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The prognostic and predictive value of melanoma-related microRNAs using tissue and serum: a microRNA expression analysis.

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Abstract

The overall 5-year survival for melanoma is 91%. However, if distant metastasis occurs (stage IV), cure rates are < 15%. Hence, melanoma detection in earlier stages (stages I-III) maximises the chances of patient survival. We measured the expression of a panel of 17 microRNAs (miRNAs) ('MELmiR-17') in melanoma tissues (stage III; n=76 and IV; n=10) and serum samples (collected from controls with no melanoma, n=130; and patients with melanoma (stages I/II, n=86; III, n=50; and IV, n=119)) obtained from biobanks in Australia and Germany. In melanoma tissues, members of the 'MELmiR-17' panel were found to be predictors of stage, recurrence, and survival. Additionally, in a minimally-invasive blood test, a seven-miRNA panel ('MELmiR-7') detected the presence of melanoma (relative to controls) with high sensitivity (93%) and specificity (≥82%) when ≥4 miRNAs were expressed. Moreover, the 'MELmiR-7' panel characterised overall survival of melanoma patients better than both serum LDH and S100B (delta log likelihood = 11, p<0·001). This panel was found to be superior to currently used serological markers for melanoma progression, recurrence, and survival; and would be ideally suited to monitor tumour progression in patients diagnosed with early metastatic disease (stage IIIa-c/IV M1a-b) to detect relapse following surgical or adjuvant treatment.

Abbreviations

AGO2, argonaute RISC catalytic component 2; AJCC, American Joint Committee on Cancer; AUC, area under the curve; AUROC, area under the receiver operator curve; CI, confidence interval; Ct, threshold cycle; DOR, diagnostic odds ratio; FFPE, Formalin-fixed paraffin-embedded; HR, hazard ratio; LDH, lactate dehydrogenase; M1a, metastasis to skin, subcutaneous (below the skin) tissue, or lymph nodes in distant parts of the body, with a normal blood LDH level; M1b, metastasis to the lungs, with a normal blood LDH level; M1c, metastasis to any other organs, OR distant spread to any site along with an elevated blood LDH level; MIA, Melanoma Institute of Australia; miR, microRNA; miRNA, microRNA; N stage, nodal or number of lymph nodes stage; NA, not applicable; NM, nodular melanoma; OR, odds ratio; PD1, Programmed cell death protein; RNA, Ribonucleic acid; S100B, S100 calcium-binding protein B; USA, United States of America; SMM, superficial spreading melanoma.

Highlights

- A seven-miRNA panel ('MELmiR-7') detected the presence of melanoma with high sensitivity (93%) and specificity (≥82%).
- In serially collected stage IV specimens, members of the 'MELmir-7' panel confirmed tumour progression in 100% of cases
- The 'MELmir-7' panel is superior to currently used serological markers for melanoma progression, recurrence, and survival.

Research in context

The use of melanoma progression markers have been used for many years however it is clear from the survival rates (5-year survival of Stage IV patients is <15%) that melanoma must be detected before disease progresses thus highlighting that the current methods of progression detection are inadequate. We have identified a seven-miRNA panel ('MELmiR-7') that has the ability to detect the presence of melanoma with high sensitivity and specificity which is superior to currently used markers for melanoma progression, recurrence, and survival. This panel may enable more precise measurement of disease progression and may herald an increase in overall survival.

Introduction

Melanomas are among the most commonly occurring cancers. Crude incidence rates in Australia[1] and the USA[2] were approximately 50 cases (in 2010) and 20 cases (in 2011) per 100,000 respectively. With the number of new cases rising each year, melanoma is currently is listed as the 4th and 6th most common cancer in Australia and the USA respectively.[1, 2]Current clinical staging criteria classify melanoma progression from a pre-invasive lesion, confined to the epidermis (stage 0), a series of early stages of local invasion (I and II), a stage involving regional lymph nodes (stage III) and finally metastasis to distant sites (stage IV). The overall 5-year survival for melanoma is 91%, which is largely due to curative surgery for early stage disease. However, cure rates are < 15%[3] if distant metastasis occurs (stage IV; AJCC 7th edition). We now have evidence that current therapeutic options for late stage disease are more effective if the disease treated with lower disease burden.[4, 5] Hence, melanoma must be treated in earlier stages to maximise the chances of patient survival. Therefore, the ability to identify signs of melanoma progression sooner would be a valuable clinical tool.

Melanoma progression biomarkers have been studied intensively with varying levels of success. Serum lactate dehydrogenase (LDH) levels have been integrated into current staging regimens[3] and elevation of LDH levels increases in specificity as disease progresses (stage II (83%), III (87%), and IV (92%)). However the sensitivity of this marker is reduced during progression (stage II (95%), III (57%), and IV (79%)).[6-11] S-100B, a calcium binding protein, is raised in serum of stage III and IV melanoma patients.[12, 13] However, the proportion of patients with elevated S100B levels varies by stage: 0-9% in stage I/II, 5-98% in stage III, and 40-100% in stage IV (reviewed in[14]). As such, serum S100B is not routinely used in the clinic,[15] highlighting the fact that the current serological methods of progression detection, whilst relatively specific, are inadequate due to variability in sensitivity across all stages of

disease. To date, there are no biomarkers that are sensitive or specific enough to be beneficial for early detection of melanoma (all stages). A blood test ('circulating' biomarkers) that detected melanoma with regional spread, prior to clinically evident distant metastasis, could improve treatment and outcomes for melanoma patients.

For a circulating biomarker to be effective, not only must it be sufficiently sensitive and specific, but it must also be highly stable and resistant to degradation. In recent years, circulating microRNAs (miRNAs) have been studied for their utility as biomarkers in a wide range of malignancies and disorders.[16, 17] miRNAs are small (20-22 nt) non-coding RNAs which function to regulate gene expression in the cell. Recently, tumour cells have been shown to release miRNAs into the circulation,[18] contained primarily in micro-vesicles or exosomes (extracellular vesicles), or bound to AGO2 - a part of the miRNA-mediated silencing complex.[16, 17] Due to the 'encapsulation' of these miRNAs in serum or plasma they are highly resistant to degradation by RNases (highly concentrated in the blood), thus their potential usefulness as a 'biomarker' is relatively high. To date, circulating melanoma-related miRNAs have been rarely studied.[19, 20]

Herein we report a multi-centre study that identifies a panel of 'melanoma-related' miRNAs that offer superior sensitivity to currently used serological markers for melanoma progression, recurrence, and survival.

Materials and Methods

Patient specimen details

Formalin-fixed paraffin-embedded (FFPE) melanoma tissues and serum (melanoma and control patients) were obtained from prospectively collected biobanks in Australia and Germany.

Tissue validation cohort

FFPE melanoma tissues, collected at diagnosis of stage III ('PAH-tissue') were obtained via a database of prospective stage IIIA-C cutaneous melanoma cases, presenting to the Princess Alexandra Hospital (PAH) Melanoma Unit and affiliated private hospitals, which has been maintained since 1997. Permission to collect and use information was approved by the hospital ethics committee (HREC Reference number: HREC/11/QPAH/650; SSA reference number: SSA/11/QPAH/694). Inclusion criteria were the same as those previously presented.[21] An additional collection (collected at diagnosis) of stage III and IV melanoma tissues ('MIA-tissue') were obtained via a database of prospectively recruited melanoma cases, presenting to the Melanoma Institute Australia and affiliated private hospitals, which has been maintained since 1967. Informed written consent was obtained for each patient under approved protocols (Protocol No X10-0305 &HREC/10/RPAH/539 and Protocol No X10-0300 HREC/10/RPAH/530) governed by the Human Research Ethics Committee of the Royal Prince Alfred Hospital (Sydney NSW, Australia). Inclusion criteria were the same as those previously presented.[21] See Table 1 for participant descriptive statistics. Supplementary Table 2 shows the mean, median, and range of follow-up times.

