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# Progressive Disease in Sentinel-negative Melanoma Patients: Biological Differences and Importance of Sentinel Lymph Node Biopsy

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**Abstract.** *Background/Aim: Among the most important prognostic factors in melanoma is the sentinel lymph node (SLN) status. Materials and Methods: Using our electronic database we identified 109 of 890 SLN-negative patients with progressive disease (PD). These patients were characterized for melanoma type, molecular type, sequence and extent of metastatic spread. Results: A total of 61 of 109 SLN-negative patients had PD in the SLN-basin indicating false-negative SLN (group-1). Forty eight of 109 patients had PD at distant sites and were therefore impossible to be identified using SLN biopsy (group-2). Despite distant spread these patients had significantly more single organ metastasis ($p<0.001$) and significantly longer disease-free-survival ($p=0.001$) compared to group-1. Additionally, to significant differences on a molecular basis between the two groups ($p=0.01$), all lentigo maligna and spindle-cell-melanomas belonged to group-2 and all, except one lentigo maligna melanoma, had single visceral metastasis. Conclusion: Two different biological groups among SLN-negative patients with PD were demonstrated. Extravascular-migratory-metastasis, rather than hematogenous spread, might be responsible for the observed PD with single organ involvement.*

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*Key Words:* Melanoma sentinel lymph node, melanoma metastases, melanoma progression, extravascular-migratory-metastasis.

One of the most important prognostic parameters in predicting patient outcome in cutaneous melanoma is sentinel lymph node (SLN) status. Sentinel lymph node biopsy (SLNB) has therefore become the recommended staging procedure for patients at higher metastatic risk (1-7). SLNB helps identifying patients with potential benefit from further immune-modulating or tumor-targeting therapies. However, SLN biopsy is a challenging interdisciplinary effort, requiring different specialists to collaborate closely. False-negative SLN results are an important issue, partly owing to difficulties associated with successfully performing SLNB.

Several studies have investigated progressive disease (PD) in SLN-negative patients. The reported incidence of false-negative SLN varies widely between 2% and 22.7%. This wide range is at least partly due to varying definitions of false-negative SLN. Reported risk factors for SLN-negative PD are older age, male sex, melanoma located on the head and neck region, melanoma type and presence of ulceration (8-13).

In this study, we compared the clinical courses of patients with false-negative SLNs to those with truly-negative SLNs and progression at distant sites. False-negative SLN was defined as follows: a patient with apparent negative SLNB and subsequent recurrence of melanoma in the SLN-basin or in a non SLN-basin but in close relation to the primary melanoma indicating an initially missed or additional second SLN-basin. We examined whether there were differences in primary tumor location, tumor type, molecular type, distant metastasis in multiple or only one organ, disease-free-survival and survival.

## Materials and Methods

The study population consisted of all consecutive melanoma patients who underwent SLN biopsy at the University Hospital Zurich (USZ) between 1999 and 2014. Using the Department of Pathology's electronic database, as well as USZ's electronic clinical database, patients diagnosed with negative SLNs and later PD were further investigated for sex, age at diagnosis, melanoma type, Breslow thickness, Clark level and possible molecular analysis. Furthermore, the sequence and extent of metastatic spread was analyzed in detail. PD was defined as histologically confirmed melanoma lymph node (LN) metastasis or visceral metastasis until August 2017. We differentiated between patients with nodal recurrence in the SLN-basin or a possible initially missed SLN-basin (false-negative SLN; group-1) and patients with nodal or visceral recurrence at distant sites (group-2). A possible missed SLN-basin was defined when the nodal recurrence occurred close to the primary melanoma. SLN-negative patients with local recurrence or in-transit metastases alone were not included in this study. Survival information was collected both from the database and from the Cancer Registry Zurich and Zug, Switzerland.

The SLN technique and pathological work-up used at USZ has been described in detail in previous publications (3, 14, 15).

In accordance with national guidelines, patients with negative SLNB underwent quarterly skin examinations including clinical investigation of LN stations (16). Additionally, LN station ultrasound was performed on an annual basis. Patients with thick primary tumors (Breslow >4 mm) also underwent FDG-PET/CT every year (alternating ultrasound and PET/CT every 6 months). Patients with LN PD were immediately restaged using FDG-PET/CT.

