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Clinical value of combined determination of plasma L-DOPA/tyrosine ratio, S100B, MIA and LDH in melanoma

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ABSTRACT

Aim of the study: L-DOPA/tyrosine ratio (an index of tyrosinase activity), melanoma antigens S100B and MIA, lactate dehydrogenase (LDH) and their combinations were evaluated for clinical value as tumour markers in melanoma.

Methods: Blood samples were obtained in 170 melanoma patients (stage I–II: $n = 57$, III: $n = 54$, IV: $n = 59$) at inclusion and in a sub-group of 82 subjects during follow-up for up to 4 years. Laboratory analyses were performed by HPLC (L-DOPA, L-tyrosine), immunoassays (S100B, MIA) and colourimetry (LDH).

Results: All markers, except LDH, were elevated in stage IV versus other stages. S100B and MIA highly correlated, especially in stage IV (r_s : 0.849, $p < 0.001$). The combination of L-DOPA/tyrosine ratio with S100B displayed the highest sensitivity/specificity (73/70%) to confirm stage III–IV or stage IV alone (69/75%) (ROC optimised cut-off). Only the L-DOPA/tyrosine ratio significantly increased (+36% over 5 months, $p = 0.001$) during progression from stage I–III to higher stages. S100B, MIA and LDH, but not the L-DOPA/tyrosine ratio, responded to progression towards death in stage IV. All markers exhibited a prognostic value in deceased patients ($n = 44$); S100B and MIA were the best predictors of survival time by Cox proportional-hazards regression.

Conclusion: The combination of plasma L-DOPA/tyrosine ratio and S100B appears an attractive approach for the biological follow-up of melanoma patients.

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1. Introduction

A wide range of molecules has been investigated for potential use as serologic tumour marker in melanoma. They all have

limited roles in screening and early diagnosis due to limited sensitivity.¹ In metastasised disease, however, specific mRNAs (like tyrosinase mRNA),² antigens S100B and MIA (Melanoma Inhibitory Activity protein)^{3–8} and metabolites of the melano-

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genesis pathway (like 5-S-Cysteinyldopa and L-DOPA)^{2,9–14} have demonstrated a correlation with tumour burden, successful therapy, disease progression and/or prognosis. In stage IV,⁷ however, S100B and MIA seem to provide no additional useful information to the non-specific lactate dehydrogenase (LDH), with respect to clinical outcome. This marker of cell death has been introduced in the revised AJCC melanoma TNM staging system (M3: elevated serum LDH).^{15,16}

The aim of this prospective study was to evaluate four serologic markers for clinical value in melanoma: the L-DOPA/tyrosine ratio, melanoma antigens S100B and MIA, the non-specific LDH, and their different combinations. Their concentrations were measured in the plasma of 170 stage I–IV melanoma patients at inclusion and for a sub-group of them ($n = 82$) during follow-up for up to 4 years. Results are interpreted with regard to disease stage and progression, presence and number of metastases, and survival.

2. Material and methods

2.1. Patients and blood sampling

A group of 170 melanoma patients (91 males, 79 females, median age: 58 years, 25th–75th percentiles: 50–69 years) diagnosed between 01/2000 and 12/2004 in the dermatology departments of Saint-Louis hospital (Paris, France) prospectively entered this study. Exclusion criteria were: absence of staging at inclusion, haemolysed blood samples, and one or more serologic tumour marker missing.

Staging was performed using the tumour-node-metastasis (TNM) system of the American Joint Committee on Cancer Classification (AJCC).¹⁵ At inclusion, there was 57 stage I–II ‘localised melanoma’ (T1–4N0M0), 54 stage III ‘regional metastases’ (anyTN1,2M0), and 59 stage IV ‘distant metastases’ (anyT,anyN,anyM). In 35 stage IV patients, the number and anatomic localisation of metastases was clearly identified by imaging techniques. A blood sample was drawn by venous puncture in 7 mL glass tubes with lithium heparinate as an anticoagulant (Becton Dickinson, Meylan, France). Blood was centrifuged (3000g for 10 min at +4 °C) and plasma separated in two aliquots stored at –80 °C before analysis.

