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10.1111/bjd.12057

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TITLE: Markers of circulating tumour cells in the peripheral blood of melanoma patients correlates with disease recurrence and progression.

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Funding sources- Ziman, NHMRC Application number 1013349; Cancer and Palliative Care Research and Evaluation Unit WAPCN Small Grants 2010/11; and Cancer Council of WA Research Grant.

Frank, Grant support MHF: National Institutes of Health (USA) grant numbers 5R01CA113796, 1R01CA138231 and 2P50CA093683.

Conflict of interest

None declared

Manuscript word count – Questions: 73 & 80 words, Abstract: 239 words, Body: 3,387

Manuscript table count –5 tables, 1 figure, 1 figure legend and 3 supplementary tables

Questions

What is already known about this topic?

- Considerable effort has been devoted to developing an effective non-invasive method of monitoring disease progression and outcome among cancer patients. One method is multimarker qRT-PCR used to detect circulating tumour cells in patient blood. In breast, colorectal and prostate cancer circulating tumour cells correlate with both disease progression and poor treatment outcome. Circulating cell analysis in melanoma has had varied success; despite the capacity to detect cells the appropriate markers remain undefined.

What does this study add?

- This is the first application of ABCB5 as a marker of circulating melanoma cells, incorporated into a multi marker qRT-PCR test of melanoma patient blood. Expressed by a subset of melanoma cells with a stem cell phenotype, its detection in this study, along with MLANA a melanocyte marker, correlated with disease recurrence, suggesting both stem and non-stem cell phenotypes are present in patient peripheral blood. MCAM expression is shown for the first time to correlate with negative treatment outcomes.

ABSTRACT

Background: Multimarker quantitative real-time PCR (qRT-PCR) represents an effective method for detecting circulating tumour cells in the peripheral blood of melanoma patients.

Objectives: The aim of this study was to investigate whether the phenotype of circulating melanoma cells represents a useful indicator of disease stage, recurrence and treatment efficacy.

Methods: Peripheral blood was collected from melanoma patients (n=230) and healthy controls (n=152) over a period of three years and nine months. Clinical data and blood samples were collected from patients with primary melanoma (early stages 0-II (n=154)) and metastatic melanoma (late stages III-IV (n=76)). Each specimen was examined by qRT-PCR analysis for the expression of five markers: MLANA, ABCB5, TGFβ2, PAX3d and MCAM.

Results: Two hundred and twelve melanoma patients (92%) expressed markers in their peripheral blood. Two markers, MLANA and ABCB5, had the greatest prognostic value, and were identified as statistically significant among patients who experienced disease recurrence within our study period, being expressed in 45% (MLANA) and 49% (ABCB5) of patients with recurrence (p=0.001 & p=0.031). For patients administered non-surgical treatments, MCAM expression correlated with poor treatment outcome.

Conclusion:

Circulating tumour cells were detectable at all stages of disease and long after surgical treatment even when patients were considered disease free. Specifically, expression of ABCB5 and MLANA had significant prognostic value in inferring disease recurrence while MCAM expression was associated with poor patient outcome after treatment, confirming multimarker qRT-PCR as a potential technique for monitoring disease status.

Introduction

Cutaneous melanoma is a tumour originating from normal skin melanocytes. Global incidence of melanoma is increasing[1], with an estimated 70,230 new cases annually in the USA [2]. Melanoma has a high metastatic potential [3] and metastatic melanoma is difficult to treat . It is important therefore to find methods of detecting melanoma spread at early stages for more accurate treatment and prediction of prognosis.

A staging system was devised by the American Joint Committee on Cancer (AJCC) based on histological features of the primary lesion, and the presence of nodal and/or systemic metastases in advanced disease [3]. Due to increased awareness, most patients present with thin, localised melanoma which is usually curable by surgical resection. However, approximately 5% of patients with lesions <1 mm thick, 25-40% with 2 to 4 mm lesions and 50-75% with lesions > 4 mm develop metastatic disease and die within ten years [3]. Patients diagnosed with regional and distant metastases have 10 year survival rates of 64% and 16% respectively [4].

