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Circulating melanoma cells and distant metastasis-free survival in stage III melanoma patients with or without adjuvant interferon treatment (EORTC 18991 side study)

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ABSTRACT

Aim: To evaluate the prognostic and predictive importance of detection of melanoma cells in peripheral blood using reverse transcriptase polymerase chain reaction (RT-PCR) in stage III cutaneous melanoma patients after sentinel or regional lymph node dissection.

Patients and methods: Serial testing for *tyrosinase* and *Mart-1/Melan-A* transcripts in peripheral blood was performed every 6 months over a maximum period of 60 months in a subset of patients enrolled in EORTC 18991 phase 3 trial, comparing pegylated interferon-α-2b with observation. Univariate and multivariate analyses were performed to estimate the role of RT-PCR as prognostic and predictive factor for distant metastasis-free survival (DMFS).

Results: Among 299 patients who underwent RT-PCR analyses, 109 (36.5%) had at least one positive sample, either at time of randomisation ($N = 17$) or subsequently ($N = 92$). The cumulative rate of positive results was similar in the two treatment groups, as the DMFS from first RT-PCR positivity. RT-PCR result, positive versus negative, at a given time point, had no prognostic impact on subsequent DMFS. Cox time-dependent analysis indicated a significantly higher risk of developing distant metastasis in patients with a positive sample as compared to those with a negative one: hazard ratio (HR) of 2.23 (95% confidence interval (CI), 1.40–3.55; $p < .001$). These results were comparable in the 2 treatment groups, indicating that RT-PCR assessment was not predictive for treatment outcome.

Conclusion: Detection of circulating tumour cells by RT-PCR for *tyrosinase* and *Mart-1/Melan-A* was a time-dependent moderate prognostic factor for subsequent development of distant metastasis in stage III melanoma patients.

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1. Introduction

In contrast to the favourable outcome in earlier stages, the estimated 5-year survival for stage III melanoma patients is heterogeneous and ranges between 67% for American Joint Committee on Cancer (AJCC) stage IIIA and 26% for stage IIIC.[1] The number of metastatic lymph nodes, ulceration of the primary melanoma and the presence of satellite or in-transit metastases are the three most powerful clinical prognostic factors regarding overall survival within this population.[2]

In order to further assess the risk for relapse, the search for minimal residual disease in peripheral blood has been extensively evaluated in melanoma patients.[3–15] Detection of melanoma cells in blood has been mainly done using reverse transcriptase polymerase chain reaction (RT-PCR), but specificity, sensitivity as well as clinical implications of RT-PCR results are still under debate because of conflicting results.[16] Some of the disparities in the literature can be explained by differences in methodology and quality assurance, but insufficient sample size and patient cohort heterogeneity with respect to stage as well as treatment may have further contributed to the conflicting results.

In order to investigate the prognostic and potentially predictive importance of RT-PCR assessment of circulating melanoma cells in a homogeneous patient population, we conducted a multicentre study of serial RT-PCR analysis of the two melanoma markers *tyrosinase* and *Mart-1/Melan-A* in 299 stage III melanoma patients after regional lymph node dissection within the framework of the EORTC trial 18991, comparing prolonged administration of pegylated interferon alpha2b (PEG-IFN) with observation only.

2. Patients, materials and methods

2.1. EORTC 18991 trial and RT-PCR study

The EORTC 18991 trial was an international multi-institutional prospective randomised phase 3 trial that evaluated the efficacy and toxicity of 5 years of PEG-IFN versus observation in resected stage III cutaneous melanoma patients.[17] Centres were invited to participate in the RT-PCR study and therefore drawing of blood samples for RT-PCR melanoma markers detection was left to the centres' choice. This side study was approved by the institutional review boards and blood samples were obtained after informed consent. Blood sample collection was prospectively performed concomitantly to tumour staging procedures at the time of the enrolment and every 6 months thereafter until discontinuation of a given patient from the study or up to a maximum of 60 months.

