Title:
Circulating microRNAs as biomarkers for early diagnosis of cutaneous melanoma

Running title:
Circulating miRNAs in melanoma diagnosis

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Abstract

Introduction: Cutaneous melanoma is the deadliest form of skin cancer, with a dramatically increasing incidence worldwide over the past decades. Early detection has been shown to improve outcome of melanoma patients. The identification of non-invasive biomarkers able to identify melanoma at an early stage remains an unmet clinical need. Circulating miRNAs (c-miRNAs), small non-coding RNAs, appear as potential ideal candidate biomarkers due to their stability in biological fluids and easy detectability. Moreover, c-miRNAs are reported to be heavily deregulated in cancer patients.

Areas covered: This review examines evidence of the specific c-miRNAs or panels of c-miRNAs reported to be useful in discriminating melanoma from benign cutaneous lesions.

Expert opinion: Although the interesting reported by published studies, the non-homogeneity of detection and normalization methods prevents the individuation of single c-miRNA or panel of c-miRNAs that are specific for early detection of cutaneous melanoma. In the future, prospective wide and well-designed clinical trials will be needed to validate diagnostic potential of some of c-miRNA candidates in clinical practice.

Keywords: circulating microRNA, melanoma, exosomes, biomarker, diagnosis.

Article Highlights

- Cutaneous melanoma is a skin cancer with increasing incidence
- To date no diagnostic biomarker has yet been identified
- The search of novel potential biomarkers represents an unmet clinical need
- Circulating miRNAs (c-miRNAs) are small non-coding RNAs found in biofluids
- C-miRNAs represent promising diagnostic biomarkers due to their features such as stability, easy sampling, capacity to discriminate between patients with cutaneous melanoma and healthy subjects
- Well-designed clinical trials are required before to transfer knowledge of c-miRNAs in the clinic routine

1.0 Introduction

Cutaneous melanoma is the most lethal type of skin cancer [1]. Melanoma population is approximately 10-15/100000 in Europe, 20-30/100000 in North America and 40-50/100000 in Australia [2]. Cutaneous melanoma is unique in its feature as originating from the skin, it can be easily accessible, although often neglected and diagnosed in advanced stage when the probability of local and distant metastases increases. Early detection and surgical removal of melanoma before its growth develops in the deep skin layers and reaches the lymphatic and blood streams, is the only procedure with curative intent that can be proposed to melanoma patients [1].

Although new drugs able to prolong survival of patients with metastatic melanoma are now available, they can provide clinical benefit only in a small proportion of patients [1,3]. International guidelines recommend a screening for early detection in people at risk of developing a melanoma; however, how to recruit the population at risk remains unclear. In Australia a vast program of education has been demonstrated to be effective in decreasing melanoma mortality [4]. However, in 2017, mortality from thin melanomas exceeded that from thick melanomas [4], suggesting that a substantial number of removed lesions during early screening will probably never metastasize. Since no sensitive or specific diagnostic biomarker has yet been identified [5], the search of novel potential biomarkers represents an unmet clinical need.

MicroRNAs (miRNAs or miRs) are short non-coding RNAs that regulate a number of biological processes including carcinogenesis and are strongly deregulated in cancer cells [6,7]. MiRNAs are localized in the cytoplasm of cells but can also be detected in peripheral blood as circulating miRNAs (c-miRNAs) [8,9].

C-miRNAs could potentially serve as promising predictive biomarkers due to a number of properties such as stability, easy sampling, capacity to discriminate between normal and tumor tissues, different subtypes of a particular cancer, or even specific oncogenic abnormalities [10,11]. Since miRNAs were found to regulate important cellular process, it is conceivable that they can be useful in predicting cancer diagnosis, as well as prognosis and response to drugs [12,13].

1.1 Circulating miRNAs

C-miRNAs have been identified for the first time in 2008 in blood samples [8,9], and subsequently in other body fluids including cerebrospinal fluid, breast milk, saliva and others [9,11,14].

In peripheral circulation, c-miRNAs are packaged and transported in microparticles (microvesicles, exosomes, apoptotic bodies), or complexed with either RNA-binding proteins (Argonaute, Ago) or lipoproteins (high-density lipoprotein, HDL). C-miRNAs may be released in a passive way from

apoptotic and necrotic cells or in an active way by cell-secretion of exosomes, microvesicles, Ago and HDL. These complexes make c-miRNAs stable and protect them from degradation by RNase [11,15,16]. C-miRNAs are known to be remarkably stable under harsh conditions, such as extreme pH values, high temperatures, multiple freeze-thaw cycles, prolonged sitting at room temperature, and long-term storage [8,11].

Several studies reported that c-miRNAs may originate from immune cells, tumor cells, blood cells, endothelial cells, as well as cells of the tumor microenvironment [9,11,17–19]. The vast majority of c-miRNAs in blood originate from blood cells and endothelial cells [11,20] and differences between cancer patients and healthy subjects seem to be due to the release of c-miRNAs by cancer and immune cells as a consequence of pathophysiological mechanisms and tissue injuries. Systemic (non-immune) response may also contribute to c-miRNA levels in blood. Theoretically, one may argue that in non-metastasizing melanomas the low mass of the tumor and its relative detachment from circulation should prevent it to significantly affect c-miRNA levels in blood directly, thus systemic and immune response could be the main culprit of any detected changes.

The different c-miRNA expression patterns between physiological and pathological conditions may discriminate, with significant specificity and sensitivity, cancer patients from healthy subjects [8,17,21–23]. Although biological functions of c-miRNAs are not completely understood, scientists speculate that c-miRNAs may play a role in promoting cancer cell growth by controlling multiple cellular functions as well as immune response. Indeed, c-miRNAs have been identified as modulators of cellular pathway and mediators of intercellular communication [15,24] and immune regulation [25].

For these reasons, the study of c-miRNAs as diagnostic tool is very promising, even though a more comprehensive validation is required [7,8,13,26–30]. The following paragraphs will be focused on circulating single or panel c-miRNAs that have been shown to be related with diagnosis of cutaneous melanoma and for each c-miRNA with significance at circulating level, knowledge of its expression (Table 1) and its oncogenic or tumor suppressor biological role in melanoma, when already described in literature are reported.

2.0 Circulating miRNAs: comparison between melanoma patients and healthy subjects

Table 2 and figure 1 summarize data from publications showing c-miRNAs that can discriminate between melanoma patients and healthy subjects. Most studies performed analysis on plasma, serum or whole blood; however, only two of them evaluated the miRNA levels in exosomes and no data have been reported about miRNAs content in circulating tumor cells in patients with cutaneous melanoma, although evidence already exists in other cancers [31–33].

The first study from Leidinger and collaborators [34] described a c-miRNA panel that the authors considered to be useful for melanoma diagnosis. In particular, signature was composed by the following 16 up- or down-deregulated c-miRNAs: c-miR-186-5p, c-let-7d-3p, c-miR-18a-3p, c-miR-145-5p, c-miR-99a-5p, c-miR-664-3p, c-miR-501-5p, c-miR-378a-5p, c-miR-29c-5p, c-miR-1280, c-miR-365a-3p, c-miR-1249, c-miR-328, c-miR-422a, c-miR-30d-5p, and c-miR-17-3p. This panel was identified by analyzing almost 900 human c-miRNAs in whole blood samples from 11 melanoma patients, as independent validation set, 24 melanoma patients as test set and 20 healthy individuals [34]. The analysis revealed that the c-miRNA panel was able to discriminate melanoma patients from healthy controls with high accuracy (97.4%), specificity (95%) and sensitivity (98.9%). Some c-miRNAs included in the panel, i.e., let-7d-3p, miR-501-5p, miR-18a-3p, miR-422a, miR-99a-5p, miR-378a-5p, miR-30d-5p, miR-29c-5p and miR-1249, have not been described in other studies, while others have been reported for their involvement in melanoma development and progression. MiR-186-5p was found to be up-expressed in melanoma tissues and cell lines where it can promote cell proliferation via a decrease in cylindromatosis tumor suppressor (CYLDI) protein expression [35]. A tumor suppressor role was instead suggested for miR-664-3p and miR-1280, two miRNAs that may act on proteolipid protein 2 (PLP2) [36] and proto-oncogene SRC [37], respectively. MiR-328 was less expressed in melanoma cells compared to human epidermal melanocytes, where its ectopic over-expression inhibited proliferation and induced G1 phase arrest by targeting transforming growth factor-beta 2 (TGFβ2) [38]. Recently, Liu *et al*. [39] reported that miR-145-5p, in melanoma cell lines, could inhibit proliferation, migration and invasion while increasing apoptosis and such effects occurred through inhibition of the mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol 3-kinase (PI3K)/protein kinase B (AKT) pathways. Furthermore, miR-17-3p was shown to be down-expressed in B-RAF-resistant cells compared to B-RAF sensitive cells [40].

