

Circulating cell-free microRNAs in cutaneous melanoma staging and recurrence or survival prognosis

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Running title: Circulating cell-free microRNAs in cutaneous melanoma

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26 **Summary**

27 Cutaneous melanoma is a skin cancer with increasing incidence. Identification of novel clinical biomarkers
28 able to detect the stage of disease and suggest prognosis could improve treatment and outcome for melanoma
29 patients. Cell-free microRNAs (cf-miRNAs), are the circulating copies of short non-coding RNAs involved
30 in gene expression regulation. They are released into the interstitial fluid, are detectable in blood and other
31 body fluids and have interesting features of ideal biomarker candidates. They are stable outside the cell,
32 tissue specific, vary along with cancer development and are sensitive to change in the disease course such as
33 progression or therapeutic response. Moreover, they are accessible by non-invasive methods or
34 venepuncture. Some articles have reported different cf-miRNAs with the potential of diagnostic tools for
35 melanoma staging, recurrence and survival prediction. Although some concordance of results is already
36 emerging, differences in analytical methods, normalization strategies and tumour staging still will require
37 further research and standardization prior to clinical usage of cf-miRNA analysis.

38 This article reviews this literature with the aim of contributing to a shared focusing on these new promising
39 tools for melanoma treatment and care.

40 **Keywords**

41 Circulating cell-free microRNA, cutaneous melanoma, biomarker, staging, prognosis, recurrence, blood.
42

Introduction

Cutaneous melanoma is a skin cancer whose incidence is increasing by 3% every year (Garbe, Peris & Eggermont, 2016). In the AJCC staging system, patients with melanoma are categorized into three groups: localized (stage I–II, no evidence of metastases), regional (stage III, spread to regional lymph nodes), and distant (stage IV, metastatic sites) disease (NCCN Melanoma, 1. 2017). Cancer stage at diagnosis influences survival, with a 5-year survival rate of 98.5, 62.9, and 19.9% for localized, regional, and distant cutaneous melanoma, respectively (SEER Cancer Statistics Factsheets, 2017).

Elevated circulating lactate dehydrogenase (LDH) levels are the sole validated independent prognostic marker with high specificity (92%) and sensitivity (79%) in stage IV melanoma (Weinstein et al., 2014; Deichmann, Benner & Naher, 1999). Although other circulating markers have been investigated, none of them was easily measurable and suitable for dynamic monitoring of the disease. Therefore, the search for novel clinical biomarkers represents one of the most intriguing topics in melanoma research.

MicroRNAs (miRNAs or miRs) are short non-coding RNAs able to regulate gene expression. They are released into extracellular space and subsequently appear in body fluids due to cell death, blebbing of apoptotic bodies, budding and shedding of macrovesicles, active secretion in the form of exosomes and of miRNA complexes with proteins (argonaute 2 (AGO2), nucleophosmin 1 (NPM1), and others) and high-density lipoproteins (HDLs) (Zandberga, Kozirovskis & Line, 2013; Sun et al., 2012). All these forms of cell-free miRNA (cf-miRNA) are highly stable in the bloodstream and other body fluids. They can be found in plasma, serum, urine, saliva, and milk, where they are protected by membranes in exosomes and other microparticles, by various proteins, lipids and, possibly, other molecules. Due to diffusion and circulation, their organ, tissue, or cell origin is difficult to determine *in vivo*, but even so they may become suitable novel markers in the future because of the following interesting properties: i) cf-miRNAs expression is related with tumour features (Peng & Croce, 2016) ii) cf-miRNAs are stable in biofluids and after extraction (Schwarzenbach et al., 2014) iii) cf-miRNAs can be profiled by quantitative real-time PCR (qRT-PCR), microarray or sequencing technology (Pritchard et al., 2012). Therefore, circulating cf-miRNAs may represent easily accessible markers helping clinicians in monitoring cutaneous melanoma progression and treatment response.

This article reviews the literature regarding melanoma related circulating cf-miRNAs with the aim of contributing to harmonization and rationalization of research results coming from different experimental approaches.

General features of miRNAs

In the mammalian genome, approximately 60–70% of total DNA is transcribed in RNA and only about 2% in protein-coding RNA. In the past 25 years, increasing evidence has been obtained that genome sequences traditionally considered as non-functional have instead gene regulatory capacity (Lee et al., 1993). Indeed, multiple types of non-coding RNAs, both long and small, regulate the functional status of chromatin and transcription in eukaryotes (Ghildiyal & Zamore, 2009; Mattick & Makunin, 2006), among them the miRNAs. They are a conserved class of small non-coding RNAs of 19–25 nucleotides, whose biogenesis is

80 tightly controlled (Figure 1). These miRNAs also function as regulators of phenotype expression (Ha &
81 Kim, 2014). Currently, nearly three thousand human miRNA sequences have been identified in the human
82 genome (miRBase release 22 march 2018) but their number is expected since long time to be higher
83 (Friedländer, Lizano & Estivill, 2014). MiRNA biogenesis begins with transcription of a long primary-
84 miRNA precursor (pri-miRNA, several hundred nucleotides long) by RNA polymerase II in the cell
85 nucleus. The pri-miRNA with a stem-loop structure is then cropped into a shorter second miRNA
86 precursor, the pre-miRNA (70 nucleotides stem-loop) by the nuclear ribonuclease DROSHA (a RNase III
87 family member) and by double-stranded RNA-binding proteins (e.g., DGCR8). Pre-miRNA is transported
88 into the cytoplasm by Exportin-5/Ran-GTP and then cleaved into a double-stranded miRNA molecule with
89 driver and passenger strands, the miRNA-5p-miRNA-3p duplex (21 nucleotides each strand). This second
90 processing step is carried out by the ribonuclease DICER (an endoribonuclease of the RNase III family)
91 and by the transactivation-responsive RNA-binding protein (TRBP). Both strands can act as mature
92 miRNAs. Generally, only the driver (guide) strand (miRNA-5p) is incorporated into a miRNA-induced
93 silencing complex (miRISC) becoming a functional miRNA, while the passenger strand (miRNA-3p,
94 previously indicated as miRna*) is quickly degraded most of the time (Jonas & Izaurralde, 2015; Ha &
95 Kim, 2014). Indeed, even the passenger strand (miRNA-3p) can be functional and the guide strand
96 (miRNA-5p) is not always the major functional strand (Jonas & Izaurralde, 2015).