2.2. RT-PCR analysis

Peripheral blood (9 ml) was collected in EDTA-containing tubes and was processed on site within 2 h. Blood was centrifuged at 3000 rpm for 10 min. Serum was removed except for 2 mm serum layer, and the cell pellet was resuspended in 5 ml of guanidium thiocyanate (GTC) buffer and was stored at −80 °C. Samples were subsequently shipped for analysis to the central laboratory at the Charité, which was blinded to clinical and pathological data. Further processing of the samples was performed as described previously.[11] Quantitative RT-PCR assays were performed using a LightCycler (Roche Diagnostics, Grenzach, Germany) with specific primer and probe sets for *tyrosinase*, *Mart-1/Melan-A* and the housekeeping gene porphobilinogen deaminase (*PBGD*). We decided not to include *gp-100* in the analysis because of its high background expression in normal donors.[11] All the samples were analysed in duplicate, and the average value was used as a quantitative value. Any detection of *tyrosinase* was considered positive; *Mart-1/Melan-A* transcripts were considered elevated if they exceeded the value of 4.4×10^{-5} ratio to PBGD in accordance with the cut-offs previously established in our laboratory.[11]

2.3. Statistical analysis

The primary statistical hypothesis was whether sample positivity for any of the two markers at a given time point would be of prognostic relevance for the subsequent risk of developing distant metastasis. Because blood samples were not always exactly drawn on planned time points, only blood samples with collection dates ranging from 30 d before the date of randomisation to 15 d after the last date of follow-up or the reported date of distant metastasis were considered for the analysis.

Distant metastasis-free survival (DMFS) was calculated from the date of randomisation to the date of the first report of distant metastasis or the date of death, and all patients alive without distant metastasis at the last follow-up were considered censored. Time to blood positivity was the time from randomisation until the first date one of the blood markers was recorded as being positive; the follow-up of patients for whom no blood positivity has been recorded, has been censored at the latest date of the assessment of melanoma markers transcripts, distant metastasis or last follow-up.

The actuarial curves were computed using the Kaplan–Meier technique, and were compared using the log rank test.[18]

Cox proportional hazards models with either time-independent or time-dependent covariates were used to obtain the estimate and the 95% confidence interval (CI) of the hazard ratio (HR) of each covariate (e.g. RT-PCR positive versus RT-PCR negative). In the time-independent Cox model if a patient had at least one positive blood sample for *tyrosinase* and/or *Mart-1/Melan-A*, the patient was included in the RT-PCR positive group, if not (all samples were negative) the patient was included in the RT-PCR negative group. This model provided a biased estimate of the prognostic importance of the positivity of *tyrosinase* and/or *Mart-1/Melan-A* due to a guarantee-time bias[19]: only patients who were alive and free of distant metastasis for a longer time period had the possibility to become positive for *tyrosinase* and/or *Mart-1/Melan-A*. A Cox model with time-dependent covariates was used to avoid this guarantee-time bias. This model allows to compare the risk of relapse according to the latest information of the RT-PCR status (e.g. positive versus negative) in patients who are still at risk of relapse; for this reason it is called time-dependent. It includes a patient in the RT-PCR positive group only from the moment he/she becomes positive, and compares his/her

subsequent prognosis to that of the remaining patients. If subsequently, such a patient becomes *tyrosinase* and *Mart-1/Melan-A* negative, he/she is switched back to the RT-PCR negative group. Adjustments for nodal status (microscopic involvement [N1] versus palpable nodes [N2]), for a number of lymph nodes involved at the time of regional lymph node dissection and for sex, were also performed.

The landmark method, which circumvents guarantee-time bias, was used to evaluate the association between RT-PCR results and DMFS with all blood samples available at a fixed time point. Fixed time points were chosen according to the theoretical time points of blood sampling. As all the samples were not exactly drawn every 6 months, four time intervals around the theoretical date were used: ±15 d, ±30 d, ±60 d and ±90 d. The 60-d time-interval window (±30 d around the theoretical date) will be reported in this paper.

The clinical database, located at the EORTC Headquarter, was frozen in December 2006 (cut-off date was 31st March 2006), and all RT-PCR results were included in March 2008. SAS version 9.1 software (SAS Institute Inc., Cary, NC) was used for statistical analyses.

3. Results

3.1. Patient characteristics

A total of 299 patients of 1256 patients enrolled in the EORTC 18991 trial had at least one blood sample and were evaluable for the RT-PCR study. One hundred forty-six patients had been randomly assigned to the treatment arm and 153 to the control arm. The median actuarial follow-up was 3.6 years. Clinical characteristics of patients in the RT-PCR study are listed out in Table 1.

There were no marked differences between the entire patient population and the patient subset included in the RT-PCR study for the most important variables (treatment arm, type of nodal involvement and number of positive lymph nodes; data not shown). However, there were more patients with microscopic nodal involvement in the RT-PCR-study population compared to the entire population (56.2% versus 43.2%, respectively).