Of the above-mentioned miRNAs, c-miR-145-5p, c-miR-10b-5p, c-miR-195-5p, c-miR-21-5p and c-miR-155-5p were found to be significantly down regulated in whole blood samples from melanoma patients in a study from Heneghan and coworkers [41]. Although some previously contrasting data on c-miR-145-5p have been published [34], its down regulation is in line with its tumor suppressor role of miR-145-5p demonstrated in human melanoma cells [39]. The expression levels of miR-10b-5p in primary melanoma tissues were higher in patients developing metastasis within 5 years than in those metastasis-free [42]. Noteworthy, c-miR-10b-5p was lower in melanoma patients than healthy subjects [41], suggesting a different diagnostic and prognostic significance for this specific miRNA.

Decreased circulating levels were unexpected for other miRNAs (c-miR-195-5p, c-miR-21-5p and

c-miR-155-5p) in the panel studied by Heneghan and co-workers [41], since they were described as oncogenic in previously published studies [43–48]. For example, miR-195-5p has been reported to be involved in cell proliferation, invasiveness and migration of melanoma cells through the regulation of the cell cycle checkpoint kinase Wee1-like protein kinase (WEE1) [43]. Furthermore, miR-21-5p in melanoma tissues was associated with invasive depth, tumor mitotic index, lympho-vascular invasion, and correlated with the AJCC stage of patients [45,46]. Moreover, it was described to promote proliferation and migration in melanoma cells by targeting metallopeptidase inhibitor 3 (TIMP3), programmed cell death protein 4 (PDCD4), phosphatase and tensin homolog (PTEN), BTG, B-cell lymphoma 2 (BCL-2) and p-AKT [47,48]. As regard miR-155-5p , it was found to be overexpressed in melanoma tissues compared to benign nevi [48] and correlated with melanoma patient outcome [49], but its oncogenic role was not clearly demonstrated in melanoma cells. In fact, it was mostly down regulated in melanoma cell lines compared to melanocytes and its ectopic overexpression was associated to impaired cellular proliferation, an effect mediated by v-ski avian sarcoma viral oncogene homolog (SKI) gene targeting [50].

In the study of Kanemaru and collaborators [51], c-miR-221-3p was identified as possible single diagnostic biomarker for melanoma. They observed a highly significant up-regulation of c-miR-221-3p in serum from melanoma stage I-IV patients compared to healthy subjects [51]. Since a significant modulation of c-miR-221-3p was not observed in patients with melanoma stage 0 compared to healthy controls [51], it cannot be a useful biomarkers for early melanoma diagnosis. A tumorigenic role of both miR-221 and its homologue miR-222 have been proposed in melanoma since they down-regulate c-Kit receptor and p27Kip1/cyclin dependent kinase inhibitor 1B (CDKN1B), leading to arrest of differentiation and enhancement of proliferation of melanoma cells, respectively [51–53]. Nevertheless, miR-221 and miR-222 were found to be also up regulated in other cancers, suggesting that these miRNAs are not specific for melanoma [54].

Greenberg and collaborators [55] conducted a pilot study on serum samples obtained from stage IV melanoma patients and healthy controls showing that the absence of c-miR-29c-5p and c-miR-324-3p levels in serum were highly indicative of metastatic melanoma. While decrease levels of c-miR-29c-5p was reported also by Leidinger and collaborators in patients with stage 0 to IV melanomas [34], no data were previously reported for miR-324-3p in bloodstream and cells of this disease.

Another source of circulating miRNAs is represented by exosomes [11,15]. Data from a comparison study demonstrated that c-miR-125b-5p was significantly down regulated in blood exosomes of melanoma patients compared to healthy subjects, suggesting that this miRNA might

be considered as a potential diagnostic biomarker in melanoma [56]. Interesting to note, the same authors suggested that exosome-derived miRNAs is most likely released from tumor cells, while serum miRNAs can also originate from blood cells or endothelial cells [56]. Reduced expression levels of miR-125b-5p were recently described in formalin-fixed and paraffin-embedded melanoma tissue samples compared to benign melanocytic nevi [45]. Moreover, miR-125b was found to be associated with cell senescence [57] and capable of regulating melanoma progression by targeting the transcription factor c-Jun [58].

It has been reported that c-miR-210 was significantly higher in plasma of melanoma patients than healthy subjects [59]. Accordingly, miR-210 expression levels in melanoma tissues were higher in metastatic melanoma tissues than primary tumors [60], confirming the previously demonstrated oncogenic role of this miRNA in melanoma and other cancer types [61,62], but also suggesting that this miRNAs is not specific for melanoma diagnosis.

Using matched serum and formalin-fixed tissue samples, Stark and collaborators [18] identified seven c-miRNAs (i.e., c-miR-16-5p, c-miR-211-5p, c-miR-4487, c-miR-4706, c-miR-4731, c-miR-509-3p/5p) able to distinguish melanoma patients from healthy subjects with high sensitivity (93%) and specificity (≥82%). miR-16-5p is a member of the miR-15/16 family that showed strong tumor suppressive abilities [63] and its ectopic over-expression was found to inhibit cell proliferation *in vitro* and tumor growth *in vivo* [64]. Down-regulation of miR-211-5p was associated with cancer cell survival under hypoxic conditions [65], whereas over-expression reduced melanoma invasiveness [66] and cell proliferation by targeting interleukin-10 receptor alpha (IL- 10R) [67]. This miRNA was also found to inhibit epithelial-mesenchymal transition (EMT) by targeting RAB22A, a member of the Ras-related small GTPase family [68]. miR-4731 regulates multiple genes associated with cell cycle (e.g., cyclin-A2 coding gene, the origin recognition complex 5l, the proliferating cell nuclear antigen) and some members of melanoma growth promoters belonging to the synovial sarcoma X breakpoint family [69]. miR-4731 was found to be down-expressed in melanoma cells [69]. miR-509-3p/-5p regulates different oncogenes related to cellular development, cell-to-cell interactions, and EMT pathways in melanoma [44].

Another panel of c-miRNAs (c-miR-374a-5p, c-miR-204-5p, c-miR-27a-3p, c-miR-200c-3p and c-miR-373-5p) able to discriminate between healthy subjects and melanoma patients was identified [70] analyzing whole blood miRNome. Among them, miR-200c has been extensively studied and found to have tumor suppressor activity in melanoma cells and its down-regulation has been involved in melanoma progression and drug resistance [70,71]. As a member of the miR-

200 family, it has a recognized role in different aspects of cancer biology, including EMT, tumor angiogenesis and chemo-resistance [72]. In particular, it can act by repressing several key messenger RNAs, such as those coding for the Notch ligand JAG1 and for β-catenin [73]. Wang et al. [44] found that miR-374a-5p is negatively correlated with EMT gene expression in melanoma cells. The selective BRAF inhibitor, Vemurafenib, up-regulated miR-204-5p in A375 melanoma cells, with the consequent inhibition of cell motility mediated by targeting the Adaptor Protein complex1 subunit Sigma 2 (AP1S2) [74]. MiR-27a-3p levels were significantly higher in melanoma cell lines than melanocytes, whereas no difference was found between melanoma tissues and melanocytic nevi [75].

Ferracin and collaborators [76] analyzed nine c-miRNAs (i.e., c-miR-320a, c-miR-21-5p, c-miR-378a-3p, c-miR-181a-5p, c-miR-3156-5p, c-miR-2110, c-miR-125a-5p, c-miR-425-5p and c-miR-766-3p) in plasma and serum samples from healthy subjects and melanoma patients. They found that c-miR-21-5p was consistently increased in plasma but not in serum samples [76], a result also confirmed in whole blood [41]. Both a lack of deregulation [76] and a significant increase in miR-378a-5p levels in melanoma [34] were observed, and contrasting data were also reported on miR-125a-5p [57]. Plasma-derived c-miR-320a appeared to be significantly up regulated in melanoma patients compared to healthy subjects. Moreover, c-miR-181a-5p was the only c-miRNA significantly deregulated in both plasma and serum of melanoma patients compared to healthy subjects. It has been hypothesized that differences in absolute c-miRNAs levels in serum and plasma might be due to the permanence of microvesicles and exosomes in plasma [76].

