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microRNAs in Uveal Melanoma: in vivo findings and computational analysis

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Abstract

Background: Uveal melanoma (UM) is the most prominent primary eye cancer in adults, featuring a high mortality rate and poor prognosis. The role of miRNAs as potential biomarkers in UM patients has drown increasing attention and recent computational analyses have shown the prognostic significance of a cluster of deregulated miRNAs in UM patients.

Purpose: The aim of the study was to assess whether the evaluation of the expression levels of the up-regulated miRNA hsa-miR-199a-5p and of down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p could be useful biomarker in uveal melanoma (UM) patients.

Methods: Expression profile of miRNAs hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p was performed in serum of 30 patients newly diagnosed with UM and compared to 10 healthy controls. Of the 30 UM patients, 11 underwent enucleation and Formalin-Fixed Paraffin-Embedded (FFPE) UM samples were analyzed to assess the expression levels of the three miRNAs under investigation. Droplet digital PCR (ddPCR) was used for both liquid biopsy and tissue samples. A novel computational analysis for the three selected miRNAs was performed in order to corroborate the experimental results.

Results: Significantly higher expression levels of miRNAs hsa-miR-199a-5p were found in serum samples of UM patients compared with healthy controls. Receiver Operating Characteristics (ROC) analysis revealed that a diagnostic test based on the evaluation of hsa-miR-199a-5p circulating levels would have a moderate accuracy (AUC=0.7433), and limited specificity and sensitivity (63.33% and 70%, respectively). No circulating levels of both miRNAs hsa-miR-508-3p and hsa-miR-514a-3p were detected. ddPCR analyses confirmed significantly upregulated expression of miRNA hsa-miR-199a-5p in UM tissue samples, whereas miRNAs hsa-miR-508-3p and hsa-miR-514a-3p were found significantly downregulated. The bioinformatics results confirmed the prognostic value of the three miRNAs under investigation and showed a plethora of targeted genes involved in different physio-pathological processes.

Conclusions: The present study demonstrated the diagnostic value of hsa-miR-199a-5p in liquid biopsy samples from UM patients. While hsa-miR-508-3p and hsa-miR-514a-3p have no role as circulating biomarkers, they were found significantly down-regulated in FFPE UM samples, suggesting their potential prognostic role in those patients treated with enucleation.

1 Background

1.1 Epidemiology

Uveal melanoma (UM) represents the most frequent primary eye cancer in adults, although it is relatively rare, being the 3-5% of all melanomas.[1,2] The largest proportion of UM, roughly the 85-90%, arises from the choroid, while the 5-8% arises from the ciliary body and the 3-5% from the iris.[2,3] The incidence of UM in the United States, between 1973 and 2008, amounted to 5.1 cases per million per year.[4] In Europe, a cancer registry-based study showed a north-to-south decreasing gradient in UM incidence, with an incidence of more than 8 cases per million in Denmark and Norway compared with about 2 cases per million in Spain and Southern Italy.[5] Likewise, in Africa and Asia the incidence is low, amounting to 0.2-0.3 cases per million per year.[6] This latitude-related decreasing trend in UM incidence has been associated with the protective role of ocular pigmentation, which increases in the southern countries compared to northern ones.[5] Similarly, the dark skin pigmentation could have a role in protecting Black population: the ratio of UM in Black versus White population is from 1:15 to 1:50.[7–9] A population-based study investigated the relative risk of UM in several racial cohort, showing a 5-fold higher risk in Hispanic and 19-fold higher risk in non-Hispanic White compared to Black population.[9]

Uveal melanoma is typically an adult malignancy, affecting older age groups.[10] Median age of diagnosis has been reported as roughly 62 years,[2] with an incidence rate that tends to increase progressively up to 70 years and then levels off after 75 years.[4,5,10,11] Mean age of diagnosis of UM seems to decrease from 59-62 years in Caucasians, [4,12] to 55 years in Japanese, 51 years in Taiwanese, 45 years in Chinese.[13–15] Uveal melanoma is uncommon in children and extremely rare in newborn or congenital.[11,16,17] Shields et al investigated incidence rate of UM in children and teenagers, showing that 50% of cases were older than 15 years, 35% between 10 and 15 years, 11% between 5 and 10 years, and only 3% of cases were between 0 and 5 years old at time of diagnosis.[17]

Incidence of UM appears to be related to the gender as well.[2,10] Population-based studies showed a higher age-adjusted incidence in male gender compared to females, with a 20-30% greater rate in males.[1,4] An Australian population-based study found this difference more prominent in the population ≥ 65 years old, whilst there was no significant gender difference in incidence of UM when considering the population younger than 65 years.[18]

1.2 Risk factors

Several risk factors have been associated with UM. Host susceptibility variables, such as fair skin color, inability to tan and light eye color have been significantly associated with UM, with a risk ratio of 1.80, 1.64 and 1.75, respectively.[19] This association is likely to be related to the poor amount of melanin in the skin and eyes. It has been assumed that a poor amount of melanin is present in the choroid and retinal pigment epithelium, leading to an increased susceptibility to ultraviolet light and higher risk of UM.[19] Oculodermal melanocytosis, also known as Nevus of Ota, represents a relevant risk factor for developing UM.[20,21] This condition is characterized by an abnormal congenital hyper-pigmentation within V1/V2 trigeminal nerve area, and can involve periocular skin, orbit, uvea, sclera, conjunctiva, as well as palate, meninges and tympanic membrane.[20,21] Usually this condition is unilateral and, mostly, confined to the eye. Oculodermal melanocytosis is found 35 times more commonly in UM patients compared to healthy population: incidence rate in White population is 0.04% versus 1.4% to 3% in UM patients.[20,21] A White patient affected by oculodermal melanocytosis presents a lifetime risk for developing UM equal to 1:400.[22] Presence of atypical cutaneous nevi and intraocular nevi has been associated with UM as well. In particular, risk for developing UM is 4-10 times higher in patients affected by atypical cutaneous nevi than healthy population.[23,24] Intraocular nevi, such as iris nevi and choroidal nevi, are considered risk factors for UM. Iris nevi have been reported to have a potential risk of malignant transformation, but the rate of this transformation has not been clearly established, ranging from 2-5%.[25,26] Predictive factors for an iris nevus to transform into an iris melanoma have been summarized in the ABCDEF acronym: A stands for young age; B stands for blood; C stands for clock-hour (inferior location); D stands for diffuse flat shape; E stands for ectropion uveae; F stands for feathery margins.[26] Choroidal nevus is a common finding in healthy population, with an incidence rate of roughly 5% in United States.[27] Basing on the concept that all melanomas originate from a nevus, the rate of transformation of a choroidal nevus into melanoma has been reported as 1:8845, increasing to 1:3664 in older age cohort (80-84).[28] Predictive factors for a choroidal melanoma to become malignant are a >2 mm thickness, presence of subretinal fluid, presence of orange pigment, proximity to optic disc, absence of drusen or halo, ultrasonographic hollowness.[29,30]

A further relevant risk factor for UM is the mutation of the onco-suppressor gene BAP1.[10,31] BRCA1 associated protein 1 (BAP1) is located on chromosome 3. The mutation of this gene has been associated with a hereditary cancer syndrome. Tumors, such as malignant mesothelioma, basal cell carcinoma, cutaneous melanomas, uveal melanomas and renal cell carcinoma, could be developed following either somatic or germline mutation of BAP1.[31] In case of germline mutation, the tumors seem to be less aggressive than those without this mutation.[31] BAP1 was found mutated in up to 47% of UM.[32] Of note, patients affected by UM present a higher risk compared to general population (about 11% more) of a second cancer, including renal cell carcinoma and cutaneous melanoma, which could be related to germline BAP1 mutations.[2]

Sunlight ultraviolet exposure has been clearly identified as a risk factor for skin melanomas,[33] but it is still debated whether this could represent a risk factor for UM: some authors support this hypothesis, others refuse this.[34,35] A meta-analysis study revealed that chronic occupational natural ultraviolet light exposure was a borderline non-significant risk factor for UM, whereas geographic latitude and outdoor leisure UV exposure were not significant.[34] Conversely, welding was found to be a significant variable associated with UM development.[34] With regards to other artificial lights, blue light exposure has been hypothesized to play a role in the oncogenetic process and progression of UM.[36] Also occupational cooking seems to be associated with higher UM risk.[37]

1.3 Clinical characteristics

Patients affected by UM can be asymptomatic in up to 30% of cases, being this malignancy an incidental finding at the time of diagnosis.[38] Symptoms, when preset, are related to the location of the tumor. Iris melanomas are quite uncommon (3-5% of UMs) and diagnosis is mostly secondary to heterochromia, i.e. changes in iris color, and corectopia, i.e. abnormality in pupil shape, which is present in about 45% of cases.[10,39] Usually the tumor is located in the inferior quadrant of the iris (45% of cases) and can cause secondary glaucoma (direct or indirect obstruction to trabecular outflow), ectropion uveae, angle seeding and bleeding with hyphema.[10] In some cases, it can be complicated by extraocular extension (3%).[39] Extraocular extension as well as high intraocular pressure were shown to be variables associated with metastatic disease.[39] Clinically, an iris melanoma could present several types of configuration and levels of pigmentation

(from amelanotic to pigmented). In most cases iris melanoma is circumscribed, while in a few cases, about 10%, could be diffuse.[39,40] Diffuse iris melanoma is an infiltrative form, undefined and flat, which could prove difficult to diagnose: presence of ipsilateral ocular hypertension and acquired heterochromia of the iris are typically associated with this condition.[40] A rare subtype of diffuse iris melanoma is the ring melanoma of the anterior chamber, a tumor that arises from the angle and tends to infiltrate the angle structures over 360 degrees, with a ring pattern of growth (minimal extension towards iris or ciliary body).[41] The main symptom is unilateral glaucoma and diagnosis is very difficult (depends on careful gonioscopy and ultrasound biomicroscopy).[41]

When it comes to choroidal and ciliary body melanomas, also called posterior uveal melanomas, main symptoms are blurriness (38% of cases), floaters and flashing lights (7% and 9%, respectively). Less commonly, visual field loss, metamorphopsia and pain are reported.[38] Diagnosis of ciliary body melanomas is usually delayed due to their location and because symptoms tend to appear when the tumors are large.[10] A study including 492 ciliary body melanomas found at baseline a mean tumor base of 11.7 mm and a mean thickness of 6.6 mm, proving that the size of these tumors at the diagnosis is quite large.[42] However, the same study included also 7256 choroidal melanomas, presenting a baseline mean base of 11.3 mm and a mean thickness of 5.5 mm.[42] The average choroidal melanoma thickness at diagnosis showed a decreasing trend from 5.5 mm in the 1970s, to 4.5 mm in the 1990s, to 4 mm in more recent times.[43] This demonstrates the efforts in improving an early diagnosis of the tumor. Choroidal melanoma appears as a pigmented lesion in 55% of cases; in 15% of cases is non-pigmented and in 30% of cases has mixed pigmented and non-pigmented features.[43] The most common configuration of choroidal melanoma is dome-shaped, in 75% of cases. When the tumor grows breaking through Bruch's membrane, it gets a typical mushroom-shaped configuration (20% of cases).[43] Less commonly, in about 5% of cases, choroidal melanoma presents a diffuse configuration, which could make more challenging the diagnosis.[43] Orange pigment and subretinal fluid are typically associated with choroidal melanomas. The tumor can cause bleeding with subsequent vitreous haemorrhage, which can obscure the view of the fundus. Neovascular glaucoma can develop in advanced cases.[43]

1.4 Diagnosis

Diagnosis of uveal melanoma is based on clinical examination and ancillary tests. Diagnosis of iris melanoma relies on slit lamp biomicroscopy, anterior segment- Optical coherence tomography (as-OCT) and Ultrasound Biomicroscopy (UBM). In particular, UBM and as-OCT are helpful tools for the assessment of the posterior extension of the tumor.[10] Gonioscopy is important to evaluate possible angle involvement.[10] Transillumination may help to evaluate ciliary body involvement.[10] A thorough fundus examination is also important to assess retinal and choroidal condition. Diagnosis of iris melanoma could be challenging, in particular in cases of small/circumscribed lesions and diffuse melanomas. Differential diagnosis includes most commonly iris nevus as well as less common lesions such as cyst, metastasis, leiomyoma, melanocytosis, inflammatory conditions (granulomas).[44,45] Differential diagnosis of diffuse melanomas includes diffuse iris nevus, congenital heterochromia, congenital, ectropion iridis, melanocytomalytic glaucoma, pigmentary glaucoma, siderosis, iridocorneal endothelial syndrome.[40] Photographic documentation has a relevant role in case of small lesion with a basal diameter < 3 mm, providing information on tumor growth during the follow-up. As reported above, the ABCDEF rule is useful in differentiating an iris nevus from a melanoma (see risk factor section). In doubtful cases, such as small lesions, fine-needle biopsy could be very helpful in the diagnostic process, with a low risk of complications and a good rate of adequate sampling.[46]

Diagnosis of ciliary body melanomas could prove difficult when the lesion is small because the location does not allow a good visualization.[10] A good scleral indentation during fundus examination could be useful to bring into the view these tumors. However, in the majority of cases tumors are diagnosed when are large with choroidal or iris invasion.[10] Transillumination may help to visualize large lesions. A valuable exam for detecting small ciliary body melanomas (< 4 mm) is the UBM, which is useful in follow-up as well.[47]

Diagnosis of choroidal melanoma depends mostly on fundus examination with indirect ophthalmoscopy. The most important test in the diagnostic process is represented by ocular ultrasonography.[10,48] In particular, B-scan ultrasonography provides information on tumor size and extension. A-scan ultrasonography provides valuable information on lesion's reflectivity. The presence of acoustic hollowing is a typical characteristic of UM.[10] Ossoinig considered as one of the cardinal hallmark of a melanoma lesion the

presence of low-medium reflectivity.[49] Other A-scan characteristics could be a quite regular internal structure with spikes showing a similar height or a decreasing height, solid consistency and sign of vascularization such as a spike showing fast, vertical motion with flickering.[49] Of note, A-scan ultrasonography has a limited use in case of very shallow lesions, with a thickness less than 1.5/2 mm. Clinical examination and information provided by ultrasonography, when carried out by an ocular oncology expert, ensure a high level of accuracy, minimizing the necessity for biopsy.[48] Other useful tests are OCT imaging, fluorescein angiography and indocyanine green angiography. Enhanced deep imaging OCT is helpful for studying small lesion (less than 3 mm diameter) which are difficult to study with other methods.[50] Furthermore, it has a high accuracy in detecting subretinal fluid and may help to differentiate small choroidal malanomas from other lesion, including nevi.[10] However, its use may be limited when it comes to lesion thicker than 3 mm.[10] Fluorescein angiography may be characterized by a progressive hyperfluorescence which may last for more than 30-40 minutes. In case of Bruch's membrane break, the exam can show a typical 'double circulation' pattern, due to the presence of tumor vessels underneath the retinal vasculature.[51] Indocyanine green angiography is more useful in showing intra-lesion vasculature, with the average peak of hyperfluorescence at 18 minutes.[52] The use of computed tomography and magnetic resonance imaging with the purpose of studying choroidal melanomas has been investigated,[10] but their application in clinical practice is very limited. The most common differential diagnosis of choroidal melanomas is the choroidal nevus. Others can be congenital hypertrophy of the retinal pigment epithelium (CHRPE), peripheral eccentric choroidal neovascular membrane, choroidal hemangioma, hemorrhagic detachment of pigment epithelium/ retina.[53] Differentiating a small choroidal melanoma from a choroidal nevus may prove very difficult in some cases. Shields et al provided a mnemonic acronym which can be useful in daily practice: TFSOM UHHD ('To Find Small Ocular Melanoma Using Helpful Hints Daily'), which stands also for thickness (> 2 mm), fluid (subretinal fluid), symptoms, orange pigment (lipofuscin), margin (≤ 3 mm from optic disc), ultrasonographic hollowness, halo (absent), drusen (absent).[30] Additionally, it has to be taken into account that a few number of choroidal nevi may transform into choroidal melanomas (less than 1:8000).[28] When one of the TFSOM UHHD factor is present, there is a 38% risk for the lesion to transform into melanoma at 5 years, increasing to 50% when at least two factors are present.[29,30] If the lesion has the following 3 TFSOM UHHD factors, such as > 2 mm thickness, a location close to the

disc and symptoms, the risk for transformation into melanoma at 5 years goes up to 69%.[29,30] A choroidal nevus with drusen (signs of chronicity), thickness less than 2 mm and no other TFSOM UHHD factor, could be considered 'low-risk'. The presence of one or more TFSOM UHHD factors indicates a 'high-risk' nevus.[10] Lesions with 2 or more TFSOM UHHD factors are likely to represent small choroidal melanomas and treatment should be indicated.[29,30] Therefore, documenting with fundus photograph a choroidal nevus/indeterminate lesion, which looks suspicious, has a relevant role for detecting lesion growth during the follow. Data from the Collaborative Ocular Melanoma Study (COMS) showed a misdiagnosis rate of about 0.5%.[54] This finding suggests that diagnosis of UM can be based on clinical examination and tests. However, the COMS applied strict eligibility criteria, which could have had an influence on misdiagnosis rate. Indeed, other studies found diagnostic fine-needle biopsy necessary in 1-9% of cases.[5556] Biopsy of intraocular tumors is debated because of the risk of tumor dissemination as well as the risk of ocular complications and inadequate sampling. However, nowadays tumor sampling has become more diffuse, usually not for confirming the diagnosis, but with the purpose of analyzing the genetic profile for assessing metastatic risk and prognosis.[57] For choroidal melanomas, fine needle aspiration biopsy (FNAB) is performed using particular precautions to prevent tumor seeding as well as subsequent application of radiotherapy, which can help to sterilize seeded cells.[57]

1.5 Staging

The 8th edition of the American Joint Committee on Cancer (AJCC) classification has been published in 2017 and provides the classification of uveal melanoma as well.[58] This is an updated version of the 7th edition.[59] However, the differences between the two editions are minimal. The widespread T (tumor), N (node), M (metastasis) staging has been used also for uveal melanoma. Two classifications have been developed, one for iris melanoma and one for choroidal and ciliary body melanoma due to different primary tumor staging (T). In either case, T0 refers to cases with no evidence of primary tumors and Tx to cases where primary tumor cannot be evaluated. Iris melanoma primary tumor (T) classification is show in Table 1.

Table 1. *Iris melanoma primary tumor (T) classification according to the American Joint Cancer Committee (AJCC 8th edition)*[58]

Primary tumor (T)			
T1	Tumor limited to the iris	T1a	not more than 3 clock hours in size
		T1b	more than 3 clock hours in size
		T1c	T1 with secondary glaucoma
T2	Tumor confluent with or extending into the ciliary body, choroid, or both	T2a: confluent with or extending into the ciliary body, without secondary glaucoma	
		T2b: confluent with or extending into the ciliary body and choroid without secondary glaucoma	
		T2c: confluent with or extending into the ciliary body, choroid, or both, with secondary glaucoma	
T3	Tumor confluent with or extending into the ciliary body, choroid, or both, with scleral extension		
T4	Tumor with extrascleral extension	T4a	extrascleral extension ≤5 mm in diameter
		T4b	extrascleral extension >5 mm in diameter

From: Kivelä T, Simpson RE, Grossniklaus HE, et al. Uveal melanoma. In: AJCC Cancer Staging Manual. 8th ed. New York, NY: Springer; 2016:805-817.

