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EXPLORING THE ROLE OF NICOTINAMIDE N-METHYLTRANSFERASE IN SKIN CANCERS: ANALYSES PERFORMED ON TISSUE SAMPLES AND CELL CULTURES.

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1 INTRODUCTION

1.1 ANATOMY OF THE SKIN

The skin, the largest organ of the body, covers an area of approximately 2 m^2 and accounts for about 20% of total adult body weight. It consists of three layers of different embryologic origin: the epidermis and its appendages develop from the ectoderm, while the dermis and the hypodermis are of mesodermal origin (1).

The integument shows regional differences in terms of thickness (varying from 1 to 4 mm), distribution of epidermal appendages and density of melanocytes (2-4). It represents the interface between human body and environment, and for this reason it functions as a protective barrier against the ultraviolet rays as well as chemical, physical, and microbial insults (1). Moreover, the skin is the largest sense organ, thanks to the presence of many tactile and thermal receptors and nociceptors. As concerns its metabolic function, it is carried out by subcutaneous adipocytes containing very large reserves of triglycerides, which provide the body's energy needs for a very long time. In addition, the skin plays an important role in thermoregulation: the adipose tissue and hairs prevent heat loss while sweat production by glands and dermal blood flow allow its dispersion (5).

1.1.1 Epidermis

The outermost layer of the skin is the epidermis, a stratified squamous keratinised epithelium (Malpighian) that renews itself continuously and it is composed by several layers (5). It is rather thin even if, in specific areas, such as the palms and the soles of the feet, its thickness can reach 1.4 mm. Epidermal thickenings (called Rete ridges or Rete pegs) extend downward between dermal papillae in order to achieve a better cohesion. Epidermis itself has no blood supply and capillaries are found in dermis, where they are linked to arterioles and venules (6). It consists of different cell types, among which keratinocytes are the most abundant, accounting for 90-95% of all epidermal cells. The remaining 5-10 % is represented by Merkel cells, Langerhans cells and melanocytes. Keratinocytes are ectoderm-derived cells which differentiate in corneocytes, anucleate and flattened cells. They are interconnected with desmosomes and organized in 4 or 5 *strata*, depending on the skin region. In particular, from the bottom to top, epidermal layers are: basal (*stratum basale*, also called *stratum germinativum*), spinous (*stratum spinosum*), granular (*stratum granulosum*), clear/translucent (*stratum lucidum*) and cornified layer (*stratum corneum*) (7) (Figure 1).

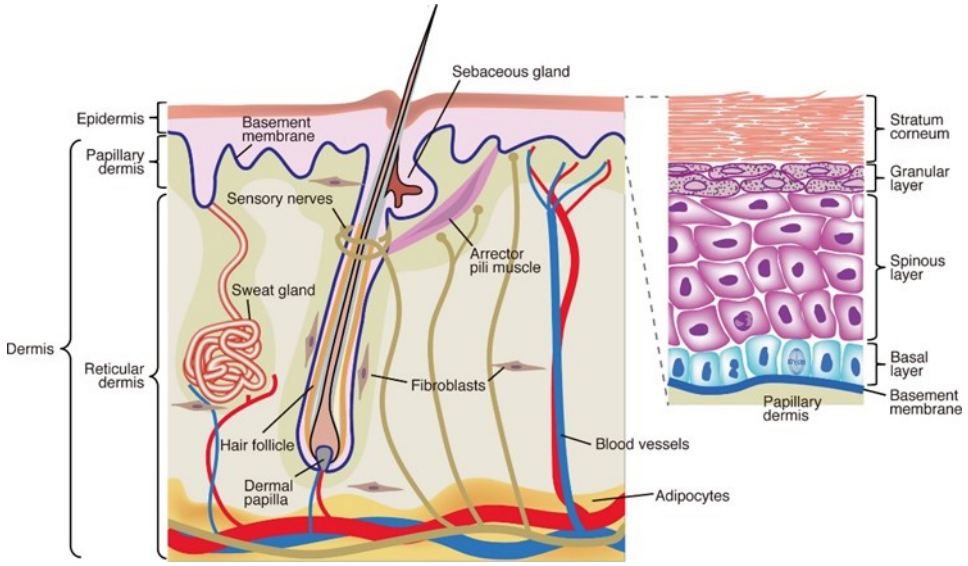

Figure 1. Mammalian skin architecture. The skin is composed of two major layers, the epidermis and dermis, which are separated by the basement membrane (dark blue line). The epidermis is a stratified epithelium maintained by the proliferation of stem cells residing in the basal layer and their progressive differentiation to form the spinous, granular and terminally differentiated cornified layers (right panel) (8).

The *stratum basale* is a monolayer of cuboidal or columnar cells connected to each other, to spinous cells and to the basal membrane via desmosomes and hemidesmosomes. Their large and ovoid nucleus occupies most of the cell and their cytoplasm is basophilic. Basal keratinocytes also contain melanosomes and loose bundles of tonofilaments, electron-dense cytoplasmic intermediate filaments consisting of keratin polypeptides which contribute to the cytoskeleton structure and insert into the desmosome plaques. This layer represents the germinative or proliferative compartment, responsible for the regeneration of epidermis through mitotic divisions of basal keratinocytes. These latter generate a mixture of progeny, including daughter cells that remain undifferentiated like their parent as well as others that migrate through the upper layers while differentiating. This journey takes approximately thirty days.
Unlike basal cells, spinous keratinocytes are large and polygonal. Their nucleus is vesicular, and they have an eosinophilic cytoplasm rich in keratin tonofilaments. The spiny appearance of this layer is an artefact of the staining process and it depends on the fact that during fixation these cells shrink slightly but the desmosomes from neighbouring cells remain tightly bound to each other (9). Keratinization begins in the *stratum spinosum* and continues in the *stratum granulosum*, which represents the most superficial layer of the epidermis containing living

cells. This layer consists of flattened keratinocytes migrated from the *stratum spinosum.* Their cytoplasm is characterised by the presence of many basophilic keratohyalin granules made up of an assembly of histidine- and cysteine-rich proteins (mainly profilaggrin) involved in the aggregation of keratin filaments (10,11).

Moreover, keratinocytes of the upper *stratum spinosum* and of the *stratum granulosum* are rich in lamellar bodies containing lipids and proteins, which are delivered to the extracellular spaces and are involved in the desquamation process and in the formation of a lipidic pericellular coat. In addition to secreting these bodies, keratinocytes start to lose their nuclei and organelles, thus becoming non-viable corneocytes in the *stratum corneum,* the outermost layer of epidermis, composed of 15–20 layers of non-viable flattened cells rich in keratin filaments and surrounded by a lipid extracellular matrix, which contributes to the skin's barrier function. During cornification process, the cell membrane is replaced by a layer of ceramides which become covalently linked to an envelope of structural proteins (the cornified envelope). Corneodesmosomes (modified desmosomes) facilitate cellular adhesion. These complexes are degraded by proteases, eventually permitting cells to be shed at the surface. As concerns the *stratum lucidum*, it is a thin, clear layer of flattened dead keratinocytes located between the granular and cornified layers of thick skin (11-15).

Unlike keratinocytes, melanocytes are neural crest-derived cells (5), mainly located in the epidermal basal layer, but also in the middle layer of the eye (the uvea), the inner ear, vaginal epithelium, meninges, bones, and heart (16-21). These dendritic cells produce melanin, a pigment displaying important biological function because of its ability to act both as an oxidant scavenger and as UV absorbing system, thus protecting neighbouring cells from UV-mediated DNA damage. In humans, melanocytes synthesize two different classes of melanin: the brown-black pigment eumelanin, and the yellow-red pigment pheomelanin. Melanin is contained in specialized organelles called melanosomes, which can be transferred to surrounding keratinocytes, thus inducing pigmentation (5).

5-8% of epidermal cells is represented by Langerhans cells, myeloid cells originated from monocytes and located in the squamous layer. They also occur in the papillary dermis, mainly around blood vessels, as well as in the oral mucosa, foreskin, and vaginal epithelium. Moreover, these cells can be observed in other tissues and organs, such as lymph nodes, particularly in association with Langerhans cell histiocytosis (22,23). Their peculiarity lies in the presence of rod-shaped or "tennis-racket" cytoplasmic organelles with a central linear density and a striated appearance, called Birbeck granules. In skin infections, the local Langerhans cells take up and process microbial antigens to become fully functional antigen-presenting cells. They can also

take on a dendritic cell-like phenotype and migrate to lymph nodes to interact with naive T-cells (24).

As regards Merkel cells, they are found in the *stratum basale* as well as rete ridges and they are considered as modified keratinocytes with epithelial origin. These oval-shaped cells function as mechanoreceptors and are essential for light touch sensation. For this reason, they are abundant in highly sensitive skin and make synaptic contacts with somatosensory afferent nerve fibres (25,26).

1.2 NON-MELANOMA SKIN CANCERS

Each epidermal cell type can undergo neoplastic transformation with the subsequent development of skin cancers, which can be divided into melanoma and non-melanoma skin cancers (NMSCs). NMSCs represent a broad group of cutaneous malignancies, including both common keratinocyte carcinomas and rare neoplasms such as Merkel cell and adnexal carcinomas as well as cutaneous sarcomas. As regards keratinocyte carcinomas, they are the most frequently diagnosed cancers worldwide and they show a constantly increasing incidence that exceeds all other malignancies combined, thus becoming a rising global healthcare problem. The most common forms of NMSCs are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which account for 80% and 20% of NMSCs, respectively, although recent studies have showed an increasing SCC incidence moving the historical 4:1 ratio to 2,5:1. Even if most cancer registries exclude keratinocyte cancers because of their ubiquity and relatively low mortality rates, numerous studies provide evidence for a growing incidence worldwide (27).

1.2.1 Basal cell carcinoma

BCC derives from basal keratinocytes and appears as a painless raised and shiny cutaneous area surrounded by small blood vessels (28). Despite its low mortality, linked to its slow growth and low metastatic potential, BCC can cause significant morbidity because of its capability to invade and destroy the surrounding tissues. This cancer is more common among white population and the incidence in men is 30% higher than in women. The lifetime risk of developing a BCC is 30%. Moreover, there is a positive correlation between BCC incidence and proximity to the equator. A study performed in the USA on a sex-stratified cohort of people between 1986 and 2016, showed that BCC incidence rate increased from 519 to 1019 cases per

100,000 person-years for women and from 606 to 1488 cases per 100,000 person-years for men. The same results have been observed in Europe, Canada, Asia and Australia. As regards the age, it seems to be an independent risk factor. BCC can develop in early life, but the incidence rate doubles from 40 to 70 years of age. In addition, after age 40 gender differences do not exist (29). BCC arises in sun-damaged skin and present well-defined subtypes with distinct clinical and histopathological findings (Table 1). All variants are more common after the sixth decade of life and their identification is important for the development of an appropriate treatment (30).

Table 1. Clinical and histopathologic findings of basal cell carcinoma subtypes (Cameron et al. 2019).

	Clinical features	Histopathologic features
Nodular	Shiny, pearly papule or nodule with a smooth surface, rolled borders, and arborizing telangiectasias with a predilection for head and neck	Discrete nests of malignant basaloid cells in the dermis, peripheral palisading, and mucoid stroma containing plump spindle cells
Superficial	Well-circumscribed and erythematous thin plaque or patch with scale, central clearing, and thin rolled borders; most common on the trunk	Multiple lobular foci of basaloid palisading keratinocyte tumors attached superficially to the epidermis with a myxoid stroma and band-like lichenoid infiltrate
Infundibulocystic	Well-circumscribed pearly papule commonly found on the head and neck of the elderly	Well-circumscribed, anastomosing strands of basaloid cells and scattered infundibulum-like cystic structures
Fibroepithelial	Skin-colored or erythematous sessile plaque or pedunculated papulonodule with a predilection for the trunk	Multiple collections of delicate strands of epidermal basaloid keratinocytes arranged in a reticular pattern within a spindle cell stroma
Morpheaform	Infiltrated plaque with poorly defined borders and shiny surface commonly found on the head and neck	Thin cords of basaloid cells surrounded by a sclerotic collagenous stroma, with mostly absent peripheral palisading and stromal cleft formation; positive staining of tumor stroma with smooth muscle alpha-actin
Infiltrative	Poorly defined, indurated, flat or depressed plaque with white, yellow, or pale pink color that may have overlying crusts, erosions, ulcerations, or papules	Thin cords with angulated ends of few basaloid keratinocytes, embedded in a classic mucinous/ myxoid stroma
Micronodular	Erythematous macule or thin papule/plaque	Multiple small aggregates of basaloid cells within the dermis, with subtle peripheral palisading and retraction artifact
Basosquamous	Majority found on the head and neck	Well-defined nodular or superficial BCC component overlying an invasive front showing BCC and SCC histologic features

The most common but least aggressive subtype is the nodular one, which accounts for about 50% to 80% of lesions. This variant is more frequent in the head and neck region and typically present as a shiny, pearly and pinkish papule or nodule with a smooth surface, a slightly elevated rolled border, and arborizing telangiectasias. Nodular BCC grows slow but in advanced cases it can become large and ulcerate (Figure 2, A-C). Histopathologically, this variant shows large

dermal nodules of malignant basaloid keratinocytes, peripheral palisading and mucoid stroma containing plump spindle cells (Figure 2, D).

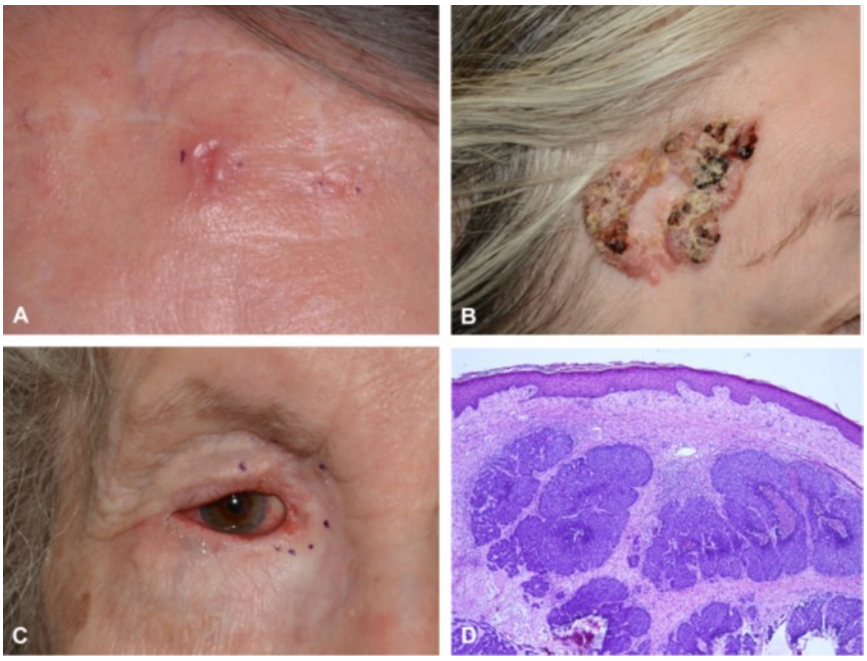

Figure 2. **A**) Nodular BCC presenting as a shiny, pearly papule with a smooth surface, rolled borders, and overlying arborizing telangiectasias. **B**) Large, advanced nodular BCC on the temple. **C**) Nodular BCC of the right eye causing free margin distortion. **D**) Haematoxylin and eosin staining of nodular BCC showing large dermal nodules of malignant basaloid keratinocytes, peripheral palisading, and mucoid stroma containing plump spindle cells (Cameron et al 2019).

The superficial subtype represents the second most common BCC variant. It accounts for about 10% to 30% of BCCs and present as a well circumscribed and erythematous thin plaque or patch with scale, central clearing, and thin rolled borders (Figure 3, A). Similar to nodular subtype, it can contain melanin (Figure 3, B). This variant mainly occurs on the trunk and legs, while it is less frequently observed in the head and neck region (29). Microscopically, superficial BCCs show multiple lobular foci of basaloid palisading neoplastic keratinocytes attached superficially to epidermis with a myxoid stroma and band-like lichenoid infiltrate (Figure 3, C).

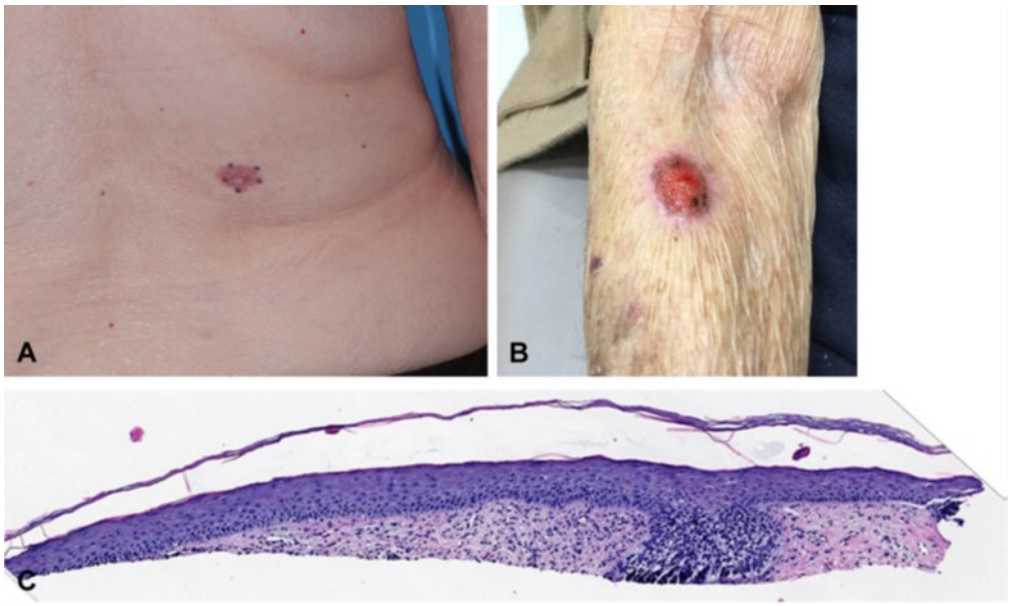

Figure 3. **A**) Superficial BCC. **B**) Pigmented superficial BCC. C) Haematoxylin and eosin staining of superficial BCC (Cameron et al 2019).

Another subtype with the predilection for the trunk is the fibroepithelial one. This uncommon lesion presents as a skin-colored or erythematous sessile plaque or pedunculated papulonodule (Figure 4, A). The histopathologic examination reveals strands of basaloid keratinocytes arranged in a reticular pattern (Figure 4, B) (31).

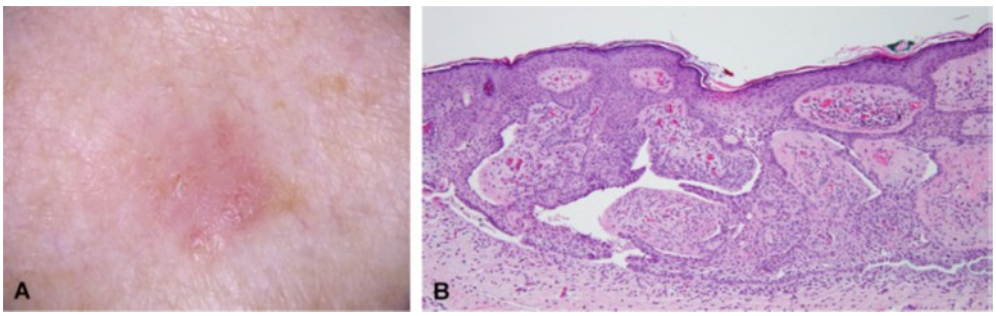

Figure 4. **A**) Fibroepithelial BCC. **B**) Haematoxylin and eosin staining demonstrating delicate strands of epidermal basaloid keratinocytes arranged in a reticular pattern within a spindle cell stroma (Cameron et al 2019).

On the contrary, infundibulocystic BCCs usually arise on the head and neck and present as well-circumscribed tumours composed of anastomosing strand of basaloid cells and scattered small infundibulum-like cystic structures (Figure 5) (32).

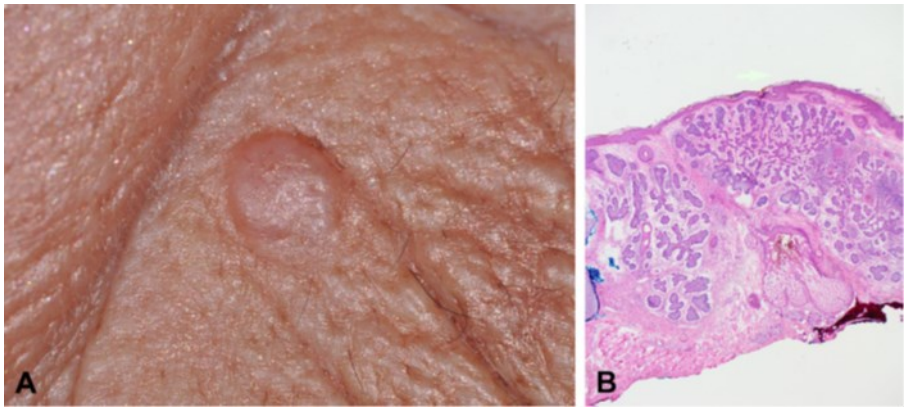

Figure 5. **A**) Infundibulocystic BCC presenting as a well-circumscribed pearly papule. **B**) Haematoxylin and eosin staining of infundibulocystic BCC showing anastomosing strands of basaloid cells and scattered small infundibulum-like cystic structures (Cameron et al 2019).

Less than 10% of BCCs has morpheaform and infiltrative characteristics. These lesions are difficult to treat, because of their aggressive behaviour with local destruction and subclinical extension as well as recurrence (9). Clinically, morpheaform BCC presents as a infiltrated plaque with shiny surface and without distinct borders (Figure 6, A) that rarely ulcerates, while infiltrative subtype appears as a poorly-defined, indurated, flat or depressed plaque with white, yellow or pale pink colour, which can be characterized by crusts, erosions, ulcerations and papules. The most common localization is in the upper part of the trunk or the face (Figure 6, B). Both variants are characterised by basaloid keratinocytes forming thin cords with angulated ends. In infiltrative BCC, however, these cells are embedded in a classic mucinous/myxoid stroma (Figure 6, C), while morpheaform lesions show a heavy stromal collagenization (Figure 6, D).

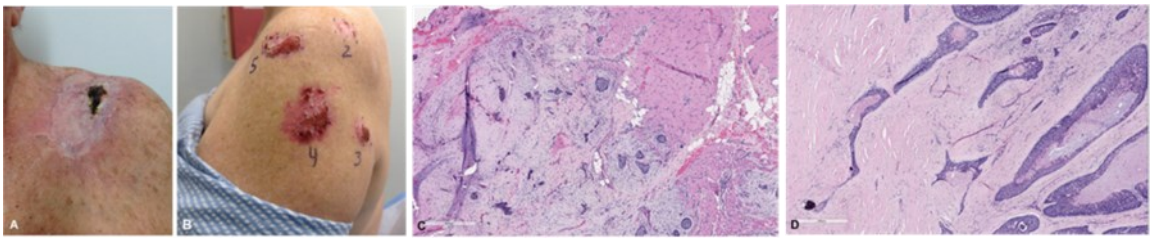

Figure 6. **A**) Morpheaform BCC. **B**) Multiple infiltrative BCCs. **C**) Haematoxylin and eosin staining of infiltrative BCC. **D**) Haematoxylin and eosin staining of morpheaform BCC (Cameron et al 2019).

Micronodular and basosquamous are two additional descriptors with primarily histopathologic significance. Micronodular BCCs are characterised by multiple aggregates of basaloid cells within the dermis and show peripheral palisading and retraction artifact, like nodular lesions. They are more difficult to treat because of their multifocal nature. Moreover, it is hard to distinguish them from superficial and nodular variants (Figure 7, A and B). As regards basosquamous lesions, they share histologic features with both BCC and SCC. In particular, they show a well-defined nodular or superficial BCC component overlying an invasive basal or squamous front (Figure 7, C) (33). In addition, immunohistochemical analysis evidenced a transition zone between BCC and SCC areas, thus suggesting the differentiation of one of the tumours into the other. This variant generally occurs on the head and neck region and is characterised by an aggressive behaviour, because of its elevated recurrence and metastatic rate (29).

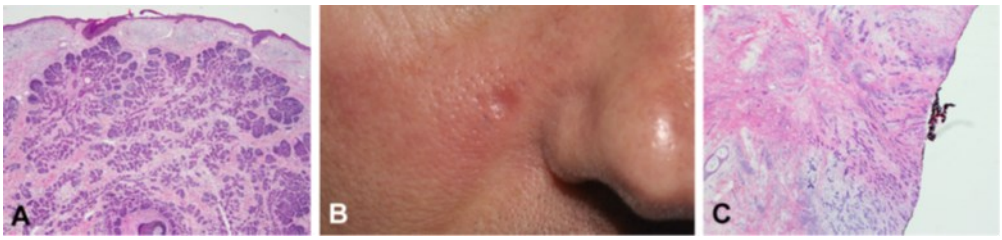

Figure 7. A) Haematoxylin and eosin staining of micronodular BCC. **B**) Micronodular BCC. **C**) Haematoxylin and eosin staining of basosquamous BCC (Cameron et al 2019).

While BCCs progress indolently through local invasion, a small subset develops into locally advanced BCC or metastatic BCC tumours. Metastases are extremely uncommon, and they can occur haematologically or through subcutaneous invasion, although lymphatic spread accounts for about 70% of metastatic cases. Micronodular, infiltrative, morpheaphorm and basosquamous BCCs represent the variants which are more likely to recur and metastasize. The metastatic sites include lymph nodes, bone, lungs, and skin. Although median metastatic BCC survival has traditionally been deemed 8 months, (29) perineural involvement (PNI), associated with metastasis production, is rare, with an incidence of less than 1% but it is an independent risk factor for recurrence and is more common with aggressive subtypes (34).

Sporadic BCCs arise from long-term resident keratinocyte progenitor cells of the interfollicular epidermis and the upper infundibulum undergoing UV-mediated mutagenesis and all BCCs show a constitutive activation of Hedgehog signalling pathway (Hh) that promotes tumorigenesis via its effector protein sonic hedgehog (Shh). In particular, this pathway is involved in neural, musculoskeletal, haematopoietic, and skin development through the regulation of embryonic development and adult tissue homeostasis, as well as cell type differentiation, patterning, and proliferation. In addition, it maintains the stem cell population of epidermis (35). Normally, hedgehog ligand activates the pathway by binding and inhibiting PTCH1, allowing the depression of smoothened (SMO) which can activate zinc finger transcription factors GLI. GLI controls Hh pathway responsibilities regulating the expression of Hh target genes (36). Moreover, this pathway is involved in cell cycle regulation at the G2/M checkpoint. Shh binding to PTCH1 allows cyclin B1 nuclear translocation and cell cycle progression to mitosis (34). Moreover, Shh also exerts its activity at G1/S checkpoint by upregulating cyclin D1 and inhibiting cyclin-dependent kinase inhibitor 1A (37). The most common mutations in BCCs are inactivating mutations of PTCH1 or SMO activating mutations, which cause an aberrant activation of Hh pathway which can lead to tumour formation. Furthermore, a loss-of-function mutation in SUFU (suppressor of fused homolog), a negative regulator of the hedgehog pathway, has been identified in a small portion of BCCs. In addition, UV-specific defects in the p53 tumour suppressor gene have been detected in about 50% of basal cell carcinomas (38). An aberrant upregulation of the Hh pathway, resulting from loss of PTCH1 protein function, is typical of basal cell nevus syndrome (BCNS), characterized by the development of multiple neoplasms (including BCCs), 26% to 50% of which are *de novo* (39). BCC incidence is higher in patients affected by xeroderma pigmentosum, a rare autosomal recessive disorder due to defects in nucleotide excision repair pathway proteins, Bazex-Dupre-Christol syndrome, a rare X-linked dominant disorder, and Rombo syndrome (29). Other

genodermatoses characterized by increased BCC risk include disorders of DNA replication/repair functions (Werner, Rothmund-Thomson, Bloom, and Muir-Torre syndromes), those affecting the immune response (cartilage-hair hypoplasia, epidermodysplasia verruciformis), folliculo-sebaceus unit (Brooke-Spiegler, Schöpf-Schulz-Passarge, and Cowden syndromes), melanin biosynthesis (oculocutaneous albinism and Hermansky-Pudlak syndrome), and some epidermal nevus syndromes (40).

Recent studies have demonstrated that BCC shows the greatest number of mutations of any human cancer, because of the ubiquity UV light which represents the primary source of mutagenesis. Intermittent sun exposure is a predominant environmental risk factor predisposing to the development of BCC. Nevertheless, other studies demonstrated an increased risk in subjects with chronic and cumulative exposure signs. Moreover, it has been observed a dose-related association between tanning bed use and skin cancer risk, especially in the young population (41-42). Even skin phenotype plays an important role. In particular, due to the protective role of skin pigmentation, a 30% risk of developing a basal cell carcinoma is linked to Fitzpatrick skin types I and II, and fair skin, red or blond hair, light eye colour, an inability to tan, and a propensity to freckle are independent BCC risk factors. Other risk factors include childhood sunburns, family history of skin cancer, chronic immunosuppression, photosensitizing drugs, ionizing radiation, exposure to carcinogenic chemicals (especially arsenic) and oral contraceptive use (43-45). As regards immunosuppression, the BCC incidence is double in HIV-positive patients and 5-times to 10-times higher in transplant recipients. About 50% of subjects who have undergone transplantation is interested by BCC in the 10 years after surgery, mainly younger patients (46-47).

A formal staging system for BCC is not available, and before the seventh edition of the American Joint Committee on Cancer (AJCC) manual it was grouped with all NMSCs. A distinct staging system for SCC excluding BCC was then created. The National Comprehensive Cancer Network (NCCN) currently differentiates localized tumours at low risk from lesions at high risk of recurrence. In particular, the NCCN describes three different body areas for risk stratification based on primary tumour location. Lesions occurring on area H (central face, eyelids, eyebrows, periorbital area, nose, lips, chin, jaw, preauricular and post-auricular skin/sulci, temporal region, ear, genitals, hands and feet) are considered at high risk, independently of tumor size. Area M (consisting of cheeks, forehead, scalp, and neck) and area L (including trunk and extremities) can be classified as high-risk locations depending on tumour size and histological features (48-49). Moreover, location in the head and neck region, a tumour depth beyond subcutaneous fat, and a diameter larger than or equal to 4 cm are predictor factors

of metastasis and death. A T classification (T1, T2 and T3) based on these three characteristics has been therefore developed. T3 lesions are greater than or equal to 2 cm and contain two of the three risk factors (50).

