

Detection of perioperative circulating melanoma cells: a clinical trial in patients with malignant melanoma undergoing lymph-node dissection

Short title: Perioperatively detected malignant melanoma cells

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Abstract

Background. Resection of malignant melanoma (MM) during spinal anaesthesia may lead to a better long-term prognosis, with fewer metastases, due to reduced compromise of the immune system with regional anaesthesia. This study investigated whether the immune system is a major factor and whether MM is a systemic disease.

Methods. In patients requiring spinal or general anaesthesia for inguinal lymph-node dissection after primary MM in the lower extremity, blood was sampled before anaesthesia induction (M1), at the end of surgery (M2), 24 hours later (M3) and on postoperative day 4–6 (M4). A nested polymerase chain reaction test for melanoma-associated antigen recognized by T-cells 1 (MART-1) was used to detect circulating melanoma cells.

Results. Forty-one patients (aged 56.80 ± 16.9 years) underwent surgery with either general anaesthesia ($n = 23$, 56.1%) or spinal anaesthesia ($n = 18$, 43.9%). The mean thickness of the primary melanoma was 4.20 ± 3.41 mm. The resected lymph nodes were cancer-free in 31 patients (76%). Nine patients (22%) had one metastatic lymph node and one patient had two. MART-1 was positive in 25 patients (63%) at any time point, and thicker primary melanomas were found in these patients. Lymphatic cancer spread was independent of circulating melanoma cells.

Conclusions. MM is a systemic disease. Circulating tumour cells are detected even in stage III, when no extralymphatic metastases are expected. An adequate immune status is therefore needed to eliminate malignant cells and an anaesthetic technique that does not impair perioperative immune responses is important. Regional anaesthesia may therefore be the preferable technique.

Keywords: anaesthesia; malignant melanoma; circulating malignant melanoma cells

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Introduction

Malignant melanoma is the most lethal form of skin cancer. It can metastasize rapidly. It represents only 4% of all skin malignancies, but nearly 80% of deaths from skin cancer.¹ While local disease in the early stages has a relatively good prognosis, later stages with positive lymph-node findings or distant metastases are associated with a poor outcome and a high mortality rate.²

A sentinel lymph-node (SLN) biopsy is recommended for staging of the disease in some cases. If the test indicates that the tumour has already metastasized to the lymph nodes, a radical lymph-node dissection is carried out. However, tumour cells may spread via the lymphatic or vascular systems. It has been shown that resecting malignant melanoma (MM) with the patient under spinal anaesthesia may be associated with a better long-term prognosis, with fewer metastases.³ It was postulated that this might be due to the reduced degree of immunocompromise present with regional anaesthesia. If the immune system is a cornerstone in the process, then MM must be a systemic disease.

There is a lack of clinical studies in humans to investigate potential perioperative changes in the immune status in patients with malignant melanoma. The present study therefore investigated the following issues:

- Is malignant melanoma a systemic disease at the time of surgery? This would imply that tumour cells may spread via the bloodstream even before lymph-node surgery. At present, however, there have been no comprehensive studies on the possible vascular spread of malignant melanoma at the very early time point of lymph-node surgery. The first major question needing to be investigated is therefore: must we – at the stage of the disease at which intralymphatic metastases are present, but there are no distant metastases (stage III) – expect there to be circulating melanoma cells in a significant number of patients who are undergoing radical inguinal lymph-node dissection?
- What is the clinical relevance of the finding if circulating melanoma cells are detected? Does the spread into the circulation depend on the thickness of the primary tumour? Is there a correlation between the observed spread of melanoma – i.e., the number of metastatic lymph nodes – and the detection of circulating melanoma cells?

Methods

After approval had been received from the ethics committee of the Medical Council of Westphalia-Lippe and from the Medical School of the University of Münster, Germany (trial no. 2006-088-f-S), patients who required anaesthesia for inguinal lymph-node dissection after a diagnosis of a primary malignant melanoma in the lower limb between 2012 and 2013 were included in this observational trial. The study was carried out at the Department of Anaesthesia, Intensive Care and Pain Medicine, Hornheide Hospital, Münster, Germany.

