observed "in vivo". MM resists to the attack of the immune system through a set of TCE mechanisms, which might be responsible of the immunosuppressive tumor microenvironment, indicated by abundant regulatory T-cells and a low ratio of CD8+ effector/regulatory T-cells, and, consequently, might explain the resistance to immunotherapy in non-negligible proportion of MM patients (84).

Although "biomarkers" able to predict response to immunotherapy has yet to be found in MM, the presence of tumor-infiltrating T cells, PD-L1 overexpression, tumor mutational burden, and the occurrence of NRAS and/or BRAF mutations, have been frequently cited as predictors of response to immunotherapy in CM (19, 22, 30).

Factor	Methodology	Treatment implications	Prognostic Role
CD45+ cells CD8+ cells CD3+ cells	immunohistochemistry	- High levels: better response to immunotherapy	High levels: better prognosis
CD163+ cells CD66b+ cells	immunohistochemistry	n.a.	High levels: poor prognosis
PD-1 expression	immunohistochemistry	- Diffuse expression: better response to anti-PD1/L1 immunotherapy	n.a.
PTEN expression	immunohistochemistry	- PTEN preserved: better response to anti-PD1/L1 immunotherapy - PI3K targeting drugs can synergize with immunotherapy in the treatment of melanoma with PTEN loss	PTEN loss may be a relevant mechanism of TCE and associated with poor prognosis
NRAS mutations	Direct sequencing, NGS	- MEK-inhibitors target therapy - PI3K-AKT-mTOR inhibitors associated with CDK4/6 inhibitors target therapy	Better prognosis
KRAS mutations	Direct sequencing, NGS	- MRTX849 (KRASG12C inhibitor) target therapy	Poor prognosis
KIT mutations	Direct sequencing, NGS	- tyrosine kinase inhibitor (Imatinib, Nilotinib, Avapritinib) target therapy - multikinase inhibitor (Regorafenib) target therapy	n.a.
BRAF mutations	Direct sequencing, NGS	- BRAF-inhibitors associated with MEK-inhibitors target therapy	n.a.
Copy number alterations: p1 loss, 3p-3q loss, 8p loss	MLPA analysis	n.a.	Poor prognosis; high risk for systemic metastasization

Table 10. Summary of most relevant findings emerged from the present study. Abbreviations: n.a., not available data; MLPA, Multiplex Ligation-dependent Probe Amplification; TCE, T-cells exclusion.

In the cohort of MM patients analyzed in our study, PDL-1 expression is almost absent and BRAF mutation was never observed. Conversely, tumor-infiltrating T cells were found in a small fraction of cases, which were characterized by better prognosis both

in terms of OS and DFS. Similarly, NRAS mutations were observed in 32.2% of cases and they were associated with improved prognosis in terms of OS. In addition, we found PTEN loss in 48% of MM samples, with very low immunoreactivity scores also in cases which were positives. Remarkably, we observed a better clinical response to anti-PD1 immunotherapy in patients affected by metastatic SN-MM if PTEN expression was preserved. This might support the hypothesis that PTEN loss can represent a T cell exclusion mechanism in MM and may be involved immunotherapy resistance in this subset of patients. Weiyi Peng etc. first proved that loss of PTEN was associated with reduced number and impaired function of tumor-infiltrating T cells, and poor response to anti-PD-1 treatment in cutaneous melanoma patients (80). Emerging studies tried to tackle the specific mechanism of T cell exclusion mediated by PTEN, and found that PTEN loss was associated with increased expression of certain immune suppressive genes like forkhead box P3 (FOXP3), IDO1, and VEGFA (85). There is also evidence suggesting that PI3K pathway activation after PTEN loss may contribute to T cell exclusion, as PI3K targeting drugs can synergize with immunotherapy in the treatment of melanoma with PTEN loss (86). In this regard, a recent study performed on multiple solid tumor types found that both PTEN loss and activation of PI3K pathway were associated with reduced T cell infiltration and enhanced immune suppressive status (81). The correlation of PTEN loss with poor response to immunotherapy was also verified with publicly available data from immunotherapy trials currently ongoing for several cancer types. In the era of immunotherapy and precision medicine, our findings, together with evidences emerging from literature, will translate into the molecular approach to classify the subpopulation of MM patients that have greater chance of responding to immunotherapy. Also, with the implication on the mechanism by which PTEN modulate the immune microenvironment, we might be able to develop strategy to manipulate immune landscape and augment the therapeutic efficacy of immunotherapy in the clinical practice. Inhibitors targeting PI3K-Akt-mTOR pathway had shown promising efficacy in improving immunotherapy response in selected cancer type (80, 86-87). However, considering the multifaceted function of PTEN in all kinds of biological process, further study is warranted to bring in-depth