Patients may suffer relapse following disease free periods of ten years or more [5,6]. Evidently then, diagnosing patients as clinically disease free after surgical removal of early stage melanomas, may be inaccurate, since tumour cells dispersed into the bloodstream may remain dormant, prior to formation of distant metastases [7].

Considerable research into the detection of circulating tumour cells in the blood of patients with breast, colorectal and prostate cancer has shown that the presence of these cells correlates with both disease progression and poor outcome[8-16]. For melanoma, circulating cell analysis has not been successfully utilised clinically. Although several articles have indicated their usefulness[17-22], markers and methodology remain controversial. Moreover, it is unclear

whether circulating melanoma stem cells are prognostic [23] or whether all circulating melanoma cells have metastatic potential [24].

Here we used multimarker qRT-PCR to investigate the phenotype of circulating cells in melanoma patients. Given qRT-PCR sensitivity, it is suitable for assessment of rare circulating melanoma cells [25] and multiple markers are essential given the heterogeneous nature of melanoma cells [18,26]. Our markers included melanocyte (PAX3d [27] and MLANA [28]), tumour (TGFβ2 [29] and MCAM [30]) and stem cell (ABCB5 [31]) markers. PAX3 regulates melanocytic development [32-34], and the PAX3d isoform is highly expressed in melanoma [35]. ABCB5 a stem cell marker, marks a subset of rare, chemoresistent melanoma stem cells [24,31,36] , while MCAM is a marker of melanoma tumour progression [34].

We show that circulating tumour cells are detectable at all stages of disease and persist long after treatment. Certain circulating cells, specifically those marked by MCAM expression, correlated with a poor treatment outcome, while ABCB5 and MLANA, correlated with disease progression and could aid in monitoring disease status.

Material and Methods

Patient cohort

Patient (230) and healthy control (152) participants provided written consent. Patients were recruited from the Medical Oncology Department of Sir Charles Gairdner Hospital and the Perth Melanoma Clinic at Hollywood Hospital (Perth, Western Australia) and consisted of 81 females and 149 males aged 24 – 96 (mean 69 years) while the aged matched, healthy cohort, recruited from the general population, contained 86 females and 66 males aged 18-99 (mean 51 years). The Human Research Ethics Committees of Edith Cowan University (No. 2932) and Sir Charles Gairdner Hospital (No. 2007-123) approved the study.

The study included 154 patients with primary cutaneous melanoma and 76 with metastatic melanoma. AJCC Clinical staging categorised 16.5% of our patients as stage 0, 33.5% as stage I, 17% as stage II, 13% as stage III and 20% as stage IV (Table 2). Patients were sampled only once and clinical history recorded, however a subset of patients (n=140) were sampled serially throughout the 3.9 year study. Tumour burden was calculated from pathology reports for a subset of 32 patients, by calculating the sum of maximal tumour diameters for all tumours within the patient, at the time of sampling.

Melanoma cell lines

Human melanoma cell line A2058 cells (EACC: 91100402), were grown in DMEM media containing 10% heat inactivated foetal bovine serum (Invitrogen) in a T75-cm^2 flask (Greiner) until cells were ~70% confluent.

Blood Collection, RNA Isolation and Amplification Protocols

At venipuncture, the first 4 mL of blood was discarded to avoid epithelial contamination [37]. 2.5mL of whole blood was then collected into a PAXgene Blood RNA Tube (PreAnalytiX), containing RNA stabilisers [38].

Total RNA was isolated using the PAXgene Blood RNA kit (Qiagen) and DNase treated (DNA-free Kit, Ambion). RNA was quantified and the integrity was qualified (Agilent 2100 Bioanalyser, Agilent Technologies). Samples with insufficient RNA levels (RIN <8) were excluded.