Patients were treated by tumour excision, lymphadenectomy, immunotherapy (interferon) or chemotherapy according to their disease stage and progression.¹⁷ Follow-up (for up to 4 years) was obtained in 82 subjects including clinical examination, abdominal ultrasounds, chest X-rays, CT-scans, standard chemistry blood tests and tumour markers (total number of blood samples: $n = 331$). In stage I–III, disease progression was defined as a move to higher stage(s); impact on tumour markers was evaluated within the shortest blood sampling interval available (median: 150 days, 25th–75th percentiles: 106–245). Patients receiving treatments at the time of sampling (chemotherapy: $n = 7$, immunotherapy: $n = 1$) were excluded from this analysis. Marker levels in stable disease (no change from initial staging) were assessed within the longest sampling interval (329 days, 147–523). In stage IV, influence of disease progression and stability on serologic markers was evaluated using the last two blood samples before death (interval: 84 days, 28–284) and the longest preceding period within this stage (99 days, 39–312), respectively. A

total of 44 patients deceased from melanoma within the study period (survival time from inclusion: 260 days, 55–429).

This study was in accordance with the ethical standards of the Helsinki declaration of 1975 as revised in 1983; an informed consent was obtained from each patient.

2.2. Serologic marker analysis

Within 2 months from sampling, analysis were performed in a blind fashion between the two laboratories measuring L-DOPA, L-tyrosine, LDH, and melanoma antigens (S100B, MIA), respectively.

2.2.1. L-DOPA and L-tyrosine

Serologic L-DOPA and L-tyrosine analysis have been previously developed in our laboratory.¹³ Briefly, 1 mL of plasma is treated by alumina extraction for L-DOPA and 1 mL deproteinised by 1 M trichloroacetic acid for L-tyrosine. Separation is obtained by HPLC (515 HPLC pump, Waters, Milford, USA) on a C₁₈ reversed-phase analytical column (150 × 4 mm internal diameter) filled with 5 µm Lichrospher particles (Merck, Darmstadt, Germany). L-DOPA is measured using a 5100 A coulometric electrochemical detector equipped with an analytical cell operating in oxidative mode (potential set at +0.35V) (ESA, Bedford, USA). L-tyrosine is measured using a RF 535 fluorimetric detector (Shimadzu, Kyoto, Japan) (excitation at 275 nm, emission at 305 nm). All procedures are carried out at room temperature with a total analysis time of about 3 h. Method precision was: CV <2.5% (intra-assay) and <4.6% (inter-assay) for L-DOPA and L-tyrosine with a detection limit of 0.25 nM for L-DOPA and 2.5 nM for L-tyrosine.¹³ The L-DOPA/tyrosine ratio is calculated for each sample and the upper normal cut-off derived from a group of 35 healthy subjects is 16.0×10^{-5} .¹³

2.2.2. Melanoma antigens

S100B plasma concentration was measured using the monoclonal two-site immunoluminometric assay LIA-mat® Sangtec® 100 (Sangtec Medical, Stockholm, Sweden) on a Berilux 400® analyser (Dade-Behring, Eschborn, Germany).^{12,14} According to the manufacturer, the detection limit is 0.02 µg/L, the calibration curve is linear until 20 µg/L and the upper normal cut-off is 0.12 µg/L.

Melanoma Inhibitory Activity (MIA) protein was measured in plasma using a quantitative ELISA kit (Roche Diagnostics, Mannheim, Germany). Absorbance was measured in duplicates at 405 nm on a microtitre plate reader. According to the manufacturer, the detection limit is 0.5 µg/L, the calibration curve is linear until 50 µg/L and the upper normal cut-off is 7.5 µg/L.

2.2.3. Lactate dehydrogenase

LDH activity was measured by a colourimetric assay adapted on a Modular® multi-parametric analytical system (Roche Diagnostics, Meylan, France). According to the manufacturer, the upper normal cut-off is 439 UI/L.

