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Circulating microRNAs are associated with docetaxel chemotherapy outcome in castration-resistant prostate cancer

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Background: Docetaxel is the first-line chemotherapy for castration-resistant prostate cancer (CRPC). However, response rates are ~50% and determined quite late in the treatment schedule, thus non-responders are subjected to unnecessary toxicity. The potential of circulating microRNAs as early biomarkers of docetaxel response in CRPC patients was investigated in this study.

Methods: Global microRNA profiling was performed on docetaxel-resistant and sensitive cell lines to identify candidate circulating microRNA biomarkers. Custom Taqman Array MicroRNA cards were used to measure the levels of 46 candidate microRNAs in plasma/serum samples, collected before and after docetaxel treatment, from 97 CRPC patients.

Results: Fourteen microRNAs were associated with serum prostate-specific antigen (PSA) response or overall survival, according to Mann–Whitney U or log-rank tests. Non-responders to docetaxel and patients with shorter survival generally had high pre-docetaxel levels of miR-200 family members or decreased/unchanged post-docetaxel levels of miR-17 family members. Multivariate Cox regression with bootstrapping validation showed that pre-docetaxel miR-200b levels, post-docetaxel change in miR-20a levels, pre-docetaxel haemoglobin levels and visceral metastasis were independent predictors of overall survival when modelled together.

Conclusions: Our study suggests that circulating microRNAs are potential early predictors of docetaxel chemotherapy outcome, and warrant further investigation in clinical trials.

Docetaxel is the first-line chemotherapy for castration-resistant prostate cancer (CRPC), providing modest survival and palliative benefits (Petrylak *et al*, 2004; Tannock *et al*, 2004). Response to docetaxel treatment in clinical practice is usually determined by changes in serum prostate-specific antigen (PSA) levels and radiological scans after three treatment cycles (~2 months).

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The response rate is ~50%, and a high percentage of patients experience significant toxicity (Tannock *et al*, 2004). Given the advent of alternative effective therapies (e.g. abiraterone, enzalutamide, cabazitaxel, radium-223) (Bishr and Saad, 2013) and the predominantly elderly status of patients, there is a need for therapeutic response biomarkers that can identify non-responders earlier in the treatment schedule. Biomarkers associated with docetaxel response may also provide insights into mechanisms of docetaxel resistance, thus leading to the development of new therapeutic strategies.

MicroRNAs are short non-coding RNAs that negatively regulate gene expression by binding to messenger RNA, causing inhibition of protein translation or messenger RNA destabilisation (Lujambio and Lowe, 2012). Many microRNAs have dysregulated expression in cancer, contribute to tumour progression, and are involved in drug resistance (Kutanzi *et al*, 2011; Lujambio and Lowe, 2012). Cells are capable of secreting microRNAs, encapsulated in microvesicles or bound to proteins, potentially as a way of influencing other cells, similar to the action of hormones (Cortez *et al*, 2011). The high stability of microRNAs (Mitchell *et al*, 2008) and abnormal circulating microRNA profiles in cancer patients (Cortez *et al*, 2011) have generated a lot of interest regarding their potential as prognostic biomarkers in various cancers including prostate cancer (Sita-Lumsden *et al*, 2013). To the best of our knowledge, only three studies to date have investigated the potential of circulating microRNAs as therapeutic response biomarkers in metastatic prostate cancer but were limited by the size of patient cohorts and the number of microRNAs tested (Gonzales *et al*, 2011; Zhang *et al*, 2011; Cheng *et al*, 2013).

The aim of our study was to determine if there is an association between a panel of 46 circulating microRNAs and docetaxel chemotherapy outcome in CRPC patients. We report our discovery of circulating microRNAs that may have potential as early therapeutic response biomarkers of docetaxel treatment.

MATERIALS AND METHODS

Cell line profiling. PC3 and DU145 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA), and were treated with escalating doses of docetaxel (Sanofi-Aventis, Surrey, UK) to produce docetaxel-resistant sublines (PC3Rx, DU145Rx) (Zhao *et al*, 2009; Lee *et al*, 2014). The sublines were significantly more resistant to docetaxel than their parental cell lines, with an increase in IC50 of more than 10-fold, without any change in P-glycoprotein activity (Zhao *et al*, 2009; Lee *et al*, 2014). The identity of the cell lines were validated by CellBank Australia (New South Wales, Australia).

Total RNA was extracted from cells that were non-treated or treated with 8 ng ml^{-1} docetaxel for 24 h (three replicates per treatment group), using the mirVana microRNA Isolation Kit (Ambion, Foster City, CA, USA), followed by additional cleanup with sodium acetate precipitation and ethanol wash. From each sample, 600 ng of total RNA was reverse transcribed using Taqman MicroRNA Reverse Transcription Kit with Megaplex Primer Pools A & B (Applied Biosystems, Foster City, CA, USA). The cDNA was analysed with Taqman Array Human MicroRNA A + B Cards Set version 3 (Applied Biosystems) on the Applied Biosystems 7900HT qPCR system. The data was normalised by global mean normalisation (Supplementary information).

Patients and blood collection. Patients receiving docetaxel chemotherapy (75 mg m^{-2} every 3 weeks = 1 cycle) were recruited for the study from Royal Prince Alfred Hospital, Westmead Hospital, Concord Repatriation General Hospital, Sydney Adventist Hospital, Calvary Mater Newcastle Hospital, and Royal North Shore Hospital. Castration-resistant prostate cancer was defined as PSA or clinical progression after maximal androgen blockade, with a minimum of 4 weeks having elapsed between the withdrawal of anti-androgens and commencement of chemotherapy. Blood samples were collected before the first cycle of docetaxel, and after the first, second, or third cycle just prior to the next cycle (Figure 1A), according to a standardised protocol using BD Vacutainer tubes (BD, Plymouth, UK) containing K_2EDTA for plasma separation, or clot activator and gel for serum separation. The blood samples were centrifuged at 3000 **g** for 5 min at room temperature to obtain plasma or serum, aliquoted and stored at −80 °C within 30 min of collection. The collection period was from October 2005 until October 2012.