Total RNA (250ng) was converted to cDNA (Omniscript Reverse Transcriptase Qiagen). Each reaction (20 µL), contained, 2µL of RT Buffer (1x), 2 µL of dNTPs (5mM), 2 µL of oligo (dT) primer (0.4 ug/ul), 1 µL of RNase inhibitor (10U/ µL) (Invitrogen) and 1 µL of omniscript reverse transcriptase (4U/ µL). Incubation was 37 °C for 1 hour; then 5 mins at 95 °C and included a no template control (NTC). Quantitative RT-PCR assays assessed the number of mRNA transcripts (level of expression) for six genes, MLANA, ABCB5, TGFβ2, PAX3d, MCAM and GAPDH. Each 15 µL reaction contained 1µL of cDNA template, 1x SYBR GreenER qPCR SuperMix (Invitrogen) and 200nM of primer (Supplementary Table 1). Using an iCycler iQ5 Real-Time Thermocycler (BIO-RAD) incubation was as per the manufacturer's instructions.

To prevent contamination all PCR reactions contained Uracil-N-glycosylase to prevent reamplification of carryover PCR products [39] and different steps were performed in separate UV treatable areas. Tm determination and gel electrophoresis confirmed the expected size and identity of PCR products.

GAPDH, our internal reference for RT efficiency, is not up regulated in melanoma tissues or cultured cells relative to normal samples [40]; samples with insufficient levels were excluded. Every assay included a standard curve, negative controls (NTC and reverse transcription control), positive control (A2058 cell line) and cDNA from a single healthy control.

Standard Curve

A standard curve to quantify mRNA copy number, was constructed using larger PCR products that included the target sequence used in qRT-PCR assays. For each standard, an end point PCR primer set was designed (Supplementary Table 2). A2058 cDNA, synthesized from 250ng of RNA, was used as a template. Using a *Taq* DNA polymerase kit (Qiagen), each 20µL reaction consisted of 1x Coral Load buffer, 0.2mM of each dNTP, 2.5U of *Taq* DNA polymerase, 1µL cDNA and 200nM of primer. Incubation involved: 94°C for 5 minutes, then 45 cycles of 94°C for 30 seconds, then 55°C for 30 seconds and 72°C for 1 minute. PCR products were purified after agarose gel electrophoresis using the freeze-squeeze method [41]. Resulting stock template solutions were quantified by spectrophotometric analysis (Nanodrop, Thermo Scientific) and diluted into eight tenfold serial dilutions (10^1 - 10^8 copies/µL) from which a standard curve for each target was generated on the iCycler iQ5 Real-Time Thermocycler. Amplification efficiency of our target genes were between 96.4 and 100%. Samples were analysed in duplicate with averaged cT values used for relative quantification. Relative mRNA copies for each transcript were calculated by dividing absolute mRNA copies of the target by that of GAPDH.

Statistical Analysis

Pearson chi-square test and Fishers exact test were used to examine statistical significance of marker expression between patient groups and controls. The Cochrane-Armitage trend test

examined correlation between clinical stage, Clark level and/or expression level. Positive and negative predictive values were calculated to examine the sensitivity of our test.

The relationship between level of marker expression and stage or presence of disease, was investigated by Mann-Whitney U test and Kruskal-Wallis H test. Generalised linear modelling was used to examine marker consistency. SPSS was used for analysis and differences of $p<0.05$ were considered statistically significant.

Results

Multimarker RT-PCR analysis was used to estimate the phenotype and quantity of circulating cells in patient blood. Marker positivity and level of expression were analysed for correlation with disease presence, stage, recurrence and treatment outcome.