Recently, a combination of two c-miRNAs, i.e., c-miR-1246 and c-miR-185-5p, was significantly associated with metastatic melanoma with a sensitivity of 90.5% and a specificity of 89.1% [77]. The authors analyzed plasma samples from patients with metastatic melanoma and healthy subjects and subsequently validated the results in two independent cohorts of stage III-IV patients and healthy subjects. miR-1246 has been very recently associated with acquired resistance to BRAF inhibitors in melanoma cells [40], while miR-185-5p has been involved in cellular proliferation and migration, and may act as an enhancer for apoptosis induced by ionizing radiation [78].

In a recently study carried out in our laboratory by analyzing plasma levels of five highly deregulated c-miRNAs in melanoma patients (at different disease stages) and healthy subjects, we found that c-miR-15b-5p, c-miR-149-3p, and c-miR-150-5p were up-regulated, while c-miR-193a-3p and c-miR-524-5p were down-regulated [79]. Although each of these markers showed a significant ability to discriminate melanoma patients from healthy subjects, the significance improved when a signature of three (i.e., c-miR-149-3p, c-miR-150-5p and c-miR-193a-3p) of

these miRNAs was considered with a high sensitivity (94.8%), specificity (83.9%) and accuracy (91%) [79]. Interesting to note, all three miRNAs in the panel had an oncogenic role in melanoma cells. For example, miR-149-3p can confer resistance to apoptosis by increasing the expression of the anti-apoptotic protein Myeloid cell leukemia 1 (Mcl-1), an effect mediated by Glycogen synthase kinase (GSK)-3α [78]. Furthermore, miR-150-5p has been suggested as to regulate melanoma immune response being linked to maturation and activity of lymphocytes [80]. An immunomodulation activity was also described for miR-193a-3p, that was found to be significantly down regulated, particularly in B-RAF mutated melanoma tissue samples [81,82].

Recently, Van Laar and colleagues [4] profiled whole-miRNome in plasma samples from healthy subjects and melanoma patients identified 38 circulating microRNAs (MEL38: c-miR-424-5p, c-miR548I, c-miR-34a-5p, c-miR-497-5p, c-miR-299-3p, c-miR-205-5p, c-miR-1269a, c-miR624-3p, c-miR-138-5p, c-miR1-5p, c-miR-152-3p, c-miR-1910-5p, c-miR-181b-5p, c-miR-3928-3p, c-miR-3131, c-miR-301a-3p, c-miR1973, c-miR520d-3p, c-miR-548a-5p, c-miR-548ad-3p, c-miR-454-3p, c-miR-4532, c-miR-1537-3p, c-miR-553, c-miR-764, c-miR-1302, c-miR-1258, c-miR-522-3p, c-miR-1264, c-miR-1306-5p, c-miR-219a-2-3p, c-miR-431-5p, c-miR-450a-5p, c-miR-2682-5p, c-miR-337-5p, c-miR-27a-3p, c-miR-4787-3p, c-miR-154-5p) with biologically and statistically significant differences between the two group. As reported by the authors, a majority of MEL38 genes have been previously associated with melanoma and are known regulators of angiogenesis and inflammation, cancer cell invasion and metastasis, immune system and treatment resistance, and tumor suppression or oncogene regulation, with a number of genes having dual functions [4,40,46,83–94]. MEL38 panel is recently examined in benign naevi, primary and metastatic melanoma biopsies, showing diagnostic and prognostic values [95] and in plasma of melanoma patients before and after surgical excision, showing clinical utility [96].

In a recent paper from Tengda and co-workers [97], serous exosomes from melanoma patients and healthy individuals were used as test study for the evaluation of 5 exosomes miRNAs, previously found to be aberrantly expressed in tissues or in the peripheral system. Two miRNAs, i.e., c-miR-532-5p and c-miR-106b were differently expressed between the two groups, a result also confirmed in a blinded validation study in a wider cohort of patients and healthy volunteers. From the functional point of view, miR-532-5p can regulate RUNX3 expression and miR-532-5p expression levels were shown to be significantly higher in human melanoma cells and metastatic melanoma tissues than in normal melanocytes and primary tumors, respectively [98]. MiR-106b plays a crucial role in melanoma growth and it has been found overexpressed in various melanoma cell lines as compared to melanocytes [99].

The last article published was conduced by Solé and collaborators, they identified eleven plasma derived c-miRNAs (c-miR-134-5p, c-miR-320a-3p, c-miR-21-5p, c-miR-92b-3p, c-miR-98-5p, c-miR-16-3p, c-let-7b, c-miR-1827, c-miR-1180, c-miR-628 and c-miR-486) expressed in significantly different way between healthy controls and different disease stages (0 to IV) in cutaneous melanoma patients. As regard of each miRNAs: miR-134 have a role in proliferation, apoptosis, invasion, metastasis and drug resistance in a wide range of cancer types, including melanoma, but its mechanism of action are still not clear [100,101]. MiR-320a acts as inhibitor of cell proliferation and it is down expressed in melanoma cells compared to healthy skin samples [102,103]. The levels of c-miR-320a are reported down-expressed compared to control by Solé and collaborators while upregulated by Ferracin and collaborators [76]. Indeed, the increase of c-miR-21-5p is also reported by Ferracin [76] and its functional activity is previously reported. C-miR-92b is elevated also in plasma of patients with uveal melanoma [104]. This miRNA appertains to a cluster of miRNAs that regulate T cells, including regulatory T cells [105]. MiR-98 is down-expressed in melanoma tissues and its expression is also negatively associated with melanoma patient survival. Furthermore, ectopic overexpression of miR-98 inhibits melanoma metastasis in part through a negative feedback loop with its target gene interleukin-6 (IL-6) [106]. Elevated levels of let-7b are correlated with the response to radiotherapy of uveal melanoma cells and with the decrease of proliferation in melanoma cells [107,108].

The expression of miR-486 in patient derived primary melanoma cell lines is significantly correlated with acral, compared to non-acral, melanomas [109]. Currently, no literature data are reported for miR-16, miR-1827, miR-1180 and miR-628.

2.1 Circulating miRNAs: comparison between melanoma and other cancer

Further support to the role of c-miRNAs as biomarkers for melanoma diagnosis may derive from the comparison of data obtained from patients with different cancer types. The levels of a single miRNA may, in fact, vary across different cancer types and subtypes, revealing tissue specificity [110]. This is indeed a big issue that is further aggravated by the fact that c-miRNAs as biomarkers possess only limited tumour/tissue specificity. Furthermore, many c-miRNAs are down-regulated and this down-regulation is not ideal for blood biomarkers, since there is little knowledge about the reproducibility of c-miRNA levels in health and disease. Indeed, to detect a measurable change in biomarker levels are require a strong systemic response as otherwise it could be masked by molecules normally occurring in the bloodstream. For these reasons the use of panels, as MEL38 [95], is more promising.

Until now, the specificity of c-miRNAs for melanoma has been poorly investigated. At present, only four articles (aforementioned for their comparison between melanoma patients and healthy subjects) studied a comparison among different cancer types (Table 3) [41,55,76].

Heneghan and co-workers [41] analyzed blood collected from cancer patients before surgery (i.e., breast, melanoma, prostate, colon and renal cancer) and healthy subjects. Although they did not find c-miRNAs able to discriminate melanoma from other cancers, findings of that study suggested that c-miR-195-5p could be capable of selecting breast cancer patients from other cancer types or controls.

In 2013, Greenberg and co-workers [55] analyzed sera samples obtained from patients with melanoma stage IV, colon cancer (n=20) and renal cancer (n=23). Although they identified loss of c-miR-29c-5p in sera from metastatic melanoma patients compared to renal and colon cancer patients as a possible discriminating profile, their results derived from small cohorts of metastatic patients and should be confirmed in prospective clinical trials.

Ferracin and colleagues [76] analyzed plasma and serum samples from patients with melanoma (n=8), breast (n=18), lung (n=18), colorectal (n=18) and thyroid (n=27) cancers. Plasma-derived c-miR-320a was suggested to be melanoma-specific; however, no further independent validation in larger cohorts was carried out [76].

In 2018, Van Laar and collegues [4] compared MEL38 with data generated by Keller et al [111], on others eight malignancies (colon, lung, ovarian, prostate, breast, renal, stomach, and Wilms tumor; N 1⁄4 393, GEO ID GSE61741). They observed that fifteen of c-miRNAs in MEL38 panel could be considered as melanoma-specifics, as compared to others cancer types.