Patients were seen by the clinicians every 3 weeks and serum PSA levels were measured from the blood collected during these visits. Radiological assessment was inconsistent in this cohort, prohibiting its use as a response criterion. Prospective Eastern Cooperative Oncology Group (ECOG) performance status was also not available for all cases. Prostate-specific antigen response (that is, changes in serum PSA) after three cycles of docetaxel was used to classify chemo response – partial response, at least 50% decrease in PSA from pre-docetaxel levels; progressive disease, increase of at least 25% from pre-docetaxel levels; stable disease, decrease of less than 50% or an increase of less than 25% from pre-docetaxel levels (Petrylak *et al*, 2004; Tannock *et al*, 2004). Patients with partial response are considered as responders, whereas patients with stable or progressive disease are grouped together as non-responders.

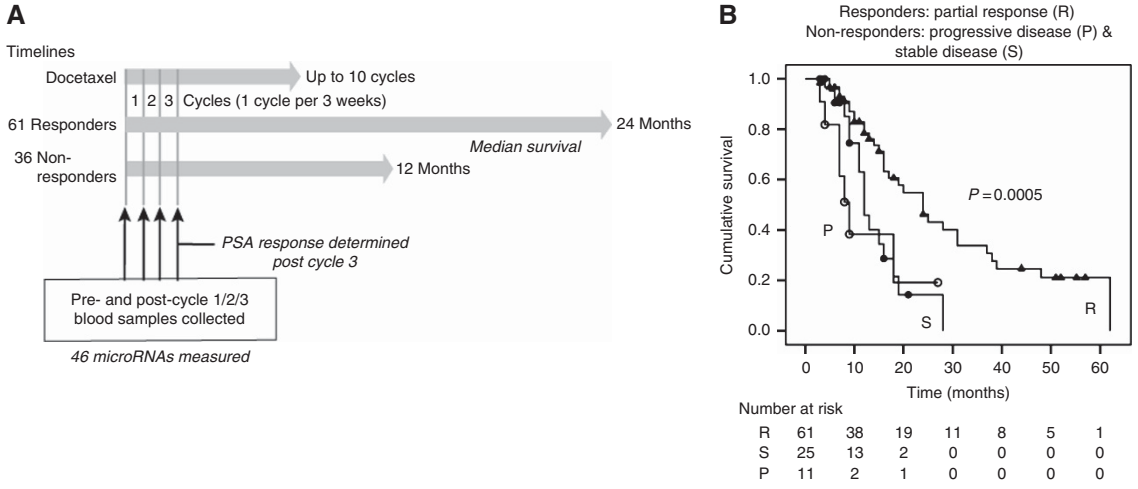

Figure 1. Study schema. (**A**) Timeline of collection from patients; (**B**) Kaplan–Meier plot of overall survival according to PSA response.

Overall survival was defined as the time from initiation of the first cycle of docetaxel to the time of death or last follow-up.

All patients provided written informed consent, and the study was approved by relevant human research ethics committees (Approval no. 13663) and registered with the Australian New Zealand Clinical Trials Registry and the Australian Cancer Trials Registry.

MicroRNA profiling of plasma/serum. Total RNA were extracted from 200 μl of plasma/serum and eluted in 14 μl RNase-free water, using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) according to the kit's instructions, except 1 ml of Qiazol and 200 μl of chloroform were used per sample. Prior to RNA extraction, the plasma/serum was centrifuged at 12000 **g** for 1 min and the supernatant transferred to a new tube for the extraction. Extracted RNA was prepared for real-time PCR (qPCR) on Custom Taqman Array microRNA cards (Part No. 4342253, configuration 4, Applied Biosystems) with pre-amplification of cDNA using custom pre-amplification primer pools provided with the cards, according to the manufacturer's protocol, with a few modifications – total RNA input was 4 μl for the 15 μl reverse transcription reaction volume; cDNA input was 8.75 μl for the 25 μl pre-amplification reaction volume; nuclease-free water was omitted for both reverse-transcription and pre-amplification reactions; and 25 μl of undiluted pre-amplified cDNA was combined with 31.25 μl nuclease-free water in the 112.5 μl qPCR reaction volume. The microRNA cards were analysed on the Applied Biosystems 7900HT system. The data was normalised by global mean normalisation (Supplementary information). The final values of microRNA levels are in logarithm scale, relative to global mean and thus referred to as relative levels.

The custom Taqman array microRNA cards in this study were designed to have 47 unique microRNA assays and a default control assay (small nuclear RNA U6), for the analysis of four biological samples per array without any replicates. The microRNA assays selected for the arrays were for 46 microRNAs based on cell line profiling results (Supplementary Table S1), and ath-miR-159a, which was a spike-in control during plasma/serum extraction.

Single qPCR validation. Single qPCR of miR-20a, miR-20b, miR-21, miR-146a, miR-200a, miR-200b, miR-200c, miR-222, miR-301b and miR-429 was performed on pre- and post-docetaxel plasma/serum samples from 20 patients (14 serum and 26 plasma samples) to validate the performance of the Taqman arrays. For each sample, 6.25 μl of surplus cDNA from the previous reverse transcription of plasma/serum RNA was pre-amplified in a 18 μl final reaction volume using custom pre-amplification primer pools provided with the cards as described above and diluted 1 : 4 with nuclease-free water. Single qPCR was performed on the diluted pre-amplified cDNA using Taqman probes from single tube Taqman microRNA assays (Cat #4427975), with 4.5 μl of the diluted cDNA in a 10 μl reaction volume, in duplicate for each assay, on the Applied Biosystems 7900HT system.

Statistical analyses. Multiple *t*-tests were performed on cell line profiles to identify differences in microRNA levels between treatment groups, using MultiExperiment Viewer version 4.8 (Saeed *et al*, 2003). MicroRNAs with less than 1.4-fold difference between comparisons were filtered. Mann–Whitney *U*, Kaplan–Meier, log-rank statistic, Receiver Operating Characteristic (ROC), Cox regression and bootstrapping analyses were performed using SPSS Statistics version 21 (IBM). Analyses of changes in microRNA levels after the first chemotherapy cycle were performed on fold differences between matched pre- and post-cycle microRNA levels of individual patients. Multivariate Cox regression of microRNAs and clinicopathologic factors was performed using the forward stepwise (conditional LR) method. The predictive performance of the final Cox regression model was assessed by internal validation using bootstrap resampling. One-thousand bootstrap samples were selected from the original cohort with replacement, and the Cox regression parameters of the final model were applied to each sample. The mean of the hazard ratios for each variable were calculated from the 1000 samples. *P*-value $\leqslant 0.05$ was required for statistical significance in all statistical analyses.