97 Commonly, miRISC binds target mRNA in complete or incomplete way acting as translational repressors,
98 regulating $\approx 50\%$ of all protein-coding genes (Schwarzenbach et al., 2014; Krol et al., 2010). In case of
99 complete complementarity, miRISC induces degradation of mRNA target. In case of incomplete
100 complementarity, miRISC induces inhibition of translation or mRNA deadenylation (Jonas & Izaurralde,
101 2015). MiRNAs can also regulate gene expression at the transcriptional level by direct binding to DNA
102 (Khraiwesh, Arif & Frank, 2010; Gonzalez et al., 2008; Kim et al., 2008) and act as expression activators
103 by targeting gene regulatory sequences (Portnoy et al., 2011). It has been reported that miRNAs can also
104 bind to ribonucleoproteins in a RISC-independent manner and interfere with their RNA binding functions
105 (decoy activity) (Beitzinger & Meister, 2010). Finally, very recently, the capability of miRNAs of binding
106 to protein receptors, in particular Toll-like receptors (TLRs) named also miRceptors, triggering their
107 downstream signaling pathways, has been reported (Fabbri, 2018). The core element responsible for
108 miRNA-TLRs recognition is unknown but some structural features able to determine miRNA-TLRs
109 interactions have been described, i.e. GU-rich motifs (GUUG for miR-21, GGUU for miR-29a, and
110 GUUGUGU for let-7b). The ability of miRNAs to activate TLRs on immune cells may be relevant in
111 modulation of tumor microenvironment biology (Vannini, Fanini & Fabbri, 2018).

112 A key feature to fully appreciate the biological relevance of miRNAs is that any miRNA may have the
113 potential to regulate the expression of hundreds of target genes, highlighting the important role of this
114 specific form of regulation in cell identity (Jonas & Izaurralde, 2015). As a matter of fact, gene regulation
115 by miRNAs has been linked to proliferation, differentiation, apoptosis, survival, immune response and
116 response to stress, that is biological events primarily altered during the pathophysiological processes

117 underlying many human diseases, including cancer (Tuna et al., 2016). The abnormal expression of genes
 118 coding for miRNAs in malignant *versus* normal cells could be due to their location in cancer-associated
 119 genomic regions, epigenetic mechanisms and/or alterations in the miRNA processing machinery (Iorio &
 120 Croce, 2017; Krol et al., 2010; Calin & Croce, 2006). A few decades ago, alterations in oncogenes and/or
 121 tumour suppressor genes were considered the causes of tumorigenesis. The discovery of small non-coding
 122 RNAs has shed light on the larger complexity of the mechanisms involved in cancer development and
 123 progression (Calin & Croce, 2006). Of note, miRNAs' expression profiles are markedly deregulated in
 124 solid and hematopoietic tumours, suggesting the existence of tumour-specific miRNA fingerprints (Iorio &
 125 Croce, 2017; Mirzaei, Gholamin & Avan, 2016).

126 While approximately 300 miRNAs have demonstrated a role in carcinogenesis, more than 2000 miRNAs
 127 have been found differentially expressed in human cancers (Yang et al., 2017) and evidence that different
 128 types of cancer can be discriminated by miRNAs profiling has been reported (Iorio & Croce, 2017;
 129 Petrovic et al., 2017). In cutaneous melanoma, there is increasing evidence for a significant role of specific
 130 miRNAs as oncogenes or tumour suppressor genes (Varamo et al., 2017; Mirzaei, Gholamin & Avan,
 131 2016; Mione & Bosserhoff, 2015) as well as their involvement in other aspects of melanoma biology (Luo,
 132 Weber & Eichmuller, 2014; Sun, Zhou & Dar, 2014; Voller et al., 2013a; Leibowitz-Amit et al., 2012).

133 The diagnostic and prognostic potential of miRNA profiling in solid specimens of the primary or secondary
 134 tumour lesions emerged in parallel with progress in understanding their role in melanoma biology but still
 135 today without application in routine clinical practice. A new perspective of clinical interest has instead been
 136 opened by the discovery that the circulating forms of miRNA can be representative of different types of
 137 tumors and can indicate the stage of the tumor and its response to treatment (Kinoshita et al., 2016;
 138 Schwarzenbach et al., 2014; Wittmann & Jäck, 2010). Indeed, research has shown that the quantitative
 139 analysis of the cf-miRNAs may assist the clinician's decision in cutaneous melanoma both in terms of
 140 diagnosis and staging and recurrence prediction and prognosis (Carpi et al, 2016).

141 We review the current knowledge on cf-miRNAs' profile in melanoma staging, recurrence prediction and
 142 prognosis (Fig. 2) and discuss some theoretical and technical aspects of cf-miRNAs' analysis with the aim
 143 to determine whether or not measurements of miRNAs, whether singly or in a panel, will add additional
 144 useful information over standard clinical measures currently in use, such as stage, sex, age, LDH and S100
 145 calcium-binding protein B (S100B).

146 In this perspective, rational reviewing cannot disregard the evidence that full knowledge of disease biology
 147 is not necessary for diagnosis. For example, detection of Congo Red-stained β -amyloid substance allows
 148 the diagnosis of Alzheimer's disease even if the aetiology and pathophysiology of the disease are not yet
 149 fully elucidated.

150 Indeed, most of the studies dealing with the cf-miRNA diagnostic potential took advantage from the
 151 evidence of miRNA dysregulation in melanoma cells, in terms of selection of potential miRNA candidates
 152 as diagnostic tools, but they were essentially heuristic in nature. Knowledge of miRNA role in cancer and
 153 melanoma is still incomplete and in progress and a logical framework rationalising miRNA changes in

neoplastic cells is still lacking. Nevertheless, the number of intra-assay measurements that can be obtained by dedicated techniques, such as RT-PCR on microarrays, and correctness of statistical analysis, both in terms of statistic tool selection and appropriate data management, can lead to diagnostic affordable conclusions also in absence of full understanding of disease biology and even in contrast with its current knowledge.

Circulating cf-miRNAs in cutaneous melanoma staging

Seven articles have evaluated the use of circulating cf-miRNAs as staging biomarkers (Table 1). The first study (Shiyyama, Fukushima & Ihn, 2013) identified a cf-miRNA panel useful to detect the presence of metastasis in patients with melanoma. It consists of serum cf-miR-9-5p, cf-miR-145-5p, cf-miR-150-5p, cf-miR-155-5p, and cf-miR-205-5p. This combination discriminated between primary and metastatic melanoma patients while improving the area under the receiver operating characteristic (ROC) curve to 0.77 (95% confidence interval, 0.58–0.96) compared to each cf-miRNA alone. The panel includes miRNAs for which a role in melanoma has been described also by other authors.

A functional role of miR-145-5p in the inhibition of proliferation, migration, invasion and apoptosis via mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) pathways in melanoma cell lines has been reported by Liu, Gao & Zhao, 2017. Moreover, evaluated in whole blood as diagnostic biomarker, it showed an opposite trend in melanoma patients compared to healthy controls (Leidinger, Keller & Meese, 2010; Heneghan et al., 2010).

MiR-150-5p has been associated with recurrence-free survival (RFS) in melanoma patients and associated with patient outcome (Jayawardana, Schramm & Yang, 2016; Friedman, Shang & Osman, 2012) although its role in cutaneous melanoma is unclear (Latchana et al., 2016).