3.0 Conclusion

In conclusion, the examination of literature evidence the possibility of using c-miRNAs in discriminating patients with melanoma from benign cutaneous lesions. An exciting time in the research area of c-miRNA is started and the use of nucleic acids as diagnostic biomarkers in clinical practice is not far away. However, some unmet needs, as a common normalization strategy, must be reached before to transfer knowledge of c-miRNAs in the clinic routine.

4.0 Expert Opinion

c-miRNAs might have the potential to become biomarkers to be used for the early diagnosis of melanoma. However, even though several line of evidence have been provided on this topic, most of them come from small, low-powered studies that used different biological samples (e.g., whole blood, serum, plasma or exosomes) and analytical protocols, making data interpretation difficult.

With regards for this, it is noteworthy that the use of different analytical standards (i.e., miRNAs) or normalization procedures may account for the contradictory results reported in the literature.

These discrepancies need to be thus clarified before designing reliable validation studies. In this preliminary phase, the robustness and reproducibility of measurements of the most promising candidates should be determined and the analytical variables affecting miRNA detection reduced. Larger clinical trials are also required to reduce the amount of false positive while increasing the diagnostic power of the miRNA signature.

Furthermore, it should be noted that the majority of published works were carried out on plasma- or serum-derived miRNAs, while only two was focused on exosomes-derived miRNAs (Table 1). Exosomes are key regulators in cell-to-cell communication in normal as well as in pathological conditions [112] and represents attractive biomarkers both themselves and with their content of miRNAs [113].

Nowadays, there are numerous methods for isolation of exosomes, besides differential ultracentrifugation, ultrafiltration, precipitation, immunoaffinity and microfluidics-based techniques [114] are available and could contribute to expander the studies of exosomes-derived miRNAs in patients with melanoma.

This is most probably due to difficulties in separating exosomes from plasma or serum samples. With regards for this, some different methods have been developed to overcome the ultracentrifugation step during exosomes isolation; however, analysis of circulating miRNAs from plasma or serum, may present the advantage of a direct extraction thus simplifying protocols while reducing analytical variables.

The performance of different detection platforms aimed at optimizing methods for miRNA extraction and quantification need also to be tested [115]. For example, exogenous normalizations is an interesting approaches proposed by Vigneron and co-workers [116]. In particular, the use of the geometrical mean obtained from three exogenous cell-derived miRNAs added at the beginning of the extraction step was found to reduce inter-assay variability, as compared to strategies based on using endogenous components (e.g., c-miR-16-5p) [117–119] or total RNA [120]. Another approach is represented by ratio-based normalization [121].

Among different methods used to analyze expression of c-miRNAs, the Digital Droplet PCR (ddPCR) may provide some advantages. In particular, this technique is specific and sensitive and does not require a standard curve or endogenous controls.

Another interesting point that is necessary to better understand to translate c-miRNAs in clinical practice is represented by the lack of understanding of the factors affecting circulating miRNA levels.

Nonetheless, many efforts still need to be done to standardize the experimental approach before using new c-miRNAs signatures as diagnostic biomarkers in melanoma patients. A multidisciplinary approach to this topic might help creating international cooperation among different research groups aimed at accelerating the bench-to-bedside transition.

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References

[1] Eggermont AMM, Spatz A, Robert C. Cutaneous melanoma. Lancet. 2014;383:816–827.
[2] Garbe C, Peris K, Hauschild A, et al. Diagnosis and treatment of melanoma. European consensus-based interdisciplinary guideline – Update 2016. European Journal of Cancer. 2016;63:201–217.
[3] Millet A, Martin AR, Ronco C, et al. Metastatic Melanoma: Insights Into the Evolution of the Treatments and Future Challenges. Med Res Rev. 2017;37:98–148.
[4] Van Laar R, Lincoln M, Van Laar B. Development and validation of a plasma-based melanoma biomarker suitable for clinical use. British Journal of Cancer. 2018;118:857–866.
[5] NCCN Evidence Blocks. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines).
[6] Ross CL, Kaushik S, Valdes-Rodriguez R, et al. MicroRNAs in cutaneous melanoma: Role as diagnostic and prognostic biomarkers. J. Cell. Physiol. 2018;233:5133–5141.
[7] Carpi S, Polini B, Fogli S, et al. Circulating MicroRNAs in cutaneous melanoma: Diagnosis and Prognosis. 2016.
[8] Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences. 2008;105:10513–10518.
[9] Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006.
[10] Peng Y, Croce CM. The role of MicroRNAs in human cancer. Signal Transduction and Targeted Therapy [Internet]. 2016 [cited 2018 May 21];1. Available from: http://www.nature.com/articles/sigtrans20154.
[11] Schwarzenbach H, Nishida N, Calin GA, et al. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol. 2014;11:145–156.
[12] Tuna M, Machado AS, Calin GA. Genetic and epigenetic alterations of microRNAs and implications for human cancers and other diseases. Genes Chromosomes Cancer. 2016;55:193–214.
[13] Polini B, Carpi S, Romanini A, et al. Circulating cell-free microRNAs in cutaneous melanoma staging and recurrence or survival prognosis. Pigment Cell Melanoma Res. 2018;
[14] Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. Clin.

Chem. 2010;56:1733–1741.
[15] Hannafon BN, Ding W-Q. Intercellular communication by exosome-derived microRNAs in cancer. Int J Mol Sci. 2013;14:14240–14269.
[16] Han L, Xu J, Xu Q, et al. Extracellular vesicles in the tumor microenvironment: Therapeutic resistance, clinical biomarkers, and targeting strategies. Med Res Rev. 2017;37:1318–1349.
[17] Wittmann J, Jäck H-M. Serum microRNAs as powerful cancer biomarkers. Biochim. Biophys. Acta. 2010;1806:200–207.
[18] Stark MS, Klein K, Weide B, et al. The Prognostic and Predictive Value of Melanoma-related MicroRNAs Using Tissue and Serum: A MicroRNA Expression Analysis. EBioMedicine. 2015;2:671–680.
***** multicentre study that identified a signature of seven c-miRNAs as tool for the diagnosis of patients with melanoma**
[19] Kinoshita T, Yip KW, Spence T, et al. MicroRNAs in extracellular vesicles: potential cancer biomarkers. J. Hum. Genet. 2017;62:67–74.
[20] Pritchard CC, Kroh E, Wood B, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila). 2012;5:492–497.
[21] Xin Y, Li Z, Chan MTV, et al. Circulating epigenetic biomarkers in melanoma. Tumour Biol. 2016;37:1487–1492.
[22] Nakamura K, Sawada K, Yoshimura A, et al. Clinical relevance of circulating cell-free microRNAs in ovarian cancer. Mol Cancer [Internet]. 2016 [cited 2019 Mar 13];15. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4921011/.
[23] Endzeliņš E, Melne V, Kalniņa Z, et al. Diagnostic, prognostic and predictive value of cell-free miRNAs in prostate cancer: a systematic review. Mol. Cancer. 2016;15:41.
[24] Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ. Res. 2012;110:483–495.
[25] Mehta A, Baltimore D. MicroRNAs as regulatory elements in immune system logic. Nat. Rev. Immunol. 2016;16:279–294.
[26] Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med. 2012;4:143–159.
**** the present review discusses the impact of miRNA dysregulation in cancer**
[27] De Guire V, Robitaille R, Tétreault N, et al. Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: promises and challenges. Clin. Biochem. 2013;46:846–860.
[28] Aravalli RN, Steer CJ. Circulating microRNAs: novel biomarkers for early detection of colorectal cancer. Transl Res. 2015;166:219–224.
[29] Powrózek T, Krawczyk P, Kowalski DM, et al. Plasma circulating microRNA-944 and microRNA-3662 as potential histologic type-specific early lung cancer biomarkers. Transl Res. 2015;166:315–323.
[30] Mumford S, Towler B, Pashler A, et al. Circulating MicroRNA Biomarkers in Melanoma: Tools and Challenges in Personalised Medicine. Biomolecules. 2018;8:21.
[31] Zhou H, Guo J-M, Lou Y-R, et al. Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using microRNA as a marker. Journal of Molecular Medicine. 2010;88:709–717.
[32] Mostert B, Sieuwerts AM, Martens JW, et al. Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients. Expert Review of Molecular Diagnostics. 2011;11:259–275.
[33] Riethdorf S. Detection of microRNAs in circulating tumor cells. Translational Cancer Research. 2018;7:S197–S208.
[34] Leidinger P, Keller A, Borries A, et al. High-throughput miRNA profiling of human melanoma blood samples. BMC Cancer. 2010;10:262.
[35] Qiu H, Yuan S, Lu X. miR-186 suppressed CYLD expression and promoted cell