RESULTS

Biomarker selection from cell line models. The levels of 754 microRNAs were profiled in PC3 and DU145 cell lines and their docetaxel-resistant sublines (PC3Rx and DU145Rx), treated or non-treated with docetaxel, to identify potential microRNA biomarkers. The total number of microRNAs detected was 241 and 246 in the cell lines of the PC3 and DU145 models, respectively. Multiple *t*-test comparisons of the microRNA levels revealed a higher number of differentially expressed microRNAs between docetaxel-resistant and sensitive cell lines (53 and 28 for PC3 and DU145 models, respectively), compared with those between docetaxel-treated and non-treated cells (15 and 37 for PC3 and DU145 models, respectively) (Supplementary Table S2). *P*-value adjustment was considered too stringent and not used to filter the results.

Eight differentially expressed microRNAs were in common for both cell line models, in the comparisons between docetaxel-resistant and sensitive cell lines, suggesting their regulation of common docetaxel-resistance pathways (Table 1). Interestingly, three members of the miR-200 family – miR-200a, miR-200b, miR-429, were downregulated in docetaxel-resistant cells relative to sensitive, for both models (Table 1). Two other miR-200 family members were also downregulated in docetaxel-resistant cells relative to sensitive, but were not in common for both models – miR-200a-5p for the PC3 model, and miR-200c for the DU145 model (Table 1).

To reduce the chances of false positives, candidates for measurement in blood were chosen according to their high statistical ranking among the different comparisons (e.g. miR-9, miR-105); their overlap between the two cell line models (e.g. miR-200 family members); or their biological significance such as known associations with cancer prognosis and chemoresistance (e.g. miR-21 (Krichevsky and Gabriely, 2009), which was differentially expressed between docetaxel-treated and non-treated resistant cells of the PC3 model). A total of 46 microRNAs was selected for measurement in blood samples (Supplementary Table S1).

Characteristics of patients. Ninety-seven CRPC patients were recruited for the study (Table 2). Patients with stable disease and progressive disease were considered as non-responders to docetaxel, as the median survival time was similar for these two groups compared with patients with partial response (Figure 1B). The characteristics of our patient cohort were generally similar to those of TAX327 (Tannock *et al*, 2004) and SWOG 99-16 (Petrylak *et al*, 2004), the two landmark Phase 3 studies, which demonstrated the survival and palliative benefits of docetaxel in CRPC patients. A notable exception in our cohort is the higher rate of responders (63%), than those two studies (45–50%).

Post-cycle 1 blood samples were only available for 58 (60%) patients, thus post-cycle 2 and post-cycle 3 blood samples for the other 29 (30%) and 10 (10%) patients, respectively were used as substitutes. It is assumed that any changes in microRNA levels after one cycle of docetaxel will have the same trend in subsequent cycles, thus all post-cycle changes in microRNA levels were analysed as dichotomous variables in terms of the direction of the change in the microRNA levels (i.e. increase or decrease/no change

Table 1. Top 28 differentially expressed microRNAs between docetaxel-resistant and sensitive cell lines

PC3 model				DU145 model			
Rank	**microRNA**	**Fold**	***P*-value**	**Rank**	**microRNA**	**Fold**	***P*-value**
Upregulated in docetaxel-resistant cell lines							
1	miR-598	54	3×10^{-8}	1	miR-30e-3p	2.3	6×10^{-5}
2	miR-105	4.7	1×10^{-5}	2	miR-191-3p	23	1×10^{-4}
3	miR-767-5p	18	2×10^{-5}	3	miR-30a-5p	1.9	7×10^{-4}
4	miR-24	3.1	3×10^{-5}	4	miR-152	1.9	1×10^{-3}
5	miR-342-3p	2.5	1×10^{-4}	5	miR-34b	2.2	2×10^{-3}
6	miR-125a-5p	2.0	2×10^{-4}	6	miR-301b	2.2	2×10^{-3}
7	miR-27a	2.9	2×10^{-4}	7	miR-100	2.2	3×10^{-3}
8	miR-194	2.7	3×10^{-4}	8	miR-301a	2.2	4×10^{-3}
9	miR-454	2.4	4×10^{-4}	*9*	*miR-99a*	*1.8*	6×10^{-3}
10	miR-26b	1.7	4×10^{-4}	*10*	*miR-151-3p*	*1.4*	7×10^{-3}
11	miR-146a	2.5	6×10^{-4}	*11*	*miR-30a-3p*	*2.5*	7×10^{-3}
12	miR-99b	2.0	7×10^{-4}	*12*	*miR-146b-5p*	*1.9*	8×10^{-3}
13	miR-455-3p	5.0	9×10^{-4}	*13*	*miR-210*	*1.7*	2×10^{-2}
14	miR-301a	3.3	1×10^{-3}	*14*	*miR-15a-3p*	*2.8*	3×10^{-2}
Downregulated in docetaxel-resistant cell lines							
1	miR-9	−29	2×10^{-7}	1	miR-135b	−2.0	1×10^{-6}
2	**miR-200a**	−176	4×10^{-7}	2	**miR-200b**	−2.5	1×10^{-4}
3	miR-1267	−15	1×10^{-6}	3	miR-590-3p	−1.5	5×10^{-4}
4	miR-205	−8	4×10^{-6}	4	**miR-429**	−2.3	5×10^{-4}
5	miR-95	−30	8×10^{-6}	5	miR-590-5p	−1.5	2×10^{-3}
6	**miR-200a-5p**	−46	1×10^{-5}	6	miR-20b	−2.2	2×10^{-3}
7	**miR-200b**	−65	7×10^{-5}	7	**miR-200c**	−1.9	2×10^{-3}
8	miR-203	−19	4×10^{-4}	8	**miR-200a**	−2.6	4×10^{-3}
9	miR-34a	−20	8×10^{-4}	*9*	*miR-25*	*−1.6*	7×10^{-3}
10	miR-375	−13	8×10^{-4}	*10*	*miR-146a*	*−2.8*	8×10^{-3}
11	miR-191	−1.7	1×10^{-3}	*11*	*miR-196b*	*−1.7*	1×10^{-2}
12	miR-100	−1.7	1×10^{-3}	*12*	*miR-489*	*−3.5*	2×10^{-2}
13	miR-135b	−3.0	1×10^{-3}	*13*	*miR-96*	*−2.7*	4×10^{-2}
14	**miR-429**	−116	1×10^{-3}	*14*	*miR-486-5p*	*−6.8*	5×10^{-2}

Shaded, microRNAs in common in both models (only 6 are among these top 28; miR-25 not shown among microRNAs downregulated in docetaxel-resistant PC3 as its ranking is 23rd); Bold, miR-200 family members; italicised, Benjamini–Hochberg false discovery rate adjusted *P*-value > 0.05; Negative fold, downregulated in docetaxel-resistant relative to sensitive cell lines.

from baseline levels) in survival and ROC analyses. Henceforth, post-cycle 1, 2 and 3 samples are collectively referred to as 'post-docetaxel' samples.

