

Original Investigation

Potential Role of Soluble c-Met as a New Candidate Biomarker of Metastatic Uveal Melanoma

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IMPORTANCE Conventional melanoma serum biomarkers (S100 and lactate dehydrogenase [LDH]) perform poorly in patients with uveal melanoma, and the search for new biomarkers is needed. A high expression of the oncoprotein c-Met in primary uveal melanoma is associated with metastatic progression, and c-Met is released as a soluble ectodomain through ADAM10- and ADAM17-mediated cleavage, suggesting a possible role as biomarker.

OBJECTIVE To determine the potential role of soluble c-Met (sc-Met) as a biomarker of uveal melanoma progression in comparison with S100 and LDH.

DESIGN, SETTING, AND PARTICIPANTS Soluble c-Met was studied in the conditioned medium of 9 uveal melanoma cell lines and in the blood serum samples of 24 mice with uveal melanoma xenografts, 57 patients with uveal melanoma (17 patients whose tumors metastasized and 40 patients whose tumors did not metastasize), and 37 healthy donors. We collected blood samples for as long as 5 years after treatment of the primary tumor. The concentration of sc-Met was measured using enzyme-linked immunosorbent assays, and the receiver operating characteristic curve was used to evaluate sensitivity and specificity in the identification of metastatic uveal melanoma. The study began on May 2, 2011, and the last samples were collected in January 2015

MAIN OUTCOMES AND MEASURES Levels of sc-Met in uveal melanoma cell cultures and in the blood serum samples of xenotransplanted mice, of healthy donors, and of patients with uveal melanoma during follow-up.

RESULTS The conditioned medium of uveal melanoma cell lines and the blood serum samples of mice with uveal melanoma xenografts contained significant levels of sc-Met. Patients with metastatic disease had significantly higher serum levels of sc-Met (median level, 590 ng/mL [range, 246-12 856 ng/mL]) than did patients without metastatic disease (median level, 296 ng/mL [range, 201-469 ng/mL]) ($P < .001$) and healthy donors (median level, 285 ng/mL [range 65-463 ng/mL]) ($P < .001$). Analysis of receiver operating characteristic curves for sc-Met levels in patients with nonmetastatic uveal melanoma vs patients with metastatic uveal melanoma yielded an area under the curve of 0.82 (95% CI, 0.68-0.95) ($P < .001$), which was superior to the areas under the curve achieved with S100 or LDH markers. Patients with progressive metastatic disease showed further increases in sc-Met level, whereas stable patients did not.

CONCLUSIONS AND RELEVANCE The present pilot study suggests that sc-Met should be further exploited as a biomarker for monitoring of uveal melanoma.

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Uveal melanoma is the most common primary tumor of the adult eye, with an incidence of 2 to 8 cases per million per year.¹ Despite excellent rates of local control of the primary tumor, the survival rate of patients with uveal melanoma is not good, with a mortality rate of up to 50%.¹ Indeed, metastatic spread to the liver, the preferential site of metastasis,² is followed by death for 80% of the patients within 1 year.³ Although there is no standard of care for patients with metastatic disease, new targeted therapies are presently under investigation,⁴ and local therapy of the liver seems to significantly improve survival, in the presence of a limited number of metastatic lesions.^{5,6} However, current screening methods, including liver function tests and ultrasonography, do not identify metastases until the tumor burden is already large, with widespread development of liver metastases. Some liver function test results,^{7,8} an increase in serum levels of S100,^{9,10} tissue polypeptide-specific antigen,¹¹ growth differentiation factor 15,¹² osteopontin,^{13,14} melanoma inhibitory antigen,¹⁵ vascular endothelial growth factor,^{14,16} and DJ-1,^{17,18} and a decrease in serum levels of insulinlike growth factor 1¹⁹ have been correlated with the development of metastasis. However, there is still a need for a good biomarker that indicates the presence of early metastasis of uveal melanoma.

The hepatocyte growth factor receptor c-Met is highly expressed in patients with uveal melanoma, and it plays an important role in tumor growth and invasion.²⁰⁻²⁴ We recently reported that surface c-Met is cleaved by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and ADAM17 and is highly expressed in patients with uveal melanoma, and that the c-MET ectodomain is released by uveal melanoma cell lines in vitro.²⁵ Moreover, both c-Met and ADAM10 messenger RNA and proteins are highly expressed in prognostically bad primary uveal melanoma. On the basis of these data, we hypothesize that during the progression of uveal melanoma to metastatic disease, an elevated level of soluble c-Met (sc-Met) may be released into the serum. Therefore, the present study analyzes the potential role of sc-Met as a biomarker of metastatic uveal melanoma.