2.3. Statistical analysis

Statistical analyses were conducted with Sigmapstat® (Jandel Scientific, San Jose, USA) and Medcalc® (Medcalc, Mariakerke,

Belgium) software. Results are presented as median and 25th–75th percentiles (non-Gaussian distribution of data by Kolmogorov–Smirnov test). Extreme values were present for all serologic markers (e.g. up to $n = 36$ > upper quartile plus 1.5 times the inter-quartile range for MIA, at inclusion). A non-parametric approach was selected to evaluate marker correlation (Spearman rank order test), influence of disease stage (Kruskal–Wallis one way analysis of variance) and status (progressive versus stable) (Wilcoxon signed-rank test) on plasma marker levels. Their efficacy in confirming the presence of metastases was evaluated by ROC analysis; the area under curve (AUC) and marker sensitivity/specificity were calculated. Marker prognostic value was tested by linear regression on log-transformed data (marker levels, survival time) and multivariate Cox proportional-hazards regression. A p value less than 0.05 was considered statistically significant for all tests.

3. Results

3.1. At inclusion

Serologic markers, except LDH, were significantly elevated in stage IV versus other stages (Table 1). The highest correlations were observed in stage IV: r_s : 0.849 for S100B with MIA, 0.679–0.694 for LDH with melanoma antigens, and 0.489–0.605 for the L-DOPA/tyrosine ratio with other markers ($n = 170$, $p < 0.001$ for all markers).

Marker efficacy to confirm the presence of ‘regional or distant’ metastases (stage III–IV) is presented in Fig. 1 and Table 2. Using ROC optimised cut-offs, L-DOPA/tyrosine ratio sensitivity/specificity was 73/70% when combined with S100B versus 69/69% with LDH and 69/68% with MIA. It was 74/68% with the addition of LDH (three markers); there was no gain with MIA in a three or four marker combination. For ‘distant’ metastases (stage IV), sensitivity ranged from 39% (LDH) up to 56% (L-DOPA/tyrosine ratio) (specificity: 81–93%). The combination of L-DOPA/tyrosine ratio (ROC optimised cut-off: 20.1×10^{-5}) and S100B (0.40 $\mu\text{g/L}$) reached a 69% sensitivity (with 75% specificity) versus 58% with MIA or 53% with LDH. Further adding MIA and/or LDH (three or four markers) added up to 3% to sensitivity, but decreased specificity by 6%.

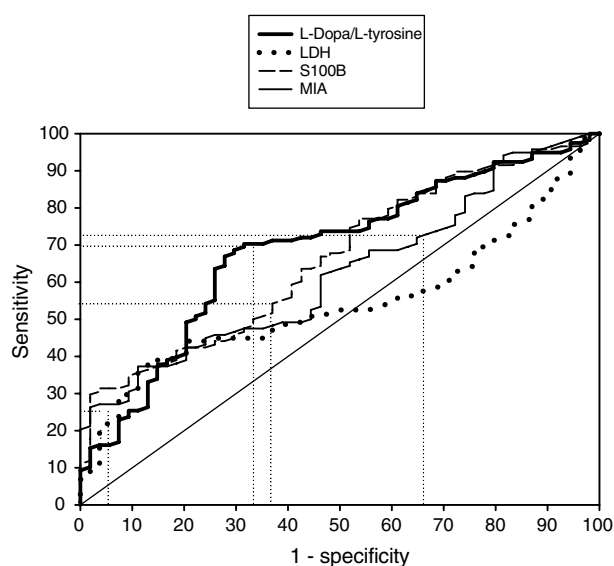


Fig. 1 – Plasma marker efficacy to confirm stage III–IV at inclusion. A total of 113 patients were classified as having ‘regional or distant’ metastases (54 stage III, 59 stage IV); 57 had ‘localised’ (stage I–II) melanoma (1992 AJCC staging system). ROC calculated AUC was 0.706 for S100B ($p < 0.001$), 0.686 for the L-DOPA/tyrosine ratio ($p < 0.001$), 0.619 for MIA ($p < 0.01$) and 0.555 for LDH ($p = 0.231$, NS). Manufacturer (LDH, S100B, MIA) or previously established cut-offs (L-DOPA/tyrosine ratio)⁹ are indicated by dotted lines.