MiR-155-5p has been observed to have significantly lower expression in plasma of melanoma patients than in controls (Heneghan et al., 2010). Moreover, miR-155-5p expression in tissues has been correlated to melanoma patient outcome (Jayawardana, Schramm & Yang, 2016) in accordance with the finding that this miRNA is over-expressed in melanoma tissues (Latchana et al., 2016). However, a decreased expression of this miRNA in melanoma cell lines has been observed *in vitro* where its experimental overexpression regulated cellular proliferation and apoptosis, in part, through inhibition of SKI (v-ski avian sarcoma viral oncogene homolog) protein (Levati, Pagani & D'Arti, 2011).

MiR-9-5p, on the contrary, is significantly down regulated in melanoma tissues compared to adjacent normal ones (Xu et al., 2016). A role of miR-9-5p as tumour suppressor is also suggested by its inhibition of melanoma development and progression through down-regulation of neuropilin-1 (NRP1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB1)-Snail1 pathway (Xu et al., 2016; Liu, Kumar & Xu, 2012).

A down regulating activity in melanoma tissues and a role as tumour suppressor has been reported as well for miR-205-5p, whose low levels are significantly associated with worse clinical outcome (Xu et al., 2012; Hanna et al., 2012; Dar, Majid & Kashani-Sabet, 2011). The tumour suppressor function of miR-205-5p is

clearly shown in the study of Dar et al. (2011) where a stable ectopic overexpression of this miRNA suppressed melanoma cell proliferation, colony formation and tumour cell growth *in vivo* in a melanoma animal model and induced a senescence phenotype accompanied by elevated expression of p16^{INK4A} protein, the main member of the Ink4 family of cyclin-dependent kinase CDK inhibitors, and of other markers of senescence. The effect on melanoma cell growth and senescence was partially reversed by overexpressing the E2 factor 1 (E2F1), a key regulator of G1/S transition phase in the cell cycle that is, in turn, a target of miR-205 (Dar, Majid & Kashani-Sabet, 2011).

The staging potential of single cf-miRNAs has been found as well. A unique plasma cf-miRNA, cf-miR-21-5p, was studied by Saldanha and collaborators and a positive trend from controls to advanced melanoma patients was found (Saldanha, Potter & Pringle, 2013). Although the decrease in this miRNA in whole blood of melanoma patients reported by Heneghan and collaborators was not statistically significant (2010), the increase of miR-21-5p levels in plasma of melanoma patients in respect to healthy subjects is in line with data reported by Ferracin and collaborators (Ferracin, Lupini & Negrini, 2015). A significant increase of miR-21-5p was observed also in melanoma tissues (Wandler, Riber-Hansen & Steiniche, 2017; Babapoor, Wu & Dadras, 2017). The correlation of this miRNA with melanoma aggressive phenotype has been linked to its targeting proteins that negatively modulate in proliferation, invasion, migration and apoptosis (Latchana et al., 2016; Mao, Chen & Xu, 2017) and its expression in melanoma tissues may thus have implications also as independent melanoma prognostic factor (Jiang, Lv & Li, 2012).

In the paper of Li et al., (2014), I-II melanoma stages were differentiated with respect to III-IV stages by circulating cf-miR-221-3p. Its serum levels were significantly lower in patients at I-II compared to III-IV stages ($p < 0.001$), suggesting that this miRNA may be a useful biomarker for staging. MiR-221-3p indeed is involved in proliferation of melanoma (Das, Sokhi & Fisher, 2010; Felicetti, Errico & Care, 2008) as well as many other cancer cell types representing a possible new target for innovative nucleic acid-based anticancer therapy (Di Martino, Rossi & Tassone, 2016). MiR-221, already described as potential diagnostic and prognostic biomarker in melanoma (Mirzaei et al., 2016), is part of the oncomiR-221&222 that increases along with the progression of the disease targeting AP2 α , a transcriptional activator of the tumor suppressor miR-126 (Felli et al. 2016). Moreover, the *in vitro* antimelanoma activity of resveratrol was mediated by decreased expression of miR-221 achieved through inhibition of the NF- κ B (RELA) activity and by the increased expression of the tumor suppressor gene tropomyosin receptor kinase [TRK]-fused gene (TFG), identified as target of miR-221 (Wu & Cui, 2017).

MELmiR-7, a panel composed by 7 cf-miRNAs, i.e. cf-miR-16-5p, cf-miR-211-5p, cf-miR-4487, cf-miR-4706, cf-miR-4731, cf-miR-509-3p, and cf-miR-509-5p, may discriminate among stage I-II with 93% sensitivity, stage III with 86% sensitivity and stage IV with 95% sensitivity (Stark, Klein & Hayward, 2015). The authors claimed MELmir-7 panel as a tool more efficient than LDH and S100B in predicting melanoma progression and recurrence, with a good relevance also in diagnosis and prognosis of melanoma. Of the cf-miRNAs in this panel, miR-16-5p and miR-211-5p have been reported to act as tumour suppressors in different reports (Guo, Guo & Li, 2016; Mazar, Qi & Perera, 2016; Yu & Yang, 2016; Poell, Venza,

228 Visalli & Venza, 2015; van Haastert & Cuppen, 2012), as well as miR-4731 in a single report (Stark, Tom &
229 Hayward, 2016). Lastly, both the miR-509 filaments, -3p and -5p, were found to control the expression of
230 proteins involved in epithelial-mesenchymal transition (EMT) in melanoma cells (Wang, Li & Zhao, 2015).

231 Research on cf-miRNAs in melanoma takes advantage also of a heuristic approach. Margue and
232 collaborators (Margue, Reinsbach & Kreis, 2015) performed qRT-PCR on a 1066 miRNAs array (Qiagen,
233 miRBase v.16) and compiled a reference data set they assumed as the healthy cf-miRNome, by integrating
234 information on differentially expressed miRNAs by multiple comparisons, previous data and literature.
235 Among the investigated miRNAs in healthy subjects, 63 % were not expressed in serum samples, 3%
236 fluctuated between individuals and only 8% (82 cf-miRNAs with a cycle quantification (Cq) values below 27
237 in 80% of samples) showed high expression values in serum samples, without evidence of circadian rhythm
238 or gender difference. This non-fluctuating standardized healthy cf-miRNome was used as the “baseline
239 control” for the profiling studies. The following cf-miRNAs panels resulted then from comparison of patient
240 sera and data of the Margue’s cf-miRNome: cf-miR-301a-3p and cf-miR-451a for stage 0 (AUC of ROC
241 curve 0.73); cf-miR-200c-3p, cf-miR-126-5p and cf-miR-373-5p for stage I (AUC 0.91); cf-miR-374-5p, cf-
242 miR-1260a and cf-miR-211-5p for stage II (AUC 0.80); cf-miR-204-5p, cf-miR-301a-3p, cf-miR-200c-3p,
243 cf-miR-182-5p, cf-miR-193b-3p, cf-miR-720, cf-miR-205-5p, cf-miR-206, cf-miR-550a-3p and cf-miR-22-
244 3p for stage III (AUC 0.99); cf-miR-200c-3p, cf-miR-16-5p, cf-miR-211-5p, cf-miR-627-5p and cf-miR-
245 629-5p for stage IV (AUC 0.97) (Margue, Reinsbach & Kreis, 2015).

