serUM-Px Liquid Biopsies Predicts Mortality in Uveal Melanoma

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Abstract

Purpose

To develop a prognostic test based on serum samples obtained at diagnosis of uveal melanoma (UM).

Design

Retrospective cohort study.

Subjects

Eighty-three patients diagnosed with primary melanoma in the choroid or ciliary body at St. Erik Eye Hospital, Stockholm, Sweden between 1996 and 2000. Serum samples from peripheral blood were obtained at diagnosis and kept at -80 °C until this analysis.

Methods

Proteome profiling of 84 different cancer-related proteins was used to screen for potential biomarkers. ELISA was then performed to evaluate the serum levels of the best candidates. Receiver operating characteristics were used to define thresholds for metastatic risk. A prognostic test that stratifies patients into low, intermediate, and high metastatic risk categories was developed (serUM-Px) in a training cohort and tested in a validation cohort.

Results

Of the 83 included patients, 43 (52 %) were female. The mean tumor diameter and thickness was 9.8 mm (SD 3.7) and 4.9 mm (SD 2.3), respectively. Twenty-two patients succumbed to metastatic UM and forty-two patients died from other causes. The median follow-up for the 19 survivors was 22.7 years (IQR 22.3-23.0). In proteome profiling, five proteins (Leptin, Osteopontin, Progranulin, Tenascin C and DLL1) were included for further analysis. After ELISA of individual proteins, Leptin and Osteopontin were selected for inclusion in the serUM-Px prognostic test. The test was developed in a training cohort (*n*=17) and then validated in an independent cohort (*n*=62). Patients had gradually shorter metastasis-free survival and greater incidence of UM-related mortality in competing risk analysis with increasing metastatic risk category (p = 0.02 and 0.027, respectively). In multivariate Cox regression, serUM-Px was an independent predictor of metastasis with tumor diameter, tumor thickness and patient age at diagnosis as covariates (hazard ratio 2.4, 95 % CI 1.0 to 5.3).

Conclusions

serUM-Px, a prognostic test based on a single peripheral venous blood sample at the time of UM diagnosis, stratifies patients into low, intermediate and high metastatic risk categories. It predicts metastases many years in advance in a validation cohort with very long follow-up. Future studies should aim to confirm these findings prospectively.

Introduction

At the time of uveal melanoma (UM) diagnosis, about 2% of patients have radiologically detectable metastases.[1] Within 15 years, this proportion increases to 32–45% even with successful treatment of the eye.[2, 3] Presumably, this is caused by subclinical dormant micrometastases that most frequently locate to the liver.[4] Once these leave their dormant state and grow into clinically detectable lesions, few effective treatment alternatives are available and the median patient survival is about one year.[5, 6] Only recently was some success reached, when treatment with the bispecific fusion protein Tebentafusp was shown to prolong median overall survival to 22 months in a group of previously untreated HLA- A*02:01–positive patients with metastatic disease.[7]

Almost all patients diagnosed with UM want prognostic information.[8] Further, patients that undergo testing experience lower levels of decision regret than patients who opt-out, even if the result indicates a very high metastatic risk.[8] There are several existing methods for prognostication of this risk. Traditionally, it may be estimated by clinical features such as tumor thickness, diameter, and location, by cytogenetic aberrations such as monosomy 3, and by presence of histopathological features such as epithelioid tumor cells and vasculogenic mimicry.[9–12] More recently, sequencing of the *BAP1* gene, manual and digital assessments of immunohistochemical stains of the BAP-1 protein and gene expression tests have shown great prognostic utility.[13–17] Samples for these tests are obtained either from enucleated eyes or by biopsy with transvitreal or transscleral techniques.[18] Although complications are rare, such invasive procedures may lead to hemorrhage, retinal detachment, and cataract.[19–22] Furthermore, gene expression tests are associated with significant costs and may not be universally available. Less invasive and expensive tests are therefore desirable. The ideal test should reflect the risk of a lethal course, be inexpensive, well-tolerated, and minimally invasive.[19]

Liquid biopsies have recently been investigated as an alternative approach to detect and monitor disease progression for patients with UM.[23–42] Liquid biopsy involves the sampling of tumor-derived molecules in body fluids such as blood.[19] This technique includes various components such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free microRNAs, as well as tumor-derived extracellular vesicles (EVs).[19] Some of these techniques have shown promise in UM; cell free-micro RNAs where miR-211 had the ability to distinguish metastatic from localized UM.[36] Others have not shown any significant correlations with prognosis, such as ctDNA which seems to be more suitable for the use of monitoring treatment response and disease course rather than for prediction of metastases.[19]

In this study, we develop the serUM-Px prognostic test based on a sample of peripheral blood obtained at UM diagnosis, that meets the criteria of being prognostically useful, inexpensive, and minimally invasive. The test is validated in an independent cohort of UM patients with long follow-up.