Methods

Cell Cultures

The human uveal melanoma cell lines MEL270, OMM2.5, MEL202,²⁶ 92.1,²⁷ OMM1,²⁸ OCM1,²⁹ MEL15675,³⁰ UPMM2,³¹ and UPMM3³¹ were cultured in Roswell Park Memorial Institute 1640 medium (Gibco/Life Technologies), supplemented with 10% fetal bovine serum.

Patients and Serum Samples

Forty patients with nonmetastatic uveal melanoma, 17 patients with metastatic uveal melanoma, and 37 healthy donors were used in our study. The participants in our study provided written informed consent. All procedures were performed according to the guidelines agreed on by the ethics committee of the Istituto di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliera Universitaria San Martino-Istituto Nazionale per la Ricerca sul Cancro in Genoa, Italy, and

At a Glance

- This pilot study explores the potential role of soluble c-Met (sc-Met) as a serum biomarker of metastatic uveal melanoma.
- Patients with metastatic uveal melanoma had significantly higher serum levels of sc-Met than did patients with nonmetastatic uveal melanoma and healthy donors.
- Analyses of receiver operating characteristic curves suggest that sc-Met may perform better than S100 and lactate dehydrogenase as a biomarker of metastatic uveal melanoma.
- These data suggest that for the follow-up of patients with uveal melanoma, sc-Met is a potential biomarker for metastatic uveal melanoma and should be further tested for the monitoring of uveal melanoma in prospective studies.

are consistent with the principles established by the latest revision of the Declaration of Helsinki (World Medical Association Declaration of Helsinki; ethical principles for medical research involving human subjects).

Blood samples were kept at 37°C for 30 minutes, then centrifuged at 300g for 10 minutes, and serum aliquots were kept at -80°C. A second serum sample was obtained during follow-up from 8 of the 40 patients with nonmetastatic uveal melanoma and from 5 of the 17 patients with metastatic uveal melanoma, based on clinical evidence of disease progression. Each patient underwent a clinical examination, a liver function test, and abdominal ultrasonography every year to determine whether the liver was metastasized. The median follow-up was 60 months (range, 6-324 months) for the patients with nonmetastatic uveal melanoma and 48.5 months (range, 10-189 months) for the patients with metastatic uveal melanoma. The median survival time of patients with metastatic uveal melanoma was 10.5 months from diagnosis of metastasis. Of the 17 patients with metastatic uveal melanoma, 13 were men and 4 were women, and the median age was 66 years. Of the 40 patients with nonmetastatic uveal melanoma, 26 were men and 14 were women, and the median age was 64.1 years. Of the 37 healthy donors, 17 were men and 20 were women, and the median age was 44 years.

Depletion of Microvesicles From Cell Line-Conditioned Medium

Cell debris was removed by centrifugation at 3000g for 20 minutes and at 10 000g for 30 minutes. Depletion of microvesicles was obtained by further centrifugation at 100 000g for 2.5 hours.