246 Some of the miRNAs in the above panels have been analysed in blood also by other authors. Cf-miR-16-5p
247 and 211-5p have been already dealt with in this review since they are both included in the panel suggested by
248 Stark, Klein & Hayward, 2015 and cf-miR-16-5p is also reported below from Guo et al., 2016. Analogously,
249 cf-miR-205-5p was one of the cf-miRNAs in the staging panel suggested by Shiiyama, Fukushima & Ihn,
250 2013 (upper) as well as cf-miR-200c-3p is reported as single miRNA with staging potential by Fogli, Polini
251 & Nieri, 2017 (below). Cf-miR-550a-3p was found over-expressed in the whole blood of melanoma patients
252 compared to healthy subjects by Leidinger, Keller & Meese, 2010, while cf-miR-22-3p was reported by
253 Saldanha, Potter & Pringle, 2013 for its prognostic value (see the following paragraph).

254 Margue and collaborators (2015) have also described two additional cf-miRNA panels able to select
255 healthy subjects and cutaneous melanoma stage 0, I and II patients (cf-miR-30b-5p, cf-miR-28-5p, cf-miR-
256 204-5p, cf-miR-27a-3p and cf-miR-1260a, AUC 0.83) or stage III and IV patients (cf-miR-204-5p, cf-miR-
257 182-5p, cf-miR-197-3p, cf-miR-720, cf-miR-211-5p, cf-miR-193b-3p, cf-miR629-5p, cf-miR-432-3p and cf-
258 miR-1280, AUC 0.81) (Margue, Reinsbach & Kreis, 2015). Among these miRNA panels, cf-miR-1280 was
259 already described to be over-expressed in the whole blood of melanoma patients by Leidinger, Keller &
260 Meese, 2010.

261 For other miRNAs in the panels, functional evidences on melanoma tissues/cells have been recently
262 reported. Cui, Li & Li, 2016 described the up-regulation of miR-301a-3p in melanoma tissues compared to
263 benign melanocytic nevi and its correlation with metastasis and poor prognosis. Moreover, they found that
264 miR-301a-3p could promote growth, colony formation, migration, invasion and chemo-resistance in

265 melanoma cells via activation of AKT and focal adhesion kinase (FAK) signals, at least partially by targeting
 266 phosphatase and tensin homolog protein (PTEN).

267 A down-regulation of cf-miR-126-5p, on the contrary, was observed in melanoma tissues and a significant
 268 difference was reported between dysplastic nevi and primary cutaneous melanoma and between primary and
 269 metastatic cutaneous melanomas (Lin et al., 2015). Furthermore, Felli, Felicetti & Caré, 2013 demonstrated
 270 that miR-126-5p plays a tumour suppressor role in melanoma through the direct or indirect repression of
 271 several key oncogenic molecules, for example disintegrin and metalloproteinase 9 (ADAM9) and matrix
 272 metalloproteinase-7 (MMP-7).

273 Also miR-204-5p has recently been reported to act as tumour suppressor in melanoma, decreasing cell
 274 proliferation, migration and invasion, and promoting cell apoptosis in this cancer (Luan, Qian & Xu, 2017).
 275 The same study identified two direct targets of miR-204-5p in MMP-9 and B-cell lymphoma-2 (Bcl-2)
 276 protein.

277 Another suggested tumour suppressor is miR-193b-3p whose ectopic overexpression induced cell
 278 proliferation decrease in melanoma cell lines through regulation of cell cycle progression inducing down
 279 expression of cyclin-D1 and MCL-1 proteins (Chen, Zhang & Tron, 2011; Chen, Feilotter & Tron, 2010).
 280 Moreover, miR-204-5p expression was found at significantly lower levels in primary melanoma than in
 281 benign nevi (Chen, Feilotter & Tron, 2010).

282 Even miR-206 may be hypothesized to be a tumour suppressor because it was able to reduce growth and
 283 migration/invasion of different melanoma cell lines (Georgantas, Sreicher & Ranade, 2014). A G1 arrest in
 284 melanoma cell lines was induced by its ectopic overexpression, targeting directly cyclin C, cyclin D1, and
 285 cyclin-dependent kinase 4 (CDK4) (Georgantas, Sreicher & Ranade, 2014).

286 Further three miRNAs, miR-200c-3p, miR-211-5p and miR-1280 showed activity as tumour suppressor in
 287 melanoma (Xu et al., 2012; Liu et al., 2012; Mazar, Qi & Perera, 2016; Sun, Zhou & Dar, 2015).

288 Although not a direct tumour suppressor, miR-28-5p may decrease melanoma progression by enhancing
 289 cancer immunity (Li, Johnston & Min, 2016). Li and collaborators observed that miR-28-5p was able to
 290 regulate T cell exhaustion, increasing the T cell level of interleukin 2 (IL-2) and tumour necrosis factor- α
 291 (TNF- α), and probably restoring the cytokine secretion function of exhausted T cells in tumour.

292 An oncogenic role has been suggested instead for miR-182-5p. Its over-expression in melanoma cell lines
 293 promoted metastatic potential by decreasing forkhead box 3 (FOXO3) and microphthalmia-associated
 294 transcription factor (MITF) (Segura, Hanniford & Hernando, 2009).

295 Even miR-30b-5p was reported as oncogenic miRNA because its overexpression increased the invasive
 296 capacity of melanoma cells *in vitro* and metastatic potential *in vivo*, by suppressing polypeptide N-
 297 acetylgalactosaminyltransferase 7 (GALNT7) (Gaziel-Sovran, Segura & Hernando, 2011). A functional role
 298 was reported also for miR-374a-5p in the control of EMT in melanoma cells (Wang, Li & Zhao, 2015).

299 On the other hand, the functional role of other miRNAs whose cell-free circulating copies emerged as
 300 potential diagnostic tools in heuristic studies is still unknown or uncertain. In particular, miR-27a-3p was
 301 reported to be over-expressed in melanoma cell lines compared to melanocytes but not in melanoma biopsy

302 samples with respect to melanocytic nevi (Satzger, Mattern & Gutzmer, 2010), miR-720 was found up-
303 regulated in melanoma tissues compared to melanocytic nevi but its functional role was not investigated
304 (Sand, Skrygan & Bechara, 2013) and miR-373-5p tissue expression resulted not significantly deregulated in
305 melanoma tissues compared to melanocytic nevi (Satzger, Mattern & Gutzmer, 2010). On the other hand, the
306 role of other miRNAs in the panel identified by Margue and coll., i.e. miR-451a, miR-374-5p, miR-1260a,
307 miR-550a-3p, miR-627-5p, miR-629-5p, miR-432-3p, miR-197-3p and miR-22-3p, has not yet been
308 investigated in melanoma cells/tissues.