Methods

Patients and serum samples

This study was approved by the Ethical Review Committee at Karolinska Institutet with an amendment from the Swedish Ethical Review Authority (Dnr 2019–04297) and adhered to the tenets of the Declaration of Helsinki. Eighty-tree patients that were diagnosed with primary melanoma in the choroid or ciliary body between February 17th, 1996 and February 17th, 1999 were included. At diagnosis, radiological exams of the thorax and abdomen are performed by routine. No patient had radiologically detectable metastases at the time of diagnosis, was pregnant or was known to have liver dysfunction. During or within a few from diagnosis, a peripheral 10 ml venous blood sample was obtained from all patients after informed consent. These samples were drawn from the antecubital fossa and collected in hydrophobic plastic tubes, in which the blood was allowed to clot by leaving it undisturbed at room temperature for 15 to 30 minutes. After collection, the blood was allowed to clot by leaving it undisturbed at room temperature for 15 to 30 minutes. The clot was then removed by centrifugation at 1500 × g for 10 minutes in a refrigerated centrifuge. The resulting supernatant was transferred into clean polypropylene cryotubes and stored at -80°C within two hours of collection. All samples were preserved frozen at -80°C without thawing until the analyses.

At the time of diagnosis, clinicopathological data including patient age, sex, tumor location, diameter, thickness, eye laterality, ciliary body involvement, extrascleral extension, symptoms, and visual acuity were recorded in medical journals. Patients were then treated by either plaque brachytherapy or enucleation, depending on tumor size and location at the discretion of the patients' wishes and the attending ophthalmologist.

Follow-up

After diagnosis, screening for metastases by ultrasonography of the liver or computed tomography (CT) of the abdomen was repeated semi-annually for a 5-year period. Thereafter, radiological exams were not performed routinely, but when motivated by patients' symptoms, palpable masses, or deteriorating health. Ocular exams were scheduled at 1, 3, 6, and 12 months and then annually for the remainder of a patient's life. Data on the date of detection of metastases, date of death, and cause of death were obtained from patients' medical journals. In some instances, the exact date of the detection of a metastasis could only be approximated within a few days or weeks. Metastasis-free and overall survival curves were therefore generated by the actuarial life table method in half-year intervals, as described below. Follow-up data was complete even for patients that resided outside the Stockholm area, as their medical journals were available either via digitalized systems or at request by post.

Proteome profiling and ELISA

Serum total protein content was assessed for each patient by a Bradford assay. The sample concentration (μg/μl) was determined using a calibration curve prepared with standard bovine serum albumin (BSA, Sigma-Aldrich Corp., St. Louis, MO, USA) dilutions (μg/μl). Two μl of each BSA dilution and serum sample were mixed with 200 μl of diluted Protein assay dye reagent (Bio-Rad Laboratories Hercules, CA, USA) in a 96-well plate. The absorbance was measured at 595 nm.

Serum levels of 84 cancer-related proteins were assayed by Proteome Profiler Human XL Oncology Array (BioTechne Corp., Abingdon, UK; cat. no. ARY026) in pooled serum samples collected as described above, according to the manufacturer's instructions. The blots were developed using ECL max chemiluminescent reagent and the images were acquired by ChemiDoc XRS$^+$ (both Bio-Rad Laboratories). Protein expression was determined by optical density (OD) of the dot blots corrected with the three positive controls, as recommended by the manufacturer, using Image Lab 3.0 software (Bio-Rad Laboratories). Data are presented on a log2 scatter plot (GraphPad Prism software, San Diego, CA, USA) with an interval of 2-fold difference. Relevant proteins were quantified from single-patient serum samples by ELISA kits for Human Progranulin (cat.no. ab252364), Human Delta-like canonical Notch ligand (DLL1) (cat.no. ab193698), Human Leptin (cat.no. ab179884), Human Osteopontin (cat.no. ab269374), Human Tenascin C (cat.no. ab213831), all from Abcam (Cambridge, UK), according to manufacturer's instructions. Data is presented as protein concentration (ng/ml).