T1 includes tumors confined to the iris: T1a tumors are ≤ 3 clock hours in size; T1b > 3 clock hours in size; T1c tumors are associated with secondary glaucoma. T2 includes tumors with ciliary body and/or choroid involvement; T2a tumors are lesions confluent with or extending into the ciliary body, without secondary glaucoma; T2b are lesions confluent with or extending into the ciliary body and choroid without secondary glaucoma; T2c are lesions confluent with or extending into the ciliary body, choroid, or both, with secondary glaucoma. T3 includes tumors with ciliary body and/or choroid involvement, with scleral extension. T4 includes tumors with extrascleral extension: ≤ 5 mm extrascleral extension are T4a; >5 mm extrascleral extension are T4b.[58] Primary tumor (T) classification for choroidal and ciliary body melanomas depends on tumor size (thickness and largest basal diameter) as well as ciliary body involvement and extraocular extension.[58] Primary tumor classification according to tumor size is displayed in Table 2.

Table 2. *Primary tumor (T) classification for choroidal and ciliary body melanoma based on thickness and largest diameter.*[58]

Thickness	**Largest basal diameter, mm**						
	≤3	3.1-6	6.1-9	9.1-12	12.1-15	15.1-18	>18
≤3 mm	1	1	1	1	2	2	4
3.1-6 mm	1	1	1	2	2	3	4
6.1-9 mm	2	2	2	2	3	3	4
9.1-12 mm	3	3	3	3	3	3	4
12.1-15 mm	3	3	3	3	3	4	4
>15 mm	4	4	4	4	4	4	4

From: Kivelä T, Simpson RE, Grossniklaus HE, et al. Uveal melanoma. In: AJCC Cancer Staging Manual. 8th ed. New York, NY: Springer; 2016:805-817.

T1 includes tumors with a ≤ 3 mm thickness and ≤ 12 mm largest basal diameter, and tumors with 3.1-6 mm thickness and ≤ 9 mm largest basal diameter. T2 includes: tumors with a ≤ 3 mm thickness and 12.1-18 mm largest basal diameter; tumors with 3.1-6 mm thickness and 9.1-15 mm largest basal diameter; tumors with 6.1-9 mm thickness and ≤ 12 mm largest basal diameter. T3 includes: tumors with 3.1-6 mm thickness and 15.1-18 mm largest basal diameter; tumors with 6.1-9 mm thickness and 12.1-18 mm largest basal diameter; tumors with 9.1-12 mm thickness and ≤ 18 mm largest basal diameter; tumors with 12.1-15 mm thickness and ≤ 15 mm largest basal diameter. T4 includes: tumors with ≤ 12 mm thickness and >18 mm largest basal diameter; tumors with 12.1-15 mm thickness and >15 mm largest basal diameter; tumors with >15 mm thickness and any diameter.[58] All T value can be featured by a letter from ‘a’ to ‘d’: ‘a’ means nor ciliary body involvement neither extraocular extension; ‘b’ means ciliary body involvement without extraocular extension; ‘c’ means no ciliary body involvement but a ≤ 5 mm extraocular extension; ‘d’ means both ciliary body involvement and a ≤ 5 mm extraocular extension.[58] Additionally, the T4e category includes any tumor size with an extraocular extension > 5 mm.[58] Regional lymph nodes (N) include preauricular, submandibular and cervical sites. Node assessment applies to tumors with extrascleral growth and conjunctival involvement. Nx includes cases where nodes cannot be evaluated; N0 means absence of node metastasis; N1 means presence of node metastasis or discrete tumor deposits in the orbit; N1 stage is classified into N1a (metastasis in one or more regional lymph nodes) and N1b (no positive regional lymph nodes, but presence of discrete tumor deposits in

the orbit that are not contiguous to the eye). Distant metastases are evaluated in the 'M' category: M0 means no metastasis; M1 means presence of distant metastasis ('a' ≤ 3 cm metastasis; 'b' 3.1-8 cm metastasis; 'c' >8.1 cm metastasis).[58] The AJCC anatomic stage is shown in Table 3.

Table 3. *Anatomic stage according to AJCC cancer staging manual, 8th edition.*[58]

	T	**N**	**M**
Stage I	T1a	N0	M0
Stage IIA	T1b-d	N0	M0
	T2a	N0	M0
Stage IIB	T2b	N0	M0
	T3a	N0	M0
Stage IIIA	T2c-d	N0	M0
	T3b-c	N0	M0
	T4a	N0	M0
Stage IIIB	T3d	N0	M0
	T4b-c	N0	M0
Stage IIIC	T4d-e	N0	M0
Stage IV	Any	N1	M0
	Any	N1	M1a-c

From Kivelä T, Simpson RE, Grossniklaus HE, et al. Uveal melanoma. In: AJCC Cancer Staging Manual. 8th ed. New York, NY: Springer; 2016:805-817.

A further classification depends on the histologic grade (G) of the tumor. Basically, there are three histopathologic types of UMs, according to their cytological composition: spindle cell UM (>90% spindle cells); epithelioid cell UM (> 90% epithelioid cells); mixed UM, consisting of <90% spindle cells and >10% epithelioid cells.[58] Spindle cells feature ovoid nuclei and their growth shows a compact and cohesive pattern. Epithelioid cells are pleomorphic, with larger and irregular shape compared to spindle ones. The growth pattern of their nuclei and nucleoli is less cohesive compared to spindle type; their cytoplasm is acidophilic.[58] Gx indicates cases where the grade cannot be evaluated. G1 includes spindle cell UM; G2 mixed cell UM; G3 epithelioid cell UM.[58]

At time of diagnosis it is mandatory to carry out imaging tests for searching systemic metastases as the presence of metastases has a relevant influence on the management plan.

In the past, baseline imaging consisted of abdominal ultrasonography and chest radiography. Given the low sensitivity of those tests,[60] baseline modern imaging work-up for ruling out metastases includes usually one of the following protocols: computed tomography (CT) of chest and abdomen; chest CT and liver magnetic resonance imaging (MRI); whole body positron emission tomography- CT.[61]

1.6 Prognosis

Uveal melanoma presents a high mortality rate and up to 50% of cases could metastasize.[2,62] A large study including both iris and posterior melanomas showed a metastasis rate of 15% at 5 years and 25% at 10 years.[42] The most common metastasis site is the liver (60-89%), followed by lung (24-29%), skin and soft tissue (11-12%), bone (8-17%), lymph node (11%).[62,63] Prognosis of UM has been related to several variables. First, location has been shown to have an influence on prognosis. Iris melanoma has a mortality rate 5 to 10 times lower than posterior uveal melanoma. In a review of more than 8000 cases of UM, 10-year metastasis disease was 33.4% for ciliary body melanomas, 25% for choroidal melanomas and 6.9% for iris melanomas.[42] The better prognosis of iris melanoma could be explained by factors including a lower biologic activity, younger age and smaller size.[64,65] The cumulative proportion of metastatic disease and death at 5 years was found 5.2% and 2.2%, respectively, raising to 8.8% and 3.3%, at 10 years respectively.[39] In case of iris melanoma, factors predicting melanoma-related metastasis are older age, increased thickness, secondary glaucoma, angle involvement, extraocular extension.[39,42,66] A study investigating very long-term prognosis in patients with posterior uveal melanoma reported a melanoma-related mortality rate of 31% at 5 years, 45% at 15 years, 49% at 25 years.[67] The COMS study, which included choroidal melanomas and featured strict eligibility criteria excluding peripapillary tumors and predominately ciliary body tumors, reported a cumulative metastasis rate of 25% at 5 years and 34% at 10 years.[63] Following metastasis development, the mortality rate was 80% within one year and 92% within two years.[63] Several factors have been investigated with the purpose of assessing their possible influence on the prognosis of uveal melanomas, and the role of some is still debated.[10] For instance, whether age and gender might have an influence on the prognosis is not completely clear. It seems that younger age may have a protective role from metastatic disease because the immune response is more robust, lesions tend to be smaller and genetic mutations are less common compared

to older patients.[17,65] In study including more than 8000 melanoma patients, Shields et al[68] reported a cumulative rate of metastasis at 10 year of 9% in patients < 20 years old, 23% in 21-60 years old patients and 28% in patients > 60 years old; cumulative 10-year death rate was 5% in patients < 20 years old, 11% in 21-60 years old patients and 16% in patients > 60 years old; at 10 years, metastases were found in 0% of 0-10 years old patients, 10% of 11-20 years old patients, 21% of 41-50 years old patients, 30% of 71-80 years old patients. The authors concluded that young patients had a lower rate of metastatic disease. However, the proportion of iris melanoma was 21% in young (≤20 years old), 4% in mid adult (21-60 years old) and 2% in older adult (>60 years old).[68] Whether female gender could have a better prognosis compared to male is still controversial: in one study mortality rate at 10 years was found two times greater in males compared to females, and time to develop metastatic disease was shorter in males compared to females (metastatic disease at 5 years from diagnosis of UM in 84% of males compared to 50% of females);[69] while no gender-related difference in survival analysis was shown in the COMS study.[70] Tumor size has been demonstrated to have a significant influence on the development of metastases. In a large study including both iris and posterior UM, 10-year metastasis rate was 6% for 0-1 mm thickness, 12% for 2.1-3 mm thickness, 16% for 3.1-4 mm thickness, 27% for 4.1-5 mm thickness, 41% for 7.1-8 mm thickness, 51% >10 mm thickness.[42] An hazard ratio of 1.06 was found for 1 mm increase in thickness.[42] The COMS report on mortality outcome of medium choroidal melanomas (2.5-10 mm thickness and ≤16 mm largest basal diameter) showed a similar rate of melanoma metastasis-related death at 10 years in both brachytherapy and enucleation arms (18% and 17%, respectively), and depicted a larger maximum basal tumor diameter as a primary predictor of melanoma metastasis-related death.[71] Likewise, the COMS report on large choroidal melanomas (>10 mm thickness and >16 mm diameter) showed a melanoma metastasis-related mortality of 40% in enucleation arm at 10 years, and depicted a larger maximum basal tumor diameter as a primary predictor of melanoma metastasis-related death.[70] A meta-analysis study on choroidal melanomas treated with enucleation reported a 5-year death rate of 16% in case of tumors with a thickness < 2 or 3 mm and a basal diameter < 10 or 11 mm, 32% in case of tumors with 3-8 mm thickness and a basal diameter < 15 or 16 mm, and 53% in case of tumors with a >8 mm thickness and a >16 mm basal diameter.[72] Not surprisingly, the AJCC staging has been demonstrated to have a prognostic role. At 10-year follow-up, tumors with a T1 stage had a 15% metastatic rate, increasing to 25% for T2 tumors and to 49% for T3 tumors.

Melanomas with a T4 stage presented a 63% metastatic rate.[73] As reported above, the location of the melanoma has a noticeable relevance in terms of metastatic risk. Overall, ciliary body melanoma could be considered the most aggressive. Ciliary body melanomas present a 2 to 4-fold higher risk of metastasis than choroidal ones.[74] Possible reasons for this higher tendency to metastasize could be related to the delay in diagnosis (ciliary body tumors are less symptomatic and, usually, are large at time of diagnosis), the relevant vascularization of ciliary body, and the higher incidence of chromosomal predisposing alterations.[10,75] Presence of oculodermal melanocytosis represents a risk factor for uveal melanoma development as well as for metastasis development in patients affected by UM.[75] Other tumor-related features that may affect prognosis are 'diffuse' configuration and extraocular extension. Risk of melanoma-related metastasis has been demonstrated to be higher in diffuse iris melanoma and diffuse choroidal melanoma compared to non-diffuse iris and choroidal melanomas, respectively.[75] Extraocular extension has a significant negative influence on the prognosis when larger than 5 mm: mortality rate at 5 years was 37%, 24% and 78% for patients with microscopic, small (1-4 mm) and large extrascleral extension, respectively.[76] The histopathology has been shown a relevant role in prognostication. With regards to cell type, the best prognosis has been associated with spindle cell melanoma, the worst with epithelioid cell type, while mixed type has an intermediate prognosis.[75] Uveal melanomas with a high mitotic activity have a worse prognosis compared to those with a low mitotic activity.[77] Nucleoli size is another histopathologic variable which affects the prognosis. The mean diameter of the 10 nucleoli with the largest size (MLN) is used for prognostication. A large MLN predicts a poor prognosis.[75] Of note, epithelioid cells are characterized by larger MLN, but MLN was shown to be an independent factor as well.[78] Tumor vascularity has an influence on prognosis. High microvascular density as well as specific microvascular patterns, such as presence of networks or loops, have been identified as predictors of worse prognosis.[75] Unfavorable prognosis has been also associated with the presence of numerous tumor-infiltrating macrophages, high insulin-like growth factor-1 receptor expression, high expression of human leukocyte antigen (HLA) Class I and II.[75] Recently, when it comes to prognostication, more attention has been given to cytogenetic characteristics. Usually, genetic tests are carried out on samples obtained from FNAB or enucleation specimen. Most relevant cytogenetic alterations include chromosome 3, 1, 8 and 6.[75] Chromosome 3 loss represents a predictor of poor prognosis. In particular, the complete loss of one chromosome 3, known as monosomy 3, has been identified as the most relevant

prognostic factor, being associated with increased risk of metastatic disease.[75] In a series of 54 uveal melanomas, monosomy 3 was found in 56% of cases. Those with monosomy 3 presented a 3-year mortality rate of 50% versus 0% of those without monosomy 3.[79] Monosomy 3 was found in association with other unfavorable prognostic factors, such as epithelioid type, vascular loops, high mitotic activity, extrascleral extension, ciliary body location and large diameter.[75] Furthermore, BAP1 has been located on the short arm of this chromosome (3p21.1) and BAP1 mutation turned out to be a prognostic factor for metastatic disease.[32] Partial or complete loss of chromosome 1p predict a negative prognosis. It is usually associated with monosomy 3, but can also occur alone.[75] Concomitant monosomy 3 and chromosome 1p loss is by far a stronger predicting factor for metastatic disease compared to loss of either chromosome 3 or chromosome 1p.[80] The most common alteration affecting chromosome 8 is a gain in chromosome number. Indeed, chromosome 8q gain was found in 41-53% of UM, whereas a loss of chromosome 8q is rare.[75] Similarly to chromosome 1p loss, chromosome 8q gain could be alone or in combination with monosomy 3. Chromosome 8q gain in combination with monosomy 3 has a poorer prognosis compared to each alteration alone: 5-year mortality rate was reported 31% in cases of 8q gain, 40% in cases with monosomy 3, 66% in cases of concomitant 8q gain and monosomy 3.[81] Conversely to chromosome 8 gain, chromosome 6 gain is a predictor of good prognosis and tend to be mutually exclusive with monosomy 3:[75] occurrence of both monosomy 3 and chromosome 6 gain was reported only in 4% of UM.[82] On the contrary, chromosome 6 loss is a predictor of unfavorable prognosis: 6q loss was found in 40% of tumors with metastatic disease versus 7% of metastasis-free melanomas.[83] Over the last years, considerable efforts have been focused on epigenetic and transcriptomic analyses. Gene expression profiling provided a prognostic classification of UM. This classification consists of two main classes: class I melanomas with a low risk of metastasis development and Class II melanoma with high risk of metastasis development.[84] These results were based on the analysis of microRNA expression of 15 genes (12 target genes and 3 controls) and have been validated in clinical setting.[85] A test analyzing these 15 genes is available and can be used in clinical practice with ease, on samples obtained from enucleation, tumor resection and FNAB.[86] Class I could be divided in Class IA with 2% metastatic risk at 5 years and Class IB with 20% metastatic risk at 5 years. Class II presents 72% metastatic risk at 5 years.[87] These data could allow patients to be offered a personalized management based on risk stratification.[87] A further point that needs to be mentioned with regards to prognostication

is the concept of micrometastasis. Eskelin et al investigated metastasis doubling time and postulated that micrometastases could start up to 5 years before primary tumor treatment.[88] Taking into account all these considerations, early diagnosis and treatment of UM, including small melanomas, could represent a key strategy for a positive long-term prognosis.[10]

1.7 Treatment

Management of UM represents a multi-disciplinary challenge, involving a variety of physicians specialized in ocular oncology, such as ophthalmologists, radiologists, medical and radiation oncologists.[89] It is important to remark that despite improvements in primary tumor treatment, metastasis rate and overall survival has remained unchanged over the last decades.[4,90] Once metastatic disease has been diagnosed, overall survival is as short as roughly one year, as shown by a recent meta-analysis.[90] In fact, patients who show metastasis at diagnosis of primary tumor often do not undergo aggressive treatment of primary tumor. [61] Primary uveal melanoma treatment can be divided into two types: globe-preserving treatment and enucleation. The former one includes radiation therapy, laser and surgical therapy. For many years the only available treatment for UM was enucleation. In 1970s efforts have been done to develop globe-preserving alternatives.[91] Then, with the introduction of radiation therapy, there has been a shift towards a globe-sparing approach rather than enucleation surgery, in particular since the COMS study showed comparable survival rate between enucleation and plaque radiotherapy in patients with medium choroidal melanomas.[71] Radiotherapy tends to be the preferred treatment for small and medium UMs, whilst enucleation is usually performed for larger and more advanced melanomas.[10] Tumor characteristics as well as patient characteristics must be taken into account when choosing the appropriate treatment. In this paragraph, the treatment options for primary tumor will be described first, and will be classified according to the location; then, metastatic disease therapy will be discussed.