Serum validation cohorts: Control sera

Sera from 'Healthy Controls' were ascertained from a cohort of participants collected as part of the Australian Cancer Study (ACS) (QIMR Berghofer HREC approved project no. P399). As part of the ACS, potential controls were randomly selected from the Australian Electoral Roll (enrolment is compulsory). Controls were prospectively sampled from within strata of age (in 5 year age-groups) and state of residence. Of 3,258 potentially eligible control participants, 41 could not be contacted and 175 were excluded because they were deceased (16), too ill (61), or unable to read or write in English (98). Of 3,042 controls meeting the inclusion criteria, 1680 (55%) gave their consent to take part. Completed questionnaires were returned by 1580 controls (48% of all potentially eligible controls selected from the roll). See Table 2 for participant descriptive statistics.

Sera from 'High naevus count' and 'History of melanoma, disease-free' participants were prospectively collected from cohorts who were enrolled in the study: 'Pigmentation genotypes and phenotypic correlations with dermoscopic naevus types and distribution'. These samples were included as 'controls' to determine the level of expression measurable in sera derived from patients with a high melanocyte burden. All study participants were enrolled in the following human ethics approved projects: QIMR HREC/P1237, The Metro South Health District HREC/09/QPAH/162, and UQ HREC approval number is 2009001590. Participants with a history of melanoma (clinically free of disease at time of blood draw) were recruited through the Melanoma Unit and Dermatology Department of the Princess Alexandra Hospital, Brisbane, Queensland, Australia, between May 2012 and November 2012. Control participants, with no personal history of melanoma, were recruited from the Brisbane Twin Naevus Study between August 2012 and November 2012. All participants had 16-panel full-body images and dermoscopic images of significant naevi recorded2. Significant naevi were defined as naevi greater than or equal to 5mm on all body sites except the scalp, buttocks, mucosal surfaces and genitals, and greater than or equal to 2mm on the

back of both males and females and on the legs of females. All significant naevi were classified by the predominant dermoscopic pattern (reticular, globular, or non-specific), colour, and profile (flat, raised, domed or papillomatous). See Table 2 for participant descriptive statistics.

The description of 'controls' used in the analyses refers to a combined cohort of 'Healthy Controls', 'High naevus count', and 'History of melanoma, disease-free' participants.

Serum validation cohorts: Melanoma patient sera

Sera from stage I-IV melanoma patients (at time of blood draw and staged according to the current AJCC staging manual3) had blood drawn and serum stored as part of a large prospectively collected cohorts from the university department of dermatology in Tubingen, Germany ('Tubingen' cohort) and Melanoma Institute of Australia, Sydney ('MIA' cohort). Usage of the 'Tubingen' bio-bank with corresponding patient data was approved by the Ethics Committee, University of Tübingen (approvals 657/2012BO2). Serially-collected stage IV patients ('MIA' cohort only) had blood drawn at time of diagnosis or at lower disease burden and then at higher disease burden (determined by routine diagnostic tests). All samples from the 'MIA' cohort had informed written consent obtained from each patient under approved protocols (Protocol No X10-0305 &HREC/10/RPAH/539 and Protocol No X10-0300 HREC/10/RPAH/530) governed by the Human Research Ethics Committee of the Royal Prince Alfred Hospital (Sydney NSW, Australia). See Table 2 for participant descriptive statistics. Supplementary Table 2 shows the mean, median, and range of follow-up times.

All serum samples were collected in 10-mL BD serum tubes then centrifuged for 10 minutes at 1,500 x g. The supernatant serum was then aliquoted into 1.5 mL cryovials and stored at −80°C until further use.

Total RNA extraction from validation cohorts

A sterile disposable biopsy punch (Kai Medical, Japan) was used to retrieve tumour content from blocks that had been scored and marked for content via H&E histological staining. The extraction of total RNA from FFPE tissue and serum was performed respectively using miRNeasy FFPE Kits (QIAGEN) as per manufacturer's instructions or as previously described.[21]

Selection criteria for 'melanoma-related' miRNAs

In our previously published miRNA microarray data[22] we found a total of 233/1898 miRNAs ('Discovery' set) (Figure 1) that were differentially expressed (DE; corrected $p \leq 0.05$ and ≥ 2 fold) between the melanoma cell lines (n=55) and the 'other' solid cancers (n=34). We applied filtering criteria to the 233 DE miRNAs to identify which miRNAs would be suitable to measure in patient derived serum. The following strict criteria were used to filter the 'Discovery' set: ≥ 15 fold higher expression in cutaneous melanoma vs. 'other' solid malignancies (n=14/14), or ≥ 2 fold higher expression in cutaneous melanoma vs 'other' solid malignancies with no detectable expression in melanocytes or melanoblasts (n=3/6). In addition, miR-16, which is known to be highly expressed in blood, was assessed for its suitability as an endogenous control. The 18 miRNA panel ('MELmiR-18') comprising: miR-211-5p, miR-514a-3p, miR-509-3p, miR-204-5p, miR-509-5p, miR-513b, miR-145-5p, miR-146a-5p, miR-508-3p, miR-506-3p, miR-513c-5p, miR-4731-5p, miR-508-5p, miR-363-3p, miR-4487, miR-4469, miR-4706, and miR-16. This panel was carried forward for testing in independent cohorts of FFPE melanoma tumours and patient derived sera.

Reverse transcription, pre-Amplification, Taqman assays and Fluidigm real-time PCR.

We performed a custom Taqman assay combined with a sensitive method of detection (Fluidigm, HD Biomark) as previously described.[21] Briefly, a custom reverse transcription (RT) primer pool consisting of equal amounts of miRNA-specific RT primers contained within each TaqMan® Assay (Life Technologies, Carlsbad, USA; miR-211-5p (000514), miR-514a-3p (001147), miR-509-3p (002236), miR-204-5p (000508), miR-509-5p (002235), miR-513b (002757), miR-145-5p (002278), miR-146a-5p (000468), miR-508-3p (001052), miR-506-3p (001050), miR-513c-5p (002756), miR-4731-5p (464084_mat), miR-508-5p (002092), miR-363-3p (001271), miR-4487 (462492_mat), miR-4469 (465059_mat), miR-4706 (464518_mat), and miR-16[#] (000391) along with cel-miR-39 (000200; serum spiked-in control) and RNU-6 (001973; FFPE endogenous control)) plus an additional pool of the corresponding TaqMan® MicroRNA Assay (Pre-Amp Primer Pool) were used to pre-amplify the RT reaction). Each assay had a serial dilution of a positive control sample (known expression for all miRNAs in panel) that had a total input of 1, 3, 15, and 45 ng in the original cDNA reaction.