3.2. During follow-up

In stage I–III, disease stability (17 stage I–II, 16 stage III patients) was associated with no significant change for MIA (+5%), L-DOPA/tyrosine ratio (+4%), and S100B (–22%); LDH levels increased by +9% ($p < 0.001$). Disease progression (11 stage I–II, 7 stage III patients) increased the L-DOPA/tyrosine ratio by +35.7% (15.7 to 21.3×10^{-5} , $p = 0.001$), but not other marker levels (MIA: +18%, S100B: –20%, LDH: –9.7%; all NS).

In stage IV, disease stability was associated with no significant change in all marker levels (Table 3). Progression significantly increased S100B, MIA and LDH, but not the L-DOPA/tyrosine ratio (+6%, NS). S100B and MIA were significantly

Table 1 – Marker plasma concentrations at patient inclusion

	AJCC melanoma stage		
	I–II ($n = 57$)	III ($n = 54$)	IV ($n = 59$)
L-DOPA/tyrosine Ratio $\times 10^{-5}$	15.6	17.5	21.4*
($N: <16.0$)	(13.0–17.7)	(15.1–19.6)	(15.8–33.7)
LDH	303	299	351
($N: <439$ UI/L)	(271–337)	(254–375)	(186–777)
S100B	0.07	0.10	0.42*
($N: <0.12$ $\mu\text{g/L}$)	(<0.02 –0.16)	(0.04–0.27)	(0.11–6.31)
MIA	8.6	8.4	15.3*
($N: <7.5$ $\mu\text{g/L}$)	(6.9–10.7)	(6.8–11.7)	(8.3–51.5)

Results are presented as median and 25th–75th percentiles in brackets. Normal upper cut-off: manufacturer for LDH, S100B and MIA, Ref. [9] for the L-DOPA/tyrosine ratio.

* Significantly different from AJCC stage I–II and stage III at $p < 0.001$ by Kruskal–Wallis one-way analysis of variance followed by the Dunn’s test.

Table 2 – Efficacy of plasma markers to confirm stage III–IV at patient inclusion

Cut-off	L-DOPA/tyrosine ratio $\times 10^{-5}$	LDH	S100B	MIA
Established ^a	16.0	439 UI/L	0.12 $\mu\text{g/L}$	7.5 $\mu\text{g/L}$
Sensitivity	69.0% (59.6–77.4)	25.7% (17.9–34.7)	55.8% (46.1–65.1)	71.7% (62.4–79.8)
Specificity	66.7% (52.9–78.6)	96.5% (87.9–99.5)	64.9% (51.1–77.1)	33.3% (21.4–47.1)
ROC optimised	16.5	353 UI/L	0.33 $\mu\text{g/L}$	20.3 $\mu\text{g/L}$
Sensitivity	64.6% (55.0–73.4)	41.6% (32.4–51.2)	36.3% (27.4–45.9)	28.3% (20.2–37.6)
Specificity	71.9% (58.5–83.0)	84.2% (72.1–92.5)	96.5% (87.9–99.5)	98.2% (90.6–99.7)

a By the manufacturer (LDH, S100B, MIA) or from Ref. [9] (L-DOPA/tyrosine ratio). The calculated ROC optimised cut-off corresponds to the level with the highest accuracy (sensitivity/specificity) to confirm stage III–IV (Fig. 1); 95% confidence intervals are in brackets.

Table 3 – Marker plasma levels during follow-up in stage IV patients

	Stable disease (n = 21)		Progressive disease (n = 16)	
	First sampling	Last sampling	First sampling	Last sampling
L-DOPA/tyrosine ratio $\times 10^{-5}$	22.8 (N: <20.1)> (15.2–26.1)>	23.4 (15.8–29.9)	19.5 (15.6–33.6)	20.7 (16.9–48.0)
LDH>	26 1 (N: <438 UI/L) (163–368)	335 (172–408)	310 (152–384)	407* (310–467)
S100B	0.19 (N: <0.40 $\mu\text{g/L}$) (0.11–0.24)	0.26 (0.07–1.25)	0.16 (0.05–1.17)	1.28** (0.09–3.35)
MIA	11.8 (N: <14.6 $\mu\text{g/L}$) (7.9–26.3)	10.6 (7.3–28.4)	9.6 (7.6–26.3)	16.5* (9.1–83.0)

Results are presented as median and 25th–75th percentiles in brackets. All normal upper cut-offs were ROC optimised for stage IV. Significantly different from the first sampling level at $p < 0.05^*$ or $p < 0.01^{**}$ by the Wilcoxon signed-rank test.