Marker Expression in Patient Blood Relative to Controls:

Melanoma patients (92%, n=212) were significantly more likely to express a melanoma cell marker in blood than were controls (59%, n=89) (p= 0.000, Odds ratio = 4.206, 95% CI 2.305-7.676). The number of markers expressed by participants was important, with 83% (n=126) of healthy controls expressing either no marker or just one marker, in contrast to melanoma patients, who were highly likely to express two or more markers (72%, n=165)(p=0.012)(Table 1). The positive and negative predictive value of expressing a marker, was 70% and 78% respectively. Expression of two or more melanoma markers improved the positive predictive value to 86%, with a negative predictive value of 66%.

To identify the most informative markers, we analysed expression incidence of each marker in patients. MLANA, ABCB5, MCAM, PAX3d, and TGFβ2 were detected in 30% (n=68), 40% (n=90), 47% (n=108), 40% (n=91), and 66% (n=152) of melanoma patients, respectively.

Marker Expression in Patients Relative to Clinical Stage:

To assess marker utility, we analysed marker expression relative to clinical stage. MLANA and ABCB5 were significantly more likely to be expressed in late stage patients (Stages III-IV) (42% and 51%) than in early stage patients (0-II) (23% and 33%) (p=0.003 and p=0.006). No other markers appeared to be stage related (Table 2).

Clinical factors including primary location, Clark level, Breslow thickness and ulceration did not correlate with marker expression (Table 3).

Pearson chi-square test was used to analyse whether the incidence of marker expression in patient blood samples taken within one month of clinical diagnosis differ from samples taken at times greater than one month following diagnosis. For most markers, expression did not differ between samples taken at times less than and greater than one month following diagnosis. TGFβ2 however, was expressed slightly more frequently in samples obtained more than a month following diagnosis (47%, n=187) relative to samples collected within in the first month following diagnosis (32%, n=50) (p=0.001). Samples taken at times greater than one month after diagnosis were as likely to express a marker (79%, n=311), than samples taken within the first month following diagnosis (71%, n=110).

Marker Expression Correlates to Disease Recurrence:

To investigate the prognostic value of our markers, the relationship between marker expression and disease recurrence was analysed. Seventy three patients (32%) experienced recurrence during our study and in these patients, both MLANA and ABCB5 were expressed significantly more frequently (45%, n=33 and 49%, n=36) than in patients without recurrence (23%, n=35, 34%, n=54) (p=0.001 and p=0.031) (Table 4). Patients with recurrence expressed 5 markers more commonly (8%, n=6) than patients who experienced no recurrence during our study (1%, n=2) (p=0.013). Patients with recurrence during our study were more likely to express both ABCB5 and MLANA (26%, n=19) than patients without recurrence during the study period (10%, n=15) (p=0.002). Co-expression of MLANA and ABCB5 produced a positive predictive value of 56% and a negative predictive value of 72%.

When divided into stages, 48% of stage III-IV patients with recurrence (n=29) expressed MLANA compared to only 19% of stage III-IV patients without recurrence (n=3) (p=0.033). There was no correlation between MLANA expression and recurrence in early stage (0-II) patients. Analysing the presence and level of markers relative to time since diagnosis in patients who developed recurrence during our study, revealed markers were present in patient blood at all stages, many years after diagnosis or surgical removal of the primary tumour, even when there were no clinical signs of disease (Table 4).

Marker Expression in relation to Tumour Burden:

To examine if marker expression correlated with tumour burden, stage III-IV patients were divided into those with one metastasis (n=16) (mean of tumour diameter: 19.8mm±13.2mm (mean±SD)) or multiple metastases (mean of sum of tumour diameter: 76.5mm±36.06mm) at the time of sampling (n=16). Only MCAM expression was significantly related to tumour burden, with the level of expression greater among stage III-IV patients with multiple metastases (1.23±1.66, n=4) than in those with only a single metastasis (0.67±0.49 (mean±SD), n=8,) (p=0.027).