[#] The miR-16 Taqman primer assay (00039) is specifically designed to bind to mature miRNA sequence of miR-16-5p which is derived from hsa-mir-16-1 and hsa-miR-16-2 stem-loop sequences. The alias for miR-16-5p is miR-16 hence the reasoning for the shortened name.

qRT-PCR analysis

The expression of the 'MELmiR-18' panel (Figure 1) was assayed in each sample with at least 4 technical replicate Taqman assays to determine their expression. Real-time expression data was extracted and analyzed as previously described.[21]

Statistical Methods

The marker level differences (e.g. univariate analysis of each miRNA in each cohort comparison represented in Table 3) were assessed using the Mann-Whitney U-test and adjusted for multiple comparisons using the Benjamini & Hochberg method. Significant markers' predictive ability was evaluated using receiver operating characteristic (ROC curve) and area under the curve (AUC) or AUROC. Univariate and multivariate logitistic regressions with backward covariate search based on AIC[23, 24] were performed to identify significant markers which were associated with melanoma status/disease stages, when time to event information was missing. For survival and recurrence analyses, univariate and multivariate Cox proportional hazard model with backward covariate search based on AIC[23, 24] was performed. Time to follow-up was measured from date of blood collection which was ≤1 month of staging. The proportional hazards assumption was also evaluated for each Cox regression.[25] The model fits were compared using likelihood ratio test. The predictive abilities of the selected significant markers were evaluated using AUROC. The selected markers were then used to classify patients using conditional inference tree analysis.[26] For serum markers, the cutoff point of each marker that characterized the melanoma status was determined to maximize AUROC statistics.

5000 nonparametric bootstraps were performed, per cohort (e.g. controls versus stage IV) & microRNA analysis pairs, to obtain robust effect size estimates, p values (for univariate analysis) and AUROC. To reflect the uncertainties of the values greater than Ct 36, the values above 36 were replaced by random values from 37 to 40 during the bootstrap. The original data was analysed without this consideration and the final models were rerun using 5000 bootstrap runs to generate robust outcomes.

For the analyses, OptimalCutpoints (v1·1-3), boot, and party packages on R version 3·0·2 were used to find cutpoints in univariate analyses (cohort vs. markers), boostrapping and tree analyses respectively.

ROC curves and scatter plots were drawn using Graph Pad Prism 6. Survival analysis was performed using R version 3·0·2.

Diagnostic inclusion criteria, score assignment, and test evaluation

To maximize the chances of having a positive signal in the patients serum, a combined stage IV cohort (n=119; 'TUBINGEN' and 'MIA') was compared with disease-free 'controls' (n=130; no history of melanoma or nevi, prior history of melanoma but disease-free, high nevus count with no melanoma). Initially, all members of the 'MELmiR-17' (miR-4469 was excluded due to assay failure) panel underwent a simple Mann–Whitney U test to identify the highly significant (p.<0·0001) miRNAs to be included in the next step (Figure 1). Those miRNAs that met these criteria then underwent AUROC analysis to determine their Area under the Curve (AUC) (Figures 1 and 2). AUC scores ≥0·70 were deemed to be diagnostically useful.[27] The miRNAs that had an AUC ≥0·70 were interrogated further to classify the median-normalized Ct values as 'high' or 'low' expression (interpretation of the median normalized Ct expression values used to determine ROC curves were evaluated with the Optimal Cutoff algorithm ('OptimalCutpoints' R package v1.1-3). For those miRNAs that met the criteria for inclusion in the diagnostic panel (Figures 1 and 2), the patient was given a diagnostic score (ranging from 0-7) determined by the number of miRNAs that were present as 'high' and 'low' or 'normal' (most like the 'control' cohort). To be deemed positive for melanoma, the patients sample must have had a score ≥4 (max 7). A negative test was a score of 0-3.

The following formulas were used to determine diagnostic test ability: Positive Predictive Value (PPV) or Precision = True Positive (TP)/(TP + False Positive (FP)); Negative Predictive Value (NPV) = True Negative (TN)/(False Negative (FN) + TN); Sensitivity = TP/(TP + FN); Specificity = TN/(FP + TN) False Positive Rate = 1 – Specificity; False

Negative Rate = 1 – Sensitivity; Likelihood Ratio Positive = Sensitivity/1-Specificity; Likelihood Ratio Negative = 1-Sensitivity/Specificity; Diagnostic Odds Ratio (DOR) = (TP/FN)/(FP/TN).

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Results

Members of the 'MELmiR-17' panel are predictors of stage, recurrence, and survival in patient tissue.

To confirm that miRNA expression was detectable in melanoma tissues prior to the serum assessment, we first measured an 18-miRNA panel ('MELmiR-18') in a prospective collection (Tissue validation cohort, see Materials and Methods) of melanoma tissues derived from stage III (n=76) and stage IV (n=10) melanoma patients (Table 1 and Figure 1). Expression was detected in all dilutions of a positive control (except miR-4469 which had assay failure thus the panel herein will be referred to as 'MELmiR-17') and in tissue samples (Figure 1), which indicated that even at low input levels, the assay and detection method was adequate (data not shown). We observed that thirteen miRNAs were differentially expressed when stage III tissues were compared with stage IV tissues (logistic regression, $p<0{\cdot}05$; Supplementary Table 1 and Supplementary Figure 1). All but one of these miRNAs (miR-204) showed higher levels in stage III compared with stage IV tissues (Supplementary Table 1 and Supplementary Figure 1). Of these miRNAs, seven (miR-506-3p, miR-508-3p, miR-508-5p, miR-509-3p, miR-509-5p, miR-513c, and miR-514a) were members of the miR-506-514a cluster.[28] Supplementary Table 1 summarises the associated AUROC analyses. Members of the miR-506-514 cluster, had AUC scores ranging from (0·65-0·79) with the highest scores being shared by miR-506-3p and miR-509-5p. To determine the minimum number of miRNAs required to discriminate stage III from stage IV, we next performed a multivariate logistic regression and illustrated this using a conditional inference tree (Supplementary Figure 2). These analyses revealed that only miR-4731 ($p=0{\cdot}003$, OR=3·0, CI 1·45-6·2) and miR-204 ($p=0{\cdot}015$, OR=0·63, CI 0·43-0·92) were required to discriminate the tissue stage (in general, higher Ct values = lower expression). Subsequent AUROC

analysis (AUC=0·89) showed an improved score than individual miRNAs (Supplementary Table 1).

We next used multivariate Cox regression modeling using the 'MELmiR-17' panel together with available records of pathology of the primary melanoma (SMM, NM) and number of involved nodes (N stage) to determine its value as a prognostic marker at stage III in the PAH tissue cohort (Table 1). These analyses showed that only nodular histotype (NM; p=0·002; HR=3·5; CI 1·57-7·81) and expression of miR-509-5p (p=0·015; HR=0·85; CI 0·75-0·97) were associated with overall survival. Expression levels of the 'MELmiR-17'panel was not significantly different between the two largest pathology classes (SMM and NM) (data not shown). Furthermore, using the same multivariate analysis, N-stage (p=0·014; HR=1·52; CI 1·09-2·12) and lower expression of miR-513b (p=0·038; HR=1·08; CI 1·00-1·17) and higher miR-513c expression (p=0·020; HR=0·92; CI 0·86-0·99) were related to recurrence.

A seven-miRNA panel identifies melanoma with high sensitivity and specificity using patient sera.