309 A recent article reported that serum cf-miR-16-5p level was remarkably decreased in patients at
310 advanced stages (III and IV AJCC stages vs. I and II AJCC stages) (Guo, Guo & Li, 2016). In the same
311 study, a tumour suppressor role of miR-16-5p was demonstrated in melanoma by *in vitro* and *in vivo*
312 experiments (Guo, Guo & Li, 2016).

313 Finally, a work from our laboratory (Fogli, Polini & Nieri, 2017) revealed the melanoma
314 oncosuppressor miR-200c-3p (Liu, Tetzlaff & Xu, 2012), to be significantly down-expressed in plasma of
315 melanoma patients at stage III-IV compared to early melanoma patients and healthy donors.

316 **Circulating cf-miRNAs as predictors of cutaneous melanoma recurrence and prognosis**

317 Biomarkers able to distinguish drug-responder from non-responder patients and predict melanoma recurrence
318 and progression may have profound impact on clinician decisions about patient therapy and care.
319 Seven articles have focused on circulating cf-miRNAs as prognostic biomarkers (Table 2).

320 A longitudinal study identified cf-miR-221-3p as a biomarker for melanoma with significantly
321 different levels between stage I-IV melanoma patients and healthy controls and reported a decrease of its
322 levels after surgical removal of the primary tumour followed by re-establishment of altered values in disease
323 recurrence (Kanemaru, Fukushima & Ihn, 2011).

324 The same circulating miRNA, cf-miR-221-3p, has been described, as a negative prognostic factor for
325 both overall survival (OS) and disease-free survival (DFS) in melanoma patients. Patients with high cf-miR-
326 221-3p serum levels had a significantly lower 5-year OS rate (22.1% vs. 54.6%) and recurrence free state
327 (RFS) rate (12.5% vs. 45.2%) than those with low serum levels (Li et al., 2014).

328 A panel of 5 serum miRNAs (cf-miR-150-5p, cf-miR-15b-5p, cf-miR-199a-5p, cf-miR-33a-5p, and
329 cf-miR-424-5p) was reported by Friedman, Shang & Osman, 2012 to be significantly associated with RFS in
330 a longitudinal evaluation on sera derived from melanoma patients (Friedman, Shang & Osman, 2012). This
331 cf-miRNA panel discriminated significantly between patients with high and low disease recurrence risk in
332 both discovery and validation cohorts. Friedmann and collaborators also reported on a longitudinal
333 evaluation of cf-miRNA expression in pre- and post-recurrence serum samples of 17 melanoma stage II
334 patients evidencing a statistically significant increase between expression levels of cf-miR-103a-3p and cf-
335 miR-221-3p at the time of primary diagnosis and at recurrence. The increase of cf-miR-221-3p is in line with
336 the data of Kanemaru, Fukushima & Ihn, 2011 mentioned above, and Li et al. (2014). Friedmann and
337 collaborators in the same paper also reported three cf-miRNAs (cf-miR-423-5p, cf-miR-424-5p and cf-miR-
338 199a-5p) (AUC = 0.89) able to increase the ability of thickness parameter alone (AUC=0.75) to distinguish

339 patients with melanoma recurrence (Friedman, Shang & Osman, 2012).
 340 MiR-199a-5p has been suggested to be a tumour suppressor since it is expressed at lower levels in
 341 melanoma than in control tissues. Moreover, ectopically over-expressed, it decreased melanoma proliferation
 342 targeting hypoxia-inducible factor 1 α (HIF-1 α) (Yang et al., 2016).
 343 MiR-33a-5p, down-expressed in melanoma cell lines compared to melanocytes (Zhou, Xu & Cao, 2015),
 344 functions as a tumour suppressor in melanoma cells by targeting HIF-1 α (Zhou, Xu & Cao, 2015) and the
 345 serine/threonine-protein kinase PCTAIRE1 (Tian et al., 2016).
 346 MiR-15b-5p was found over-expressed in melanoma tissues compared to melanocytic nevi and its
 347 deregulation induced an increased cell proliferation and a decreased apoptosis in melanoma cell line,
 348 supporting a possible oncogenic role (Satzger, Mattern & Gutzmer, 2010).
 349 MiR-423-5p was reported to be differentially expressed between thin and thick melanomas (Babapoor, Wu
 350 & Dadras, 2017).
 351 MiR-424-5p was significantly increased in invasive compared with *in situ* melanomas and dysplastic nevi
 352 (Babapoor, Wu & Dadras, 2017). On the contrary no data are reported about the involvement of miR-103a-
 353 3p in melanoma.

354 A list of ten plasma cf-miRNAs (cf-miR-211-5p, cf-miR-660, cf-miR-22-3p, cf-miR-579, cf-miR-
 355 215, cf-miR-296, cf-miR-425-5p, cf-miR-99a-5p, cf-miR-486-5p, cf-miR-486-3p) deregulated between
 356 blood samples of preoperative and postoperative patients having regional node resection for stage III
 357 melanoma was published by Saldanha, Potter & Pringle, 2013. MiR-211-5p and miR-425-5p were reported
 358 to be tumour suppressors in melanoma (Mazar, Qi & Perera, 2016; Liu, Hu & Hu, 2015). On the other hand,
 359 miR-296 was only reported as ineffective in vascular endothelial growth factor receptor 2 (VEGFR2) post-
 360 translational control in melanoma (Langenkamp, Zwiers & Molema, 2012) and miR-486 filaments, was
 361 significantly correlated with acral as compared to non-acral melanomas (Chan, Patel & Weidhaas, 2011). To
 362 date, miR-22-3p, miR-660, miR-579, miR-215 and miR-99a-5p do not result investigated in melanoma
 363 cells/tissues.

364 Similarly to what we described for cf-miR-221-3p, higher circulating levels of the cf-miR-210
 365 correlated with poorer prognosis associated with melanoma recurrence. This correlation was particularly
 366 interesting since higher circulating levels were identifiable before the clinical evidence of recurrence (Ono et
 367 al., 2015). In this study, samples from high number melanoma patients (n=218, stage III and IV) and 35
 368 healthy controls were used to assess the levels of cf-miR-210 and a significant prediction of disease
 369 recurrence was obtained while LDH levels did not show a significant predictive trend (Ono et al., 2015). In
 370 the same paper, the tissue miR-210 was found over-expressed in metastatic melanoma tissues compared to
 371 primary tumours (Ono et al., 2015). Of note, miR-210 has been reported to act as oncogene in different types
 372 of tumours (Gee, Camps & Harris et al., 2010; Campus, Buffa & Tagoussis, 2008) and the hypoxia-induced
 373 expression of miR-210 potentiated the immunosuppressive, tumor-promoting effects of myeloid-derived
 374 suppressor cells, resulting in increased tumor growth (Noman et al., 2015).