The blood samples from 65 (67%) patients were plasma, whereas those from 32 (33%) patients of the same hospital were serum. Both sample types were used in this study, as other studies have shown that microRNA levels in matched plasma and serum samples from the same individual are highly correlated (Mitchell *et al*, 2008; Wang *et al*, 2012).

Quantitation of circulating microRNAs. The microRNA levels in plasma and serum samples were measured by qPCR using custom Taqman Array MicroRNA Cards, which contain pre-loaded Taqman Gene Expression assays for the 46 candidate microRNAs. All of these candidate microRNAs, except miR-767-5p, were detectable in the plasma and serum samples. The majority of microRNAs have low Ct (number of cycles required for fluorescent signal to cross the set threshold; inversely proportional to microRNA levels) (median 22, 1st quartile 18, 3rd quartile 26, range 4–40) indicating the high sensitivity of the method (Supplementary Figure S1).

Exogenous addition of non-human microRNAs or endogenous small nuclear RNA such as U6 are proposed as normalisers by some studies (Meyer *et al*, 2010). However, the disadvantages of these methods are that exogenous additives only control for technical variability during the RNA extraction method and U6 levels in blood samples were reported to be inconsistent (Qi *et al*, 2012; Wang *et al*, 2012). We found that global mean normalisation was better at reducing data variation compared with ath-miR-159a spike or U6 (Supplementary Figure S2). The global microRNA levels in serum samples were lower than plasma samples, but these differences were adjusted by global mean normalisation (Supplementary Figure S3).

The qPCR performance of the custom Taqman Array MicroRNA Cards were compared with single qPCR of 10 microRNAs in surplus cDNA from the Taqman Array MicroRNA Cards' analysis of pre- and post-docetaxel plasma/serum samples from 20 patients (total of 14 serum and 26 plasma samples). The microRNA profiles of the patients were very similar for both techniques, as indicated by high Pearson correlation coefficients (R) ranging from 0.83 to 0.99 for all samples except one which had $R = 0.53$ (Supplementary Figure S4A). The poor correlation of that sample was due to undetected expression of two microRNAs by single qPCR. The correlation of microRNA levels measured by both techniques was also generally high when the levels were examined according to each microRNA with R ranging from 0.75 to 0.98 (Supplementary Figure S4B). Overall, the high correlation of microRNA profiles between the two techniques indicates that the patient's microRNA

Table 2. Characteristics of patient cohort

	Number of patients (%)	Median (range)
Age (years)	97 (100)	68 (46–87)
Follow-up (months)		12 (3–62)
PSA response		
Partial response	61 (63)	
Stable disease	25 (26)	
Progressive disease	11 (11)	
Status		
Alive	42 (43)	
Dead	55 (57)	
Median survival (months)		
Partial response		24
Stable disease		12
Progressive disease		9
Gleason score at diagnosis		8 (5–10)
<7	7 (7)	
7	22 (23)	
>7	47 (48)	
Unknown	21 (22)	
Serum PSA ($\mu g\,l^{-1}$)[a]	95 (98)	145 (0.6–3882)
Haemoglobin ($g\,l^{-1}$)[a]	96 (99)	124 (67–162)
Alkaline phosphatase ($U\,l^{-1}$)[a]	88 (91)	141 (15–2962)
Metastasis[a]		
None	5 (5)	
Bone	48 (50)	
Visceral	13 (13)	
Both	25 (26)	
Unknown	6 (6)	

Abbreviation: PSA = prostate-specific antigen.
[a]Before chemotherapy.

profile is reproducible with both techniques, and confirms the high accuracy and sensitivity of the custom Taqman Array MicroRNA Cards for profiling circulating microRNA levels.

Circulating microRNAs associated with PSA response. Six microRNAs – miR-200c, miR-200b, miR-146a, miR-222, miR-301b and miR-20a, were significantly associated with PSA response (Mann–Whitney U test, $P<0.05$), according to their pre-docetaxel levels, or the direction of post-docetaxel change from baseline levels (Figure 2A). Non-responders tended to have higher pre-docetaxel levels of miR-200c or miR-200b, or lower pre-docetaxel levels of miR-146a (Figure 2A). After docetaxel treatment, non-responders tended to have a decrease/no change in post-docetaxel levels of miR-222 or miR-20a, or an increase in post-docetaxel levels of miR-301b (Figure 2B).

The ability of these six microRNAs to predict chemoresponse was assessed by ROC. High pre-docetaxel levels of miR-200c or miR-200b, or low pre-docetaxel levels of miR-146a predicted non-responders when analysed as a continuous variable (Figure 2C). The fold change in miR-222, miR-20a or miR-301b after docetaxel treatment also predicted non-responders when analysed as a continuous variable (Figure 2D). When the change in miR-222, miR-20a or miR-301b was analysed as a categorical variable to take into account the different post-cycle collections, a decrease/no change in miR-222 or miR-20a, or an increase in miR-301b after docetaxel treatment also predicted non-responders (Figure 2E). The combined predictive ability of the microRNAs was assessed by using the sum of scores as variables, where the pre-docetaxel levels or post-docetaxel change for each microRNA was dichotomised and its categories represented by the score of 0 or 1 (Figure 2F). The combined scores of all six microRNAs produced an AUC of 0.73 (95% CI 0.62–0.84), which was higher than those of individual microRNAs (Figure 2F), indicating that a combined panel of microRNAs could be a better predictor than single microRNAs.