BAP-1 immunohistochemistry

Metastatic risk categories according to our serum-based prognostic test were correlated to BAP-1 expression in tumor cell nuclei in a subset of formalin-fixed and paraffin-embedded (FFPE) enucleated eyes available in the archives of the ocular pathology service, St. Erik Eye Hospital. Each tumor was cut into a 4 µm section, pretreated in ethylenediaminetetraacetic acid (EDTA) buffer at pH 9.0 for 20 minutes, and incubated with mouse monoclonal antibodies against BAP-1 at dilution 1:40 (clone C-4; Santa Cruz Biotechnology, Dallas, TX) and a red chromogen secondary antibody kit (Leica Biosystems, Nußloch, Baden-Wurttemberg, Germany), and finally counterstained with hematoxylin and rinsed with deionized water. The deparaffinization, pretreatment, primary staining, secondary staining, and counter-staining steps were run in a Bond III automated IHC/ ISH stainer (Leica, Wetzlar, Germany). The dilutions had been gradually titrated until optimal staining was achieved, according to manual control. The level of nuclear BAP-1 expression was assessed by GS according to a previously described method.[43] To be classified as positive, tumor nuclei had to be uniformly positive and accumulation of chromogen in nucleoli or similar did not suffice.

Statistical methods

P values below 0.05 were considered statistically significant, all p values being two-sided. For tests of continuous variables that did not deviate significantly from a normal distribution (Shapiro–Wilk test $p > 0.05$) Student's *t*-tests were used. For non-parametrical data, Mann–Whitney *U* tests were used. For comparisons of continuous variables across three categories or more, we used one-way ANOVA. In comparisons of categorical variables, we used contingency tables and Pearson chi-square (χ^2) tests (if all fields had a sample of > 5) or Fisher's exact tests (if any field had a sample of < 5). In comparison of categorical and ordinal variables, the Kruskal-Wallis test was used. The serUM-Px prognostic test was developed in three steps: Firstly, biomarker candidates were identified with proteome profiling of 84 different cancer-related proteins in a pooled sample of serum from 10 randomly selected patients that later developed metastases and 12 patients that did not. Secondly, ELISA of identified candidates was then performed in a training cohort. Thresholds for classification into metastatic risk categories were

determined with receiver operating characteristics in a training cohort. Thirdly, the final serUM-Px prognostic test was then validated in an independent cohort (Fig. 1).

Metastasis-free and overall survival curves were generated by the actuarial life table method in half-year intervals, and the Wilcoxon (Gehan) test was applied. For comparisons of association with metastasis, multivariate Cox regression hazard ratios (HR) were calculated. Considering the risk of influence from competing risks on metastasis-free survival (i.e., death from other causes before the development of metastases), cumulative incidence function estimates from competing risks data was plotted with the cmprsk package for R, and the equality of survival distributions was tested with Gray's test for equality. [44] All statistical analyses were performed using IBM SPSS statistics version 27 (Armonk, NY, USA) and GraphPad Prism version 9.3.0 (San Diego, CA, USA). The Sankey diagram (Fig. 5) was generated with SankeyMATIC (https://sankeymatic.com).

Results

Descriptive statistics

Of the 83 included patients, 43 (52%) were female. Their mean age at diagnosis was 65 years (SD 12). The mean largest basal tumor diameter was 9.8 mm (SD 3.7) and the mean apical thickness was 4.9 mm (SD 2.3). No patient had iris melanoma and the primary tumor location was the choroid in 80 patients (96%) and the ciliary body in 3 (4%), as determined with slit-lamp biomicroscopy, wide-field fundus imaging, and ultrasonography. Seventy-one patients (86%) were primarily treated with plaque brachytherapy and 12 (14%) with enucleation. Twenty-two patients developed metastases during follow-up and all of these 22 patients succumbed to their disease. Forty-two patients died from other causes. The median follow-up for the 19 survivors was 22.7 years (IQR 22.3–23.0, Table 1). The data met the proportional hazards assumption ($p = 0.55$).