Given its low metastatic potential, BCC treatment is directed al local control. Low-risk lesions are treated with standard excision with postoperative margin assessment (SEPMA) (clinical margins: 4 mm) (51). BCCs of any size occurring on neck, trunk, and extremities have high 5-year cure rate, while surgical excision is less effective for H-area tumours (52). Treatment of recurrent and metastatic lesions involves Mohs micrographic surgery (MMS), given its long-term cure rate due to the complete peripheral and deep margin assessment (CCPDMA). In particular, the 5-year recurrence rates for primary and recurrent BCCs treated with MMS are 1% and 5.6%, respectively, compared with 10.1% and 17.4%, respectively, for SEPMA. The 10-years recurrence rate for primary lesions is 4.4% for MMS and 12.2% for SEPMA, while for relapses is 3.9% and 13.5%, respectively (53-55). For low-risk BCCs, the NCCN recommends the curettage and electrodessication (CE), a fast and cost-effective technique. Nevertheless, CE does not allow for histologic margin assessment and is operator-dependent. Moreover, patients subjected to CE have worse cosmetic outcomes compared to those treated with MMS. In addition, cryosurgery represents a suitable strategy for the treatment of low-risk lesions (56-58).

Patients with superficial BCCs, and those with extensive/multifocal disease or diffuse actinic damage, are treated with aminolevulic acid (ALA) or methyl aminolevulinate (MAL) photodynamic therapy (59). As regards unresectable tumours or those arisen in subjects for whom surgery is contraindicated, they are treated with radiation therapy (RT), recommended by NCCN in case of large-diameter lesions or extensive PNI and age over 60 (60-61). Another strategy for low-risk superficial BCCs that cannot undergo more definitive therapies, is represented by local treatment with imiquimod and fluorouracil creams, approved by the US Food and Drugs Administration (FDA). The imiquimod cure rate is up to 81% and its efficacy is higher than that of fluorouracil and MAL-PDT (62).

Although basal cell cancers are easily cured with local therapies, patients with BCNS and advanced or metastatic lesions need systemic treatment. BCC chemotherapeutic drugs are specific Hh pathway inhibitors. Vismodegib was the first approved by FDA, but nearly all patients experienced at least one less severe adverse effect, including muscle spasms, alopecia, dysgeusia, weight loss, fatigue, or diarrhoea, while the 25% of them shows severe adverse effects. Although BCC incidence and size of existing tumours in BCNS patients is significantly reduced with vismodegib, this drug is not well tolerated. The second Hh pathway inhibitor

approved by FDA for advanced BCCs that recur after surgery or RT is sonidegib. The limitations of these two chemotherapeutic treatments are represented by the high frequency of adverse effects and the development of resistance. As concerns advanced and metastatic BCC patients, they show lower overall response rates and approximately 20% develop resistance during the first year. Another emerging treatment for the most aggressive forms of BCC could be the anti-programmed death 1 (anti-PD-1) immunotherapy, whose trial is under way (63-66).

1.2.2 Squamous Cell Carcinoma

SCC is the second most common NMSC. It derives from keratinocytes of the *stratum spinosum* and it usually presents as a hard lump with a scaly top, although it can also form ulcers. SCC is becoming an emerging public health problem due to the increase of its incidence and mortality rate. In the United States, at least 200000 to 400000 new cases are detected each year, resulting in up to 3000 expected deaths and the lifetime risk is estimated between 9% and 14% for men and 4% to 9% for women (67). From 1976 to 1984 and from 2000 to 2010, SCC incidence showed an increase of more than 263% in both genders (68). According to a European study, SCC incidence ranges from 9 to 96 per 100000 males and from 5 to 68 per 100000 females. Even in Australia it is more common in men than in women, and the mortality rate is 2 per 100000 patients (69-71). Moreover, in the USA its incidence is higher in southern and central states. In particular, 5604 to 12,572 SCC patients developed nodal metastasis, and 3932 to 8791 died from SCC in the United States in 2012 (72).

Most SCCs arise from sun damaged skin of pre-existing precursor lesions. Actinic keratosis (AK) represents and early precursor lesion that can accumulate mutations and, in some cases, can progress to squamous cell carcinoma *in situ* (SCCIS) and then to invasive SCC. Histologically, AK consists in a proliferation of atypical keratinocytes confined in the lower layers of epidermis. These cells show an increased size, a loss of polarity, pleomorphic and hyperchromatic nuclei, and an augmented nuclear/cytoplasm ratio. The neoplastic keratinocytes crowd the basal layer and the papillary dermis without breaching the basement membrane. Moreover, an hyperkeratotic area can be observed in the *stratum corneum* overlying the atypical cells. From a histological point of view, AK appears as areas of parakeratosis alternated with orthokeratosis, since the preneoplastic process does not affect the adnexal structures.

The SCCIS represents the intermediate step in the progression from AK to invasive SCC. The epidermis shows full-thickness involvement by atypical keratinocytes, which spare the adnexal structures, and, in some cases, hyperkeratosis can occur. SCCIS cells are characterised by a

pleomorphic and hyperchromatic nucleus and show frequent mitoses. Although the terms SCCIS and Bowen's disease are frequently used interchangeably, the latter occurs in the anogenital region and is unrelated to UV-induced AK (73).

In invasive SCC, the neoplastic epithelial squamous cells invade the dermis. It can arise from sun-damage skin or *de novo* and the invasive component can take the form of infiltrating cords or sheets, group pf single cells, circumscribed nodules, squamous islands, or cystic structures. Unlike AK and SCCIS, neoplastic keratinocytes can have a cytomorphology that varies from normal appearance to highly anaplastic one (74-75). SCC can be histologically divided into well, moderately, and poorly differentiated variants. Different factors must be taken into consideration for this grading system, including the keratinization degree, atypia of nuclei and degree of architectural atypia. In particular, the well-differentiated variant is characterized by slightly enlarged keratinocytes with abundant, glassy pink to eosinophilic cytoplasm and it usually shows keratinized structures known as "keratin pearls". The identification of parakeratosis at keratin pearl level is useful to discriminate between a well-differentiated SCC and a squamoproliferative lesion. Unlike well-differentiated lesions, which tends to be circumscribed with pushing margins and a lobulated appearance, the poorly differentiated variant has a highly infiltrative pattern and is characterised by atypical keratinocytes with pleomorphic and hyperchromatic nuclei, several mitoses and no keratinization (74, 76) (Figure 8).

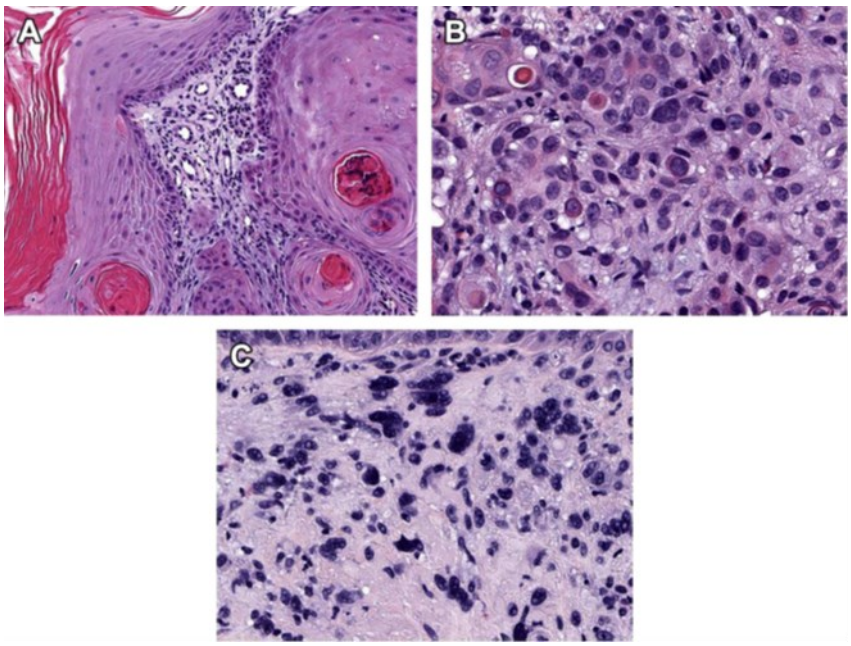

Figure 8. **A**) Well-differentiated SCC. **B**) Moderately differentiated SCC. **C**) Poorly differentiated SCC (Parekh et al 2017).

Several histologic variants of SCC have been described and their identification has a diagnostic and prognostic significance. The acantholytic squamous cell carcinoma (Figure 9, A), also known as adenoid SCC, adenoacanthoma of the sweat glands or pseudoglandular SCC, has three different rare subtypes: small-cell, pseudovascular and pseudoangiosarcomatous SCC. Neoplastic keratinocytes show a variable degree of desmosome disruption, resulting in rounded cells with a centrally located nucleus. Acantholysis gives the appearance of pseudoglandular, pseudoalveolar or pseudovascular spaces. These lesions can be classified according to the degree of involvement of the follicular epithelium alone or with the interfollicular epidermis. The identification of this variant is possible through the differential diagnoses including adenoid BCC, eccrine carcinoma, metastatic adenocarcinoma and angiosarcoma. In particular, the presence of specific areas with basaloid cells, peripheral palisading, necrosis, artifactual clefting and stromal mucin indicates an adenoid BCC. Moreover, the immunopositivity for smooth muscle actin, p63, calponin or S100 protein of the basal or myoepithelial layers in ductal structures, the immunoreactivity for carcinoembryonic antigen in luminal borders and the presence of luminal secretions that stain with periodic acid–Schiff diastase are useful to distinguish the eccrine carcinoma. As regards metastatic adenocarcinoma, it can be suspected on the basis of the clinical history, the presence of a multiplicity of lesions, and a lack of epidermal connection, while the diagnosis of angiosarcoma is based on the identification of blood-filled spaces and it is confirmed by the positivity for various endothelial markers.

Unlike the acantholytic variant, the adenosquamous carcinoma has a glandular differentiation. From the histological point of view, it is characterised by interconnecting nests of tumour cells forming keratocysts with focal or diffuse areas of gland formation. The glands are coated by cuboidal and low columnar epithelium with a luminal positivity for carcinogenic antigen. This variant usually invades the deep dermis (73). The differential diagnosis includes primary cutaneous mucoepidermoid carcinoma and metastatic adenocarcinomas. The first represents a controversial entity and there is no technique to distinguish it from adenosquamous carcinoma (77), while the diagnosis of metastatic adenocarcinoma requires a thorough clinical history and imaging studies to identify the primary site and is based on the presence of multiple lesions, the histologic demonstration of a lack of epidermal connection, and, when necessary, the judicious use of IHC markers (73).

The spindle cell squamous carcinoma, also known as sarcomatoid SCC, is characterized by a haphazard growth of spindle-shaped cells in the dermis without connection with the epidermis. Tumour can be entirely constituted by the atypical spindle cells or partly also by conventional neoplastic squamous cells. Tumour bulk can also show pleiomorphic giant cells as well as heterologous elements. This variant usually infiltrates the deep dermis, subcutis, muscle and bone and there is no stromal desmoplasia (Fig 9, B) (78-80).

As concerns the desmoplastic variant, it is characterized by infiltrating cords of spindle–squamoid tumour cells surrounded by a dense collagenous (desmoplastic) stroma, and it can show single-cell keratinization. Keratin pearls and perineural invasion are usually present (Fig 9, C) (73). Regarding the clear cell subtype, it is extremely rare and is divided into three categories: type I (keratinizing lesion characterized by sheets or islands of clear cells with peripherally displaced nuclei or central nuclei with bubbly cytoplasmic appearance, and focal areas of keratinization, even forming keratin pearls), type II (dermal nonkeratinizing tumour without connection to the epidermis, characterized by cells with a cytoplasm with a finely reticulated clear appearance and arranged in parallel or anastomosing cords separated by a fibrotic stroma with a heavy inflammatory infiltrate) and type III (pleomorphic neoplasm with extensive ulceration). Another rare variant is represented by the signet-ring squamous cell carcinoma, composed of signet-ring cells with a clear cytoplasm pushing the nucleus towards the periphery (Fig 10 d). On the contrary, the pigmented squamous cell carcinoma is characterized by lobules, nests, and cords colonized by benign pigmented dendritic melanocytes (73). As regards verrucous carcinoma, it has an exo-endophytic growth pattern, and it shows prominent acanthosis, papillomatosis, and hyperkeratosis.

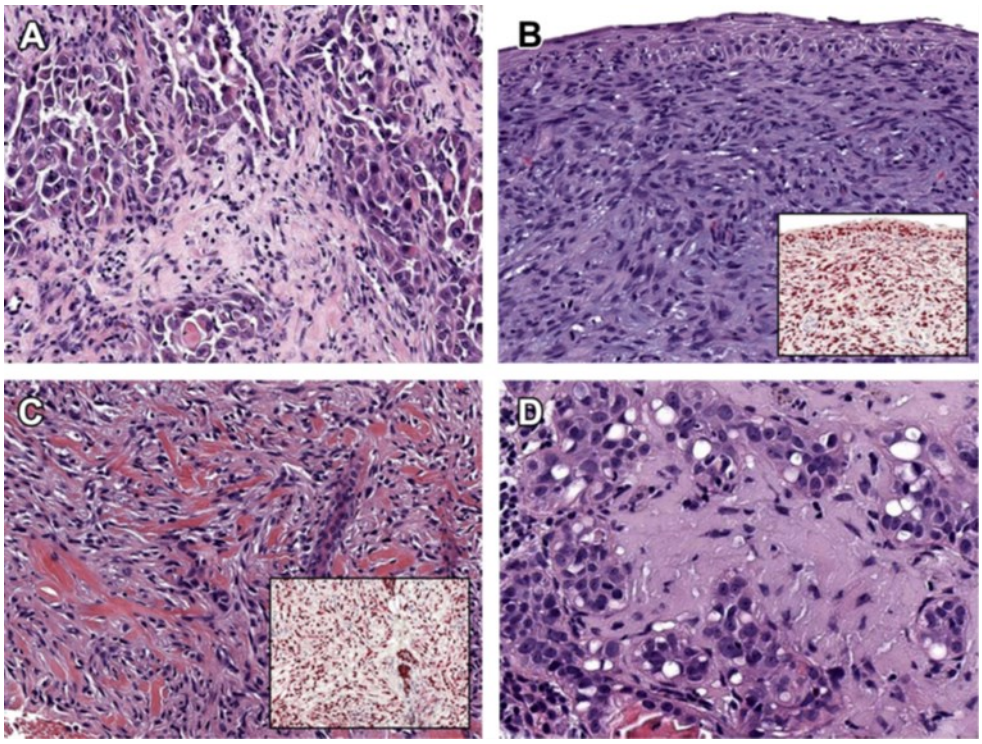

Figure 9. A) Acantholytic SCC. **B**) Spindle cell SCC. Insert: p63 immunostaining. **C**) Desmoplastic SCC. Insert: p63 immunostaining. **D**) Signet-ring SCC (Parekh et al 2018).

As concerns keratoacanthoma (KA), it has a rapid development and produces a dome-shaped nodule with a keratin-filled crater. KA usually undergoes spontaneous resolution, and its classification is unclear. Some authors classify it as a benign squamoproliferative lesion, a continuum between benign and malignant proliferation, while others describe it as an outright SCC that has the biological capacity to regress (81-83). There are several clinical variants of AK, composed of mature-appearing keratinocytes with a characteristic pink, glassy cytoplasm. The pleomorphism and atypia seen in conventional SCC are lacking (Figure 10) (73).

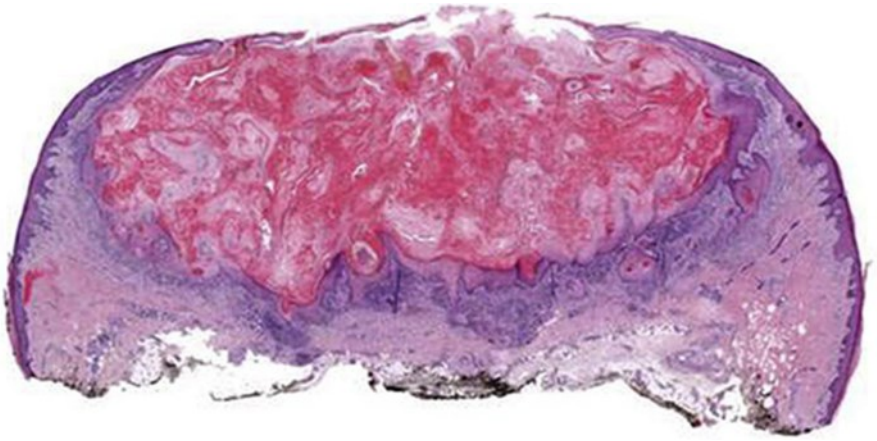

Figure 10. Keratoacanthoma. Dome-shaped nodule with a central keratin-filled crater (Parekh et al 2017).

In response to UV light damage, a skin area can progress to dysplasia and SCC, as a result of gene mutation accumulation. The mutation frequency is higher in squamous cell carcinoma than in other common malignancies (72). In particular, the most mutated gene is TP53 gene, encoding for tumour suppressor protein 53 (p53). In SCC patients this gene is affected by C/T single-base transition mutations at dipyrimidine sites and tumour cells become therefore resistant to apoptosis and clonally expand at the expense of neighbouring normal keratinocytes (84). Other mutations affect the cyclin-dependent kinase inhibitor 2A (CDKN24), involved in cell cycle control, Ras, important for cellular signal transduction, and Notch homolog 1, a tumour suppressor gene playing a fundamental role in carcinogenesis. Mutations in TP53 and Ras have also been found in AK, thus suggesting that they are the first result of UV light damage that kick off the SCC development, although they are not sufficient for tumour formation and growth. Understanding the role of SCC gene mutation can help to develop a specifically designed targeted therapy for this neoplasm. In particular, treatments with epidermal growth factor receptor (EGFR) inhibitors, which impact the Ras/mitogen-activated protein kinase (MAPK) pathway, and programmed cell death protein 1 (PD1) inhibitors, which stimulate T cells attack, are under investigation (72). Up to now, other skin cancers therapies are unsuccessful since they lead to SCC development. For example, the previously described treatment with vismogedib causes an increase by 8 times of the incidence of SCC compared to control subjects (85), probably because it selects tumour cells that proliferate through the MAPK pathway. Moreover, the use of B-Raf protein inhibitors for metastatic melanoma treatment induces the eruption of squamoproliferative lesions activated by the MAPK pathway (86-87).

Many factors can increase the risk for developing SCC, but cumulative sun exposure, especially in childhood and youth, is of greatest importance. Moreover, SCCs are more common in men than in women and their incidence increases with age. In addition, they are more frequent in fair skinned individuals, although in black patients they show a higher mortality rate (about 18%), because of a not prompt diagnosis and the occurrence of lesions on previous scars or trauma (88-89).

Another important risk factor is represented by immunosuppression. When compared with the general population, solid organ transplant recipients (SOTRs) are in fact 65 to 250 times more likely to develop SCC, and the rate of lesion formation is proportional to the number and quantity of immunosuppressive agents administrated to them (90-92). In addition, patients affected by chronic lymphocytic leukaemia, who lack a competent cell-mediated and humoral immunity, have an 8- to 10-fold increased risk, thus suggesting that SCC is highly immunologically mediated (72).

SCC development is also associated with the infection of the oncogenic human papillomavirus (HPV). HPV types 16 and 18, in particular, possess the E6 and E7 proteins, which inhibit the apoptotic process and stimulate a continuous replication of viral DNA by regulating p53 and retinoblastoma (93). Moreover, SOTRs with SCCs commonly show HPV types 8, 9 and 15 infection (94). However, HPV in SCC lesions is not transcriptionally active, thus suggesting the hypothesis that these viruses are involved in tumour induction but not in its progression and maintenance (95).

In addition, ionizing radiation exposure is associated with more aggressive SCCs and environmental exposures that increase SCC incidence include arsenic, polycyclic aromatic hydrocarbons, nitrosamines, and alkylating agents (72). The individual predisposition to multiple SCCs in young age is due to the presence of rare familial syndromes, such as xeroderma pigmentosum, albinism, epidermolysis bullosa, epidermolysis verruciformis, Ferguson-Smith epithelioma, Rothmund-Thomson and Bloom syndromes (96).

Even if most of SCCs are treated with a complete surgical excision, a subset of patients is affected by SCCs with specific histological and clinical findings that increase the risk of relapse and metastasis, thus resulting in poor prognosis (97-99). Several staging systems have been proposed, including a TMN (Tumour-node-metastasis) staging system proposed by the AJCC in 2002, the revised AJCC and International Union Against Cancer staging systems (Table 2), Brigham and Women's Hospital (BWH) tumour staging system (Table 3), NCCN guidelines (Table 4) and European Organization for Research and Treatment of Cancer guidelines (100-104). Until 2014, the AJCC staging manual included SCC in the cutaneous malignancy group,

while in the recently published eighth edition of the manual it was described in the chapter entitled “cutaneous squamous cell carcinoma of the head and neck” and although the chapter focuses primarily on SCC, the staging system applies to all histologic subtypes of carcinoma limited to the head and neck.

Several studies have evaluated the prognostic potential of the AJCC grading system, which is found to be unsatisfactory. The alternative BWH staging system classifies tumour categories by analysing several clinical and pathologic risk factors and appears to provide a more accurate prognostication for patients with localized SCCs. Nowadays, the NCCN guidelines provide an approach similar to that used for BCC to stratify high-risk and low-risk lesions. These guidelines represent a practical guide for health care providers, useful to identify the correct treatment for SCCs with different aggressiveness degree. Current treatment recommendations are therefore based on NCCN risk stratification (67).

Table 2. AJCC cutaneous SCC staging system for tumours of the head and neck, 8th edition 2017.

T category	T criteria	N category	N criteria for pathologic N	M category	M criteria
TX	Primary tumor cannot be identified	NX	Regional lymph nodes cannot be assessed	M0	No distant metastasis
Tis	Carcinoma in situ	N0	No regional lymph node metastasis	M1	Distant metastasis
T1	Tumor <2 cm in greatest dimension	N1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension and ENE^{-}*		
T2	Tumor ≥2 cm but <4 cm in greatest dimension	N2	Metastasis in a single ipsilateral lymph node ≤3 cm in greatest dimension and ENE^{+}; or >3 cm but not >6 cm in greatest dimension and ENE^{-}; or metastases in multiple ipsilateral lymph nodes, none >6 cm in greatest dimension and ENE^{-}; or in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension and ENE^{-}		
T3	Tumor ≥4 cm in clinical diameter OR minor bone erosion OR perineural invasion OR deep invasion†	N2a	Metastasis in single ipsilateral or contralateral node ≤3 cm in greatest dimension and ENE^{+}; or in a single ipsilateral node >3 cm but not >6 cm in greatest dimension and ENE^{-}		
T4	Tumor with gross cortical bone/marrow, skull base invasion, and/or skull base foramen invasion	N2b	Metastasis in multiple ipsilateral nodes, none >6 cm in greatest dimension and ENE^{-}		
T4a	Tumor with gross cortical bone/marrow invasion	N2c	Metastasis in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension and ENE^{-}		
T4b	Tumor with skull base invasion and/or skull base foramen involvement	N3	Metastasis in a lymph node >6 cm in greatest dimension and ENE^{-}; or in a single ipsilateral node >3 cm in greatest dimension and ENE^{+}; or multiple ipsilateral, contralateral, or bilateral nodes, any with ENE^{+}		
		N3a	Metastasis in a lymph node >6 cm in greatest dimension and ENE^{-}		
		N3b	Metastasis in a single ipsilateral node >3 cm in greatest dimension and ENE^{+}; or multiple ipsilateral, contralateral, or bilateral nodes, any with ENE^{+}		

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ENE, Extranodal extension.
*Extension through the lymph node capsule into surrounding connective tissue, with or without stromal reaction.
†Deep invasion is defined as invasion beyond subcutaneous fat or >6 mm (as measured from granular layer of adjacent normal epidermis to the base of the tumor). Perineural invasion is defined as tumor cells within the nerve sheath of a nerve deeper than the dermis or measuring ≥0.1 mm, or presenting with clinical or radiographic involvement of named nerves without skull base invasion.

Table 3. BWH tumour classification system.

Category	Definition
T0	In situ SCC
T1	0 risk factors*
T2a	1 risk factor
T2b	2-3 risk factors
T3	4 risk factors or bone invasion

SCC, Squamous cell carcinoma. *Risk factors include tumour diameter 2 cm or larger, poorly differentiated histology, perineural invasion, and tumour invasion beyond the subcutaneous fat (excluding bone, which automatically upgrades to T3).

Table 4. NCCN stratification of low versus high risk SCC.

Parameters	Low risk	High risk
Clinical		
Location*/size†	Area L <20 mm Area M‡ <10 mm	Area L ≥20 mm Area M ≥10 mm Area H§
Borders	Well defined	Poorly defined
Primary vs recurrent	Primary	Recurrent
Immunosuppression	No	Yes
Site of prior radiation therapy or chronic inflammatory process	No	Yes
Rapidly growing tumor	No	Yes
Neurologic symptoms	No	Yes
Pathologic		
Degree of differentiation	Well to moderately differentiated	Poorly differentiated
High-risk histologic subtype‖	No	Yes
Depth (thickness or Clark level)¶	<2 mm, or I, II, III	≥2 mm or IV, V
Perineural, lymphatic, or vascular involvement	No	Yes

cSCC, Cutaneous squamous cell carcinoma.
*Area L consists of trunk and extremities (excluding hands, feet, nail units, pretibia, and ankles); area M consists of cheeks, forehead, scalp, neck, and pretibia; and area H consists of central face, eyelids, eyebrows, periorbital skin, nose, lips, chin, mandible, preauricular and postauricular skin/sulci, temple, ear, genitalia, hands, and feet.
†Greatest tumor diameter, including peripheral rim of erythema.
‡Location independent of size may constitute high risk.
§Area H constitutes high-risk on the basis of location, independent of size.
‖Adenoid (acantholytic), adenosquamous (showing mucin production), desmoplastic, or metaplastic (carcinosarcomatous) subtypes.
¶A modified Breslow measurement should exclude parakeratosis or scale/crust and should be made from base of the ulcer is present. If clinical evaluation of incisional biopsy suggests that microstaging is inadequate, consider narrow-margin excisional biopsy.

The identification of a high-risk lesion is essential for the development of a correct treatment plan. There are different clinical high-risk features, such as the tumour location. In particular, lesions occurring in the head and neck region show an increased rate of local recurrence and metastasis. In addition, neoplasms arising in chronic wounds, scars and sites of prior burns or radiation therapy are more likely to metastasize and relapse (67). Tumour recurrence itself is a high-risk predictor since recurrent cancers tend to be wider and invasive. Moreover, they show an increased metastatic rate and are associated with a poorer disease survival. Patients with multiple SCCs as well as those who have undergone solid organ transplantation (105-106) have an augmented risk of local recurrence and metastasis, Squamous cell carcinomas with a diameter and/or a depth greater than or equal to 2 cm are more likely to recur and to produce metastasis. Perineural invasion independently predicts increased rate of local recurrence and metastasis. In particular, PNI of large-caliber nerves (>0.1 mm) is associated with a greater likelihood of lymph node metastasis and a higher mortality rate. In addition, also tumour differentiation is an independent predictor of metastasis and relapse. In particular, poorly differentiated lesions have the highest recurrence rate and the lowest 5-year metastasis-free and overall survival rate (67). As regards the histologic subtypes, some of them are associated with a poor prognosis, but data are not sufficient. For example, the acantholytic SCC is thought to be highly aggressive, even if this is not convincingly supported by published studies. Moreover, desmoplastic variant and tumours with infiltrative and desmoplastic growth patterns are associated with an aggressive behaviour in terms of local recurrence and metastasis. The NCCN designates acantholytic, adenosquamous, and desmoplastic SCC subtypes as high-risk factors and the current European Organization for Research and Treatment of Cancer guidelines list acantholytic, spindle, and desmoplastic subtypes as the high-risk prognostic factors. KA is not regarded as a subtype when it is identified with certainty, based on clinical and histologic features (103, 104).