Iris melanoma treatment depends on the size of the lesion as well as on its characteristics. A small lesion with a basal diameter < 3 mm, with no other sign and no symptoms, that could be a nevus or a small melanoma (indeterminate lesions), can be monitored periodically with photographic documentation for evaluating possible growth.[10] Small circumscribed lesions with documented growth can be treated with sector iridectomy.[92] If there is an involvement of the anterior chamber, a portion of the trabecular meshwork

needs to be removed as well; this type of resection is called iridotrabeculectomy. If there is an involvement of the ciliary body, an iridocyclectomy can be performed, resecting a portion of the iris and ciliary body.[10] Intraocular surgery could be associated with complications, such as hypotony, retinal detachment, lens subluxation, phthisis, endophthalmitis, sympathetic ophthalmia.[92–94] Larger melanomas are usually non-resectable and treatment is based on radiotherapy or enucleation.[10,95–97] Lloyd and Ellis described in 1952 the use of radioactive wires (tantalum), inserted into the eye, for the treatment of small iris melanoma.[98] Afterwards, external beam and plaque radiotherapy have become available for the treatment of iris melanomas. Anterior segment irradiation can be beneficial for non-resectable lesions and for extensive seeding because treatment margins are larger compared to simple resection.[99,100] Small series of patients with iris melanoma showed that proton beam and plaque radiotherapy can achieve local tumor control in up to 93%[101] and 97%[102] of cases, respectively. A larger study on 144 patients reported local recurrence in about 15% of cases at 7 years, showing an adequate local tumor control; metastasis rate at 7 years was 1%.[103] Even if radiation treatment is a globe-spearing approach, complications may be severe and sight-threatening, including corneal opacities, cataract and iris neovascularization, culminating in vision loss.[10,100] Enucleation surgery is usually reserved for large tumors, poor visual function, recurrent tumors, multifocal iris melanoma and diffuse iris melanoma.[10] However, radiation therapy has recently shown good local tumor control also for both diffuse and multifocal iris melanomas.[99,100]

The treatment of posterior uveal melanoma can be surgery, radiation therapy or laser. In general, the most common treatments are radiotherapy, including plaque brachytherapy or external beam radiation therapy, mostly used for small/medium melanomas, and enucleation surgery, mostly used for large melanomas and poor visual function.[10] Other possible treatment options include surgical resection and laser treatments, such as transpupillary thermotherapy and photodynamic therapy.[2] Importantly, in case of indeterminate lesion, which could be either a choroidal nevus or a small melanoma, observation could represent the first approach: the patient is monitored for documented growth or TFSOM UHHD[30] risk factors (as reported above). If there is evidence of documented growth or presence of TFSOM UHHD[30] factors, treatment should be considered.[61] In some selected patients affected by small choroidal melanomas (thickness < 3 mm and largest basal diameter < 10 mm), usually presenting with low-grade tumor (stable or growing slowly), advanced age, multiple comorbidities, limited life expectancy,

observation could represent an alternative to the treatment. However, patients must be informed about both the risks of treatment (visual loss) and the risk of metastasis (unquantified albeit small) for observation.[61,104]

Radiotherapy for uveal melanoma includes plaque brachytherapy, proton beam radiotherapy and photo beam radiation therapy (stereotactic radiotherapy). Radiotherapy has gained increasing popularity for the UM treatment and replaced enucleation surgery for melanoma of suitable size and location.[61] It is a globe-preserving treatment, which ensures excellent local tumor control.[2] After the introduction of radiotherapy for UM treatment, the main concern of physicians was whether there was a difference in survival between radiotherapy and enucleation.[105] Therefore, the COMS group conducted from 1986 to 2003 two large multicenter clinical trials comparing survival between radiotherapy and enucleation in patients affected by medium and large choroidal melanoma.[71] Patients affected by large choroidal melanoma (apical height > 10 mm and maximum basal tumor diameter > 16 mm) were randomized to enucleation alone or external beam irradiation (20 G) preceding enucleation surgery; patients affected by medium choroidal melanoma (2.5-10 mm apical height and maximum basal tumor diameter ≤ 16 mm) were randomized to iodine-125 brachytherapy or enucleation.[106] The COMS was the largest RCT performed in ocular oncology, with more than 2000 patients enrolled.[106] The results showed no survival differences at 5, 10 and12 years between plaque brachytherapy and enucleation in patients with medium choroidal melanoma: 5-, 10- and 12-year all-cause mortality rate was 19%, 35% and 43% in brachytherapy arm and 19%, 35% and 41% in enucleation arm, respectively; 5-, 10- and 12-year metastasis-related death rate (histopathologically confirmed) was 10%, 18% and 21% in brachytherapy arm and 11%, 17% and 17% in enucleation arm, respectively.[71] This reassured that brachytherapy is 'as safe as enucleation'.[107] However, in many cases metastases developed so soon that systemic spread was supposed to be present at time of primary lesion treatment: this could have led to a lack of statistical power.[107] Nonetheless, the conclusion of comparable efficacy in terms of survival between brachytherapy and enucleation could be considered correct.[105] With regards to the large choroidal melanoma arms, no difference in 5- and 10-year tumor-related mortality was found between enucleation alone and enucleation with preoperative irradiation:[70,106] this finding confirmed that primary enucleation alone does not increase mortality from metastatic disease as hypothesized by Zimmerman et al.[108]

Brachytherapy is one of the most largely used conservative treatment for uveal melanoma.[91] After the publication of COMS reports, brachytherapy has become the treatment of choice for suitable tumors.[61] Brachytherapy is used for posterior uveal melanomas with <10 mm thickness and <18 mm maximum basal diameter. Selected iris melanomas and ciliary body melanomas (< 10 mm thickness and no extensive circumferential growth) can be considered for brachytherapy as well.[61] The radiation dose delivered to tumor apex is 80-100 Gy.[10] According to the 2014 consensus guidelines from the American Brachytherapy Society, apex dose can range from 70-100 Gy.[104] The apex dose in the COMS trial was 85 Gy.[109] The plaque features a saucer shape and contains the radioisotope. The plaque is sutured to the sclera (positioned corresponding to the tumor) until the dose has been delivered. Plaque size has to physically exceed tumor margin by at least 2 mm (free-margin).[105] During the surgery the plaque has to be positioned adequately, in relation to tumor location: intraoperative US or transillumination are used to ensure a proper positioning; a notched plaque is used in case of juxta-papillary lesions.[105] The most largely used radioisotopes are iodine-125, ruthenium-106 and palladium-103.[61] Ruthenium-106 emits beta radiation, while iodine-125 gamma radiation. Beta radiation has a lower depth of penetration compared to gamma radiation. As a consequence, ruthenium-106 can be used for tumor with a thickness <6 mm.[2,105] The advantage of this limited depth of penetration should be a reduced damage to eye structures.[91] A study on 400 eyes treated with palladium-103 plaque, showed favorable visual outcome and local tumor control compared with ruthenium-106 and iodine-125; mean apex dose was 73.3 Gy (for an equivalent dose, more radiation was delivered in palladium-treated tissue compared to iodine-treated).[110] Local recurrence rate has been reported as 3% for palladium-103, 7-10% for iodine-125 and 14.7% for ruthenium-106.[2] Local recurrence can be either re-treated with brachytherapy or treated with enucleation. A further therapeutic option suitable for minimal margin recurrence can be transpupillary thermotherapy (TTT).[61] Proton beam radiotherapy delivers high dose radiation by using charged particles and relatively sparing superficial tissues.[105] Proton beam radiotherapy can be used for the treatment of both posterior uveal melanomas and iris melanomas.[95,111] Tantalum markers are sutured to the sclera and their distance from tumor margins, limbus and from each other, is measured for proper localization and treatment planning. Usually, a 2-mm safety margin is used. Following a simulation phase, treatment is delivered in 4 consecutive day-sessions, with a total dose of 56 Gy.[105] In the past, the total dose amounted to 60-70 Gy, whereas more recent studies used a dose

ranging between 50 and 60 Gy.[111] Proton beam radiotherapy for posterior UM presents comparable outcomes in terms of tumor control, systemic prognosis and visual result compared to brachytherapy.[112] Proton beam radiotherapy is considered as an effective and safe treatment for UM, with a rate of local tumor control over 90%, and 5-year overall survival of 70-85%.[111] Chraged-particle radiotherapy could be preferred to brachytherapy for tumors with a location that may challenge plaque positioning, with also risk of suboptimal immobilization of the plaque (for instance, posterior pole).[111,113] Proton beam radiotherapy can be used to treat also large tumors, but it could be challenged by high rate of local recurrence and high risk of radiation-induced complications that can lead to vision loss and/or secondary enucleation. Additionally, a 'toxic tumor syndrome' has been described following radiotherapy for large tumors as a result of severe intraocular inflammation, which causes exudative and ischemic complications.[114] A study on more than 300 patients affected by large choroidal melanoma (> 10 mm thickness or > 16 mm largest basal diameter; >8 mm thickness in case of optic nerve involvement) showed that proton beam radiotherapy allowed to retain the eye in 70% of cases at 10 years; 10-year mortality (60%) was comparable with enucleation; 10-year local tumor control was 87%.[115] However, visual outcome was poor, with only 8.7% of cases with a visual acuity of 20/200 or better at 10 years; additionally, 25% of cases developed neovascular glaucoma; this rate increased to roughly 35% by 5 years after treatment.[115] Stereotactic photon beam radiotherapy with gamma knife, cyber knife or linear accelerator, delivers high doses from multiple directions, trying to spare surrounding tissues.[105] Tumor control, survival outcome and visual outcome have been reported comparable with those of proton beam radiotherapy.[116]

Common sight-threatening complications from radiotherapy are radiation retinopathy, called radiation maculopathy when affecting the macula, and radiation papillopathy, when affecting the optic disc. Radiation maculopathy and optic nerve atrophy can lead to visual loss.[117] These complications are related to the radiation-induced damage to the retina and optic nerve: tumor size and location, as well as dosimetric parameters have an influence on their development.[111,117] For instance, cumulative incidence rate of radiation maculopathy has been reported as high as 64% at 5 years for tumors located within 4 disc diameters to the macula.[117] Other complications of radiotherapy include glaucoma, neovascular glaucoma, cataract, vitreous bleeding, ocular surface problems, radiation-induced dry eye, keratitis, diplopia/strabismus, scleral necrosis.[10,105,111]

Enucleation represented the mainstay treatment for UM before the advent of radiotherapy.[91] After that, enucleation has been the second most common treatment for UM.[118] In 1970s, a concern as to whether enucleation could increase risk of metastasis was raised due to the diffusion of the 'Zimmerman hypothesis', which based this assumption on potential dissemination of tumor cells into the blood system at the moment of optic nerve cutting.[108] As reported above, this hypothesis was disproved following the publication of COMS findings. Indications for enucleation include the presence of large tumor, poor visual potential, and extraocular growth.[10,113] Enucleation is the preferred treatment for tumors with a thickness > 10 or 12 mm and/or a basal diameter > 18 mm.[61,118] For these lesions, charged-particle radiotherapy can be still offered, but high-dose irradiation carries a high risk of complications that can lead to vision loss and, possibly, to eye loss; furthermore, these patients should cope with the anxiety related to possible recurrence.[61,118] In a series of 1632 patients treated for UMs from 1993 to 2002, 35% underwent primary enucleation, 31% plaque brachytherapy and 17% proton beam radiotherapy. Factors associated with primary enucleation were tumor size, proximity to the optic disc, extensive involvement of iris, angle or ciliary body.[119] Orbital exenteration can be required in case of extensive extraocular growth or orbital invasion.[10,105]

Local resection of the tumor represents a globe-sparing surgical treatment which could be suitable for selected patients affected by posterior UMs. Tumor resection can be based either on an external approach, called exoresection, or on an *ab-interno* approach, called endoresection. Exoresection of a posterior UM needs to be performed under hypotensive general anesthesia because of high hemorrhagic risk.[105] A lamellar, partial thickness, scleral flap is created around the lesion, which is 'en-bloc' excided together with the inner scleral layer. The superficial scleral flap is, then, used to close the deep opening. [120–123] This surgery is not commonly performed and can be associated with sight-threating complications, such as bleeding, retinal detachment, cataract and tumor recurrence. Shields et al reported outcomes of 95 posterior UMs treated with exoresection: retinal haemorrhage, retinal detachment and tumor recurrence/ residual occurred in 35%, 28% and 15% of cases, respectively.[120] Adjuvant plaque brachytherapy can be associated with exoresection to reduce risk of recurrence.[124] Recently, Caminal et al.[125] described outcomes of transcleral resection performed without hypotensive anesthesia and combined with vitrectomy with silicone oil and plaque brachytherapy. The author concluded that this demanding procedure, when successful, could provide a better visual outcome compared to plaque brachytherapy. However, submacular haemorrhage, retinal

detachment and ocular hypertension occurred in 16%, 21%, and 21% of cases, respectively.[125] Endoresection involves piecemeal removal of the tumor by using a vitreous cutter during a pars plana vitrectomy.[126,127] Tumor residual can be destroyed using endolaser photocoagulation. Silicone oil is used as endotamponade. Timing of silicone oil removal is variable, ranging from 3 to 8.8 months.[126,127] Because of concerns about tumor seeding during surgical manipulation, adjuvant radiotherapy has been associated with endoresection surgery.[127,128] Endoresection has been proposed to treat posterior uveal melanomas with a juxta-papillary location, because radiotherapy is likely to cause radiation-induced optic neuropathy.[126] Konstantinidis et al[126] reported on 71 patients with juxta-papillary uveal melanoma treated with endoresection. Over a median follow-up of 4 years, local recurrence occurred in 3% of cases, and retinal detachment in 22% of cases. All-cause mortality was 9% and 20% at 5 and 10 years, respectively. The authors concluded that the procedure could be a useful alternative to irradiation for juxta-papillary melanomas.[126] Endoresection has been used also to treat large uveal melanomas, in combination with preoperative stereotactic radiotherapy and adjuvant plaque brachytherapy.[127] Over a mean follow-up of 32 months, 15% of cases required a further vitrectomy, mainly because of retinal detachment, 5% of cases had a local recurrence, and 15.5% of cases died for metastatic disease.[127]

Laser treatment for posterior UMs includes transpupillary thermotherapy (TTT) and photodynamic therapy (PDT). Transpupillary thermotherapy delivers an infrared laser light (810 nm) to the tumor surface through a dilated pupil. The laser causes an increase in tumor temperature, heating its cells to 45-60°. As a result, tumor abnormal vessels are obliterated, leading to a necrotic process.[129] This treatment could be used for small choroidal melanomas (thickness $\leq$ 3 mm), because of limited laser penetration (maximum penetration of 4 mm).[129] Tumor pigmentation may also have an influence on treatment outcome because amelanotic lesions feature poor heat absorption.[130] Therefore, small pigmented lesions could be suitable for TTT. However, the use of primary TTT as solely treatment of small choroidal melanomas has been questioned due to high rate of local recurrence, reported up to 29% of cases.[131] As a consequence, TTT should be preferably used in combination with radiotherapy.[105] A study including 143 patients with choroidal melanoma compared brachytherapy alone versus brachytherapy combined with TTT: combined treatment provided lower recurrence rate, while metastasis rate and overall survival were comparable.[132] However, a larger study including 449 patients with choroidal melanoma showed no difference in tumor control and vital prognosis between

brachytherapy and brachytherapy combined with TTT; actually, brachytherapy alone provided a better visual outcome.[133] Treatment of large uveal melanomas with proton beam radiotherapy combined with TTT could reduce the number of secondary enucleations.[134] It's worth mentioning a large study on 391 patients with choroidal melanoma treated with primary TTT: tumor recurrence occurred in 28% of patients and its predictive factors were the presence of ocular symptoms, proximity to the optic disc, subretinal fluid, greater thickness and elevation of post-treatment tumor scar.[129] Common complications following TTT for UM were: retinal vein occlusion (26%), macular epiretinal membrane (23%), macular edema (9%) and vitreous haemorrhage (10%).[129] In general, TTT or PDT are used only if the lesion is very small and there is a high risk of visual loss from radiotherapy.[105] Of note, TTT represents a treatment option in case of tumor recurrence after brachytherapy.[135]

Photodynamic therapy is a non-thermal laser treatment which involves the administration of a photosensitizer activated with laser light.[136] This minimally invasive therapy has been also described for the treatment of ocular tumors, including choroidal melanoma.[136] Following the intravenous administration of the photosensitizer and its accumulation into the tumor tissue, laser light is delivered to activate the photosensitizer. This, once activated, has a direct cytotoxic effect on the tumor, causing peritumoral vasculature destruction and local inflammation, with subsequent autophagy.[136] It is important to highlight that the presence of pigmented tumor is a contraindication for PDT.[136,137] Pigmentation seems to prevent light penetration into the lesion.[136] Thus, PDT could be used for the treatment of small amelanotic melanomas (<4 mm thickness).[10] Most commonly, PDT is performed using verteporfin as photosensitizer. Verteporfin PDT for choroidal melanoma treatment can be with either standard fluence (50 j/cm^2 in 83 seconds) or a double fluence (100 j/cm^2 in 166 seconds).[136] A review of 6 reports including a total of 38 choroidal melanoma cases primarily treated with verteporfin PDT showed 80% tumor control over 31 months.[136] A more recent study on 12 eyes with choroidal melanoma reported tumor control in 67% of cases, while 33% failed to regress.[137] Photodynamic therapy with indocyanine green was used for the treatment of choroidal melanoma, showing high rate of tumor control.[136] Interestingly, a study on 25 patients showed good local tumor control combining both TTT and indocyanine green PDT for the treatment of small and medium choroidal melanomas.[138]

1.8 Surveillance and metastatic disease treatment

Following primary tumor treatment, UM patients need to receive periodical ocular and systemic surveillance. The aim of ocular follow-up is the early detection and management of possible local tumor recurrence and treatment-related complications. In general, close follow-up visits are scheduled in the early post-operative period, which, then, are extended to a 3- to 6-month interval for a few years; thereafter, if the clinical condition is stable, follow-up could be arranged every year.[105] Local tumor recurrence is usually managed in the same way as primary tumor.[105] Treatment of secondary orbital involvement is challenging and includes radiotherapy and surgery (excision, debulking, or exenteration).[139] Early detection of radiation-induced complications can allow their early treatment. Anti- vascular endothelium growth factor agents have been used for the treatment of radiation retinopathy, radiation maculopathy, radiation-induced optic neuropathy and neovascular glaucoma, and could help to stabilize or, in some cases, to improve clinical conditions.[140–143]

With regards to systemic surveillance, an ideal surveillance protocol which would define timing, duration and type of examinations according to patient characteristics has not yet been developed.[61,105] Systemic monitoring is aimed at early detection of metastasis, which could have some clinical relevance because highly selected cases of hepatic metastasis could be managed with surgical resection, resulting in improved survival.[144] In addition, no adjuvant therapy has been demonstrated effective in reducing metastatic risk.[145] In this scenario, risk stratification for metastasis development could play a key role and allow to plan a surveillance protocol which could be suited to individual risk.[145,146] As aforementioned, prognosis depends on multiple factors, including clinical variables and genetic prolife (GEP class). According to GEP class, patients could be classified in low-risk (Class I) and high-risk (Class II).[87] Surveillance imaging tends to be focused on hepatic monitoring because UM tendency to metastasize to liver.[145] Low-risk patients can be monitored with hepatic US at 6-month interval.[145] Hepatic ultrasound features a good specificity (100%), but poor sensitivity (14%).[60] Therefore, high-risk patients are recommended to undergo a more frequent (less than 6-month interval) and a more intensive hepatic monitoring, which would include more sensitive and specific imaging tests, such as liver CT/MRI.[145] However, other authors recommend annual liver US and physical examination for low-risk patients, and 6-monthly liver imaging (US alternated with liver/abdomen MRI) plus annual physical examination for high-risk patients.[61] Low-

risk and high-risk patients can be transitioned to the GP at 5 years and 10 years, respectively.[61]

So far, no therapy has been demonstrated effective for the treatment of metastatic disease in UM patients. Metastatic UM has a sadly poor prognosis. Several chemotherapeutic drugs, including dacarbazine, cisplatin, trosulfan, temozolomide and fotemustine, have been investigated, showing low response rate and disappointing outcomes.[113,146] While immunotherapy has noticeably improved outcomes in metastatic cutaneous melanoma, this has not been the case for metastatic UM.[147] A possible reason for such a different response to immunotherapy could be related to the different biological characteristics between cutaneous and uveal melanomas, as well as their different immunogenicity.[148] Ipilimumab showed a response rate of about 5-10%, with an overall survival ranging from 6 to 9.7 months.[146] Nivolumab showed in a prospective trial a 6% response rate and an overall survival of 11 months.[149] A real word retrospective study reported outcomes of 89 patients treated with ipilimumab plus nivolumab, showing a 11.6% response rate and an overall survival of 15 months.[150] Understanding of the molecular mechanisms involved in UM carcinogenesis and progression contributed to the development of targeted therapy for the treatment of metastatic UM. While BRAF inhibitors, such as dabrafenib and vemurafenib, have been used in cutaneous melanoma, which typically harbors BRAF and NRAS mutations, there is no rationale for the use of these agents in UM due to the different molecular profile compared to cutaneous ones.[151] Given the commonly harbored GNAQ/GNA11 mutations in UM, agents targeting downstream effectors of biological pathways GNAQ/GNA11-related, such as MEK and protein kinase C (PKC), have been investigated. However, likewise to other therapeutic approaches, disappointing results have been reported and response rates, in general, are lower than 10-15%. [113,146]MEK inhibitors include selumetinib and trametinib. Initially, the use of selumetinib in metastatic uveal melanoma seemed to provide promising results: a randomized clinical trial enrolling 101 patients compared selumetinib with traditional chemotherapy and reported longer progression free survival (PFS) and higher response rate (14% vs 0%).[152] However, these quite positive outcomes failed to be achieved in a subsequent phase III randomized trial: the SUMIT trial compared selumetinib plus dacarbazine with dacarbazine alone in 129 patients with metastatic UM and showed no significant difference in PFS between the two interventions (median PFS, 2.8 months in selumetinib plus dacarbazine group vs 1.8 months in dacarbazine alone group), and no difference in response rate (3% in selumetinib plus dacarbazine group vs 0% in dacarbazine group).[153]