understanding into its role in immune microenvironment and immunotherapy before we can actually exploit this knowledge in clinical practice.

Tumor mutational burden (TMB) represents another factor that can be used as a potential prognostic biomarker for cancer patients treated with immune checkpoint inhibitors (88). The total number of mutations per megabase (excluding synonymous mutations) in tumor tissue is called TMB, which reflects the overall burden of tumor antigens. The key point of immunotherapy is to arouse and strengthen the host's immune system to kill the tumor. Theoretically, the higher the TMB or mutation rate in a cancer cell, the more likely it is to be recognized by the immune system, consequently improving immunotherapy efficacy (88). Generally, in presence of a strong driver mutation, the TMB is low. In this regards, lower TMB was observed in BRAF-V600-mutated cutaneous melanoma than in BRAF wild type tumors (89). A possible explanation includes the fact that BRAFV600 melanoma arises early in life with less opportunity for cumulative ultraviolet damage, whereas high TMB is associated with increased age and UV exposure. Further investigation into such possibility is warranted. When considering MM, data available in literature are scarce but seems to suggest that it is characterized by a lower TMB while has a much higher level of structure variation and chromosomal instability compared to CM.

Our analysis has shown that MM has a diverse range of genetic and genomic alterations in several biological pathways. However, somatic mutation rates, at least considering those genes that we addressed in this study, are considerably lower in MM and do not display the UV mutational signatures, as compared to UV-exposed cutaneous melanoma. In details, in our cohort of SN-MM, we found NRAS mutations in 32.2% of cases, KRAS mutations in 6.5% of cases, KIT mutations in 6.5% of cases, while PIK3CA, RET and CTNNB1 mutations in 3% of cases, each. Remarkably, no BRAF mutations were found, which is significantly in contrast with the classical molecular profile of CM and strongly argues against the clinical efficacy of BRAF inhibitors in such groups of patients. Conversely, other forms of target therapy specifically addressing the mutated genes may be used in MM.

NRAS mutations in melanoma, although considered as undruggable targets until recently, are emerging as potential actionable driver mutations using MEK-inhibitors target therapy and PI3K-AKT-mTOR inhibitors associated with CDK4/6

inhibitors. Data on MM are lacking so far but clinical trials are ongoing in order to investigate the efficacy of such drugs in selected MM patients (58, 60).

Mutations in KRAS, such as the G12C mutation, are found in most pancreatic, half of colorectal and a third of lung cancer cases and is thus responsible for a substantial proportion of cancer deaths (90). Consequently, KRAS has been the subject of exhaustive drug-targeting efforts over the past decades. These efforts have included targeting the KRAS protein itself but also its post-translational modifications, membrane localization, protein-protein interactions and downstream signalling pathways. Most of these strategies have failed and no KRAS-specific drugs have yet been approved. However, for one specific mutation, KRASG12C, there is light on the horizon. MRTX849 was recently identified as a potent, selective and covalent KRASG12C inhibitor that possesses favorable drug-like properties. MRTX849 selectively modifies the mutant cysteine residue in GDP-bound KRASG12C and inhibits GTP-loading and downstream KRAS-dependent signalling. The drug inhibits the in vivo growth of multiple KRASG12C -mutant cell line xenografts, causes tumour regression in patient-derived xenograft models and shows striking responses in combination with other agents. It has also produced objective responses in patients with mutant-specific lung and colorectal cancer (90). Future studies on CM and MM are required in order to investigate the potential of such drug in the treatment of KRAS-mutated MM.