proliferation in human melanoma. Oncol Lett. 2016;12:2301–2306.
[36] Ding Z, Jian S, Peng X, et al. Loss of MiR-664 Expression Enhances Cutaneous Malignant Melanoma Proliferation by Upregulating PLP2. Medicine (Baltimore). 2015;94:e1327.
[37] Sun V, Zhou WB, Nosrati M, et al. Antitumor activity of miR-1280 in melanoma by regulation of Src. Mol. Ther. 2015;23:71–78.
[38] Li J-R, Wang J-Q, Gong Q, et al. MicroRNA-328 inhibits proliferation of human melanoma cells by targeting TGFβ2. Asian Pac. J. Cancer Prev. 2015;16:1575–1579.
[39] Liu S, Gao G, Yan D, et al. Effects of miR-145-5p through NRAS on the cell proliferation, apoptosis, migration, and invasion in melanoma by inhibiting MAPK and PI3K/AKT pathways. Cancer Med. 2017;6:819–833.
[40] Kim J-H, Ahn J-H, Lee M. Upregulation of MicroRNA-1246 Is Associated with BRAF Inhibitor Resistance in Melanoma Cells with Mutant BRAF. Cancer Res Treat. 2017;49:947–959.
[41] Heneghan HM, Miller N, Kelly R, et al. Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. Oncologist. 2010;15:673–682.
[42] Saldanha G, Elshaw S, Sachs P, et al. microRNA-10b is a prognostic biomarker for melanoma. Mod. Pathol. 2016;29:112–121.
[43] Bhattacharya A, Schmitz U, Wolkenhauer O, et al. Regulation of cell cycle checkpoint kinase WEE1 by miR-195 in malignant melanoma. Oncogene. 2013;32:3175–3183.
[44] Wang D, Li Y, Ding N, et al. [Molecular networks and mechanisms of epithelial-mesenchymal transition regulated by miRNAs in the malignant melanoma cell line]. Yi Chuan. 2015;37:673–682.
[45] Wandler A, Riber-Hansen R, Hager H, et al. Quantification of microRNA-21 and microRNA-125b in melanoma tissue. Melanoma Res. 2017;27:417–428.
[46] Babapoor S, Wu R, Kozubek J, et al. Identification of microRNAs associated with invasive and aggressive phenotype in cutaneous melanoma by next-generation sequencing. Lab. Invest. 2017;97:636–648.
[47] Mao X-H, Chen M, Wang Y, et al. MicroRNA-21 regulates the ERK/NF-κB signaling pathway to affect the proliferation, migration, and apoptosis of human melanoma A375 cells by targeting SPRY1, PDCD4, and PTEN. Mol. Carcinog. 2017;56:886–894.
[48] Latchana N, Ganju A, Howard JH, et al. MicroRNA dysregulation in melanoma. Surg Oncol. 2016;25:184–189.
[49] Jayawardana K, Schramm S-J, Tembe V, et al. Identification, Review, and Systematic Cross-Validation of microRNA Prognostic Signatures in Metastatic Melanoma. J. Invest. Dermatol. 2016;136:245–254.
[50] Levati L, Pagani E, Romani S, et al. MicroRNA-155 targets the SKI gene in human melanoma cell lines. Pigment cell & melanoma research. 2011;24:538–550.
[51] Kanemaru H, Fukushima S, Yamashita J, et al. The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker. Journal of Dermatological Science. 2011;61:187–193.
[52] Felicetti F, Errico MC, Bottero L, et al. The Promyelocytic Leukemia Zinc Finger-MicroRNA-221/-222 Pathway Controls Melanoma Progression through Multiple Oncogenic Mechanisms. Cancer Research. 2008;68:2745–2754.
[53] Das SK, Sokhi UK, Bhutia SK, et al. Human polynucleotide phosphorylase selectively and preferentially degrades microRNA-221 in human melanoma cells. Proc. Natl. Acad. Sci. U.S.A. 2010;107:11948–11953.
[54] Di Martino MT, Rossi M, Caracciolo D, et al. Mir-221/222 are promising targets for innovative anticancer therapy. Expert Opin. Ther. Targets. 2016;20:1099–1108.
[55] Greenberg E, Besser MJ, Ben-Ami E, et al. A comparative analysis of total serum miRNA profiles identifies novel signature that is highly indicative of metastatic melanoma: a pilot study. Biomarkers. 2013;18:502–508.

[56] Alegre E, Sanmamed MF, Rodriguez C, et al. Study of circulating microRNA-125b levels in serum exosomes in advanced melanoma. Arch. Pathol. Lab. Med. 2014;138:828–832.
**** The first study that identified circulating exosomal miRNAs in patients with melanoma**
[57] Nyholm AM, Lerche CM, Manfé V, et al. miR-125b induces cellular senescence in malignant melanoma. BMC Dermatol. 2014;14:8.
[58] Kappelmann M, Kuphal S, Meister G, et al. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression. Oncogene. 2013;32:2984–2991.
[59] Ono S, Lam S, Nagahara M, et al. Circulating microRNA Biomarkers as Liquid Biopsy for Cancer Patients: Pros and Cons of Current Assays. J Clin Med. 2015;4:1890–1907.
[60] Ono S, Oyama T, Lam S, et al. A direct plasma assay of circulating microRNA-210 of hypoxia can identify early systemic metastasis recurrence in melanoma patients. Oncotarget [Internet]. 2015 [cited 2018 Apr 10];6. Available from: http://www.oncotarget.com/fulltext/3142.
[61] Ivan M, Huang X. miR-210: fine-tuning the hypoxic response. Adv. Exp. Med. Biol. 2014;772:205–227.
[62] Gee HE, Camps C, Buffa FM, et al. hsa-mir-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer. 2010;116:2148–2158.
[63] Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death & Differentiation. 2010;17:215–220.
[64] Poell JB, van Haastert RJ, de Gunst T, et al. A functional screen identifies specific microRNAs capable of inhibiting human melanoma cell viability. PLoS ONE. 2012;7:e43569.
[65] Mazar J, Qi F, Lee B, et al. MicroRNA 211 Functions as a Metabolic Switch in Human Melanoma Cells. Mol. Cell. Biol. 2016;36:1090–1108.
[66] Levy C, Khaled M, Iliopoulos D, et al. Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. Mol. Cell. 2010;40:841–849.
[67] Venza I, Visalli M, Beninati C, et al. IL-10Rα expression is post-transcriptionally regulated by miR-15a, miR-185, and miR-211 in melanoma. BMC Med Genomics. 2015;8:81.
[68] Yu H, Yang W. MiR-211 is epigenetically regulated by DNMT1 mediated methylation and inhibits EMT of melanoma cells by targeting RAB22A. Biochem. Biophys. Res. Commun. 2016;476:400–405.
[69] Stark MS, Tom LN, Boyle GM, et al. The "melanoma-enriched" microRNA miR-4731-5p acts as a tumour suppressor. Oncotarget. 2016;7:49677–49687.
[70] Margue C, Reinsbach S, Philippidou D, et al. Comparison of a healthy miRNome with melanoma patient miRNomes: are microRNAs suitable serum biomarkers for cancer? Oncotarget. 2015;6:12110–12127.
[71] Xu Y, Brenn T, Brown ERS, et al. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. British Journal of Cancer. 2012;106:553–561.
[72] Liu S, Tetzlaff MT, Cui R, et al. miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1. Am. J. Pathol. 2012;181:1823–1835.
[73] Chen Y, Zhang L. Members of the microRNA-200 family are promising therapeutic targets in cancer. Exp Ther Med. 2017;14:10–17.
[74] Vitiello M, Tuccoli A, D'Aurizio R, et al. Context-dependent miR-204 and miR-211 affect the biological properties of amelanotic and melanotic melanoma cells. Oncotarget. 2017;8:25395–25417.
[75] Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. International Journal of Cancer. 2010;NA-NA.
[76] Ferracin M, Lupini L, Salamon I, et al. Absolute quantification of cell-free microRNAs in cancer patients. Oncotarget. 2015;6:14545–14555.
[77] Armand-Labit V, Meyer N, Casanova A, et al. Identification of a Circulating MicroRNA Profile as a Biomarker of Metastatic Cutaneous Melanoma. Acta Derm. Venereol. 2016;96:29–34.