Trametinib was investigated in a phase I trial enrolling 16 patients with metastatic UM and 81 patients with cutaneous or unknown primary melanoma. In patients affected by UM, trametinib showed no objective response (0% response rate) and a median PFS of 1.8 months.[154] Trametinib was also used in combination with Akt inhibitor: a randomized trial compared trametinib alone (18 patients receiving ≥1 study drug dose) versus trametinib combined with the Akt inhibitor GSK2141795 (21 patients receiving ≥1 study drug dose), showing no difference in median PFS (15.7 vs 15.6 weeks) and only one partial response in each group.[155] Another biological pathway which has been studied as possible target in UM is the MAPK pathway, through PKC inhibition. Sotrastaurin, a PKC inhibitor, showed a median PFS of 15.4 weeks, a partial response in one patient and stable disease in 55 patients (47%).[156] Furthermore, growth factor receptors which have been found overexpressed in UM, have been studied as possible target. Sunitinib, a C-kit inhibitor, was used in a pilot study on 20 patients with C-kit expressing metastatic UM and showed a partial response in one patient and 12 stable disease. Median PFS was 4.2 months.[157] A retrospective study evaluated the use of sunitinib as adjuvant therapy in high risk UM and reported a longer overall survival in those receiving adjuvant sunitinib compared to historical controls.[158] Cabozantinib is a multiple tyrosine kinase receptor inhibitor, including c-MET, Axl, and VEGF. This has been used for the treatment of metastatic UM. Very recently, the results of a RCT comparing cabozantinib versus dacarbazine or temozolomide, have been published and showed no improvement in PFS in patients treated with cabozantinib.[159] Sorafenib, multikinase inhibitor, was used in a trial enrolling 152 patients with metastatic UM: of the 118 patients evaluable for response, 32.2% showed a progression, 1.7% had a partial response, 66.1%. Patients with stable disease were randomized to sorafenib continuation or placebo, with a median PFS significantly longer in sorafenib arm (5.5 vs 1.9 months).[160]

Since the liver represents the most common site for UM metastasis, several liver directed treatments have been investigated. A recent meta-analysis of trials (phase Ib-III) in metastatic UM showed that liver directed treatments has longer overall survival and PFS compared to other therapies (chemotherapy, immunotherapy, targeted therapy).[161] Liver directed treatments include surgical resection, stereotactic radiotherapy, radiofrequency ablation, regional chemotherapy and embolization.[113,162] Surgical resection could be a therapeutic option in very selected cases. A study including 155 patients with liver metastases from UM reported that 11% of patients underwent liver resection, with a better survival compared with those who did not receive surgical resection.[144] Regional

chemotherapy includes hepatic intra-arterial infusion and isolated hepatic perfusion (IHP). Fotemustine showed better response rate (10.5% vs 2.5%) and longer PFS (4.5 vs 3.5 months) when administered as intra-arterial perfusion compared with intravenous, but with no difference in overall survival.[163] Isolated hepatic perfusion is based on the isolation of liver blood supply from systemic circulation with the purpose of delivering high dose chemotherapy and reducing at the same time systemic exposure. A retrospective study on 18 patients treated with percutaneous IHP with melphalan reported a median overall survival of 9.6 months and a median PFS of 12.4 months.[164] Embolization treatment modalities include chemoembolization, radioembolization, and immunoembolization.[162] A recent prospective trial investigated radioembolizaton in two groups of patients: treatment-naïve patients and patients who showed progression after immunoembolization. In the treatment-naïve group, median PFS was 8.1 months and median overall survival 18.5 months; no case of complete response was found, but 20 out of 23 patients had a partial response or stable disease. In the group resistant to immunoembolization, median PFS was 5.2 months, median overall survival was 19.2 months; there was no case of compete response, but 14 out of 24 patients had partial response or stable disease.[165]

2 Genetic and epigenetic features in uveal melanoma

Genetic and epigenetic characteristics of tumors have been given ever-increasing attention in the last years, not only because of their relevant role in carcinogenesis process, but also because they can provide new insights into understanding tumor behavior.[166] This can potentially allow the development of reliable biomarkers and new therapeutic targets, leading to new breakthroughs in uveal melanoma management. Genetic alterations affect directly and permanently the DNA sequence and include the following: chromosomal aberration, copy number variation (CNV), mutations either somatic or germline, and single nucleotide polymorphism (SNP). Conversely, epigenetic alterations modulate gene activity and expression without involving any changes in the DNA sequence; these include the alteration of microRNAs expression levels, DNA methylation and histone modifications.[166] The interaction between genetic, epigenetic and other possible factors involved in UM carcinogenesis is shown in Figure 1.

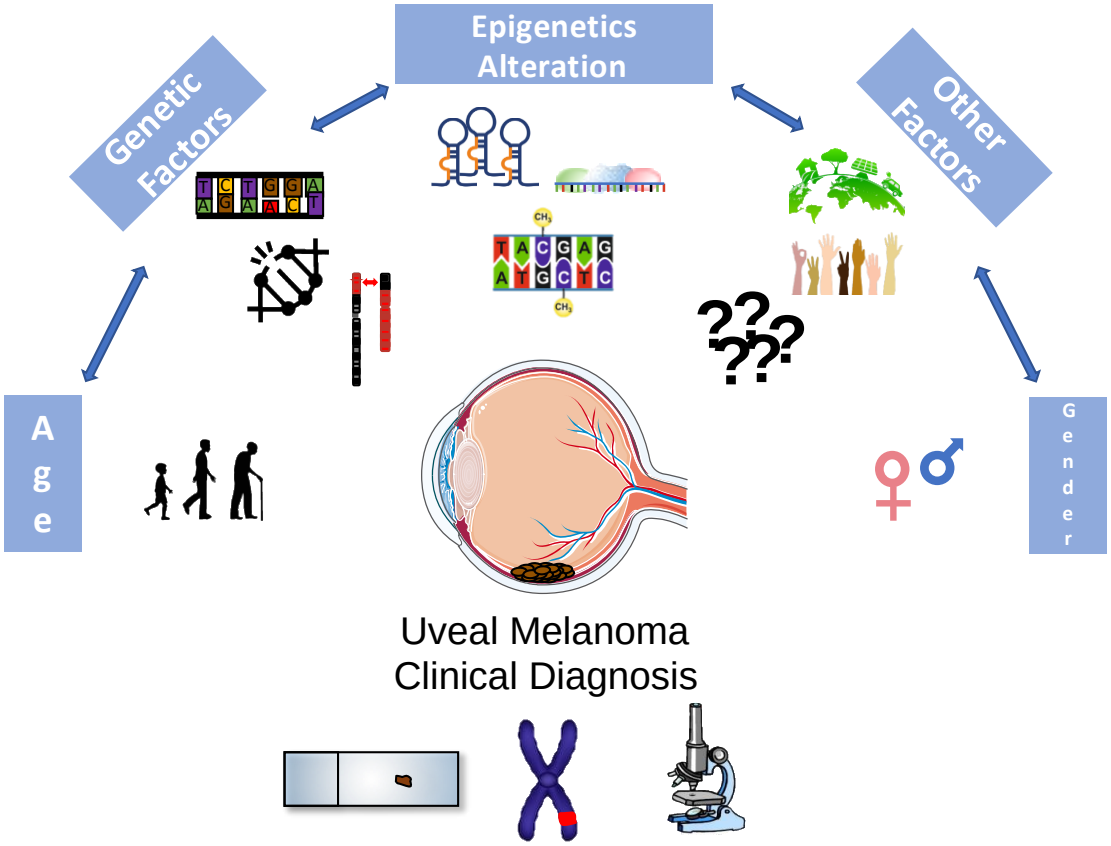

Figure 1. Schematic representation of genetic and epigenetic alterations and risk factors involved in uveal melanoma development. Environmental and individual risk factors (including age, gender and ethnics) are able to induce both genetic and epigenetic modifications responsible for the malignant transformation of choroid cells. Clinical and prognostic assessment could benefit from the analysis of genetic and epigenetic factors associated with uveal melanoma development.

Basically, UM is a sporadic tumor, rarely showing a familial inheritance.[166] This is the case of tumor syndromes, such as Lynch syndrome[167] and BAP1-tumor predisposition syndrome.[168] As reported above, chromosomal alterations in uveal melanoma mainly affect chromosomes 1,3,6 and 8, and present a meaningful prognostic relevance (see paragraph 1.6). With regard to somatic mutations, the most commonly mutated genes that have been identified in UM patients are GNAQ, GNA11 mainly affected by specific point mutations (p.Q209P and p.Q209L, respectively) and BAP1 that is subjected to several mutations occurring in the whole gene sequence.[87,169] Unlike cutaneous melanoma, uveal melanoma does not harbor typical mutations in BRAF and NRAS genes.[151] The GNAQ and GNA11 genes encode for the Gα11 subunits and the Gαq subunits of G proteins, respectively.[169] G proteins are involved in signal transduction, controlling gene transcription and, subsequently, cell survival, growth and mortality.[169] GNAQ and GNA11 are located on chromosome 9 (q21.2) and chromosome 19 (p13.3), respectively.[166] These genes have been considered as driver oncogenes, representing early or initiating mutations in UM.[166,169] Oncogenic mutations of GNAQ and GNA11 genes are usually mutually exclusive and were found in up to 83% of UMs.[170] Oncogenic mutations of these genes determine a constitutive activation of G proteins, which, in turn, affect downstream signaling.[169] Several intracellular pathways are regulated by GNAQ and GNA11 genes, including the RAF/mitogen‐activated protein kinase kinase (MEK)/extracellular signal‐regulated kinase (ERK) pathway.[171] RAF/MEK/ERK pathway activation determines an overexpression of cell‐cycle regulatory protein cyclin D 1 (CCND1), leading to the inactivation of the retinoblastoma tumor suppressor gene.[171] Mutations in this pathway have been assumed to be early or initiating events in UM carcinogenesis.[171] The BAP1 gene is an onco-suppressor gene located on chromosome 3 (p21.1). Inactivating somatic mutations of BAP1 have been associated with metastatic disease: up to 84% of metastasizing UMs harbor inactivating mutations of this onco-suppressor gene.[32] Looking at BAP1 function, this is a deubiquitinating enzyme that regulates specific proteins. In particular, BAP1 regulates genes involved in melanocyte function and differentiation: inactivating mutations can lead to melanocytic de-differentiation, promoting a pro-metastatic behavior. Loss of tumor suppressor activity of BAP1 has been identified in breast and lung cancers as well.[172] Of note, BAP1 mutations can also occur in germline featuring the BAP1 familial cancer syndrome, which predisposes to several cancers, including UM.[173]

With regard to epigenetic alterations, biological mechanisms related to microRNAs and DNA methylation have been identified in UM, whereas modest evidence on histone modification is available. The role of histone modification has been associated with BAP1 loss. It has been shown that BAP1 depletion determines a loss of differentiation in cancer cells through an hyperubiquitination of histone H2A.[172] The use of histone deacetylases inhibitors proved to reverse H2A hyperubiquitination, inducing cell differentiation and inhibiting tumor growth.[172] Indeed, nuclear expression of several histone deacetylases was found in UMs, confirming the role of this epigenetic alteration as potential therapeutic target.[174]

DNA methylation represents an important epigenetic mechanism which regulates the expression of several genes involved in UM carcinogenesis. This regulation is based on methylation/demethylation mechanisms, that is the addition/removal of a methyl group (CH_3) to/from a DNA sequence mediated by DNMT3A and TET1. It has been widely demonstrated that certain methylated DNA regions can be used as stable biomarkers, considering that DNA methylation is maintained almost unchanged during the cell replication process by DNA methyltransferase 1 (DNMT1).[175,176] The functional alteration of this enzyme, together with that of other methyltransferases (DNMT3A and DNMT3B) that catalyze the de novo DNA methylation, is responsible for the global hypomethylation and hypermethylation of widespread regions of the tumor cell genome.[177]

Identifying the gene regions affected by methylation phenomena is important to establish the effect that this modification determines at the transcriptional level. It is now known that promoter methylation determines gene suppression by blocking the access of transcription factors to binding sites on the promoter,[178,179] while it has not yet been fully clarified the functional role of intragenic and intergenic methylation in the control of gene expression.[180] On this matter, several studies have described how DNA methylation can modulate the expression of non-coding RNA, the alternative splicing, the recruitment of enhancers, and the increase of RNA polymerase activity that influences the expression levels of the genes involved. As regards UM, hypomethylation of sites close to the Preferentially Expressed Antigen in Melanoma (PRAME) promoter was shown to promote PRAME activation with subsequent increase in metastatic risk.[181] Hypomethylation of the Deleted in Split hand/Split foot 1 (DSS1) promoter was found to be a frequent event in uveal melamoma.[182] Hypermethylation of the following oncosuppressor gene promoters has been demonstrated in uveal melanoma: p16,

RASSF1A, RASEF, TIMP3, EFS.[183–188] This event leads to inactivation of these genes. Methylation of human telomerase reverse transcriptase (TERT) promoter was described in UM. Human TERT is an oncogene which was found upregulated in UM.[184] TRAIL receptors DcR1 and DcR2 were found hypermethylated in both uveal and cutaneous melanomas.[189] Interestingly, hypermethylation of a site on chromosome 3 at BAP1 locus determined BAP1 down-regulation, showing the epigenetic regulation of this gene.[190]

MicroRNA-based epigenetic mechanisms have been also investigated in UM. microRNAs (miRNAs) are noncoding RNA molecules, consisting of a single-stranded sequence of 18-22 nucleotides. miRNAs regulate gene expression, playing important roles in both physiological and pathological processes, such as cell proliferation, differentiation, apoptosis, organ formation, angiogenesis, extracellular matrix remodeling, etc. Dysregulation of specific miRNAs has been associated with onset and progression of many cancers, including UM. Research in this field has been progressing tremendously. Venza et al found 96 miRNAs dysregulated in both uveal and cutaneous melanoma cell lines.[191] Radhakrishnan et al found specific microRNAs associated with metastatic UM, which were in association with chromosome 1,3 and 8 aberrations.[192] However, Larsen et al failed to show the role of this association between chromosome alterations and miRNAs expression in predicting metastatic disease in UM.[193] Worley and coworkers showed that the expression of 6 miRNAs (let-7b, miR-199a*, miR-199a, miR-193b, miR-143, miR-652) could be used to distinguish Class1 UM (low metastatic risk) from Class 2 ones (high metastatic risk) with maximum sensitivity and specificity.[194] Among the miRNAs involved in cell proliferation and apoptosis, which were found abnormally expressed in UM, noteworthy are the following: miR-137 (downregulated); MiR-144 (downregulated); miR-145 (downregulated); miR-92a-3p (upregulated); miR-181b (upregulated).[195–200] Among miRNAs involved in cell migration and invasion, which were found abnormally expressed in UM, noteworthy are the following: miR-20a (upregulated); miR-155 (upregulated); miR-296-3p (downregulated); miR-454 (upregulated); miR-367 (upregulated); miR-21 (upregulated); miR-23a (downregulated); miR-224-5p (downregulated).[201–208] Additionally, miR-204 and miR-145 were found downregulated, whereas miR-20a, miR-17, miR-106a, miR-34a and miR-21 were found upregulated in UM samples.[209] Circulating levels of the following miRNAs were found dysregulated in metastatic UM: miR-125b, miR-20a, miR-146a, miR-181a, miR-155 and miR-223.[210] miRNAs are also involved in regulation of immune mediators, which can modulate UM behavior. For instance, IL-10 could have a role in promoting cancer as can

suppress immune response against the tumor. Specific microRNAs seem to be involved in IL-10 modulation.[211] miRNAs could also have a promising role for the development of therapeutic target. For instance, Genistein, an antitumor drug, was shown to inhibit miR-27a expression and, as a result, to increase ZBTB10 gene expression (miR-27a target gene). The antitumor action could be related to miR-27a regulatory mechanism.[212] Overall, all these studies give important insights on miRNAs involvement in UM development and progression as well as in UM patients' prognosis. However, some data are still conflicting, therefore there is an urgent need to clarify which miRNAs are effectively involved in UM development or in specific clinical-pathological features of patients.