The 'MELmiR-17' panel was next assessed in independent cohorts of patient sera (Serum validation cohorts, see Materials and Methods) with different stages of disease at time of blood collection (from no melanoma to stages I-IV) (Table 2 and Figure 1). All expression values (Ct) were normalized to *cel-miR-39* (synthetic 'spike-in' control) according to previously published methods[21] prior to statistical analysis. In miRNA derived from serum, expression of 13 miRNAs was detected (Figure 1 and Table 3). Notably, the expression of the miR-506-514a cluster was generally quite low (miR-506-3p and miR-514a) or not detected (miR-508-3p, miR-508-5p, miR-513b, and miR-513c (data not shown)). In the 13 detected miRNAs, seven (miR-16, miR-211-5p, miR-4487, miR-4706, miR-4731, miR-509-3p, and miR-509-5p) showed highly significant differences (Mann-Whitney; corrected p<0·0001)

between 'controls' (no melanoma) and patients with stage IV disease (Table 3 and Supplementary Figures 3 and 4). The same miRNAs were also differentially detectable in stages I/II and stage III, compared with 'controls', with the exception of miR-211 (Table 3 and Supplementary Figures 3 and 4). While miR-16 was originally included as a blood control, our data show that it is significantly associated with disease in both tissue and serum, as observed previously in a study of colorectal cancer.[29] Intriguingly, levels of expression of most of these differentially expressed miRNAs were lower in melanoma patients compared to patients without disease (controls). The miRNAs -4487, -4706, -4731, 509-3p, and 509-5p all showed lower expression (on average) and miRNAs -16 and -211 (stage IV only) had higher expression (on average) in melanoma patients (Supplementary Figure 3). The observed lower serum expression of miR-4487, miR-4706, miR-4731, miR-509-3p, and miR-509-5p in the melanoma cases was associated with melanoma (presence of or recently removed tumour; see Discussion), and in the case of miR-509-3p, has been noted previously by Leidinger *et al.*[30]

AUROC analysis revealed which of the differentially detected miRNAs (Table 3 and Supplementary Figure 5) have the potential to be used for diagnostic purposes. Particular attention is paid to those miRNAs that were able to discriminate stage IV disease (i.e. distal metastatic deposits) from disease-free controls (Table 3). These were: miR-16, miR-211-5p, miR-4487, miR-4706, miR-4731, miR-509-3p, and miR-509-5p (herein referred to as 'MELmiR-7'). Multivariate logistic regression identified the five most robust markers of the 'MELmiR-7' panel (miR-211, $p<0{\cdot}0001$; miR-509-3p, $p=0{\cdot}0014$; miR-509-5p, $p<0{\cdot}0001$; miR-4706, $p=0{\cdot}028$; and miR-4731, $p<0{\cdot}0001$). Subsequent AUROC analysis revealed these five markers produced a near perfect AUC score of 0·9907 (cf. 'MELmiR-7'= 0·9911). Furthermore, a conditional inference tree analysis highlighted that patients could be discriminated into categories based on combinations of expression levels by members of the MELmiR-7 panel. Supplementary Figure 6 illustrates that only four miRNAs (miR-509-5p,

miR-miR-4731, miR-211, and miR-509-3p) were required to discriminate the stage IV samples (AUC=0·9738) from controls. Further comparisons showed that 'MELmiR-7' panel members can also discriminate stages I/II (AUC=0·991) and stage III (AUC=0·9722) from 'controls' (Supplementary Figures 7 and 8).

The sensitivity and specificity of the 'MELmir-7' was then assessed by assigning a diagnostic score to the data. The expression values graphed in the Supplementary Figure 3 were used to observe the direction of the data (i.e. higher or lower expression in 'controls' vs. all stages). The optimal cut points[24] in the AUROC datasets were identified which allowed the expression values to be categorised as positive or negative for melanoma (see Materials and Methods). A diagnostic score (see Materials and Methods)) was then applied to each sample which ranged from 0 to 3 (low likelihood of melanoma) and 4 to 7 (high likelihood of melanoma). Upon applying the derived diagnostic score, the 'MELmiR-7' panel was evaluated as a group. We found that it had the ability to identify melanoma (independent of stage), when ≥4 miRNAs (93% sensitivity and ≥82% specificity) reached or exceeded their optimal cut point (Table 4). The sensitivity of the 'MELmiR-7' panel increased to 95% in the stage IV cohort. Table 4 provides a summary of the effectiveness of the 'MELmiR-7' panel in relation to other stages. The diagnostic odds ratio (DOR) was used to determine the lowest diagnostic score possible for the 'MELmiR-7' panel while still maintaining very high sensitivity and specificity. Moreover, upon comparison with currently used serological tests (LDH and S100B), we found that the 'MELmiR-7' panel was more sensitive than the combined power of both tests. Using the available data (Tubingen cohort), elevated levels of LDH and S100B were found in 40% (27/67) and 63% (42/67) of these patients (Table 2). In the same patients, the 'MELmiR-7' panel achieved 91% (63/67) (when ≥4 miRNAs reached or exceeded their optimal cut-points) sensitivity and ≥82% specificity (specificity could not be determined for serum LDH and S100B as 'controls' were not assayed). The sensitivity of

the 'MELmiR-7' panel was confirmed in an independent serial collection of stage IV patients (initial blood draw at lower disease burden and one at a higher disease burden) (Figure 2). Figure 2 highlights that the 'MELmir-7' panel can be used to monitor tumour progression in 100% of the patients assessed (≥2 miRNAs with ≥1·5 fold relative expression). Subsequent AUROC analysis (Figure 2) highlights that if measured in isolation, the most informative markers would be miR-509-5p and miR-4731 (AUC=0·84 respectively).

Members of the 'MELmiR-17' differentiate stage and are associated with survival in patient sera.

To discern whether significant differences in stage could be found, we first assessed the 'MELmiR-17' panel using Mann-Whitney tests (with corrected *p* values) combined with AUROC analysis for stage I/II vs. IV and stage III vs. IV. Table 3 summarizes the associated corrected *p* values and AUROC scores. Next, multivariate logistic regression was used to identify the minimum miRNAs required to predict differences in the melanoma stages which was illustrated using conditional inference trees (Supplementary Figures 9 and 10) and AUROC analysis (AUC scores for stage I/II vs. IV and stage III vs. IV were 0·989 and 0·9945 respectively). There were no markers, however, that were significantly associated with time to recurrence when Cox regression was performed.

The 'MELmiR-17' panel was next assessed to identify miRNAs related to OS in the serum cohorts from Tubingen (n=131) and Melanoma Institute of Australia (MIA; n=124) first separately and then jointly (n=255) (Table 2). The predictive performance of the joint model on each cohort was statistically equivalent to that of the best separate analysis on each cohort (likelihood ratio test; p=0·34 for MIA cohort; p=0·22 for Tubingen cohort), hence the joint model was used to analyse the combined Tubingen and MIA cohorts. Furthermore, the miR-

4706 marker was dichotomized at Ct 37 to meet the proportionality assumption. The outcome from the combined analysis is summarised in Table 5.

A conditional inference tree analysis for the survival data was then performed which showed that stage at blood draw together with miR-211 expression could be used to triage patients based on overall survival (OS) status (Figure 3 with Kaplan-Meier plots per each classification). Importantly, upon diagnosis with stage IV, miR-211 expression was able to discern survival based on high (Ct ≤24; median survival= 4·8 months, CI 4·5-5·9) and low expression (Ct ≥24·01; median survival= 2.7 yrs, CI 1·7-NA).

Finally, the MELmiR-7 panel was further assessed for its utility in terms of predicting OS in serum cohorts having LDH and S100B status available (Tubingen) (Table 2). The 'MELmiR-7' panel performed significantly better than both serum LDH and S100B (delta log likelihood = 11, p<0·001).