Approval for the study was obtained from the ethics committee of Zurich, Switzerland, (approval number KEK-ZH-Nr. 2014–0193 and Stv. 16-2007, amendment 2014). In addition, the majority of patients provided written informed consent to use their medical data for studies in accordance with the Declaration of Helsinki. By those who were not able to do so the ethical approval allowed us to use them if the patients were not explicit against.

Statistical analysis was performed using IBM SPSS Statistics, version 25.0 (IBM Corp., Armonk, NY, USA). Results of the descriptive statistics were expressed as numbers, percentages and mean (± standard deviation) or medians with interquartile ranges. The false-negative rate for SLNB was calculated using the following: # false-negative SLNB/(# positive SLNB + # false-negative SLNB)*100. Data were analyzed using the binominal test, chi-square Fishers exact test and Mann–Whitney *U*-test. Variables were checked for correlations with Pearson's and Spearman's rho. Overall survival and disease-free survival were analyzed using Kaplan–Meier curves and log-rank testing, and are reported with 95% confidence intervals (CI). Groups were compared using the log-rank test. All statistical tests were two-sided and exact *p*-value <0.05 was considered to indicate statistically significant differences.

## Results

*Characterisation of SLN patients with PD*. We identified 1151 melanoma patients who underwent SLNB at the University Hospital Zürich from 1999 to 2014. SLNs were positive in 261 (22.7%) and negative in 890 (77.3%) patients. One hundred nine (12.2%) SLN-negative patients later developed metastatic disease until August 2017. Follow-up, defined as the time from primary melanoma diagnosis to death or last clinical visit, was available for 97 patients, with a median of 77 months (range=9-204 months) (Figure 1). Sixty-nine (63%) patients were males and 40 (37%) females. Mean age at melanoma diagnosis was 57 years (range=13-87 years). Twenty-eight primary melanomas (25.7%) were on the back, 26 (23.9%) on the leg, 25 (22.9%) on the head, 20 (18.3%) on the arm and 10 (9.2%) on the abdomen/chest. Breslow thickness was known (with the exception of one patient) and had a mean of 2.6mm (range 0.8mm-9mm). Thirty-six melanomas (33%) were nodular malignant melanomas (NMM), 31 (28.4%) superficial spreading melanomas (SSM), 23 (21.1%) not otherwise specified (NOS), 12 (11%) acral lentiginous melanomas (ALM), 5 (4.6%) lentigo maligna melanomas (LMM) and 2 (1.8%) spindle cell melanomas. A molecular analysis was carried out in 59 (54.1%) melanoma patients: 29 (49.2%) were *BRAF* mutated, 14 (23.7%) *NRAS* mutated, 2 *C-KIT* mutated (3.4%) and 14 (23.7%) *BRAF/NRAS* wild type (Table I).

Eleven of 109 (10%) patients with PD had SLN biopsies of more than one SLN-basin (ten patients with 2 and one with 3 SLN-basins). The primary melanomas of these patients were located on the head (5), the back (4) and on the abdomen/chest (2).

*Characterisation of local recurrence, satellite metastases and in-transit metastases*. As most patients had a resection margin of at least 1 cm, we did not distinguish between local recurrence and satellite metastases. Twenty-five of 109 (22.9%) patients had in-transit metastases and 8 (7.3%) had satellite metastases/local recurrence. There was an association between anatomical site and occurrence of in-transit metastases or satellite metastases/local recurrence ($p$=0.056). In 10 (40%) patients in-transit metastases were located on the leg and in 7 (28%) on the arm. Four of 10 (40%) satellite metastases/local recurrences were located on the head.

Nineteen of 25 (76%) patients with in-transit metastases had also visceral spread. There was a strong correlation between the occurrence of in-transit metastases and visceral metastases ($p$<0.001; corr. coeff. 0.9) (Figure 2A).

*Characterisation of LN and visceral tumour recurrence*. We detected PD mostly in the SLN-basin in 50 of 109 (45.9%) patients. Recurrence in a non SLN-basin, but in close relation to the primary, was interpreted as an initially possible missed second SLN-basin or misinterpreted SLN-basin. This was found in 11 (10.1%) patients. Considering the initially 261 SLN positive patients, the false negative SLN rate is 19%.

Distant non SLNs (excluding possibly missed second basin LNs) were found in 32 (29.4%) and visceral metastases without LNs in 26 (23.8%) patients. In total, 48 (44 %) patients with PD could not have been identified using SLN biopsy.