3 Aim of the study

Epigenetic mechanisms responsible for the alteration of miRNA expression levels have been shown to have a relevant role in carcinogenic processes.[166,213] Research in this area sought to investigate clinical applications of miRNAs epigenetic alterations, with diagnostic and prognostic purposes, as well as for the identification of potential therapeutic target.[166,213] This ever-increasing interest, in association with the development of innovative high-throughput technologies, has led to the production of a huge amount of data on molecular mechanisms involved in cancer development and progression. Public electronic databases have been set up with the purpose of collecting molecular data on cancers, including uveal melanoma. However, this huge amount of data are often the results of multiple independent studies subjected to significant bias that make difficult the correct understanding of the molecular profile of tumors. In this context, International Consortia, such as The Cancer Genome Atlas (TCGA) [214] and ENCODE are collecting standardized bioinformatics data, however, their interpretation could be difficult in absence of specific bioinformatics tools.[215–217]

In recent years, several computational tools have been developed in order to facilitate the analysis of the so-called "Big Data", in a process named "data mining".[218]

By using both bioinformatics data and computational tools, researchers have tried to unveil the molecular mechanisms involved in tumor development, as well as to identify novel biomarkers or useful molecular targets for the management of tumors.[219]

As mentioned above, molecular data has been collected also for uveal melanoma, including genetic, proteomic and epigenetic data. Indeed, several bioinformatics studies have investigated the TCGA database aiming at identifying genes and miRNAs which could represent useful biomarkers in uveal melanoma.[220–222]

In particular, the research group of the Translational Oncology & Functional Genomics Laboratory of the University of Catania, where this Ph.D. project has been conducted, recently analyzed the TCGA database with the purpose of identifying which miRNAs were found dysregulated in UM according to tumor stage and patient survival.[220] The first step of this analysis involved the identification of putative miRNAs potentially implicated in the development and progression of UM by searching uveal melanoma microarray datasets containing the expression levels of miRNAs related to cancerous tissues.[220] The UM datasets containing the clinical-pathological features of patients and miRNA expression profiles were obtained by consulting the UCSC Xena Browser

(https://xenabrowser.net/). Noteworthy, the TCGA UVM database does not contain any healthy individuals, but only data obtained from UM patients. A total of 80 UM patient-related datasets were found on the TCGA portal and downloaded. These datasets included the expression levels of almost 2 thousand different mature miRNAs, but only those miRNAs with the expression data reported in at least 40 UM patients (50% of cases) were taken forward to further analyses.[220] As a result, a total of 795 mature miRNAs were interrogate for analysis purpose. Differential analyses were conducted according to two clinical variables; namely, tumor stage (T3-T4, considered as 'high-grade' versus T1-T2, considered as 'low-grade') and survival (dead vs alive).[220] The expression levels of a total of 38 miRNAs and 128 miRNAs were found significantly dysregulated in high grade tumors compared to low grade and in dead patients compared to alive, respectively. The findings of these computational analyses were eventually illustrated as a list of 20 dysregulated miRNAs (10 upregulated and 10 downregulated) according to tumor grade and a list of 20 dysregulated miRNAs (10 upregulated and 10 downregulated) according to survival, which are shown in Table 4 and Table 5, respectively.[220] As shown in the two tables, seven miRNAs were found dysregulated both in the tumor grade list and in the vital status list. Among these seven miRNAs, two were up-regulated in both lists, i.e. hsa-miR-592 and hsa-miR-199a-5p, while five miRNAs, hsa- miR- 508- 3p, hsa-miR-514a-3p hsa- miR- 509-3-5p, hsa-miR-513a-5p and hsa- miR- 513c- 5p were downregulated showing comparable expression levels in both groups. Through the use of miRCancerdb and mirDIP software, 53 genes were shown to correlate with the dysregulation of the top 20 tumor stage-related miRNAs, and a clear division pattern of correlation was found (Figure 2 and 3). Additionally, the molecular pathways modulated by these 20 miRNAs were identified through the DIANA-mirPath bioinformatics tools (Table 6).[220] Kaplan-Meier log-rank analyses were performed to assess prognostic significance of the 20 dysregulated mi-RNAs in high-grade vs low-grade UMs and 12 dysregulated mi-RNAs were found significantly associated with a worse prognosis (Figure 4): the hsa-miR-592, hsa-miR-452-5p, hsa-let-7b-3p, hsa-miR-224-5p, hsa-miR-199a-5p (log-rank test $P<0.01$) and hsa-let-7b-5p (log-rank test $P=0.0110$) were associated with unfavorable prognosis when up-regulated; the hsa-miR-514a-3p, hsa- miR- 508- 3p, hsa- miR- 513c- 5p, hsa-miR-513a-5p, hsa- miR- 509- 3- 5p (log-rank test $P<0.01$) and the hsa-miR-211-5p (log-rank test $P=0.0301$) were significantly associated with a lower survival when down-regulated.[220] These results demonstrated that the miRNAs identified might play a key role in the progression of uveal melanoma and that could represent novel

effective diagnostic and prognostic biomarkers for uveal melanoma patients.[220] However, these preliminary computational data need to be validated by *in vivo* studies.

Therefore, the aim of the present study was to assess whether the evaluation of the expression levels of the predicted up-regulated miRNA hsa-miR-199a-5p and of the predicted down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p could be useful to early diagnose UM and could give information about the aggressiveness of tumor and the prognosis of patients. For these purposes, liquid biopsy samples and Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples were obtained from UM patients and healthy donors in order to assess the circulating levels of the candidate biomarkers and the tissue expressions of the selected miRNAs, respectively. Due to the unavailability of normal choroid samples, FFPE normal tissues were obtained by separating the normal choroid from the neoplastic tissue. Finally, in order to corroborate the experimental results, a novel computational analysis for the three selected miRNAs was performed.

Table 4. *"Top 20 list of dysregulated miRNAs according to tumor stage". From Falzone et al 2019.*[220]

	miRNA	**FC High-Grade vs Low-Grade**	**p_Value**
up-regulated miRNAs	hsa-miR-1247-5p	2.548	2.38E-03
	hsa-miR-199a-5p	2.390	5.74E-03
	hsa-miR-767-5p	1.945	8.82E-03
	hsa-miR-210-3p	1.832	2.18E-03
	hsa-let-7b-3p	1.649	1.01E-03
	hsa-miR-592	1.644	7.09E-03
	hsa-miR-224-5p	1.624	6.47E-03
	hsa-miR-452-5p	1.569	8.59E-03
	hsa-miR-143-5p	1.546	5.79E-03
	hsa-let-7b-5p	1.477	5.83E-03
down-regulated miRNAs	hsa-miR-29c-3p	-1.746	4.95E-03
	hsa-miR-374b-5p	-1.746	1.86E-03
	hsa-miR-211-5p	-1.759	1.82E-03
	hsa-miR-507	-2.739	5.22E-03
	hsa-miR-513a-5p	-3.031	7.84E-03
	hsa-miR-509-5p	-3.774	1.80E-04
	hsa-miR-513c-5p	-3.926	3.33E-03
	hsa-miR-509-3-5p	-4.332	2.40E-03
	hsa-miR-508-3p	-4.376	9.44E-03
	hsa-miR-514a-3p	-4.538	5.14E-03

In bold dysregulated according to both tumor stage and vital status

Table 5. *"Top 20 list of dysregulated miRNAs according to tumor stage". From Falzone et al 2019.*[220]

	miRNA	**FC Dead vs Alive**	**p_Value**
up-regulated miRNAs	**hsa-miR-199°-5p**	3.080	1.18E-05
	hsa-miR-10b-5p	2.629	3.46E-05
	hsa-miR-199°-3p	2.629	3.45E-05
	hsa-miR-199b-3p	2.185	0.009078
	hsa-miR-155-5p	2.076	0.008769
	hsa-miR-212-3p	1.983	1.66E-05
	hsa-miR-142-5p	1.903	0.000399
	hsa-miR-708-5p	1.855	0.001027
	hsa-miR-887-3p	1.811	0.006292
	hsa-miR-592	1.776	0.001731
down-regulated miRNAs	hsa-miR-513b-5p	-4.446	1.58E-05
	hsa-miR-514°-5p	-4.753	1.83E-05
	hsa-miR-513°-5p	-6.703	4.1E-08
	hsa-miR-513c-5p	-7.937	1.66E-07
	hsa-miR-509-3-5p	-8.562	7.81E-08
	hsa-miR-506-3p	-9.124	1.78E-07
	hsa-miR-514°-3p	-14.126	8.95E-08
	hsa-miR-508-5p	-14.657	3.12E-08
	hsa-miR-509-3p	-19.826	2.13E-08
	hsa-miR-508-3p	-21.957	3.02E-08

In bold dysregulated according to both tumor stage and vital status

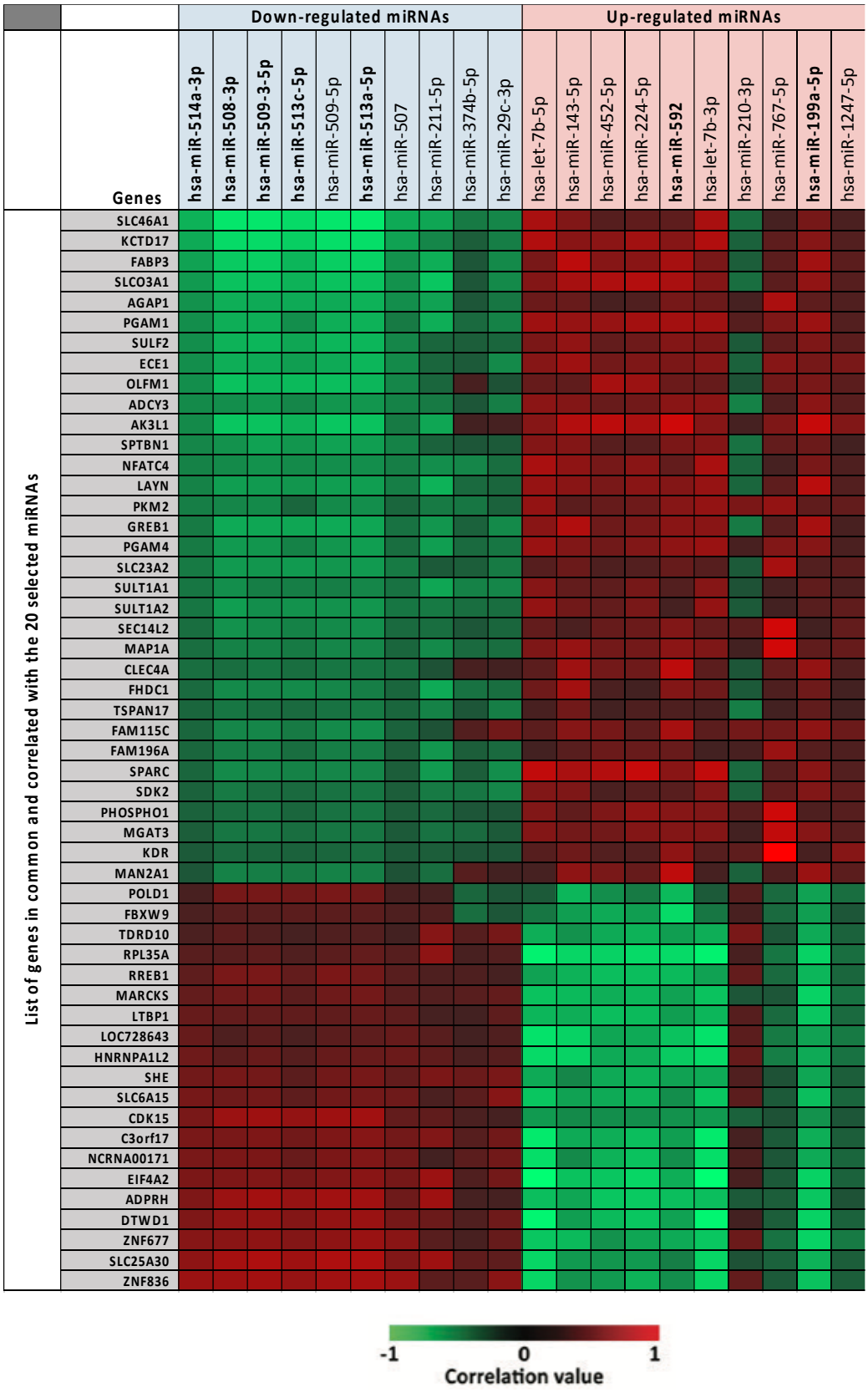

Figure 1. "miRCancerdb correlation analysis results. In bold are reported the miRNAs with prognostic significance for the definition of the overall survival of patients with uveal melanoma. The miRNAs in common between tumor stage and vital status stratification are marked with an asterisk. On the left side of the heatmap all the genes shared and correlated with all the 20 miRNAs are listed. The green

squares indicate negative correlations while the red squares positive correlations." From Falzone et al 2019.[220]

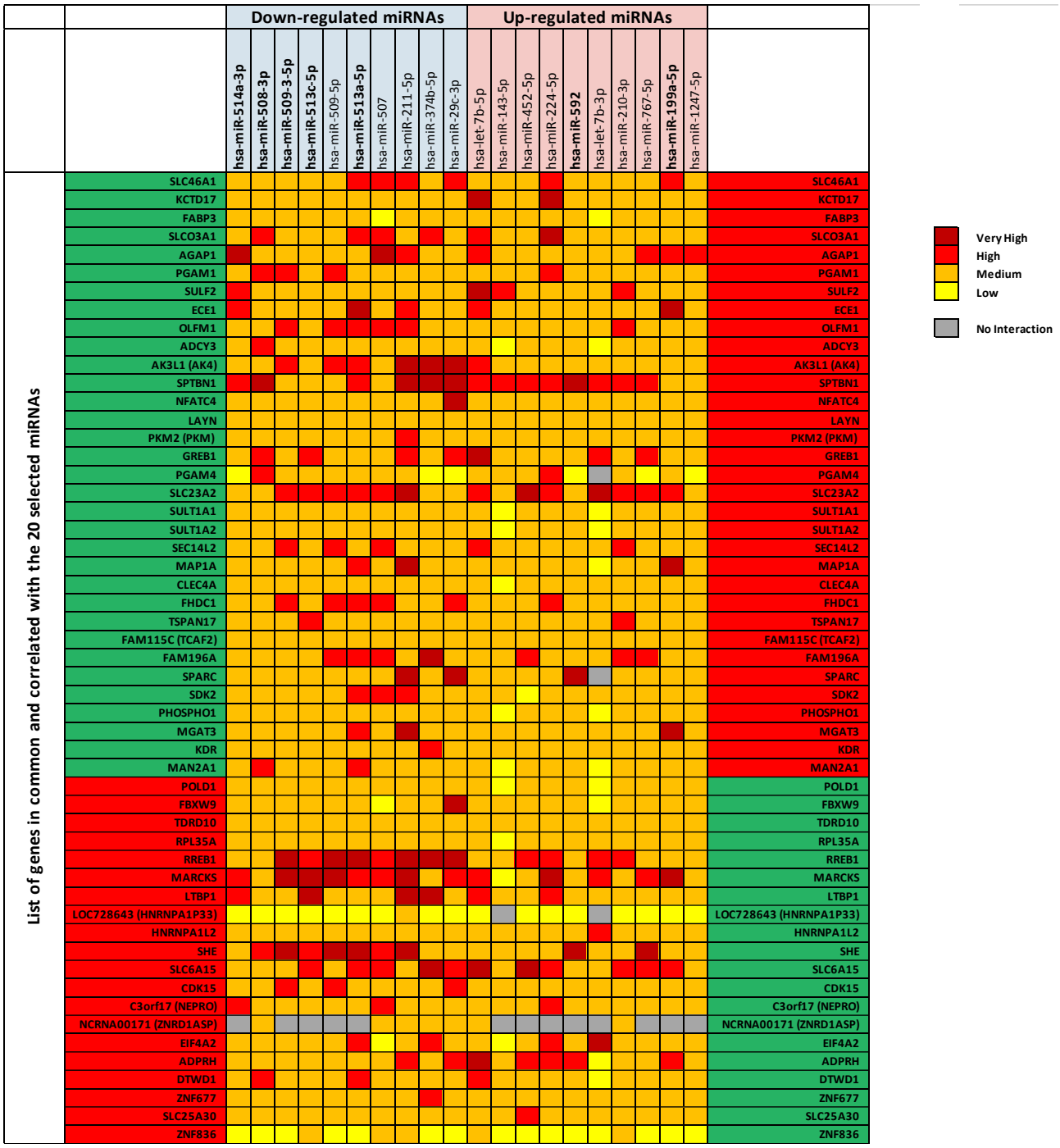

Figure 2. "mirDIP analysis of miRNA-gene interactions. Interaction between selected miRNAs and the 53 genes identified through miRCancerdb. For each miRNA is reported the level of interaction with the 53 genes positively (Red) and negatively (Green) correlated. The intensity of miRNA-gene interaction is highlighted with a color scale ranging from dark red (very high interaction) to yellow (low interaction). Gray boxes indicate no miRNA-gene interactions." From Falzone et al 2019.[220]

Table 6. "*Molecular pathways and number of genes modulated by the 20 dysregulated miRNAs in High-Grade UM". From Falzone et al 2019.*[220]

No.	KEGG pathway	p-value	No. Of genes	No. Of miRNAs
1	Proteoglycans in cancer (hsa05205)	7.25E-09	90	17
2	FoxO signaling pathway (hsa04068)	4.91E-05	65	17
3	Pathways in cancer (hsa05200)	2.03E-04	150	17
4	Adherens junction (hsa04520)	1.84E-08	42	17
5	PI3K-Akt signaling pathway (hsa04151)	4.05E-02	120	17
6	Viral carcinogenesis (hsa05203)	8.36E-11	89	16
7	Chronic myeloid leukemia (hsa05220)	5.00E-05	39	16
8	Glioma (hsa05214)	8.05E-05	32	16
9	TGF-beta signaling pathway (hsa04350)	2.03E-04	39	16
10	Prostate cancer (hsa05215)	7.38E-04	44	16
11	Hippo signaling pathway (hsa04390)	3.24E-10	70	15
12	Cell cycle (hsa04110)	6.49E-10	68	15
13	Endometrial cancer (hsa05213)	3.50E-03	25	15
14	HIF-1 signaling pathway (hsa04066)	1.85E-02	44	15
15	Bladder cancer (hsa05219)	3.14E-02	20	15
16	Non-small cell lung cancer (hsa05223)	3.53E-02	24	15
17	Melanoma (hsa05218)	4.39E-02	29	15
18	Renal cell carcinoma (hsa05211)	1.46E-06	35	14
19	p53 signaling pathway (hsa04115)	5.19E-05	38	14
20	Central carbon metabolism in cancer (hsa05230)	3.67E-03	28	14
21	Small cell lung cancer (hsa05222)	7.16E-05	45	13
22	Pancreatic cancer (hsa05212)	5.12E-04	35	13
23	Thyroid cancer (hsa05216)	9.72E-03	14	13
24	Transcriptional misregulation in cancer (hsa05202)	3.14E-02	66	13
25	Colorectal cancer (hsa05210)	3.93E-05	34	12

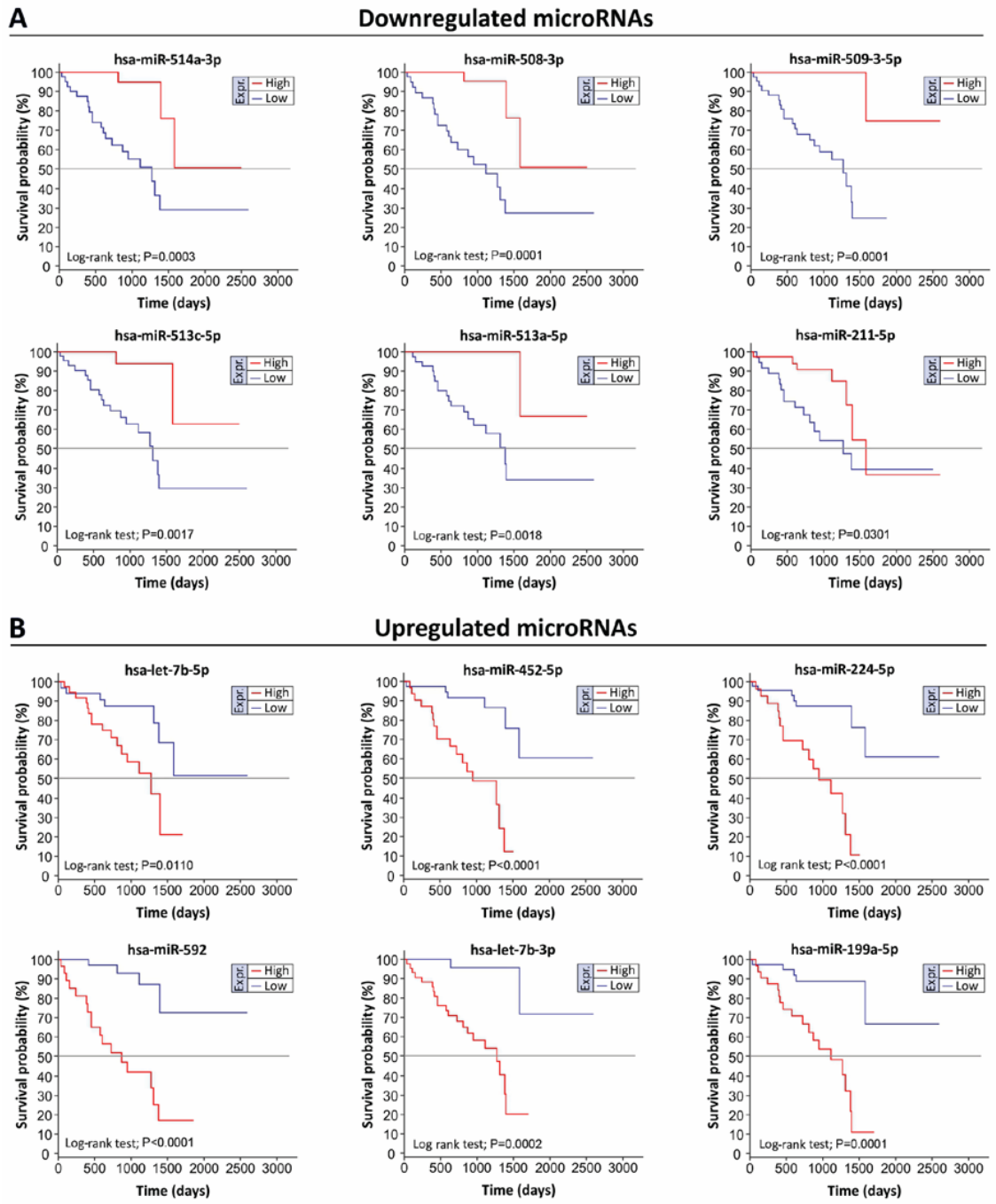

Figure 3. "Prognostic significance of High-Grade UM dysregulated miRNAs. (A) OS of patients with uveal melanoma according to the expression levels of the 10 tumor stage down-regulated miRNAs; (B) OS of uveal melanoma patients according the expression levels of the 10 up-regulated miRNAs. Only Kaplan-Meier curves with a log-rank test value of P<0.05 were reported." From Falzone et al 2019.[220]