[78] He J, Tian N, Yang Y, et al. miR-185 enhances the inhibition of proliferation and migration induced by ionizing radiation in melanoma. Oncol Lett. 2017;13:2442–2448.
[79] Fogli S, Polini B, Carpi S, et al. Identification of plasma microRNAs as new potential biomarkers with high diagnostic power in human cutaneous melanoma. Tumor Biology. 2017;39:101042831770164.
****identified a signature of five c-miRNAs as tool for the diagnosis of patients with melanoma by using two different normalisation approaches**
[80] Zhou B, Wang S, Mayr C, et al. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proceedings of the National Academy of Sciences. 2007;104:7080–7085.
[81] Caramuta S, Egyházi S, Rodolfo M, et al. MicroRNA Expression Profiles Associated with Mutational Status and Survival in Malignant Melanoma. Journal of Investigative Dermatology. 2010;130:2062–2070.
[82] Carpi S, Polini B, Poli G, et al. Anticancer Activity of Euplotin C, Isolated from the Marine Ciliate Euplotes crassus, Against Human Melanoma Cells. Mar Drugs. 2018;16.
[83] Li F, Li X, Qiao L, et al. MALAT1 regulates miR-34a expression in melanoma cells. Cell Death & Disease [Internet]. 2019 [cited 2019 Nov 14];10. Available from: http://www.nature.com/articles/s41419-019-1620-3.
[84] Chai L, Kang X-J, Sun Z-Z, et al. MiR-497-5p, miR-195-5p and miR-455-3p function as tumor suppressors by targeting hTERT in melanoma A375 cells. Cancer Management and Research. 2018;Volume 10:989–1003.
[85] Mueller DW, Rehli M, Bosserhoff AK. miRNA Expression Profiling in Melanocytes and Melanoma Cell Lines Reveals miRNAs Associated with Formation and Progression of Malignant Melanoma. Journal of Investigative Dermatology. 2009;129:1740–1751.
[86] Donato AL, Huang Q, Liu X, et al. Caspase 3 Promotes Surviving Melanoma Tumor Cell Growth after Cytotoxic Therapy. Journal of Investigative Dermatology. 2014;134:1686–1692.
[87] Larsen A-C. Conjunctival malignant melanoma in Denmark: epidemiology, treatment and prognosis with special emphasis on tumorigenesis and genetic profile. Acta Ophthalmologica. 2016;94:1–27.
[88] Etnyre D, Stone AL, Fong JT, et al. Targeting c-Met in melanoma: Mechanism of resistance and efficacy of novel combinatorial inhibitor therapy. Cancer Biology & Therapy. 2014;15:1129–1141.
[89] Haflidadóttir BS, Bergsteinsdóttir K, Praetorius C, et al. miR-148 Regulates Mitf in Melanoma Cells. Jin D-Y, editor. PLoS ONE. 2010;5:e11574.
[90] Mouawad R, Khayat D, Merle S, et al. Is there any relationship between interleukin-6/interleukin-6 receptor modulation and endogenous interleukin-6 release in metastatic malignant melanoma patients treated by biochemotherapy?: Melanoma Research. 1999;9:181–188.
[91] Venturelli S, Sinnberg TW, Berger A, et al. Epigenetic Impacts of Ascorbate on Human Metastatic Melanoma Cells. Frontiers in Oncology [Internet]. 2014 [cited 2019 Nov 14];4. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2014.00227/abstract.
[92] Galore-Haskel G, Nemlich Y, Greenberg E, et al. A novel immune resistance mechanism of melanoma cells controlled by the ADAR1 enzyme. Oncotarget [Internet]. 2015 [cited 2019 Nov 14];6. Available from: http://www.oncotarget.com/fulltext/4905.
[93] Adinolfi B, Carpi S, Romanini A, et al. Analysis of the Antitumor Activity of Clotrimazole on A375 Human Melanoma Cells. Anticancer Res. 2015;35:3781–3786.
[94] Stark MS, Tyagi S, Nancarrow DJ, et al. Characterization of the Melanoma miRNAome by Deep Sequencing. Soyer HP, editor. PLoS ONE. 2010;5:e9685.
[95] Van Laar R, Lincoln M, Fereday S. Characterisation and validation of Mel38; A multi-tissue microRNA signature of cutaneous melanoma. Ahmad A, editor. PLOS ONE. 2019;14:e0211504.
[96] van Laar RK, Lincoln MT, van Laar BJ. A plasma microRNA biomarker of melanoma as a personalised assessment of treatment response: Melanoma Research. 2019;29:19–22.

[97] Tengda L, Shuping L, Mingli G, et al. Serum exosomal microRNAs as potent circulating biomarkers for melanoma. Melanoma Res. 2018;28:295–303.
[98] Kitago M, Martinez SR, Nakamura T, et al. Regulation of RUNX3 tumor suppressor gene expression in cutaneous melanoma. Clin. Cancer Res. 2009;15:2988–2994.
[99] Prasad R, Katiyar SK. Down-regulation of miRNA-106b inhibits growth of melanoma cells by promoting G1-phase cell cycle arrest and reactivation of p21/WAF1/Cip1 protein. Oncotarget. 2014;5:10636–10649.
[100] Pan J-Y, Zhang F, Sun C-C, et al. miR-134: A Human Cancer Suppressor? Molecular Therapy - Nucleic Acids. 2017;6:140–149.
[101] Venkatesan N, Kanwar J, Deepa PR, et al. Clinico-Pathological Association of Delineated miRNAs in Uveal Melanoma with Monosomy 3/Disomy 3 Chromosomal Aberrations. Li Z, editor. PLOS ONE. 2016;11:e0146128.
[102] Kozubek J, Ma Z, Fleming E, et al. In-Depth Characterization of microRNA Transcriptome in Melanoma. Slominski AT, editor. PLoS ONE. 2013;8:e72699.
[103] Solé C, Tramonti D, Schramm M, et al. The Circulating Transcriptome as a Source of Biomarkers for Melanoma. Cancers. 2019;11:70.
[104] Triozzi PL, Achberger S, Aldrich W, et al. Association of tumor and plasma microRNA expression with tumor monosomy-3 in patients with uveal melanoma. Clinical Epigenetics [Internet]. 2016 [cited 2019 May 29];8. Available from: http://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-016-0243-0.
[105] de Kouchkovsky D, Esensten JH, Rosenthal WL, et al. microRNA-17-92 Regulates IL-10 Production by Regulatory T Cells and Control of Experimental Autoimmune Encephalomyelitis. The Journal of Immunology. 2013;191:1594–1605.
[106] Li F, Li X, Qiao L, et al. miR-98 suppresses melanoma metastasis through a negative feedback loop with its target gene IL-6. Experimental & Molecular Medicine. 2014;46:e116–e116.
[107] Zhou Y, Zhang L, Fan J, et al. Let-7b overexpression leads to increased radiosensitivity of uveal melanoma cells: Melanoma Research. 2015;25:119–126.
[108] Xu D, Tan J, Zhou M, et al. Let-7b and microRNA-199a inhibit the proliferation of B16F10 melanoma cells. Oncology Letters. 2012;4:941–946.
[109] Chan E, Patel R, Nallur S, et al. MicroRNA signatures differentiate melanoma subtypes. Cell Cycle. 2011;10:1845–1852.
[110] Petrovic N, Ergün S, Isenovic ER. Levels of MicroRNA Heterogeneity in Cancer Biology. Mol Diagn Ther. 2017;21:511–523.
[111] Keller A, Leidinger P, Vogel B, et al. miRNAs can be generally associated with human pathologies as exemplified for miR-144. BMC Med. 2014;12:224.
[112] Samanta S, Rajasingh S, Drosos N, et al. Exosomes: new molecular targets of diseases. Acta Pharmacologica Sinica. 2018;39:501–513.
[113] Avgeris M, Panoutsopoulou K, Papadimitriou M-A, et al. Circulating exosomal miRNAs: clinical significance in human cancers. Expert Review of Molecular Diagnostics. 2019;19:979–995.
[114] Li P, Kaslan M, Lee SH, et al. Progress in Exosome Isolation Techniques. Theranostics. 2017;7:789–804.
[115] Moret I, Sánchez-Izquierdo D, Iborra M, et al. Assessing an Improved Protocol for Plasma microRNA Extraction. PLOS ONE. 2013;8:e82753.
[116] Vigneron N, Meryet-Figuière M, Guttin A, et al. Towards a new standardized method for circulating miRNAs profiling in clinical studies: Interest of the exogenous normalization to improve miRNA signature accuracy. Mol Oncol. 2016;10:981–992.
[117] Bihrer V, Waidmann O, Friedrich-Rust M, et al. Serum MicroRNA-21 as Marker for Necroinflammation in Hepatitis C Patients with and without Hepatocellular Carcinoma. PLOS ONE. 2011;6:e26971.
[118] Cookson VJ, Bentley MA, Hogan BV, et al. Circulating microRNA profiles reflect the presence of breast tumours but not the profiles of microRNAs within the tumours. Cell Oncol.