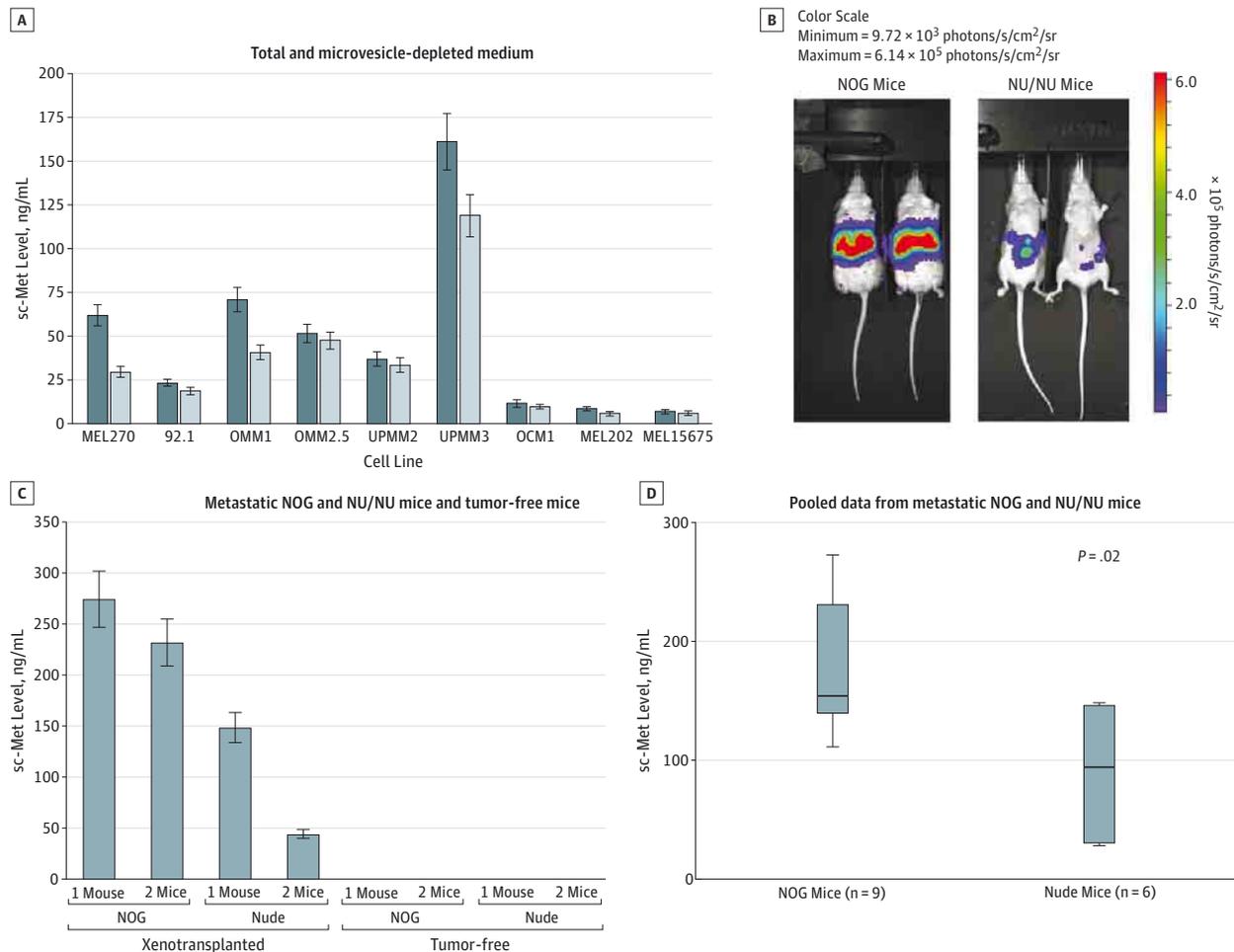
Enzyme-Linked Immunosorbent Assay for Detection of sc-Met

The level of sc-Met was measured using the Human Total HGF R/c-MET DuoSet IC enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), following the manufacturer's instructions. Optical density was measured at dual wavelengths of 450 and 540 nm (400 ATC; SLT Laboratory Instruments). The intra-assay coefficient variation of the ELISA was less than 8.3%.

Statistical Analysis

The Mann-Whitney *U* test was performed to compare the sc-Met serum levels of the patients with metastatic uveal melanoma with the sc-Met serum levels of the patients with non-

Figure 1. Soluble c-Met (sc-Met) Levels in Conditioned Medium of Uveal Melanoma Cell Lines and in Serum Samples of Mice With Xenografts



A, The mean sc-Met levels in total (dark blue bars) and in microvesicle-depleted medium (light blue bars). Error bars indicate SD. B, Liver metastases at 21 days in NOG and NU/NU mice. C, The mean serum sc-Met levels in metastatic NOG and NU/NU mice (left) or tumor-free mice (right). Error bars indicate SD.

D, Pooled data from metastatic NOG and NU/NU mice. The horizontal line in the middle of each box indicates the median, while the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below the box mark the minimum and maximum values.

metastatic uveal melanoma and healthy donors. The area under the empirical receiver operating characteristic curve was calculated to study the sensitivity and specificity of serum markers. Survival curves were constructed using the Kaplan-Meier method, and the log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test were used to compare the rates of death. The survival time of patients with metastatic uveal melanoma was defined as the elapsed time between diagnosis of metastasis and death or last follow-up. Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc).

Animal Experiments

All the mice used in our study were anesthetized with intraperitoneal injections of ketamine hydrochloride and xylazine hydrochloride. Liver metastases were obtained by injecting either 92.1 or MEL270 uveal melanoma cells (10^6 cells in 100 μ L of culture medium) that expressed the luciferase gene, under the spleen capsule of 6 NU/NU and 9 NOD/SCIDIL2Ry null (NOG) mice.³² Six