Among stage III-IV patients, the surgical removal of a clinically measureable metastatic tumour also influenced marker expression. MLANA and Pax3d were expressed less frequently in patients following the surgical removal of a clinically measureable metastatic tumour (18%, n=29; 19%, n=30), compared to patients with an existing tumour burden (48%, n=16; 42%, n=14) (p=0.000 & p=0.003, respectively). A significant reduction in the level of ABCB5 expression was observed in stage III-IV samples corresponding to the removal of a clinically measureable metastasis (0.19±0.79), compared to samples with an existing tumour burden (0.22±0.42)(p=0.011).

Marker Expression in Relation to Clinical Treatment:

To analyse the effect of non-surgical treatment on circulating tumour cell phenotypes, marker expression was investigated in 34 patients (15%) who received non-surgical treatment. Treatments included: radiotherapy (n=29), interferon alfa 2b (n=5), limb infusion (n=1), vaccine (n=4), chemotherapy (n=9) and radio frequency ablation (RFA) (n=1).

Three patients were sampled both before and after their non-surgical treatment, whereas the majority of non-surgically treated patients were sampled during or following treatment. The condition of two patients deteriorated post treatment, while the third remained stable (Figure 1). Patient 1 was sampled at diagnosis of a stage III melanoma, two months prior to radiotherapy treatment of the left inguinal node, and expressed only ABCB5. Seventeen months later, with their left axilla node identified as positive, the patient gained MLANA and MCAM expression and the level of ABCB5 expression was maintained. No markers were detected in patient 2, three months prior to RFA treatment for a lung metastasis. Sampled three months after treatment, MLANA, ABCB5 and Pax3d were detected; the patient died three months later. Patient 3 had positive lymph nodes and expressed no markers the day before interferon alfa 2b treatment. Serial blood collections were then made at six, twelve, twenty-one, and twenty-six months post treatment. TGFβ2 was detected at six, and twelve months and TGFβ2, MCAM and ABCB5 were detected by twenty-six months with a 5 fold decrease in MCAM between the last two serial samples.

The majority of the non-surgically treated stage IV samples were collected after non-surgical treatment (n=26). To examine whether the phenotype of circulating melanoma cells reflects the effectiveness of treatment, samples were divided into those from patients who had a negative treatment outcome (n=40) (progression and/or death) and those with a positive outcome (n=22) (disease remained stable or tumour size reduced). The expression of MCAM

was significantly more common in patients with a negative treatment outcome with 43% expressing MCAM (n=17), while only 9% of samples from patients with a positive outcome, expressed this marker (n=2) (p=0.006)(Table 5). The positive predictive value for MCAM expression as a marker of poor clinical outcome in patients with stage IV melanoma is 89.5%, while the negative predictive value is 46.5%.

Marker Consistency and Serial Blood Collection

21.7% of all acquired blood samples had a technical repeat (second PAXgene Blood RNA tube) taken on the same day. Expression in these samples was highly consistent, with 70% of duplicate samples having identical marker expression (n=273).

Generalised linear modelling was employed to examine serially collected samples (n=140), acquired for each patient at intervals of six months over a period of two years. For the majority of our markers, time was not a significant variable, with marker expression remaining consistent, regardless of sampling time and regardless of stage (Supplementary Table 3). For ABCB5 however, expression incidence significantly reduced over time (Wald-Chi = 16.209, p=0.000) in stage IV patients (Odds ratio: 0.2518, p=0.000, 95% CI 0.131, 0.483), which may represent the effect of non-surgical treatment on ABCB5 expression in stage IV patients.

Discussion:

This study showed that a multi marker qRT-PCR test for melanoma circulating cells correlated with melanoma diagnosis. Very few melanoma patients expressed no markers at all, only 7%. A significant number of healthy controls, however also expressed one marker. Therefore, patients and controls are best differentiated by the number of markers expressed i.e. by a multi marker test.