3.2. RT-PCR results

Between November 2000 and March 2006, 1422 consecutive peripheral blood samples had been collected. Of these, 1250 samples (87.9%) had a sufficient level of housekeeping gene expression and thus were suitable for analysis. A median of four samples (range: 1–12) was available per patient. Overall, 134 samples (10.7%) yielded positive RT-PCR results, with 50 blood samples positive for *tyrosinase* and 93 for *Mart-1/Melan-A* and an overlap of nine blood samples being positive for both markers.

A total of 109 of 299 patients (36.5%) had at least one positive sample and 18 patients had more than one positive sample. When comparing patients with at least one positive sample to patients with no positive samples, no marked differences for the most important pretreatment variables of the trial were observed (Table 1), with the exception of a higher percentage of patients with microscopic nodal involvement (62.4% versus 52.6%) in the subgroup of patients with at least one positive sample.

3.2.1. Time from randomisation to RT-PCR positivity

At 2 years, 33.8% (95% CI, 27.8–39.7%) of patients had a positive *tyrosinase* and/or *Mart-1/Melan-A* detection; no significant difference between the treatment arms was observed (Fig. 1).

Among 109 patients who had a RT-PCR sample positive, 17 (16%) patients were positive at randomisation, 64 (59%) within 1 year and 84 (77%) within 2 years after randomisation.

3.2.2. Time from randomisation to distant metastasis or death

DMFS probabilities from randomisation are shown in Fig. 2A. At 4 years, 67.6% of patients with at least one positive sample and 51.3% of patients with no positive samples were still alive and without distant metastasis. Such analysis indicates that the patients with at least one positive blood sample seemed to be at a lower risk of developing distant metastasis. The delay before the first event in the group of patients with at least one positive blood sample indicated that such a kind of analysis was extremely biased (guarantee-time bias) in favour of the patients included in this subgroup: patients were considered, unduly, to be at risk of developing distant metastasis from the time of randomisation (starting point of the Kaplan–Meier curves), when in fact they should be considered to be at risk only from the moment they became RT-PCR positive.

3.2.3. Time from RT-PCR positivity to distant metastasis or death

Kaplan–Meier curves corresponding to DMFS from first RT-PCR positivity by treatment arm were quite similar (Fig. 2B). Approximately 5–10% of patients who were RT-PCR positive developed distant metastasis at the same time. For 8 patients, the date of the first positive blood sample was 1 d before the detection of distant metastasis, which explains why the curve begins below the 100% point on the vertical axis. At 2 years, the percentage of RT-PCR positive patients who developed distant metastasis reached 25% in both treatment groups, indicating that the risk of occurrence of distant metastasis following RT-PCR positivity was quite modest. There was therefore a subgroup of patients who remained alive and distant metastasis free for a sufficient period of time before becoming RT-PCR positive ('guarantee-time bias').

3.3. Prognostic impact on DMFS

3.3.1. Classical Cox model

Using improperly a time-independent Cox model to determine whether the patients with at least one positive blood sample over time had a different outcome from randomisation compared to those who always had a negative blood sample, we found that the first group had a lower risk of developing distant metastasis or of death than the latter one: the estimated hazard ratio was 0.61 (95% CI, 0.41–0.90; $p = 0.012$; Table 2). Similar results were observed when the patients of both randomisation arms were analysed separately (Table 3).

Table 1 – Clinical and pathological characteristics of the patients with regard to the positivity of all valid blood samples.

Characteristic	RT-PCR status					
	Total (*N* = 299)		At least once positive (*N* = 109)		Always negative (*N* = 190)	
	No.	%	No.	%	No.	%
Age, years						
Median	48		48		48	
Range	18–70		18–69		18–70	
Q25–Q75	38–57		38–55		38–57	
Sex						
Male	179	59.9	66	60.6	113	59.5
Female	120	40.1	43	39.4	77	40.5
Ulceration						
Present	89	29.8	28	25.7	61	32.1
Absent	160	53.5	66	60.6	94	49.5
Unknown	50	16.7	15	13.8	35	18.4
Nodal involvement						
microscopic	168	56.2	68	64.2	100	52.6
palpable	131	43.8	41	37.6	90	47.4
Number of positive nodes						
1	178	59.2	65	59.6	113	59.5
2–4	82	27.4	35	32.1	47	24.7
>5	34	11.4	9	8.3	25	13.2
Unknown	5	1.7	0	0.0	5	2.6
Randomisation arm						
Observation	153	51.2	56	51.4	97	51.1
PEG-IFN	146	48.8	53	48.6	93	48.9

Abbreviations: Q25, first quartile (i.e. 25% of patients have values below or equal to Q25); Q75, third quartile (i.e. 75% of patients have values below or equal to Q75); RT-PCR, reverse transcriptase polymerase chain reaction; PEG-IFN, pegylated interferon alfa2b.