Circulating microRNAs associated with overall survival. Twelve microRNAs were significantly associated with overall survival (log-rank $P<0.05$) according to their pre-docetaxel levels or the direction of change in their post-docetaxel levels (Table 3, Figure 3). High pre-docetaxel levels of miR-200b, miR-429, miR-200a, miR-21, miR-200c, miR-375, miR-132 or miR-20a, or low pre-docetaxel levels of miR-590-5p were associated with shorter survival (Table 3, Figure 3). A decrease/no change in post-docetaxel levels of miR-20a, miR-222, miR-20b, miR-132 or miR-25 was associated with poor prognosis (Table 3, Figure 3).

Among the top five microRNAs with pre-docetaxel levels associated with overall survival were four members of the miR-200 family – miR-200b, miR-429, miR-200a and miR-200c (Table 3, Figure 3). The risk of death was 2.3- to 3.5-fold higher in patients with high pre-docetaxel levels of any of these microRNAs, than those with low levels ($P<0.007$). Among the top microRNAs with post-docetaxel changes associated with overall survival were two members of the miR-17 family – miR-20a and miR-20b (Table 3, Figure 3). The risk of death was 2.6- to 3.3-fold higher in patients with a decrease/no change in any of these microRNAs after docetaxel treatment than those with an increase ($P\leqslant0.004$).

Certain microRNAs were consistently associated with outcome measures in more than one setting. Both pre-docetaxel levels and post-docetaxel changes for miR-132 and miR-20a were associated with overall survival (Table 3). Pre-docetaxel levels of miR-200b and miR-200c, and post-docetaxel changes in miR-222 and miR-20a, were associated with both PSA response and overall survival (Figure 2, Table 3). The associations were in the same direction, where high pre-docetaxel levels, or a decrease/no change in post-docetaxel levels were associated with poor PSA response or shorter survival.

Although circulating microRNA levels in plasma and serum from matched individuals were shown to be generally highly correlated in healthy individuals (Mitchell *et al*, 2008; Wang *et al*, 2012), it is unknown if this correlation also applies to patients with tumours or diseases. In our study, statistical analyses of patients according to their respective sample types were biased by differences in patient characteristics and cohort size. For example, the serum cohort is smaller and has a higher death rate and longer follow-up time (Supplementary Information Table S3). Interestingly, despite these differences, most microRNAs significantly associated with PSA response or overall survival in the whole cohort were also significant in either the serum or plasma cohort, when these cohorts were analysed separately (data not shown). More importantly, four microRNAs (miR-200a, miR-429, miR-375 and miR-222) were in common for the serum, plasma and the combined cohorts in their association with overall survival ($P<0.05$), indicating that these microRNAs are likely to be robust biomarkers that are not influenced by the blood sample type.

Prognostic model combining microRNAs and clinicopathologic factors. The ability of the microRNAs to predict overall survival in comparison with established prognostic clinicopathologic factors was evaluated by multivariate Cox regression. Stepwise regression analysis of the top 10 microRNAs associated with survival, together with clinicopathologic factors that were significant in univariate Cox regression, revealed that high pre-docetaxel levels ofmiR-200b, decreased/unchanged post-docetaxel levels of miR-20a, low