It is generally accepted that most SCCs are successfully treated with standard treatments, such as surgical excision. In particular, surgical excision with a minimum margin of 4 mm around the tumour border is appropriate in 95% of cases to completely remove primary low-risk lesions with a diameter of less than 2 cm. On the contrary, bigger neoplasms with high risk features should be removed with margins greater than 6 mm (107). As for BCC, MMS has a better cure rate than standard surgical excision and it is indicated for lesions occurring on the scalp, nose, ear, eye, lip, hand or nail unit, aggressive histological subtypes of SCC, poorly differentiated cancers or tumours of >2.0 cm at any location, and recurrent cancer at any location. After MMS treatment, local recurrence and metastasis rate are less frequent. In particular, 86% of SCCs

with PNI has a 5-year survival after treatment with MMS, whereas the percentage drops to 76% for those treated with surgical excision. In addition, only 3.1% of SCCs of the ear recurs after MMS, while 10.9% recurs after standard excision, and the same results were obtained for lesions occurring on the lip. The recurrence rates of SCCs measuring greater than 2 cm, treated with MMS or standard excision were 25.2 and 41.7%, respectively. Poorly differentiated lesions treated with MMS have a recurrence rate of 32.6 %, whereas that of tumours which have undergone standard resection is 53.6%. MMS is better than standard excision also for recurrent SCCs, but it cannot be used for lesions with micrometastasis (108). Well-differentiated, slow-growing tumours with a diameter of less than 1 cm can be removed by electrodessication and curettage (ED&C) but this technique is not appropriate for high-risk lesions. Moreover, it may be associated with a longer healing time and inferior cosmetic outcomes compared to standard excision (108). If surgical therapy is not feasible or elected, nonsurgical approaches may be considered for low-risk tumours. For example, PDT, a two-part treatment consisting in a topical application of a photosensitizer, ALA or MAL, followed by visible light irradiation, may be used in combination with curettage or surgical treatment (108). Locally applied treatments that are occasionally utilized but for which sufficient evidence is lacking are imiquimod and 5-FU. The available data do not support the use of topical modalities for SCC because of the severe effects. In particular, the topical use of 5-FU results in marked erythema, erosions, and crust lasting for a month or longer, and it decreases patient compliance with treatment regimens that may result in reduced effectiveness. Similarly, imiquimod can induce erythema, oedema, erosions, ulceration, and crust and the application on larger surface area can be associated with systemic symptoms (67). RT can be used when surgery is not feasible or contraindicated, but its effectiveness is higher in smaller and thinner lesions. Another treatment for low-risk SCCs is represented by the cryosurgery, which is not recommended for tumours with high-risk features. For these lesions, in particular for metastatic SCCs, the therapeutic recommendations are based on the extent of disease and consist primarily of surgical resection with possible lymph node dissection and consideration of adjuvant radiation therapy, with or without concurrent systemic therapy (67). For inoperable lymph node metastases, a combination therapy should be considered. Moreover, for patients with advanced disease, it is also appropriate to provide or refer to best supportive and palliative care to optimize symptom management and maximize quality of life. Existing data on the treatment of patients with distant metastatic SCCs are sparse and limited to phase II clinical trials. Chemotherapy, including cisplatin as a single agent or combined with 5-FU, has shown some activity, but the results have not been confirmed in larger cohort. In other phase II trials, EGFR inhibitors, also

combined with classic chemotherapeutic agents, have shown a good efficacy in patients with advanced disease. Great interest currently exists in ongoing clinical trials evaluating the efficacy of immune checkpoint inhibitors for locally advanced and metastatic SCCs, while careful consideration must be given to immunosuppressed individuals (67).

1.2 CUTANEOUS MELANOMA

1.2.1 Genesis and biology of melanoma

Melanoma is a tumor resulting from the neoplastic transformation of melanocytes. It mainly occurs in the skin, where it represents the most aggressive form of cancer and one of the leading causes of cancer-related mortality due its metastatic power, although it may also develop in the mucous membranes, the uveal tract and the leptomeninges (110). Melanocytes are responsible for the pigmentation of skin and hair, and thereby contribute to the appearance of skin and provide protection from damage by ultraviolet radiation. In particular, in response to UV-induced damage, epidermal keratinocytes produce the melanocyte stimulating hormone (MSH), which binds to the melanocortin 1 receptor (MC1R) located on the surface of melanocytes, thus stimulating them to produce and release melanin, which operates as a shield for UV radiation preventing further DNA alterations. Cutaneous melanoma can originate from chronically or intermittently sun-exposed skin and it shows different sites of onset, age at diagnosis, types of oncogenic drivers and mutational load (111-112). The main genetic drivers are B-Raf proto-oncogene (BRAF), neurofibromin 1 (NF1) and NRAS protooncogene mutations. Melanomas associated with chronically sun-exposed skin usually have high mutational load related to UV exposure, whereas lesions associated with an intermittent sun exposure arise in younger patients and exhibit a lower mutational load. Melanomas can evolve from preneoplastic lesions through different patterns. Moreover, each melanoma subtype can result from different precursor lesions and can show different gene mutations (113). UV light can induce melanocyte malignant transformation either directly or indirectly (Figure 11). It is noteworthy that although 80% of benign nevi has a BRAF mutation, they remain indolent for decades under immune surveillance and rarely progress to melanoma, thus suggesting that oncogenic BRAF alone is not sufficient for melanoma development. However, UV radiation can determine the onset of additional genetic mutations, such as telomerase reverse transcriptase (TERT) and CDKN2A, that lead to the malignant transformation of the nevi. The direct transformation of normal melanocytes in neoplastic cells is due to several mutations affecting both proto-oncogenes and tumour suppressor genes (113).

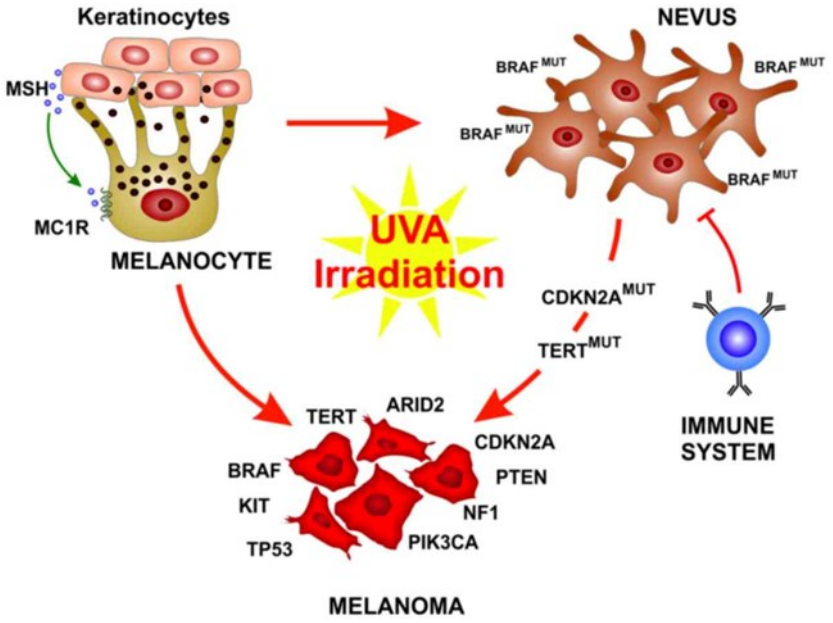

Figure 11. Melanocyte malignant transformation (Leonardi et al, 2018)

Several studies demonstrate that melanoma development and spreading is the result of different genetic mutations and alterations in the tumour microenvironment provoked by the dysregulation of protein expression which can promote tumour infiltration into the surrounding tissues. In particular, the overexpression of matrix metalloproteinases 9 and 2 (MMP-9, MMP-2), mediated by the genetic dysregulation of nuclear factor (NF)-kB pathway, induces the degradation of the extracellular matrix, thus causing tumour cell spreading through the bloodstream (114-116). Melanomas show the greatest burden of genetic alteration of all human neoplasms, affecting genes that control cell proliferation (BRAF, NRAS and NF1), growth and metabolism (phosphatase and tensin homolog-PTEN and KIT-proto-oncogene receptor tyrosine kinase), resistance to apoptosis (TP53), cell cycle control (CDKN24) and replicative lifespan (telomerase reverse transcriptase-TERT) (117-118). Two main signalling pathways are aberrantly activated by these genetic alterations, the MAPK and phosphoinositol-3-kinase (PI3K)/AKT(protein kinase B) pathways (119). The first is physiologically involved in the transduction of extracellular signals inducing the expression of fundamental genetic drivers of cell proliferation, differentiation, and survival and it is aberrantly activated in 90% of melanoma cases (120-122). The second is normally involved in cellular homeostasis and its activation has been observed in different cancer types including melanoma. BRAF is by far the most frequently altered gene in the MAPK pathway. In fact, 37% to 50% of melanomas carry a somatic mutation in the BRAF gene, with the highest frequency in those derived from intermittent sun exposure damage (113). They are usually missense mutations resulting in the

substitution of valine at amino acid position 600. In particular, in approximately 80%-90% of patients valine 600 is substituted with glutamic acid (V600E), while 5% to 12% are valine-to-lysine substitution (V600K) and less than 5% are valine-to-acid aspartic (V600D) or to-arginine substitution (V600R) (123-124).

BRAF protein is a serine/threonine protein kinase with 766 amino acids and consists of two domains with regulatory function and a catalytic domain, responsible for mitogen-activated protein kinase kinase (MEK) phosphorylation in the MAPK pathway as well as for maintaining the protein in its inactive conformation, through a hydrophobic interaction between the "so-called" glycine-rich loop and the activation segment, making it inaccessible for ATP binding.

Several studies demonstrate the involvement of BRAF in the intracellular signal transduction pathway. Initially, the ligand binding causes an autophosphorylation of the receptor itself, thus allowing the interaction between the phosphorylated receptor and RAS protein. This results in the activation of RAS protein and in the subsequent phosphorylation of BRAF, which becomes in turn capable of binding and phosphorylating MEK. This activation cascade culminates with the phosphorylation and subsequent translocation of ERK into the nucleus, where, finally, it phosphorylates specific transcription factors, with consequent repercussions on the expression of genes involved in cell growth and differentiation processes. The MAPK pathway plays therefore an important role in normal cells, but it is essential to prevent a persistent activation state. For this purpose, in physiological conditions the cascade is inhibited upstream by means of a negative feedback mechanism. However, in tumour cells having BRAF gene mutations, the activation process of the MAPK pathway is different since the absence of a physiological negative feedback induces the permanent activation of the cascade, which leads to uncontrolled cell proliferation. In particular, in $BRAF^{V600E}$ mutation, valine is replaced with a glutamic acid, that is an hydrophilic and polar amino acid, and this results in an abnormal flip of the catalytic domain which generates a constitutive active conformation with a kinase activity 500-fold higher than wild-type BRAF kinase. BRAF non-V600E mutations act in a similar way (125-126).

The second most common cause of aberrant activation of MAPK pathway is represented by NRAS activating missense mutations arising on codon 12, 13 or 61 and interesting 15% to 30% of melanomas. These mutations lead to the prolongation of the NRAS-active GTP-bound state, thus abnormally maintaining NRAS signalling through both the MAPK and the PI3K pathways. NRAS and BRAF mutations are considered mutually exclusive, although co-mutations can rarely occur (113).

The third most frequently mutated gene in melanoma is NF1, that encodes for a protein regulating the RAS family by converting the active RAS-guanosine triphosphate (RAS-GTP) to the inactive RAS-guanosine diphosphate (RAS-GDP), thus inhibiting RAS signalling downstream. NF1 inactivation leads to a hyperactivation of NRAS protein and a subsequent stimulation of the MAPK and PI3K signalling pathways. These mutations, which occur in 10-15% of melanoma cases, are more common in chronically sun-exposed skin and are usually associated with those affecting BRAF and NRAS (127-130).

As concerns the mutations in the gene encoding for tyrosine-protein kinase KIT, involved in the growth of cancer cells, in metastatic invasion and in the inhibition of apoptosis through the MAPK and PI3K pathways, they are found in 2-8% of melanomas. (131-132).

In addition, a number of other genetic mutations is involved in the characterization of invasive and metastatic melanoma genotype. For example, TERT promoter mutations, which are associated with an increased melanoma cell proliferation, have been frequently observed in *in situ* lesions. The same goes for heterozygous CDKN24 alterations, while biallelic mutations in the same gene are associated with aggressive phenotypes and rarely occur in precursor lesions. Moreover, PTEN dysregulation is usually detected in vertically growing melanomas and metastases with a frequency of 10–30% of cutaneous melanoma. In particular, missense and frameshift mutations or chromosomal deletions are the most frequent alterations, even if epigenetic mechanisms and microRNAs post-transcriptional regulation of PTEN expression have also been found. PTEN alterations are usually mutually exclusive with NRAS mutations, although they frequently co-occur with those affecting BRAF. The link between PTEN mutations and the aggressive behaviour of melanoma depends on the involvement of this tumour suppressor gene in PI3K/AKT and MAPK pathway aberrant activation. Moreover, β-catenin-mediated WNT (wingless-related integration site) signalling activation has been shown to be associated with melanoma formation and metastatic dissemination (113).

1.2.2 Histologic classification of cutaneous melanoma

There are four major subtypes of cutaneous melanoma. In particular, the superficial spreading melanoma (SSM) (70%) represents the most common histological variant of melanoma and it could arise from healthy skin or it evolves from a precursor lesion, usually a dysplastic nevus. A vertical growth phase, where the lesion becomes thick and nodular, may follow a prolonged radial growth phase, where the lesion remains thin. Early SSM is more frequently cured than late nodular melanoma, since the risk of spread is strictly related with lesion depth.

Histologically, SSM is characterized by large melanocytic cells with nest formation along the dermo-epidermal junction and usually invades the upper epidermis in a pagetoid fashion. The pattern of rete ridges is often effaced, and the dermis is usually invaded by atypical pleomorphic melanocytes.

Nodular melanoma (NM) (15%–30%) is the most aggressive form, generally appears as a new spot on normal skin. Moreover, it shows a vertical growth pattern since it tends to grow in depth more quickly than it does in width. The prognosis is therefore often worse than other subtypes, since it takes longer for a person to be aware of the changes. NM is most often darkly pigmented, although some lesions can be light brown, multicoloured or even non-pigmented. A light-coloured or non-pigmented NM lesion may escape detection because the appearance is not alarming, even if an ulcerated and/or bleeding lesion is common. Polypoid melanoma represents a virulent variant of NM. Unlike SSM, these tumours are sharply circumscribed, and they do not show the epidermal features of disarrangement and pagetoid infiltration seen in SSM. Within the epidermal component, nodular melanomas are characterized by epithelioid melanocytes with abundant cytoplasm and large vesicular nuclei with prominent nucleoli.

Lentigo maligna (LM) (4%–10%) usually appears in chronically sun-exposed skin of elderly patients. It typically begins as a small freckle-like macule. Over time it becomes darker and asymmetric, and it exhibits a vertical growth phase. LM consists of confluent rows or nests of atypical melanocytes arranged in the basal portion of the epidermis. Pagetoid spread is uncommon in early phase although it may be seen when dermal invasion develops. The cells are often arranged perpendicular to the surface. The melanocytes show prominent artifactual retraction of the cytoplasm. Nuclear pleomorphism and multinucleation are frequently present.

Acral lentiginous melanoma (<5%) is a form of melanoma arising most commonly on palmar, plantar, subungual, and occasionally, mucosal surfaces. Its main characteristic is continuous proliferation of atypical melanocytes at the dermo-epidermal junction. Other histological signs of acral lentiginous melanoma include dermal invasion and desmoplasia (133).

Even if cutaneous melanomas represent 91.2% of all diagnosed melanomas, these neoplasms may also develop in noncutaneous sites where melanocytes usually occur, including the respiratory, digestive, and genitourinary tracts as well as the eye (134).

1.2.3 Epidemiology and risk factors

The worldwide incidence of cutaneous melanoma has been increasing annually at a more rapid rate compared to any other type of cancer. In 2012, 232,000 new cases of melanoma and 55,000 deaths were reported, ranking 15th among most common cancers worldwide. Its incidence varies greatly between countries because of variability in race-related skin pigmentation phenotypes, as well as differences in sun exposure. In particular, in the United States, European countries, and other regions where Caucasian population is predominant the incidence is higher, since Caucasians are at a 10-fold increased risk of developing this neoplasm. Queensland, Australia, has the highest incidence of cutaneous melanoma, which is the leading malignancy and represents the first cause of death for cancer in Australians aged 15 to 44 years. As concerns the United States, melanoma is the sixth most common cancer in women and the fifth most common in men and the incidence has risen by 270% over the past 30 years, due to a variety of factors, including the augmented exposure to UV radiation. It is noteworthy that melanoma development can be influenced by sun exposure patterns, as well as by exposure to artificial UV light (135).

Melanoma mostly affects young and middle-aged adults, with a median age at diagnosis of 57 years. After the age of 25 years until the age of 50 years its incidence increases linearly.

However, melanoma risk differs by gender as well as age. Indeed, women between ages 25 and 40 years are more likely to develop a melanoma compared with men of the same ages, but after 75 years, men have three times increased risk to develop the malignancy with the respect to women. Overall, men are 1.5 times more likely to develop melanoma (136).

Melanoma has a variety of presentations depending on site of occurring. Noncutaeous lesions, as well as cutaneous melanomas arising in location difficult to visualize, are usually diagnosed in advantage stage. Moreover, superficial spreading and nodular variants tend to have a more classic presentation including irregular borders, asymmetry, and enlarged diameter and are easy to recognize. Lentigo maligna melanomas are more common on the face while acral lentiginous melanomas are most commonly found at the palms of the hands, soles of the feet, and nail beds. Body location of melanoma onset is strictly linked to sun exposure, history of sunburn and gender. Men usually develop lesions on the back, whereas women are more likely to develop melanoma on the arms and legs. Melanomas on the trunk have been associated with a history of severe sunburn, and patients with a family history of melanoma were more likely to develop lesions on the limbs than those with no family history (57% versus 42%) (137).

UV radiation is the main environmental risk factor for NMSC and melanoma and in 1992 the International Agency of Research on Cancer (IARC) classified it as a carcinogen. The classification was then updated in 2009 to include UV radiation from indoor tanning. Subsequently also UV radiation from arc welding was considered to be a carcinogen for humans, and in 2017 also welding fumes were included (138-139). UV exposure occurred during sun exposure, indoor tanning, and welding has long been associated with increased development of skin cancer and in particular melanoma. Both sun exposure patterns and timing are associated with an increased risk to develop this neoplasm. Indeed, chronical sustained exposure is linked to NMSCs whereas intermittent sun exposure and sunburns are more closely associated with melanoma (140). Furthermore, a history of sunburn in childhood or adolescence is associated with the highest risk of developing melanoma and individuals experiencing >5 episodes of severe sunburn have a 2-fold increased risk (141-142). In the United States more than 6,000 melanomas/year are thought to be caused by indoor tanning. Moreover, an increased duration and an earlier age at initiation of indoor tanning is associated with an even higher risk of melanoma development compared with individuals who do not engage in indoor tanning. In particular, in women with a history of indoor tanning, melanomas are more likely to develop on the trunk, while in they occur more commonly on the head and neck region. In addition, the use of psoralen and ultraviolet A (PUVA) for the treatment of psoriasis, eczema, and vitiligo, is associated with an augmented possibility to develop skin cancers, especially melanoma (137, 143-145).

As regards genetic risk factors, the freckles, light-coloured eyes, red hair, fair skin, and inability to tan, associated with different polymorphisms of MCR1, increase the individual risk for developing melanoma by nearly 50%. Moreover, individuals with light-coloured skin are more likely to develop amelanotic lesions, which often go unnoticed until the lesion becomes more advanced (146). In other cases, the genetic component is not completely clear. Nevertheless, a family history of melanoma and mutations that alter the efficacy of UV-induced DNA repair mechanisms and cell damage–induced signalling pathways are known to influence melanoma onset. In particular, among Caucasian population individuals with a positive family history of melanoma have a relative risk of 2.24 for melanoma compared with individuals without a positive family history, but this risk was independent of age, nevus count, hair and eye colour, and freckling (147). The most common germline mutations identified in familial forms of melanoma are CDKN2A mutations, which results in changes altering the function of p16, a negative regulator of cell cycle progression, and CDK4 mutation, which determines a phenotype similar to that caused by CDKN2A mutations (148-149). Other genetic syndromes

that increase overall cancer risk also confer a higher risk of melanoma. For example, BRCA1 and BRCA2 mutations are associated to an increased risk of several cancers among which also melanoma, as well as variants of p53 and RB1 (150-151). Moreover, in patients with xeroderma pigmentosum (XP), a rare autosomal recessive disorder of DNA repair characterized by increased sensitivity to UV, the possibility to develop nonmelanoma skin cancer and melanoma were found to be 10,000-fold and 2000-fold higher, respectively, than in the general population (152).Moreover, other inherited conditions associated with an increased risk of developing melanoma are familial retinoblastoma, Lynch syndrome type II and Li-Fraumeni cancer syndrome. In addition, the presence of dysplastic nevi is associated with a 1.5- to 10-fold increased incidence of melanoma and it is approximately 1.5 times higher in people with 11 to 25 nevi and seems to be doubled with every increase of 25 nevi (136). A personal history of melanoma is associated with an increased risk for a second one and is greater within the first year of index diagnosis. A validated association between smoking and melanoma has not been established but a direct positive correlation between current smoking and sentinel lymph node metastasis, ulceration, and increased Breslow thickness in smokers who developed melanoma was noted. As for NMSC, SOTRs have a 2-4-fold increased risk of developing melanoma, as well as patients with HIV infections (137).

1.2.4 Prevention and diagnosis

As described above melanoma risk is increased by different factors among which UV radiation exposure, thus suggesting that most of melanoma cases could be prevented. Its prevention can be divided into primary, aimed at reducing sun exposure and ensuring a stricter labelling protocols for sun cream, and secondary, including body examination by an expert (154-156). In high-incidence countries, several measures have been taken to reduce UV exposure. For example, in 2003 in United Kingdom (UK) a national campaign called SunSmart was distributed to raise public awareness to the use of lotions with sun protector factor (SPF) ≥15, to wear protective clothing and hats, and to avoid sun exposure during the hours when the UV rays are the most intense (157). In Norway and Sweden similar campaigns has been launched, but none of these campaigns achieved the expected results. Only in Australia the awareness campaign has been successful, as witnessed by the slowing melanoma incidence (158-160).

As regard the secondary prevention, the principal method is represented by the visual examination of the skin. For this reason, in 2003 in Germany the SCREEN program (Skin Cancer Research to Provide Evidence for Effectiveness of Screening) was started. It consisted

in a population screening project based on the examination of the skin of the whole body (WBE or Whole-Body skin Examination). The adoption of this program allowed a 48% decrease in melanoma mortality. Australia also undertook a general population screening campaign in 2008, with a consequent decrease in mortality attributable to melanoma (161-162).

An early diagnosis and a prompt treatment are strictly linked to a better prognosis. Cutaneous melanoma is usually diagnosed via visual skin inspection. Diagnostic clues include asymmetry, border, colour, diameter, and evolution (ABCDEs), as well as the "ugly duckling sign" (Figure 12). The diagnostic accuracy can be improved by the use of dermatoscopy. Moreover, several new technologies seek to enhance prebiopsy diagnostic accuracy, including artificial intelligence image analysis, whole-body 3-D imaging, reflectance confocal microscopy (RCM), optical coherence tomography (OCT), and epidermal genetic information retrieval from adhesive tape stripping. Despite their advantages, these techniques are not routinely used, except for digital photographic monitoring that allows to detect thinner lesions. A diagnosis of melanoma is confirmed by skin biopsy typically performed with local anaesthesia by using one of these three techniques: saucerization shave biopsy, punch biopsy, or narrow excision with 2-mm margins. The biopsy should assess the invasion depth (Breslow thickness), representing the most important prognostic indicator and treatment guide, as well as the whole lesion, even if it may be impractical for certain locations or large neoplasms. Moreover, for patients with an unknown primary skin lesion presenting with a bulky lymph node or metastatic disease, fine-needle aspiration may be used with sensitivity and specificity above 90%. If clinical suspicion is high and the fine-needle aspiration is nondiagnostic, an excisional biopsy should be performed to confirm the diagnosis. Tissue confirmation is needed even in the setting of distant metastatic disease, and molecular sequencing for common mutations is now standard of care (163-165). Several characteristics are considered during histologic exam. Besides tumour thickness as described by Breslow, measured in millimetres by means of an ocular micrometre, additional parameters of the primary lesion should be reported due to their prognostic significance. These parameters include the histologic subtype, mitotic rate, degree of atypia, tumour infiltrating lymphocytes, lymph vascular invasion, neurotropism, ulceration or tumour regression, microsatellitosis, margins and Clark level of invasion. The latter led to classify lesions in five levels: level 1 (melanoma cells are confined to the epidermis), level 2 (invasion of single cells or very small nests of melanoma into the papillary dermis), level 3: (melanoma cells "fill and expand" the papillary dermis), level 4 (invasion into the reticular dermis), level 5 (invasion into the subcutaneous fat).

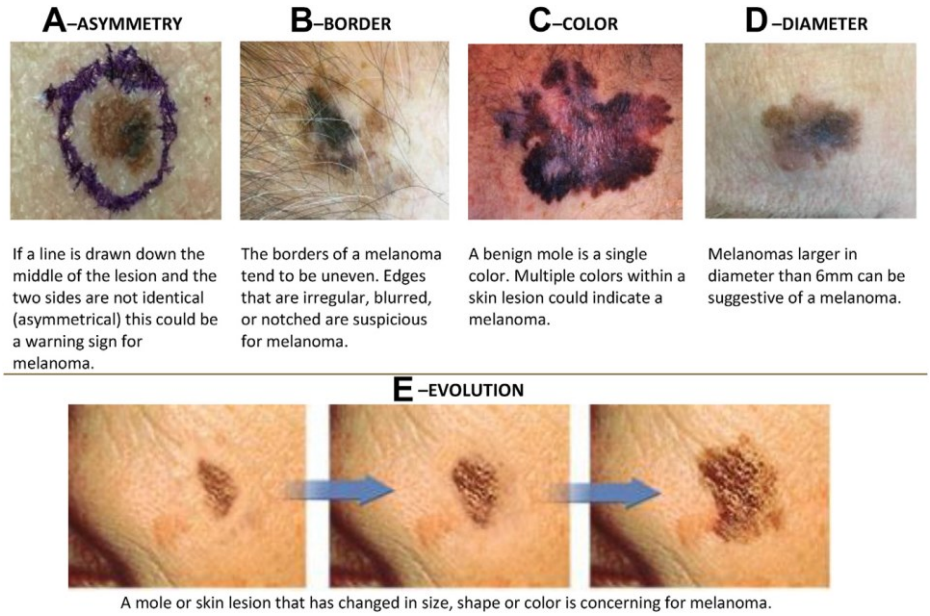

Figure 12. ABCDEs of melanoma. These characteristics may be used by health care providers and patients to identify skin lesions that may necessitate a biopsy due to concerning features (Carr et al, 2019).

After biopsy, several more quantitative and semiquantitative techniques are being used more frequently to improve melanoma detection and diagnosis, such as fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), sequencing, mass spectrometry (MS) and immunohistochemistry (IHC) . In particular, the most used immunohistochemical biomarkers are melanocytic markers, such as melan-A and MART-1 (which are two different antibodies against the same antigen) and proliferative markers, as Ki-67 and phosphohistone H3 (PHH3). As concerns the melanocytic markers, the combination melan-A/MART-1 is more sensitive than HMB-45 (human melanoma black 45), an antibody recognizing the melanocytic antigen gp100. Other common melanocytic markers include S100 protein, microphthalmia transcription factor (MITF), tyrosinase and SOX10. Despite all, diagnosis is not always completely clear, and lesions difficult to distinguish from melanoma, such as Spitz tumours, cellular and/or epithelioid blue nevi, and deep penetrating nevi, which are termed “melanocytic tumour of uncertain malignant potential” should be excised completely (166-167).

1.2.5 Staging System

Understanding the staging of melanoma is essential for developing the correct treatment strategy. The pathological staging is described in the seventh edition of AJCC staging system, implemented by the eighth edition of 2018, that includes both the anatomic "TNM" (Tumour-Node-Metastasis) prognostic factors and the nonanatomic factors such as lactate dehydrogenase (LDH) levels (Table 5). In particular, Tis is used for melanoma in situ with no invasive component, whereas T0 indicates no evidence of primary tumour and Tx is assigned when tumour thickness cannot be assessed. In the seventh edition mitotic rate was used to subcategorize T1 but it is not included in the eighth edition. The current subcategorization of T1 is based on a tumour thickness threshold of 0.8 mm. The concept of using tumour thickness to subcategorize T1 melanoma was considered and evaluated by the melanoma expert panel, based on the evidence seen in studies that reported poorer prognosis associated with thicker T1 melanoma compared with thin T1 melanoma. The evidence for subcategorizing T1 based on lesion thickness comes from the multivariate analysis of cancer depth, ulceration, mitotic rate, and melanoma-specific survival (MSS). Sentinel lymph node (SLN) positivity for tumours of thickness less than 0.8 mm (<5%) is much less compared to lesions of thickness of 0.8 to 1.0 mm (5%–12%). However, mitotic rate remains an important prognostic factor across all the T stages.

As regards N staging subcategories, in the seventh edition the terminology "a" and "b" for microscopic and macroscopic nodal disease, respectively. was used, while in the eighth edition these terms were replaced with "clinically occult" nodal diseases, which are identified by SLN and "clinically evident" nodal disease, identified by clinical and/or radiological examination. Moreover, the eighth edition of melanoma staging system gives a better definition of not-modal metastases, such as microsatellite, satellite, and in-transit lesions. A microsatellite is defined as any microscopic focus of metastatic tumour cells in the skin or subcutaneous tissue, adjacent to, or deep to but discontinuous from the primary lesion. Whereas a satellite metastasis is defined as any focus of clinically evident cutaneous and/or subcutaneous metastasis, occurring discontinuous, but within 2 cm from the primary site. As regards the in-transit lesion, it is a clinically evident cutaneous and/or subcutaneous metastasis, occurring beyond 2 cm from the primary lesion, in the region between the primary site and draining regional lymph nodes.

The melanoma expert panel concluded that it is premature to perform a broad-based analysis for M categorization because the treatment of metastatic melanoma is rapidly evolving. However, according to the current edition, the metastatic site remains the main criteria based on which M staging is subcategorized. M1a refers to a non-visceral metastasis: that is distant

cutaneous, subcutaneous, or nodal metastases. M1b includes patients with pulmonary metastases only. M1c includes patients with metastases to sites other than non-visceral, the lungs, and central nervous system (CNS). Moreover, the eighth edition has included an additional subcategory of “M1d,” which exclusively includes patients with CNS metastases, with or without metastases at other sites, because of their worst prognosis. In addition, M subcategories has been dichotomized into “0” and “1” based on LDH level. Patients with normal LDH levels are therefore labelled as “0”, while those with elevated LDH levels are labelled as “1”. Ultimately, melanoma is characterized by 5 stages. Stage 0 refers to melanoma in situ and is characterized by the presence of microscopically identified tumour cells, which have not penetrated the epidermis. Stages I and II refer to a lesion that grows in the area where the primary tumor (T) has developed, without involvement of neighboring (lymph nodes) or distant (other parts of the body) structures. Stage III indicates the spread of the primary tumor to the lymph nodes (N), while stage IV certifies the presence of distant metastases (M).