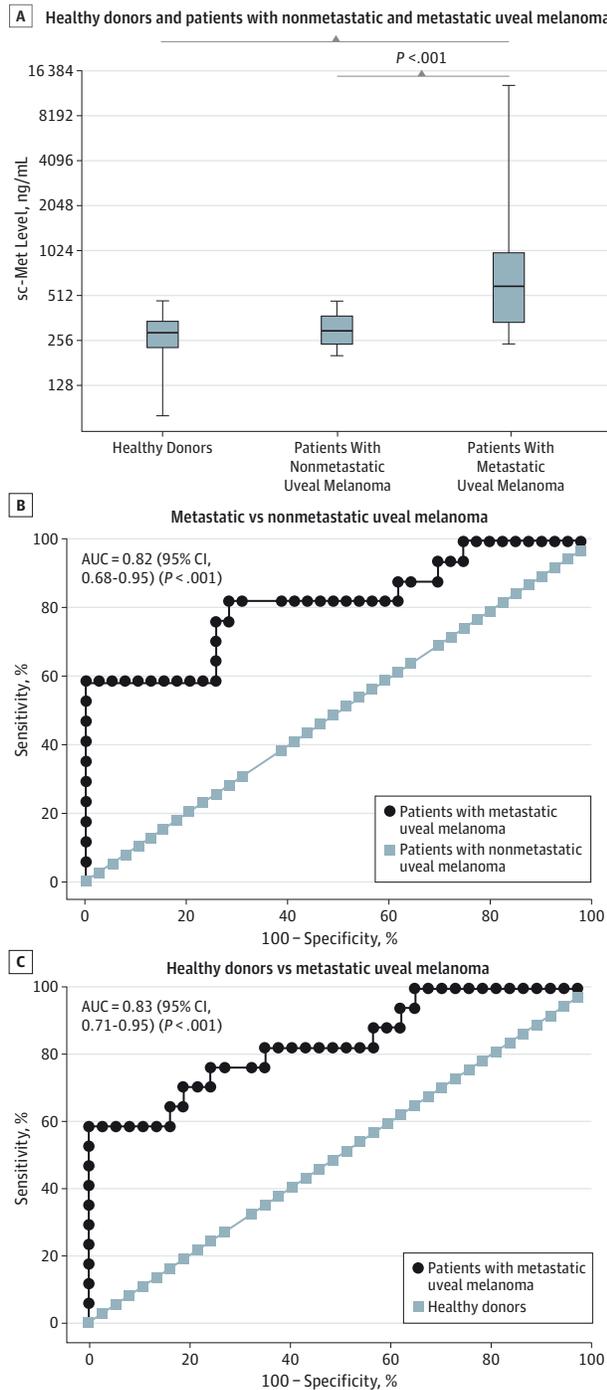
NOG and 3 nude tumor-free mice were used as controls. Development of metastases was monitored weekly by use of the In Vivo Imaging System. Blood samples were obtained from the tail vein of mice at the time of detection of metastasis in the liver.³² The experiments were performed according to the National Regulation on Animal Research Resources and approved by the institutional review board for animal experimentation at the Istituto di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliera Universitaria San Martino-Istituto Nazionale per la Ricerca sul Cancro in Genoa, Italy (approval identification No. IST 284).

Results

Sc-Met in Conditioned Medium of Uveal Melanoma Cell Lines and Serum of Mice With Uveal Melanoma Xenografts

We have previously shown that sc-Met is detectable in the conditioned medium of uveal melanoma cell lines,²⁵ owing to the

Figure 2. Soluble c-Met (sc-Met) Levels in the Serum Samples of Patients and Healthy Donors



A, Box-and-whisker plots of sc-Met levels in the serum of 37 healthy donors, 40 patients with nonmetastatic uveal melanoma, and 17 patients with metastatic uveal melanoma. The horizontal line in the middle of each box indicates the median, while the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below the box mark the minimum and maximum values. Receiver operating characteristic curves for sc-Met levels in patients with metastatic uveal melanoma vs patients with nonmetastatic uveal melanoma (B) and patients with metastatic uveal melanoma vs healthy donors (C). AUC indicates area under the curve.