Discussion

Five-year survival proportions for melanoma are poor for patients with metastatic disease, however if disease is detected in its early stages, then survival is one of the highest for all cancers. Even for those with metastases, survival differs depending on the extent of disease spread. Patients with metastases confined to regional lymph nodes (stage III disease) have 5-year survival of ~50%, whereas patients with widely disseminated metastases (stage IV disease) have 5-year survival of <15%. Thus better monitoring of a patient's tumour burden may improve survival by precipitating earlier therapeutic interventions. In support of this, clinical trials in stage III unresectable and stage IV melanoma patients, treated with ipilimumab,[5] vemurafenib,[4] combined dabrafenib and trametinib[31] or anti-PD1 pembrolizumab,[32] have observed improved overall survival and response rate in patients with lower disease volume (M1a/M1b) as compared to those with distal disease (M1c). Moreover, it is believed that there is potential for long-term survival if relapses are identified promptly with treatment initiated without delay.[33] In clinical practice, there is currently a lack of reliable, sensitive and specific predictive biomarkers for detecting early melanoma progression. This study aimed to identify a more effective biomarker that was sensitive and specific enough to identify early metastatic disease. Since commencement of this study there have been a number of studies investigating the utility of miRNAs to serve as melanoma blood and tissue biomarkers. [19-21, 30, 34-36] For example, a study by Friedman *et al.* [20] screened 355 miRNAs in sera from 80 melanoma patients using a previously characterised panel of serum-expressed miRNAs. The authors found detectable expression for 170 miRNAs and a panel of five miRNAs (miR-150, miR-15b, miR-199a-5p, miR-33a, and miR-424) showed a significant association with recurrence-free survival. This five-miRNA signature was able to classify the patients into high and low recurrence risk. Our approach was to identify a panel of melanoma-related miRNAs that involved first screening a panel of melanoma cell lines

(n=55) in comparison with a group of other solid malignancies (cell lines were derived from breast, ovarian, colorectal, prostate, etc). [22] Interestingly, the five-miRNA panel indentified by Friedman *et al*. [20] was not present in our dataset which may indicate that this panel is not specifically melanoma-related but instead related to the tumourigenic process. We focused on miRNAs that were highly expressed or more predominantly expressed in melanoma with the premise that these may be both 'diagnostic' for melanoma and/or more easily detectable in patient serum. Our current approach differed from the aforementioned studies as: 1) this study harnessed the power of our previous comprehensive analysis of known miRNAs (n=1898) in relation to melanoma; [22] 2) this study validated the cell-line derived miRNA panel (MELmiR-17) in a large of panel of stage III and IV melanoma tissues prior to serum analysis to confirm they were expressed; 3) this study used an ultra-sensitive method of detection (see Materials and Methods) to ensure that lowly expressed miRNAs could be detected. We have successfully used these approaches in a previous study where a panel of miRNAs were identified that was related to good and poor prognosis in stage III melanoma patients. [21] However, our current study was limited by the lack of available serially collected specimens (to detect recurrence as in Friedman *et al.* [20]) at time of study design. To address this limitation, further studies in larger, independent, prospectively collected melanoma cohorts will be required to strengthen these data.

In sum, we found that a 'melanoma-related' panel of miRNAs was expressed in metastatic melanoma in a stage-specific manner and, together with the tissue pathology and nodal status, was prognostic for recurrence and OS. These markers may therefore also be useful to support histopathologic diagnosis of metastatic deposits suspected of being melanoma. We further observed that expression of the various miRNAs from the MELmiR-17 panel in stage IV tissues was often lower than in stage III tissue, which is in keeping with previous studies. For example, miR-211 expression is commonly lost in subsets of melanoma cell lines,[22, 37] and

miR-506, a member of the miR-506-514 cluster, has been shown to be lost during metastatic colonisation despite being up-regulated in early melanoma progression.[28, 38] We have also recently reported that inhibition of miR-514a leads to increased cell proliferation.[22] These data indicate that expression of this cluster reduces during melanoma progression.

Currently there is an unmet need for a minimally invasive, highly specific, and predictive serum biomarker of melanoma burden. For many years the use of the seroprotein markers S100B and LDH has been disputed, due to reported inconsistencies in sensitivity and specificity.[6-10, 12-14] Despite this lack of consensus, a recent study did find that elevated levels of S100B were prognostic of survival times in patients with unresectable melanoma.[39]

Here, we present data that shows that our 'MELmiR-7' panel has the potential to be used as a primary screening tool for clinically undetected metastatic melanoma due to its high sensitivity (93%) and specificity (≥82%). However, detection of early melanoma lesions (*in situ* and stage I/II melanoma) is currently being adequately achieved (as evident by high survival rates) via clinical strategies. The 'MELmiR-7' panel could be utilized during routine follow-up (i.e. post primary excision of melanoma and later in advanced disease) of melanoma patients. In comparison with serum LDH and S100B, expression levels of the 'MELmiR-7' panel performed better than both markers in predicting overall survival. We have shown that the 'MELmir-7' panel was measurable at time of progression in 100% of stage IV melanoma patients. These data suggest that this panel would therefore be suited to monitor tumour burden. Better monitoring of a patient's tumour burden could improve survival by precipitating earlier therapeutic interventions. In support of this notion, clinical trials in stage III unresectable and stage IV melanoma patients, treated with ipilimumab,[5] vemurafenib,[4] combined dabrafenib and trametinib[31] or anti-PD1 pembrolizumab,[32] have observed improved overall survival and response rate in patients with lower disease volume (M1a/M1b) as compared to those with distal disease (M1c).

According to the AJCC Staging committee, stage III melanoma patients have a 50% chance of survival beyond 5 years;[3] these patients also remain the most difficult for whom to provide effective treatments/surveillance regimens and accurate survival estimates. Following treatment, stage III patients are subjected to a series of physical examinations, scans and serology at regular intervals. The frequency of these tests is deemed necessary for early detection of recurrence; however this causes a burden to both the patient and the healthcare system. It is important to note that these guidelines are not universally accepted and differ from centre to centre.[15] We foresee that the 'MELmiR-7' panel could be offered to patients to complement physical examination. If the diagnostic score for melanoma positivity has changed from earlier measurements, then this may indicate the presence of disease recurrence and as such, these patients may qualify earlier for adjuvant, systemic, or targeted therapies that would otherwise be only offered to stage IV patients. As previously discussed, due to the panel's high sensitivity and specificity, the use of this miRNA panel in this manner has the potential to increase the chances of survival, by earlier and more precise detection of the presence of metastases.

In terms of prognosis, elevated miR-211 expression levels were associated with poorer survival in stage IV patients. Therefore, miR-211 measurement might allow better triaging of patients diagnosed with stage IV disease, into good and poor prognosis which would be highly informative for not only the treating clinician but also for the quality of life of the patients.