The role of KIT as an oncogene and its potential for target therapy is well known in GIST and recently applied also in selected cases of KIT-mutated metastatic melanoma using Imatinb or Nilotinib drugs (62-63). Responses were only seen in patients harboring KIT mutation and not in those with KIT amplifications (62-63). In a case-report of a vulvar MM harboring an uncommon mutation in KIT exon 17 (N822K) activating the loop domain of the receptor, avapritinib proved to be effective even in the presence of a pretreated disease (failure of the combined immunotherapy ipilimumab/nivolumab) and brain metastases (91).

In the future, targeted therapy could offer an alternative adjuvant therapy option for a group of patients based on their gene sequencing results. If actionable driver mutations are identified in an individual MM, targeted therapies for the driver genes or proteins could be utilized on a patient-by-patient basis. Since the number of MM

patients harboring druggable mutations is limited so far, future efforts on developing other alternative targeting strategies based on mutated genes such as splicesome complex components, telomerase, and DNA repair pathway are required.

The genetic and molecular profile of melanoma might have also an impact on the immune composition of the tumor microenvironment. Increasing numbers of studies have explored how somatic alterations influence the response of immunotherapy through immunogenicity and the immune microenvironment (89-92).

Constitutive activation of the mitogen-activated protein kinase pathway in BRAFV600 melanoma regulates cytokine production, which may ultimately influence immune regulation within the TME (92). A recent retrospective analysis of patients included in the CheckMate 066 and 067 phase III clinical trials evaluating response to immuno-oncology therapies in advanced melanoma, improved survival was observed with high TMB and absence of BRAF mutation (89).

Similarly, tumors with NRAS mutation are reported to have low tumor-infiltrating lymphocyte grades, suggesting a more immunosuppressive microenvironment (92). Activation of the RAS pathway can decrease antigen-presenting major histocompatibility complex (MHC) I molecule expression and reduce the number of infiltrating immune cells in tumors (92), which may weaken the antitumor activity of immune checkpoint inhibitors. In this regards, the combination of immunotherapy with MEK inhibitors for NRAS mutated melanoma is controversial. MEK inhibition not only resulted in an accumulation of intratumoral antigen-specific T cells but also impaired T cell priming in lymph nodes (92). The phase 3 clinical trial of atezolizumab (anti-PDL-1) combined with cobimetinib (MEK-inhibitor) in metastatic melanoma failed to demonstrate superior survival over anti-PD-1 monotherapy (93). Further studies are focusing on the sequence of immunotherapy and targeted therapy and dosing schedules such as intermittent versus continuous dosing of MAPK inhibitors (94). Other combination strategies, including drugs targeting RAS, the PI3K/AKT/mTOR pathway, CDK, and alternative immune checkpoint inhibitors, are also being investigated.

The impact of an activating KRAS mutation on the immune composition of the melanoma tumor microenvironment is not well described. It has been reported that activating KRAS mutations has been shown to facilitate immune suppression via

metabolic reprogramming, which leads to increased glycolysis and myeloid-derived suppressor cell recruitment in colon cancer (95) and triple negative breast cancer (96).

A comprehensive understanding of the mechanisms by which somatic mutations influence the immune landscape and molecular network in the tumor microenvironment will be critical in the future to clarify this problem.