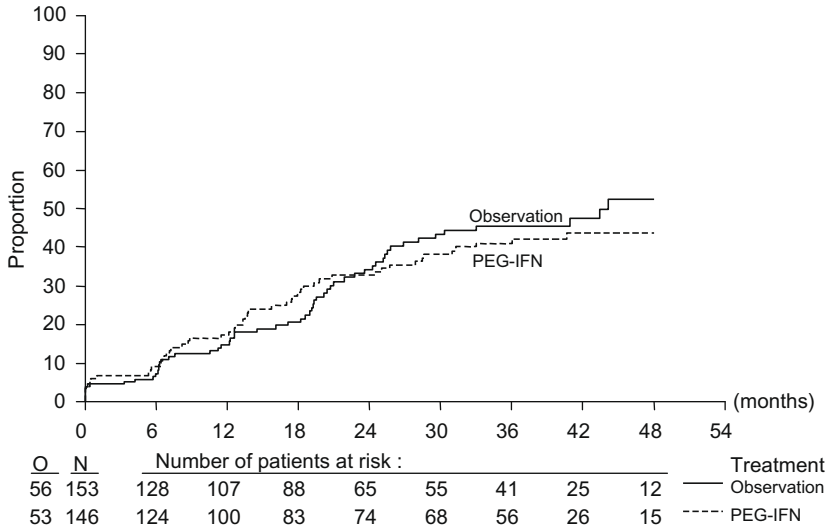

Fig. 1 – Cumulative rate of first positive blood sample from randomisation date by treatment arm.

3.3.2. *Landmark method*

Analysis of baseline blood samples revealed no differences in terms of DMFS between patients with a positive sample and patients with a negative sample within 30 d around the date of randomisation (Fig. 3, 1st left-hand side panel). Using other time windows similar results were obtained (data not shown). Also for later time points (every 6 months until 60 months), no significant differences were obtained between the group of patients with a positive and patients with a negative blood sample regarding DMFS (Fig. 3). In these analyses, only those patients alive and free of disease at the given time points and for whom a blood assessment was obtained were considered. Other time windows around these time points provided similar results (data not shown).