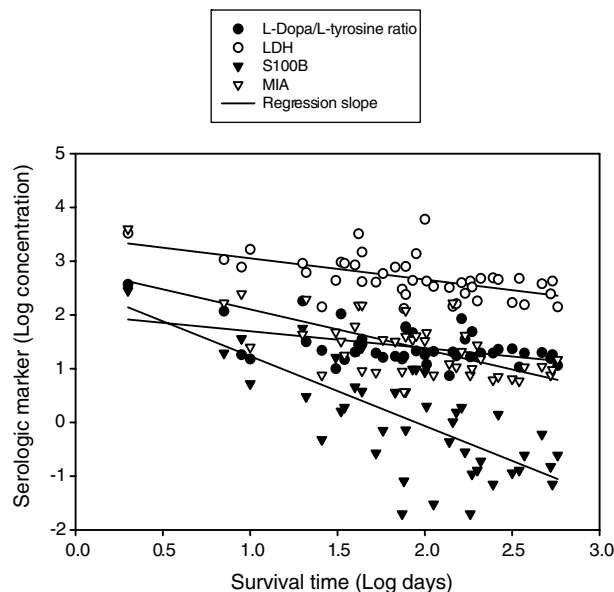


Fig. 2 – Plasma markers levels and survival time. A linear regression on log-transformed data (survival time, marker level) was performed in patients deceased from melanoma ($n = 44$). Survival time could be predicted from marker plasma levels (last blood sample) as follows: Log survival time (days) = $2.0096 - 0.3134 \text{ Log L-DOPA/tyrosine ratio}$, $r: 0.496$ ($p = 0.001$); $= 3.4252 - 0.3969 \text{ Log LDH}$, $r: 0.558$ ($p < 0.001$); $= 2.5304 - 1.2983 \text{ Log S100B}$, $r: 0.712$ ($p < 0.001$); $= 2.8501 - 0.7464 \text{ Log MIA}$, $r: 0.684$ ($p < 0.001$).

higher in stage IV patients with two or more metastases versus a single metastasis (+550% and +138%, respectively, $p < 0.05$).

In deceased patients ($n = 44$), all markers (last blood sample value) correlated with survival time (median: 87 days, 41–194) (Fig. 2). Patients with four markers elevated had significantly shorter survival (median: 45 days; 20–78) than those with two (180 days; 134–238) or only one marker (253 days;

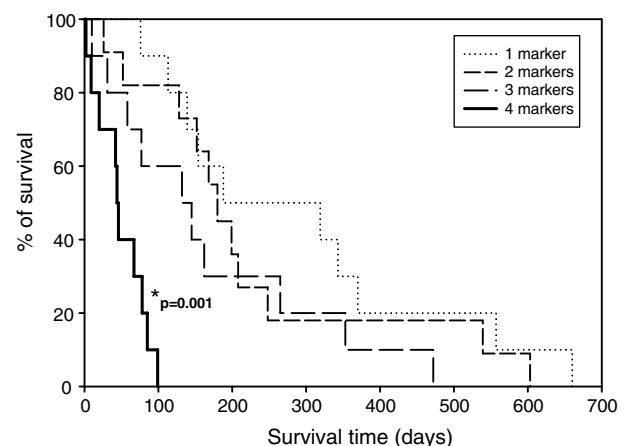


Fig. 3 – Number of elevated plasma markers and survival time. In deceased patients ($n = 44$), there was $n = 3$ with no marker elevated (last blood sample), $n = 10$ with one marker elevated, $n = 11$ with two markers elevated, $n = 10$ with three markers elevated and $n = 10$ with all four markers elevated above stage III–IV ROC optimised cut-offs (see Table 2).