Table 5. The eighth edition of AJCC staging system for melanoma (2018).

Stage	Classification	5-year survival
Stage 0	Tis: Melanoma *in situ*	>98%
Stage I (A/B)	T1a: <0.8 mm and nonulcerated	97–92%
	T1b: ≥0.8 mm or <0.8 mm with ulceration	
	T2a: >1.0–2.0 mm without ulceration	
Stage II (A, B, C)	T2b: >1.0–2.0 mm with ulceration	81–53%
	T3a: >2.0–4.0 mm without ulceration	
	T3b: >2.0–4.0 mm with ulceration	
	T4a: >4.0 mm without ulceration	
	T4b: >4.0 mm with ulceration	
Stage III (A, B, C, D)	N1a: 1 clinically occult (in SLN biopsy)	78–40%
	N1b: 1 clinically detected	
	N1c: Presence of in-transit, satellite, and/or microsatellite mets	
	N2a: 2–3 clinically occult (in SLN biopsy)	
	N2b: 2–3, at least 1 clinically detected	
	N2c: 1 clinically occult or detected, with in-transit, satellite, and/or microsatellite mets	
	N3a: 4 or more clinically occult (in SLN biopsy)	
	N3b: 4 or more, at least 1 of which clinically detected, or presence of any number of matted nodes	
	N3c: 2 or more clinically occult or clinically detected with in-transit, satellite, and/or microsatellite mets	
Stage IV	M1a: Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph nodes. LDH not recorded or unspecified	20–15%
	M1a(0): LDH not elevated	
	M1a(1): LDH elevated	
	M1b: Distant metastasis to lung with or without M1a sites of disease. LDH not recorded or unspecified	
	M1b(0): LDH not elevated	
	M1b(1): LDH elevated	
	M1c: Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease. LDH not recorded or unspecified	
	M1c(0): LDH not elevated	
	M1c(1): LDH elevated	
	M1d: Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease. LDH not recorded or unspecified	
	M1d(0): LDH not elevated	
	M1d(1) LDH elevated	

1.2.6 Surgical resection

Surgical removal of cutaneous melanoma and surrounding healthy tissue is the primary treatment for localized lesion. The wide local excision (WLE) is necessary for reducing recurrence and specific melanoma mortality. The recommended peripheral clinical margins for WLE depend on the Breslow thickness and range from 0.5 cm to 2.0 cm (there are no additional benefits from deeper excision). Breslow thickness is a predictor parameter for the possibility to develop sentinel lymph node metastases and SNL biopsy (SLNB) is performed when tumours have a depth greater than 0.8 mm or are ulcerated. Historically, patients with a positive SLNB

subsequently underwent complete lymph node dissection (CLND). CLND increases the rate of regional control and slightly improved disease-free survival, although melanoma-specific survival was unaffected. Unfortunately, patients undergone to CNLB develop lymphedema. Therapeutic lymphadenectomy is performed when there is nodal recurrence and no evidence of distant metastases, or if there is a bulky nodal disease. For patients with resectable in-transit metastases without distant metastases, surgical excision is recommended to obtain pathologic clearance, while for those affected by metastases not amenable to resection, treatment options include topical treatment, and radiation therapy (RT). In patients with in-transit as well as distant metastatic disease, systemic treatment options should be considered (168).

1.2.7 Radiation Therapy

RT is usually used for patients with advanced disease, particularly when they cannot undergo surgery or in the case of LM. RT may also be used in the adjuvant setting for primary melanomas, where anatomic constraints prevent clear surgical margins. Moreover, for desmoplastic melanoma, it may have an adjuvant role both for positive and negative surgical margins in the setting of high-risk features, such as neurotropism or Breslow thickness of 4 mm or greater. As concerns patients with metastatic disease, radiotherapy guarantees an effective reduction of symptoms and it is used as palliative cure (168).

1.2.8 Systemic therapy

Systemic therapy, including cytotoxic chemotherapy, immunotherapy, or a combination approach such as biochemotherapy, represents the mainstay of therapy for most patients with stage IV melanoma. Moreover, many novel therapies are currently under investigation.

1.2.8.1 Dacarbazine and temozolomide

For patients with metastatic disease, surgical treatment alone is not curative. Drug therapies represent therefore the next line of defence. Until recently, the only treatment options for these patients was chemotherapy. In particular, the standard of care for metastatic melanoma is dacarbazine, (5-[3,3-dimethyl-1-triazenyl]-imidazole-4-carboxamide, or DTIC), an alkylating agent which results in DNA adducts and is cytotoxic to cells. Alkylating agents are capable of

inserting alkyl groups within macromolecules of organic compounds, such as proteins and DNA. At DNA level these agents can cause an incorrect chain pairing, as well as breaks within the strands, which can generate errors during the cell replication phase, thus having a genotoxic effect. The cytotoxic action of alkylating agents occurs with much greater intensity in reproductive cells and has a linear dose / response effect. DTIC gained FDA approval for the treatment of melanoma in 1975. It is administered by intravenous infusion and it is metabolized by the liver into its intermediate metabolite, 3-methyl-(trianzen-1-yl)-imidazole-4-carboxamide (MTIC). Its side effects consist in nausea and vomiting and myelosuppression. Nevertheless, it is generally well tolerated. Several studies demonstrated that DTIC treatment has partial response (PR) rates of approximately 15–28 %, complete response (CR) rates of approximately 3–5 %, and very few durable responses of <2 %. The addition of other agents in order to increase DTIC efficacy, including chemotherapeutic drugs, immunotherapy agents (IFN and IL-2), and anti-oestrogens, showed no improvements in overall survival and at the cost of increased side effects and decreased quality of life (169-171). Dose and schedules of dacarbazine vary widely, the most used regimen is 800 to 1000 mg/m^2 intravenously repeated every 3–4 weeks, or 200 mg/m^2 intravenously for 5 days every 3–4 weeks (172).

A dacarbazine analogue orally administered is temozolomide. Like dacarbazine, it is an alkylating agent, which is FDA-approved in the treatment of glioblastoma, and it is converted to the active alkylating metabolite MTIC as well as DTIC. Unlike dacarbazine, however, this conversion is spontaneous, nonenzymatic, and occurs at a physiologic pH in all tissues to which the drug is distributed. Besides having excellent oral bioavailability, temozolomide penetrates the CNS and may potentially prevent or treat melanoma brain metastases. Temozolomide has several side effects, among which headache, nausea and vomiting, and myelosuppression, including lymphopenia and thrombocytopenia. Different studies demonstrated that there are no differences between these two agents, which are generally believed to be interchangeable, since they have an equivalent median overall survival and similar response rates (173). Since temozolomide does not have the FDA approval for advantaged melanoma treatment, it is used for patients with CNS metastases. Two different schedules have been evaluated: 1) a 5-day regimen with a daily dose of 150–200 mg/m^2 on days 1–5, repeated every 3–4 weeks. The major side effect is represented by mild to moderate myelosuppression. Mild nausea and vomiting are also common but they can be readily controlled with standard antiemetic therapy; 2) a second regimen (known as extended dosing of temozolomide), which consists in a lower daily dose for prolonged periods (75 mg/m^2 daily for 6 weeks on, 2 weeks off) has been investigated. This

dosing regimen of temozolomide is associated with more lymphopenia, and opportunistic infections, specifically pneumocystis pneumonia, have been reported (174).

1.2.8.2 Nitrosoureas and Carboplatin/Taxanes

In addition to DTIC and temozolomide, the nitrosoureas are used for advanced-stage melanoma. In particular, the combined use of two alkylating agents, carmustine (BCNU) and lomustine (CCNU), was investigated. BCNU is administered intravenously, while CCNU is taken orally. It is believed that treatment with BCNU and CCNU provides benefit to those patients with brain metastases, but side effects are greater than those of DTIC treatment. As regards carboplatin, it is used for the treatment of different solid tumours, exploiting its cytotoxic effect resulting from DNA crosslink formation and inhibition of both replication and transcription, It has been tested for metastatic melanoma treatment, as well as taxanes, both paclitaxel and docetaxel. This class of chemotherapy agents functions as microtubule inhibitors. In particular, they act through the stabilization of tubulin polymerization and microtubule formation, resulting in dysfunctional mitotic spindle complexes and cell death, and they are tested to be used alone or in combination. The response rates obtained with carboplatin, paclitaxel and docetaxel are comparable to those observed with dacarbazine, but taxanes show significant side effects. Combinations of platinums and taxanes have also been the examined for the treatment of advanced-stage melanoma, in the attempt to identify more effective chemotherapy regimens. Several trials demonstrated that the combination of carboplatin and paclitaxel can be used as a second-line treatment (172).

1.2.8.3 Immunotherapy

Cytotoxic chemotherapy may have a palliative benefit in some patients with metastatic melanoma, but it usually does not lead to durable responses and its survival benefit has not been proven. Preclinical and clinical data have revealed the susceptibility of melanoma to approaches designed to modulate the immune system. Some immunotherapeutic approaches have led to durable complete responses in a small subset of patients, although it has been challenging to predict patient responsiveness to immunotherapy. In 1998, FDA approved the high-dose bolus intravenous IL-2 (HD IL-2) as a therapy for metastatic melanoma. IL-2 is secreted by T helper cells and promotes T-cell proliferation, as well as the development of lymphokine-activated killer (LAK) cells, which have the ability to directly lyse tumour cells (175). HD IL-2 is

administered at a dose of 600,000 to 720,000 IU/kg by IV bolus every 8 hours on days 1 to 5 (cycle 1) and on days 15 to 19 (cycle 2), with a maximum of 28 doses per each two-cycle course. Response evaluation is usually performed 4 weeks after the second cycle. Courses of HD IL-2 may be repeated in patients with evidence of tumour regression. Th administration of HD IL-2 requires the hospitalization of the patient. Major toxicities associated with this therapeutic approach include fever, chills, hypotension, increased capillary permeability, cardiac arrhythmias, oliguria, volume overload, delirium, and rash. Bacterial sepsis can also complicate HD IL-2 administration, and antibiotic prophylaxis is therefore recommended. The risk of multiorgan complications usually limits its use to younger patients with excellent performance status and organ function. To reduce systemic toxicity and increase local therapeutic effects, many groups have adopted an intralesional approach for IL-2 alone or in combination. Several studies suggest that intralesional IL-2 can expand regulatory T cells (Tregs), an inhibitory T-cell population with a high affinity for IL-2 receptor, CD25, which allows them to readily expand in response to IL-2. Intralesional IL-2 may therefore promote a systemic suppressive response by Tregs. A phase II clinical trial reported no additional deaths from melanoma in patients who had survived for at least 25 months after this therapy. These results are promising, given that the 5-year survival for patients with cutaneous melanoma metastases is approximately 18%. Intralesional IL-2 therapy is usually administrated in combination with the topical application of imiquimod (176-177), which induces the production of a variety of cytokines, including interferon alfa-2b (IFN-α), TNF, and IL-12, through the activation of immune cells via the toll-like receptor 7 (TLR7)-MyD88-dependent signalling pathway. This mechanism contributes to the strong anti-tumour properties of this small molecule. Despite all, subcutaneous and dermal melanomas develop resistance to imiquimod, and for this reason the use of this therapeutic agent alone is not recommended (177). On the contrary, the FDA approved INF-α for adjuvant therapy of resected high-risk melanoma. This agent is associated with a moderate antitumour activity in metastatic melanoma patients. Responses, however, are limited to patients with low volume disease in cutaneous or soft-tissue sites and are sometimes delayed, with onset many months after initiation. The common toxicities associated with IFN-α, such as fever, chills, fatigue, myalgias, psychocognitive impairment, and autoimmune events, adversely affect patient quality of life, especially with long-term administration, whereas pegylated IFN-α (PEG-Intron) allows a more convenient administration with a good tolerability, thus suggesting that interferon cannot be used alone, but its antitumour activity may be important for developing a combination of therapeutic agents (178-179). Moreover, specific antibodies are used as immunotherapeutic agents. In particular, the cytotoxic T-lymphocyte

antigen (CTLA)-4 is a transmembrane receptor, expressed on activated T lymphocytes, that induce the downregulation of T cell activation by provoking cell cycle arrest, inhibiting IL-2 secretion and by downregulating T-cell cytokine receptors. Monoclonal antibodies that bind to CTLA-4 may therefore potentiate immune responses against cancer cells, allowing for unopposed T-cell activation, breaking of tolerance to tumour antigens, and ultimately tumour lysis (180-183). Anti-CTLA-4 antibodies (ipilimumab and tremelimumab) are currently being evaluated in clinical trials in melanoma and they have been associated with encouraging antitumor activity. Responses to anti-CTLA-4 antibodies are sometimes delayed in onset and are often associated with development of immune-related adverse events. Anti-CTLA-4 antibodies may be particularly useful in combination with other strategies designed to stimulate antitumor immune responses, such as adoptive T-cell therapy or vaccination approaches (173). The programmed cell death 1 receptor (PD-1), expressed by T cells, has two primary ligands: PD-L1, found on cancer cells and tumour-infiltrative macrophages, and PD-L2, found on antigen-presenting cells. When PD-1 binds PD-L1 it acts as a negative regulator of T cells. As well as for CTLA-4 therapy, antibodies against both PDL1 and PD-1 have been developed to inhibit this down-regulatory pathway. Recent phase I trials of nivolumab (anti-PD-1) in combination with ipilimumab and BMS936559 (anti-PD-L1) have showed promise in treating patients with advanced melanoma. A second anti-PD-1 agent, pembrolizumab, has been just recently approved by FDA, although some side effects are reported (173, 184).

Another immunotherapeutic strategy is represented by the adoptive cell therapy (ACT), which involves collection of lymphocytes from the patient (peripheral blood lymphocytes or tumor-infiltrating lymphocytes [TIL]), *in vitro* selection/expansion/ activation of collected lymphocytes, and subsequent infusion of processed lymphocytes back into the patient to induce an immune response against the cancer cells. A recent report demonstrates that ACT may be useful for treating metastatic melanoma. In particular, good results has been obtained by a clinical trial, which involved host lymphodepletion (using cyclophosphamide plus fludarabine with or without total-body irradiation) followed by autologous TIL transfer and HD IL-2. Another example of successful use of ACT involved a novel approach that isolated and expanded autologous CD4+ T-cell clones with specificity for the melanoma-associated antigen NY-ESO-1. When these cells were infused into a patient with refractory metastatic melanoma, who had not undergone any previous conditioning or cytokine treatment, the transferred CD4+ T cells mediated a durable clinical remission and led to endogenous responses against melanoma antigens other than NY-ESO-1 (185-186).

Cancer vaccines are included into the immunotherapeutic strategies. The primary goal of this therapy is to activate the immune system in order to stimulate the recognition and disruption of cancer cells. Cancer vaccines can be univalent and polyvalent, the first inducing the immune response to a unique antigen, the second allowing the host to mount an immune response against multiple antigens. Peptides derived from the melanoma antigens MART-1, Melan-A, gp100, and tyrosinase have all been employed in cancer vaccines. Moreover, dendritic cells, nucleic acids (Allovectin-7), and heat shock protein complexes (vitespen) were used to develop a melanoma vaccine. Several trials show that the combined used of vaccination and IL-2 has a better result than IL-2 treatment alone (173).

1.2.8.4 Chemotherapeutic combination

Given the low response rates to single chemotherapy agents, combinations of three to four chemotherapy agents have been investigated for the treatment of advanced-stage melanoma. Several regimens have been investigated, including combinations of cisplatin, vinblastine or vindesine, and dacarbazine (CVD) and what is known as the Dartmouth regimen (carmustine, dacarbazine, cisplatin, and tamoxifen). This latter showed a not significantly increased tumour response and no long-term or survival benefits were observed. Moreover, there were significantly increased toxicities, suggesting that it should not replace dacarbazine as a standard chemotherapy regimen. Concerning CVD, it is used as a second line treatment and as a backbone for combining with IL-2 and interferon to develop biochemotherapy regimens. In addition, the combination chemotherapy regimen of bleomycin, vincristine, lomustine, and dacarbazine (BOLD) with INF-a in the treatment of advanced-stage melanoma has also been investigated. The study results show that BOLD do not show differences in response rate and overall survival compared to DTIC treatment, as well as combined with immunotherapy has the same response rate and overall survival of dacarbazine administration. The combination of paclitaxel and carboplatin (PC) has been reported to have antitumor activity in patients with metastatic melanoma. Although combinations of cytotoxic agents may yield somewhat higher response rates than dacarbazine monotherapy they are associated with greater toxicity and the survival is not significantly extended. Currently, the optimal chemotherapy regimen has not yet been identified (172-173).

1.2.8.5 Biochemotherapy

The term biochemotherapy refers to regimens that combine cytotoxic agents with IFN-α and/ or IL-2. Early single-institution trials of biochemotherapy demonstrated promising antitumor activity. This led to the rapid development of many such regimens and subsequently to an extensive investigation comparing these regimens to chemotherapy alone. Several trials showed a higher response rate compared to chemotherapy alone, even if there is not an improvement of the overall survival. It was tested in the treatment of melanoma, in the metastatic setting and as neoadjuvant and adjuvant therapy in stage III disease. Biochemotherapy is associated with considerable toxicity, including myelosuppression, nausea, vomiting, rash, hypotension, and fluid retention. Given the lack of survival benefit, which has been evaluated in a randomized clinical trial, along with the toxicity, biochemotherapy is not the standard of care for patients with stage IV melanoma. This therapeutic strategy may provide a palliative benefit in patients who are symptomatic and/or have rapidly progressive disease (172, 177).

1.2.8.6 Targeted therapy

BRAF mutations are present in 40‑70% of melanomas, inducing a constitutive activation and uncontrolled cellular proliferation. Vemurafinib and dabrafenib are BRAF inhibitors specific to melanomas harboring the BRAF V600E and V600E/K mutations, respectively. The development of BRAF inhibitors treatment led to a significant improvement in survival. Furthermore, the inhibition of BRAF has a radio-sensitizing effect. Although the increase in survival with vemurafenib alone has been modest, combination therapies using this agent along with other targeted therapies and immunotherapies, are currently under investigation. The understanding of the mechanisms involved in vemurafenib resistance and the subsequently design of multidrug regimens that block these survival pathways will be useful to develop an effective targeted therapy treatment. The identification of these resistance pathways in a given patient may allow for a more "personalized medicine" approach. Patients treated with vemurafenib, especially older patients with chronic sun damage, show an increased incidence of squamous cell carcinoma, as a result of comorbid mutations in the RAS gene in patients having a mutation in BRAF. A concomitant treatment with a MEK inhibitor can therefore be used. However, the targeted therapy increases the risk of radiation toxicity to the skin, an event that is averted by administering reduced doses of the drug. Several studies have reported increased survival in patients with brain metastases undergoing combination therapy of BRAF

inhibitor and surgical excision, due to the fact that BRAF inhibitor administration allows the reduction of tumour mass (168, 187-188).

1.3 NICOTINAMIDE N-METHYLTRANSFERASE

1.3.1 Drug metabolism

The term “xenobiotic” refers to a molecule foreign to the physiologic metabolism. This substance, therefore, once introduced into the body, tends to be expelled. Drugs belong to the category of xenobiotic compounds that, once introduced, can be excreted as such or undergo structural changes, aimed at promoting their excretion. The biochemical modifications of drugs, occurring through specific enzymatic systems, constitute a complex of reactions included within drug metabolism. After being introduced into the body, the drug must be equipped with a chemical structure that counteracts its elimination, to reach the target organ and perform its function. The lipophilic nature of xenobiotic compounds favors their absorption, while the hydrophilic one facilitates their excretion. For this reason, lipophilic compounds are converted into high-hydrophilic substance that can be excreted into urine or bile. Drug metabolism occurs principally in the liver, followed by lung, kidney and intestine. The reactions constituting drug metabolism can be grouped into 2 pathways, each provided with a specific set of enzymes. Phase I biotransformation (redox) reactions determine the introduction or the disclosure of functional groups allowing drug polarization, and are performed by cytochrome P450 monooxygenases, flavin monooxygenases, monoamine oxidases, aldehyde dehydrogenase, alcohol dehydrogenase, and hydrolysis by cholinesterase, arylesterase and epoxide hydrolase. In Phase II metabolism, endogenous compounds, or metabolites of phase I reactions are conjugated to molecules with a marked polar character. These reactions are catalyzed by cytosolic enzymes, such as glutathione S-transferases, N-acetyltransferases, sulfotransferases, UDP-glucuronyltransferases and methyltransferases (189). Methyltransferases play a role of primary importance in Phase II metabolism of drugs and xenobiotic compounds. This class of enzymes can allow the methylation of sulfur (S), oxygen (O) and nitrogen (N). Specifically, enzymes such as thiol methyltransferase (TMT) and thiopurine methyltransferase (TPMT) catalyze the S-methylation of pharmacologically active compounds such as 6-mercaptopurine, D-penicillamine and captopril. The O-methylation reactions, involving the phenolic groups of catecholamines and structurally similar compounds, such as some neurotransmitters, are catalyzed by enzymes such as catechol-O-methyltransferase (COMT) and phenol O-methyltransferase (POMT). Finally, the transfer reactions of a methyl group to nitrogen are catalyzed by enzymes such as histamine N-methyltransferase (HNMT) and nicotinamide N-methyltransferase (NNMT) (190-191).

1.3.2 Nicotinamide homeostasis

Vitamin B3 also known as nicotinamide, niacinamide, niacin and vitamin PP, owes its biological effect to two functionally related forms: nicotinamide and nicotinic acid. It represents the precursor of pyridine compounds, such as NAD+ (Nicotinamide Adenin Dinucleotide) and NADP+ (Nicotinamode Adenin Dinucleotide Phosphate). These cofactors play a leading role in cell energy metabolism since they are involved in most of redox reactions, mediating the transfer of hydrogen ions and electrons for ATP production. In addition, these pyridine compounds are also involved in non-redox processes, which lead to the breaking of the β-N-glycosidic bond, with consequent release of nicotinamide. Among them, it is possible to distinguish:

- deacetylation of histones and transcription factors, catalysed by NAD(+)-dependent histone deacetylases (sirtuins) (192-193).
- ADP-ribosylation of proteins, catalysed by mono-ADP-ribosyltransferase (ARTs) and poly ADP-ribose polymerases (PARPs) (194).
- production of effector molecules, such as cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NaADP), that originate from CD38 molecule catalysis and are able to mobilize intracellular calcium stores (195).

Concerning its absorption, nicotinamide introduced with the diet is assimilated at gastrointestinal level, according to a dose-dependent transmembrane transport mechanism. In addition to being introduced in the diet, nicotinamide can be synthesized via the kynurenine pathway that leads to the conversion of L-tryptophan into quinolinic acid and NAD+. However, the contribution of this "salvage pathway" to the synthesis of nicotinamide is rather poor, as the precursor:vitamin conversion rate is 60:1 (196-198). Another source of nicotinamide is represented by the reserves of NAD+ in the liver (storage NAD+). The catalysis carried out by the hepatic NAD glycohydrolases determines the release of nicotinamide directly into the bloodstream. The liver also catabolizes the nicotinamide in excess, by means of pyridine nitrogen methylation. Following this modification, nicotinamide is concentrated in the kidney and excreted trough urine. In humans, methylated nicotinamide may undergo further oxidation reaction by hepatic enzymes, an event that leads to the formation of pyridones that are also excreted trough urine. In the light of these evidences, the liver is a fundamental organ for maintenance of nicotinamide levels in serum (199).

1.3.3 Human NNMT characterization

Among enzymes of Phase II drugs metabolism, a key role is played by nicotinamide N-methyltransferase (NNMT), a cytosolic enzyme that catalyse the N-methylation of nicotinamide and other structural analogues of pyridine, using S-adenosyl-L-methionine (SAM), as methyl donor. Methylated nicotinamide (N1-methylnicotinamide or MNA) can be eliminated as it is in the urine or can undergo oxidation, through the catalysis exerted by the enzyme aldehyde oxidase. The products, resulting from the accomplishment of this catalysis, are N1-methyl-2-pyridone-5-carboxyamide and N1-methyl-4-pyridone-3-carboxyamide, also excreted through urine. S-adenosyl-L-methionine, following donation of the methyl group to nicotinamide, is converted into S-adenosyl-L-homocysteine (SAH) and subsequently hydrolyzed into homocysteine and adenosine (Figure 13). Although the aza-heterocyclic compounds are N-methylated by several N-methyltransferases, only NNMT uses nicotinamide as methyl acceptor, thus suggesting its important role in nicotinamide catabolism (200-203).

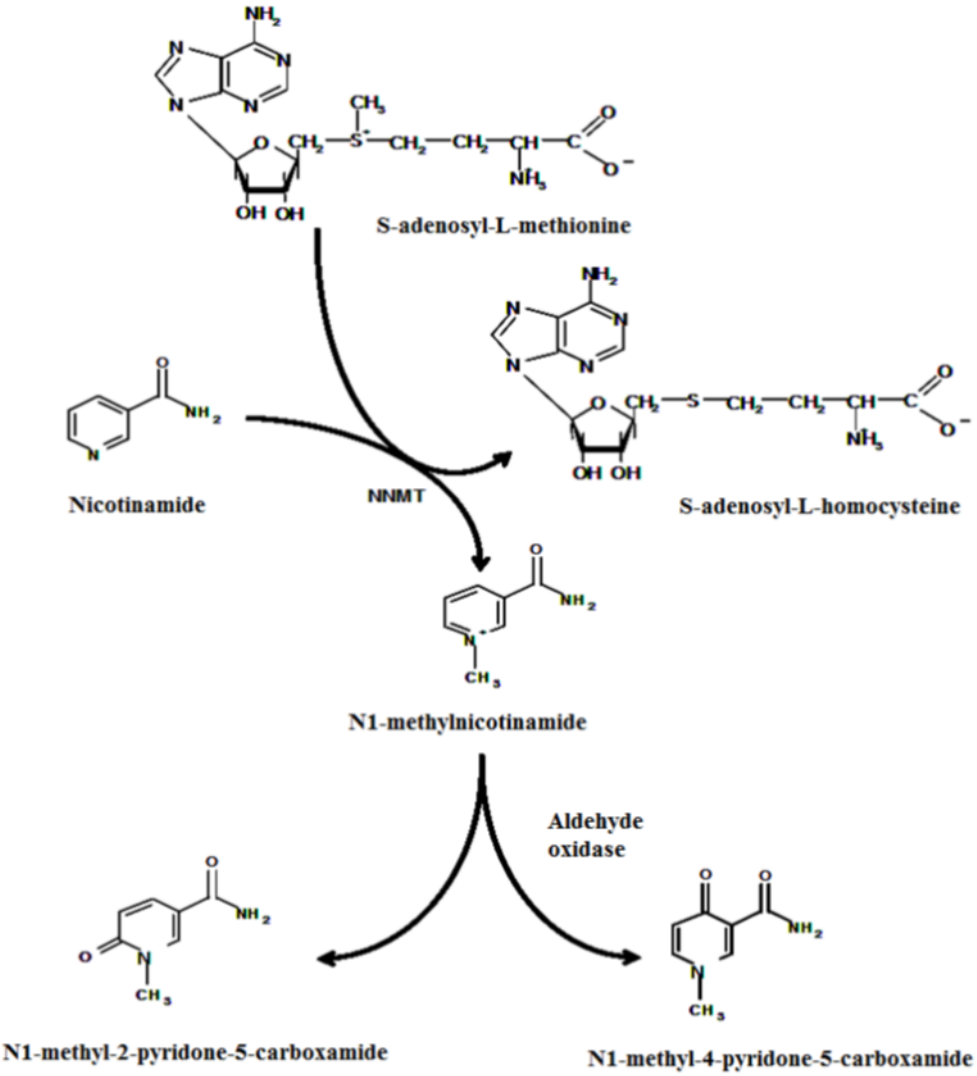

Figure 13: Reaction of methylation of nicotinamide and oxidation of N1-methylnicotinamide, catalyzed by NNMT and aldehyde oxidase, respectively.

Human NNMT characterization was firstly performed on hepatic tissue extracts and required the development of a 4-step assay to determine the catalytic activity. The enzyme assay includes:

- the preparation of a mixture containing [$_{14}$CH3] S-adenosyl-L-methionine,
- the extraction of N1-methylnicotinamide using 60% isoamyl alcohol in toluene, with 1-heptane sulfonic acid, as a phase modifier,
- the separation and identification of N1-methylnicotinamide, through reverse phase chromatography in HPLC,
- the measurement of radioactivity with a liquid scintillator.

In particular, NNMT showed a cytoplasmic localization and worked under optimal conditions at a pH value close to 7.4. Km values calculated for nicotinamide and S-adenosyl-L-methionine were 347μM and 1.76μM respectively. The specific activity of the enzyme in hepatic tissue extracts was found to be 51.5± 32.5U/mg, but individual tissue samples showed poor homogeneity. The frequency distribution found within the population analyzed in this study showed a bimodal trend and 26% of the samples showed high values of specific activity. The differences observed in NNMT activity were thought to be due to individual metabolic variations of metabolism, thus suggesting that enzyme activity could be regulated by genetic polymorphism (204-206). The native protein obtained from liver tissue was partially purified by ion exchange chromatography followed by gel filtration. The sample was subsequently subjected to SDS-PAGE and, following photoaffinity labeling, it was possible to determine a molecular mass of 29.6 kDa, corresponding NNMT. The last step of this process involved a chemical and enzymatic proteolysis of the partially purified protein, and after the analysis of the amino acid sequence of generated fragments it was possible to identify the cDNA sequence. As shown in Figure 14, NNMT cDNA consists of a 792bp region coding for a 264 amino acid protein, with a molecular mass of 29.6 kDa. For the identification of NNMT kinetic parameters, cDNA was inserted into a specific eukaryotic vector, which was used to transfect COS-1 cells. Data (substrate kinetic and enzyme inhibition) obtained for recombinant NNMT were compared to those related with protein partially purified from human liver. In particular, the following parameters were identified:

- Km for nicotinamide were 0.43mM and 0.38mM for the native and recombinant protein, respectively;
- Km for S-adenosyl-L-methionine were 1.8μM and 2.2μM for native and recombinant protein, respectively.
- IC_{50} for N1-methylnicotinamide were 60μM and 30μM for the native and recombinant protein, respectively (207).