375 In the multicentre study conducted by Stark, Klein & Hayward, 2015, ‘MELmiR-7’ panel was

suggested as useful also in the routine follow-up of melanoma patients because it was more predictive than the two classical markers LDH and S100B.

Finally, cutaneous melanoma patients with low serum cf-miR-16-5p levels had a significantly shorter survival compared with those with high serum miR-16 levels suggesting the potential of cf-miR-16-5p in the prediction of melanoma patients prognosis (Guo, Guo & Li, 2016).

Conclusions

In the last years, several studies have correlated circulating cf-miRNA expression with disease stage and outcome in cutaneous melanoma patients. These must be considered pilot studies, conducted in small cohort of patients. However, the potential of circulating cf-miRNAs as biomarkers clearly is emerging in melanoma as in other cancers and diseases. Many cf-miRNAs have been at significantly higher or lower concentration in blood of melanoma patients than in healthy controls. These differences seem to correlate with stages of the disease or to be stage specific and seem to be of clinical relevance to predict recurrence and survival. Some cf-miRNAs have been identified as biomarkers in different and independent studies. Serum cf-miR-221-3p and cf-miR-211-5p have been identified as biomarkers in three articles published by different groups of researchers (Stark, Klein & Hayward, 2015; Margue, Reinsbach & Kreis, 2015; Li et al., 2014; Saldanha, Potter & Pringle, 2013; Friedman, Shang & Osman, 2012; Kanemaru, Fukushima & Ihn, 2011;) where they appeared significantly correlated with cutaneous melanoma aggressiveness and recurrence. Serum cf-miR-205-5p, miR-200c-3p and cf-miR-16-5p may be useful staging biomarkers (Fogli, Polini & Nieri, 2017; Stark, Klein & Hayward, 2015; Margue, Reinsbach & Kreis, 2015; Shiiyama, Fukushima & Ihn, 2013) while cf-miR-150-5p, cf-miR-22-3p and cf-miR-16-5p were reported each in two articles to be related to melanoma staging or melanoma survival prediction (Guo, Guo & Li, 2016; Margue, Reinsbach & Kreis, 2015; Stark, Klein & Hayward, 2015; Shiiyama, Fukushima & Ihn, 2013; Friedman, Shang & Osman, 2012; Saldanha, Potter & Pringle, 2013).

This convergence of results has occurred, despite differences in terms of biological matrix (serum or plasma) and use of internal standards for normalization of analytical results, although a clear trend towards shared methods is emerging, suggesting the results are relatively robust.

Concerning possible matrix effects on the analytical performance, it is well recognised that the correlation of cf-miRNA levels in matched plasma and serum can vary for each miRNA species and rely strongly on preparation. Every change in tissue collection steps (like source type, blood tube used, tissue centrifugation and conservation) determines changes in cf-miRNA levels (Ferracin, Lupini & Negrini, 2015; Zheng et al., 2013; Pritchard, Kroh & Tewari, 2012;). In particular, differences between serum and plasma could be linked to the permanence in plasma of microRNAs' containing microvesicles and exosomes (Skog, Wurdinger & Breakefield, 2008). Thereby comparison of miRNA profiles across studies in which tissues have been collected using different protocols is a potential methodological bias.

Analysis of very low copy number molecules like circulating cf-miRNAs relies on retrotranscription and pre-amplification. Due to the exponential amplification of the PCR-based methods, accuracy of these analytical

413 steps is a critical factor to attain accuracy of the overall analytical performance. Indeed, several variables,
414 inherent to the qRT-PCR workflow, may influence accuracy, including the sample matrix, the amount of
415 starting material, storage and manipulation of samples, enzymatic efficiencies and overall transcriptional
416 activity (Vandesompele, De Preter & Speleman, 2002). Consequently, accuracy of results in qRT-PCR-based
417 or microarray-based cf-miRNA expression profiling is largely dependent on a proper normalization strategy
418 (Brattelid, Aarnes & Jonassen, 2011; Mestdagh, Van Vlierberghe & Vandesompele, 2009). This is usually
419 accomplished by using internal reference standard sequences and/or computational methods (see Table 1 and
420 2) but at this “work in progress” stage of the research field we are discussing, no strategy has become a “gold
421 reference” among the laboratories.

422 Appropriate normalization reduces the technical variation within a dataset and minimizes the experimental
423 biases. Nevertheless, any variation in gene expression levels is always composed of both experimentally
424 induced (technical) and true biological variation. Currently, normalization of the experimental data through
425 internal reference standard sequences and/or computational methods addresses the problem of technical
426 variability (interassay variability). In particular:

427 - in microarray-based analyses, the large number of experimental data allows the use of normalisation
428 strategies based on algorithms such as RefFinder (search for the 5 most stable miRNAs) or NormFinder
429 (global mean normalization),

430 - in conventional qRT-PCR, specific primers may be used to amplify the spike-in added *Caenorhabditis*
431 *elegans* miRNA cel-miR-39-3p, cel-miR-54 and/or cel-miR-238 (sometimes referred as cel-39, cel-54 and
432 cel-238) or miR-16 or miR-191 to control for variations in recovery and amplification efficiency between
433 RNA preparations (Margue, Reinsbach & Kreis, 2015),

434 - specific primers may be used as well to amplify miR-451a (highly expressed in blood cells) (Koberle, Pleli
435 & Reid, 2013; Kirschner, Kao & Piiper, 2011) and miR-23a-5p (relatively stable in serum and not affected
436 by hemolysis) to estimate the occurrence of hemolysis (Blondal, Jensby Nielsen & Dahlsveen, 2013).

437 - specific primers may be used to amplify SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and
438 RNU6-2, that are highly expressed in blood cells and normally absent in serum to control for blood cell
439 contamination of the serum.

440 Different research groups use different normalisation strategies enabling them to attain self-consistent and
441 affordable results, in terms of control of the interassay variability in their own laboratory. Nevertheless,
442 quantitative comparison of data from different laboratories remains difficult or impossible due to
443 methodological differences that may influence the efficiency of RNA extraction, retrotranscription,
444 amplification and accuracy of quantitation. This is of importance from a clinical perspective, where a
445 diagnostic decision is to be taken for individuals and at different times for a single patient with no
446 comparison with “control” groups.

447 In addition, the problem of biological variability remains to be addressed. Indeed, unpredictable changes in
448 plasma composition and volume occurs during the homeostatic events of the ordinary life or in presence of
449 disease. In example, up to 10% variation may occur in volemia even in healthy condition, due to water

450 restriction or loss in perspiration and even larger in the presence of diseases such as anaemia, congestive
 451 heart failure, oedema, diarrhoea and many others. In the case of common blood chemistry analyses, the
 452 effect of plasma volume enlargement or restriction may often be of no critical importance in clinical
 453 decision, but it certainly cannot be neglected in the case of low copy number molecules that are substrates in
 454 amplification procedures, such as cf-miRNAs, since small differences in the initial number of molecules per
 455 unit volume will result in large differences after the amplification procedure.