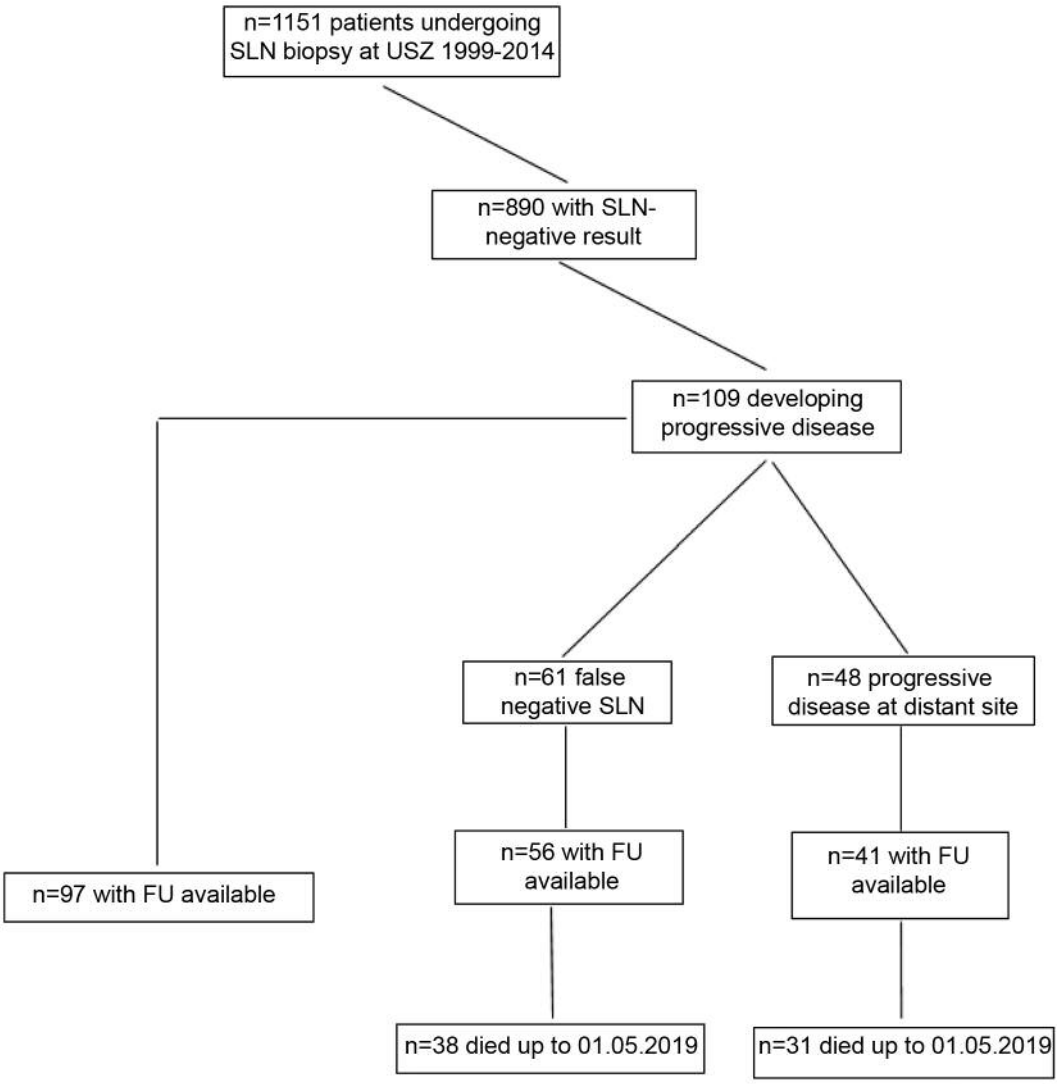


Figure 1. *Progressive disease in sentinel lymph node (SLN)-negative patients. FU (follow-up).*

Only 16 out of 109 (14.7%) patients had LN PD without visceral spread. Ten of them had PD in the SLN-basin and 6 in an initially missed SLN-basin. Thirteen of these patients had a median follow-up of 118 months (range=59-191 months) and are still all alive.

There was a significant difference in primary melanoma localisation between SLN-basin and non-SLN-basin recurrence ($p$=0.015). Primary melanomas of the extremities and abdomen/chest region had mostly recurrence in the SLN-basin, whereas primary melanomas of the head and back had in over half of the cases a non-SLN-basin metastasis.