Subsequent molecular analyzes showed that NNMT gene is located on chromosome 11, in the q23.1 region and its nucleotide sequence (16.5Kb) consists of 3 exons and 2 introns. The transcription start site was identified 108 nucleotides upstream the first coding triplet, 30 nucleotides downstream of an atypical TATA box sequence (TCTAA) (208). Transcription activity is controlled by a strong promoter, located 700 nucleotides upstream of the transcription start site. Further studies evidenced the involvement of HNF-1β, IL6, TGF-β1, STAT3 as molecules able to regulate the activity of the NNMT promoter. Signal transducer and activator of transcription 3 (STAT3) was found to be activated after phosphorylation. Studies performed

in embryonic kidney cells have highlighted an increased activity of the NNMT promoter following activation/phosphorylation of STAT3 induced by leukemia inhibitory factor (LIF) and interleukin 6 (IL6). Research studies carried out in breast, liver and colorectal cancer cells have shown elevated levels of NNMT following treatment with IL6. Moreover, both STAT3 inhibition or downregulation in colon cancer cells resulted in a reduction of NNMT expression level (209).

An association between the overexpression of HNF-1β and NNMT was found in papillary thyroid carcinoma cell lines. Following the introduction of point mutations in NNMT promoter region hosting the HNF-1β binding site, a decrease in promoter activity was observed, reflecting the importance of the HNF-1β, as a transcription factor of NNMT. In addition, NMMT protein and mRNA levels were decreased in BHP 18-21 papillary thyroid cancer cells after treatment with the histone deacetylase inhibitor depsipeptide that mediates the downregulation of HNF-1β (210-211). A further molecule, involved in the regulation of NNMT expression, is TGF-β1. This hypothesis arose following the results of a study conducted in insulinoma, in which mRNA levels of both TGF-β1 and NNMT are reduced compared to healthy tissue. Moreover, high NNMT levels seem to be associated with the upregulation of TGF-β1 in clear cell renal cell carcinoma (ccRCC) compared to those of control tissue, thus confirming that this cytokine is able to positively regulate the NNMT expression (212-213).

NNMT mRNA level is particularly elevated in the liver, but also in other organs and tissues such as placenta, kidney, lung, skeletal muscle, heart, spleen, prostate, thymus, testis, intestine, ovary, stomach, thyroid, spinal cord, lymph nodes, trachea, adrenal gland, bone marrow and in particular areas of the nervous system.

Analysis by X-ray diffraction, conducted on crystals obtained from the purified human recombinant NNMT, led to the resolution of the 3D structure of the enzyme. Subsequent site-directed mutagenesis experiments allowed the identification of the amino acid residues mainly involved in catalytic activity: tyrosine 20 (Y20) and aspartic acid 197 (D197) (Figure 15) (207-208,214).

```
1   atggaatcaggcttcacctccaaggacacctatctaagccattttaac   48
1    M  E  S  G  F  T  S  K  D  T  Y  L  S  H  F  N    16
49  cctcgggattacctagaaaaatattacaagtttggttctaggcactct   96
17   P  R  D  Y  L  E  K  Y  Y  K  F  G  S  R  H  S    32
97  gcagaaagccagattcttaagcaccttctgaaaaatcttttcaagata  144
33   A  E  S  Q  I  L  K  H  L  L  K  N  L  F  K  I    48
145 ttctgcctagacggtgtgaagggagacctgctgattgacatcggctct 192
49   F  C  L  D  G  V  K  G  D  L  L  I  D  I  G  S    64
193 ggccccactatctatcagctcctctctgcttgtgaatcctttaaggag 240
65   G  P  T  I  Y  Q  L  L  S  A  C  E  S  F  K  E    80
241 atcgtcgtcactgactactcagaccagaacctgcaggagctggagaag 288
81   I  V  V  T  D  Y  S  D  Q  N  L  Q  E  L  E  K    96
289 tggctgaagaaagagccagaggcctttgactggtccccagtggtgacc 336
97   W  L  K  K  E  P  E  A  F  D  W  S  P  V  V  T   112
337 tatgtgtgtgatcttgaagggaacagagtcaagggtccagagaaggag 384
113  Y  V  C  D  L  E  G  N  R  V  K  G  P  E  K  E   128
385 gagaagttgagacaggcggtcaagcaggtgctgaagtgtgatgtgact 432
129  E  K  L  R  Q  A  V  K  Q  V  L  K  C  D  V  T   144
433 cagagccagccactgggggccgtcccctttaccccggctgactgcgtg 480
145  Q  S  Q  P  L  G  A  V  P  L  P  P  A  D  C  V   160
481 ctcagcacactgtgtctggatgccgcctgcccagacctccccacctac 528
161  L  S  T  L  C  L  D  A  A  C  P  D  L  P  T  Y   176
529 tgcagggcgctcaggaacctcggcagcctactgaagccaggggcttc  576
177  C  R  A  L  R  N  L  G  S  L  L  K  P  G  G  F   192
577 ctggtgatcatggatgcgctcaagagcagctactacatgattggtgag 624
193  L  V  I  M  D  A  L  K  S  S  Y  Y  M  I  G  E   208
625 cagaagttctccagcctccccctgggccgggaggcagtagaggctgct 672
209  Q  K  F  S  S  L  P  L  G  R  E  A  V  E  A  A   224
673 gtgaaagaggctggctacacaatcgaatggtttgaggtgatctcgcaa 720
225  V  K  E  A  G  Y  T  I  E  W  F  E  V  I  S  Q   240
721 agttattcttccaccatggccaacaacgaaggacttttctccctggtg 768
241  S  Y  S  S  T  M  A  N  N  E  G  L  F  S  L  V   256
769 gcgaggaagctgagcagacccctgtga  792
257  A  R  K  L  S  R  P  L  -   264
```

Figure 14: Coding sequence of human NNMT. Different colors represent the different exons. In addition, the amino acid sequence of the translated protein is also indicated.

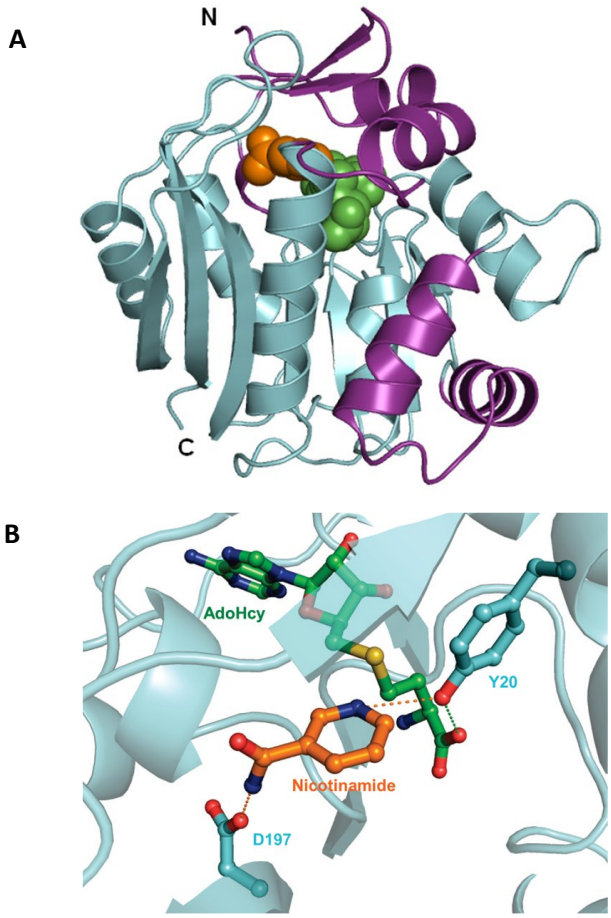

Figure 15: 3D structure of human recombinant NNMT bound to nicotinamide and S-adenosyl-L-homocysteine (A). Enzyme active site and principal amino acids (Y20 e D197) involved in catalytic activity (B).

As previously reported, nicotinamide is the substrate of NNMT. However, a study conducted on native porcine protein purified to homogeneity evidenced the ability of the enzyme to methylate pyridine-like compounds (211). Concerning NNMT substrate specificity, a recent study described enzyme methylation activity towards β-carbolines. These molecules are pyridine-derived compounds, capable of exerting a marked inhibitory effect against respiratory chain complex I, whose accumulation in the central nervous system (dopaminergic neurons) represents an etiopathogenetic trait of Parkinson's disease. The methylation activity of human recombinant NNMT was assayed against β-carboline norharman (NH). The results obtained showed that the enzyme can exert its catalysis towards this substrate, resulting in the formation of 2-N-methylnorharman (MeNH), showing a greater affinity towards NH compared to that related with nicotinamide (215-216). Identification of alternate substrates and inhibitors of the

enzyme, has been performed following the development of a new and rapid assay for the determination of NNMT activity, coupling liquid chromatography to mass spectrometry. The application of this methodology allowed the evaluation of a wide spectrum of pyridine-like compounds, as potential substrates of the enzyme, and represented the basis for the identification of enzyme inhibitors (217).

1.3.4 NNMT polymorphisms

As previously hypothesized, differences in NNMT activity could be associated to genetic polymorphism within different regions of the related gene. Therefore, the presence of polymorphisms in three exons, intron 1, and 5'-flanking region of NNMT gene was evaluated in samples obtained from human liver biopsies, where enzyme activity displayed wide variability. No single nucleotide polymorphism (SNP) or deletion/insertion was found in exons and 5'-flacking region. On the contrary, eight SNPs were evidenced in intron 1, although none of them is related to a change in activity, thus, suggesting that individual variation in enzyme activity is due to the differences of NNMT mRNA and protein levels (218-219).

In a study focusing on genetic determinants of venous thrombosis, a genome-wide linkage scan was performed for genes influencing homocysteine plasmatic levels. Results obtained revealed the presence of a genetic determinant at chromosome 11q23, corresponding to NNMT gene. Moreover, one SNP (rs694539) on NNMT intron 1 was found to be strongly related with homocysteine levels, potentially acting as regulator of NNMT transcription (220).

Given the important role of NNMT in homocysteine metabolism, the relation between NNMT polymorphism and the risk of developing congenital heart defects (CHDs) was analysed. The results of this study showed that children carrying the nicotinamide N-methyltransferase polymorphism rs694539 (AG/AA genotype) and exposed to periconception medicines and low nicotinamide intake have an eight-fold increased risk for CHDs (221). Even if maternal hyperhomocysteinemia is a risk factor for neural tube defects, there is no correlation between NNMT SNP and spina bifida (222).

Abdominal aortic aneurysm (AAA) is also associated with hyperhomocysteinemia, thus suggesting a potential involvement of NNMT gene variants in the development of this disease. To explore this hypothesis, 56 polymorphisms of genes involved in methionine metabolism were assessed through a primer extension-based microarray technology, and seven haplotypes, including one screened for NNMT, were significantly associated with AAA. Nevertheless, subsequent studies showed no significant association between AAA and NNMT polymorphism

(223-224). Moreover, NNMT IVS-151 TT variant is associated with the increased risk to develop acute lymphoblastic leukaemia (ALL) (225). As previously described, rs694539 is associated to a hyperhomocysteinemia, while rs1941404 variant is strictly related to schizophrenia (SZ). In addition, in post-mortem frontal cortex of patients affected with SZ, NNMT mRNA levels were 35% lower than control subjects, thus suggesting that the enzyme could be involved in SZ aetiology (226). In Han Chines female population an association between the NNMT rs694539 variant and SZ was detected, confirming the key role of the enzyme in the disease (227).

Other studies evidenced that NNMT polymorphism rs694539 is a risk factor of several diseases including non-alcoholic steatohepatitis, epilepsy, and bipolar disorders. Moreover, AA genotype is associated with an increased risk for epilepsy and non-alcoholic steatohepatitis, while GG genotype displayed protection against both diseases. Despite the role of rs694539 NNMT variant in different diseases is still not clear, epigenetic alterations, increase of homocysteine and variation of nicotinamide levels seem to be associated with the development of the above described pathologies (228-230).

1.3.5 NNMT and non-neoplastic diseases

Nicotinamide N-methyltransferase is mostly expressed in liver but its function in hepatic cells is still unclear. A recent study demonstrated that in mice liver, MNA mediated the regulation of glucose, lipid, and cholesterol metabolism through the stabilization of SIRT1 protein (231). Moreover, in cirrhotic patients, MNA serum levels are increased compared to those of control subjects (232). An upregulation of the enzyme was evidenced in biopsies of human renal allograft after acute cellular rejection, and in endometrial biopsies of pregnant women subjected to intracytoplasmic sperm injection cycles (233-235). Moreover, endometrial stromal cells showed an augmented enzyme expression in response to the production of cytokines by macrophages, thus suggesting that during the process of endometriosis there is an association between NNMT high level and migration and invasion of endometrial cells (236).

As previously described, NNMT SNPs are associated with several non-communicable chronic diseases. In particular, rs694539 is linked to hyperlipemia and high levels of plasmatic homocysteine, while rs10891644 is correlated with obesity (237-238). Moreover, an increased NNMT activity and homocysteine release are evidenced in murine embryo fibroblasts subjected to adipogenic differentiation, and in murine and human adipose tissue. These results suggest that NNMT activity could influence homocysteine level in bloodstream, as well as

cardiovascular disease risk, being hyperhomocysteinemia is a major factor for the development of these disorders (239).

Elevated levels of NNMT mRNA were detected in quadricep muscle samples of patients affected by chronic obstructive pulmonary disease (COPD). Subsequent induction of enzyme overexpression in myoblasts stimulated cell proliferation and migration, and decreased protein oxidation and cell death induced by H_2O_2, thus demonstrating a protective role exerted by NNMT against oxidative stress (240-241). In addition, NNMT might be potentially involved in COPD severity, given the high NNMT levels in lung tissues isolated from COPD patients (242). The protective role of the enzyme against mitochondrial reactive oxygen species (ROS) generation was evidenced also in in the pathogenesis of kidney proximal tubular cell damage and consequent renal dysfunction, in patients with refractory proteinuria. Lipotoxicity-induced oxidative stress and cell damage were reduced in proximal tubular cells treated with MNA. This evidence supports the hypothesis that MNA administration may have a beneficial effect for refractory proteinuria (243).

In mice treated with phenobarbital, a compound involved in in ROS generation via cytochrome P450 enzymes stimulation, NNMT activity was enhanced. The decrease of NAPH-dependent antioxidant enzymes activity was due to the depletion of pyridine nucleotides, that is strictly correlated with a high activity of the enzyme. Reasonably, NNMT overexpression might inhibit protective system against ROS generation (244).

The overexpression of NNMT was evidenced also in obese and diabetic mice, in white adipose tissue (WAT) and liver. In addition, mice subjected to gene silencing of NNMT showed protection against diet-induced obesity. Subsequently, NNMT levels were measured in WAT of individuals with type 2 diabetes and of insulin-resistant patients. Results showed a higher enzyme expression in samples obtained from insulin resistance subjects and type 2 diabetes patients compared to the controls, and a positive correlation between the NNMT expression and the degree of insulin resistance (245-246). Moreover, NNMT expression was evaluated in adipose tissue of Wistar Ottawa Karlsburg W (WOKW) and Dark Agouti (DA) rats, being the first considered animal models for metabolic syndrome and the second controls. Higher levels of NNMT, in terms of mRNA, protein, and enzyme activity, were measured in WOKW rats compared with DA ones, suggesting that the enzyme could have an important role in the pathogenesis of metabolic syndrome (247). A recent study demonstrated that NNMT overexpression in mice fed with a nicotinamide-supplemented high fat diet (HFD) promoted the development of hepatic steatosis and fibrosis, thus suggesting that enzyme inhibition could represent a new therapeutic strategy for treating both fatty liver and fibrosis (248).

Another study evidenced a negative relation between serum MNA level and ventricular ejection fraction and preload recruitable stroke work (249). Moreover, as regard cardiovascular diseases, an overexpression of NNMT, both in terms of mRNA and serum protein level, was detected in individuals with peripheral occlusive arterial disease, suggesting the hypothesis that enzyme could be a novel biomarker for this disease (250).

It is noteworthy that MNA could be used as topical therapy for several pathological conditions, such as dermatitis and *acne vulgaris*. The anti-inflammatory effect of MNA could be due to its ability to reduce adherence of pro-inflammatory cells and molecules to the vascular endothelium (251-252). In addition, in rats with arterial thrombosis, MNA showed an anti-thrombotic effect, indirectly participating to a mechanism that involved cyclooxygenase-2 (COX-2) and prostacyclin (PGI2). In this light, the demonstration that MNA is capable to regulate thrombotic and inflammatory processes, known to promote cardiovascular diseases, has been a starting point for important therapeutic implications related with these disorders (253). Since the development and progression of atherosclerotic plague are induced by vascular inflammation and thrombosis, and being these processes modulated by MNA, it was suggested that both this compound and NNMT could have a potential role in atherosclerosis. In atherosclerotic mice, the increased levels of hepatic NMMT activity and plasmatic MNA were associated with the progression of the disease. Given the antithrombotic and anti-inflammatory role played by MNA, enzyme overexpression could play a compensatory role in the disease (254). Subsequent analysis showed that MNA has a potential hepatoprotective effect through a PGI2-dependent mechanism. In particular, the progression of murine concavalinA (ConA)-induced hepatitis was associated with an increase level of NNMT activity in liver and the subsequent increase of plasmatic MNA (255). The activation of NNMT-MNA pathway in mice was found to be caused by the endurance exercise. In addition, exercise-induced activation of NNMT in the liver involved IL6, while plasmatic MNA levels were not totally IL6-dependant. Consequently, NNMT activity in liver could be regulated by IL6 but MNA plasma level was also modulated by other tissues (256).

NNMT activity in liver and lung was positively correlated with the progression of pulmonary arterial hypertension (PAH), being this enzyme upregulation associated with elevated plasma MNA level. These evidences suggest that the activation of NNMT-MNA pathway has a compensatory role also in PAH, partly due to the vasoprotective activity exerted by MNA (257).

1.3.6 NNMT and Parkinson's disease

N-methylation is an important mechanism for the detoxification of several xenobiotic compounds, but in some cases this process can increases the toxicity of some substrates. For this reason, it is conceivable that high NNMT activity in brain may induce the production of neurotoxic methylpyridinium ions, whose accumulation represents an etiopathological trait of Parkinson's disease (PD). The analysis of regional expression of NNMT in healthy human brain tissue, showed that enzyme is constitutively expressed in different areas of the central nervous system. Physiologically, NNMT mRNA levels are elevated in the spinal cord, medulla and temporal lobe, while are reduced in the subthalamic and caudate nuclei, as well as in the cerebellar portion. In addition, NNMT, both in terms of protein and its catalytic activity, is detectable in the temporal lobe and spinal cord. An increased protein level was found in the cerebellar area of subjects who died from Parkinson's disease. This data laid the basis for research aimed at identifying a potential involvement of NNMT in this neurodegenerative disorder. Moreover, several studies showed an inverse correlation between NNMT expression and the duration of the disease, suggesting a causative effect in the pathogenesis of PD (258-259). Numerous studies have speculated on the possible neurotoxicity of MNA and metabolic consequences resulting from the catalysis carried out by the NNMT towards alternative substrates (260-263). Recent researches demonstrated that the enzyme induces a cellular response that counteracts the stress condition associated with Parkinson's disease. In particular, the induction of NNMT expression in the human neuroblastoma-derived cell line SH-SY5Y inhibited cell death and led to increased activity of the mitochondrial complex I. Furthermore, within these cells, there was an increase in the intracellular levels of ATP and in the ATP/ADP ratio, as an index of energy availability. Subsequently, SH-SY5Y, lacking endogenous NNMT expression, were incubated with MNA. Results obtained showed that, treatment with MNA gave the cells a greater protection against the cytotoxic effect induced by complex I inhibitors, such as 1-methyl-4-phenylpyridinium ion (MPP+) and rotenone (264). Moreover, NNMT diaplayed a protective role against mitochondrial toxicity induced by potassium cyanide (KCN), 2,4-dinitrophenol, and 6-hydroxydopamine *in vitro* and this function is was carried out only through the production of MNA (265).

It was demonstrated that NNMT has also a role in neuron morphology and differentiation, since in SH-SY5Y and N27 rat mesencephalic dopaminergic cell lines the enzyme expression increases neurite branching and the production of functional synapses via the activation of the Akt-EFNB2 signaling pathway (266). Subsequently, to understand the molecular mechanism

behind the cytoprotective role of the enzyme, Sirt-3 was silenced in NNMT-expressing SH-SY5Y. Results showed that Sirt-3 mediates the NNMT-induced complex I activity and ATP production (267). As previously described, NNMT catalyses the N-methylation of several BCs, such as NH. The reaction product, MeNH, was shown to be less toxic than its precursor, since it was able to increase cell viability and intracellular ATP concentration in NNMT-expressing SH-SY5Y cells. In the light of these results, the overexpression of NNMT represent a molecular event exerting a cytoprotective effect featuring the pathogenesis of PD (216).

1.3.7 NNMT and cancer

Gene expression analysis on glioblastoma multiforme (GBM) samples revealed elevated levels of NNMT mRNA in pathological samples compared to controls (268). In addition, NNMT gene silencing in glioblastoma, allowed to demonstrate that the enzyme alters biochemical and cellular function, inhibiting the activity of the tumour suppressor enzyme PP2A (269). A recent study showed that NMMT is overexpressed in mesenchymal glioblastoma stem cells (GSCs) and its silencing induced a decreased cellular proliferation and *in vivo* tumour growth (270). Moreover, high NNMT activity and MNA levels were also found in human glioma cell lines treated with interferon gamma (271).

DNA microarray was used to analyse the gene expression profile of different human thyroid cancer cell lines. In particular, in papillary cancer cells NNMT level was found to be higher than in other subtypes, where the enzyme expression was low or absent. Moreover, immunohistochemical analysis of human thyroid tissues evidenced a strong NNMT positivity in follicular and papillary tumour, while no stain or faint immunoreaction was detected in other subtypes (272).

Concerning human breast adenocarcinoma, NNMT expression was found in adriamycin-resistant MCF-7 cells but not in parental adriamycin-sensitive cells (273-274). Subsequently, NNMT was downregulated in Bcap-37 and MDA-MB-231 breast cancer cell lines and silenced cells showed a decreased cellular growth *in vitro* and tumorigenicity *in vivo*. In addition, NNMT downregulation was associated to an augmented ROS generation and induced apoptosis via the mitochondria-mediated pathway. To confirm this hypothesis, the enzyme was overexpressed in MCF-7 and SK-BR-3 cell lines and results obtained candidate the enzyme to be a new promising molecular target for breast cancer treatment (275).

The overexpression of NMMT was detected also in pancreatic cancer (PC). Indeed, high NNMT level was observed in pancreatic juice of PC patients compared to controls (276).

Moreover, a metabolomic analysis conducted in PANC-1 PC cell line and miR-1291-expressing counterpart evidenced that expression of miR-1291 increased levels of MNA and those of NNMT mRNA expression. PANC-1 cells stably expressing miR-1291 also showed a reduced cell migration, invasion, and xenograft tumorigenesis. In addition, an inverse relationship was found between NNMT mRNA levels and xenograft pancreatic tumour size, upon cutaneous injection in athymic mice, thus suggesting that NMMT expression may affect the extent of pancreatic cancer progression (277). The involvement of NNMT in PC was demonstrated also by the enzyme silencing in PANC-1 cells. Transfected cells showed a decreased cell proliferation, invasion, and migration. On the contrary, enzyme overexpression induced an increase of PANC-1 survival subjected to glucose deprivation, or glycolytic inhibition, or treated with rapamycin, confirming the important role of NNMT in metastatic process and survival, under metabolic stress condition (278). The comparative analysis of pancreatic cancer tissue samples, paracancerous tissues and chronic pancreatitis, showed a high cancer-specific NNMT expression. Moreover, enzyme levels were positively correlated with unfavourable clinicopathological findings (279).

The overexpression of the enzyme was evidenced also in colorectal cancer tissue compared to adjacent normal tissue samples, using a proteomics approach. Moreover, high NNMT levels were also detected in serum of colorectal cancer patients compared to that of healthy subjects, suggesting that the enzyme could be a biomarker for the early and non-invasive detection of this type of tumour (280). To investigate the role of NNMT in colorectal cancer cell metabolism, the upregulation of the enzyme was induced in SW480 cell line, lacking constitutive NNMT expression. Results obtained showed that NNMT increased tumorigenicity both *in vitro* and *in vivo*, promoted cell cycle progression and inhibited apoptosis (281). Moreover, SW480 colorectal cancer cells showed a decreased 5-FU-induced apoptosis and reduction of caspases levels, as result of NNMT overexpression. In particular, enzyme activity inhibited the activation of the ASK1-p38 MAPK pathway, reducing cancer cells sensitivity to 5-FU (282).

NNMT involvement in cancer metabolism was also confirmed in gastric cancer, as following a comparative proteome analysis, the overexpression of the enzyme was evidenced in stomach adenocarcinoma with respect to control tissues (283-284). It is noteworthy that gastric cancer tissue subjected to two-dimensional electrophoresis and western blot (WB) showed multiple spots of NNMT, while healthy tissue and gastric ulcer samples showed a unique spot, thus suggesting that NNMT in cancer cells may undergo post-translational modifications (285). Furthermore, NNMT was found to be overexpressed in gastric cancer tissues compared to

healthy margins and enzyme levels were correlated with tumour size, lymph node infiltration and distant metastasis, TNM stage, and overall survival. Subsequent NNMT silencing in human gastric cancer cell lines (MGC803 and BGC823) induced a decrease of cell proliferation, invasion, and migration *in vitro* and *in vivo.* Taken together these results demonstrated the potential role of the enzyme as novel prognostic factor of gastric cancer (286). The NNMT-induced increase of invasive ability of gastric cancer cells was demonstrated upon the induction of enzyme overexpression in BGC-823 cell line. In particular, NNMT upregulation determined an increased expression of TGF-β1 with consequent activation of TGF-β1/Smad signaling that promoted the epithelial-mesenchymal transition (EMT) (287).

Profiling gene expression of ccRCC showed a higher NNMT expression in cancerous tissue compared than in normal kidney, being enzyme mRNA levels inversely correlated with tumour size, suggesting a potential role for the enzyme in cancer progression (288, 289). Immunohistochemical analysis confirmed enzyme upregulation in renal cancer and high NNMT expression was found to correlate with poor prognosis (290). Moreover, increased protein level was also evidenced in plasma and tissue interstitial fluid of RCC patients compared to those of controls, thus suggesting that the enzyme may be used as diagnostic biomarker for this cancer (291-293). Subsequent experiments of NNMT gene silencing in ccRCC cells showed that enzyme downregulation was associated with a reduced invasive capacity *in vitro* and an inhibited tumour growth and metastatic potential *in vivo* (294).

In order to investigate the role played by the enzyme in prostate cancer, immunohistochemistry was performed in benign and malignant tumour tissue samples. High NNMT level was detected in pathological samples compared to benign lesions and an inverse correlation between NNMT expression and Gleason score was demonstrated. Moreover, high enzyme level was associated with prolonged progression-free survival and overall survival in patients with advanced prostate cancer (295). Subsequently, NNMT level was found to be higher in peripheral blood of prostate cancer patients compared to healthy individuals and the induction of enzyme overexpression in human prostate cancer cell line PC-3 increased viability, invasion, and migration ability of cancer cells through SIRT1, demonstrating that the enzyme is a regulator of SIRT1 expression in prostate cancer cells and can be a potential therapeutic target for this neoplasm (296).

NNMT overexpression was also highlighted in bladder cancer (BC). Gene expression profile of stress-related and DNA repair genes was analysed in BC cell line MGH-U1 and its radiosensitive subclone (S40b). Results showed higher enzyme level in MGH-U1 compared to S40b, hypothesizing that the enzyme could play a potential role in predicting radiation response in this neoplasm (297).

In the context of cancer stem cells (CSCs), NNMT overexpression enhanced resistance to radiation treatment of tumorigenic mesenchymal CSCs. The increase of NNMT activity may reduce the intracellular amount of nicotinamide, thus removing nicotinamide-mediated PARP inhibition and consequent activation of DNA repair processes. In addition to NNMT, the enzyme nicotinamide phosphoribosyltransferase (NAMPT), responsible for the conversion of nicotinamide into NAD+, was also overexpressed in CSC clones after radiation treatment. High NAMPT activity resulted in an increase of intracellular level of NAD+, being substrate for PARP-mediated DNA repair activity (298).

In CSC-enriched population obtained from Hep-2, T24, MG63, A549 and CaCo2 cell lines NNMT was found to be upregulated at both mRNA and protein level, as well as concerning catalytic activity. Since CSCs have a crucial role in early tumour development and maintenance, these observations suggested that NNMT could represent an interesting molecular target for cancer therapy (299-300).

A positive correlation between NNMT expression and cancer cell migration and tumour stage was found in human bladder cancer cell lines. Moreover, NNMT silencing in bladder cancer cells induced a decrease of cell migration and proliferation (301).

NNMT expression as well as its activity were found to be higher in bladder urothelial carcinoma (UC) samples compared with adjacent healthy tissues specimens. Subsequently, NNMT level was measured also in exfoliated urinary cells collected from urine from bladder UC patients and control subjects. Results obtained evidenced an augmented NNMT expression, in terms of both mRNA and protein level, in pathological samples with the respect to healthy urines, suggesting that the enzyme can be a novel biomarker for early and non-invasive detection of bladder cancer (302).This hypothesis was confirmed by a recent study that analysed NNMT mRNA level in urine from bladder cancer patients and controls, by using Real-Time PCR. Receiver operating characteristic (ROC) analysis and area under the curve (AUC) values showed an excellent diagnostic accuracy associated with this analysis, thus laying the basis for the development of potential urine-based NNMT test for BC detection (303). Moreover, the upregulation of the enzyme was recently reported in in muscle-invasive bladder cancer compared to non-invasive one (304).