4 Materials and Methods

4.1 Study design

The present observational study was conducted from November 2017 to August 2020 at the Department of Biomedical and Biotechnological Sciences of the University of Catania -Translational Oncology & Functional Genomics Laboratory- and at the Eye Clinic of the University of Catania. The study protocol was approved by the Institutional Review Board and conformed to the tenets of the Declaration of Helsinki. Participants were fully informed on the study protocol and an informed consent was signed. All consecutive patients referred to the Ocular Oncology service of the Eye Clinic of the University of Catania and newly-diagnosed with uveal melanoma were assessed for eligibility. Diagnosis of uveal melanoma was carried out by a senior ophthalmologist with expertise in ocular oncology, and was based on a complete eye examination, including best corrected visual acuity measurement, slit-lamp exam, Goldmann applanation tonometry, dilated fundus examination, as well as ancillary tests, including A-scan and B-scan ocular ultrasound, fluorescein angiography and indocyanine green angiography, optical coherence tomography. Inclusion criteria included as follows: a) new diagnosis of uveal melanoma; b) age $\geq$ 18 years. Patients with history of previous/concomitant cancers affecting other sites, and/or history of chronic inflammatory diseases were excluded. At time of UM diagnosis, from each enrolled patient one peripheral blood sample was obtained during routine blood test.

Healthy controls were enrolled among patients referred to Eye Clinic of the University of Catania for routine cataract and/or ocular adnexa surgery and a blood sample was obtained during routine blood test.

Blood samples obtained from 30 UM patients and 10 healthy donors were centrifuged at 2000 g for 10 minutes at room temperature in order to separate serum and from blood clot. About 2 mL of serum (divided into two aliquots of 1 mL) were obtained from each subject.

Clinical management of enrolled patients was not affected by the study protocol. As per clinical practice, patients with diagnosis of uveal melanoma received a CT-based imaging work-up to rule out metastases. Primary tumor treatment could be either enucleation surgery or radiotherapy (plaque brachytherapy or proton beam radiotherapy).

The following clinical data were collected from each UM patient: demographic data (age, gender), tumor location (choroid, iris, ciliary body), baseline ultrasound tumor size

(thickness, longitudinal and transverse diameters), AJCC TNM stage, type of treatment.
Eleven UMs and three normal choroid FFPE tissue samples were obtained from the bio-bank of the Pathology Section of the University of Catania and used to validate the expression levels of the selected miRNAs in tissue samples. Due to the unavailability of normal choroid samples, FFPE normal tissues were obtained by separating the normal choroid from the neoplastic tissue.
The primary outcome of the study was to evaluate whether the expression levels of circulating miRNAs, including hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p, were found dysregulated in patients with new diagnosis of UM, compared to healthy controls. Secondary outcome measures included the tissue expressions of the selected miRNAs in FFPE in UM samples compared to normal adjacent choroid tissue and bioinformatics analyses for the three selected miRNAs.

4.2 RNA extraction, miRNAs retro-transcription and ddPCR analysis

The circulating total RNA, including miRNAs, was extracted from serum samples obtained from both UM patients and healthy donors. Briefly, serum samples were centrifuged at 2000 g x 10 minutes at room temperature in order to pool down debris and protein aggregates. Then 200 µL of serum were extracted by using the miRNeasy Serum/Plasma Kit (Qiagen – Cat. N. 217184) by adding the spike-in control mix used as exogenous control (Qiagen – Cat. N. 339390).
As regards FFPE tissues, total RNA, including miRNAs, was extracted by using the the miRNeasy FFPE kit (Qiagen – Cat. N. 217504) and following the manufacturer instruction. Briefly, RNA was extracted from two FFPE tissue sections of 10 µm thickness by using specific deparaffination and extraction solutions.
After RNA extraction, 2 µL of total RNA, including miRNAs, were retro-transcribed into cDNA by using the miRCURY LNA RT Kit (Qiagen – Cat. N. 339340).
Finally, the cDNA obtained from liquid biopsy samples was analyzed as it while the cDNA obtained from FFPE samples was diluted 1:50 in RNase-free water and then analyzed by using the miRCURY LNA miRNA PCR Assays (x200), i.e. specific primers for the three selected miRNAs and for the Unisp4 spike-in control (Qiagen - Cat. N. 339306). The miRNAs expression levels of FFPE tissue samples were normalized by using a miRCURY LNA miRNA PCR Assay (x200) specific for the snRNA U6 used as housekeeping non-coding RNA in tissue samples. For the analysis of miRNA expression

levels, a custom droplet digital PCR (ddPCR) protocol was used. Briefly, a 22 µL ddPCR reaction mix was obtained by mixing 11 µL of 2x QX200TM ddPCRTM EvaGreen® Supermix (Cat. N. 1864034 – Bio-Rad, Hercules, California, USA), 1.1 µL of miRNA specific target probe (Thermo Fisher Scientific – A25576), 4.9 µL of molecular biology-grade water and 5 µL of cDNA in order to obtain a final volume of 22 µL. Subsequently, twenty microliters of the reaction mix were processed by using the QX200 droplet generator (Bio-Rad, Hercules, California, USA) to obtain droplet nanopartitions. After transferring and sealing the obtained droplets into a 96-well plate, the plate was amplified in a C1000 Thermal Cycler (Bio-Rad, Hercules, California, USA) with the following thermal conditions: Polymerase activation at 95°C for 10 minutes, 40 cycles of amplification at 94°C for 30 seconds (denaturation) and 60°C for 1 minute (annealing), droplets stabilization at 98°C for 10 minutes followed by an infinite hold at 4°C. A ramp rate of 1.6 °C/s was used among the steps of the amplification. Following amplification process, negative and positive droplets were read in the QX200 Droplet Reader (Bio-Rad, Hercules, California, USA). All experiments were validated in triplicate.

4.3 Bioinformatics analyses

In order to better clarify the involvement of hsa-miR199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p in UM development and aggressiveness, different computational approaches have been used. Firstly, the targeted genes of the three validated miRNAs were identified using the bioinformatics tool miRWalk (http://mirwalk.umm.uni-heidelberg.de).[223] In particular, miRWalk is able to identify the genes targeted by three miRNAs by analyzing interaction data contained in 14 different resources for miRNA-target data, including miRBase, TargetScan, KEGG Pathway, etc.

For the targeted genes identified by miRWalk, the protein-protein interaction (PPI) and the biological and molecular functions were assessed by using, respectively, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and GO PANTHER software.[224,225] In addition, miRCancerdb tool was used to establish if the three miRNAs are negatively or positively correlated with the expression levels of the genes identified through miRWalk. In particular, miRCancerdb analyzes the data contained in The Cancer Genome Atlas Uveal Melanoma (TCGA UVM) database.[226]

Finally, the clinical implication of the three miRNAs was assessed by analyzing the clinical data contained in TCGA UVM database and downloaded by using the online exploration tool UCSC Xena Browser.[227]

4.4 Statistical Analyses

For the absolute quantification of miRNAs expression in serum samples and FFPE tissues obtained from UM patients and healthy individuals, the QuantaSoft software was used (Bio-Rad, Hercules, California, USA). Kolmogorov-Smirnov normality test was performed to assess the distribution of hsa-miR199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p expression levels observed with ddPCR. The same normality test was used to assess the distribution of the expression levels of these miRNAs deposited on the TCGA UVM database. Statistical differences between cases and controls were evaluated by using unpaired Student t-test. Receiver Operating Characteristic (ROC) curves were obtained by using GraphPad Prism v.6 in order to evaluate the specificity and sensitivity of the analyzed miRNAs.

Kruskal-Wallis test (and post-hoc Dunn's Multiple Comparison test) and One-way ANOVA test (and post-hoc Dunnett's Multiple Comparison test) were used for assessing the statistical differences existing between the expression levels of hsa-miR199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p in the samples analyzed and for the data reported in the TCGA UVM database by taking into account UM tumor stages. All statistical analyses were performed by using GraphPad Prism v.6.

5 Results

5.1 Characteristics of UM patients

The first part of the study was performed on liquid biopsy samples obtained from 30 uveal melanoma patients and ten healthy donors used as controls. The socio-demographic and clinical-pathological features of the 30 UM patients enrolled in the study were reported in Table 7 and 8, respectively.

Table 7*. Baseline characteristics of the 30 selected uveal melanoma patients*

Patients, n	**Female, n (%)**	**Age**	**Location, n**	**Tumor size, mm**		
				Depth.	**Height**	**Width**
Overall population						
n, 30	12 (40%)	59.5 (16)*	choroid, 26 choroid&CB, 2 iris&CB, 2	7.62 (4.21)*	12.51 (3.82)*	12.29 (3.87)*
Patients undergoing proton beam radiotherapy						
n, 17	7 (41%)	61.5 (13)*	choroid, 15 iris&CB, 2	5.09 (2.27)*	10.72 (3.15)*	10.35 (3.07)*
Patients undergoing R-106 brachytherapy						
n, 2	2 (100%)	51.5 (2.1)*	choroid, 2	5.13 (1.03)*	10.53 (2.58)*	10.7 (1.97)*
Patients undergoing enucleation						
n, 11	3 (27%)	57.5 (21.6)*	choroid, 9 choroid&CB, 2	12.43 (2.56)*	15.95 (2.63)*	15.9 (2.67)*

*= mean (standard deviation); CB= ciliary body

Table 8. *TNM classification and anatomic stage according to AJCC 8th edition*

Patients, n (%)	**T**	**N**	**M**	**Anatomic Stage**
4, (13%)	T1a	N0	M0	I
10, (33%)	T2a	N0	M0	IIa
9, (30%)	T3a	N0	M0	IIb
2, (7%)	T3b	N0	M0	IIIa
4, (13%)	T4a	N0	M0	IIIa
1, (3%)	T4b	N0	M0	IIIb

For the second part of the study, 11 FFPE UM tissues and three FFPE normal adjacent choroid tissues were used. The clinical-pathological features of the 11 UM FFPE tissue included in the study were reported in Table 9.

Table 9. *Clinical and histological characteristics of 11 enucleated eyes*

TNM	ON infiltration	Scleral infiltration	Necrosis	Infiltrating lymphocyte	Histology
T3a, 4 T3b, 2 T4a, 4 T4b, 1	1 out of 11	1 out of 11	3 out of 11	3 out of 11	Spindle, 6 Epithelioid, 2 Mixed, 3

ON: optic nerve; TNM: Tumor, Node, Metastasis classification according to AJCC 8th edition.

5.2 Analysis of miRNA circulating levels in liquid biopsy samples of UM patients and healthy donors

In the present study, a pilot case series of 40 individuals, of which 30 were UM patients and ten were healthy controls, was analyzed. The clinical validation of the diagnostic potential of UM-related miRNAs previously identified[220] was performed on liquid biopsy samples collected and stored as described in Materials and Methods section. In particular, one up-regulated miRNAs, hsa-miR-199a-5p, and two down-regulated miRNAs, hsa-miR-508-3p and hsa-miR-514a-3p were analyzed by using the innovative high-sensitive ddPCR amplification system. Although performed in a low number of samples (30 tumor samples and ten normal controls), statistically significant results were obtained by analyzing the absolute quantitative of these miRNAs.

In particular, positive signals were obtained only for one out of the three selected miRNAs, the hsa-miR-199a-5p, thus allowing its absolute quantification in both tumor and normal samples (Figure 5).

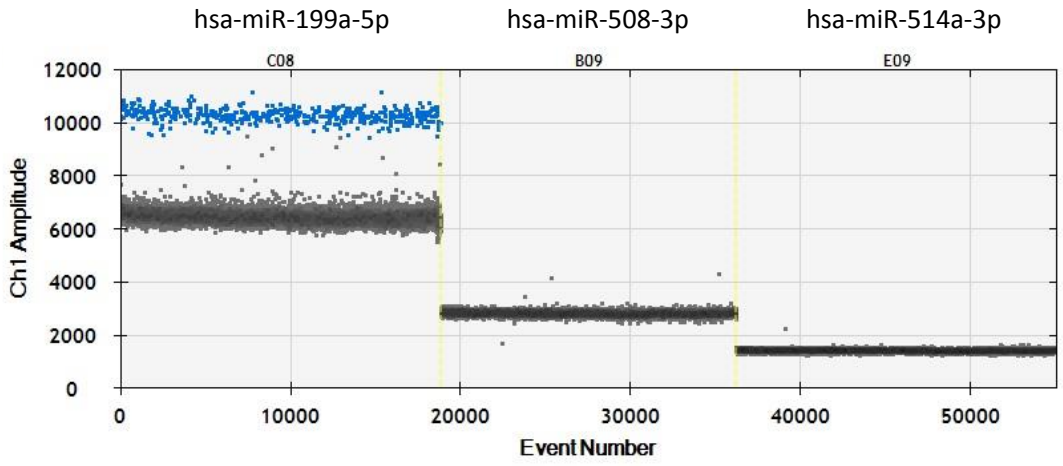

Figure 4. Representative picture of ddPCR amplification signals of circulating miRNAs. Positive signals were obtained only for the predicted up-regulated hsa-miR-199a-5p**.**

This first result has demonstrated that hsa-miR-508-3p and hsa-miR-514a-3p are not secreted in the extracellular space and, therefore, they cannot be detected in liquid biopsy samples.

By analyzing the expression levels of hsa-miR-199a-5p in UM serum samples and healthy controls liquid biopsy samples, a statistical difference of expression was observed ($p=0.0235$; Figure 6).

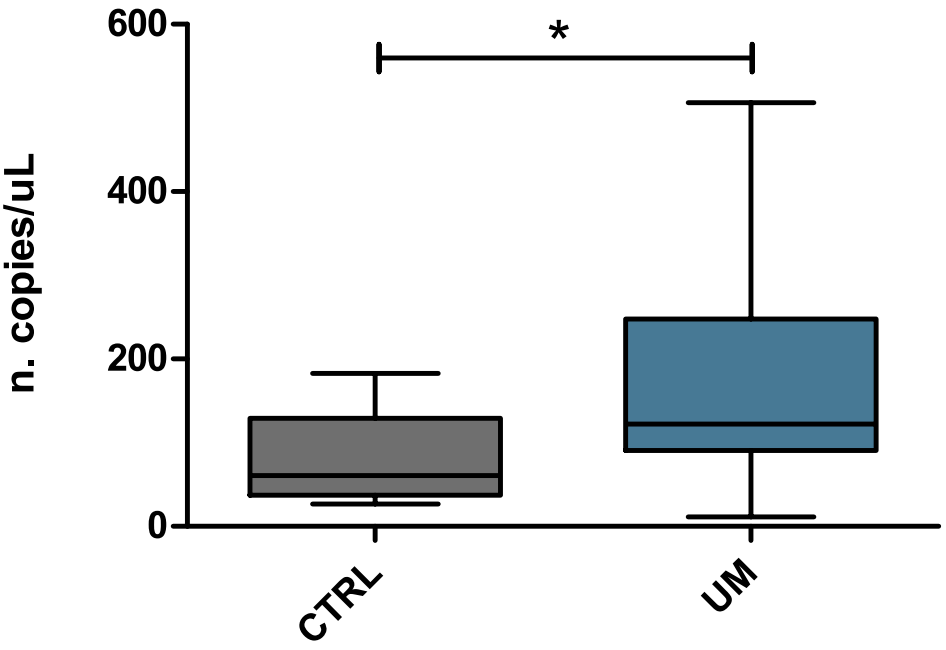

Figure 5. Serum levels of hsa-miR-199a-5p in UM patients vs healthy controls. Mann Whitney test * $p<0.05$.

As already mentioned, no signals were obtained for the circulating levels of hsa-miR-508-3p and hsa-miR-514a-3p, therefore, for these miRNAs it was not possible to evaluate any difference between UM patients and healthy controls.

5.3 Diagnostic role of hsa-miR-199a-5p in Uveal Melanoma

In order to assess the diagnostic significance of hsa-miR-199a-5p circulating levels in UM, Receiver Operating Characteristics (ROC) curve was calculated. As shown in Figure 7, ROC analysis allowed us to establish the sensibility and specificity rates of hsa-miR-199a-5p in correctly diagnose UM.

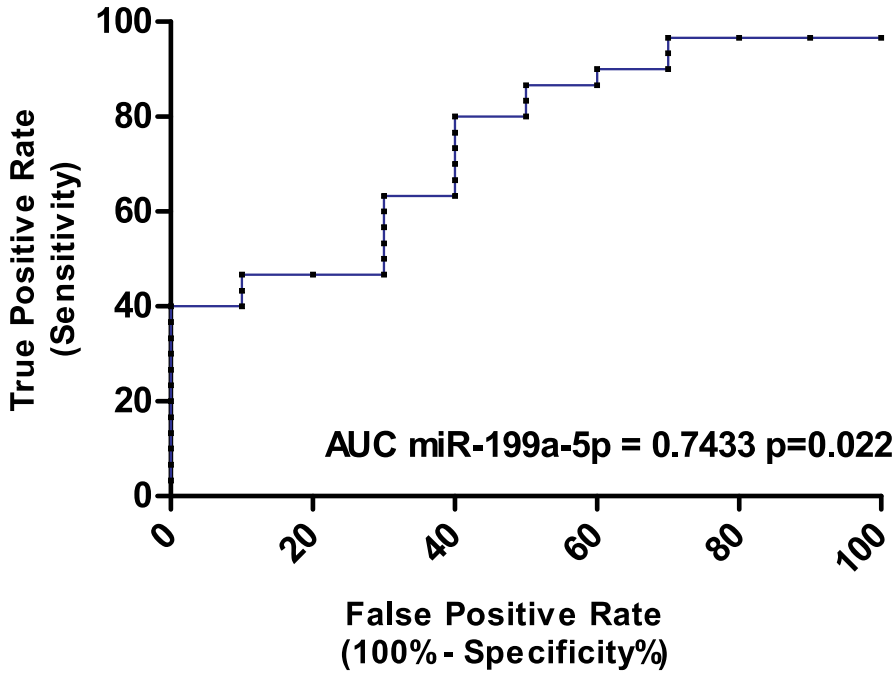

Figure 6. ROC analysis confirmed the high diagnostic value of hsa-miR-199a-5p. The observed sensitivity and specificity rates were 63.33% and 70%, respectively, with an Area Under the Curve of 0.7433.