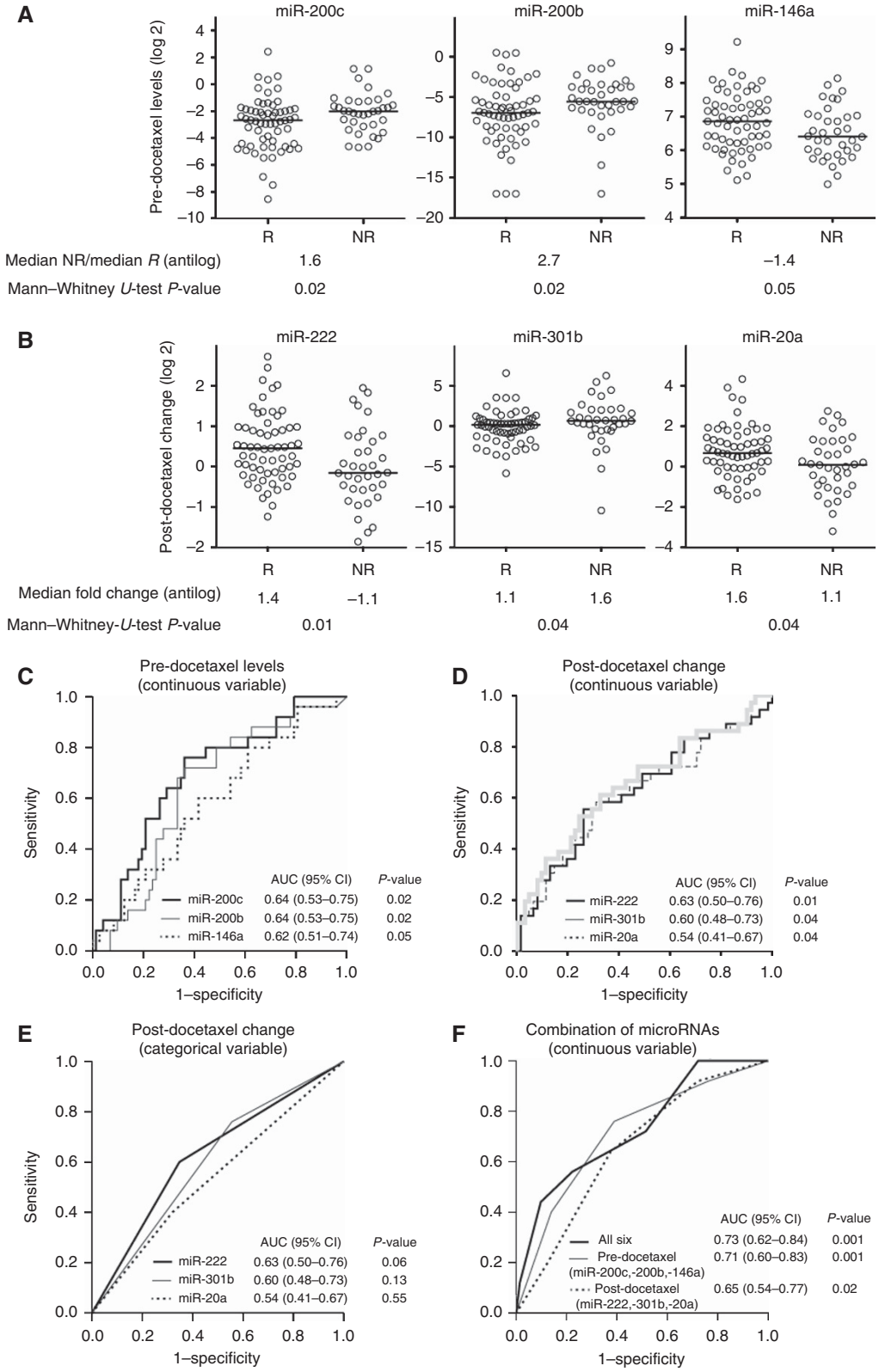

Figure 2. Circulating microRNAs associated with PSA response. Scatter plots of (**A**) pre-docetaxel levels and (**B**) post-docetaxel change in microRNA levels in responders and non-responders (negative fold refers to decrease in first group of comparison; R, responder (partial response); NR, non-responder (stable or progressive disease)); ROC analysis assessing the association of microRNAs and chemoresponse (responder or non-responder) by (**C**) pre-docetaxel microRNA levels as continuous variables, (**D**) post-docetaxel change in microRNA levels as continuous variables, (**E**) post-docetaxel change in microRNA levels as categorical variables, and (**F**) combinations of pre-docetaxel levels and post-docetaxel change using the sum of scores as variables, where each microRNA was dichotomised and its categories represented by the score of 0 or 1 as follows: score 0 (low risk) = pre-docetaxel miR-200c or miR-200b levels ⩽ median, pre-docetaxel miR-146a level > median, post-docetaxel miR-222 or miR-20a levels increased from baseline levels, post-docetaxel miR-301b levels decreased/unchanged from baseline levels; score 1 (high risk) = converse of criteria for score 0.

Table 3. MicroRNAs associated with overall survival (log-rank *P*-value < 0.05)

	Univariate Cox regression	
microRNA	***P*-value**	**Hazard ratio (95% CI)**
Pre-docetaxel levels, high (> median) *vs* low (⩽ median)		
miR-200b	0.0001	3.2 (1.7–5.9)
miR-429	0.0003	3.5 (1.8–6.9)
miR-200a	0.0004	2.8 (1.6–5.0)
miR-21	0.004	2.3 (1.3–3.9)
miR-200c	0.006	2.3 (1.3–4.1)
miR-590-5p	0.007	0.5 (0.3–0.8)
miR-375	0.02	2.0 (1.2–3.6)
miR-132	0.02	1.9 (1.1–3.2)
miR-20a	0.05	1.8 (1.0–3.3)
Post-docetaxel change, increased *vs* decreased/unchanged		
miR-20a	0.0004	0.30 (0.16–0.58)
miR-222	0.002	0.43 (0.25–0.74)
miR-20b	0.004	0.39 (0.20–0.74)
miR-132	0.005	0.45 (0.26–0.79)
miR-25	0.01	0.48 (0.28–0.84)

Abbreviation: CI = confidence interval.

pre-docetaxel haemoglobin and the presence of visceral metastasis were independent predictors of poor overall survival ($P \leqslant 0.02$) (Table 4). Lactase dehydrogenase levels and ECOG performance status were not available for comparing the prognostic ability of the regression model to the Halabi nomogram (Halabi *et al*, 2003). The regression model was validated by bootstrap analysis. The bootstrap estimates of the hazard ratios and confidence intervals for each of the factors were very similar to the original model, suggesting excellent internal validation (Table 4).

The ability of these four factors to predict death within 12 months was assessed by ROC (Figure 4). The factors were assessed individually, or in combination using the sum of scores as variables, where each factor was dichotomised and its categories represented by the score of 0 or 1 (Figure 4). Pre-docetaxel levels of miR-200b was the best single predictor with an AUC of 0.72 (95% CI 0.60–0.83) (Figure 4). The combination of all four factors was a better predictor with an AUC of 0.77 (95% CI 0.67–0.88), suggesting that combining microRNAs with clinicopathologic factors could be a better prognostic indicator than individual factors.

DISCUSSION

Our study has identified that circulating microRNAs, notably those of the miR-200 and miR-17 families, are associated with PSA response and/or overall survival in CRPC patients. These circulating microRNAs have the potential to be early therapeutic response biomarkers of docetaxel treatment and prognostic biomarkers in CRPC patients. Moreover, as regulators of gene expression, these microRNAs may also be directly involved with docetaxel resistance and therefore are potential therapeutic targets.

Our study is the only study to date that has discovered an association between multiple circulating microRNAs with docetaxel response and overall survival in CRPC patients. Other studies only assessed the levels of individual microRNAs (Gonzales *et al*, 2011; Zhang *et al*, 2011; Cheng *et al*, 2013), of which only miR-21 was significant in our study. Zhang *et al* (2011) reported that pre-docetaxel serum levels of miR-21 were higher in four CRPC patients that were resistant to docetaxel, compared with six patients with partial response (Zhang *et al*, 2011). In contrast, our study found that there was no difference in the levels of miR-21 between responders and non-responders. Instead, we found that high pre-docetaxel levels of miR-21 were associated with shorter survival. This discrepancy is likely due to differences in cohort size.

Several studies have reported that some of the identified circulating microRNAs were dysregulated in prostate cancer patients, thus indicating that these microRNAs are involved in disease progression. Higher levels of miR-21, miR-200a, miR-200b, miR-200c or miR-375 in serum, plasma or circulating microvesicles were observed in patients with metastatic disease compared with those with localised disease or healthy controls (Brase *et al*, 2011; Zhang *et al*, 2011; Bryant *et al*, 2012; Nguyen *et al*, 2012; Cheng *et al*, 2013). The exact cellular source of these microRNAs is unclear in these studies. However, other studies showed that the levels of miR-21, miR-200c and miR-375 were higher in prostate tumours compared with matched normal tissues suggesting their derivation from tumours (Ambs *et al*, 2008; Ribas *et al*, 2009; Schaefer *et al*, 2010).