In order to identify new biomarkers for lung cancer, serum from non-small lung cancer (NSCLC) patients, subjects affected by COPD and healthy donors were subjected to ELISA and increased levels of NNMT was detected in samples from cancer patients compared to controls. Moreover, the sensitivity for NSCLC detection was higher when NNMT was used in combination with the carcinoembryonic antigen (CEA) (305). In another study, NNMT

expression was evaluated in tumour, tumour-adjacent, and contiguous normal-looking tissues from patients with NSCLC. Data obtained showed that NNMT was overexpressed in tumour specimens compared to adjacent and surrounding tissue samples, thus, suggesting that NNMT could be a molecular marker for NSCLC (306-307).

A negative correlation between NNMT level and miR-449a was evidenced in Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors (EGFR-TKI)-resistant NSCLC cells. In addition, in drug-resistant NSCLC cells NNMT silencing induced the expression of miR-449a and inhibited p-Akt and tumorigenesis. These data showed that the enzyme might have a potential as new therapeutic target for drug-resistant NSCLC (308).

Conversely, NNMT mRNA levels were significantly reduced in hepatocellular cancer (HCC) samples compared to healthy surrounding tissue. However, a positive correlation was found between enzyme expression and tumour stage, while an inverse association was detected between protein level and overall survival, suggesting that the enzyme could be a prognostic factor in HCC (309). NNMT overexpression in human HCC cell line stimulated cellular invasion and adhesion (310).

The expression of NNMT was also assessed in oral squamous cell carcinoma (OSCC). A significant increased NNMT level was detected in pathological tissue compared to normal oral mucosa. Moreover, both mRNA and protein levels were inversely correlated with main clinicopathological findings of enrolled patients (311-312). These results were confirmed by a recent study that showed a higher enzyme catalytic activity in OSCC tissues than normal oral mucosa. Moreover, western blot analysis demonstrated that NMMT expression was increased in saliva of OSCC patients with respect to that of control samples, suggesting that NNMT could be a potential biomarker for non-invasive and early diagnosis of oral cancer (313).

In order to explore the role of the enzyme in oral cancer cell metabolism, KB and PE/CA-PJ15 OSCC cell lines were subjected to RNA-mediated NNMT silencing and effects on cancer cell phenotype were explored. Data obtained demonstrated that a decreased cell growth *in vitro* and tumorigenicity *in vivo* were associated to NNMT downregulation, showing the important role of the enzyme in tumorigenesis (314-315).

On the contrary, HSC-2 cells were subjected to the induction of NNMT overexpression and apoptosis and cell proliferation were evaluated. An increase in cell proliferation was detected after NNMT overexpression and a positive correlation was evidenced between enzyme level and that of survivin ΔEx3 isoform, highlighting potential involvement of NNMT in apoptosis. These data confirmed the key role played by the enzyme in tumorigenicity of OSCC cells (316).

NNMT overexpression was also described in nasopharyngeal cancer samples and enzyme level was associated with advanced tumour stage and poor prognosis (317). Also adenoid cystic carcinoma lymph node metastatic cells (ACCS-LN) showed enzyme overexpression, and cell ability to acquire stem-like phenotype involved NNMT upregulation (318).

A recent study showed that high NNMT activity influences the cancer cell methylation potential, due to enzyme-related consumption of methyl units from S-adenosyl-L-methionine. Therefore, NNMT overexpressing cancer cells undergo hypomethylation of the histones and cancer-related proteins, as well as enhanced expression of pro-tumorigenic factors (319-320).

NNMT expression induced a higher metabolic plasticity in serous ovarian cancer cells resistant to glucose starvation. Moreover, Zinc Finger E-box Binding Protein 1 (ZEB1)-induced phenotypes, such as those displaying an increased migration capacity, required the expression of NNMT and its level was found to be increased in metastatic and recurrent neoplasms compared to primary carcinomas, while normal ovary and fallopian tube tissue had no detectable NNMT expression. In addition, in a recent study was demonstrated that NNMT might reflect the degree of malignant and metastatic behaviour in ovarian cancer. Taken together these results suggested that NNMT could be an important therapeutic target against drug resistance and recurrence in high-grade serous ovarian cancer (321-322).

The overexpression of NNMT was also detected in cervical squamous cell carcinoma and elevated enzyme level were found in association with high-grade lesions, advanced stage disease and presence of lymph node metastasis (323).

1.4 AIM OF THE STUDY

As previously described, NNMT was found to be overexpressed in several neoplasms and play a crucial role in cancer metabolism. However, to date no data are available on its expression in skin cancers. Since skin cancers represent an emerging global health problem, the identification of prognostic and diagnostic factors is of primary importance. For this reason, during this research work the attention was focused on skin cancers, aiming to explore whether NNMT could be involved in these neoplasms.

The research plan was divided into two phases: the aim of the first phase was to analyze the expression of the enzyme in melanoma and NMSCs, while the second one had the purpose to evaluate the role of the enzyme in melanoma cell metabolism. In particular, during the first phase of the research work immunohistochemical analyses were carried out on 40 BCC cases and 39 SCC cases, to evaluate enzyme expression in tumour and surrounding healthy margins. Moreover, the relationship between NNMT levels in cancer tissues and clinicopathological parameters was explored. Subsequently, NNMT immunohistochemical expression was evaluated in 34 cutaneous melanoma samples and 34 nevi specimens, used as controls. To evaluate enzyme role in melanoma cell metabolism, during the second part of the work NNMT gene silencing was carried out on the human cutaneous malignant melanoma A375 cell line. Cells were transfected with plasmids encoding shRNAs targeting NNMT mRNA and, upon the evaluation of silencing efficiency, NNMT silenced cells were subjected to tests aimed at evaluating aspects strictly related to tumorigenicity, such as cell proliferation (MTT assay) and migration (wound healing assay). Subsequently, proliferation of A375 cells downregulating NNMT and controls was evaluated under treatment with dacarbazine, in order to explore the potential involvement of the enzyme in sensitivity of melanoma cells to chemotherapy. These analyzes were carried out in order to explore whether the decrease of NNMT expression was able to alter the phenotype of the neoplastic cell, in order to verify the possibility to target the enzyme as molecular approach to effectively treat cutaneous melanoma.

2 MATERIALS AND METHODS

2.1 ANALYSES PERFORMED ON TISSUE SAMPLES

2.1.1 NMSC patients and specimens

As regards NMSCs, a total of 79 formalin-fixed and paraffin-embedded (FFPE) samples were obtained from the archives of the Pathologic Anatomy and Histopathology Division of the Department of Biomedical Sciences and Public Health of Polytechnic University of Marche. Specimens were collected from patients subjected to biopsy or excisional surgery between 2016 and 2018. NMSC samples included 40 cases of BCC and 39 SCC cases. The BCC group consisted of 26 males and 14 females aged between 42 and 86 years (mean age: 68 years). BCC samples included 20 nodular and 20 infiltrative cases, three of which showed areas with nodular appearance within the same tumour. SCC specimens were obtained from 27 males and 12 females with a mean age of 75 years (age range: 54 ‐ 94 years). Twenty SCC cases arose in the head and neck region, while 19 occurred on the trunk and extremities. Demographic and clinicopathological findings are reported in Table 6. Healthy tissue margins were used as controls.

Table 6: Patients and clinicopathological findings of BCCs and SCCs.

CATEGORIES	**No.**
BCCs	
CASES	40
GENDER	
Males	26
Females	14
AGE	
Mean	68
Range	42 - 86
DIAMETER (cm)	
Mean	0.7
Range	0.2 - 1.3
SUBTYPE	20
Nodular	20
Infiltrative	
SCCs	
CASES	39
GENDER	
Males	27
Females	12
AGE	
Mean	75
Range	54 - 94
THICKNESS (mm)	
Mean	2.5
Range	0.4 - 7.5
DIAMETER (cm)	
Mean	1.1
Range	0.4 - 2.6
GRADING	
G1	28
G2	11
G3	0
SUBTYPE	
Head and Neck	20
Trunk and Extremities	19

2.1.2 Cutaneous melanoma patients and specimens

With regard to melanoma, 34 FFPE samples of primary melanoma lesions were obtained from the archives of the Pathologic Anatomy and Histopathology Division of the Department of Biomedical Sciences and Public Health of Polytechnic University of Marche. Thirty-four benign compound or dermal melanocytic nevus specimens served as controls. Samples were collected from patients subjected to biopsy or surgical excision between 2009 and 2016. The melanoma group consisted of 21 males and 13 females aged between 28 and 96 years (mean age: 62.3). Nevus specimens were obtained from age- and gender-matched patients. The demographic characteristics and the clinicopathological parameters related to the pathological samples are reported in Table 7.

Table 7: Patients and Clinicopathological findings of melanoma.

CATEGORIES	**No.**
CASES	34
SEX	
Males	21
Females	13
AGE	
Mean	62
Range	28 – 96
BRESLOW THICNESS (mm)	
Mean	1.76
Range	0.1 – 8
CLARK LEVEL	
I	2
II	5
III	12
IV	13
V	2
MITOTIC RATE (mitoses/mm^2)	
0	18
1-5	8
> 5	8
REGRESSION	
No	27
Yes	7
ULCERATION	
No	29
Yes	5
FLOGOSIS	
No	17
Yes	17
SUBTYPE	
Suoerficial spreading	21
Nodular	9
Lentiginous acral	2
Melanoma *in situ*	2

2.1.3 Immunohistochemical analyses

NNMT expression in NMSC, melanoma and nevus samples, was evaluated by immunohistochemical analysis. Five μm microtome sections obtained from FFPE tissue blocks were mounted on poly-L-lysine coated glass slides. Subsequently, after deparaffinization in xylene and rehydration in a graded series of alcohols, samples were treated with EnVision FLEX Target Retrieval Solution Low pH (Dako, Carpinteria, California, USA) for antigen retrieval. Endogenous peroxidase activity was inhibited by incubating samples with a 3% H_2O_2 solution for 7 minutes. After washing with EnVision FLEX Wash Buffer (Dako, Carpinteria, California, USA) for 5 minutes, specimens were incubated with rabbit polyclonal antibody against human NNMT (1:1500 dilution) (Sigma‐Aldrich, Saint Louis, MO) in a humified atmosphere for 1 hour at room temperature. After a further washing, sections were treated with EnVision FLEX/HRP (Dako, Carpinteria, California, USA) for 20 minutes. The slides were then washed and incubated with the diaminobenzidine contained in the EnVision FLEX DAB + Chromogen (Dako, Carpinteria, California, USA) solution for 10 minutes. Sections were counterstained with Mayer's Hematoxylin (Bio-Optica, Milan, Italy) and permanently mounted on glass slides. ccRCC sample was used as positive control, while negative controls were obtained by replacing the primary antibody with rabbit IgG isotype. All analyses were independently carried out by two experts who were blinded to the clinicopathological characteristics of the samples, using a light microscope equipped with a Nikon DS-Vi1 digital camera (Nikon Instruments, Europe BV, Kingston, Surrey, England). To assess inter- and intra-observer variability, each slide was analysed three times. The agreement between the two experts was always greater than 95% and discrepancies were resolved by means of a double-headed optical microscope, for the simultaneous assessment of the case. For cell counting, NIS Elements BR 3.22 imaging software (Nikon Instruments, Europe BV, Kingston, Surrey, England) was used. Stained cells were counted in at least ten fields per sample (field 0.07 mm^2, magnification 400×) and quantified as a percentage of the total counted cells.

2.1.4 Statistical analysis

Data were analyzed using GraphPad Prism software, version 7.00 for Windows (GraphPad Software Inc., San Diego, California, USA). The differences between groups and the correlations between NNMT expression levels and clinicopathological findings were

determined using the Mann-Whitney U test and the Spearman test. A p value less than 0.05 was considered statistically significant.

2.2 ANALYSES PERFORMED ON CELL CULTURES

In order to evaluate the role of NNMT in cancer cell metabolism, in vitro experiments were performed using the human malignant cutaneous melanoma A375 cell line as experimental model.

2.2.1 Cell line and reagents

The human malignant cutaneous melanoma cell line A375, obtained from the American Type Culture Collection (ATCC), was cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and 50μg/ml gentamicin. Cells were maintained at 37°C in a humified 5% CO_2 atmosphere.

2.2.2 NNMT shRNA-mediated gene silencing

NNMT silencing was performed on A375 cell line through RNA interference (RNAi), a post-transcriptional gene silencing mechanism based on the action of double-stranded RNA (dsRNA) molecules that bind to mRNA in a specific sequence causing its degradation. RNAi can be performed using 2 types of silencer: small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs). The first consists of a double-stranded RNA which, once acquired by the target cell through transfection, determines the sequence-specific degradation of mRNA related to the target gene (324). As regard shRNAs, they are encoded by a sequence in a plasmid used to transfect cells of interest. Once inside the cell, these plasmids are recognized by RNA Polymerase III, which can lead to the transcription of shRNA sequences. These molecules consist of a sense filament, a spacer (loop) and an antisense filament. The sense and antisense filaments pair up leading to the formation of a typical "hairpin" structure which, once recognized by specific multienzymatic complexes, first undergoes the degradation of the loop and then the unwinding process, which consists in the separation of the two complementary filaments. The antisense filament then binds to a complementary sequence present in the mRNA of the target gene, inducing its subsequent degradation (Figure 16) (325).

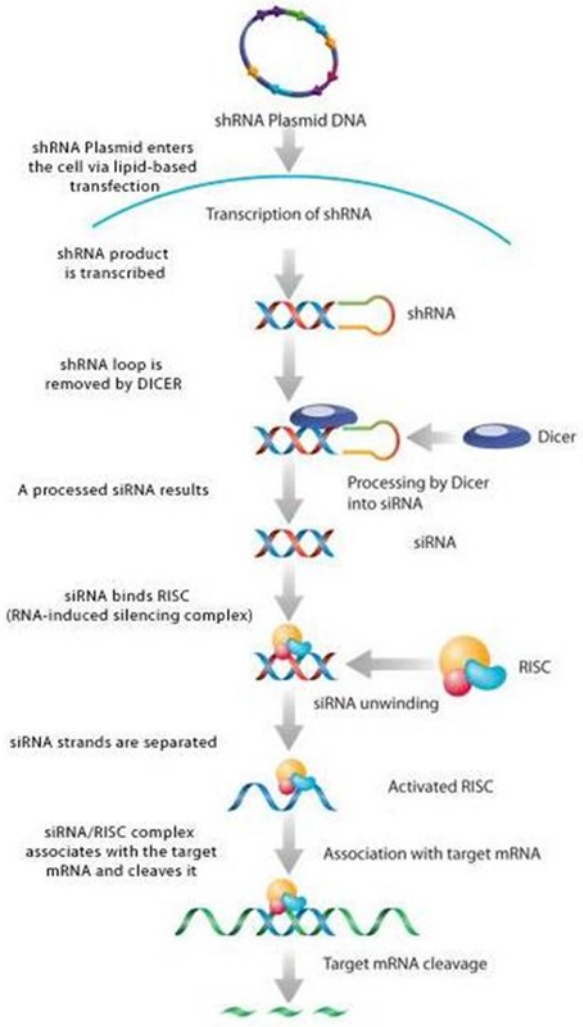

Figure 16: Gene silencing by shRNAs encoding plasmids (324).

In particular, to achieve NNMT silencing, a set of pLKO.1 vectors containing stem-loop cassettes encoding for different shRNAs targeting different region of NNMT mRNA was used (Sigma-Aldrich, Saint Louis, MO). A375 cells were seeded in 24 well plates ($4x10^4$ cells/well) the day before transfection with different plasmids (0.5 µg for each well), each encoding for a specific shRNA against NNMT (pLKO.1-164, pLKO.1-330 and pLKO.1-711) or with a combination of all three plasmids (0.5 µg of final DNA amount, named as pLKO.1-mix). Control cells were transfected with an empty vector (pLKO.1-puro) or treated with transfection reagent only (Mock) (Figure 17).

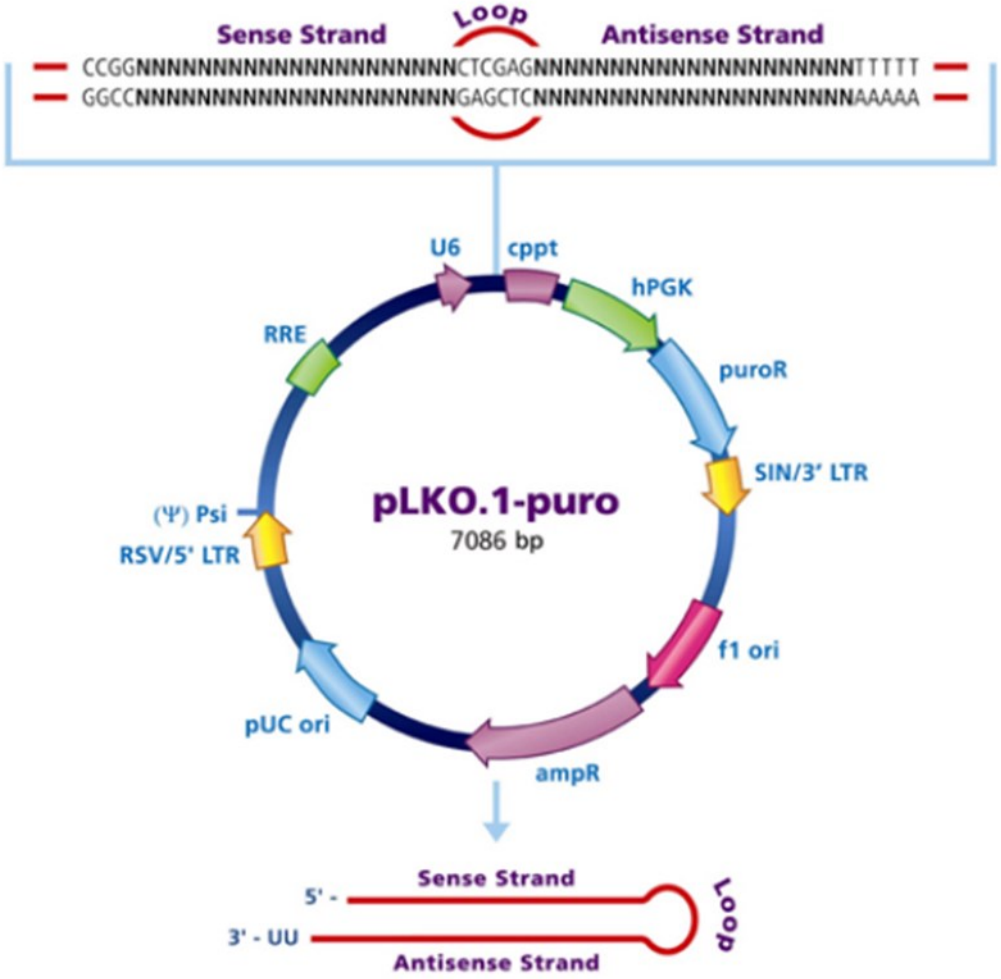

Figure 17: pLKO.1-puro plasmid. The expression of shRNA encoded by the plasmid are regulated by the human U6 promoter. The vector includes a puromycin resistance gene (PuroR) for selection in mammalian cells

Transfection was performed the day after seeding through the liposomal agent FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). For each sample, a 25 µl volume mixture was prepared, containing 0.5 µg of plasmid DNA, 1.5 µl of FuGENE and serum- and antibiotic-free DMEM medium. The mixture was then incubated at room temperature for 10 minutes and subsequently added to each well containing 500 µl of complete medium. Forty-eight hours after transfection, cellular clones stably downregulating NNMT started to be selected by treatment with 1µg/ml puromycin. The selection of puromycin-resistant cell clones lasted for a period of about two weeks and upon completion of the selection process, the cell clones were expanded. For all subsequent analysis puromycin resistant cells were cultured in complete medium with

1μg/ml puromycin. The efficiency of NNMT silencing in A375 cells was evaluated by Real-Time PCR and Western blot analysis.

2.2.3 Total RNA extraction and cDNA synthesis

Cells pellets ($1x10^6$ cells), collected after treatment with Trypsin-EDTA and counted using the Burker chamber and Trypan-Blue, were suspended and homogenized in a lysis buffer. After the isolation of total RNA through SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions, quantity and quality of RNA were tested spectrophotometrically at 260 nm, 230 nm and 280 nm using NanoDrop 1000 (Thermo Scientific). Subsequently, 1 μg of RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), using random primers. cDNA obtained was used as template for subsequent Real-Time PCR analysis.

2.2.4 Real Time PCR

NNMT silencing was verified by Real-Time PCR, using the previously synthesized cDNA as template. Real-Time PCR allows the amplification, and, at the same time, quantification of the DNA used as a template (326). The DNA is amplified by a polymerase chain reaction and the progress of the reaction is monitored through the emission of fluorescence by specific fluorescent markers, the accumulation of which in the reaction product follows the same kinetics of the amplification reaction. Therefore, as the accumulation of the reaction product increases, the fluorescent signal, which is acquired by an optical reader coupled to the thermocycler and subsequently reprocessed by specific software, becomes higher. The chemistry adopted for the detection of amplification products involved the use of EvaGreen as a fluorescent molecule capable of intercalating with the double strand of DNA.

In an amplification reaction, the amount of product formed after a thermal cycle doubles in the next cycle. Consequently, by measuring the increase in fluorescence, due to the formation of a new product as the cycles progress, it is possible to obtain the amplification curve of the template of interest, having a sigmoidal trend. In the increasing exponential phase of this curve it is possible to identify an amplification cycle corresponding to a fluorescence value which is a measure of the quantity of product formed: the threshold cycle (Ct). Graphically, the Ct is determined by the projection on the abscissa axis of the point where the curve intersects the baseline, above which any fluorescence increase becomes significant. For NNMT expression

analysis, the relative quantification method was adopted, which allows to evaluate the differences in the expression levels of a gene in a sample with respect to its control, by comparing the threshold cycles. In order to make a comparison it is necessary to choose a reference gene different from that of interest (GOI), which can act as an internal standard for both the sample and the control: the housekeeping gene (HKG). Once the Ct values relating to the GOI and HKG have been calculated, the ΔCt (ΔCt = Ct GOI - Ct HKG) is determined. The parameter that quantifies the relative expression of GOI in the sample (S) compared to the control (C) is equal to $2^{-\Delta\Delta Ct}$, where ΔΔCt = SΔCt - CΔCt (327).

The analysis of the NNMT expression in the samples was carried out using the CFX96 Real-Time PCR Detection System (Bio-Rad). For each sample, reaction mixtures of 20 µl, containing 1 µl of cDNA, 10 µl of EvaGreen Supermix (Bio-Rad) and the specific primers at a final concentration of 500 nM were prepared in duplicate. The protocol provides for the carrying out of 40 amplification cycles, each of which consists of a denaturation phase at 95 ° C for 30 seconds and an annealing-elongation phase at 58 ° C for 30 seconds. The nucleotide sequences relating to the pairs of primers used for the amplification of the genes of interest and for the housekeeping gene (β-actin), are as follows:

- NNMT (forward) → 5'-GAATCAGGCTTCACCTCCAA-3',
- NNMT (reverse) → 5'-TCACACCGTCTAGGCAGAAT-3',
- β-actina (forward) → 5'-TCCTTCCTGGGCATGGAGT-3',
- β-actina (reverse) → 5'-AGCACTGTGTTGGCGTACAG-3'.

Changes in relative NNMT expression were calculated by $2^{-\Delta\Delta Ct}$, where ΔCt was (Ct NNNMT-Ct β-actin) and ΔΔCt = ΔCt (pLKO.1-164, pLKO.1-330, pLKO.1-711, pLKO.1-mix or pLKO.1-puro)- ΔCt (Mock).

2.2.5 Western Blot

NNMT protein levels were evaluated using western blot analysis. $2x10^6$ cells pellets were suspended in 200 µl of lysis buffer (phosphate buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 1 mM phenylmethylsulphonyl fluoride and 2 µg/ml aprotinin) and then homogenized by passing (3-10 times) through a 30 gauge needle attached to a 1 ml syringe. Cell lysates were then centrifuged at 16000 x g for 10 minutes at 4 °C and supernatants represented the protein extracts. Fifty µg of the protein extract obtained from each sample were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the Laemmli method (328), using a running gel at a polyacrylamide concentration equal to 15%. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 250 mA for 30 minutes, using a wet transfer method. After blocking overnight at 4 °C in 1X phosphate buffered saline (PBS) solution containing 5% non-fat dry milk, and 0.05% tween-20, the membranes were washed three times with the washing solution (1x PBS containing 0.05% tween-20). Subsequently, PVDF membranes were incubated with rabbit polyclonal antibody against NNMT (Sigma-Aldrich, St. Louis, MO, USA) (1:1000 dilution) for 1 hour, and then treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) (1:2000 dilution) for 1 hour. A 5 minutes incubation with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) allowed the detection of NNMT protein level. NNMT-related chemiluminescent signal was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA). Beta-actin (β-actin) was used as internal control.

2.2.5 Protein assay

The evaluation of protein concentration in the samples was performed by means of the Bradford assay (329), using the Bio-Rad Protein Assay (Bio-Rad), consisting of an aqueous solution of Comassie Brilliant Blue G-250 (BBC), methanol and phosphoric acid.

The formation of the bond between the dye (BBC) present in the Bradford reagent and the proteins determines the shift of the maximum absorption of the solution from 465 nm to 595 nm, the wavelength at which the samples are read spectrophotometrically. By setting up a calibration curve using standards with known concentrations, it is possible to obtain by interpolation the protein content of the samples.

2.2.6 MTT assay

The MTT biochemical assay was used to evaluate the effect of enzyme silencing on cell proliferation. This method exploits the ability of mitochondrial dehydrogenase to cleave the tetrazole ring of the MTT compound [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] added to the culture medium, in order to generate a dark blue-violet formazan salt that accumulates inside vital cells. The samples are subsequently treated with a suitable solvent to obtain cellular lysates showing a color whose intensity is proportional to the quantity of formazan produced and, consequently, to the number of viable cells present in the well.
A375 cells downregulating NNMT as well as controls were seeded in 96-wells ($1x10^3$ cells / well) and cultured for 24 hours in humified atmosphere at 37°C and 5% of CO_2. The day after seeding, the first MTT test was performed, corresponding to the zero point of the growth curve (0h). A solution of MTT (5 mg / ml in PBS) was diluted (1:12) in complete culture medium and added to the cells. After 4 hours of incubation, the medium was discarded and 200 µl of dimethyl sulfoxide (DMSO) were added to each well to dissolve formazan salts. The reaction product was then quantified by assessing the absorbance at 540 nm using an automatic plate reader. This procedure was repeated at 24h, 48h and 72h from the first test (zero point).
Each experiment was repeated three times. Results were expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at 0h) and presented as mean values ± standard deviation of three independent experiments performed in triplicate.

2.2.6 Monolayer wound healing assay

In order to evaluate migration ability, A375 cells were subjected to the Wound Healing Assay, which mimics the migration capacity of cells during wound healing in vivo (330). NNMT downregulating cells as well as control populations were seeded into 6-well plates ($3x10^5$ cells/well) and allowed to grow up to 100% confluency. Monolayers were then wounded by using sterile 200 µl pipette tips. Subsequently, cell debris were removed by washing cell monolayers three times and medium was replaced with D-MEM supplemented with 2mM L-glutamine and 0.5% FBS. Scratched monolayers were monitored under a microscope equipped with a camera and photographed at 0h, 4h, 8h, 24h and 28h after scratching. Images were analyzed by the software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). Each experiment was run in

triplicate and and independently replicated three times. Results were expressed as percentage of wound recovery.

2.2.7 Chemotherapeutic treatment

NNMT downregulating A375 cells and controls were seeded in 96-well plates ($1x10^3$ cells/well). After 24 hours, cells started to be incubated with complete medium containing 100µg/ml dacarbazine (Sigma-Aldrich), previously dissolved in 1M HCl at 50mg/ml stock solution. Untreated cells were grown in complete medium containing HCl only, diluted at 2mM final concentration, corresponding to that obtained in samples subjected to treatment with chemotherapeutic drug. MTT assay was used to evaluate cell proliferation in untreated populations (0h) and at different time points (24h, 48h and 72h) after starting chemotherapeutic treatment.

2.2.8 Statistical analysis

The analysis of the obtained data was performed using GraphPad Prism software version 7.00 for Windows (GraphPad Software, San Diego, USA). Differences between groups were established using two-way analysis of variance (ANOVA). A p value < 0.05 was considered as statistically significant.

3 RESULTS

3.1 EXPRESSION OF NICOTINAMIDE N-METHYLTRANSFERASE IN SKIN CANCERS

3.1.1 NNMT levels in NMSCs

The immunohistochemical expression of nicotinamide N-methyltransferase was evaluated both in tumour tissues and in healthy surrounding margins.

As regards BCCs, the statistical analysis showed a no significant relationship between enzyme level and age, gender, and tumour size ($p \geq 0.05$).

Protein immunopositivity was found to be higher in pathological tissue than in normal keratinocytes. In particular, the percentage of positive cells was 9.92 ± 1.93 in tumour tissue and 5.05 ± 1.27 in healthy margins ($p < 0.0001$). Both in nodular and in infiltrative variants NNMT expression was significantly increased in tumour tissue (being 16.6 ± 2.38% for nodular BCC and 3.25 ± 0.81% for infiltrative BCC; $p < 0.0001$) with respect to the corresponding controls (10.1 ± 1.72% and no staining, respectively; $p < 0.0001$). In addition, the infiltrative subtype showed a significantly reduced NNMT immunopositivity compared to the nodular one, thus suggesting an inverse correlation with the tumour aggressiveness (Figures 18 and 20). Interestingly, this expression pattern was also found in BCCs showing both nodular and infiltrative features within a single tumour (Figure 21).