Table 1
Demographics and clinical features of study patients

n	**83**
Age at diagnosis, mean (SD)	65 (12)
Sex, n (%)	
Female	43 (52)
Male	40 (48)
Primary tumor location, n (%)	
Choroid	80 (96)
Ciliary body	3 (4)
Iris	0 (0)
Tumor eye laterality, n (%)	
Right	47 (57)
Left	36 (43)
Extrascleral extension, n (%)	
Yes	1 (1)
No	82 (99)
Presentation, n (%)	
Shadow in visual field	10 (12)
Visual impairment	19 (23)
Pain	1 (1)
Floaters	4 (5)
Photopsia	11 (13)
No symptoms	38 (46)
Visual acuity at diagnosis, mean LogMAR (SD)	0.3 (0.5)
Tumor thickness at diagnosis, mean mm (SD)	4.9 (2.3)
Tumor diameter at diagnosis, mean mm (SD)	9.8 (3.7)
AJCC T-category at diagnosis, n (%)	
T1a	36 (43)
T1b-d	0 (0)

n	**83**
T2a	29 (35)
T2b-d	0 (0)
T3a	16 (20)
T3b-d	0 (0)
T4a-c	0 (0)
T4e	1(1)
AJCC stage at diagnosis, n (%)	
I	36 (43)
IIA	29 (35)
IIB	16 (20)
IIIA	0 (0)
IIIB	0 (0)
IIIC	1 (1)
IV	0 (0)
Primary treatment, n (%)	0 (0)
Plaque brachytherapy	71 (86)
Enucleation	12 (14)
Metastasis before last follow-up (%)	
Yes	22 (27)
No	61 (73)
Melanoma related death, n (%)*	
Yes	22 (27)
No	42 (51)
Alive	19 (23)
Median follow-up or follow-up for survivors, years (IQR)	22.7 (22.3–23.0)
SD, Standard deviation. AJCC, American Joint Committee on Cancer. IQR, Interquartile Range.	

Training cohort

First, in order to assess relevant protein biomarkers that present differential expression in primary UM, we performed proteome profiling in which we screened 84 different cancer-related proteins in a pooled sample of serum from 10 randomly selected patients that later developed metastases and 12 patients that did not. A 2-fold relative regulation cut-off was applied to compare the metastatic and non-metastatic group. Five proteins with a > 2-fold deviation between metastatic and non-metastatic patients were then selected for further analysis: Leptin, Osteopontin, Progranulin, Tenascin C and DLL1 (Fig. 2). Epidermal growth factor receptor (EGF R), Receptor tyrosine-protein kinase erbB-3 (ErbB3/HER3) and metalloproteinase 3 (MMP3) deviated > 2-fold but were not selected for further analysis considering that these are mechanistically predominantly related to epithelial cancers (EGFR, ErbB3/HER3) or for overlapping functions with proteins with higher deviation (MMP3 vs. Osteopontin).[45]

Next, we performed ELISA of each of the five selected proteins in 22 individual samples per group (metastatic and non-metastatic) from the same patients as the pooled analysis, while ensuring that the remaining proportion of patients that later developed metastases (5 of 18, 28%) would be similar to the proportion in the total sample (22 of 83, 27%).

There were no significant differences in median serum concentration of the five tested proteins between patients that later developed metastases from patients that did not: Leptin (9.3 ng/ml versus 19.4 ng/ml, Mann-Whitney U p = 0.11, Fig. 3A); Osteopontin (7.2 ng/ml versus 5.5 ng/ml, p = 0.06, Fig. 3B); Progranulin (58.6 versus 51.3 ng/ml, p = 0.68, Fig. 3C); Tenascin C (7.0 versus 7.3 ng/ml, p = 0.65, Fig. 3D) and DLL1 (7.3 versus 4.9 ng/ml, p = 0.58, Fig. 3E). In receiver operating characteristics with equal emphasis on sensitivity and specificity for metastasis, prognostically meaningful cutoffs were identified for Leptin (area under the curve, AUC 0.76, 95% CI 0.52 to 1.0, Fig. 3F), and for Osteopontin (AUC 0.80, 95% CI 0.53 to 1.0, Fig. 3G). No meaningful cutoffs could be found for Progranulin (AUC 0.58, 95% CI 0.22 to 0.95, Fig. 3H), Tenascin C (AUC 0.58, 95% CI 0.31 to 0.86, Fig. 3I) or DLL1 (AUC 0.60, 95% CI 0.29 to 0.91, Fig. 3J).