139–370) elevated (Fig. 3). In the final Cox regression model, S100B and MIA were the two survival predictors (respectively $\exp(b) = 2.436$; $p < 0.01$ and $\exp(b) = 2.6159$, $p < 0.05$).

4. Discussion

Screening, early diagnosis and staging of melanoma cannot be reliably obtained by means of current tumour markers mainly due to poor sensitivity.¹ Some of these markers also lack specificity for melanoma. LDH blood levels reflect cell turnover in other malignancies and are elevated in a large variety of tissue injuries (such as hepatitis or myocardial infarction). Serum MIA is elevated in the blood of pregnant women (>38th week) and before the age of 17 years.¹⁸ Serum S100B has been proposed as a marker for central nervous system damage.¹⁹ Elevated serum levels of these two antigens have also been observed in up to 35% of patients with advanced gastrointestinal tumours.²⁰ The L-DOPA/tyrosine never exceeded 16.3×10^{-5} in a group of 17 patients with non-melanoma metastasised cancers (mainly breast cancer).⁹ However, as L-DOPA and other melanin precursors are implicated in normal melanogenesis,²¹ elevated levels may be found in other pigmentation disorders. For example, an extreme value of 184.8×10^{-5} was observed in a case of Dubreuilh melanosis with extensive cutaneous mass.² Hypermelanosis may also be associated with some genetic or endocrine disorders, as well as treatments with chlorpromazine or antimalarial drugs.

A serologic tumour marker would be useful in melanoma if able to suggest or confirm the presence of distant (stage IV) or even regional metastases (stage III). Despite some correlation with melanoma markers (especially S100B and MIA), LDH levels did not reflect melanoma stage. LDH ability to confirm the presence of metastases was poor, as reflected by its non significant ROC AUC and low sensitivity/specificity. All other markers were elevated in stage IV, but not in stage III (up to +12% increase versus stage I–II for the L-DOPA/tyrosine ratio, NS). For ‘distant’ metastases, they displayed a similar ~50% sensitivity with 80–90% specificity, thus unsuitable for clinical use as ‘stand-alone’ markers. For the detection of ‘regional or distant’ metastases (stage III–IV), the L-DOPA/tyrosine ratio possessed the highest and clinically relevant marker efficacy regardless of the cut-off selected (ROC optimised and previously published cut-offs were almost identical). By contrast, melanoma antigens (especially MIA) displayed poor specificity using their manufacturer established cut-off. A ~3 time increase of the cut-off by ROC optimisation allowed a ~95% specificity, but at the expense of sensitivity (<36%). Overall, melanoma antigens S100B and MIA appear not superior to LDH to detect melanoma metastases, at least when used as ‘stand-alone’ markers.

Combining markers with different biological behaviour (production/release, kinetics, or half-lives) and/or sensitivity/specificity could represent an attractive way to enhance metastasis detection. In a stepwise approach towards a combination suitable for stage III–IV, the L-DOPA/tyrosine ratio was first selected based on its higher efficacy and limited correlation with other markers. Adding S100B at the 0.33 µg/L ROC cut-off led to the most effective pair of marker with nine ‘false negative’ patients detected (five stage III, four stage IV) at the expense of only one ‘false positive’ (a stage II patient

with 7.0 µg/L). S100B also confirmed L-DOPA/tyrosine ratio ‘true positive’ results in 41% of cases. Further, adding LDH (three markers) detected two additional patients, but added one ‘false positive’. MIA was not superior to LDH and all new cases were detected by S100B. For distant metastases (stage IV), the L-DOPA/tyrosine ratio and S100B was a similarly effective combination when using adapted higher ROC cut-offs (20.1×10^{-5} and 0.40 µg/L). As for stage III–IV, further adding LDH and/or MIA was not associated with improved overall efficacy (data not shown).