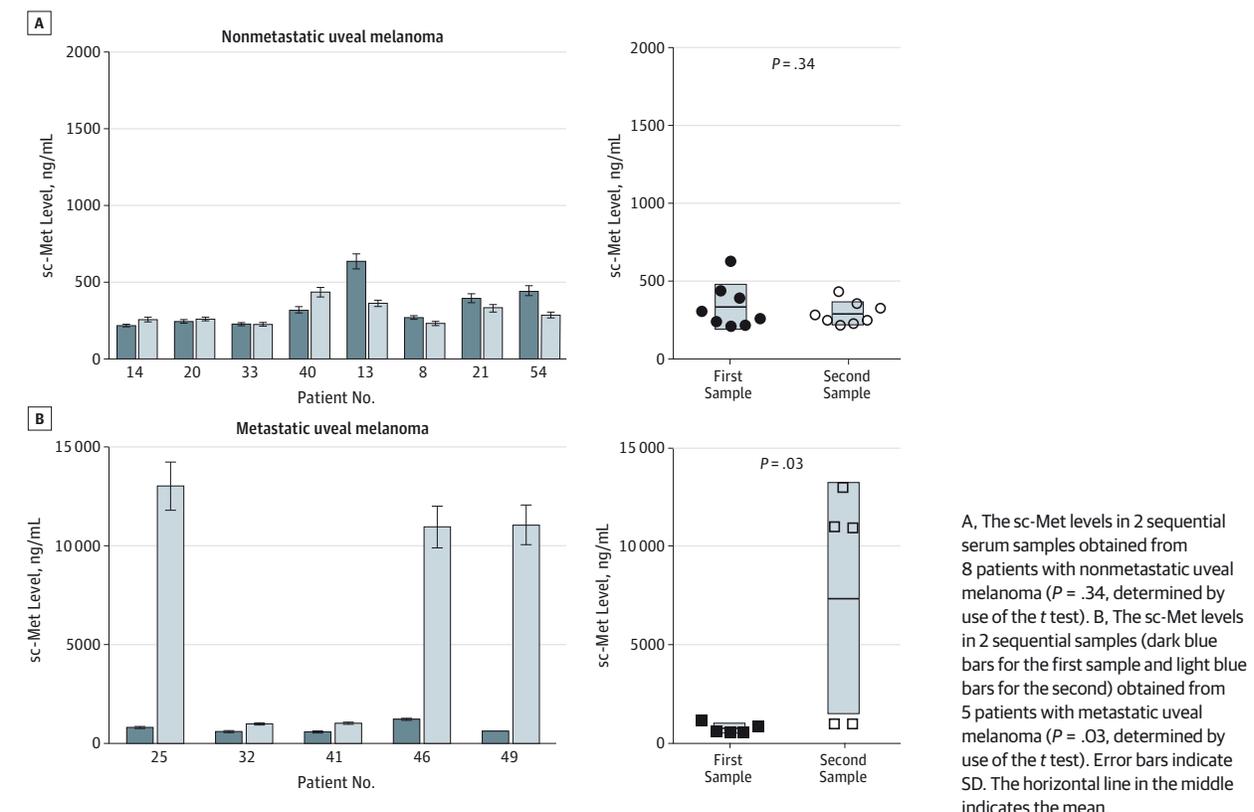
activity of ADAM10 and/or ADAM17, which cleave c-Met at the cell membrane. In the present study, we measured the level of sc-Met in the conditioned medium of a larger panel of uveal melanoma cell lines (Figure 1A). All of the cell lines tested released measurable amounts of sc-Met in their conditioned medium at levels ranging from 7.3 to 161 ng/mL, which suggests that shedding of c-Met is a common property of uveal melanoma cells. In addition, we depleted supernatants from microvesicles by ultracentrifugation and found that most of the c-Met was present in the microvesicle-depleted supernatants and, therefore, was mostly present in a soluble form (Figure 1A). To demonstrate that c-Met is released in vivo by the tumor cells and may accumulate in the serum, we implanted 2 different uveal melanoma cell lines (MEL270 or 92.1), transduced with the luciferase gene, in the spleen of NOG or NU/NU mice.³² All the mice showed the development of spleen and liver metastases, detected by In Vivo Imaging System monitoring. In Figure 1B and C, the results of a representative experiment involving both mice strains are shown; tumor development was more rapid in the highly immune-deficient NOG mice than in the nude mice. Significant levels of human sc-Met (44-260 ng/mL) were present in the serum samples of mice with xenografts, whereas the serum samples from control tumor-free mice showed no cross-reactivity with the human sc-Met ELISA (Figure 1C). Pooled data from 3 different experiments showed higher levels of sc-Met in NOG mice than in nude mice, which had smaller tumors, suggesting a possible relationship with tumor burden (Figure 1D). These data prompted us to determine whether sc-Met may be used as a potential biomarker of uveal melanoma growth in patients.

Serum Levels of sc-Met in Patients With Uveal Melanoma

We collected serum samples from a cohort of 40 patients with nonmetastatic uveal melanoma, 17 patients with metastatic uveal melanoma, and 37 healthy donors. The sc-Met levels were significantly higher in the serum samples of patients with metastatic uveal melanoma than in the serum samples of patients with nonmetastatic uveal melanoma ($P < .001$, determined by use of the Mann-Whitney test) and healthy donors ($P < .001$) (Figure 2A). We did not find any significant difference in sc-Met levels between the male and female participants in our study (data not shown). In addition, age did not significantly influence sc-Met levels. Indeed, if the different groups were stratified according to median age, the serum sc-Met levels showed a similar distribution in old and young participants (data not shown). The median level of sc-Met was 296 ng/mL for patients with nonmetastatic uveal melanoma (range, 201-469 ng/mL) and 590 for patients with metastatic uveal melanoma (range, 246-12 856 ng/mL). The sc-Met levels were not significantly different between patients with nonmetastatic uveal melanoma and healthy donors (median level, 285 ng/mL [range, 65-463 ng/mL]; $P > .30$).