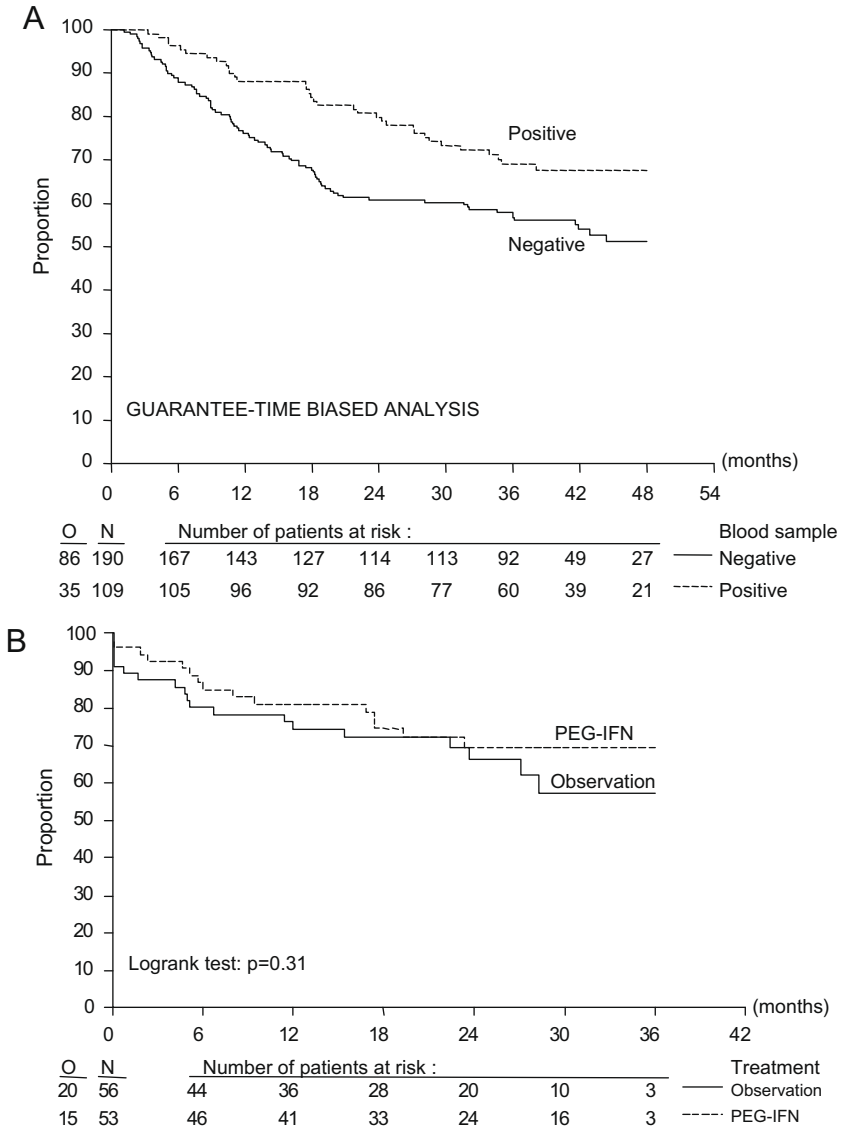

Fig. 2 – (A) Distant metastasis-free survival from the date of the first blood sampling stratified for reverse transcriptase polymerase chain reaction (RT-PCR): always negative versus at least once positive (guarantee-time biased analysis). (B) Distant metastasis-free survival from the date of the first positive blood sample by treatment arm.

3.3.3. *Cox time-dependent covariates model*

Classical statistical approaches were guarantee-time biased: only patients who survived without distant metastasis for a minimum period of time (generally 6 months or more) could be categorised in the positive group. RT-PCR testing was therefore used as a time-dependent prognostic factor in a proportional hazards model. The latest RT-PCR determination was used to compare the subsequent outcome of those who were positive versus those who were negative. Cox time-dependent model estimated the hazard ratio for the latest RT-PCR positive versus negative to be 2.23 (95% CI, 1.40–3.55; $p < 0.001$) (Table 2, right-hand side). This indicates that there is approximately twofold increase risk of developing metastasis/death subsequent to a blood determination in patients who are RT-PCR positive versus those who are negative at any given time point post randomisation, and who are still free of distant metastases. After adjustments for nodal status, number of positive nodes and sex, the hazard ratios for variable RT-PCR (positive versus negative) remained almost unchanged. In the same way hazard ratios exceeded 1 when PEG-IFN-treated patients only were considered (Table 3, upper part), or patients in the Observation Arm only (Table 3, lower part). It seemed that the RT-PCR prognostic significance was more important in the latter group (hazard ratio ± 3) than in PEG-IFN group (hazard ratio ± 1.5); however this could not be demonstrated statistically: the interaction test between positivity of blood samples and treatment arm yielded p-value > 0.15.

We performed an additional analysis for explorative purposes, in order to assess whether the degree of RT-PCR positivity had an impact on the outcome. Thus, an additional cut-off value had been chosen for the *tyrosinase* value (0.0004) and for the *Mart-1/Melan-A* (0.0002) in order to distinguish moderately from strongly positive. At each assessment, the patients were categorised as strongly positive (1–2 markers were strongly

Table 2 – Prognostic impact of RT-PCR positive detection of *tyrosinase* and/or *Mart-1/Melan-A* on DMFS using the time-independent Cox model (analysis with guarantee-time bias) or the time-dependent Cox model (analysis corrected for guarantee-time bias).

Variable	Time-independent Cox model[a]		Time-dependent Cox model[b]	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
All patients (*N* = 299, *O* = 121)				
Univariate analysis				
RT-PCR positive versus RT-PCR negative	0.61 (0.41–0.90)	.012	2.23 (1.40–3.55)	<.001
Multivariate analysis				
RT-PCR positive versus RT-PCR negative	0.62 (0.41–0.91)	.016	2.22 (1.39–3.53)	<.001
N2 versus N1	1.55 (1.08–2.22)	.018	1.61 (1.12–2.31)	.009
Male versus female	1.80 (1.22–2.67)	.003	1.75 (1.18–2.60)	.005
Multivariate analysis (N = 294)[c]				
RT-PCR positive versus RT-PCR negative	0.62 (0.42–0.93)	.021	2.27 (1.42–3.62)	<.001
No. positive nodes				
2–4 versus 1	1.39 (0.92–2.11)	.116	1.32 (0.88–2.00)	.183
>5 versus 1	1.81 (1.07–3.08)	.028	1.86 (1.09–3.16)	.022
N2 versus N1	1.40 (0.96–2.05)	.084	1.44 (0.98–2.11)	.060
Male versus female	1.72 (1.15–2.55)	.008	1.66 (1.12–2.47)	.012