A significant sub-group, stage III–IV patients, still remained ‘false negative’ even with the use of a four marker combination ($n = 29$, 26%). Limited sensitivity clearly appears as a significant limit of current marker combinations, even in advanced melanoma.¹ But should ‘false positives’ ($n = 16$ stage I–II patients with the L-DOPA/tyrosine ratio, $n = 1$ with S100B) be more closely followed and/or candidates for alternative or more aggressive therapies? In this sub-group of patients, the 22.1×10^{-5} median level of plasma L-DOPA/tyrosine ratio was within the range of those with distant metastases. For example, a 12 years stage I patient (female, melanoma of the left foot, Breslow score: 5 mm) had highly elevated L-DOPA/tyrosine ratio at inclusion (29.4×10^{-5}). She progressed to stage II 13 month later (33.8×10^{-5}) and to stage IV with bone metastasis 4 months later (62.0×10^{-5}). The patient with elevated S100B also had a particularly high marker concentration (7.0 µg/L). In a previous study,¹⁴ there was a +561% difference in S100B levels between patients with $n \leq 2$ metastases and those with $n \geq 3$ metastases. S100B here possesses the most explosive response to widespread disease (>+500%), but with extreme variability. Interestingly, the three most elevated L-DOPA/tyrosine ratios (311×10^{-5} , 195×10^{-5} , 181×10^{-5}) were observed in the presence of liver metastases, a phenomenon previously reported.^{9,12} It remains, however, elusive to rationally use any of these markers to evaluate the number of visceral metastases and/or their organ specificity.

Serial determination of tumour marker has gained wide acceptance in the follow-up of various malignancies, such as prostate, colorectal, breast, or lung cancers. In melanoma, serum L-DOPA/tyrosine ratio (but not S100B) was found significantly higher in progressive stage III patients (21.7×10^{-5}) than in stable ones (12.0×10^{-5}).¹⁴ Our results support this observation with a more than 30% increase over 5 months in stage I–III patients progressing to higher stages (no change in stable disease). If the lack of response of LDH was expected, S100B and MIA were equally insensitive at these early stages (mostly stage I–II). It could be hypothesised that the L-DOPA/tyrosine ratio more precociously rises than melanoma antigens in the course of melanoma. If some controversy exists on whether L-DOPA is formed from L-tyrosine hydroxylation or via dopaquinone reduction,²¹ plasma L-DOPA/tyrosine ratio likely reflects the metabolic activity of the melanocyte mass at the time of sampling.^{2,9,13,14} By contrast, melanoma antigens and LDH are released in blood following cell death predominant in advanced disease and during aggressive chemotherapy. For example, we found that serum S100B (but not the L-DOPA/tyrosine ratio) was higher in progressive stage IV patients (1.56 µg/L) than in stable ones (0.23 µg/L).¹⁴ Using repeated serial blood sampling,¹² S100B concentration was, however, found to strongly vary over time. This phenom-

enon has been attributed by others to a short ~30 min half-life.²² In the present study, MIA and LDH also responded to progression towards death, whereas the L-DOPA/tyrosine ratio did not.

Evaluation of prognosis in melanoma patients mostly relies on histopathology and clinical presentation. The L-DOPA/tyrosine ratio, S100B, MIA and LDH have all demonstrated some prognostic value in recent studies.^{4,5,8,14,23–25} Again, they correlated with survival time (median: 3 months) in our group of deceased patients (34 stage IV, seven stage III, three stage I–II). Prognosis was also dramatically worsened if all four markers were elevated over their stage III–IV ROC cut-off. Using a multivariate analysis (Cox regression), survival time was significantly predicted by S100B and MIA in the final model. As previously observed,^{2,14} elevated values of the L-DOPA/tyrosine ratio can be observed at distance from death (e.g. 41.8×10^{-5} in a stage IV patient treated by interferon 696 days before death, all other markers within normal range), but also low values shortly before death (15.2×10^{-5} in a stage IV patient 10 days before death, but S100B: 5.3 µg/L, MIA: 25.3 µg/L, LDH: 1671 UI/L).

5. Conclusion

The combination of plasma L-DOPA/tyrosine ratio and S100B appears an attractive approach for the biological management of melanoma. It could confirm or suggest the presence of local and/or distant metastases, help monitor disease progression (at early stages: L-DOPA/tyrosine ratio; advanced disease: S100B) and predict survival time.

Conflict of interest statement

None declared.

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