The original premise of this study was that the melanoma-enriched miRNAs identified in our previous study [22] would be translated directly to the expression observed in melanoma patient-derived serum. Evidence for this notion is apparent in the serially collected stage IV melanoma patients, when, at progression (or recurrence), the MELmiR-7 panel increases, which is reflective of increased tumour burden (i.e. the detectable miRNA expression was

from the presence of tumour cells and/or tumour derived extracellular vesicles (e.g. exosomes) in the circulation). These data strongly suggest that the expression is tumour derived and as such this panel could be considered melanoma-related. However, as we have noted, we observe a paradoxical decrease in the expression of the significantly expressed (mir-509-5p, miR-509-3p, mir-4731-5p, miR4487, miR4706) miRNAs when melanoma serum cohorts were compared with control cohorts. These data thus provide evidence that the assessed miRNAs (detectable in serum) are not restricted to the melanocytic lineage as initially thought. The source of this miRNA expression is currently unknown but could include cells of the haematopoietic lineage including T-cells, B-cells or NK cells. This loss of expression from a 'non-tumour' source has not been elucidated but warrants further investigation. An observed loss of expression of serum-derived miRNAs has been noted previously by Friedman *et al.* [20] in post-operative specimens as compared to specimens collected at disease relapse. A plausible reason for a loss of expression observed in the serum may be due to a cytokine-driven systemic response. For example, pro-inflammatory cytokines have been shown to down-regulate miRNAs present in the circulation. [40] Specifically, in a study by Hooten *et al.,* [40] the serum expression of miR-181a was found to be negatively correlated with pro-inflammatory cytokines IL-6 and TNFα and positively correlated with the anti-inflammatory cytokines TGFβ and IL-10. [40] Recently, it has been confirmed that IL-6 expression is induced in melanoma cells with mutant BRAF (V600E). Therefore a possible explanation for what we have observed is that the miRNAs of interest could be expressed by non-melanocyte derived cells where expression is down-regulated in patients with melanoma due to melanoma-related cytokines (e.g. IL-6). [41]

In conclusion, we envisage that as a growing number of miRNA-panels have been identified as potential prognostic indicators for melanoma, [19-21] it will eminently feasible to quantify circulating cell-free miRNAs directly,[42] paving the way for rapid measurements to occur in a

diagnostic laboratory. Given these advances, combined with the data presented herein, future melanoma treatment regimens should consider the utility of miRNAs as a prognostic aid in the clinical setting. Our sensitive and specific miRNA panel, in combination with newly identified panels, may enable more precise measurement of disease progression, and in conjunction with current therapy options, may herald an increase in overall survival.

CONFLICT of INTEREST

The authors state no conflict of interest except for GVL who reports personal fees from Amgen, BMS, GSK, Merck, Novartis, Provectus, and Roche, which were outside the submitted work; JFT who reports personal fees and other from Provectus, Bristol Myers Squibb, and other from GlaxoSmithKline, which were outside the submitted work; CG who reports personal fees from Amgen, MSD, Novartis, and grants and personal fees from BMS, Roche, and GSK, outside the submitted work; and PMP who reports personal fees from Novartis, outside the submitted work.

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AUTHORS' CONTRIBUTIONS

MSS designed the study, performed the experiments, data analysis and interpretation, and wrote the manuscript. KK performed the data analysis and interpretation and wrote the manuscript. BW performed the experiments, data collection, contributed specimens, and wrote the manuscript. LEH performed the data analysis and interpretation. AP, YHT, DCW, GVL performed the data collection, contributed specimens, and wrote the manuscript. JMP performed the data collection. RS, JFT, APB, HPS, CG contributed specimens and wrote the manuscript. GJM contributed specimens and data, performed data interpretation and wrote the manuscript. AH, PMP designed the study and wrote the manuscript. NKH designed the study, performed data interpretation, and wrote the manuscript.

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TABLE LEGENDS

Table 1

Descriptive statistics of all tissue cohorts used within the study.

Table 2

Descriptive statistics of all serum cohorts used within the study. **Bolded text**s are the cohorts that were used in the analysis.

Table 3

Table provides a summary of the Mann-Whitney tests and AUROC analyses that were performed in each cohort for 13 detectable miRNAs in serum derived from melanoma patients and controls. *P* values were corrected for false discovery rate (FDR) via the Benjamini–Hochberg (BH) procedure. ns = non-significant. Shading represents the MELmiR-7 panel. Brackets represent the 95% Confidence Interval (CI).

Table 4

Summaries of the diagnostic test statistics generated when AJCC staged melanoma is compared with controls.

Table 5

Summarizes the output from a multivariate survival analysis of serum miRNAs. A total of seven miRNAs contributed to overall survival with five reaching statistical significance ($p<0.05$). Stage at diagnosis was included in the analysis. HR = Hazard ratio. ns = non-significant. CI = confidence interval.

FIGURE LEGENDS

Figure 1

Melanoma-related miRNAs were first identified in a 'Discovery set' of 233 miRNAs previously found to be significantly associated with melanoma.[22] The 18-miRNA panel was measured firstly in an independent cohort of FFPE melanoma tissues (Tissue validation cohort; see Materials and Methods). The miRNA panel was then measured in cohorts of serum derived from controls in comparison to AJCC staged melanoma patients (stages I-IV) (Serum Validation Cohorts; see Materials and Methods). The detectable miRNAs (listed) were carried forward for further statistical comparisons.

Figure 2

Serially collected blood samples from stage IV melanoma patients highlight the utility of the MELmiR-7 panel as a whole in monitoring tumour progression. AUC scores were determined via an AUROC analysis. Coloured bars represent relative fold change of progression sample in relation to the initial blood draw.

Figure 3

A conditional inference tree (CI tree) illustrates that stage at blood draw together with miR-211 expression can triage patients based on known survival outcomes when melanoma was the confirmed cause of death. miR-211 expression cutpoints were determined by the CI analysis. Ct values ≤24 (or high expression) and ≥24.01 (or low expression) allowed for a significant splitting of the stage IV patients into good and poor prognosis patients. *P* values as indicated. Solid lines in the Kaplan Meier survival curves represent the observed events. Dotted lines are the 95% Confidence Intervals. n = number of patients in each group.

Table 1 Descriptive Statistics for tissue cohorts

Prognostic factors		PAH Cohort n (%)	MIA Cohort n (%)	MIA Cohort n (%)
	Totals	66 (100)	10 (100)	10 (100)
Stage at collection	III	66 (100)	10 (100)	-
	IV	-	-	10 (100)
Stage subclass	IIIB	25 (38)	-	-
	IIIC	41(62)	-	-
	Unknown	0 (0)	0 (0)	-
Sex	Male	35 (53)	7 (70)	5 (50)
	Female	31 (47)	3 (30)	5 (50)
Age at tissue collection	20-30	6 (9)	-	1 (10)
	31-40	9 (14)	-	0 (0)
	41-50	15 (23)	-	0 (0)
	51-60	11 (17)	-	2 (20)
	61+	28 (42)	-	7 (70)
	Unknown	0 (0)	10 (100)	0 (0)
Histological subtype of primary	SSM	35 (53)	-	-
	Nodular	14 (21)	-	-
	LMM	1 (2)	-	-
	DM	1 (2)	-	-
	Unknown	15 (23)	10 (100)	10 (100)
N-stage	1	34 (52)	-	-
	2	7 (11)	-	-
	3	25 (38)	-	-
	Unknown	0 (0)	10 (100)	10 (100)
Recurrence at Last FU	Yes	45 (68	-	-
	No	21(32)	-	-
	Unknown	0 (0)	10 (100)	10 (100)
Cause of death	Alive	24 (36)	-	-
	Melanoma	42 (64)	-	-
	Other Ca.	0 (0)	-	-
	Not Ca.	0 (0)	-	-
	Unknown	0 (0)	-	-
	Missing data	0 (0)	10 (100)	10 (100)