Concerning SCC, no statistically significant relationship was observed between enzyme expression and age, sex, and tumour size ($p \geq 0.05$). The immunohistochemical analysis showed a significantly lower NNMT immunopositivity in cancer cells compared to normal keratinocytes (19.38 ± 2.54% and 41.74 ± 3.48%, respectively; $p < 0.0001$). The decrease in NNMT expression can be evidenced also considering lesions occurring on the head and neck region and those of the extremities and trunk separately. Indeed, the percentage of positive cells in head and neck SCC was 9.24 ± 2.23%, while that observed in the healthy tissue margins was 45 ± 3.81% ($p < 0.0001$), while in SCC arising on the trunk and extremities the percentage of positive cells was 29.89 ± 2.57% and 38.32 ± 3.22% in the pathological tissues and controls, respectively ($p<0.0001$). It is noteworthy that NNMT levels were significantly higher in tumours occurring in the trunk and extremities than in SCC of the head and neck ($p<0.0001$). These results suggest the presence of a negative correlation between enzyme expression and tumour aggressiveness. Moreover, in well-differentiated (G1) lesions NNMT immunopositivity was increased compared with moderately-differentiated (G2) tumours (21.64 ± 2.61% and 15.00 ± 2.50%, respectively; $p < 0.0001$) (Figures 19 and 20).

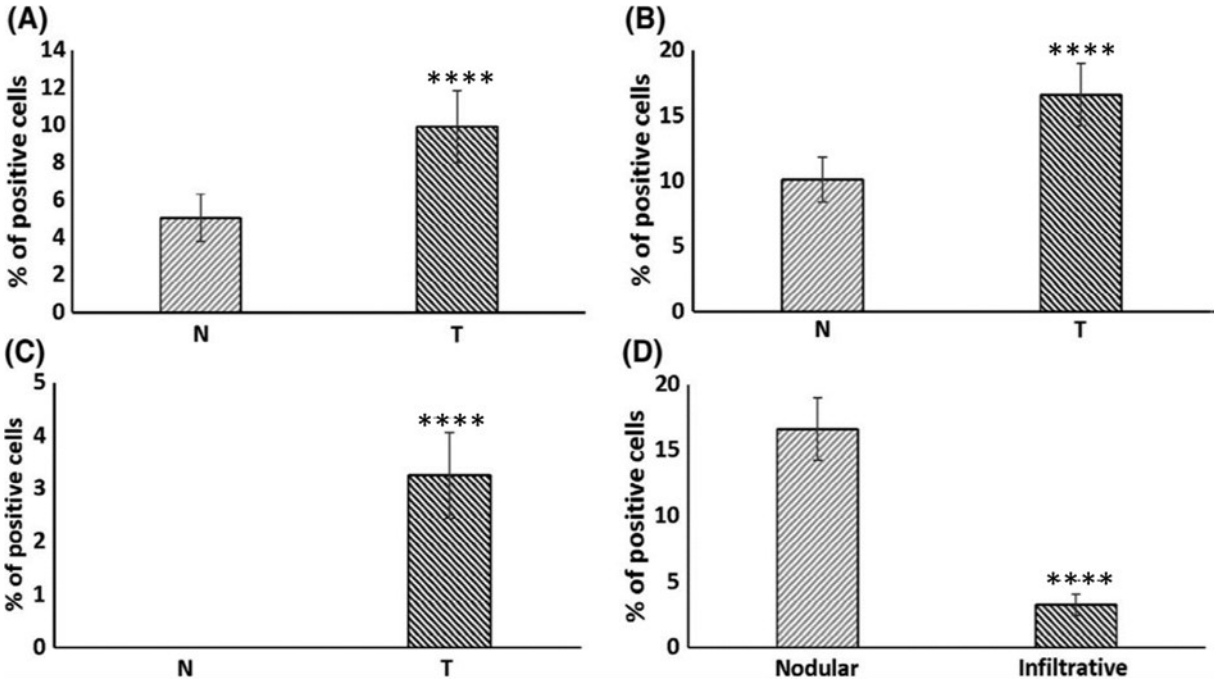

Figure 18: Immunohistochemical expression of NNMT in BCC samples (N, normal; T, tumour). Values represent the mean percentage ± standard deviation of stained cells with respect to total counted cells. **A)** NNMT positivity in pathological tissue compared to healthy margins, considering all BCC cases. **B)** Enzyme expression in nodular variant. **C)** Protein levels in infiltrative subtype. **D)** Comparison between nicotinamide N-methyltransferase immunopositivity in nodular and infiltrative BCCs (**** $p<0.0001$)

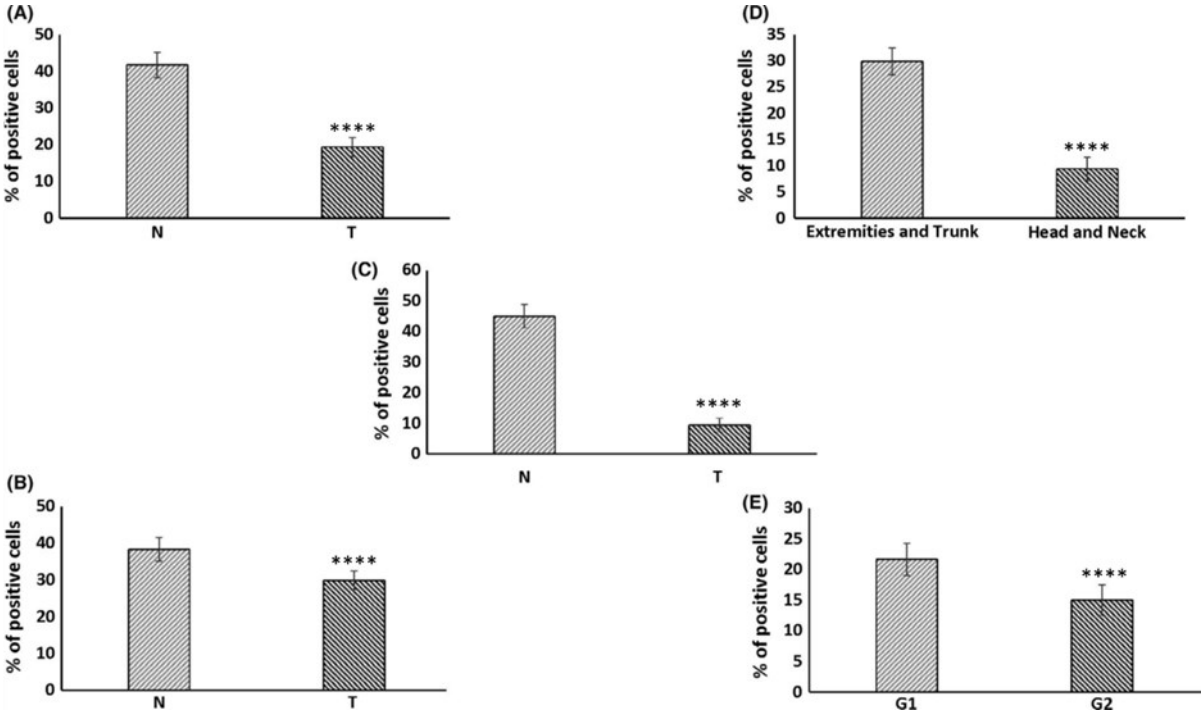

Figure 19: Nicotinamide N-methyltransferase level in SCC samples (N, normal; T, Tumour). Values represent the mean percentage ± standard deviation of stained cells with respect to total counted cells. **A)** NNMT immunohistochemical expression in pathological tissue and healthy tissue margins considering all SCC cases. **B)** Protein levels in extremities and trunk specimens. **C)** NNMT immunopositivity in SCC arising on the head and neck region. **D)** Enzyme levels in lesions occurring on the trunk and extremities versus head and neck SCCs. **E)** Comparison between protein expression in well-differentiated (G1) and moderately-differentiated (G2) forms. (**** $p < 0.0001$).

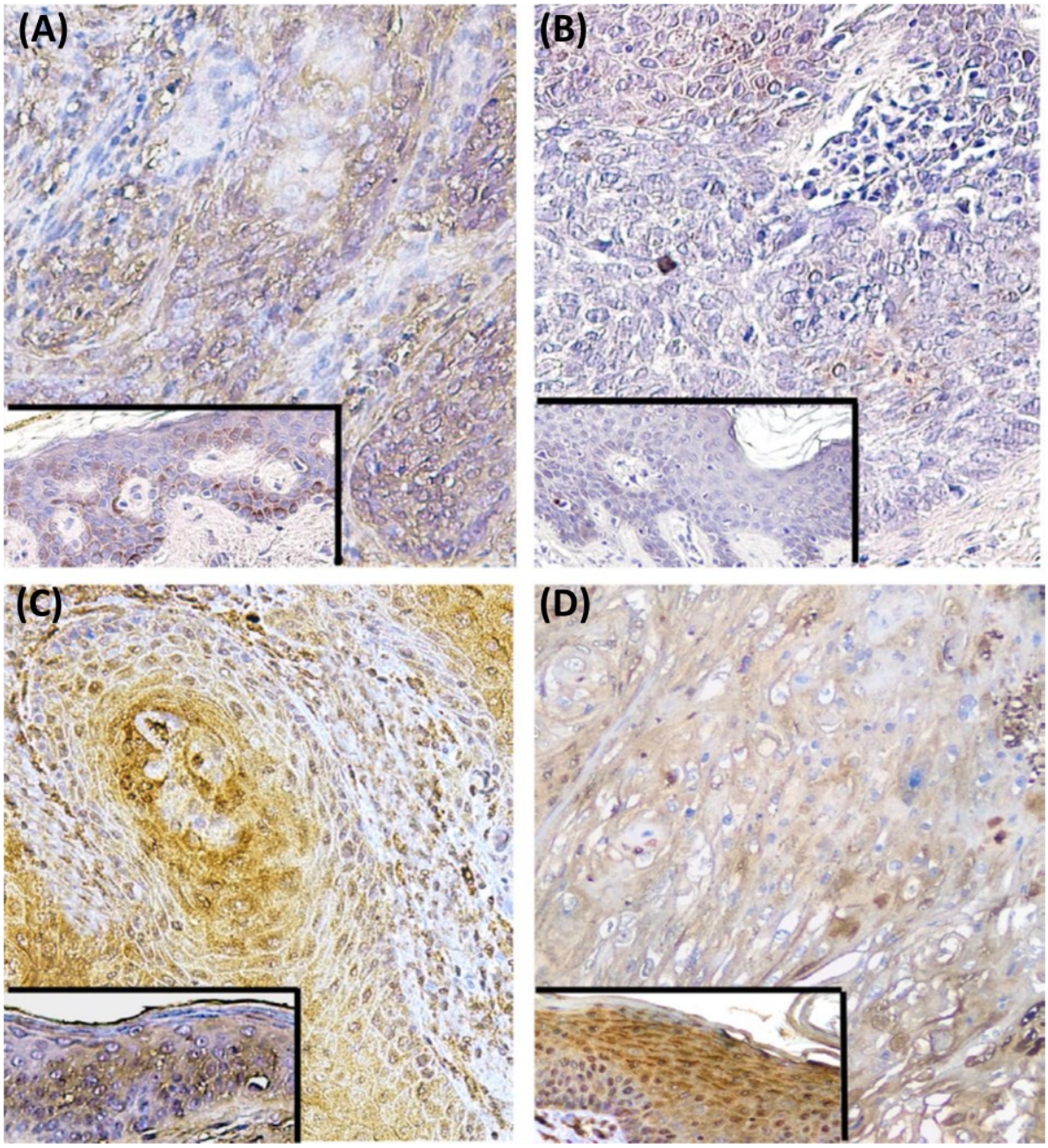

Figure 20: Nicotinamide N-methyltransferase immunopositivity in BCC and SCC sections. Inserts represent healthy tissue margins (immunoperoxidase, ×200 original magnification). **A)** Nodular BCC variant. **B)** Infiltrative BCC subtype. **C)** SCC of the trunk and extremities. **D)** Head and neck SCC.

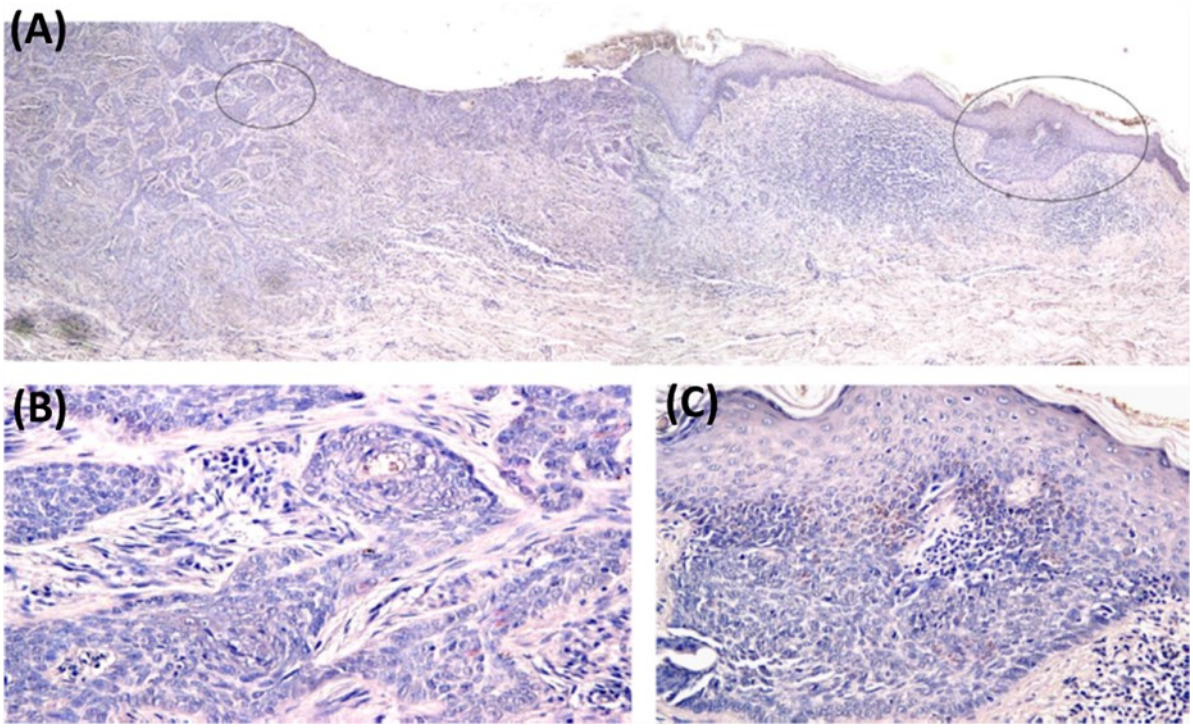

Figure 21: A) NNMT immunohistochemical expression in BCC showing both nodular and infiltrative features. (immuperoxidase, ×40 original magnification). **(B)** and **(C)** Higher magnification of infiltrative and nodular lesions, respectively (immuperoxidase, ×200 original magnification).

3.1.2 Nicotinamide N-methyltransferase in cutaneous melanoma

NNMT expression was evaluated in nevi and melanoma samples by immunohistochemical analysis. Results were expressed both as mean percentage ± standard deviation of stained cells with respect to total counted cells and as mean score ± standard deviation. In particular, to each sample was assigned a score on the basis of a five-tier grading system: 0 (0–10%), 1 (10–30%), 2 (30–50%), 3 (50–80%), and 4 (80–100%). Only seven nevus cases were immunolabeled by the anti-NNMT antibody and they showed a very weak cytoplasmic positivity, whereas in all melanoma specimens a NNMT immunopositivity was detected. Our data evidenced a significantly increased enzyme expression in tumour tissues compared with controls, both in terms of percentage and score ($p < 0.0001$) (Figure 22). The subsequent statistical analyses were conducted in order to explore the presence of a correlations between the NNMT levels and the clinicopathological parameters related to the samples examined, such as Breslow thickness, Clark level, presence and number of mitoses, flogosis, regression and ulceration. The results obtained showed the presence of a statistically significant inverse relation ($p <0.05$) between the enzyme expression and Breslow thickness, Clark level, the presence and number of mitoses and ulceration, considering both the percentage of positive cells and the score (Figures 23 and 24). In particular, an increased NNMT immunopositivity was found in thin, low Clark level, or nonulcerated lesions, compared to thick, high Clark level, or ulcerated melanomas. Moreover, protein immunoreactivity was significantly higher in samples with no mitoses compared with those showing a moderate or high mitotic rate (Figure 25). On the contrary, there was no correlation between the levels of NNMT and the presence of inflammation and regression.

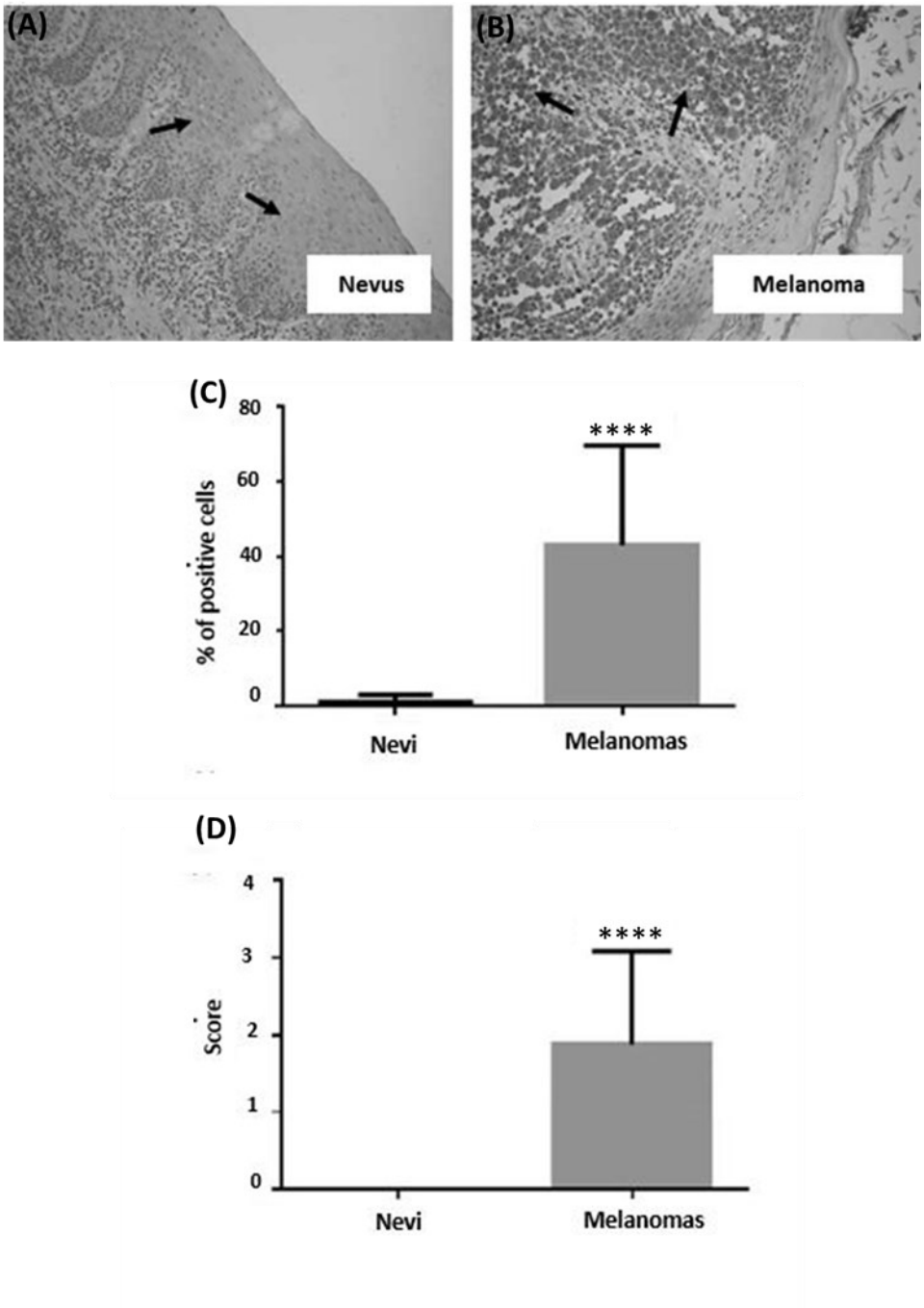

Figure 22: Immunohistochemical expression of NNMT in nevus **(A)** and melanoma **(B)** samples. (Immunoperoxidase, x200 magnification). Graphs show NNMT levels in nevi and melanomas expressed both as mean percentage ± standard deviation of stained cells with respect to total counted cells **(C)** and mean score ± standard deviation **(D)**.

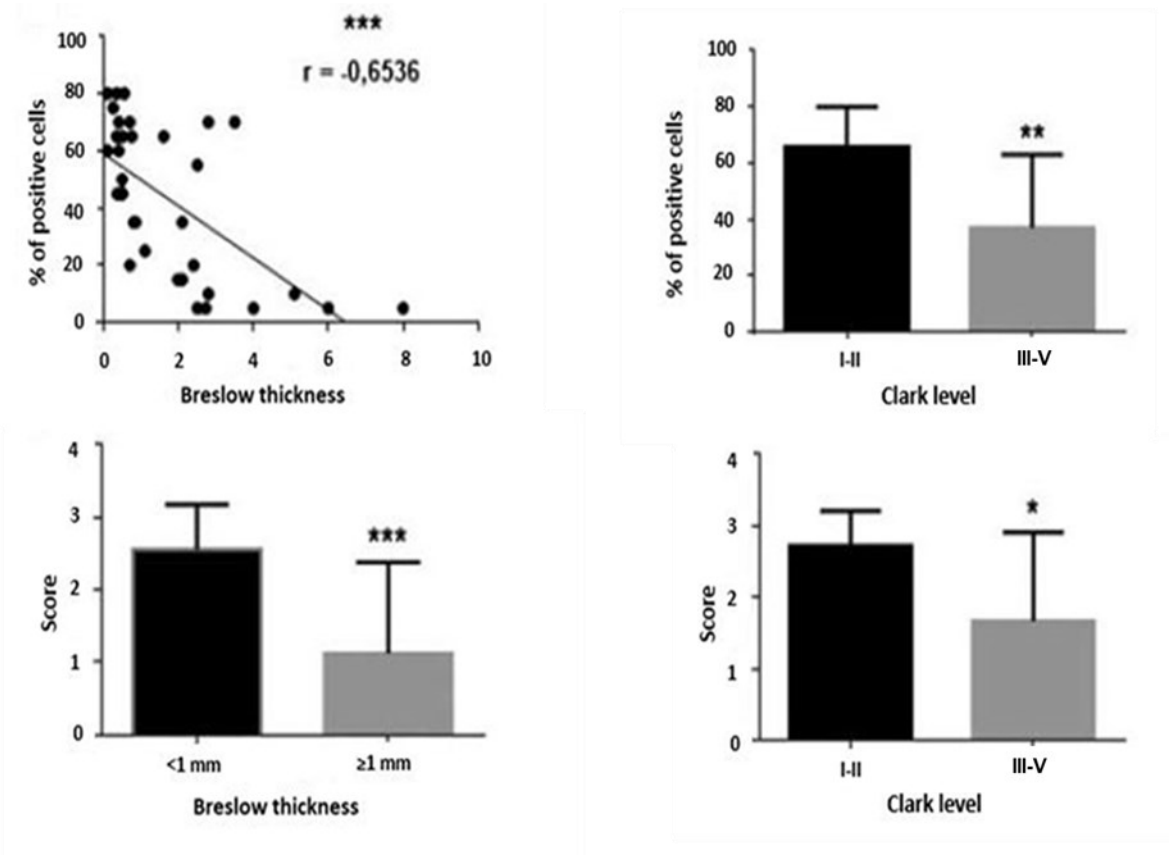

Figure 23: Correlation between NNMT levels (expressed both as mean percentage ± standard deviation of stained cells with respect to total counted cells and as mean score ± standard deviation) and clinicopathological parameters, such as Breslow thickness and Clark level (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

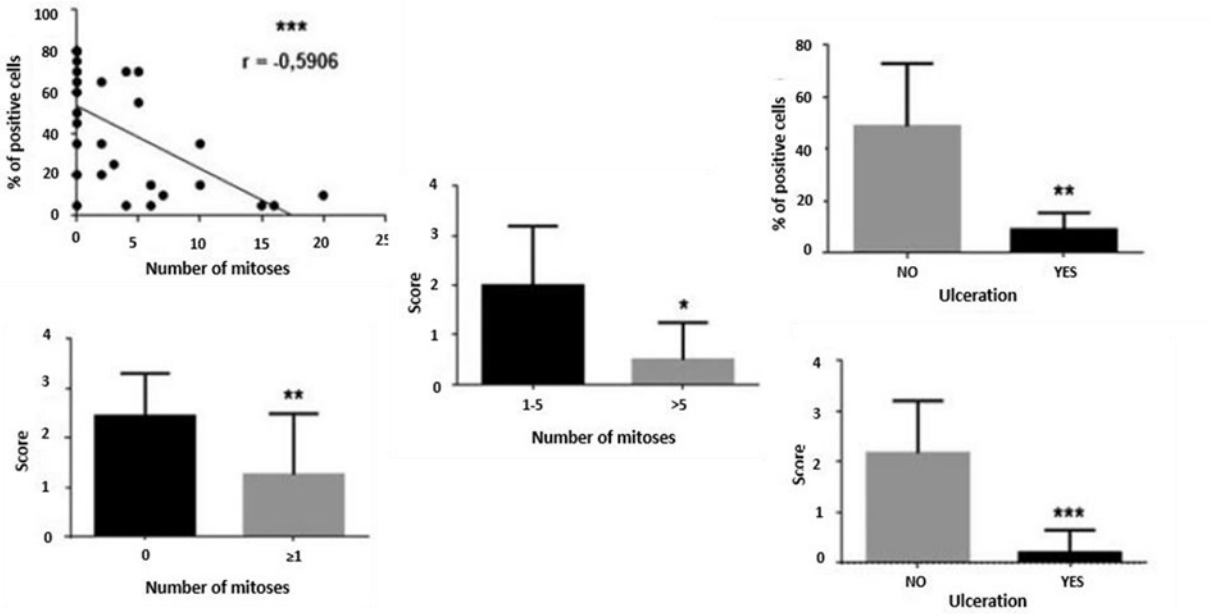

Figure 24: Correlation between NNMT levels (expressed both as mean percentage ± standard deviation of stained cells with respect to total counted cells and as mean score ± standard deviation) and clinicopathological parameters, such as presence and number of mitosis and ulceration (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

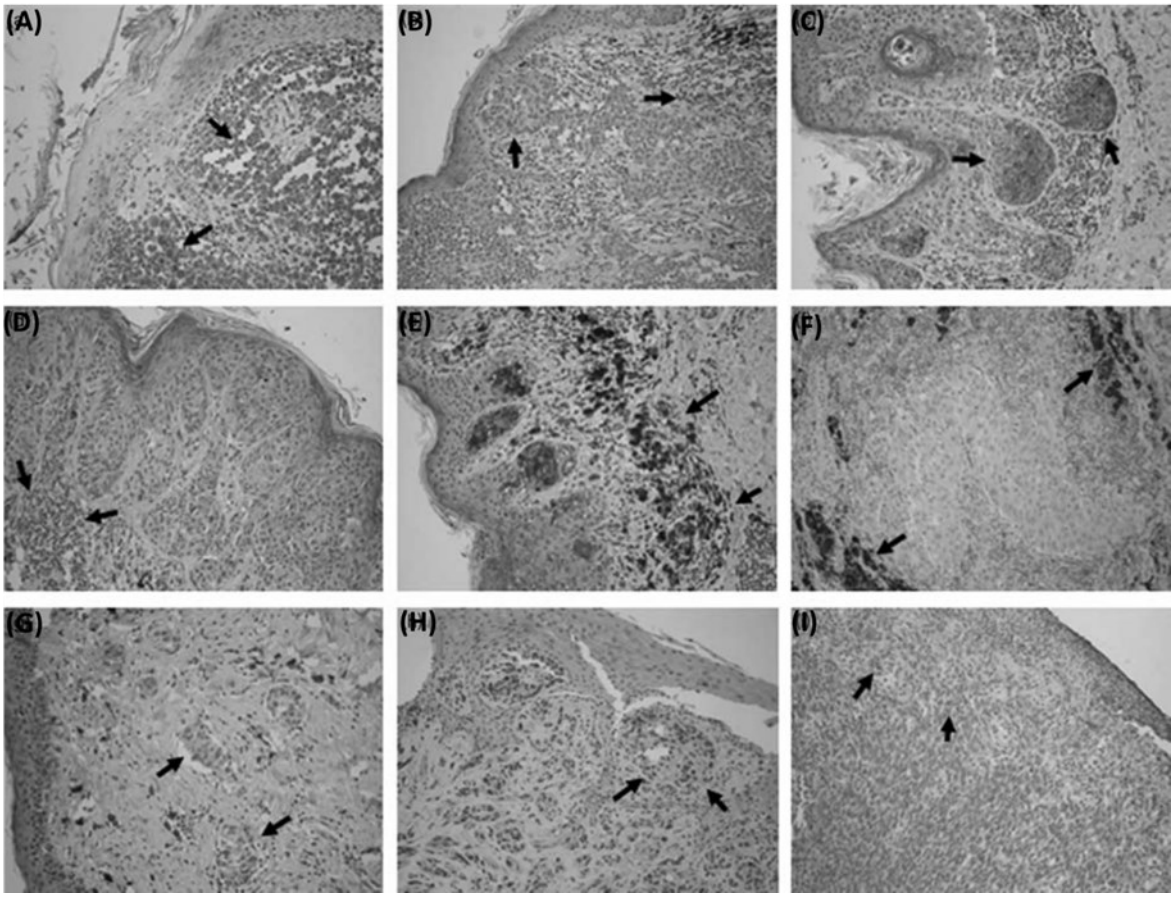

Figure 25: Nicotinamide N-methyltransferase immunopositivity in melanoma sections. Comparison between NNMT expression in melanomas with low **(A)** and high **(B)** Breslow thickness. Enzyme immunopositivity in lesions with low **(C)** and high Clark level **(D).** Protein level in nonulcerated **(H)** and ulcerated melanomas **(I)**. NNMT immunoreactivity in specimens with no mitoses **(E)**, and in those with a moderate **(F)** and high **(G)** mitotic rate. Arrows indicate the most representative areas showing NNMT cytoplasmic immunoreactivity (immunoperoxidase, ×200 original magnification).