In the light of these results, Progranulin, Tenascin C, and DLL1 were eliminated for lack of statistical significance, while Leptin and Osteopontin were included in the final prognostic test (serUM-Px). This panel was constructed so that patients could be assigned to one of three metastatic risk categories: low; intermediate; or high (Table 2).

Table 2
SerUM-Px test classification

Metastatic risk category	Definition
Low	Serum Leptin concentration high, *and* serum Osteopontin concentration low
Intermediate	Serum Leptin concentration low, *or* serum Osteopontin concentration high
High	Serum Leptin concentration low, *and* serum Osteopontin concentration high

Validation cohort

Of the 65 patients initially included in the validation cohort (83 minus the 18 patients in the training cohort), three samples were excluded because their serum total protein concentrations being outside the 1st to 99th percentile (all three had total protein concentrations > 300 µg/µl). The mean total protein concentration in serum from the remaining 62 patients was 103 µg/µl (SD 50).

Clinical variables were evenly distributed across the three metastatic risk categories. There were no significant differences in patient sex, age at diagnosis, mean tumor thickness, diameter, or median follow-up (Table 3). The tumor diameter did not gradually increase for each increased metastatic risk group but was rather largest in the intermediate group.

Table 3
Clinicopathological features across metastatic risk categories in validation cohort

	Low	Intermediate	High	p^b
n =	19	27	16	
Mean age at diagnosis, years (SD)	64 (13)	68 (13)	66 (12)	0.69
Sex, n (%)				
Female	6 (32)	16 (59)	10 (63)	0.06
Male	13 (68)	11 (41)	6 (38)	
Mean tumor thickness, mm (SD)	5.3 (3.0)	5.2 (2.0)	4.6 (2.3)	0.22
Mean tumor diameter, mm (SD)	9.9 (4.4)	11.0 (4.2)	9.8 (2.5)	0.06
Median follow-up[a], years (IQR)	13.9 (11.6)	15.5 (11.7)	14.5 (9.2)	1.0

SD, Standard deviation. [a]For non-metastatic patients. [b]One-Way ANOVA for continuous variables, chi-square for categorical variables.

Patients had gradually shorter metastasis-free survival with each increasing metastatic risk category (Wilcoxon (Gehan) p = 0.02, Fig. 4A). Nineteen patients in the low metastatic risk category had a 5-, 10-, and 20-year metastasis-free survival of 95, 95, and 87%, respectively. Twenty-seven patients in the intermediate metastatic risk category had a 5-, 10-, and 20-year metastasis-free survival of 85, 81, and 69%, respectively. Sixteen patients in the high metastatic risk category had a 5-, 10-, and 20-year metastasis-free survival of 65, 58 and 50%, respectively. However, there were no significant differences in overall survival between the three metastatic risk categories (Wilcoxon (Gehan) p = 0.26, Fig. 4B).

Metastases appeared up to 16 years after diagnosis, with 65% (11 of 17), 76% (13 of 17) and 94% (16 of 17) of metastases occurring during the first 5, 10 and 15 years after diagnosis, respectively (Fig. 4C).

In multivariate Cox regression, serUM-Px was an independent predictor of metastasis when entering tumor diameter, tumor thickness, and patient age at diagnosis as covariates (hazard ratio 2.4, 95% CI 1.0 to 5.3, Table 4A). As the risk of metastasis is not necessarily similar across all ages, and tumor thickness and diameter can be assumed to correlate, we also entered tumor diameter and serUM-Px in a separate multivariate Cox regression. Again, serUM-Px was an independent predictor of metastasis (hazard ratio 2.2, 95% CI 1.1 to 4.7, Table 4B and Fig. 4D).