Unfortunately, in our series of SN-MM, no statistically significant associations were observed between response rates to immunotherapy and somatic genetic mutations in NRAS, KRAS and KIT. This can be probably explained by the fact that immunotherapy was introduced in our current treatment protocol for SN-MM only in the last five years and was used in monotherapy (anti-PDL1) or combined therapy (anti-PDL-1/anti-CTLA4) as palliative treatment in very advanced stages of disease (unresectable cancer or metastatic disease). This may have precluded efficacy of immunotherapy, regardless the mutational status and the immune contexture of the disease. Moreover, the large majority of patients treated with immunotherapy in our cohort resulted to be wild type for somatic mutations in our retrospective molecular analysis. Further studies analyzing interaction between TMB, occurrence of driver somatic mutations and immune contexture of MM are required in order to better understand the potential of such biomarkers in predicting which patients are most likely to benefit from such innovative treatments.

In the last two years, an early double immune checkpoint inhibitors therapy with nivolumab (anti-PD1) associated to ipilimumab (anti-CTLA4) was used in three patients affected by SN-MM (not included in the present series) as adjuvant treatment after surgery (two cases) and as neoadjuvant treatment before surgery (one case). The results emerging from such protocols are preliminary but encouraging. We are convinced that moving the use of effective systemic therapy to earlier stages of disease may result in higher cure rates from MM. Trials combining targeted therapy with immunotherapy either concurrently or sequentially are currently ongoing. Results from these and other biomarker-driven trials will help shape the future armamentarium in localized and advanced MM, aiming to achieve a personalized therapy based on the molecular signature of an individual tumor.

In addition to their implications in the treatment choice using both target therapy and immunotherapy or combinations of them, the presence of somatic

mutations has been investigated also for their potential role as prognosticators in melanoma. Also in this case, data emerging from literature are controversial. Amit et al. found no association between survival outcomes and mutational status from a retrospective analysis of 66 patients affected by SN-MM, where the prevalence of somatic alterations included NRAS mutation in 30% of cases and KIT mutation in 5% of cases (97). This incidence of mutations in NRAS and KIT was in line with our findings. However, in our study, we found a significant impact of mutational status on prognosis of SN-MM. In details, the occurrence of NRAS mutations was associated with statistically significant improvements in OS ($p=0.05$) while KRAS mutations was correlated with worst OS ($p=0.01$) and DFS outcomes ($p=0.01$). Despite the dismal overall prognosis of such cancer, the occurrence of NRAS or KRAS mutation might be used in the future to stratify patients in long-survivors (NRAS-mutated/KRAS-wt), who can experience multiple recurrences at local or regional sites but late systemic dissemination of disease, and short-survivors (NRAS-wt/KRAS-mutated) with early metastatic spread of disease and OS rates less than 3 years. Therefore, given their therapeutic and prognostic impact, BRAF, NRAS, KRAS and KIT mutations should be incorporated into routine clinical testing for MM in the precision medicine era.

Finally, the analysis of specific somatic copy number alterations in our series of SN-MM was able to reveal some chromosomal abnormalities associated with poor overall prognosis (loss of 1p, loss of 3p/3q, and loss of 8p) and short DFS with early metastasization rates (loss of 1p and loss of 3p/3q). Prognostication was improved by considering chromosome 3 losses together with 1p loss and 8p loss, which was found to be an independent predictor of poor overall outcome and early metastatic dissemination of disease, and might represent a multichromosome copy number aberration signature that can add a specific HR value for classification of risk categories. This results is innovative in the field of MM and is in line with findings already described for UM where it has been demonstrated that monosomy of chromosome 3 was associated with increased risk of metastasis (69-72). At present, cytogenetic abnormalities observed both in MM and UM do not have direct implications in terms of molecular-based therapeutic approaches. However, the multichromosome copy number aberration signature described in our series may represent a step forward in the management of SN-MM because it might help in

identifying patients at high risk of early recurrence and systemic dissemination spread who could be candidate to intensified treatment strategies including radical surgical resection, carbon ion irradiation in case of involved surgical margins or unresectable disease, combined double checkpoint immune inhibitors with anti-PDL-1 and anti-CTLA4 immunotherapy in early stages of disease (neoadjuvant or adjuvant setting), and specific forms of target therapy to be developed according to the mutational status of the disease.