Table 2- Descriptive Statistics for serum cohorts

Prognostic factors		Healthy Controls n (%)	High Nevus Count n (%)	History of Melanoma n (%)	Tubingen Cohort Stage I/II n (%)	MIA Cohort Stage I/II n (%)	Combined Stage I/II n (%)	MIA Cohort Stage III n (%)	Tubingen Cohort Stage IV n (%)	MIA Cohort Stage IV n (%)	Combined Stage IV n (%)
	Totals	**102 (100)**	**12 (100)**	**16 (100)**	52 (100)	34 (100)	**86 (100)**	**50 (100)**	79 (100)	40 (100)	**119 (100)**
Sex	Male	**50 (49)**	**2 (17)**	**7 (44)**	19 (37)	20 (59)	**39 (45)**	**32 (64)**	48 (61)	28 (70)	**76 (64)**
	Female	**52 (51)**	**10 (83)**	**9 (56)**	33 (63)	14 (41)	**47 (55)**	**18 (36)**	31 (39)	12 (30)	**43 (36)**
Age at blood draw	20-30	**4 (4)**	**8 (67)**	**1 (6)**	3 (6)	3 (9)	**6 (7)**	**2 (4)**	3 (4)	1 (2)	**4 (3)**
	31-40	**5 (5)**	**4 (33)**	-	2 (4)	2 (6)	**4 (5)**	**3 (6)**	3 (4)	-	**3 (3)**
	41-50	**28 (27)**	-	**2 (13)**	12 (23)	2 (6)	**14 (16)**	**6 (12)**	19 (24)	5 (12)	**24 (20)**
	51-60	**19 (19)**	-	**6 (38)**	10 (19)	10 (29)	**20 (23)**	**15 (30)**	19 (24)	9 (23)	**28 (24)**
	61+	**46 (45)**	-	**7 (44)**	25 (48)	17 (50)	**42 (49)**	**24 (48)**	35 (44)	25 (63)	**60 (50)**
Histological subtype of primary	SSM	-	-	-	19 (36)	-	**19 (22)**	-	34 (43)	-	**34 (29)**
	Nodular	-	-	-	14 (27)	-	**14 (16)**	-	18 (23)	-	**18 (15)**
	LMM	-	-	-	4 (8)	-	**4 (5)**	-	-	-	-
	ALM	-	-	-	2 (4)	-	**2 (2)**	-	6 (7)	-	**6 (5)**
	Unknown	-	-	**16 (100)**	13 (25)	34 (100)	**47 (55)**	**50 (100)**	21 (27)	40 (100)	**61 (51)**
Breslow's thickness of	≤1 mm	-	-	-	3 (6)	-	**3 (3)**	-	18 (23)	-	**18 (15)**
	1.01-2 mm	-	-								

primary	2.01-4 mm	-	-	-	32 (61)	-	**32 (37)**	-	12 (15)	-	**12 (10)**
	>4 mm	-									
	Unknown	-	-	-	11 (21)	-	**11 (13)**	-	20 (25)	-	**20 (17)**
			-	-	5 (10)	-	**5 (6)**	-	16 (20)	-	**16 (13)**
				16 (100)	1 (2)	34 (100)	**35 (41)**	**50 (100)**	13 (16)	40 (100)	**43 (36)**
M staging	M1a	-	-	-	-	-	-	-	19 (24)	15 (38)	**34 (29)**
	M1b	-	-	-							
	M1c	-	-	-	-	-	-	-	16 (20)	9 (18)	**25 (21)**
	Unknown	-	-								
				-	-	-	-	-	44 (56)	16 (28)	**60 (50)**
					-	-	-	-	-	-	
Serum LDH	Elevated	-	-	-	-	-	-	-	27 (34)	7 (18)	**34 (29)**
	Normal	-	-	-	-						
	Unknown	-	-	-	-	-	-	-	42 (53)	5 (12)	**47 (40)**
						-	-	-	10 (13)	28 (70)	**38 (32)**
S100B	Elevated	-	-	-	-				43 (54)	-	**45 (38)**
	Normal	-	-	-	-						
	Unknown	-	-	-	-				25 (32)	-	**25 (21)**
									11 (14)	40 (100)	**55 (46)**
Cause of Death	Alive	-	**12 (100)**	**16 (100)**	48 (92)	30 (88)	**78 (91)**	**32 (64)**	15 (19)	20 (50)	**35 (29)**
	Melanoma	-	-								
	Other Ca.	-		-	2 (4)	4 (12)	**6 (7)**	**15 (30)**	62 (78)	20 (50)	**82 (69)**
	Not Ca.	-	-								
	Unknown	-		-	1 (2)	-	**1 (1)**	-	1 (1)	-	**1 (1)**
	Missing data	**102**	-	-	-	-	-	-	-	-	-
			-								
				-	1 (2)	-	**1 (1)**	**3 (6)**	1 (1)	-	**1 (1)**
			-								
				-	-	-	-	-			

Bolded texts are the cohorts that were used in the analysis

Table 3 Univariate analysis in serum cohorts for detectable miRNA

Comparison	Test	miR-145	miR-146a	miR-16	miR-204	miR-211	miR-363-3p	miR-4487	miR-4706	miR-4731	miR-506-3p	miR-509-3p	miR-509-5p	miR-514a
Controls (n=130) vs. **Stage I/II** (n=86)	Mann-Whitney U test	0·026	<0·0001	<0·0001	ns	ns	0·0088	<0·0001	<0·0001	<0·0001	ns	<0·0001	ns	ns
	AUROC score	0·64 (0·56, 0·7)	0·73 (0·67, 0·80)	0·85 (0·79, 0·90)	0·59 (0·48, 0·67)	0·53 (0·47, 0·59)	0·66 (0·58, 0·72)	0·95 (0·91, 0·98)	0·88 (0·83, 0·93)	0·89 (0·85, 0·94)	0·53 (0·48, 0·59)	0·76 (0·69, 0·82)	0·95 (0·92, 0·98)	0·53 (0·47, 0·59)
Controls (n=130) vs. **Stage III** (n=50)	Mann-Whitney U test	ns	0·039	<0·0001	ns	ns	ns	<0·0001	<0·0001	<0·0001	ns	<0·0001	<0·0001	ns
	AUROC score	0·57 (0·48, 0·66)	0·65 (0·57, 0·73)	0·87 (0·81, 0·91)	0·54 (0·46, 0·62)	0·55 (0·46, 0·65)	0·62 (0·49, 0·70)	0·93 (0·87, 0·98)	0·85 (0·79, 0·91)	0·85 (0·78, 0·90)	0·54 (0·47, 0·62)	0·72 (0·64, 0·79)	0·93 (0·89, 0·96)	0·53 (0·48, 0·61)
Controls (n=130) vs. **Stage IV** (n=119)	Mann-Whitney U test	ns	ns	0·0001	0·008	<0·0001	ns	<0·0001	<0·0001	<0·0001	ns	<0·0001	<0·0001	ns
	AUROC score	0·53 (0·47, 0·59)	0·60 (0·53, 0·66)	0·70 (0·64, 0·77)	0·65 (0·57, 0·71)	0·72 (0·65, 0·78)	0·55 (0·48, 0·61)	0·89 (0·84, 0·93)	0·85 (0·80, 0·90)	0·93 (0·89, 0·96)	0·53 (0·47, 0·59)	0·74 (0·67, 0·80)	0·91 (0·87, 0·94)	0·57 (0·47, 0·64)
Stage III (n=50) vs. **Stage IV**	Mann-Whitney U test	ns	ns	ns	0·025	0·025	0·025	0·0099	ns	0·025	ns	<0·0001	ns	ns
	AUROC score	0·55 (0·47, 0·64)	0·54 (0·47, 0·61)	0·64 (0·55, 0·72)	0·67 (0·59, 0·75)	0·66 (0·58, 0·74)	0·66 (0·58, 0·74)	0·73 (0·62, 0·82)	0·54 (0·47, 0·62)	0·67 (0·58, 0·76)	0·55 (0·47, 0·63)	0·78 (0·71, 0·84)	0·53 (0·47, 0·61)	0·57 (0·46, 0·66)