Table 4
Multivariate Cox regressions, hazard for metastasis

A	B	S.E.	Wald	P	Exp(B)	95% CI lower	95% CI upper
Patient age at diagnosis	-0.03	0.02	1.7	0.19	1.0	0.9	1.0
Tumor diameter, mm[a]	0.2	0.1	1.8	0.18	1.2	0.9	1.5
Tumor thickness, mm[a]	-0.3	0.2	1.7	0.19	0.8	0.5	1.1
SerUM-Px[b]	0.9	0.4	4.2	0.04	2.4	1.0	5.3
B							
Tumor diameter, mm[a]	0.07	0.09	0.6	0.46	1.1	0.9	1.3
SerUM-Px[b]	0.8	0.4	4.5	0.03	2.2	1.1	4.7
[a]Per increasing mm. [b]Per increasing metastatic risk category.							

Competing risks survival

In cumulative incidence function estimates from competing risks data in the validation cohort, patients had a significantly greater incidence of UM-related mortality with increasing serUM-Px metastatic risk category (Gray's test for equality p = 0.027, Fig. 5).

Primary tumor BAP-1 expression versus metastatic risk category

Sixteen eyes from 12 primary and 4 secondary enucleations were available in the archives of the ocular pathology service, St. Erik Eye Hospital. After assessment of BAP-1-stained sections, one tumor was excluded because no viable tumor cells were visible on the slide. Of the remaining 15 tumors, 5 (33%) had low nuclear BAP-1 expression (associated with high metastatic risk) and 10 (67%) had high expression (associated with low metastatic risk). The relationship between primary tumor BAP-1 expression and serUM-Px metastatic risk category was not significant (Kruskal-Wallis p = 0.056, Table 5, Fig. 6).

Table 5
serUM-Px metastatic risk category versus primary tumor BAP-1 expression

	BAP-1 high, n	BAP-1 low, n	*p*
Low, n	4	0	0.056
Intermediate, n	3	1	
High, n	3	4	

Discussion

In this study, we have shown that serum concentrations of Leptin and Osteopontin at the time of UM diagnosis can identify patients with poor metastasis-free survival. These two proteins were included in the serUM-Px prognostic test which assigns patients to a metastatic risk category. Herein, low serum Leptin concentrations and high serum Osteopontin concentrations correlate with the highest metastatic risk category at the time of UM diagnosis. Patients had gradually shorter metastasis-free survival and a greater incidence of UM-related mortality with each increasing metastatic risk category.

Previous studies have found a significant difference in plasma levels of Osteopontin between patients with and without detectable metastases.[38, 39] These studies showed that tumor marker levels increase before the existence of metastasis that can be identified by imaging.[38] Osteopontin is a 314-amino acid phosphoglycoprotein that is a component of the noncollagenous bone matrix.[39] This protein has been described in the role of diverse physiological roles such as chemotaxis, cell migration and adhesion, angiogenesis, apoptosis, cell-extracellular matrix interactions, and immune regulation.[46] Osteopontin actively promotes the tumorigenic phenotype and contributes to metastatic spread.[39] Elevated serum levels of Osteopontin have been described in patients with advanced or metastatic cancer.[39] Recently, increased Osteopontin levels have been observed in patients with metastatic UM, which correlates with our results.[47–49]

The other variable in serUM-Px – Leptin – has been described in various types of tumor cells, including breast, prostate, colon and endometrium where Leptin has been implicated as a growth factor for these cancers.[50–54] Leptin does not only play a role in food intake and energy balance but also functions as a pro-inflammatory adipokine with a broad range of activities including cytokine production, cellular immunity, and inflammation.[55–57] Leptin may also promote tumor growth by signaling through normal endocrine pathways. Physiologic binding of leptin to its receptors on hypothalamic neurons leads to Thyrotropin-releasing hormone (TRH) production by these cells.[55] Ellerhorst et al. have shown that melanoma cells express TRH and that TRH induces proliferation of these cells, which raises the possibility of Leptin as an inducer of melanoma TRH production and secretion, accounting in part for its growth-promoting effects.[58]

The serUM-Px metastatic risk categories were not associated with primary tumor BAP-1 expression, which is a well-established strong prognostic marker.[59]

Considering that this correlation was examined in a very limited sample of 15 tumors, we suspect that the non-significant correlation ($p = 0.056$) may represent a type II error, which should be investigated in a larger cohort.

Considering that most patients diagnosed with UM desire prognostic information and that most current testing alternatives entail an invasive procedure unless the tumor eye is enucleated, liquid biopsies based on peripheral blood samples are an attractive alternative. We suggest that the newly developed serUM-Px test may be seen as an alternative to FNABs and transvitreal biopsies. serUM-Px has the benefit of being

a test that reflects the risk of lethal course, is relatively inexpensive, minimally invasive, and has a low risk profile regarding complications. Consequently, prognostic testing can be made available for all UM patients, regardless of treatment modality.