2012;35:301–308.
[119] Schwarzenbach H, da Silva AM, Calin G, et al. Data Normalization Strategies for MicroRNA Quantification. Clin. Chem. 2015;61:1333–1342.
[120] Liu AM, Yao T-J, Wang W, et al. Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a retrospective cohort study. BMJ Open. 2012;2:e000825.
[121] Boeri M, Verri C, Conte D, et al. MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer. Proceedings of the National Academy of Sciences. 2011;108:3713–3718.

Circulating miRNAs	[Ref]	Evaluated in experimental models and tissues of melanoma	[Ref]
c-miR-186-5p	[34]	evaluated	[35]
c-let-7d-3p	[34]	nr	
c-miR-18a-3p	[34]	nr	
c-miR-145-5p	[34, 41]	evaluated	39]
c-miR-99a-5p	[34]	nr	
c-miR-664-3p	[34]	evaluated	[36]
c-miR-501-5p	[34]	nr	
c-miR-378a-5p	[34]	nr	
c-miR-29c-5p	[34, 55]	nr	
c-miR-1280	[34]	evaluated	[37]
c-miR-365a-3p	[34]	nr	
c-miR-1249	[34]	nr	
c-miR-328	[34]	evaluated	[38]
c-miR-422a	[34]	nr	
c-miR-30d-5p	[34]	nr	
c-miR-17-3p	[34]	evaluated	[40]
	[41]	evaluated	[42]
	[41]	evaluated	[48, 49, 50]
	[41]	evaluated	[43]
	[41,76, 91]	evaluated	[45, 46, 47, 48]
c-miR-221-3p	[51]	evaluated	[51, 52, 53]
c-miR-324-3p	[55]	nr	
c-miR-125b-5p	[56]	evaluated	[45, 56,57]
c-miR-210	[59]	evaluated	[60, 61, 62]
c-miR-16-5p	[18]	evaluated	[18, 63, 64]
c-miR-211-5p	[18]	evaluated	[18, 65, 66, 67]
c-miR-4487	[18]	evaluated	[18]
c-miR-4706	[18]	evaluated	[18]
c-miR-4731	[18]	evaluated	[18, 69]
c-miR-509-3p	[18]	evaluated	[18, 44]
c-miR-509-5p	[18]	evaluated	[18, 44]
c-miR-374a-5p	[70]	evaluated	[44]
c-miR-204-5p	[70]	evaluated	[74]
c-miR-27a-3p	[70]	evaluated	[75]
c-miR-200c-3p	[70]	evaluated	[70, 71, 72]
c-miR-373-5p	[70]	nr	
c-miR-320a	[76]	nr	
cf-181a-5p	[76]	nr	
c-miR-1246	[77]	evaluated	[40]
c-miR-185-5p	[77]	evaluated	[74]
c-miR-15b-5p	[79]	nr	
c-miR-149-3p	[79]	evaluated	[78]
c-miR-150-5p	[79]	evaluated	[80]
c-miR-193a-3p	[79]	evaluated	[81]
c-miR-524-5p	[79]	nr	
c-miR-424-5p	[4]	evaluated	[46]
c-miR-548	[4]	nr	
c-miR-34a-5p	[4]	evaluated	[83]
c-miR-497-5p	[4]	evaluated	[84]
c-miR-299-3p	[4]	nr	
c-miR-205-5p	[4]	evaluated	[46]
c-miR-1269a	[4]	nr	
c-miR-624-3p	[4]	evaluated	[85, 86]
c-miR-138-5p	[4]	evaluated	[87]
c-miR-1-5p	[4]	evaluated	[88]
c-miR-152-3p	[4]	evaluated	[89]

c-miR-1910-5p	[4]	nr	
c-miR-181b-5p	[4]	evaluated	[85]
c-miR-3928-3p	[4]	evaluated	[90]
c-miR-3131	[4]	nr	
c-miR-301a-3p	[4]	evaluated	[85]
c-miR-1973	[4]	evaluated	[91]
c-miR-520d-3p	[4]	evaluated	[85]
c-miR-548a-5p	[4]	evaluated	[92]
c-miR-548a-3p	[4]	nr	
c-miR-454-3p	[4]	evaluated	[85]
c-miR-4532	[4]	evaluated	[40]
c-miR-1537-3p	[4]	evaluated	[94]
c-miR-553	[4]	nr	
c-miR-764	[4]	nr	
c-miR-1302	[4]	nr	
c-miR-1258	[4]	nr	
c-miR-522-3p	[4]	nr	
c-miR-1264	[4]	nr	
c-miR-1306-5p	[4]	nr	
c-miR-219a-2-3p	[4]	nr	
c-miR-431-5p	[4]	nr	
c-miR-450a-5p	[4]	nr	
c-miR-2682-5p	[4]	nr	
c-miR-337-5p	[4]	nr	
c-miR-27a-3p	[4]	nr	
c-miR-4787-3p	[4]	nr	
c-miR-154-5p	[4]	nr	
c-miR-532-5p	[97]	evaluated	[98]
c-miR-106b	[97]	evaluated	[99]
c-miR-134-5p	[103]	evaluated	[100, 101]
c-miR-320a-3p	[103]	evaluated	[102, 103]
c-miR-92b-3p	[103]	nr	
c-miR-98-5p	[103]	evaluated	[106]
c-miR-16-3p	[103]	nr	
c-let-7b	[103]	evaluated	[107, 108]
c-miR-1827	[103]	nr	
c-miR-1180	[103]	nr	
c-miR-628	[103]	nr	
c-miR-486	[103]	evaluated	[110]

nr : not reported

Table 1. Expression of circulating miRNAs proposed as diagnostic biomarkers in experimental models and in tissues of melanoma.

C-miRNAs	Source	Technique	Normalization	Melanoma patients	Healthy subjects	References
c-miR-186-5p **c-let-7d-3p** **c-miR-18a-3p** **c-miR-145-5p** **c-miR-99a-5p** **c-miR-664-3p** **c-miR-501-5p** **c-miR-378a-5p** c-miR-29c-5p **c-miR-1280** **c-miR-365a-3p** **c-miR-1249** **c-miR-328** **c-miR-422a** c-miR-30d-5p c-miR-17-3p	Whole blood	qRT-PCR	RNU48	11 (independent validation set) 5 stage I 3 stage II 1 stage III 2 stage IV 24 (test set) 3 stage 0 15 stage I 4 stage II 1 stage III 1 stage IV	20	34
c-miR-10b-5p c-miR-145-5p c-miR-155-5p c-miR-195-5p c-miR-21-5	Whole blood	qRT-PCR	miR-16	10 3 stage I 5 stage II 1 stage III 1 stage IV	63	41
c-miR-221-3p	Serum	qRT-PCR	Cel-miR-54	90 8 stage 0 20 stage 1 20 stage 2 17 stage 3 12 stage IV 13 not determined	?	51
c-miR-29c-5p c-miR-324-3p	Serum	qRT-PCR	NormFinder, Genorm	28 28 stage IV	20	55
c-miR-125b-5p	Serum exosomes	qRT-PCR	Cel-miR-54	21 15 stage IV 6 ?	19	56
c-miR-210	Plasma	RT-qPCR-DP	dCq = mean Cq values (1 ng of RNA from M14) − mean Cq values (each sample)	46 (pilot study) 20 stage III 26 stage IV 218 (verification study) cohort A: 60 stage III 70 stage IV cohort B: 88 stage III	6 35	59
c-miR-16-5p **c-miR-211-5p**	Serum	qRT-PCR	Cel-miR-39	255 86 stage I-II	130	18