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; DMFS, distant metastasis-free survival; O, observed number of events; HR, hazard ratio; N1, microscopic nodal involvement; N2, palpable nodes.

a At least once RT-PCR positive versus always RT-PCR negative.

b Latest assessment: RT-PCR positive versus RT-PCR negative.

c Patients with a known number of positive nodes.

positive), moderately positive (1–2 markers were moderately positive), and negative (both markers were negative). Among 109 patients RT-PCR positive, initially or during the course of the study, 51 (17.1%) patients were or became strongly positive. Using the Cox time-dependent covariates model, the risk of developing metastasis/death was 3.15 ($p < 0.001$) higher for patients who were strongly positive versus those who were negative and was 1.45 for those who were moderately positive versus those who were negative. In the PEG-IFN group, the estimated HR for strongly positive versus negative was 2.29 ($p = 0.06$) and was 0.78 for moderately positive versus negative; the corresponding estimates in the Observation group were 4.30 ($p < 0.001$) and 1.92, respectively.

Data analyses by individual markers did not show a significant superiority of one marker over the other (data not shown).

4. Discussion

Detection of circulating tumour cells might improve current staging procedures by identifying a subgroup of patients with more aggressive disease or by indicating impending relapse.[20] Since the first description in the early 1990s by Smith and colleagues,[3] numerous reports on the prognostic significance of mRNA melanoma markers have been reported.[4–15,21–25] Most of the studies failed to demonstrate a prognostic relevance of RT-PCR results in the very favourable risk groups of stage I and II melanoma patients.[21,22] The most interesting patient population would be sentinel-node-staged stage III patients. Previous reports in stage III patients, however, had included patients not followed up within clinical trials, resulting in heterogeneities with respect to time interval between nodal resection and first RT-PCR assessment, intervals between RT-PCR assessments, scheduling of clinical follow-up examinations and treatment. These reports yielded heterogeneous results on the role of circulating melanoma cells as a prognostic factor[10,12,14,15] and, by design, were unable to address a potential role of the RT-PCR assay results as predictive factor for adjuvant IFN treatment.

The relatively large prospective study reported here specifically investigated stage III melanoma patients homogenously followed within the framework of a prospective randomised adjuvant treatment trial. In this setting, the presence of circulating melanoma cells detected by *tyrosinase* and *Mart-1/Melan-A* RT-PCR was a time-dependent prognostic factor for subsequent development of distant metastasis. However, the analysis of blood samples at baseline or at later fixed time points did not reveal significant differences in terms of DMFS according to RT-PCR results.

In accordance with our results, two of three previous studies with a single RT-PCR determination failed to demonstrate a prognostic significance of the RT-PCR assay.[7,24,25] Similarly, studies[4,10,12,13,25] with serial samples did not find any relation between baseline determinations (mostly post-operative) and prognosis, which again is in line with our findings. With the overall data of all these studies it can be concluded that with current adjuvant treatments the RT-PCR analysis for circulating melanoma cells cannot be used for treatment decisions based on single-time-point RT-PCR results and circulating melanoma cell detection should not be employed to select patients for adjuvant treatment. RT-PCR results were indeed not predictive for treatment effect in our study in multivariate analysis but, due to the low number of patients included in the two different arms, estimates are quite unreliable.

As shedding of melanoma cells has been assumed to be a discontinuous event,[26] dynamic assessment of serially collected blood specimens has been recommended for evaluation of the biology of the circulating melanoma cell phenomenon

Table 3 – Prognostic impact of RT-PCR positive detection of *tyrosinase* and/or *Mart-1/Melan-A* on DMFS according to the randomised arm, using the time-independent Cox model (analysis with guarantee-time bias) or time-dependent Cox model (analysis corrected for guarantee-time bias).