Study protocol

Patients underwent surgery either under spinal anaesthesia (SA group) or general anaesthesia (GA group). Patients were eligible for inclusion in the study if they had been diagnosed with stage III malignant melanoma.² The inclusion criteria were as follows: malignant melanoma patients scheduled for inguinal lymph-node dissection; American Society of Anesthesiologists (ASA) physical status classes I–III; and availability of signed informed consent. The exclusion criteria were: age < 18 years, female patients who were pregnant or breast-feeding, allergies to study medications, multiple organ failure, contraindications for spinal anaesthesia, unwillingness, or current inclusion in another clinical trial within 30 days.

Anaesthesia was administered as described in Gottschalk et al.³ Spinal anaesthesia (in the SA group) was administered between lumbar spaces L2–3, L3–4, or L4–5. A total of 2.5–3.0 ml hyperbaric bupivacaine 0.5% was injected. Patients allocated to general anaesthesia (the GA group) received standardized general anaesthesia with propofol (2 mg/kg i.v.) as induction medication, rocuronium (0.5 mg/kg i.v.) as a muscle relaxant and sufentanil (0.2–0.5 µg/kg i.v.) for analgesia. Sevoflurane in air/oxygen (expiratory concentration 1.8–2.0%) was used to maintain anaesthesia.

Blood was drawn before the induction of anaesthesia (M1), at the end of the operation (M2), 24 hours after surgery (M3) and at postoperative day 4–6 (M4). It was immediately transferred to the local laboratory for analysis.

To study the potential for hematogenic distribution of malignant melanoma, a check was carried out for circulating melanoma cells in the blood samples obtained at time points M1, M2, M3 and M4. A reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed and implemented for detecting the cells. The technical platform for this diagnostic melanoma test consists of two sequential RT-PCR reactions, in which the PCR products of the first reaction serve as a template for the second reaction ('nested PCR'). Tyrosinase (first PCR product: 123 bp; nested PCR product: 107 bp) and melanoma-associated antigen recognized by T-cells 1 (MART-1; first MART-1 PCR product: 261 bp, nested MART-1 PCR product: 252 bp) were used as molecular melanoma markers. Other types of tumour, as well as lymph nodes from patients who do not have cancer, do not produce MART-1.^{4,5} Following isolation of RNA from a 5-ml aliquot of the patient's blood, subsequent RNA quality controls and quantification, an aliquot of RNA was reverse-transcribed into cDNA. The cDNA served as a template for the first of the two sequential PCR steps (described above), with the aim being to identify melanoma cells in the blood sample.

Statistics

Statistical analyses were performed in accordance with the principles of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline E9, *Statistical Principles for Clinical Trials*, using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, New York, USA). Normal scale variables were described using relative and absolute frequencies, and Pearson's chi-squared test was used to assess differences between groups. Fisher's exact test was used if matched

cells were rare (frequency ≤ 5). Variables with interval or higher-scale levels were described using means and standard deviation. The covariation of variables was analysed using Pearson correlation coefficients.

A *t*-test for independent variables or repeated-measures analysis of variance was used to compare groups. Any significance tests applied were two-sided and significance was set at $P \leq 0.05$. Missing values were not replaced using any kind of statistical imputation.

RESULTS

A total of 19 women and 22 men, with a mean age of 56.80 years (SD \pm 16.9 years), were included in the study. The patients' medical histories are outlined in Table 1. In relation to the ASA classification, 14 patients (34%) were assessed as ASA I, 24 (59%) as ASA II and three (7%) as ASA III. Surgery was carried out with the patients under either general anaesthesia ($n = 23$, 56.1%) or spinal anaesthesia ($n = 18$, 43.9%).