Multiple markers were detected at all disease stages, even in early stage patients, however the incidence was lower in these patients than in later stage patients. In contrast to previous papers [18,42], the levels and number of markers were not stage related, possibly related to the use of ABCB5 and TGFβ2, both previously unused markers of circulating tumour cells. TGFβ2 was the most common marker in controls, so cannot be recommended as a single marker for patient analysis.

By contrast, ABCB5, described by Frank and colleagues in 2003 was a highly informative marker [23,24,31,36]. ABCB5 reportedly represents a subset of melanoma cells with a stem cell phenotype and is thought here to represent a subset of heterogeneous circulating melanoma cells present at all disease stages. In this study it was identified as a potential predictor of active recurrence/progression events with its expression in blood correlating with disease recurrence and progression in patients, a finding that warrants further testing of this marker. This marker was detected amongst patients regardless of whether or not they were considered clinically disease free, indicating that these stem - like circulating cells remain for long periods in the blood. A role for ABCB5 cells in disease spread has been demonstrated in mouse -to-mouse transplantation experiments, where circulating ABCB5 expressing tumour cells were shown to be capable of initiating metastatic disease after transplantation into donor mice [23].

Besides ABCB5, MLANA expression was also a potential predictor of recurrence events in our patient cohort. Since ABCB5 positive cells do not co-express MLANA[24], circulating cells must therefore include both stem and non-stem cell phenotypes during recurrence. This may reflect a change in stem cell to a non-stem cell phenotype during formation of the metastatic tumour[43]. Previous studies described MLANA expression as correlating with disease stage[25], and in this study both MLANA and ABCB5 expression correlated with disease stage, with expression more common in advanced (stage III-IV) than in early stage (stage 0-II) patients. In fact, removal of a clinically measurable metastasis significantly reduced the expression of Pax3d, ABCB5 and MLANA in stage III-IV patients, signifying the importance of metastases in maintaining the hematogenous spread of tumour cells [44,7].

Utilising these markers, circulating tumour cells were detected at all disease stages long after surgical treatment, even when a patient was considered clinically disease free. Thus, as found in previous studies of circulating tumour cells [42,45], the mere presence of circulating tumour cells does not always correlate with disease stage or metastatic recurrence, and additional markers are required to stratify those early stage patients likely to have disease progression.

In our study MCAM expression correlated with increased tumour burden, a result consistent with recent *in vivo* studies with nude mice that revealed a significant correlation between MCAM expression and the formation of metastases [46]. MCAM was also the most suitable marker for monitoring response to therapy and indicates, presumably, ineffective eradication of circulating melanoma cells, evidenced here by an increase in the incidence of MCAM expression in patients with a negative treatment outcome. MCAM expression could therefore be used to monitor treatment resistance or risk of relapse. Given MCAM expression is thought to play a role in cell-cell and cell-matrix interactions during formation of metastases, the expression of MCAM in circulating cells may aid in the spread of malignant melanoma

cells throughout the body [7,44]. A previous study found MCAM to be a predictor of poor patient survival and disease prognosis [43]. Overall, 68% of non-surgically treated patients had a negative treatment outcome, highlighting the poor response rate of patients to non-surgical treatments and reflected here by 64% of these patients expressing MCAM. Essentially, MCAM expression may aid in identifying a subset of patients with metastatic disease who respond poorly to conventional systemic treatments and may benefit from an alternative treatment regime.

Some p-values were achieved by considering several different predictors. However, corrections were not made for multiple comparisons because the markers were considered to be independent. Moreover, even when we applied Bonferroni corrections to secondary hypotheses for 5 comparisons, the majority of hypotheses remained significant ($p<0.01$); for example, comparisons between early versus late disease stage (MLANA $p = 0.003$ and ABCB5 $p=.006$), disease recurrence (MLANA $p=0.001$) and negative treatment outcome (MCAM $p=0.006$) remain significant. Only the correlation of MCAM expression and tumour burden (MCAM $p = 0.027$), and ABCB5 expression in relation to recurrence (ABCB5 $p=0.031$) and the surgical removal of a clinically measurable lesion (ABCB5 $p=0.011$) are no longer significant. Despite this, MCAM and ABCB5 remain valuable markers of clinical outcome and disease recurrence, especially ABCB5 expression in combination with MLANA.