We then performed an analysis of the receiver operating characteristic curves for serum sc-Met levels of patients with metastatic uveal melanoma vs patients with nonmetastatic uveal melanoma and healthy donors that yielded areas under the curve of 0.82 (95% CI, 0.68-0.95) ($P < .001$) and 0.83 (95% CI, 0.71-0.95) ($P < .001$), respectively (Figure 2B

Figure 3. Evaluation of Soluble c-Met (sc-Met) Levels in 2 Sequential Serum Samples of Patients With Uveal Melanoma



and C). In addition, we evaluated modifications of serum levels of sc-Met in paired samples during follow-up in 8 patients with nonmetastatic uveal melanoma and 5 patients with metastatic uveal melanoma, which showed an increase in size and/or number of metastatic lesions, based on echographic or computed tomographic scans (Figure 3A and B). There was no significant change in the sc-Met serum level in the 8 patients with nonmetastatic uveal melanoma between the first and the second evaluation (mean time lapse of 31.6 months after the first test), and only one of these patients showed a 20% increase at the second evaluation (Figure 3A). On the other hand, 5 patients with metastatic uveal melanoma who developed progressive disease showed a significantly increased level of sc-Met in the second sample ($P = .03$), which had been obtained, on average, 8 months after the first sample. The sc-Met level was more than 10 times higher at the second evaluation in 3 of the 5 patients with metastatic uveal melanoma, reaching values of greater than 10 000 ng/mL, while the other 2 patients (ie, patients 32 and 41) showed about a 50% increase in sc-Met level (Figure 3B). Of the 17 patients with metastatic uveal melanoma, 5 were alive, 3 were lost to follow-up, and 9 had died shortly after their last follow-up. The median survival time was 9 months (range, 2-24 months) for patients with high levels (above the median value) of sc-Met and 13 months (range, 1-84 months) for patients with low levels of sc-Met. Kaplan-Meier analysis suggests that patients with sc-Met

levels lower than the median value survive longer than patients with high levels of sc-Met ($P = .07$) (Figure 4A).

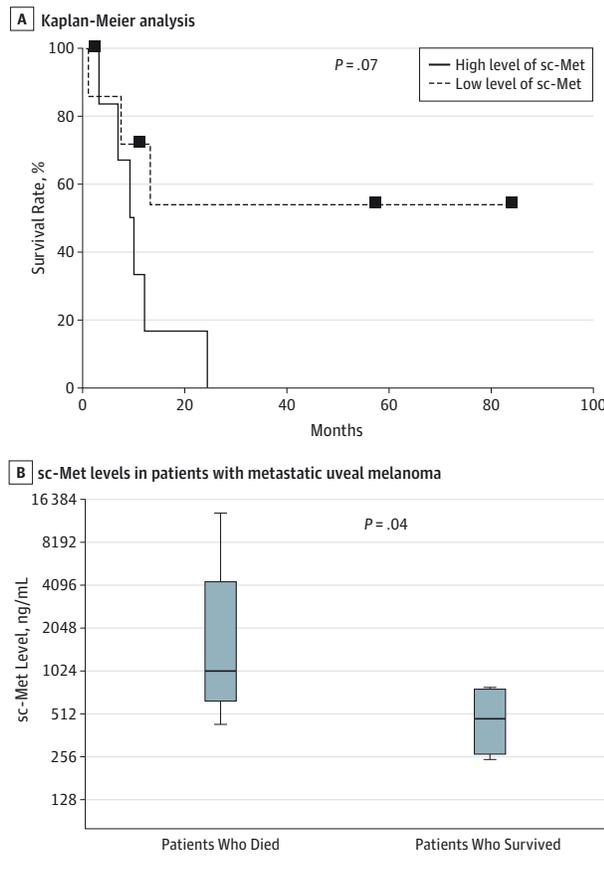
We further analyzed the relationship between sc-Met levels and survival of 12 patients with metastatic uveal melanoma with longer follow-up after sc-Met testing, stratifying these patients according to their median survival time; the levels of sc-Met in the 6 surviving patients with metastatic uveal melanoma were significantly lower than those in the 6 patients with metastatic uveal melanoma who had died ($P = .04$) (Figure 4B).

Serum Levels of LDH, S100, and sc-Met in Patients With Uveal Melanoma

Information on the lactate dehydrogenase (LDH) and S100 levels, evaluated within 7 days from blood sampling for sc-Met, were available for some of the patients with nonmetastatic uveal melanoma and some of the patients with metastatic uveal melanoma. The LDH levels were not significantly different between the 2 groups ($P = .38$), whereas significantly higher levels of sc-Met ($P = .004$) were detected in the patients with metastatic uveal melanoma compared with the patients with nonmetastatic uveal melanoma (Figure 5A). In contrast, serum levels of both sc-Met and S100 were significantly increased in patients with metastatic uveal melanoma ($P = .001$ and $.03$, respectively) (Figure 5B).