*Comparing patients with false-negative SLNs (group-1) to patients with PD at distant sites (group-2) (Table II).* Comparing group-1 and 2, the above described difference in localisation of primary melanoma persisted but was less significant ($p$=0.048). There was a significant difference in melanoma type ($p$=0.03). SSM and NMM were equally distributed in both groups. In contrast, all LMM and spindle cell melanomas and one third of NOS melanomas recurred at distant sites (Table II). Interestingly, despite distal spread, all LMM except one, and all spindle cell melanomas had metastatic spread to just one visceral organ. In total, group-2 had significantly more single organ involvement than group-1 ($p$<0.001; Table II). The organs mainly affected by single tumour spread were the lungs, the brain and the liver. There was also a significant difference on the molecular basis ($p$=0.01). Melanomas with recurrence at distant sites had mostly *BRAF* mutations (17/27; 63%), whereas *NRAS* mutations were found in just 2 patients (7.4%). In loco-

Table I. *Characteristics of SLN-negative melanoma patients with progressive disease.*

| Variables | |
|---|---|
| Gender | |
| Female | 40 (36.7%) |
| Male | 69 (63.3%) |
| Age at primary diagnosis | |
| Mean | 57 (13-87) |
| Median | 60 |
| <60 | 54 (49.54%) |
| >60 | 55 (50.46%) |
| Type of melanoma | |
| SSM | 31 (28.4%) |
| NMM | 36 (33%) |
| ALM | 12 (11%) |
| LMM | 5 (4.6%) |
| Spindle cell | 2 (1.8%) |
| NOS | 23 (21.1%) |
| Breslow (n=108) | |
| Mean (mm) | 2.9 (0.8-9) |
| <1 mm | 6 (5.5%) |
| 1.01-2 mm | 37 (34%) |
| 2.01-4 mm | 42 (38.5%) |
| >4 mm | 24 (22%) |
| Clark level (n=85) | |
| II | 1 (1.2%) |
| III | 19 (22.3%) |
| IV | 57 (67.1%) |
| V | 8 (9.4%) |
| Site of primary tumor | |
| Head | 25 (22.9%) |
| Back | 28 (25.7%) |
| Abdomen/chest | 10 (9.2%) |
| Upper extremities | 20 (18.3%) |
| Lower extremities | 26 (23.9%) |
| Molecular analysis (n=59) | |
| BRAF mutation | 29 (49.2%) |
| NRAS mutation | 14 (23.7%) |
| C-KIT mutation | 2 (3.4%) |
| BRAF/NRAS wild-type | 14 (23.7%) |
| Follow-up (n=97) | |
| Mean (±SD/month) | 78 (9-204)±45 |
| Median | 82 (95%CI=66-88) |

NMM: Nodular malignant melanoma; SSM: superficial spreading melanoma; LMM: lentigo maligna melanoma; ALM: acral lentiginous melanoma; NOS: not otherwise specified; spindle cell: spindle cell melanoma; SLN: (sentinel lymph node); SD: standard deviation.

regional recurrence, *BRAF* and *NRAS* mutations were equally distributed (Table II).

Patients with false-negative SLN (group-1) had a significantly ($p$<0.001) shorter disease-free-survival [median=24 months (95%CI=20-28 months] compared to patients with metastatic disease at distant sites (group-2; median=48 month (95%CI=32-63 months). LN metastases ($p$<0.001) and visceral metastases ($p$=0.016) occurred also significantly earlier in

Table II. *Comparison of patients with false-negative SLNs (group-1) to patients with PD at distant sites (group-2).*