It is clear that circulating tumour cells are detectable at all stages of disease and the specific detection of ABCB5, MLANA and MCAM expressing tumour cells in blood may be of prognostic value. Importantly, we demonstrated that a qRT-PCR multi-marker blood test provides a suitable method from which to develop further a reliable test for monitoring disease status without the need for specific isolation of circulating tumour cells.

Acknowledgements

The authors thank Liz Watson, Jenny Fairweather and Katrina Burton for their assistance with this study.

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Tables

Table 1 – Number of markers detected in the peripheral blood of patients and healthy controls.

Number of markers **% (n)**	**0**	**1**	**2**	**3**	**4**	**5**	**n**
CMM Patients	8 (18)	20 (47)	34 (79)	23 (54)	10 (24)	3 (8)	**230**
Healthy controls	41 (63)	41 (63)	11 (17)	4 (6)	2 (3)	0	**152**

Table 2 - qRT-PCR Marker detection in the peripheral blood of healthy controls and in melanoma patients categorised by AJCC stage.

	AJCC staged melanoma patients % (n)					
Markers	0	I	II	III	IV	Healthy Controls
MLANA	26 (10)	25 (19)	18 (7)	40 (12)	43 (20)	13 (19)
ABCB5	45 (17)	27 (21)	33 (13)	50 (15)	52 (24)	22 (33)
Pax3d	47 (18)	30 (23)	36 (14)	50 (15)	46 (21)	14 (21)
MCAM	42 (16)	49 (38)	46 (18)	53 (16)	43 (20)	14 (21)
TGFβ2	63 (24)	64 (49)	74 (29)	77 (23)	59 (27)	22 (33)
N	**38**	**77**	**39**	**30**	**46**	**152**

Table 3 - Melanoma patient characteristics

Prognostic factor		Number of patients
Gender	Males	81
	Females	149
AJCC Stage	0	38
	I	77
	II	39
	III	30
	IV	46
Primary Site	Face	36
	Torso	78
	Arms	31
	Legs	37
	Scalp	14
	No primary lesion or unknown	34
Breslow thickness (primary) mm	<1mm	103
	1-4mm	82
	>4mm	20
	Unknown	25
Clark level	I	43
	II	30
	III	20
	IV	81
	V	15
	Unknown	41
Ulceration	No	178
	Yes	34
	Unknown	18

Table 4 - qRT-PCR Marker detection in the peripheral blood of melanoma patients categorised by recurrence and time since diagnosis.

	Patients with recurrence during study period % (n)		**Time since diagnosis in relation to sampling in patients with recurrence % (n)**			
Markers	recurrence	no recurrence	< 1 month	< 1 year	< 5 years	> 5 years
MLANA	*45 (33)	23 (35)	33 (2)	18 (7)	26 (32)	22 (18)
ABCB5	*49 (36)	34 (54)	0	18 (7)	22 (27)	32 (26)
Pax3d	41 (30)	39 (61)	33 (2)	21 (8)	22 (27)	15 (12)
MCAM	49 (36)	46 (72)	0	21 (8)	21 (26)	25 (25)
TGFβ2	63 (46)	68 (106)	50 (3)	29 (11)	34 (42)	34 (34)
N	**73**	**157**	**6**	**38**	**124**	**81**

P-value* - Pearson chi-square test, $p<0.01$

Table 5 – MCAM expression in the peripheral blood of stage IV melanoma patients in relation to clinical outcome.

	MCAM expression % (n)		
Clinical Outcome	+	**-**	**N**
Negative (disease progression and/or death)	43 (17)	58 (23)	40
Positive (disease remained stable or tumour size reduced)	9 (2)	91 (20)	22