We then tested the predictive value of LDH, S100, and sc-Met for the detection of metastasis by an analysis of the

Figure 4. Analysis of Soluble c-Met (sc-Met) Levels in and Survival Status of Patients With Metastatic Uveal Melanoma



A, Kaplan-Meier analysis of 14 patients with metastatic uveal melanoma stratified for sc-Met levels above or below the median value. B, Box and whisker plots of sc-Met levels in the serum samples obtained from 6 patients with metastatic uveal melanoma who died ($n = 6$) or survived ($n = 6$) 7 months after testing. The horizontal line in the middle of each box indicates the median, while the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below the box mark the minimum and the maximum values.

receiver operating characteristic curves. The area under the curve was 0.60 (95% CI, 0.41-0.80) ($P = .20$) for LDH and 0.82 (95% CI, 0.64-1.00) ($P = .001$) for sc-Met, in a subgroup of 45 patients (Figure 5A). The area under the curve was 0.73 (95% CI, 0.53-0.92) ($P = .03$) for S100 and 0.85 (95% CI, 0.70-1.01) ($P = .001$) for sc-Met, in a subgroup of 29 patients (Figure 5B). These results confirm that elevated levels of sc-Met in the serum are predictive of progression, whereas elevated levels of LDH or S100 seem to be less reliable markers.

Discussion

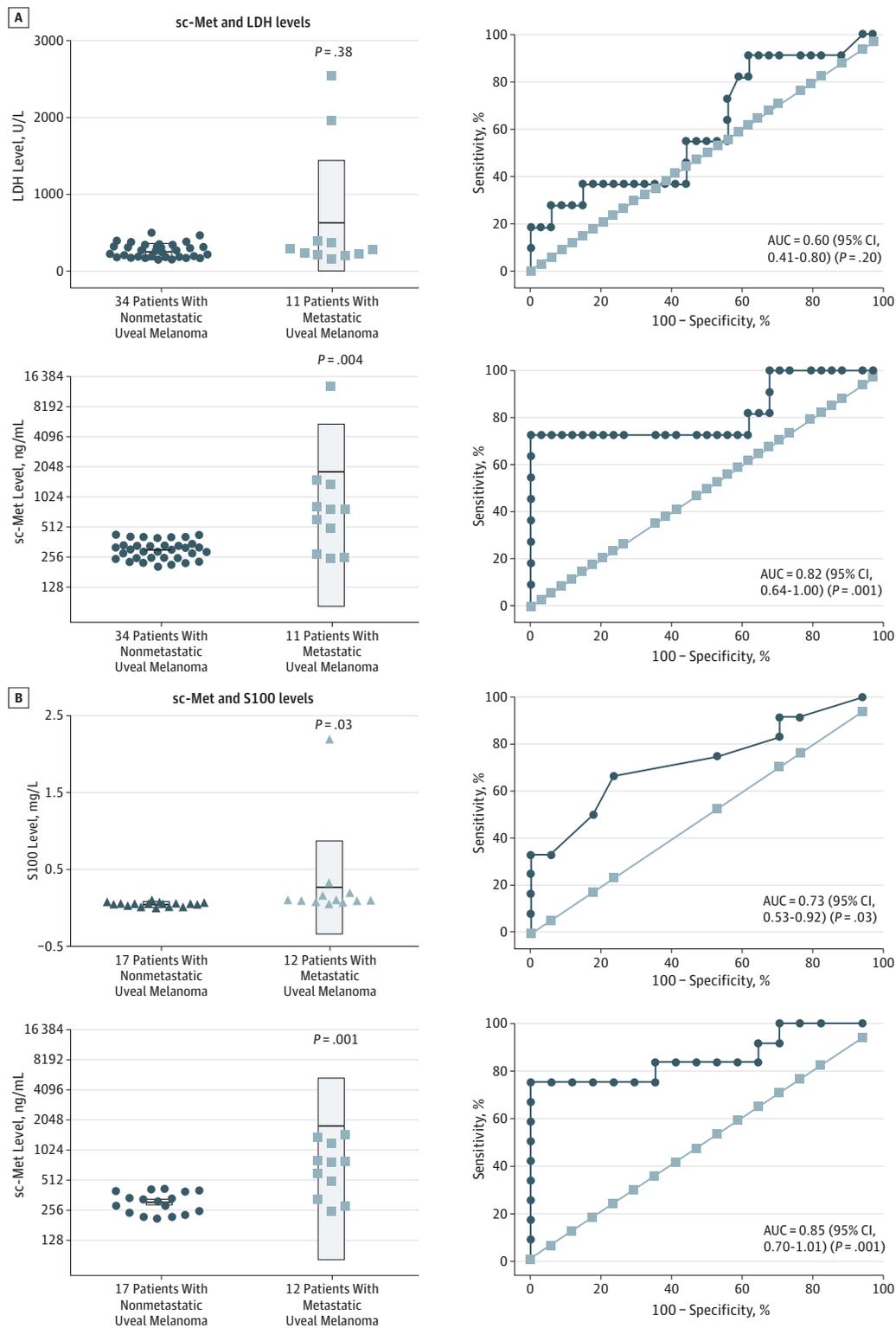
The hepatocyte growth factor receptor c-Met is a proto-oncogenic tyrosine kinase membrane receptor that works by inducing the spread of tumor cells^{20,33} in a broad range of tumors, including uveal melanoma.^{23-25,34,35} We have recently