| Variables | Group-1 n=61 | Group-2 n=48 | $p$-Value |
|---|---|---|---|
| Gender | | | |
| Female | 23 (37.70%) | 17 (35.42%) | 0.84 |
| Male | 38 (62.30%) | 31 (64.58%) | |
| Age at primary diagnosis | | | |
| Median | 59 | 62 | 0.87 |
| Mean (±SD/years) | 56.6 (±13.35) | 57.9 (±15.26) | |
| <60 | 31 (50.82%) | 23 (47.92%) | |
| >60 | 30 (49.18%) | 25 (52.08%) | |
| Type of melanoma | | | |
| NMM | 20 (32.79%) | 16 (33.33%) | 0.03 |
| SSM | 17 (27.87%) | 14 (29.17%) | |
| LMM | 0 (0%) | 5 (10.42%) | |
| ALM | 9 (14.75%) | 3 (6.25%) | |
| Spindle cell | 0 (0%) | 2 (4.17%) | |
| NOS | 15 (24.59%) | 8 (16.67%) | |
| Breslow (mm) (n=108) | | | |
| Mean (±SD/month) | 2.91 (±1.75) | 2.86 (± 1.61 | 0.89 |
| <1 mm | 3 (4.91%) | 5 (10.64%) | |
| 1.01-2 mm | 22 (36.06%) | 13 (27.66%) | |
| 2.01-4 mm | 24 (39.34%) | 18 (38.30%) | |
| >4 mm | 12 (19.67%) | 11 (23.40%) | |
| Site of primary | | | |
| Head | 12 (19.67%) | 13 (27.08%) | 0.048 |
| Back | 10 (16.39%) | 18 (37.5%) | |
| Abdomen/chest | 7 (11.47%) | 3 (6.25%) | |
| Upper extremities | 14 (22.95%) | 6 (12.5%) | |
| Lower extremities | 18 (29.51%) | 8 (16.67%) | |
| Site of SLN | | | |
| Head | 3 (4.92%) | 6 (12.5%) | 0.26 |
| Neck | 10 (16.39%) | 7 (14.58%) | |
| Axilla | 27 (44.26%) | 24 (50.00%) | |
| Inguine | 20 (32.79%) | 9 (18.75%) | |
| Number of visc. met | | | |
| 0 | 17 (27.87%) | 1 (2.08%) | <0.001 |
| 1 | 7 (11.47%) | 15 (31.25%) | |
| 2 or more | 37 (60.66%) | 32 (66.67%) | |
| Molecular analysis (n=59) | | | |
| *BRAF* mutation | 12 (37.5%) | 17 (62.96%) | 0.01 |
| *NRAS* mutation | 12 (37.5%) | 2 (7.41%) | |
| *C-KIT* mutation | 2 (6.25%) | 0 (0%) | |
| *BRAF*/*NRAS* wild-type | 6 (18.75%) | 8 (29.62%) | |
| Disease-free survival | | | |
| Mean (±SD/month) | 32.26 (± 25.83) | 57.79 (±36.1) | <0.001 |
| Median | 24 (CI 20-28) | 48.5 (CI 32-63) | |
| Occurrence of LN met | | | |
| Mean (±SD/month) | 32.16 (±26.08) | 68.45 (±41.36) | <0.001 |
| Median | 24 | 61 | |
| Occurrence of visc. met | | | |
| Mean (±SD/month) | 40.33 (±29.64) | 57.58 (±36.35) | 0.014 |
| Median | 34 | 48.5 | |
| Time from diagnosis to death (n=69) | | | |
| Mean (±SD/month) | 55.79 (±33.29) | 69 (±37.44) | 0.047 |
| Median | 44.5 | 57 | |

SLN: Sentinel lymph node; PD: progressive disease; NMM: nodular malignant melanoma; SMM: superficial spreading melanoma; LMM: lentigo maligna melanoma; ALM: acral lentiginous melanoma; NOS: not otherwise specified; spindle cell: spindle cell melanoma; LN met: lymph node metastases; visc. met: visceral metastases; g1=group-1; g2=group-2.