3.2 EFFECT OF NICOTINAMIDE N-METHYLTRANSFERASE SILENCING ON A375 CELLS

3.2.1 Efficiency of shRNA-mediated gene silencing of NNMT

With the purpose of NNMT silencing, A375 cells were transfected with three plasmids containing the sequence encoding for shRNA targeting different regions of NNMT mRNA (pLKO.1-164, pLKO.1-330 and pLKO.1-711), with a combination of these plasmids (pLKO.1-mix), with an empty vector (pLKO.1-puro) or treated with transfection reagent only (Mock), as described in the paragraph 2.2.2. Mock population and pLKO.1-puro treated cells were used as controls. The specific effect of shRNA treatment on enzyme expression was evaluated by measuring NNMT mRNA and protein levels, through Real-Time PCR and Western blot, respectively. Real Time PCR evidenced a statistically significant ($p<0.05$) decrease in enzyme expression in A375 cells transfected with plasmids encoding shRNA targeting NNMT mRNA. In particular, the NNMT relative expression was 0.48 ± 0.02 for pLKO.1-164, 0.49 ± 0.05 for pLKO.1-330, 0.37 ± 0.03 for pLKO.1-711, 0.56 ± 0.03 for pLKO.1-mix, 1.00 ± 0.07 for Mock. Moreover, pLKO.1-puro empty vector did not affect enzyme expression, which was 1.04 ± 0.03 (Figure 26 A). The reduced NNMT expression in cells transfected with pLKO.1-164, pLKO.1-330, pLKO.1-711 or pLKO.1-mix with respect to those treated with transfection reagent only (Mock) or empty vector (pLKO.1-puro) was confirmed by Western blot analysis (Figure 26 B).

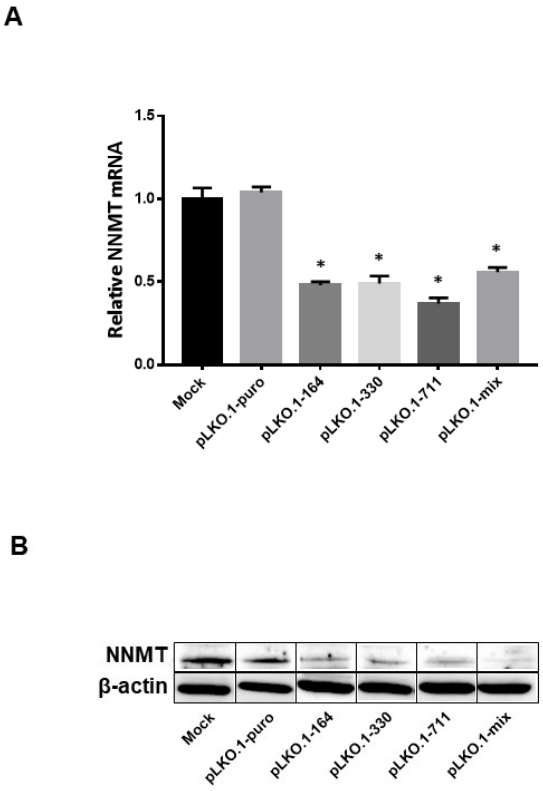

Figure 26: Nicotinamide N-methyltransferase expression levels analysed in A375 cells transfected with empty plasmid (pLKO.1-puro), shRNA plasmids (pLKO.1-164, 1-330, 1-711 and mix) or treated with transfection reagent only (Mock), by Real-Time PCR **(A)** and Western Blot **(B)**. Values are expressed as mean ± standard deviation (* $p < 0.05$).

3.2.2 Effect of NNMT silencing on A375 proliferation and migration

Cell proliferation and migration were assessed at different time points in A375 cell line, in order to evaluate the role of NNMT in melanoma cell metabolism and explore the influence of enzyme downregulation on cancer cell biology. The effect of NNMT downregulation on cell proliferation was analysed by means of MMT assay. The results of colorimetric assay were expressed as relative cell proliferation referred to the control (absorbance at 0h and equal to 100%).

Cells transfected with pLKO.1-puro had no significant difference in cell proliferation compared to Mock. Conversely, cellular clones stably encoding shRNAs against NNMT mRNA (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix), characterized by the enzyme downregulation, showed a significant ($p<0.05$) decrease in cell proliferation at 24h, 48h and 72h with respect to controls (pLKO.1-puro and Mock) (Figure 27).

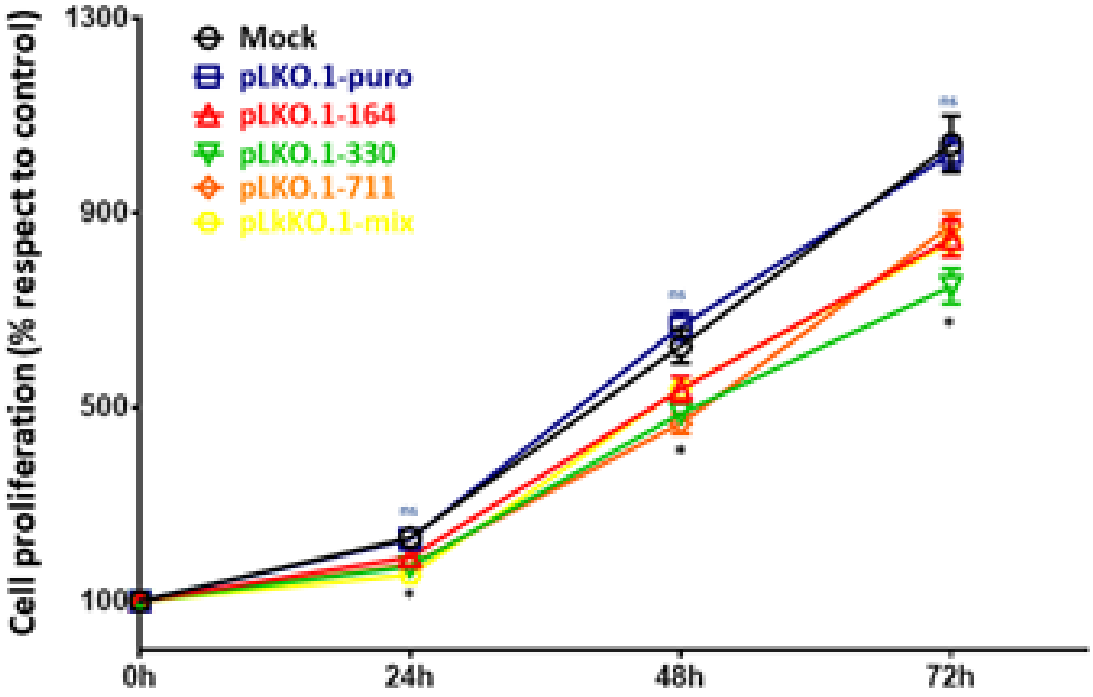

Figure 27: Effect of shRNA-mediated NNMT silencing on cell proliferation. Cell proliferation was analysed by MTT assay in Mock and NNMT downregulating cells (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) at 0h, 24h, 48h and 72h. Values were expressed as mean ± standard deviation (*$p<0.05$; ns = not significant).

The migration ability of A375 cells was evaluated using monolayer wound healing assay. Results were expressed as percentage of wound recovery with respect to 0h time point. Interestingly, the migration rate of NNMT-silenced A375 cells was significantly ($p<0.05$) decreased at all examined time points (4h, 8h, 12h, 24h and 28h) compared to Mock or cells transfected with empty plasmid (pLKO.1-puro), (Figure 28 A and B).

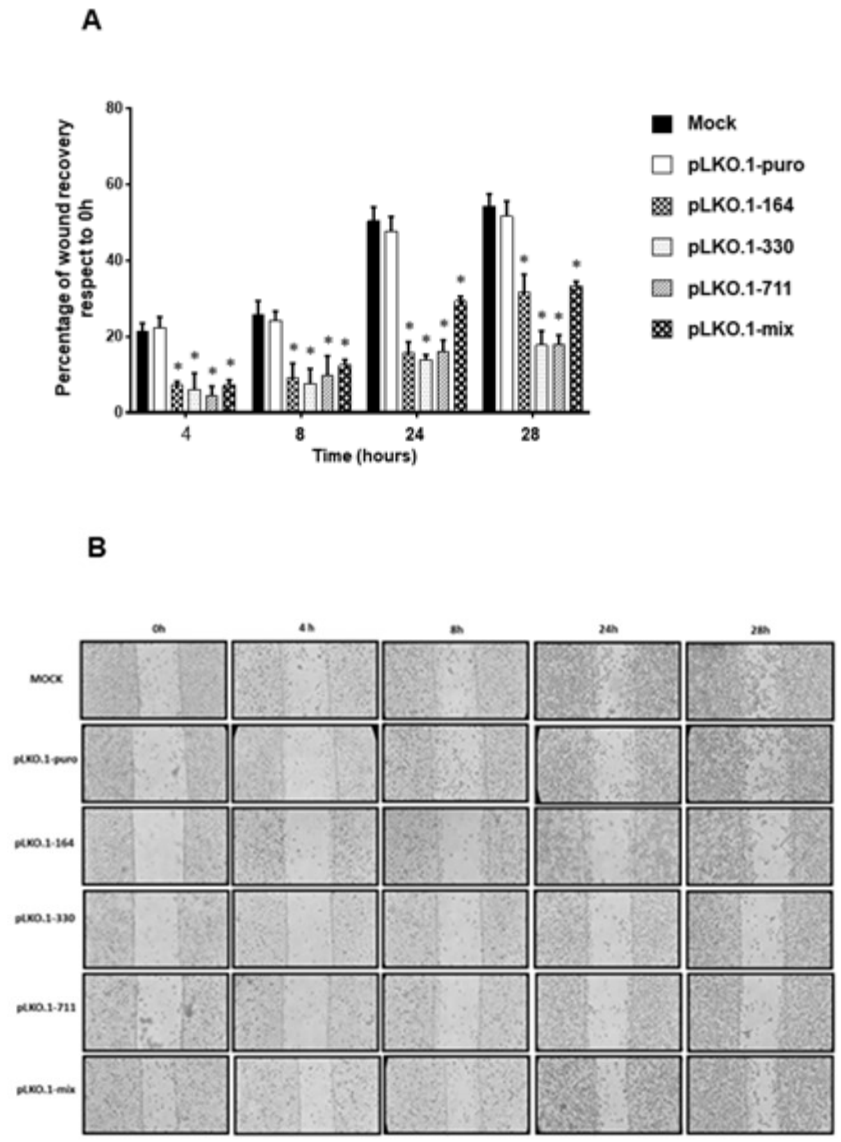

Figure 28: Effect of NNMT silencing on cell migration**.** The migration ability of A375 cells was assessed by wound healing assay. A375 cells transfected with plasmids (pLKO.1-164, pLKO.1-330, pLKO.1-711, pLKO.1-mix or pLKO.1-puro) and Mock were photographed immediately after scratch (0h) and at different time points (4h, 8h, 24h and 28h). Cell migration potential was analysed at different time points by measuring percentage of wound recovery compared with 0h. Panel A reports bar diagrams showing wound recovery of all samples at the different time points. Panel B displays images of wound recovery. Values are expressed as mean ± standard deviation (*$p<0.05$).

3.2.3 NNMT influence on A375 cell sensitivity to chemotherapeutic treatment

The effect of dacarbazine treatment on cell proliferation was monitored by means of MMT assay. The treatment with medium containing 2mM HCl (Untreated) determined no significant difference in cell proliferation compared to treatment with complete medium only (data not shown). The effect of chemotherapeutic treatment was evident at 48h and 72h time points, when the proliferation rate of both cells transfected with plasmids encoding shRNAs targeting NNMT mRNA (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) and Mock showed a statistically significant reduction. It is noteworthy that the decrease in cell proliferation upon treatment with dacarbazine was markedly enhanced in NNMT-silenced A375 cells with respect to Mock (Figure 29 A-D).

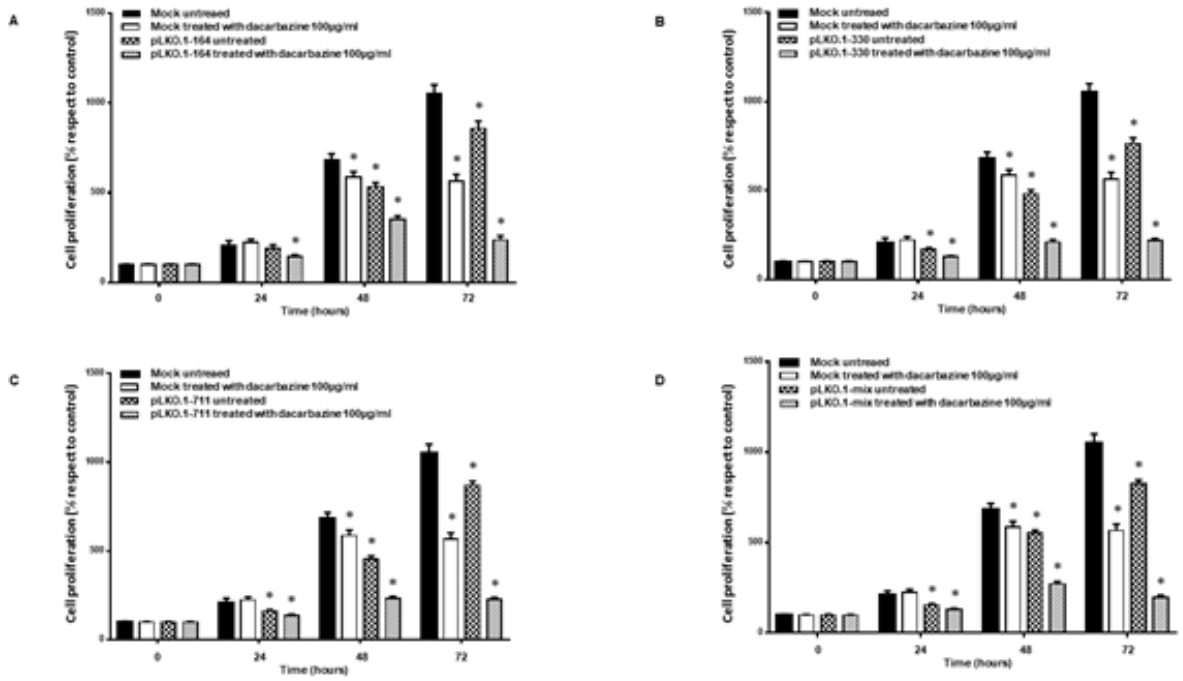

Figure 29: Effect of chemotherapy treatment on A375 cells. The effect of dacarbazine (100µg/ml) on cell proliferation of Mock and cells transfected with pLKO.1-164 (panel A), pLKO.1-330 (panel B), pLKO.1-711 (panel C) or pLKO.1-mix was evaluated using MMT assay (panel D). Measurements were performed at different time points (0h, 24h, 48h and 72h). Results were expressed as mean ± standard deviation (*$p<0.05$).

4 DISCUSSION AND CONCLUSION

Nicotinamide N-methyltransferase catalyzes the N-methylation of nicotinamide and pyridine compounds, playing a leading role in the metabolism of many drugs and xenobiotics (207, 214). An overexpression of the enzyme has been observed in several tumours, such as gastric cancer (283,285), oral squamous cell carcinoma (311 –313), ccRCC (289), colorectal cancer (280), pancreatic cancer (276), papillary thyroid carcinoma (272), bladder urothelial carcinoma (302), and non-small-cell lung cancer (306). However, until now no data in literature concerned the evaluation of NNMT expression in skin cancers. For this reason, the first part of this study was aimed to evaluate the enzyme levels in tissue samples obtained from different cutaneous neoplasms. In particular, immunohistochemical analyses were performed on BCC and SCC cases and their surrounding normal tissue, and subsequently on cutaneous melanoma samples and benign nevi. As regards BCC, the data obtained evidenced a significant overexpression of the enzyme in tumour tissue compared to controls, both considering total BCCs and distinguishing between less and more aggressive variants. Moreover, NNMT levels were found to be higher in nodular BCC than in the infiltrative subtype, which represents a more aggressive variant. These results are in agreement with previous studies exploring NNMT expression in different solid tumours (280, 286, 313). However, the significance of its overexpression in cancer, as well as the cellular effects exerted by such dysregulation, has not been totally elucidated. As regards SCC, NNMT levels were evaluated in lesions occurred on the head and neck region as well as on the rest of the body. Surprisingly, the immunohistochemical analysis detected a significantly decreased NNMT immunopositivity in tumour tissue with respect to heathy tissue margins, even discriminating between forms displaying different aggressiveness. It is noteworthy that only a few studies showed a downregulation of the enzyme in association with tumours. In particular, a lower NNMT expression was observed in insulinoma and HCC (212,309). Nevertheless, the significance of the decrease in NNMT levels detected in those tumours, and in cutaneous SCC as reported in this study, remains unknown. The differences in enzyme dysregulation found in BCC and SCC could be related with specific biological aspects and they might depend on the fact that these NMSCs arise from keratinocytes residing in different epidermal layers. Moreover, it is interesting to note that NNMT immunoexpression was inversely correlated with cancer aggressiveness. Indeed, protein levels were significantly increased in nodular BCCs compared to infiltrative lesions, and this expression pattern was also found in those neoplasms showing both nodular and infiltrative features within a single tumour. Similarly, a decreased enzyme immunopositivity was evidenced in SCC occurred on the head and neck region with respect to those arose in the trunk and extremities. In addition, a significant inverse relationship was evidenced between NNMT expression and histological grading of SCC

samples. Our results are in agreement with previous studies in which an inverse relationship between enzyme expression and tumor stage was evidenced. In particular, NNMT overexpression in ccRCC was found to be inversely related with the lesion size, leading to hypothesize that the enzyme may be implicated in cancer progression (331). Moreover, in OSCC, NNMT upregulation was negatively correlated with pT, lymph node metastasis, pathological staging and histological grading, thus supporting its potential contribution in tumour growth and differentiation (311, 312). The significant reduction in enzyme immunoexpression in poorly differentiated SCC with respect to well-differentiated ones could be due, at least in part, to the fact that during neoplastic transformation tumour cells gradually lose/modify their staining properties. However, our results suggest the effectiveness of the enzyme as potential prognostic biomarker for these neoplasms.

As concerns the immunohistochemical evaluation of NNMT expression in melanoma samples, a significant overexpression of the enzyme was found in tumour tissues with respect to benign nevi, which were used as controls. Moreover, the results obtained evidenced an inverse association between enzyme levels and clinicopathological parameters, such as Breslow thickness, Clark level, the presence and number of mitoses, and ulceration. These data agree with previous reports (311,331) and seem to indicate the potential of NNMT as a prognostic biomarker for melanoma.

The second part of this research work had the purpose of exploring the role played by NNMT in melanoma cell metabolism. The data obtained showed that the decreased enzyme expression in metastatic melanoma A375 cell line, due to the NNMT shRNA-mediated gene silencing, is associated with a reduction in both the proliferation and migratory ability of cells, thus suggesting a potential involvement of the enzyme in the cell proliferation process and in the tumorigenic mechanisms of the neoplastic cell. It has been previously observed the presence of an association between the enzyme downregulation and the decreased cell viability and anchorage-independent cell growth of HeLa-derived KB cancer cells (314), PE/CA PJ-15 OSCC cells (315) and NSCLC tumour cell line A549 (307). Moreover, NNMT silencing determined a significant reduction in tumour formation capacity of PE/CA-PJ15 cells upon subcutaneous transplantation into athymic mice (301). The role of the enzyme in cancer cell invasion and metastases was demonstrated by the reduction of chemotaxis and chemokinesis of the human BUC cell line following the treatment with siRNA against NNMT mRNA. Moreover, NNMT silencing induced a significantly reduced tumour formation and metastatic spread in ccRCC cell line subcutaneously transplanted into athymic mice (294). In addition, in breast cancer cell lines the NNMT knockdown stimulated the apoptosis via the mitochondria-

mediated pathway and it was associated with a decrease of cell growth *in vitro*, as well as tumour formation *in vivo* (275). Furthermore, NNMT downregulation in oesophageal squamous cell carcinoma cell lines significantly suppressed cell viability and migration, induced cell cycle arrest, promoted apoptosis and inhibited the epithelial-mesenchymal transition (EMT) via Wnt/β-catenin pathway (332). Conversely, the induction of enzyme overexpression in HSC-2 OSCC cell line induced the increase of cell proliferation (316). The same results were obtained in CRC and prostate cancer cell lines. In particular, the overexpression of the enzyme in HT-29 cells was able to enhance cell proliferation and colony formation ability, inhibit the apoptotic pathway, and promote the progression of cell cycle, while in PC3 cell line the induced NNMT upregulation increased cell proliferation, invasive capacity and migration ability (281, 296). Moreover, in a gastric cancer cell line NNMT upregulation induced the expression of TGF-β1, with the subsequent activation of TGF-β1/Smad signalling, which promotes EMT, a key event in tumour invasion and metastasis (287). Taken together, all these results indicate that the enzyme is a fundamental mediator of crucial events inducing malignant transformation, thus representing a potential molecular target for cancer therapy.

Patients with advanced stage lesions had a poor prognosis, since their tumours are disseminated and unresectable and typically responds weakly to traditional chemotherapy (51). Although numerous studies led to the identification of new therapeutic approaches, chemotherapy is still the best options for these patients, especially if affected with melanomas that do not harbor somatic mutations, such as those related with BRAF, NRAS, or KIT. The alkylating agent dacarbazine (DITC), which had FDA approval for the melanoma treatment in 1975, is used for advanced-stage lesions. This treatment had partial and complete response rates in 15–28% and 3–5% of patients, respectively, while durable responses were found in less than 2% of subjects (330). For this reason, the combination of dacarbazine with other chemotherapeutic agents or immunotherapies was studied in order to increase its efficacy (333). Nevertheless, these new therapeutic approaches showed no improvement in overall survival, but only an increase of side effects and the reduction of life quality (333). Dacarbazine is therefore still the standard chemotherapy treatment option for patients with metastatic melanoma (172, 169,171). In light of these considerations, the identification of the molecules involved in the initiation and progression of melanoma and associated with the resistance to chemotherapy is necessary for the development of an effective treatment for this neoplasm. The results obtained in this research work showed that the downregulation of the enzyme, besides causing a decrease in cell proliferation and invasion, was associated with an increased sensitivity to the treatment with dacarbazine. To support this hypothesis, some studies evaluated the ability of NNMT to

influence the efficacy of chemotherapeutic agents. In particular, 5-FU resistance in CRC HT-29 cell line was reduced by NNMT downregulation, whereas its overexpression in CRC cell line SW480 determined a lower response to chemotherapy. The enzyme ability to make cells resistant to drug treatment was shown to be mediated by N1-methylnicotinamide, which counteracts the ROS formation induced by 5-FU (282). Similar results were described in TE1 cells where NNMT downregulation increased 5-FU sensitivity, while the overexpression of the enzyme in EC1 and Eca109 cells had the opposite effect (334). The results of the present study suggest an involvement of the enzyme in melanoma cell resistance to chemotherapy. Further studies will clarify whether the combination of enzyme inhibitors with DITC could enhance A375 sensitivity to chemotherapeutic treatment.

In conclusion, this study evaluated for the first time NNMT expression in NMSCs and cutaneous melanoma. Further analyses carried out on a larger cohort of skin cancer samples and preneoplastic lesions will enable a more in-depth investigation of the prognostic role of the enzyme in these tumours. Moreover, this research work was the first to analyse the role of NNMT in melanoma progression and spread and to explore its potential involvement in chemoresistance. Nevertheless, additional analyses are needed to clarify the mechanisms by which the enzyme could participate in melanoma tumorigenesis and chemosensitivity. In addition, given the ubiquitous expression of the enzyme and its fundamental role in the metabolism of xenobiotic compounds, further studies should be performed in order to design an accurate drug-delivery strategy to selectively address NNMT inhibitors to the metastatic melanoma cells.

5 LIST OF ABBREVIATIONS

5-FU	5-fluoruracil
A375	Human malignant melanoma cell line
A549	Adenocarcinomic human alveolar basal epithelial cells
AAA	Abdominal aortic aneurysm
ACCS-LN	Adenoid cystic carcinoma lymph node metastatic cells
ACT	Adoptive cell therapy
ADP	Adenosine 5'-diphosphate
AK	Actinic keratosis
AKT	Protein kinase B
AJCC	American Joint Committee on Cancer
ALA	Aminolevulic acid
ALL	Acute lymphoblastic leukemia
anti-PD-1	Anti-programmed death 1
ART	Mono-ADP-ribosyltransferase
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
AUC	Area under the curve
BBC	Comassie Brilliant Blue G-250
BC	Bladder cancer
BCC	Basal cell carcinoma
BCNS	Basal cell nevus syndrome
BCNU	Carmustine
BGC823	Human gastric cancer cell line
BRAF	B-Raf protein
BWH	Brigham and Women’s Hospital

CaCo-2	Human colorectal carcinoma cell line
cADPR	Cyclic adenosine 5'-diphosphate-ribose
CCPDMA	Complete peripheral and deep margin assessment
CCNU	Lomustine
ccRCC	Clear cell renal cell carcinoma
CE	Electrodessication
CEA	Carcinoembryonic Antigen
CDKN24	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CHDs	Congenital heart defects
CLND	Complete lymph node dissection
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
ConA	ConcanavalinA
COPD	Chronic obstructive pulmonary disease
COS-1	Cercopithecus Aethiops kidney cell line
COX-2	Cyclooxygenase-2
CR	Complete response
CSCs	Cancer stem cells
CTLA	Cytotoxic T-lymphocyte antigen
DA	Dark agouti
D-MEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DTIC	Dacarbazine, 5-[3,3-dimethyl-1-triazenyl]-imidazole-4-carboxamide
ED&C	Electrodessication and curettage

EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovin serum
FDA	Food and Drugs Administration
FFPE	Formalin-fixed and paraffin-embedded
FISH	Fluorescence in situ hybridization
GDP	Guanosine diphosphate
GBM	Glioblastoma Multiforme
GOI	Gene of interest
GSCs	Glioblastoma stem cells
GTP	Guanosine triphosphate
GLI	zinc finger transcription factors GLI
HCC	Hepatocellular cancer
HD IL-2	High-dose bolus intravenous IL-2
HKG	Housekeeping gene
HFD	High fat diet
Hh	Hedgehog signaling pathway
HMB-45	Human melanoma black 45
HNF-1β	Hepatocyte nuclear factor beta 1
HNMT	Histamine N-methyltransferase
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSC-2	Human oral squamous cell carcinoma

IARC	International Agency of Research on Cancer
IHC	Immunohistochemistry
KCN	Potassium cyanide
IL-2	Interleukin 2
IL-6	Interleukin-6
INF	Interferon
LDH	Lactate dehydrogenase
LIF	Leukemia Inhibitory Factor
LM	Lentigo maligna
MAL	Methyl aminolevulinate
MC1R	Melanocortin 1 receptor
MCF-7	Adriamycin-resistant cells
MDA-MB-231	Breast cancer cell lines
MGH-U1	Bladder cancer cell line
MITF	Microphthalmia transcription factor
MNA	N1-methylnicotinamide
MMS	Mohs micrographic surgery
MAPK	Ras/mitogen-activated protein kinase pathway
MG63	Human osteosarcoma cell line
MGC803	Human gastric cancer cell line
MEK	Mitogen-activated protein kinase kinase
MeNH	Methyl-norharman
MPP+	1-methyl-4-phenylpyridinium ion
MMP-2	Matrix metalloproteinases 2
MMP-9	Matrix metalloproteinases 9

mRNA	Messenger RNA
MS	Mass spectrometry
MSH	Melanocyte stimulating hormone
MSS	Melanoma-specific survival
MTIC	3-methyl-(trianzen-1-yl)-imidazole-4- carboxamide
MTT	3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NCCN	National Comprehensive Cancer Network
NF	Nuclear factor
NF1	Neurofibromin 1
NH	Norharman
NM	Nodular melanoma
NMSC	Non melanoma skin cancer
NNMT	Nicotinamide N-methyltransferase
NSCLC	Non-small cell lung cancer
OCT	Optical coherence tomography
OSCC	Oral Squamous Cell Carcinoma
p53	Tumour suppressor p53
PAGE	Polyacrylamide gel electrophoresis
PANC-1	Pancreatic cancer cell line
PARP	Poly ADP-ribose polymerase
PBS	Phosphate-buffered saline
PC	Pancreatic cancer

PD	Parkinson’s disease
PGI2	Prostacyclin
PHH3	Phosphohistone H3
PCR	Polymerase chain reaction
PI3K	phosphoinositol-3-kinase
PNI	Perineural involvement
POMT	Phenol O-methyltransferase
PP2A	Tumour suppressor enzyme
PUVA	Psoralen and ultraviolet A
PVDF	Polyvinylidene fluoride
ROC	Receiver operating characteristic
RCM	Reflectance confocal microscopy
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT	Radiation therapy
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SCC	Squamous cell carcinoma
SCCIS	Squamous cell carcinoma *in situ*
SCREEN	Skin Cancer Research to Provide Evidence for Effectiveness of Screening
SDS	Sodium dodecyl sulfate
SEPMA	Standard excision with postoperative margin assessment
Shh	Sonic Hedgehog signaling pathway
shRNA	Short hairpin RNA
SH-SY5Y	Human neuroblastoma-derived cell line

Sir2	Sirtuin
SLN	Sentinel lymph node
SLNB	Sentinel lymph node biopsy
SMO	Smoothened
SNP	Single nucleotide polymorphisms
SOTRs	Solid organ transplant recipients
SSM	Superficial spreading melanoma
STAT3	Signal Trasducer and Activator of transcription 3
SUFU	Suppressor of fused homolog
SZ	Schizophrenia
SW480	Human colorectal carcinoma
T24	Human bladder cancer cell line
TGF-β1	Transforming growth factor beta 1
TERT	Telomerase reverse transcriptase
TLR7	Toll-like receptor 7
TMN	Tumour-node-metastasis
TMT	Thiol methyltransferase
TPMT	Thiopurine methyltransferase
UC	Urothelial carcinoma
WAT	White adipose tissue
WB	Western blot
WBE	Whole-Body skin Examination
WOKW	Wistar Ottawa Karlsburg W
ZEB1	Zinc Finger E-box Binding Protein 1

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