The miR-200 and miR-17 families and miR-222 demonstrate consistent associations with response to docetaxel and overall survival from CRPC. The miR-200 family members are well known for their regulation of epithelial-to-mesenchymal transition (EMT), a mechanism of drug resistance and metastasis, where downregulation of these microRNAs induces EMT (Gregory *et al*, 2008). However, high levels of miR-200 family members are associated with poor chemotherapy outcome in our study. A possible role of miR-200 family in CRPC is enhanced metastatic colonisation, similar to that observed in breast cancer (Korpal *et al*, 2011). Increased expression of miR-200s in breast cancer was associated with poor prognosis in patients. Mouse models and *in vitro* experiments showed that the miR-200s promoted metastatic colonisation of breast cancer cells through its gene target *SEC23A* (Korpal *et al*, 2011). SEC23A, a component of the endoplasmic reticulum, is involved in endoplasmic reticulum-Golgi protein trafficking and regulates the secretion of metastasis-suppressive proteins such as Igfbp4 and Tinagl1 in mice (Korpal *et al*, 2011). Potentially, overexpression of miR-200 family members in prostate cancer cells directly inhibits production of SEC23A, which prevents the secretion of metastasis-suppressive proteins, thus enabling the metastatic spread and colonisation of the cancer cells.

The association of miR-17 family members (miR-20a, miR-20b) with docetaxel resistance may be through the regulation of acute inflammatory pathways, as these microRNAs have immune regulatory functions (Bonauer and Dimmeler, 2009), and inflammation is known to promote tumorigenesis and chemoresistance (Mahon *et al*, 2011). The docetaxel-resistance mechanism mediated by miR-17 family members may involve regulation of macrophage differentiation, as downregulation of the miR-17 family is required for monocytes to differentiate into macrophages (Fontana *et al*, 2007), and macrophages have been implicated in chemoresistance. Tumour-associated macrophages are associated with chemoresistance in breast cancer (Shree *et al*, 2011), and macrophage-associated cytokines were associated with docetaxel response in CRPC patients (Zhao *et al*, 2009; Horvath *et al*, 2013). The miR-17 family controls monocytopoiesis by targeting RUNX1, a subunit of the transcription factor CBF that controls the expression of genes involved in myeloid differentiation such as CSF1R (Fontana *et al*, 2007). Inhibition of miR-17 upregulated RUNX1 and CSF1R levels, leading to monocytic differentiation and maturation (Fontana *et al*, 2007). Therefore, downregulation of miR-17 family members in CRPC patients suggests increased macrophage differentiation that may promote docetaxel resistance and tumour progression.

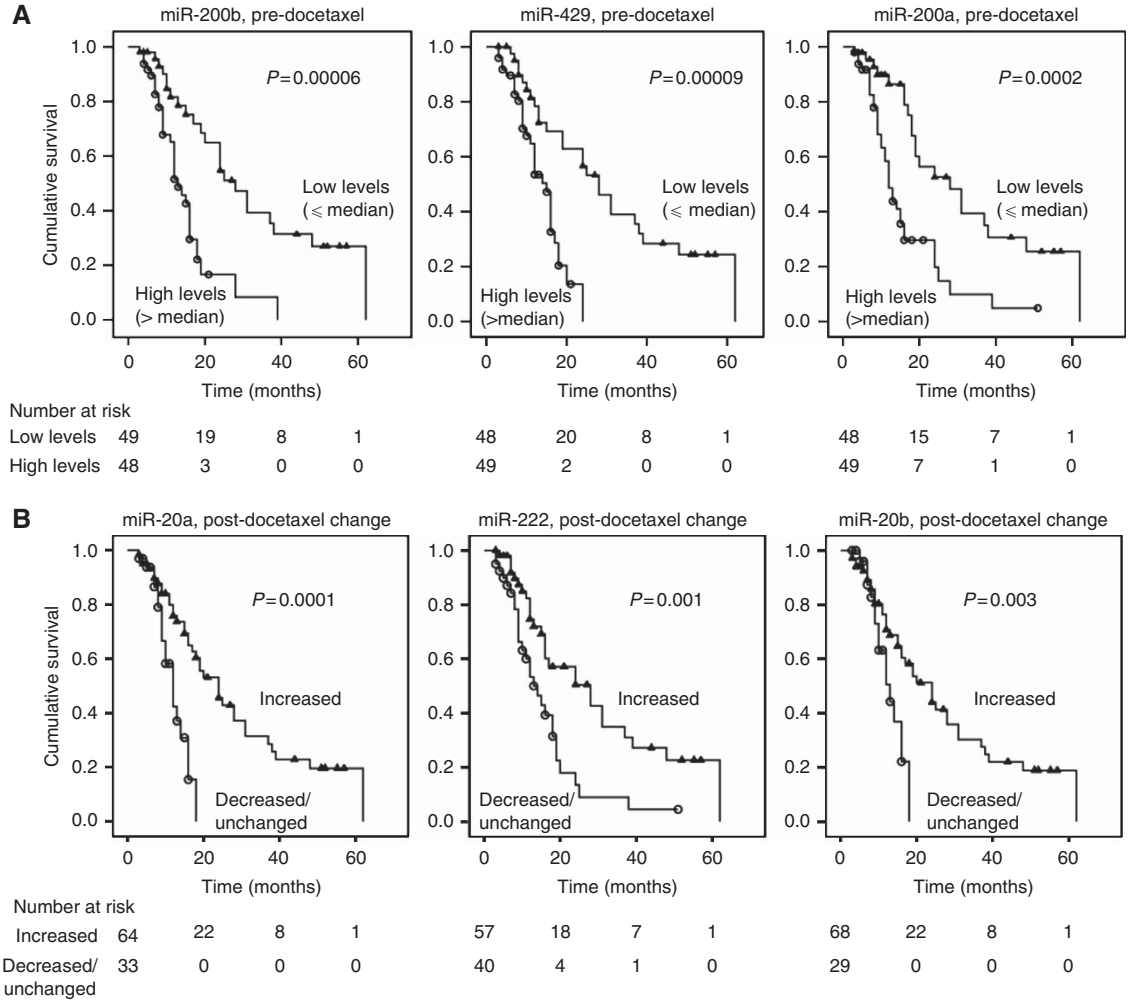

Figure 3. Kaplan–Meier plots assessing the association between circulating microRNA levels and overall survival in CRPC. (**A**) Association between pre-docetaxel levels of circulating miR-200b, miR-429 or miR-200a, and overall survival; (**B**) Association between change in post-docetaxel levels of circulating miR-20a, miR-222 or miR-20b, and overall survival.