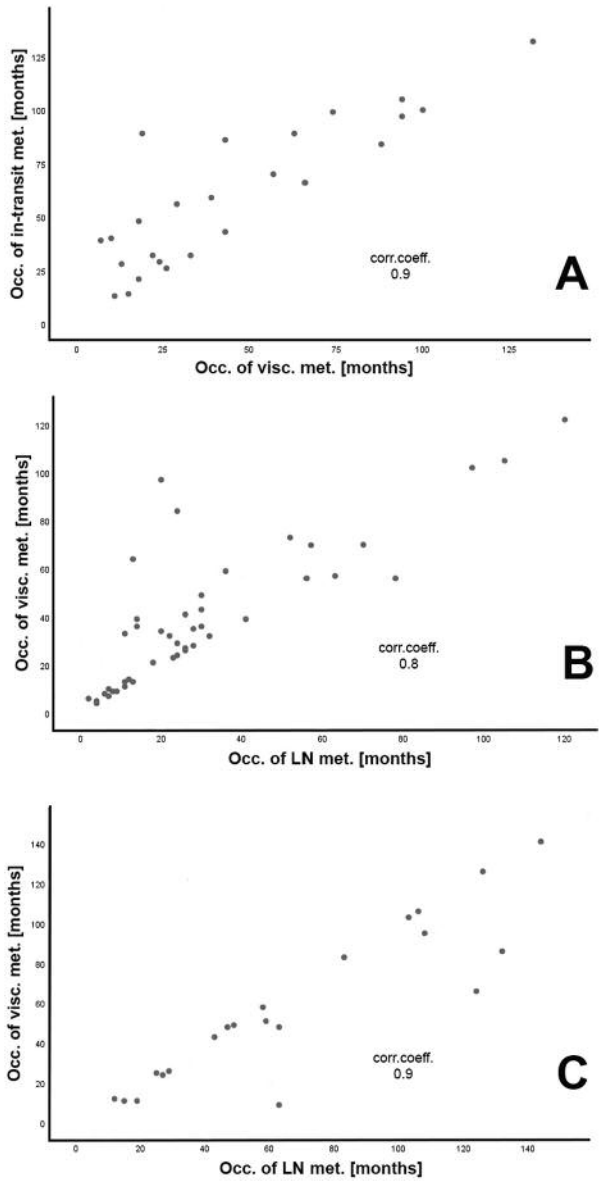


Figure 2. *Correlation plots between the occurrence of metastases at different sites over month. A: Correlation of the occurrence of in-transit metastases (in-transit met) and visceral metastases (visc. met.) in months. B: Correlation of the occurrence of lymph node (LN) metastases and visceral metastases (visc. met.) in patients with false-negative sentinel lymph nodes (group-1) in months. C: Correlation of the occurrence of lymph node (LN) metastases and visceral metastases (visc. met.) in patients with false-negative sentinel lymph nodes (group-2) in months.*

group-1 (Figure 3A-C). There was a significant correlation between the occurrence of LN and visceral metastases in both groups ($p$<0.001, corr. coeff. 0.8 group-1 and $p$<0.001, corr. coeff. 0.9 group-2). Twenty six of 61 (42.6%) with false-negative SLN and all patients with metastases at distant sites had already developed visceral metastases when LN metastases were diagnosed. In group-2, 12 patients developed LN metastases after visceral metastases (Figure 2B and C).

There was no significant difference in patient age at primary melanoma diagnosis or Breslow thickness among both groups.

*Survival of patients with tumour recurrence*. Patients treated between 1999 and 2017 received different therapies, including most recently immune-modulating and tumour-targeting therapies. Therefore, interpretation of survival data is limited. Survival information was available for 56 out of 61 (91.8%) patients with false-negative SLN (group-1) and 41 out of 48 (85.4%) patients with metastases at distant sites (group-2). Thirty-eight of 56 (67.8%) patients died with a median survival of 82 month (95%CI=73-91 months) in group-1, and 31 of 41 (75.6%) with a median survival of 83 month (95%CI=44-122 months) in group-2. Ten-year melanoma-specific survival was poor and equal to 28.3% and 25.9% for group-1 and group-2 patients, respectively. However, there was a significant difference in time between primary diagnosis and death between the two groups with longer survival in group-2 ($p$=0.047). In addition, patients with in-transit metastases had a significant better survival ($p$=0.037) (Figure 3D).

## Discussion

PD after negative SLN is a well-known problem (8-12, 17-20). In our single center study cohort of 1151 patients, we found 109 out of 890 negative SLNs (12.2%) with PD. This is in accordance with previously reported incidence rates, which range from 2% to 22,7% (8-12, 18). In contrast to other studies, we focused on tumour biology and progression in SLN-negative patients. Thereby, we differentiated between patients with false-negative SLNs (group-1) and those with recurrence at distant sites, with or without LN metastases (group-2). False-negative SLN included recurrence in the SLN-basin as well as recurrence in an initially missed or second SLN- basin. Between the two groups, we found significant differences in primary melanoma location, melanoma type, molecular type, disease-free-survival, occurrence of LN and visceral metastases. Melanomas without loco-regional LN metastases were mostly located on the back (37.5%) and head (27.08%). Head melanomas are well known as risk factors for PD in SLN-negative patients. Previous studies have suggested that this was most likely due to unpredictable drainage patterns in this anatomical region (12, 18, 21). In our study, the frequency of drainage to multiple SLN-basin was 23%, which is in accordance with a previous report (22). Interestingly, our study results demonstrate that 64.3% of melanomas on the back and 52% on the head with PD cannot be identified using SLNB because they are skipping regional LNs. This is in accordance with a previous study which reported primary melanomas located on the trunk