The mean thickness of the primary melanoma was 4.20 mm (SD \pm 3.41 mm). The mean total number of lymph nodes removed during the radical inguinal lymph-node dissection was a mean of 7.8 (SD \pm 3.1). No intralymphatic metastases were found in the specimens in 31 patients (76%). Nine patients (22%) had one positive lymph node and one patient had two metastatic lymph nodes. In all, melanoma cells were detected in 25 patients (63%) at any time point. Ten blood samples (24%) contained melanoma cells (positive for MART-1) even before the induction of anaesthesia. In addition, melanoma cells were demonstrated in eight of the blood samples (20%) obtained at the end of surgery and in 13 of the blood samples (32%) obtained after surgery (24 h or postoperatively at day 4–6) (Fig. 1).

The numbers of circulating cells detected did not increase during or after surgery. Melanoma cells were detected in most cases in only one of the three blood samples from a patient. MART-1 positive findings were obtained in only one sample in 35 patients, in two samples in five patients, and never in all three samples. There were no significant differences in the detection of circulating melanoma cells between patients with tumour-free or metastatic lymph nodes during radical dissection (Table 2). However, patients with circulating melanoma cells had significantly larger (thicker) primary tumours (Table 3).

DISCUSSION

The results of this study show that positive malignant melanoma cells in the blood are found in many patients who are undergoing inguinal lymph-node dissection due to malignant melanoma in the lower limb. Interestingly, these patients may be MART-positive before, during, or after surgery. However, it is of no importance which of these patients is MART-positive at what time point. Malignant melanoma is thus already a systemic disease even before surgery. The presence of an adequate perioperative immune reaction is consequently necessary in order to control the systemic disease and eliminate cancer cells.

In comparison with colonic cancer, in which the presence of circulating tumour cells preoperatively and postoperatively is a crucial prognostic factor in relation to postoperative relapse,⁶ it is not at present clear how malignant melanoma patients who have MART-

positive findings at any time should be managed. The present study shows that patients may be MART-positive even before any surgical intervention is carried out. In addition, some patients may be MART-negative and others MART-positive afterwards, without any relationship to their preoperative MART status. It therefore appears that some immunological crosstalk occurs. This might be crucial for the long-term prognosis in these patients, as malignant melanoma has the highest prevalence of somatic mutations among all types of human cancer.⁷

The biological background for the primary hypothesis in the present study was the observation that tumours are imprinted by the immunological environment in which they develop. There is a complex interaction between tumour cells and immune cells. The clinical outcome for cancer patients is highly dependent on this process, which shapes the capability of tumour cells to extravasate, circulate in the body and establish themselves in a distant organ. Many patients suffering from malignant melanoma already have micrometastases and scattered tumour cells at the time of lymph-node dissection. Whether this minimal residual disease impairs the prognosis after tumour surgery depends largely on the efficacy of the host's immune system. In addition, undetectable micrometastases may already exist even in localized disease. The fact that many patients were found to be MART-positive even before surgery may be evidence of this. The fate of these 'proto-metastases', which escape detection and destruction, appears to be significantly influenced by biochemical changes and disturbances of cellular signalling that occur following excision of the primary tumour.⁸ Potentially curative surgical resection may paradoxically create a window of susceptibility in which residual cancer cells (either disseminated during surgery or pre-existing micrometastases) are able to overcome host defences and become established.⁸ There is as yet no well-established information about which cancer patients this may occur in.

Study limitations. No long-term data are yet available to suggest that the immune changes described above affect the cancer prognosis for patients. It would also be of interest to know whether patients who have MART-positive findings at any time have a poorer long-term prognosis in comparison with patients who are always MART-negative. Additionally, it might also be questionable why anaesthesia was not maintained using propofol during the whole period of anaesthesia in the study. However, it should be noted that the investigation was concerned with comparing anaesthetic techniques, rather than medications.

CONCLUSIONS

The results of this study show that malignant melanoma is a systemic disease. Circulating tumour cells are detected even in stage III, when metastases outside of the lymph nodes are not expected. The presence of an adequate immune reaction is therefore necessary in order to eliminate malignant cells. In these patients, it is consequently important to select an anaesthetic technique that does not impair the perioperative immune reaction. Regional anaesthesia may therefore be the preferable technique.