Variable	Time-independent Cox model[a]		Time-dependent Cox model[b]	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
PEG-IFN group (N = 146, O = 52)				
Univariate analysis				
RT-PCR positive versus RT-PCR negative	0.63 (0.35–1.15)	.14	1.54 (0.72–3.28)	.26
Multivariate analysis				
RT-PCR positive versus RT-PCR negative	0.59 (0.32–1.08)	.086	1.37 (0.64–2.93)	.417
N2 versus N1	1.79 (1.03–3.11)	.038	1.80 (1.03–3.13)	.037
Male versus Female	2.60 (1.38–4.90)	.003	2.43 (1.29–4.57)	.006
Multivariate analysis (N = 144)[c]				
RT-PCR positive versus RT-PCR negative	0.65 (0.35–1.21)	.176	1.43 (0.67–3.08)	.358
No. positive nodes				
2–4 versus 1	0.87 (0.44–1.75)	.703	0.79 (0.40–1.57)	.503
>5 versus 1	1.33 (0.61–2.92)	.471	1.36 (0.62–2.97)	.446
N2 versus N1	1.77 (0.98–3.18)	.059	1.77 (0.98–3.18)	.059
Male versus female	2.52 (1.33–4.79)	.005	2.37 (1.25–4.48)	.008
Observation group (N = 153, O = 69)				
Univariate analysis				
RT-PCR positive versus RT-PCR negative	0.58 (0.34–0.97)	.038	2.90 (1.59–5.27)	<.001
Multivariate analysis				
RT-PCR positive versus RT-PCR negative	0.61 (0.36–1.04)	.067	3.19 (1.74–5.83)	<.001
N2 versus N1	1.47 (0.90–2.83)	.120	1.66 (1.03–2.70)	.038
Male versus female	1.34 (0.81–2.23)	.258	1.40 (0.84–2.33)	.200
Multivariate analysis (N = 150)[c]				
RT-PCR positive versus RT-PCR negative	0.62 (0.36–1.05)	.077	3.38 (1.84–6.20)	<.001
No. positive nodes				
2–4 versus 1	1.82 (1.07–3.10)	.027	1.96 (1.15–3.33)	.013
>5 versus 1	2.48 (1.20–5.09)	.014	2.50 (1.22–5.14)	.013
N2 versus N1	1.35 (0.81–2.25)	.255	1.52 (0.91–2.53)	.113
Male versus Female	1.25 (0.75–2.09)	.395	1.31 (0.78–2.19)	.307

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; DMFS, distant metastasis-free survival; O, observed number of events; HR, hazard ratio; N1, microscopic nodal involvement; N2, palpable nodes; PEG-IFN, pegylated interferon alfa2b.

a At least once RT-PCR positive versus always RT-PCR negative.

b Latest assessment: RT-PCR positive versus RT-PCR negative.

c Patients with a known number of positive nodes.

during follow-up or treatment.[10] Including the study reported here, four large studies have now evaluated RT-PCR results in a covariate model as a time-dependent covariate. In time-dependent Cox regression analysis done on 118 patients, Reynolds and colleagues[9] revealed a statistically significant correlation between the presence of circulating melanoma cells and recurrence-free survival with a risk of recurrence for patients with at least one positive RT-PCR result of 2.6, which is extremely close to our estimate. But analysis was based on resected stage IIB (N = 78), IIIA (N = 59), IIIB (N = 48) or IV (N = 11) melanoma patients enrolled in a vaccination trial. Voit and colleagues[14] enrolled 111 patients (78 stage II and 33 stage III patients) and *tyrosinase* determinations were carried out every 3–6 months. *Tyrosinase* RT-PCR was confirmed to be a significant time-dependent prognostic variable for recurrence-free survival with an unadjusted hazard ratio of 4.5. More recently Scoggins and colleagues[25] confirmed time-dependent *tyrosinase* RT-PCR as a significant prognostic variable for disease-free survival in a total of 838 stages I (N = 353), II (N = 266) and III (N = 207) melanoma patients with a hazard ratio of 5.5.

Highly sensitive methods are a prerequisite for a successful detection of rare circulating tumour cells. Combination of immunomagnetic enrichment with RT-PCR or immunocytochemistry increased both the detection rate and the effectiveness.[27] At the same time, studies using RT-PCR have also been criticised as potentially being prone to false positive results. Recent advances include the development of an automated immunomagnetic enrichment and staining system (Cell Search™) which is currently in clinical use[28] and a microfluidic platform recently established by Nagrath and colleagues capable of high selective and sensitive separation of circulating tumour cells from peripheral whole blood samples.[29] Both the techniques enabled detection of circulating epithelial cancer cells. A method for detection of circulating melanoma cells has not been validated yet.