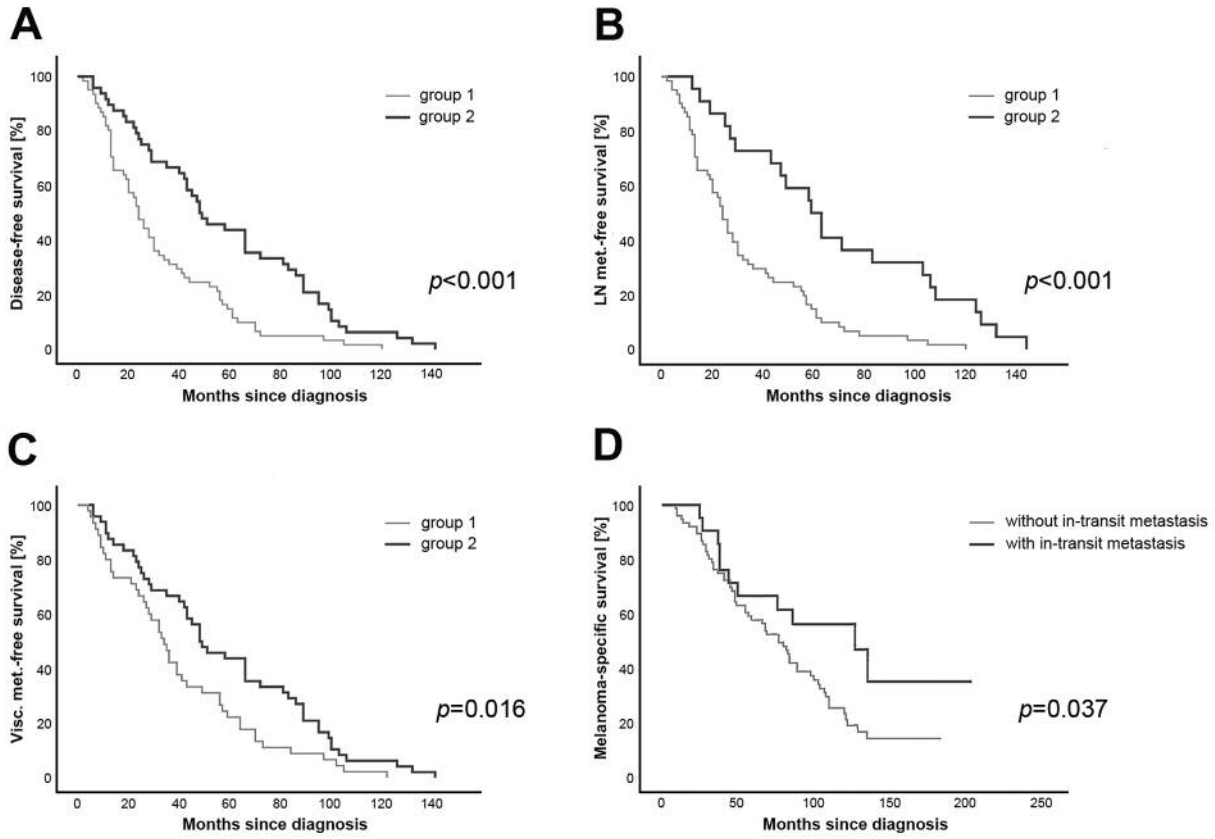


Figure 3. *Kaplan–Meier curves showing differences in metastatic-free and melanoma-specific survival. A: Kaplan–Meier curves of disease-free-survival in patients with false-negative SLNs (group-1; n=61) and patients with PD at distant sites (group-2; n=48). B: Kaplan–Meier curves of occurrence of lymph node metastases (LN met.) in patients with false-negative SLNs (group-1; n=61) and patients with PD at distant sites (group-2; n=22). C: Kaplan–Meier curves of occurrence of visceral metastases (visc.met.) in patients with false-negative SLNs (group-1; n=55) and patients with PD at distant sites (group-2; n=48). D: Kaplan–Meier curves of melanoma-specific-survival in patients with and without in-transit metastases.*

and head as significant predictors for distant recurrence in SLN-negative patients (13). In 14% of melanomas on the back, we found PD in an initially missed or second SLN-basin. Drainage to multiple SLN in truncal melanomas has also been previously reported (23).