c-miR-4487 c-miR-4706 c-miR-4731 c-miR-509-3p c-miR-509-5p				50 stage III 119 stage IV		
c-miR-374a-5p c-miR-204-5p c-miR-27a-3p c-miR-200c-3p **c-miR-373-5p**	Serum	qRT-PCR	5 most stable miRNAs by RefFinder	52 4 stage 0 11 stage I 17 stage II 11 stage III 9 stage IV	30	70
c-miR-21-5p **c-miR-320a** **cf-181a-5p**	Plasma Plasma Plasma/serum	ddPCR	Cel-miR-39-3p	8	18	76
c-miR-1246 **c-miR-185-5p**	Plasma	Microarray qRT-PCR	miSPIKE solution	14 (screening) 3 stage III 11 stage IV 29 (training) 9 stage III 20 stage IV 31 (validation) 3 stage III 28 stage IV	5 16 43	77
c-miR-15b-5p **c-miR-149-3p** **c-miR-150-5p** c-miR-193a-3p c-miR-524-5p	Plasma	qRT-PCR	Global mean Normalizatio, NormFinder	30 14 stage I-II 16 stage III-IV	32	79
c-miR-424-5p c-miR-548I **c-miR-34a-5p** **c-miR-497-5p** **c-miR-299-3p** c-miR-205-5p c-miR-1269a c-miR-624-3p c-miR-138-5p c-miR-1-5p **c-miR-152-3p** **c-miR-1910-5p** **c-miR-181b-5p** c-miR-3928-3p c-miR-3131 **c-miR-301a-3p** c-miR-1973 c-miR-520d-3p **c-miR-548a-5p** c-miR-548a-3p **c-miR-454-3p**	Plasma	Whole-microRNAome profiling	Spike-In Control	32 4 stage I 18 stage II 4 stage III 4 stage IV	16	4

c-miR-4532 **c-miR-1537-3p** c-miR-553, c-miR-764 c-miR-1302 **c-miR-1258** c-miR-522-3p c-miR-1264 c-miR-1306-5p c-miR-219a-2-3p **c-miR-431-5p** **c-miR-450a-5p** **c-miR-2682-5p** **c-miR-337-5p** **c-miR-27a-3p** c-miR-4787-3p **c-miR-154-5p**						
c-miR-532-5p **c-miR-106b**	Serum exosomes	RT-PCR	RNU6	30 (screening) 95 (training) 25 (blinded tests) 29 stage I 30 stage II 46 stage III 45 stage IV	30 (screening) 95 (training) 25 (blinded tests)	95
c-miR-134-5p c-miR-320a-3p **c-miR-21-5p** **c-miR-92b-3p** (= stage 0) **c-miR-98-5p** (down: stage II) **c-miR-16-3p** **c-let-7b** (down: stage I/II and III) c-miR-1827 c-miR-1180 (up: stage 0) **c-miR-628** (down: stage I/II and IV) c-miR-486 (up: stage 0)	Plasma	RT-PCR	NormFinder c-miR-24 c-miR-191	96 29 stage 0 17 stage I 16 stage II 27 stage III 7 stage IV	28	103

Bold: up-regulated compared to healthy subjects; Not-bold: down-regulated compared to healthy subjects; qRT-PCR = quantitative RT-PCR; NGS = Next Generation Sequencing; ddPCR = droplet digital PCR; qRT-PCR-DP = quantitative RT-PCR directly plasma

Table 2. Circulating miRNAs proposed as diagnostic biomarkers in cutaneous melanoma.

C-miRNAs	Source	Cancer Types										
		Melanoma	Colon	Renal	Breast	Lung	Thyroid	Ovarian	Prostate	Stomach	Wilms tumor	References
c-miR-29c-5p	Serum	-	+	+	nr	nr	nr	nr	nr	nr	nr	[55]
c-miR-320a	Plasma	+	-	nr	-	-	nr	nr	nr	nr	nr	[76]
15 c-miRNA of MEL38	Plasma	de	de	de	de	de		de	de	de	de	[4]

nr : not reported; de: differentially expressed; +: increase; -: decrease.

Table 3. Circulating miRNAs differentially expressed between melanoma and other cancer.

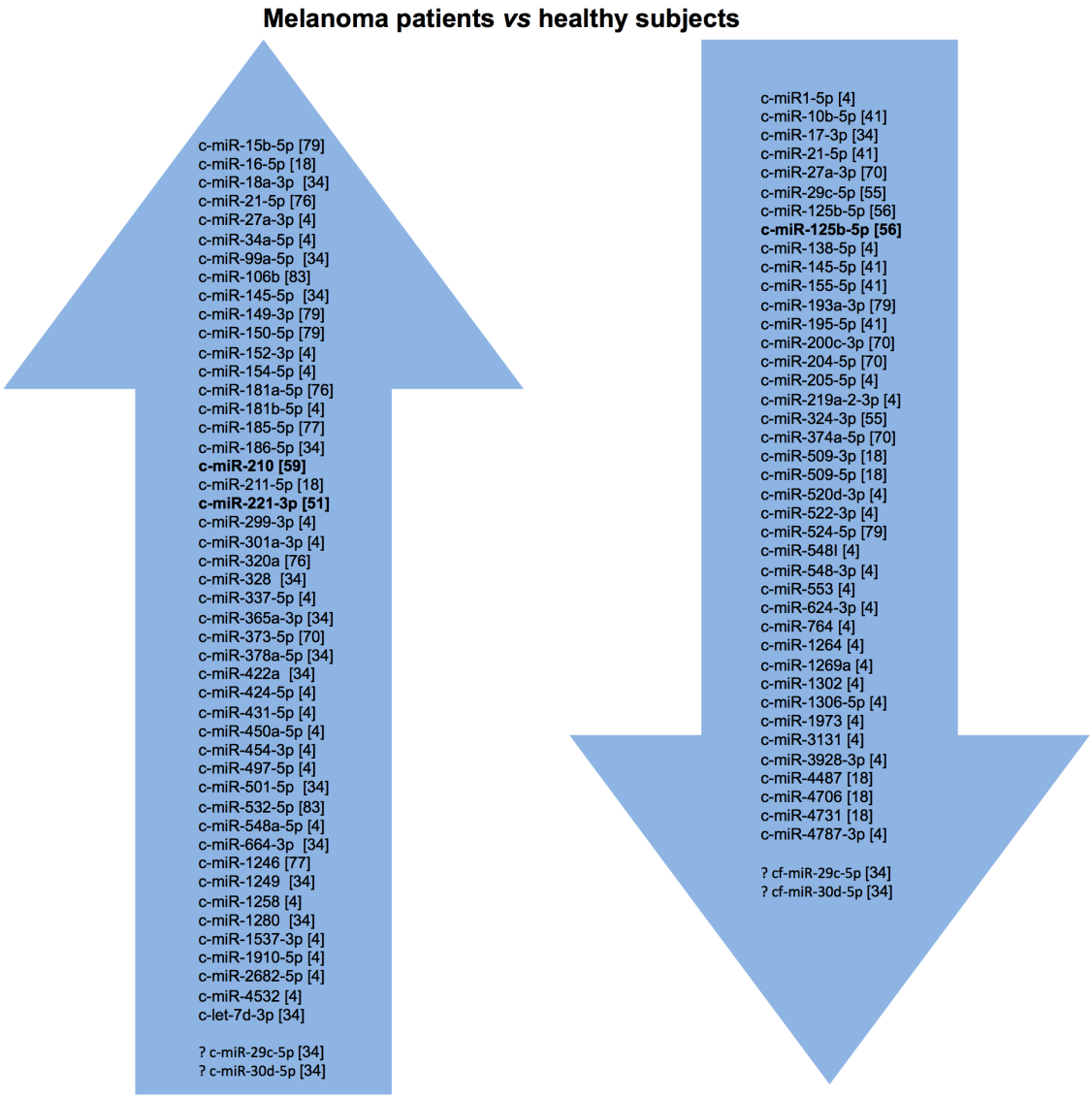

Figure 1. Circulating miRNAs up and down-regulated in melanoma patients compared to healthy subjects. In bold, c-miRNAs with diagnostic potential as single biomarkers (?: deregulation not specified).

Legend

Table 1. Expression of circulating miRNAs proposed as diagnostic biomarkers in experimental models and in tissues of melanoma.

Table 2. Circulating miRNAs proposed as diagnostic biomarkers in cutaneous melanoma.

Table 3. Circulating miRNAs differentially expressed between melanoma and other cancer.

Figure 1. Circulating miRNAs up and down-regulated in melanoma patients compared to healthy subjects. In bold, c-miRNAs with diagnostic potential as single biomarkers (?: deregulation not specified).