456 In principle, the problem of biological variability among individuals and in a given individual might be
 457 addressed by amplifying with specific primers other endogenous non-coding RNAs such as miR-103 and
 458 miR-191, that have been reported as stably expressed in different normal and cancer tissues (Peltier &
 459 Latham, 2008), or the transcription products of housekeeping genes, such as small nuclear (U6) and small
 460 nucleolar (U24, U26) RNAs. This could give an “individual normalisation standard” enabling comparison of
 461 data obtained at different times in the same patient along his clinical history. Nevertheless, this goal can be
 462 attained only if a parallel and independent assessment of volemia is performed, with methods like
 463 radioisotope dilution (Perales, 2015). Otherwise, the normalisation performance of these “biological
 464 standards of expression” will be poor.

465 As stated above, in the clinical perspective, no control groups are available for comparison. Decision is to be
 466 taken on the basis of a decision threshold value. To this respect, a shared decision criterium is still lacking.

467 Methods based on microarray technology take advantage from the parametric (i.e. aleatory and continuous)
 468 nature of the recorded signal and the large number of analytes that are quantified in a single analysis. Indeed,
 469 microarray analysis make possible to assume as decision threshold the distance from the mean/median value
 470 of the normal distribution of data, either in terms of standard deviation (i.e. ± 3 sd) or of quantile (i.e. >95%-
 471 <5%) by applying appropriate non-parametric statistics, such as the Mann-Whitney or Wilcoxon distribution
 472 or ΔC_t (the difference between C_t values (threshold cycle) of the sample and for the 5 most stable miRNAs
 473 in repeated analyses or the global mean value, with a decision criterium set at $\Delta C_t = \pm 2-3$ fold) (Stark, Klein
 474 & Hayward, 2015). The addition to the reaction mixture of specific primers may be used to minimize the
 475 potential lack of reproducibility coming from the use of primer mix, whose real composition may vary from
 476 batch to batch. On the other hand, methods based on qRT-PCR are scientifically sound, suitable for further
 477 analysis in terms of ROC curves, but less suitable in itself for use in routine clinical practice, unless a large
 478 number of unrelated miRNAs is assessed in the same analytical session to establish a reference value and the
 479 circulating volume at the time of blood sampling is not measured independently.

480 In any case, based on the evidence obtained from all the studies, the role of decision panels seems to be more
 481 relevant than single cf-miRNA measurement to correctly classify the subjects enrolled. This evidence is not
 482 surprising in terms of decision theory. Indeed, the higher the number of parameters in use to decide, the
 483 higher the ability to discriminate among the different classes of subjects (i.e. true positive, false positive
 484 *etc.*). Nevertheless, some issues remain to be addressed to. First, evidence has been given of very broad
 485 confidence intervals of the area under the ROC curve, that may range from just above the “no benefit” 0.5
 486 value to the “perfect decision” 1.0 value in the study of Shiiyama, Fukushima & Ihn, 2013 and poor attention

487 has been paid to this critical parameter in other studies. Secondly, decision criteria in the case of subjects
 488 with positive evidence of only some but not all the cf-miRNAs of the decision panel must be established.
 489 Thirdly, the heuristic nature of some experiments we are reviewing raises the need of a dedicated discussion.
 490 In principle, differences in the cf-miRNA pattern among distinct stages may have a rational understanding
 491 for miRNAs that have been proven as possible co-factors in melanoma genesis and development, as
 492 oncogenes/oncosuppressors. Nevertheless, the effect of the uncontrolled cellular duplication on the
 493 circulating cf-miRNAs cannot be neglected. Indeed, the larger the number of neoplastic cells within the
 494 organism, the larger the number of surrounding cells suffering the aggressive behaviour of the disease. Cell
 495 disruption is a source of circulating cf-miRNAs and identification, as candidate analytes for melanoma
 496 staging, recurrence prediction and prognosis, of circulating cf-miRNAs with no evidence of direct
 497 involvement of their intracellular copies in melanoma biology may have a rational basis. Indeed, detection
 498 and quantitation of cf-miRNAs rely on the amplification of low copy number sequence in a biological
 499 specimen by qRT-PCR. As a limited amount of melanoma cells in the early stages of the disease may offer
 500 enough template molecules for their detection since amplification, so even a limited number of cells
 501 surrounding still unknown metastases may give enough molecules for a correct clinical decision on
 502 melanoma staging, recurrence or survival prediction. A clear example is given by cf-miR-210 that is induced
 503 by hypoxia and it has been already described as an independent prognostic factor in breast cancer (Campus,
 504 Buffa & Tagoussis, 2008). This being the case, affordability and reproducibility of the method in use
 505 becomes of paramount importance, even greater, if possible, than for other analyses of the clinical
 506 biochemistry. Fig. 3 and 4 summarise the potential sources of analytical biases in cf-miRNA assessment in
 507 melanoma and compare the possibilities offered by methods based on real time-PCR or microarray
 508 methodology.

509 To our knowledge, no studies still reported multivariate analysis to assess specific miRNAs, or miRNA-
 510 panels, as independent markers of prognosis vs. other prognostic parameters, such as disease sub-stage (stage
 511 IIIA-C, stage IV A-C/D), performance status and LDH levels. Although, this should be of interest, in our
 512 opinion, it is only a further progress of knowledge and testing of methodology robustness but not a reason
 513 enough to delay the use of cf-miRNA analysis in clinical practice.

514 At the same time, a recent technical improvement may be potentially profitable. Indeed, RNA-Seq has the
 515 potential of discovering novel miRNAs, offering the possibility to detect more differentially expressed genes
 516 with higher fold change or relatively low abundance transcripts. Nevertheless, the expectance of new results
 517 is not a good reason to delay the use of already available information to sustain the clinical practice.

518 In conclusion, the picture obtained from the literature on the possibility of using cf-miRNAs in melanoma
 519 staging and recurrence or survival prediction seems to allow optimism for future use of these nucleic acid
 520 molecules in clinical practice. Nevertheless, further progress relies on the fruitful cooperation among the
 521 different research groups, aimed to focus on profitable technical and biological normalization strategies. In
 522 our opinion, the time has come for a “cf-miRNAs Conference for Melanoma” aimed to compile reference
 523 microarray - or qRT-PCR methods to be tested in a large cohort of patients to finally validate the clinical role

524 of cf-miRNAs assessment in staging, recurrence prediction and prognosis in melanoma.