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Table 1 The patients' medical histories

	Patients (n)
Myocardial infarction	1
Coronary artery disease	1
Arrhythmia	1
Hypertension	9
Chronic obstructive pulmonary disease	2
Liver disease	2
Coagulopathy	1
Diabetes mellitus	4

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Table 2 Detection of circulating melanoma cells (melanoma-associated antigen recognized by T-cells 1, MART-1) in patients with tumour-free or metastatic lymph nodes. No significant differences in the MART-1 findings were noted between patients with tumour-free and metastatic lymph nodes

	Before anaesthesia		At end of surgery		After surgery *		At any time point	
	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)
LNs tumour-free	23(77)	7(23)	24 (80)	6 ⁹	19 (63)	11 (37)	11 (37)	19 (63)
LNs metastatic	7(70)	3(30)	8 (80)	2 ⁹	8 (80)	2 ⁹	4 (40)	6 (60)

LNs, lymph nodes.

* After 24 hours and at postoperative day 4–6.

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Table 3 Thickness of the primary tumour in patients with circulating tumour cells (MART-1–positive) or without circulating tumour cells (MART-1–negative)

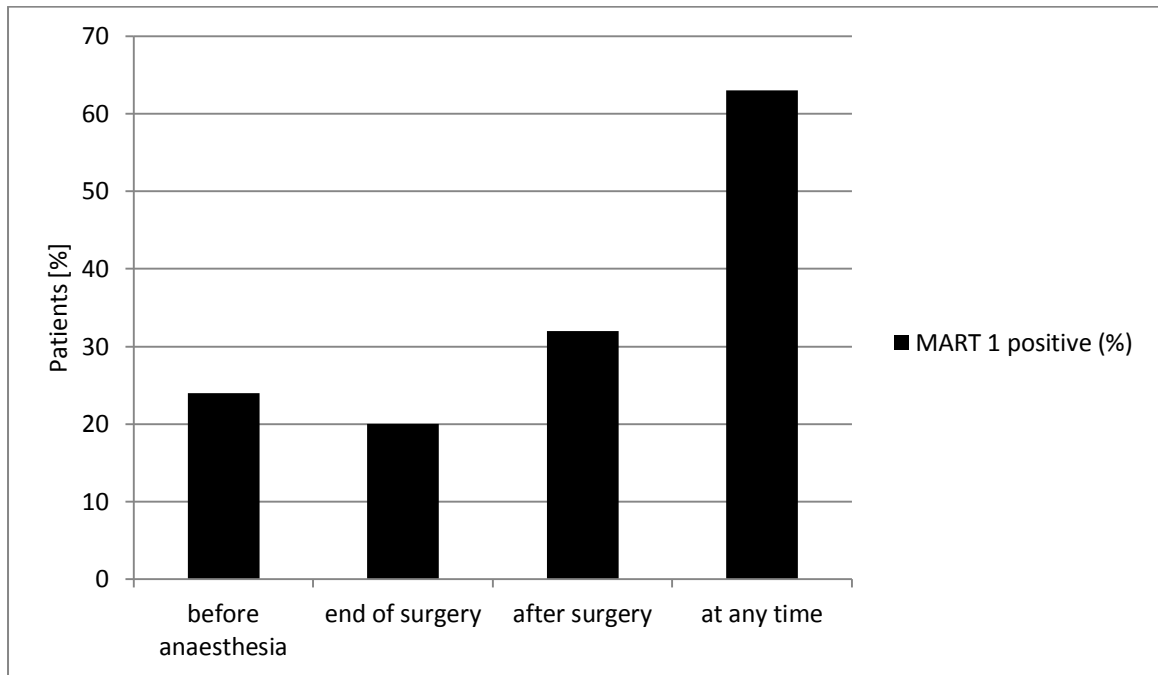
	Thickness of the primary tumour (mm)				<i>t</i>	<i>P</i>
	<i>n</i>	Mean	SD			
MART-1 negative	15	2.9	1.44		-2.19	0.035
MART-1 positive	25	4.85	4.02			

MART-1, melanoma-associated antigen recognized by T-cells 1.

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Legend

Fig 1 Percentage of patients with circulating melanoma cells.



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