CONCLUSIONS

Mucosal melanoma has been shown to be significantly different from cutaneous melanoma with regard to its pathogenesis and epidemiologic/clinical characteristics. As such, it is important to evaluate these patients as a separate subset in order to give patients realistic expectations for their disease course and to propose them specific forms of treatment.

We found that MM is a *noninflammed* tumor with an immune contexture poor of CD45, CD8 and CD3 positive cells. In addition to the scarce immune infiltration, PDL-1 expression is almost absent in MM. This "cold" immune contexture may explain the limited efficacy of immunotherapy, even in the form of double immune checkpoint inhibitor anti-PD-1/anti-CTLA4, which has been observed in MM patients. Therefore, additional prospective investigations are necessary to clarify the role of immunotherapy in MM patients. Further investigations on the role of PTEN loss as responsible of TCE from the immune contexture of MM should be performed. The molecularly characterized four SN-MM cell lines obtained in the present work will represent cellular models for further "in vitro" studies aimed to decipher mechanisms of TCE in MM. Our SN-MM cell lines are heterogeneous in term of PTEN expression (respectively PTEN sufficient for SN-MM1 and SN-MM3; PTEN deficient for SN-MM2 and SN-MM4). Loss of PTEN in cutaneous melanoma promotes resistance to immune infiltration of melanoma through the modulation of CCL21, CXCL1, CXCL10, CCL2 and VEGF (80). Currently, we are analyzing a large set of TCE-modulating genes, which will be correlated with the PTEN phenotype and the PI3K activation status. Other mechanisms of TCE from the immune contexture of MM are currently under investigation by our research group. In details, the interaction between plasmacytoid dendritic cells (PDCs) and MM cells should be explored. PDCs play an important anti-tumor role bridging the innate and adaptive immune system via the production of high amounts of type I interferons (I-IFNs) and proinflammatory cytokines (e.g. IL-6 and TNF-α), following TLR-7/9 activation. We hypothesize that the functional impairment of PDC might partially explain the TCE observed in MMs. PDC re-activation via TLR-agonist might amplify the local and systemic anti-tumor

response and execute direct elimination of melanoma cells. We are planning to perform further experiments on MM cell lines using a set of TLR-7/9 agonists (including R848 and CpG-A ODNs) as stimuli to overcome TCE. Based on future results emerging from our investigations, we will be able to understand if administration of the TLR-7/9 agonists and vaccination with PDCs might induce favorable T cell responses in patients with MM and could emerge as a new therapeutic option both in monotherapy and in addition to other well-known immune check point inhibitors.

When considering the molecular landscape of MM, it appears clear that the set of preliminary genetic alterations found in the present study, although of potential relevance for a small fraction of cases, is still insufficient to open windows of opportunity for large use of target therapies in the treatment of MM patients. We found somatic mutations only in 14/31 cases, mainly involving NRAS, KRAS and KIT. No BRAF mutations were found, in contrast with the genetic fingerprint of cutaneous melanoma. Therefore, we are convinced that additional, unknown abnormalities might drive or cooperate in sunlight-independent melanoma genesis. A future step of the research will be devoted to explore other genomic variants of MM.

From genomic viewpoint, we described in this study a multichromosome copy number aberration signature characterized by chromosome 3p-q losses together with 1p loss and 8 p loss, which is associated with poor prognosis and early systemic metastasization risk. Although this genomic signature at present does not have a direct therapeutic implication, it may be useful in identifying patients at high risk for early dissemination of disease and poor prognosis, who might benefit from intensification of systemic treatments in neoadjuvant or adjuvant setting.

Future approaches to characterize these tumors should include also a combination of transcriptomic and proteomic analyses to better understand the complexities of the cellular effects and potential vulnerabilities conferred by these key changes. This will finally support the translation of personalized cancer medicine into the clinical management of sinonasal mucosal melanoma, which will surely lead to improved survival for this rare and aggressive group of cancers.

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