Strengths of this study include the complete control of patients' follow-up. We had access to detailed data regarding the tumor and patient characteristics, as well as survival data from clinical records that were accessible regardless of where in the country the patient resides, which enabled robust correlation to the outcome where no patient was lost to follow-up. Further, our serUM-Px test predicted metastatic disease many years before macrometastases developed, whereas most other similar tests have relied on repeated sampling to reveal macrometastases at the time of or just before they become radiologically detectable.[19] Another of the foremost strengths of this study is simultaneously one of its considerable limitations; the > 20-year storage of the serum samples at -80°C allowed for long follow-up. However, no fresh samples were included. Even though previous studies indicate that serum samples can be stored deep-frozen even for decades without protein degradation, the protein concentrations observed herein do not necessarily reflect concentrations in fresh samples.[60–62]

This study has several other limitations. The results were based on a relatively small cohort of patients with moderately few metastatic events. The latter is likely a result of inclusion of a cohort with quite small tumors. Tumor size is strongly associated with virtually all other prognostic factors in UM, including ciliary body involvement, *BAP1* mutation, gene expression class 2, monosomy 3, tumor cell type, and patient age.[63–65] Several of these factors were not included in our data and we cannot assess their correlation with serUM-Px. Further, serum Leptin levels have a diurnal variation that follows the circadian rhythm with peak levels at night.[66] Even though all of our blood samples were taken in the daytime, some patients may have been classified differently if their blood sample had been drawn at a different time of the day.[67] Leptin levels also correlate to patient body mass and fat tissue volumes, with obese patients having higher serum concentrations. We did not collect data on patient weight or similar in this project, which would have helped us investigate if the patients with high Leptin levels and low metastatic risk were associated with obesity. This association merits further investigation in future studies.

Conclusions

We have developed a novel serUM-Px prognostic test based on a single peripheral venous blood sample at the time of UM diagnosis. This test stratifies patients into low, intermediate, and high metastatic risk categories and predicts metastases up to many years in advance in an independent validation cohort with long follow-up. Further prospective validation of the serUM-Px test may contribute to the implementation of non-invasive prognostic testing in UM.

Abbreviations

AJCC, American Joint Committee on Cancer

AUC, area under the curve

BAP-1, BRCA1 Associated Protein-1

BSA, bovine serum albumin

CT, computed tomography

CTCs, circulating tumor cells

ctDNA, circulating tumor DNA

DLL1, human Delta-like canonical Notch ligand

EDTA, ethylenediaminetetraacetic acid

EGF R, Epidermal growth factor receptor

ELISA, enzyme-linked immunoassay

ErbB3/HER3, Receptor tyrosine-protein kinase erbB-3

FFPE, formalin-fixed and paraffin-embedded

HR, hazard ratio

IHC, immunohistochemistry

ISH, *In situ* hybridization

IQR, interquartile range

MMP3, metalloproteinase 3

OD, optical density

ROC, receiver operating characteristics

SD, standard deviation

UM, uveal melanoma

Declarations

Ethical Approval and Consent to participate

This study was approved by the Ethical Review Committee at Karolinska Institutet with an amendment from the Swedish Ethical Review Authority (Dnr 2019-04297) and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients.

Consent for publication

Not applicable

Availability of supporting data

The dataset supporting the conclusions of this article is included within the article and its additional file.

Competing interests

No conflicting relationship exists for any author

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Authors' contributions

Christina Herrspiegel: Writing - Original Draft, Laboratory work.

Flavia Plastino: Writing - Original Draft, Laboratory work.

Emma Lardner: Handling of frozen serum samples and tumor specimens. Sectioning and immunohistochemical staining.

Stefan Seregard: Conceptualization, supervised the collection of serum samples in the 1990s.

Helder André: Conceptualization, Supervision.

Gustav Stålhammar: Conceptualization, Formal analysis, Resources, Visualization, Supervision, Project administration, Funding acquisition, Writing - Original Draft.

All authors reviewed the manuscript.