Decreased miR-222 levels after docetaxel treatment was also associated with poor outcome. Depending on the cellular context, miR-222 and its cluster member miR-221, display oncogenic or tumour-suppressive functions (Garofalo *et al*, 2012). Increased levels of miR-222 are typically associated with tumour proliferation through inhibition of its gene target p27kip1 that regulates cell cycle progression. The growth of prostate cancer xenografts in mice was suppressed by inhibition of miR-222 (Mercatelli *et al*, 2008), suggesting that high levels of miR-222 promotes prostate cancer proliferation. However, miR-222 levels in prostate carcinoma tissue were reported to be lower than surrounding normal tissue (Schaefer *et al*, 2010). Perhaps the association of decreased miR-222 levels with poor chemotherapy outcome is through its upregulation of angiogenesis via its target KIT, a tyrosine kinase receptor. Inhibition of KIT expression by miR-222 overexpression in endothelial cells prevented tube formation and cell migration, indicating that downregulation of miR-222 promotes angiogenesis (Poliseno *et al*, 2006).

While our study has identified a panel of circulating microRNAs as potential biomarkers of docetaxel response and overall survival, we recognise the limitations of our research. In the discovery phase of our study, our cell line models do not take into account tumour and microenvironment interactions or the systemic effects of chemotherapy, thus microRNAs produced by non-tumour cells and other organs would be missed as candidates. The association of circulating microRNA levels with poor chemotherapy outcome was not consistent with some of the findings from cell line profiling. For example, the levels of the miR-200 family members in the docetaxel-resistant prostate cancer cells were lower than the docetaxel-sensitive cells, which was also demonstrated by other studies on docetaxel-resistant prostate or lung cancer cell line models (Feng *et al*, 2012; Puhr *et al*, 2012). This discrepancy indicates that non-tumour cells may make a significant contribution to the identified circulating microRNAs, or the expression of microRNAs by cancer cells is influenced by the tumour microenvironment, which is not appropriately modelled by *in vitro* conditions.

Additional limitations are the different post-cycle collections, and the use of both serum and plasma samples in the human study. Presumably, the trend in the acute change of microRNA levels are the same after subsequent treatment cycles, but this assumption has to be confirmed with further studies. MicroRNA levels in matched plasma and serum samples are highly correlated in healthy individuals according to other studies, of which one study performed global profiling (Mitchell *et al*, 2008; Wang *et al*, 2012). Statistical comparison of patients in our study according to their respective sample type is biased by differences in patient characteristics and cohort size. Nevertheless, despite these differences, microRNAs (miR-200a, miR-429, miR-375, miR-222) consistently associated with overall survival could still be identified regardless of cohort type (serum, plasma or combined).

Table 4. Cox regression analysis of circulating microRNAs and clinicopathologic factors in relation to overall survival

		Univariate Cox regression		Multivariate Cox regression: Initial estimates[a]		Multivariate Cox regression: Bootstrap estimates[b]
Variables[c]	*n*	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)
microRNA						
miR-20a, post-docetaxel change (increased *vs* decreased/ unchanged)	97	0.3 (0.2–0.5)	0.00004	0.29 (0.15–0.58)	0.0005	0.30 (0.13–0.54)
miR-200b, pre-docetaxel level	97	2.8 (1.6–5.0)	0.0004	3.1 (1.6–6.0)	0.001	2.8 (1.5–6.5)
Clinicopathologic factor						
Haemoglobin, pre-docetaxel level	96	0.33 (0.2–0.6)	0.0003	0.38 (0.20–0.73)	0.004	0.42 (0.21–0.79)
Visceral metastasis (present *vs* absent)	91	1.8 (1.0–3.1)	0.04	2.0 (1.1–3.7)	0.02	2.1 (1.2–4.6)
PSA response (non-responder *vs* responder)	97	2.9 (1.6–5.2)	0.0004	—	—	—
Serum PSA, pre-docetaxel level	95	1.8 (1.0–3.2)	0.04	—	—	—
Age	97	1.4 (0.8–2.4)	0.2	—	—	—
Gleason score at diagnosis (⩽7 *vs* >7)	76	1.2 (0.7–2.2)	0.5	—	—	—
Alkaline phosphatase, pre-docetaxel level	88	1.4 (0.2–10)	0.7	—	—	—

Abbreviations: CI = confidence interval; HR = hazard ratio; *n* = number of patients; PSA = prostate-specific antigen.
[a] *n* = 89, number of events (death) = 54.
[b] Estimates from fitting the final model to 1000 bootstrap samples (*n* = 90).
[c] Low (⩽ median) *vs* high (> median) unless mentioned otherwise.

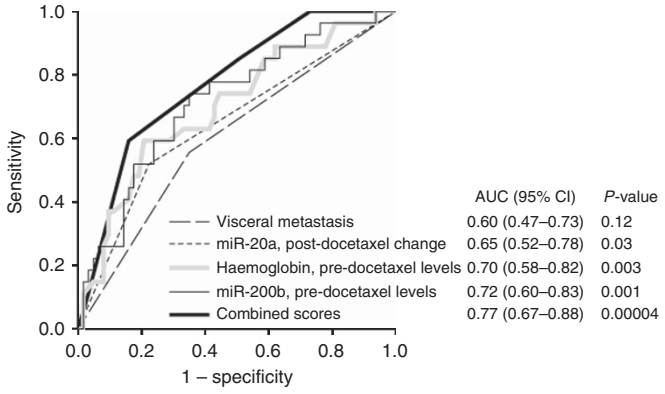

Figure 4. Receiver Operating Characteristic graphs assessing the association between independent risk factors (as defined by multivariate Cox regression) – the presence of visceral metastases, post-docetaxel change in the levels of miR-20a, baseline haemoglobin levels and pre-docetaxel levels of miR-200b; and death within 12 months. These factors are considered independently and as 'combined scores' using the sum of scores as variables, where each factor was dichotomised and its categories represented by the score of 0 or 1 as follows: score 0 (low risk) = pre-docetaxel miR-200b levels ⩽ median, post-docetaxel miR-20a levels increased from baseline levels, pre-docetaxel haemoglobin levels > median, absence of visceral metastasis; score 1 (high risk) = converse of criteria for risk score 0.

Overall, despite the variations arising from mixed sample types and different post-cycle collections, our study has identified circulating microRNAs that are associated with docetaxel chemotherapy outcome. These microRNAs are potential early therapeutic response and prognostic biomarkers that may in the future assist in the stratification of CRPC patients into appropriate treatments. Validation in a large independent cohort with consistent blood sample type and post-cycle collections are now required to confirm the findings and the clinical utility of these microRNA biomarkers in the future.

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