In conclusion, the detection of circulating melanoma cells by RT-PCR is a moderate time-dependent prognostic variable for subsequent development of distant metastasis in stage III melanoma patients and of interest for following the biology of minimal residual disease. This assay is not suitable for

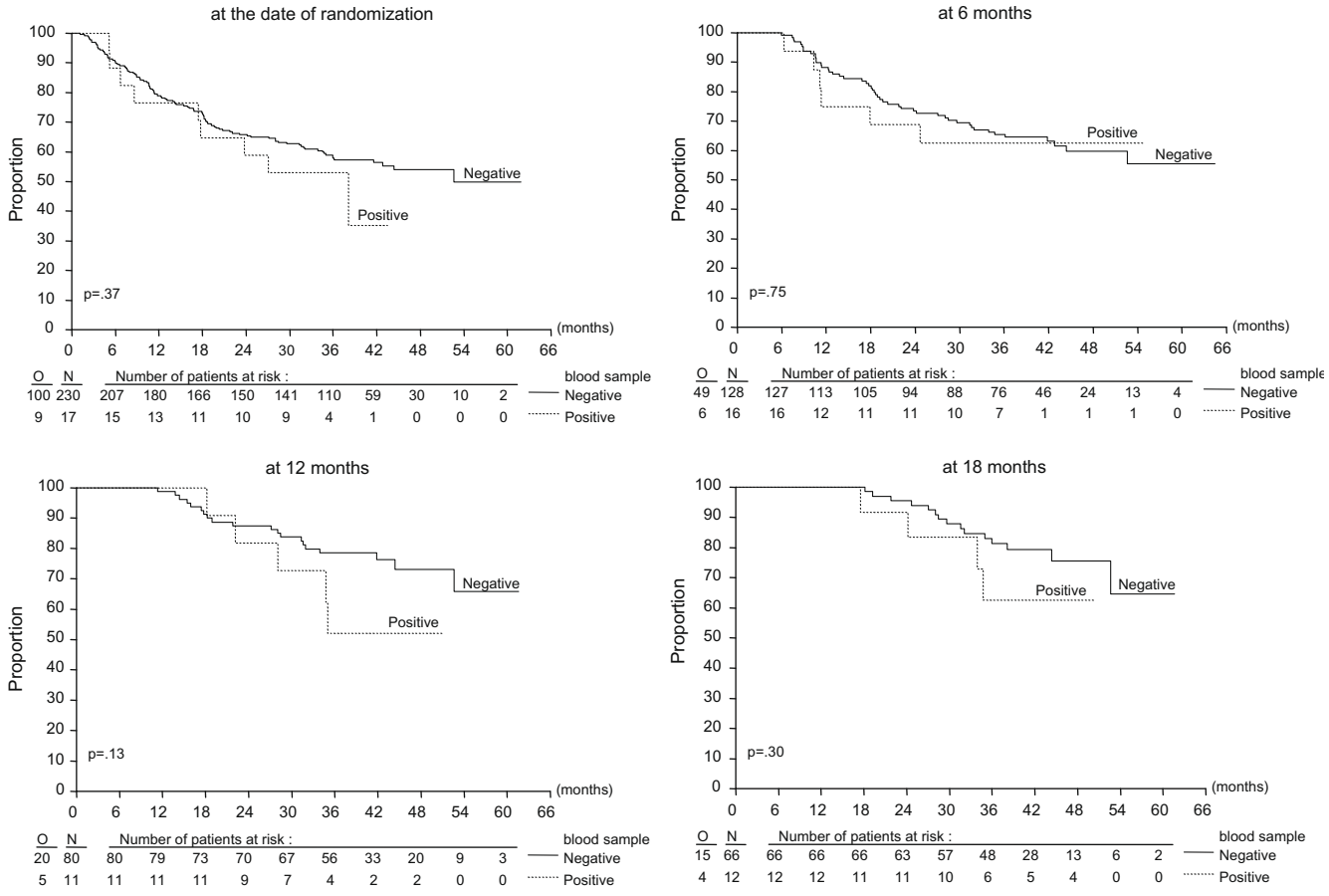

Fig. 3 – Distant metastasis-free survival according to RT-PCR results at different fixed time points with a 60-d time-interval window (±30 d around the theoretical date of sampling).

decisions on adjuvant IFN treatment. Hazard ratios for recurrence are modest, but are higher than 1. In the Observation group, only patients with strongly positive RT-PCR values showed a worse subsequent prognosis (HR = 4.3). The method has therefore a value in predicting relapse in the setting of patients presented but, at the same time, results should not be overestimated.

Conflict of interest statement

A.G. Dalgleish has served as consultant/advisor for Onyvax, received research funding from Celgene Inc. and an honoraria from GlaxoSmithKline. A.M.M. Eggermont and U. Keilholz have served as consultants/advisors for Schering Plough. The other authors declare no conflict.

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Appendix

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