(n=119)													
Stage I/II (n=86) vs. **Stage IV** (n=119)	Mann-Whitney U test	0·026	0·043	0·031	0·0002	0·0014	0·0029	0·0082	ns	ns	ns	<0·0001	ns	ns
	AUROC score	0·64 (0·58, 0·72)	0·62 (0·55, 0·70)	0·63 (0·56, 0·70)	0·72 (0·66, 0·79)	0·71 (0·65, 0·78)	0·68 (0·61, 0·75)	0·67 (0·59, 0·74)	0·58 (0·49, 0·66)	0·57 (0·48, 0·65)	0·51 (0·48, 0·59)	0·79 (0·73, 0·86)	0·56 (0·44, 0·65)	0·58 (0·47, 0·66)

Table 4 Sensitivity and Specificity summary

MELmiR-7-panel	Melanoma vs. Controls	Stage I/II vs. Controls	Stage III vs. Controls	Stage IV vs. Controls
Diagnostic Score	≥4	≥4	≥4	≥4
Sensitivity	93%	93%	86%	95%
Specificity	≥82%	≥82%	≥82%	≥82%
False Positive Rate	18%	18%	18%	18%
False Negative Rate	7%	7%	14%	5%
Positive Predictive Value (PPV)	91%	77%	64%	82%
Negative Predictive Value (PPV)	85%	95%	94%	95%
Likelihood Ratio Positive	5·01	5·04	4·66	5·14
Likelihood Ratio Negative	0·09	0·09	0·17	0·06
Diagnostic Odds Ratio (DOR)	54·86	58·89	27·13	83·18

Table 5 Multivariate survival analysis of serum miRNAs and melanoma stage

Covariate	HR	Lower 95%CI	Upper 95%CI	*p* Value
Stage III	6·03	2·33	15·56	0·0002
Stage IV	8·48	3·12	23·01	<0·0001
hsa-miR-16	1·04	0·88	1·23	ns
hsa-miR-211	0·87	0·82	0·91	<0·0001
hsa-miR-4487	0·88	0·79	0·98	0·02
hsa-miR-4706	0·45	0·29	0·69	0·0002
hsa-miR-4731	0·98	0·90	1·06	ns
hsa-miR-509-3p	1·04	1·00	1·09	ns
hsa-miR-514a	0·92	0·85	0·99	0·02

FIGURES:

Figure 1 Study summary

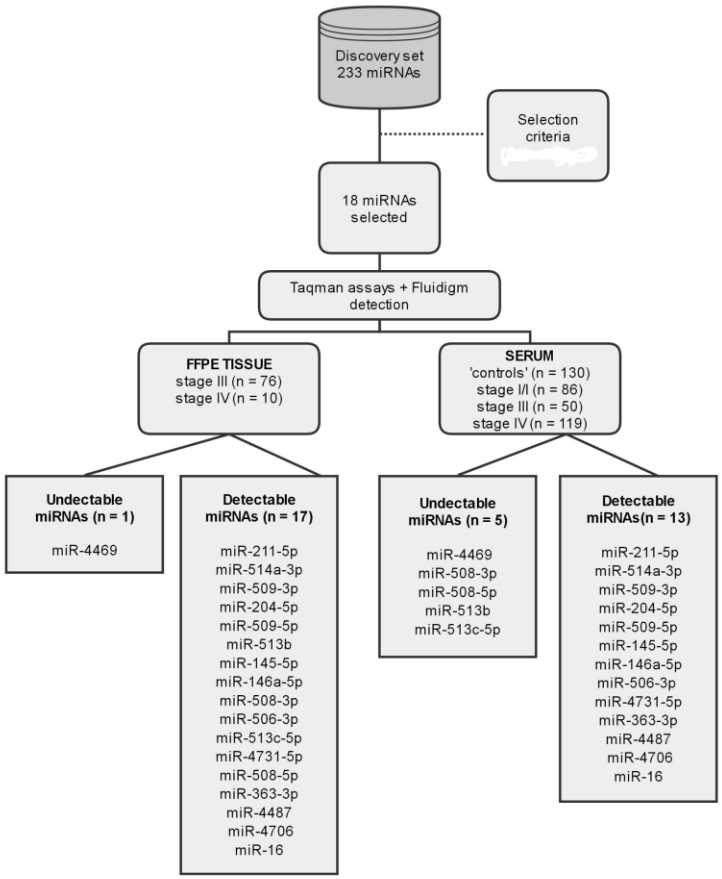

Figure 2 Expression of MELmiR-7 in stage IV progression patients

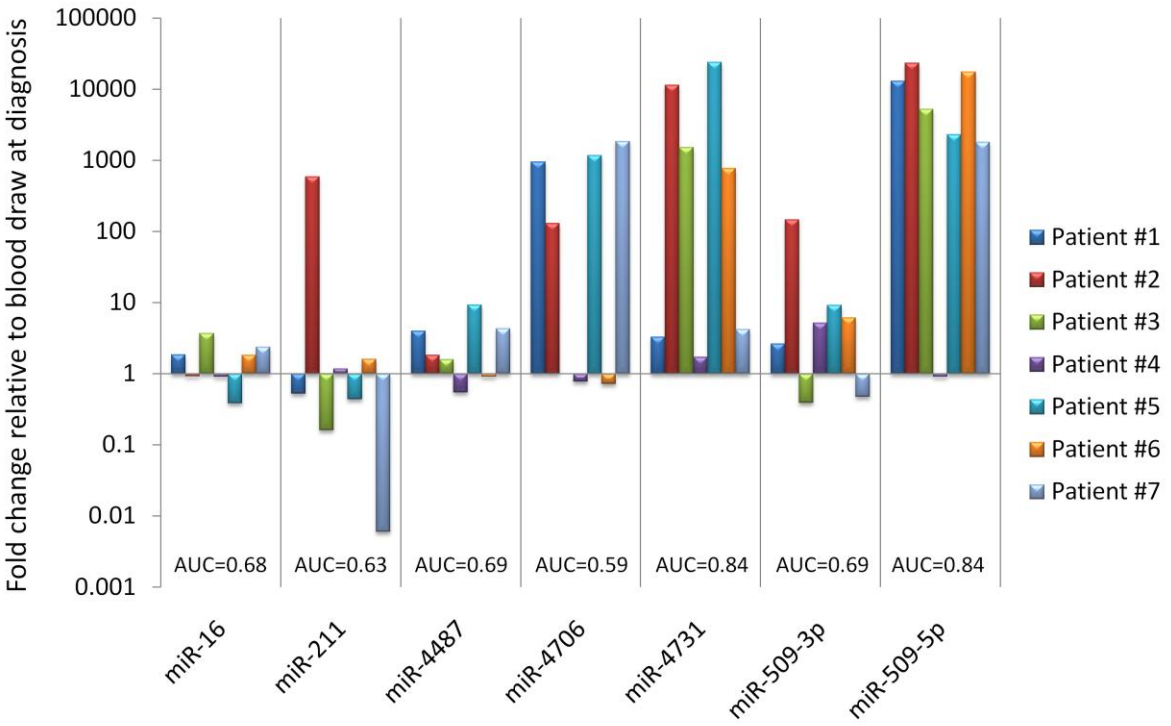

Figure 3 Conditional Inference tree predicting survival outcomes