demonstrated that high levels of c-Met, ADAM10, and, to a lesser extent, ADAM17 expression in primary uveal melanoma is associated with metastatic progression. ADAM10 and ADAM17 mediate the proteolytic cleavage of c-Met and the release of sc-Met from uveal melanoma cell cultures. Indeed, the silencing of either one of these metalloproteases has reduced the level of sc-Met released.²⁵

Herein we tested the hypothesis that the level of sc-Met might be increased in the serum samples of patients with uveal melanoma that has metastasized. The present finding that the level of sc-Met is increased in the serum samples of immune-deficient mice bearing human uveal melanoma xenografts further supports this possibility. Previous reports showed the presence of sc-Met in the supernatant of several tumor cell lines (skin melanoma, mammary gland, ductal carcinoma, colorectal adenocarcinoma, renal cell carcinoma, and MG glioblastoma) and in the serum samples of different tumor xenograft-bearing mice.³⁶ Moreover, soluble Met ectodomain correlated with tumor burden in U87MG glioblastoma, NCI-H1993 non-small cell lung cancer, and HS746T gastric cancer xenografts.^{36,37} In addition, high levels of circulating sc-Met were found in patients with non-small cell lung cancer and correlated with a worse outcome,³⁸ while urinary sc-Met levels were significantly higher in patients with bladder carcinoma than in individuals with no evidence of cancer.³⁹

In the present study, we show that patients with metastatic uveal melanoma also have significantly higher levels of sc-Met in their serum relative to metastasis-free patients and healthy donors. Analyses of receiver operating characteristic curves suggest that sc-Met may perform better than S100 and LDH in the detection of uveal melanoma liver metastases. In fact, the areas under the curve of LDH and S100 were smaller (0.60 and 0.73, respectively) than that of soluble c-Met (0.82). Similarly, a recent report¹⁷ showed that the oncoprotein DJ-1/PARK7 is a suitable serum biomarker for metastatic uveal melanoma because it performed better than the liver function test. The areas under the curve reported for DJ-1 (0.86) were slightly higher than those found for sc-Met. Therefore, further prospective studies are required to test the performance of sc-Met, eventually in association with other biomarkers such as DJ-1 to determine whether it can be used for the early prediction of uveal melanoma metastases. The levels of sc-Met remained stable (<20% change) in sequential samples from patients whose uveal melanoma did not metastasize. Interestingly, the level increased significantly in paired serum samples obtained during the progression of existing metastases. In addition, high sc-Met levels showed a trend toward a correlation with a worse survival for these patients with metastatic uveal melanoma. It would be important to identify a biomarker that allows for the early detection of uveal melanoma metastases. However, a limit of the present study is that none of the patients with nonmetastatic uveal melanoma in our cohort developed metastases during follow-up. Therefore, we could not show evidence that sc-Met levels can be helpful to identify patients at the earliest phases of metastasis development.

Figure 5. Lactate Dehydrogenase (LDH), S100, and Soluble c-Met (sc-Met) Levels in the Serum Samples Obtained From Patients With Nonmetastatic or Metastatic Uveal Melanoma



A, Scatterplot (left) and receiver operating characteristic (ROC) analysis (right) of sc-Met and LDH levels in a subset of 34 patients with nonmetastatic uveal melanoma and 11 patients with metastatic uveal melanoma. B, Scatterplot (left)

and ROC analysis (right) of sc-Met and S100 levels in 17 patients with nonmetastatic uveal melanoma and 12 patients with metastatic uveal melanoma. The horizontal line in the middle indicates the mean.

Conclusions

In conclusion, our pilot study indicates that sc-Met may be helpful for the follow-up of patients with metastatic

uveal melanoma because it appeared to correlate with disease progression and worse survival. However, further prospective follow-up studies are needed to define the prognostic value of sc-Met as a biomarker in metastatic uveal melanoma.

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Author Contributions: Drs Barisione and Gangemi had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Drs Ferrini and Gangemi contributed equally to this work. *Study concept and design:* Queirolo, Jager, Ferrini. *Acquisition, analysis, or interpretation of data:* All authors.

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