In particular, ROC analysis revealed that a diagnostic test based on the evaluation of hsa-miR-199a-5p circulating levels would have a moderate accuracy (AUC=0.7433), however, although statistically significant (p=0.022), these preliminary data showed that a hypothetical diagnostic test for the evaluation of a single miRNA would have a limited specificity and sensitivity (63.33% and 70%, respectively).

5.4 Analysis of miRNA expression levels in FFPE UM samples and normal choroid tissues

The analysis of the circulating levels of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p revealed that these latter two miRNAs are not secreted by the cells and cannot be detected in the bloodstream or other fluids. Therefore, in order to establish if the predicted hsa-miR-508-3p and hsa-miR-514a-3p miRNAs are down-regulated in UM, FFPE UM and normal adjacent choroid tissue samples obtained from UM patients were analyzed. The ddPCR analysis thus performed further confirmed the up-regulation of hsa-miR-199a-5p in UM samples compared to normal choroid ($p<0.022$). As regards the down-regulated miRNAs, the ddPCR analysis revealed that both hsa-miR-508-3p and hsa-miR-514a-3p were significantly down-regulated in UM samples compared to normal adjacent choroid ($p<0.0127$; Figure 8).

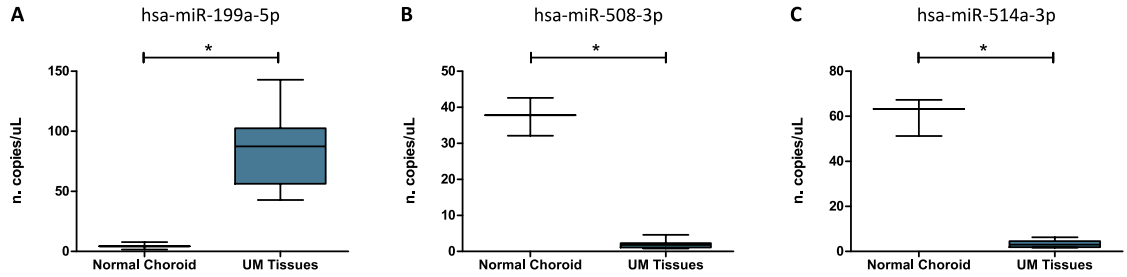

Figure 7. Panel A) Tissue levels of hsa-miR-199a-5p in FFPE UM samples compared to normal adjacent choroid tissues; Panel B) Tissue levels of hsa-miR-508-3p in FFPE UM samples compared to normal adjacent choroid tissues; Panel C) Tissue levels of hsa-miR-514a-3p in FFPE UM samples compared to normal adjacent choroid tissues. Paired Student's t-test was used for the analysis of hsa-miR-199a-5p tissue levels, while Mann Whitney test was used for the analysis of hsa-miR-508-3p and hsa-miR-514a-3p tissue expression levels. * $p<0.05$.

For the hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p no ROC analyses were performed due to the low number of samples analyzed. However, the data showed in Figure 8 confirm our previous bioinformatics findings. Indeed, also the evaluation of hsa-miR-508-3p and hsa-miR-514a-3p expression levels observed in tissue samples may give useful information to the clinicians to better diagnose UM and predict the prognosis of patients.

5.5 Functional role of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p in Uveal Melanoma

The bioinformatics analyses performed for the up-regulated miRNA hsa-miR-199a-5p and the two down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514aa-3p revealed

the strict clinical implication of these miRNAs in the pathogenesis and aggressiveness of UM.

First, miRWalk analysis allowed the identification of the genes targeted by hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p. In particular, the analysis revealed that a total of 1,653 univocal genes are targeted by hsa-miR-199a-5p, a total of 1,214 univocal genes are targeted by hsa-miR-508-3p while 523 univocal genes are targeted by hsa-miR-514a-3p. The analyses revealed also that some of these genes are targeted in different gene positions, suggesting that for some genes the regulatory action of miRNAs is stronger (data not shown). By merging the lists of targeted genes obtained for the three dysregulated miRNAs in UM, a panel of 20 univocal genes targeted by all miRNAs was identified. miRWalk analysis revealed that hsa-miR-199a-5p and hsa-miR-508-3p shared the highest number of targeted genes (175 gene targets), while only 77 and 53 genes were shared between hsa-miR-199a-5p/hsa-miR-514a-3p and hsa-miR508-3p/hsa-miR-514a-3p, respectively (Figure 9).

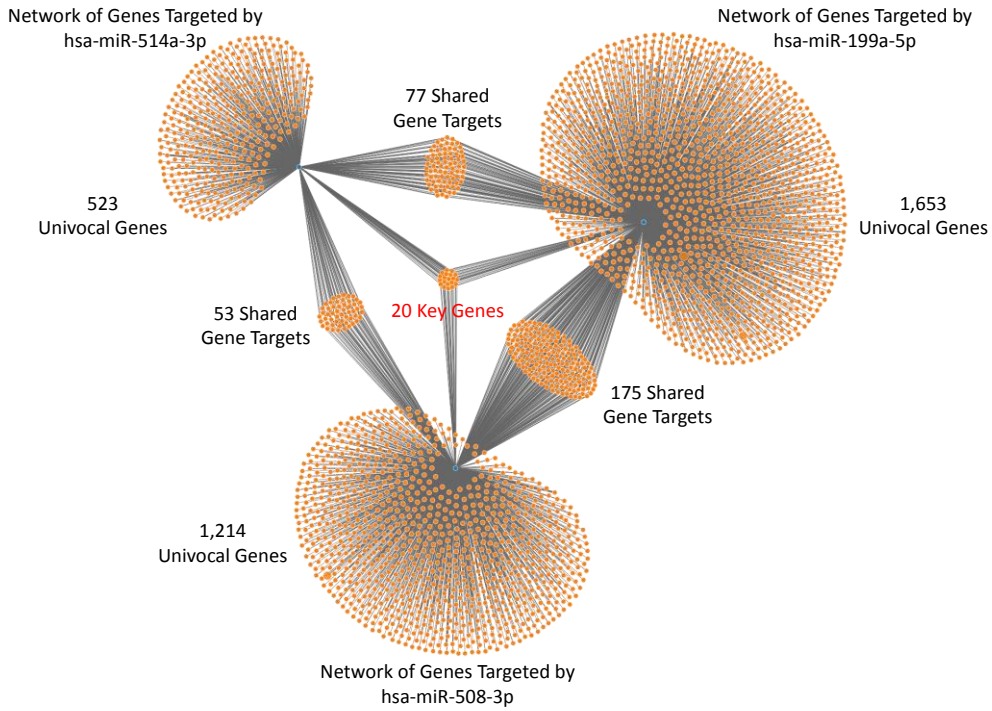

Figure 8. Network of genes targeted individually or in common by hsa-miR-199a-5p, hsa-miR508-3p and hsa-miR-514a-3p.

For the further STRING and GO PANTHER analyses only the 20 genes targeted by all miRNAs and the 53 targeted genes shared by the down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p were taken into account. The genes shared by both down-

regulated and up-regulated (hsa-miR-199a-5p) miRNAs were excluded because of the opposite action of these miRNAs in gene regulation that could generate confusing data. As regards the 20 genes targeted by the three miRNAs, STRING analysis revealed that only 14 genes were interconnected each other. Of these, MAPT gene, already associated with the development of brain tumor,[228] seems to have the highest number of interactions with the other genes. Another hub gene identified is PRKAA2 previously reported as a gene involved in melanoma development.[229] (Figure 10).

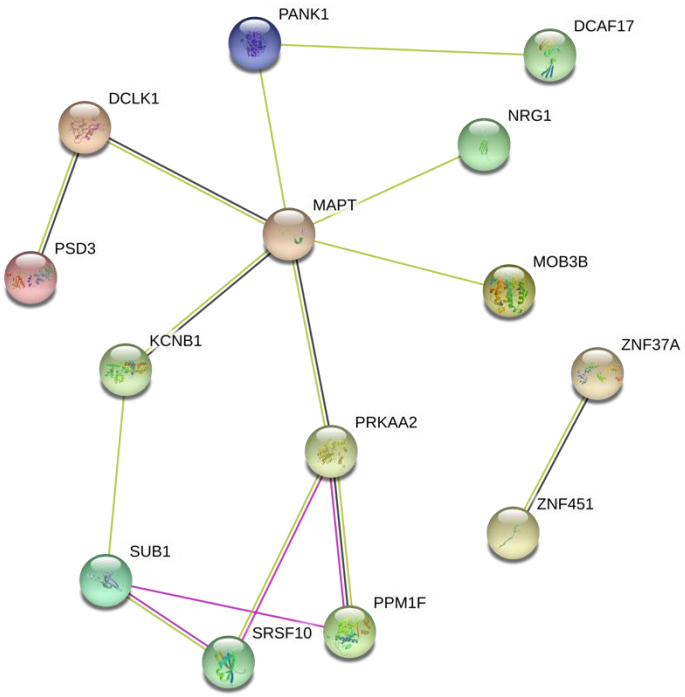

Figure 9. STRING protein interaction network of 14 out of 20 genes targeted by hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p.

In the same manner, the STRING analysis performed for the 53 genes targeted by both hsa-miR-508-3p and hsa-miR-514a-3p showed a more complex interaction network where hub genes were represented by MAPK14, HIST2H3PS2, UBE2G1, TANC2, HSPA14, TP63, and other genes already associated with the development and metastases of UM and other tumors.[230–233] In particular, 43 out of the 53 selected genes were able to establish interconnection each other (Figure 11).

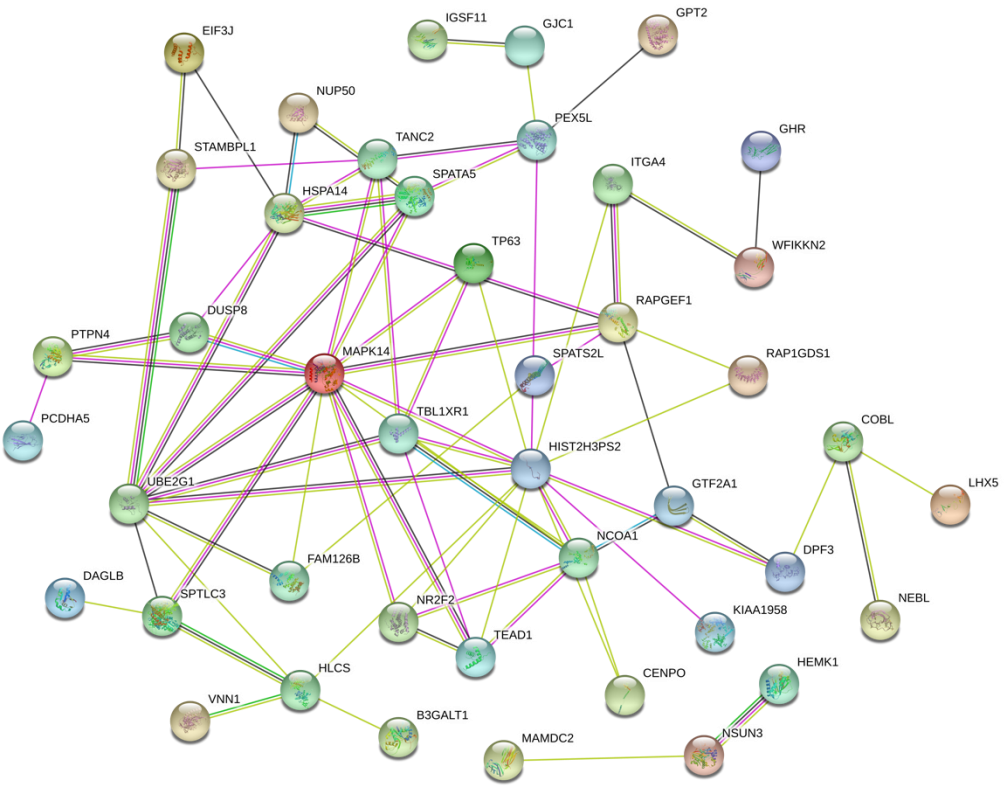

Figure 10. STRING protein interaction network of 43 out of 53 genes targeted by hsa-miR-508-3p and hsa-miR-514a-3p.

As regards the functional and molecular functions of the 20 genes targeted by all miRNAs, the GO PANTHER analysis showed that the majority of these genes are involved in binding (5 genes) and catalytic (5 genes) activities (Figure 12A); within the category "Biological process", the majority of genes were involved in the regulation of cellular processes (9 genes), metabolic processes (7 genes) and biological regulation (7 genes) (Figure 12B). In addition, GO PANTHER analysis gave information about the pathways altered by these genes (3 genes) that are involved in Coenzyme A biosynthesis (P02736), EGF receptor signaling pathway (P00018) and p53 pathway by glucose deprivation (P04397) (Figure 12C).

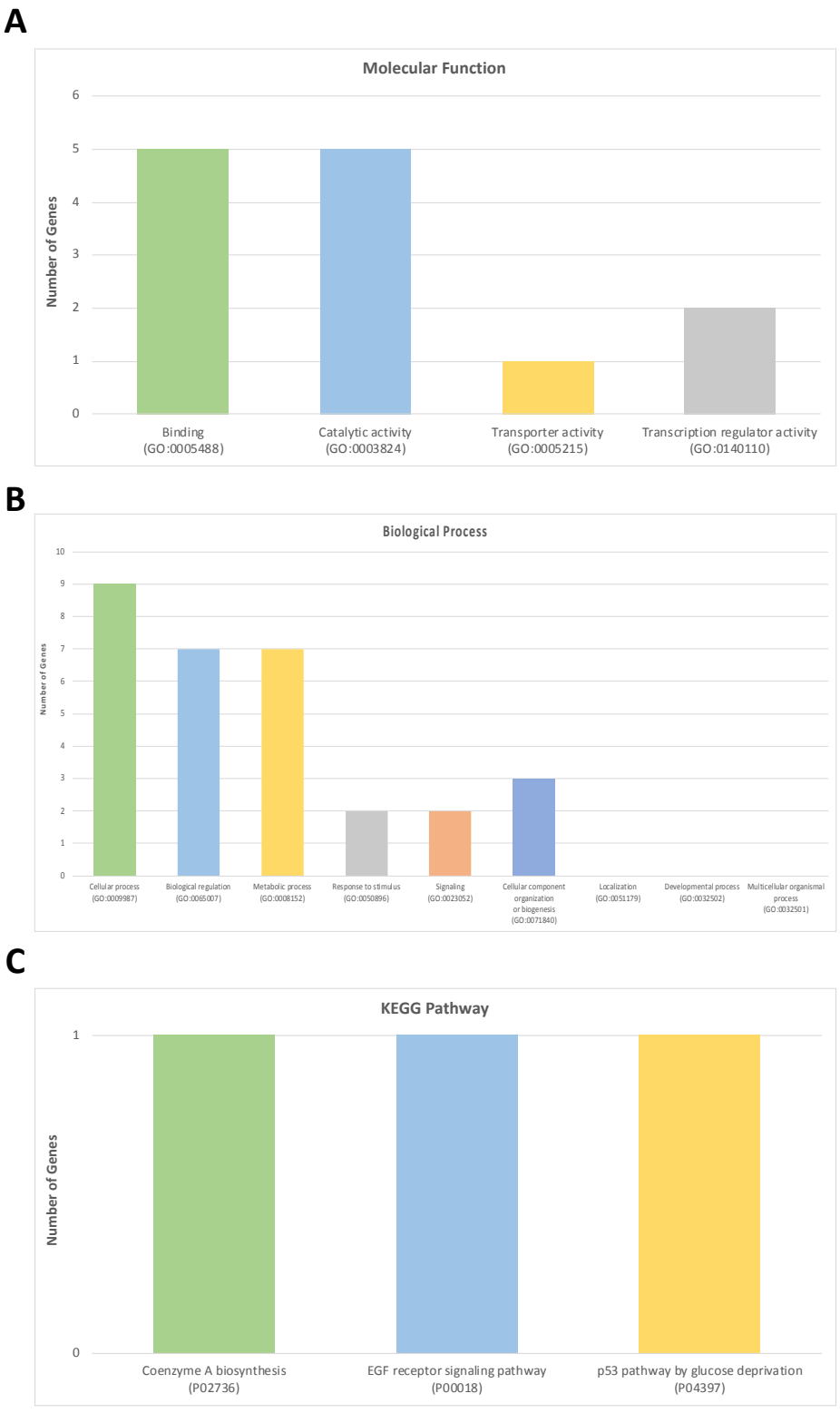

Figure 11. GO PANTHER analysis of the 20 genes targeted by all miRNAs. Panel A) Molecular Function of miRNA-targeted genes; Panel B) Biological Process of miRNA-targeted genes; Panel C) KEGG Pathways where the of miRNA-targeted genes were involved.

In the same manner, the 53 genes targeted by the down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p were mainly involved in binding (13 genes), catalytic activity

(9 genes) and transcription regulator activity (8 genes) as regards their "Molecular Function" (Figure 13A). Regarding the "Biological Process", the genes identified are involved in cellular and metabolic processes (22 and 18 genes, respectively) as well as in biological regulation (12 genes) (Figure 13B). The selected genes were also involved in the following pathways: Integrin signaling pathway (P00034) (2 genes), Oxidative stress response (P00046) (2 genes), Wnt signaling pathway (P00057) (2 genes) and p53 pathway feedback loops 2 (P04398) (2 genes) (Figure 13C). The genes were also involved in other minor pathways (data not shown).