Interestingly, patients with skipping LNs (group-2) had a significantly longer disease-free-survival; LN metastases and visceral metastases occurred later and time from primary diagnosis to death was longer (Figure 3A-C). These observations illustrate that these patients had longer dormant or slower spreading melanoma cells. The important role of immune surveillance in cancer development and progression is well known (24). Along those lines, immune-modulating medications have been developed and are now well-established and successful clinical therapies (25). The fact that tumor cells may be in equilibrium for a long time, as shown in our collective, especially in group-2, indicates that patients at high risk for metastatic disease might benefit from prophylactic immune-modulating therapy.

On a molecular basis, we found significantly more *BRAF* and less *NRAS* mutations in melanoma with PD at distant sites. This suggests their closer relationship to common nevi which also often harbor *BRAF* mutations (26).

Previous studies have reported divergent results regarding association of melanoma type and melanomas skipping regional LNs. Some authors have found an association to NMM or ALM, whereas others did not (11, 12, 18). In our study, we demonstrate a significant association to spindle cell melanomas and LMM. However, in the study by Savoia *et al*., which demonstrated no significant difference, three of four LMM patients also had PD at distant sites (11) .

In contrast to other patients with PD in multiple organs, all but one patient with spindle cell melanoma or LMM had single organ metastatic spread. Unexpectedly, single organ visceral metastases were significantly more frequent in the patient group with metastatic disease at distant sites (Table II). Extravascular-migratory-metastasis, rather than hematogenous spread, might be responsible for observed PD

with single organ involvement. Lugassy and Barnhill have shown the capacity of melanoma cells to migrate and spread along the abluminal vascular surface in a pericyte location without intravasation (27, 28). Together with these authors, we have previously shown extravascular dissemination of melanoma to the brain (29, 30).

Furthermore, the comparably high incidence (22.9%) of in-transit metastases in our cohort, as well as the survival benefit of this subgroup, despite simultaneous distant spread, might reflect the importance of extravascular dissemination as well. It should be mentioned that Rutkowski *et al*. have also reported high incidence of in transit metastasis (20.1%) in SLN-positive patients (31). However, these authors did not differentiate between in transit-metastases and local recurrence as we did. If we were to include local recurrence the incidence would amount to a percentage of 30.2%.

LN metastases of patients with PD at distant sites were detected simultaneously with visceral metastases in all cases of our cohort. LN metastases (mostly abdominal and mediastinal LNs) occurred sometimes even later than visceral metastases (Figure 2C). This is in accordance with the finding of lymphangiogenesis in visceral metastases leading to regional LN metastases (32).

Twenty-six of 61 (42.6%) patients with false-negative SLN already presented with stage IV PD (Figure 2B) and 38 (68%) died after median survival of 82 months (CI, 73-91 months). This confirms the already reported worse prognosis of false-negative SLN patients compared to positive SLN patients (10, 17). This fatal outcome is mostly due to delayed detection of LN metastases using ultrasound. A previous multicenter study has shown the superiority of SLN biopsy over ultrasound in detecting small tumour loads – the mean detectable size is 0.13 $mm^2$ for SLN biopsy, *vs*. 6.84 $mm^2$ for ultrasound (33).

Ten-year melanoma-specific survival was poor and almost equal in patients with false-negative SLNs (28.3%) and those with metastases at distant sites (25.9%). These data reflect the need of adjuvant therapy in this high-risk patient group with PD after negative SLNB.

In conclusion, we demonstrate the multifactorial components, including melanoma subtype and molecular type, second lymph node basin and direct distant spread, contributing to PD in SLN-negative patients. We show the difficulty in detecting PD in patients with false-negative SLNs at an early stage. Our data with long follow-up also mirrors the dormant state of melanoma cells indicating the importance of immune surveillance in melanoma. Last but not least, extravascular-migratory-metastasis appears to have an important role in melanoma as suggested by the PD with single organ involvement.

The strength of this study is that the data consist of a single center study. All patients had histologically confirmed melanoma metastases, identical SLNB and equal follow up. In addition, there was a long follow up. The weakness of the study is that the data set is too small to perform multivariate analysis. In addition, patients received different therapies therefore survival analysis is limited.

## Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

## Authors' Contributions

Designed study: DM. Collected data: AC. Provided clinical data: MR, DH, JM, MH, NL, DM. Analyzed data: AC, DM. Drafted the article: AC, DM. Reviewed and revised the article: DH, MW, MH, RLB, CL. All Authors read and approved the final article.

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