525 **Conflict of interest**

526 The authors declare they have no conflict of interest.

527 **Acknowledgements**

528 This work was supported by the Associazione contro il Melanoma (ACM ONLUS).

529 Authors are grateful to Dr. Jim Lin for his revision of manuscript

530

Legend

Figure 1. The canonical pathway of miRNAs biogenesis.

Figure 2. Circulating cf-miRNAs with staging or prognostic significance subdivided for their role as tumour suppressor or oncogene or actually unknown/unclear role in cutaneous melanoma.

Figure 3. Sources of analytical biases in RT-PCR based assessment of cf-MiRnas for diagnostic use in clinical practice.

Figure 4. Comparison of methods based on RT-PCR and microarray-based amplicon detection and measurement. RT-PCR may take advantage in terms of specificity from the use of Taqman probes but it implies the setting up of multiple samples with distinct primer pairs to detect multiple sequences. It is specific, accurate, cost and lab-demanding, it relies for decision on a reference gold standard, to be prepared by pooling a large number of serum/plasma from melanoma-free subjects, shared and validated by independent laboratories. The use of a primer mix and microarray makes the analysis potentially less specific and accurate and increases the analytical signal noise, due to the fluorophore in use. Nevertheless, it is less cost and lab-demanding, it allows the assessment of a virtually unlimited number of internal controls and candidate diagnostic sequences and it does not need a gold standard for clinical decision. As any other lab analysis, it requires only a periodic inter-assay quality control for reliability assessment.

Table 1. Circulating cf-miRNAs as staging biomarkers in cutaneous melanoma.

Table 2. Circulating cf-miRNAs as prognostic biomarkers in cutaneous melanoma.

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Single cf-miRNA or cf-miRNA panel	Subjects enrolled	Source	Normalisation
cf-miR-9-5p cf-miR-145-5p cf-miR-150-5p cf-miR-155-5p cf-miR-205-5p (Shiyyama, Fukushima & Ihn, 2013)	14 healthy subjects 16 primary melanoma 11 metastatic melanoma	serum	Cel-miR-39
cf-miR-21-5p (Saldanha, Potter & Pringle, 2013)	11 healthy subjects 12 stage 0-I-II 10 stage III 4 stage IV	plasma	miR-191
cf-miR-221-3p (Li et al., 2014)	27 stage I-II 45 stage III-IV	serum	miR-16
cf-miR-16-5p cf-miR-211-5p cf-miR-4487 cf-miR-4706 cf-miR-4731 cf-miR-509-3p cf-miR-509-5p (Stark, Klein & Hayward, 2015)	130 healthy subjects 86 stage I-II 50 stage III 119 stage IV	serum	Cel-miR-39
cf-miR-301a-3p cf-miR-451a (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 4 Stage 0	serum	5 most stable miRNAs by RefFinder
cf-miR-200c-3p cf-miR-126-5p (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 11 stage I	serum	5 most stable miRNAs by RefFinder
cf-miR-373-5p cf-miR-374-5p cf-miR-1260a cf-miR-211-5p (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 17 stage II	serum	5 most stable miRNAs by RefFinder
cf-miR-204-5p cf-miR-301a-3p cf-miR-200c-3p cf-miR-182-5p cf-miR-193b-3p cf-miR-720 cf-miR-205-5p cf-miR-206 cf-miR-550a-3p cf-miR-22-3p (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 11 stage III	serum	5 most stable miRNAs by RefFinder

cf-miR-200c-3p cf-miR-16-5p cf-miR-211-5p cf-miR-627-5p cf-miR-629-5p (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 9 stage IV	serum	5 most stable miRNAs by RefFinder
cf-miR-30b-5p, cf-miR-28-5p, cf-miR-204-5p, cf-miR-27a-3p cf-miR-1260a (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 32 stage 0, I, II	serum	5 most stable miRNAs by RefFinder
cf-miR-204-5p, cf-miR-182-5p, cf-miR-197-3p, cf-miR-720, cf-miR-211-5p, cf-miR-193b-3p, cf-miR-629-5p, cf-miR-432-3p cf-miR-1280 (Margue, Reinsbach & Kreis, 2015)	30 healthy subjects 20 stage III, IV	serum	5 most stable miRNAs by RefFinder
cf-miR-16-5p (Guo, Guo & Li, 2016)	120 healthy subjects 30 stage I 30 stage II 30 stage III 30 stage IV	serum	cel-miR-39
cf-miR-200c-3p (Fogli, Polini & Nieri, 2017)	32 healthy subjects 14 stage I-II 16 stage III-IV	plasma	Global mean Normalization, NormFinder

RT-PCR = Real Time-PCR

Table 1. Circulating cf-miRNAs identified as staging biomarkers in cutaneous melanoma.

Single cf-miRNA or cf-miRNA panel	Outcome endpoint	Subjects enrolled	Source	Normalisation
cf-miR-221-3p (Kanemaru, Fukushima & Ihn, 2011)	recurrence	8 melanoma patients: 1 stage I 5 stage II 1 stage III 1 not determined	serum	cel-miR-54
cf-miR-221-3p (Li et al., 2014)	survival	27 stage I-II 45 stage III-IV	serum	miR-16
cf-miR-150-5p, cf-miR-15b-5p, cf-miR-199a-5p cf-miR-33a-5p cf-miR-424-5p (Friedman, Shang & Osman, 2012)	recurrence	80 melanoma patients (discovery cohort): 39 stage I 20 stage II 21 stage III 50 melanoma patients (validation cohort): 10 stage I 28 stage II 12 stage III	serum	?
cf-miR-103a-3p cf-miR-221-3p (Friedman, Shang & Osman, 2012)	recurrence	17 stage II pre- and post-recurrence	serum	?
cf-miR-423-5p cf-miR-424-5p cf-miR-199a-5p (Friedman, Shang & Osman, 2012)	recurrence		serum	?
cf-miR-211-5p cf-miR-660 cf-miR-22-3p cf-miR-579 cf-miR-215 cf-miR-296 cf-miR-425-5p cf-miR-99a-5p cf-miR-486-5p cf-miR-486-3p (Saldanha, Potter & Pringle, 2013)	recurrence	3 stage III pre- and post-operative	plasma	miR-191

cf-miR-210 (Ono et al., 2015)	recurrence	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	plasma	ΔCq = mean Cq values (1 ng of RNA from M14) – mean Cq values (each sample)
cf-miR-16-5p cf-miR-211-5p cf-miR-4487 cf-miR-4706 cf-miR-4731 cf-miR-509-3p cf-miR-509-5p (Stark, Klein & Hayward, 2015)	survival	130 healthy subjects 86 stage I-II 50 stage III 119 stage IV	serum	cel-miR-39
cf-miR-16-5p (Guo, Guo & Li, 2016)	survival	120 healthy subjects 30 stage I 30 stage II 30 stage III 30 stage IV	serum	cel-miR-39

Table 2. Circulating cf-miRNAs identified as prognostic biomarkers in cutaneous melanoma.