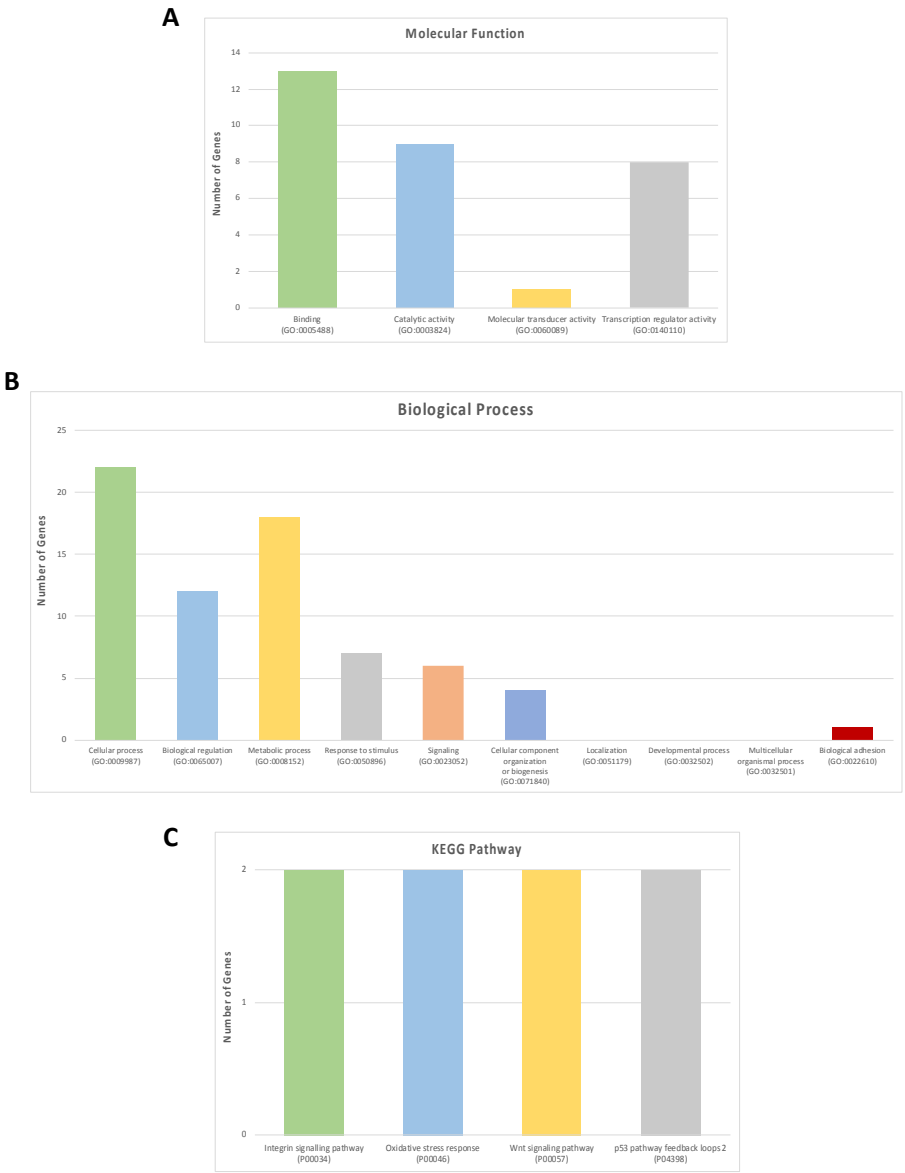

Figure 12. GO PANTHER analysis of the 53 genes targeted by the two down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p. Panel A) Molecular Function of miRNA-targeted genes; Panel B) Biological Process of miRNA-targeted genes; Panel C) KEGG Pathways where the of miRNA-targeted genes were involved.

Subsequently, to establish if the expression levels of the three miRNAs correlate positively or negatively with the expression levels of the genes identified through miRWalk, miRCancerdb analysis was performed. As regards the 20 genes targeted by all miRNAs, miRCancerdb analysis showed correlation levels only for the 50% of these genes (ten genes). As shown in Table 10, opposite correlation values were observed for the hsa-miR-199a-5p up-regulated miRNA compared to the two down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p. Among the ten genes showing significant correlation levels, SEC22C was the gene with the highest correlation values, suggesting that the expression levels of this genes may significantly depend on the expression of these altered miRNAs (Table X). In addition, other studies have demonstrated that SEC22C is dysregulated in UM due to the aberrant expression of miRNAs.[234]

Table 10. *Correlation levels of genes targeted by hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p according to TCGA UVM data*.

Gene	miRCancerdb Correlation		
	hsa-miR-199a-5p	hsa-miR-508-3p	hsa-miR-514a-3p
SEC22C	-0.48	0.47	0.34
PSD3	-0.3	0.15	0.15
PANK1	-0.21	/	/
PPM1F	-0.14	/	/
PRKAA2	-0.13	-0.15	-0.11
ZNF37A	0.15	/	/
SLC26A7	0.2	-0.15	/
MAPT	0.24	-0.32	-0.24
ZNF451	0.32	-0.22	-0.17
SUB1	0.34	-0.2	/

In the same manner, the miRCancerdb analysis performed on the 53 genes targeted by both hsa-miR-508-3p and hsa-miR-514a-3p showed that the expression of both miRNAs correlates positively with seven genes and negatively with ten genes. Noteworthy, the correlation levels in the 17 genes identified (17 out of 53 genes) were concordant between the two miRNAs, suggesting that both miRNAs cooperate in the regulation of such genes (Table 11).

Table 11. *Correlation levels of genes targeted by hsa-miR-508-3p and hsa-miR-514a-3p according to TCGA UVM data.*

Gene	miRCancerdb Correlation	
	hsa-miR-508-3p	hsa-miR-514a-3p
ZHX1	0.37	0.32
SHE	0.3	0.28
HEMK1	0.23	0.13
MAPK14	0.21	0.32
IGSF11	0.16	0.17
PALM2	0.15	0.38
PTPN4	0.15	0.18
FAM126B	-0.13	-0.11
RAPGEF1	-0.17	-0.15
CENPO	-0.18	-0.16
ITGA4	-0.18	-0.13
COBL	-0.21	-0.16
NR2F2	-0.22	-0.12
RAP1GDS1	-0.26	-0.14
DAGLB	-0.27	-0.24
GJC1	-0.3	-0.22
HLCS	-0.33	-0.25

Finally, the analysis of miRNAs expression levels according to the clinical-pathological data contained in the TCGA UVM database revealed that the expression levels of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p change significantly in the group of patients with more advanced tumors. Of note, TCGA UVM database contains molecular data of Stage II; III and IV UM patients, therefore, the expression levels of the selected miRNAs were stratified according to these three tumor stages. Overall, the expression levels of hsa-miR-199a-5p increase significantly according to tumor stage ($p=0.0057$). In particular, the post-hoc Dunn's Multiple Comparison test revealed that the expression levels of hsa-miR-199a-5p increase significantly between Stage II UM patients and Stage III ($p<0.05$) and Stage IV ($p<0.05$) UM patients (Figure 14A). Significant results were obtained also by analyzing the expression levels of hsa-miR-508-3p and hsa-miR-514a-3p. The analysis revealed the strong down-regulation of these two miRNAs in the most advanced tumors. Regarding hsa-miR-508-3p, the Kruskal-Wallis test showed high statistical significance ($p=0.0018$). In particular, the expression levels of hsa-miR-508-3p decrease mostly between Stage II and Stage IV UM patients ($p<0.01$) and with a lesser extent between Stage III and Stage IV UM patients ($p<0.05$) (Figure

14B). Similar results were obtained for hsa-miR-514a-3p, whose expression levels decrease significantly according to tumor stage (p=0.0038). However, the post-hoc Dunn's Multiple Comparison test showed statistical differences only between Stage II and Stage IV UM patients (p<0.05) (Figure 14C).

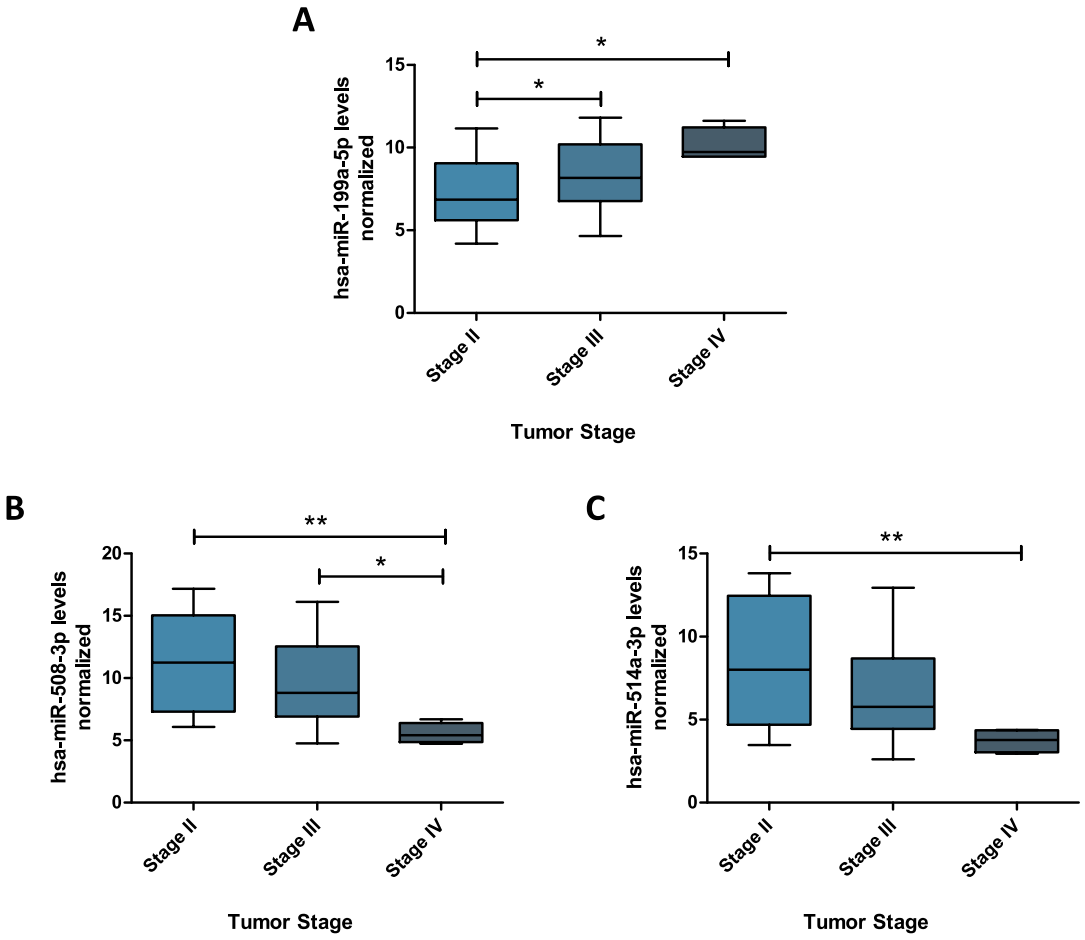

Figure 13. Panel A) Statistical difference of hsa-miR-199a-5p expression in different UM tumor stages according to the TCGA UVM data (p=0.0057); Panel B) Statistical difference of hsa-miR-508-3p expression in different UM tumor stages according to the TCGA UVM data (p=0.0018); Panel C) Statistical difference of hsa-miR-514a-3p expression in different UM tumor stages according to the TCGA UVM data (p=0.0038). * p<0.05; ** p<0.01

6 Discussion

Several studies have tried to identify novel effective biomarkers in uveal melanoma which could be useful for early diagnosis and for prognostic purposes, allowing improvements in patient management. The role of effective biomarkers could assume a great clinical value considering the high mortality of this malignancy and the fact that in some cases the diagnosis could prove challenging.[10,105]

During the last years, novel high-sensitive biomarkers for UM have been explored. Among these biomarkers, some hepatic markers are used to diagnose uveal melanoma metastatic disease (including FAL, AST, ALT and LDH) and other biomarkers mainly used for melanoma, including S–100β, OPN, and MIA.[235] However, the sensitivity and specificity of these markers are still under evaluation and their increment may be related to other pathologies (e.g. hepatitis, cirrhosis, etc.).

On these bases, it is evident the necessity of discovery novel effective biomarkers for the development of this tumor. In this context, several studies have tried to study the predictive value of several epigenetic biomarkers for UM, including DNA hyper- or hypomethylation[213,236] and the alteration of non-coding RNAs (ncRNAs).[220,237,238] However, also these studies are still in a preliminary phase, thus the results are not validated yet. In this scenario, to the best of our knowledge, the research group of the University of Catania was the first to comprehensively analyze all the bioinformatics data about miRNAs expression in UM patients through the integrated analysis of miRNAs expression, phenotype and clinical datasets obtained from The Cancer Genome Atlas database.[220] In this bioinformatics study, a panel of dysregulated miRNAs strictly involved in the development and aggressiveness of UM was identified. Among these miRNAs, the most altered and involved in the clinical features of UM were the two down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p and the up-regulated miRNAs hsa-miR-199a-5p.[220]

A few clinical studies explored the role of circulating miRNAs as biomarkers in UM has been recently given their numerous advantages including high sensitivity and specificity, non-invasive accessibility, good stability and long half-life, rapid and non-expensive detection.

Ragusa et al investigated miRNA expression in vitreous humor, serum and histologic samples of patients affected by uveal melanoma.[239] The authors showed that many miRNAs were found dysregulated in both vitreous humor and vitreal exosomes, such as

upregulated expression of miR-21, -34a,-146a, while serum miRNAs expression did not overlap with vitreous humor findings. Upregulated expression of miR-21 and -146a was also found in histologic samples. Interestingly, miR-146a was the only one to be found upregulated in vitreous humor, serum and histologic samples, suggesting its potential use as circulating biomarker of UM diagnosis.[239] This finding of upregulated expression of miR-146a in UM patients has been corroborated by Russo et al who found this miRNA significantly overexpressed in both serum and histologic samples from 14 patients affected by UM, while other 7 miRNAs differentially expressed (miR-523, -19a, -30d, -127, -451, -518f, -1274b) failed to show a statistical significance.[240]

Achberger et al[210] studied circulating miRNAs featuring immune regulatory activities in 6 patients from time of UM diagnosis up to metastatic disease development. The authors found higher plasma levels of miR-20a, -125b, -146a, -155, -181a, and -223 at diagnosis time compared to healthy controls. All these miRNAs but mi-RNA181a showed higher plasma levels at metastasis development compared to time of primary diagnosis.[210]

Very recently, Stark et al[241] assessed miRNAs expression in serum samples from 3 different cohorts of patients: choroidal naevi cohort (n= 10), localized UM cohort (n=50) and metastatic UM cohort (n=5). First, the MELmiR-17 panel was analyzed, showing no expression for 6 miRNAs while 11 miRNAs were detected in serum samples. Among these 11 miRNAs, the expression levels of 6 miRNAs (miR-16, miR-145, miR-146a, miR-204, miR-211, and miR-363-3p) were found significantly different across the three study cohorts. All these six miRNAs showed different expression levels between the naevi cohort and the localized UM cohort. When comparing the localized UM cohort and the metastatic UM cohort, only miR-211 featured a significantly different expression level. The authors concluded that this panel of 6 circulating miRNAs could be a useful tool in the diagnostic process because it could help clinicians when it comes to differentiate benign lesions from UM.[241]

Taking into account these previous studies and the bioinformatics analyses carried out at the University of Catania,[220] in the present study we wanted to confirm the diagnostic value of the miRNAs previously identified in silico focusing the attention on the expression levels of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-miR-514a-3p in liquid biopsy samples and tissue samples of UM patients and healthy individuals. For this purpose, the high-sensitive ddPCR was used to analyze the expression levels of the three miRNAs selected as candidate biomarkers for UM. We choose to use ddPCR as it represents the best methods for the analysis of liquid biopsy samples and for the detection

of low expressed circulating miRNAs as well as targets with low concentrations, circulating mutations, low amount of viral and bacterial nucleic acids, etc.[242–245]

The ddPCR analysis here performed by using the miRCURY LNA technology has revealed that in liquid biopsy samples only the expression levels of the predicted up-regulated miRNA hsa-miR-199a-5p were detected, while no expression was detected for the two down-regulated miRNAs hsa-miR-508a-3p and hsa-miR-514a-3p that are not secreted in the bloodstream or other body fluids. The results obtained from the ddPCR analysis of liquid biopsy samples are in line with those obtained by Stark and coworkers who, similar to our findings, did not detect the serum levels of hsa-miR-508-3p.[241] The results obtained for the liquid biopsy samples confirmed the over-expression of hsa-miR-199a-5p in UM patients compared to healthy donors, thus validating the dysregulation of this miRNA during the neoplastic transformation of choroid cells. In addition, ROC analyses confirmed the diagnostic value of this miRNA which showed an AUC of 0.7433, suggesting a moderate accuracy of a hypothetical diagnostic test based on the analysis of the expression levels of this miRNA. Of note, ROC analysis showed a limited sensitivity and specificity rate for hsa-miR-199a-5p in correctly diagnose UM, therefore, the analysis of this miRNA should be accompanied by the evaluation of other molecular and clinical parameters that would allow a correct diagnosis of UM. As regards the diagnostic potential of hsa-miR-508-3p and hsa-miR-514a-3p, our results demonstrated that the evaluation of these miRNAs can be performed only in tissue samples, therefore, the use of these miRNAs for the early diagnosis of UM is not suitable. However, the analysis of the expression levels of these two down-regulated miRNAs may give important information to establish the prognosis of patients. Overall, the evaluation of miRNA expression levels in FFPE tissue samples demonstrated the strong involvement of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-514a-3p in the pathogenesis of UM. In particular, despite the low number of FFPE samples analyzed, hsa-miR-199a-5p resulted significantly over-expressed in UM tissues compared to normal choroid samples, while hsa-miR-508-3p and hsa-514a-3p were down-regulated in UM with statistical significance.

To further corroborate the experimental results herein achieved, a novel computational analysis for the three selected miRNAs was performed. The bioinformatics results confirm that the three miRNAs are able to target a plethora of genes involved in different physio-pathological processes. Of these genes, some are actively involved in UM and other tumors. In particular, MAPT gene, actively targeted by all of the three miRNAs, is

known to be dysregulated and involved in brain tumor.[228] In the same manner, other genes involved in melanoma and uveal melanoma development have been identified, including PRKAA2, MAPK14, HIST2H3PS2, UBE2G1, TANC2, HSPA14, TP63.[229–233]

Finally, the analysis of clinical-pathological data and miRNAs expression data obtained from the UM patients recorded in the TCGA UVM database further confirmed the strong dysregulation of hsa-miR-199a-5p, hsa-miR-508-3p and hsa-514a-3p in an increasing manner depending on patients' tumor stage. In particular, significant differences were observed for all the miRNAs, however, the down-regulated miRNAs hsa-miR-508-3p and hsa-miR-514a-3p showed the highest statistically significant differences, especially comparing Stage II with Stage IV patients. These data further confirm that the evaluation of hsa-miR-508-3p and hsa-miR-514a-3p is of fundamental importance for the prediction of the prognosis of patients and the development of a more aggressive tumor phenotype.

This study presents the following limitations. First, a relatively small cohort of patients was enrolled. However, it is important to consider that UM is a relatively rare cancer. Additionally, this study was aimed to investigate miRNAs expression levels at baseline, with no data on the follow-up of enrolled patients. The follow-up time has a great relevance when it comes to evaluate the prognostic value of a biomarker. We indirectly evaluated the prognostic values of the miRNAs under investigation by conduction computational analyses. However, further clinical studies with a long follow-up are needed to corroborate the prognostic value of our findings.

7 Conclusions

Overall, the results of the present study confirmed the bioinformatics results previously obtained. Indeed, despite the low number of samples analyzed, we demonstrated the diagnostic value of hsa-miR-199a-5p in liquid biopsy samples. While hsa-miR-508-3p and hsa-miR-514a-3p have no role as circulating biomarkers, they were found significantly down-regulated in FFPE UM samples, suggesting their potential prognostic role in those patients treated with enucleation. In particular, bioinformatics analyses demonstrated that hsa-miR-199a-5p, hsa-miR-508-3p and hsa-514a-3p were significantly dysregulated in UM, and these differences increase according to the tumor stages of patients. Therefore, this pilot study represents a milestone for the implementation of novel diagnostic strategies based on the use of ddPCR for the analysis of liquid biopsy samples and FFPE tissues and the identification of diagnostic and prognostic biomarkers for UM. In conclusion, the innovative results obtained in the present study represent only the starting point for the development of novel effective strategies for the management of UM patients. Further experimental and functional studies on a large number of samples are needed in order to evaluate the expression levels of the miRNAs here identified and to further validate their predictive role as biomarkers for UM.

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