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Figures

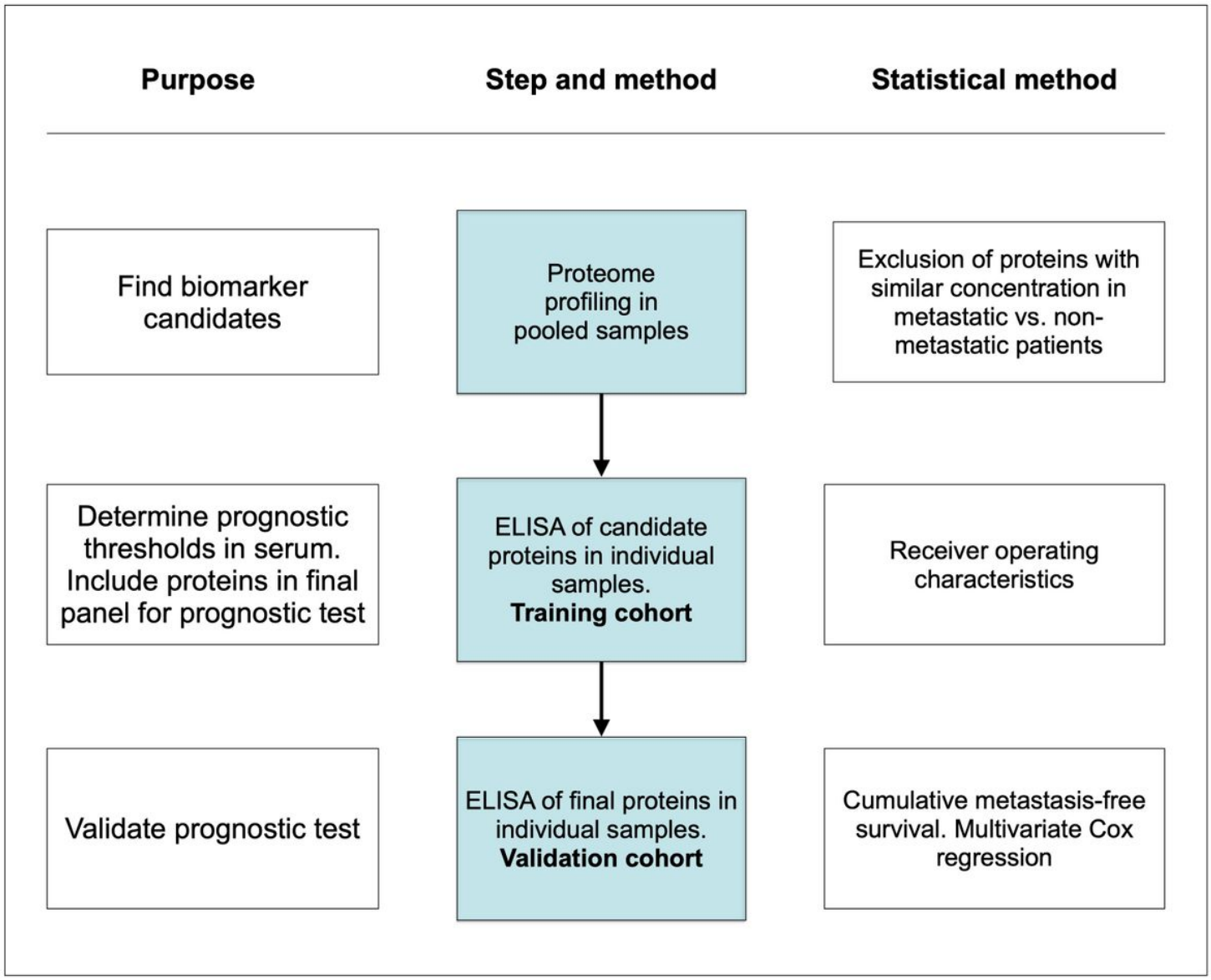

Figure 1

Steps in the development of serUM-Px. The test was developed in three steps: In step one biomarker candidates were identified with proteome profiling of 84 different cancer-related proteins in a pooled sample of serum from randomly selected patients that later developed metastases and patients that did not in order to find biomarker candidates. In step two ELISA of identified candidates from step 1 was then performed in a training cohort. The thresholds for classification into metastatic risk categories was determined with receiver operating characteristics in a training cohort. In step three the final serUM-Px prognostic test was validated in an independent cohort.

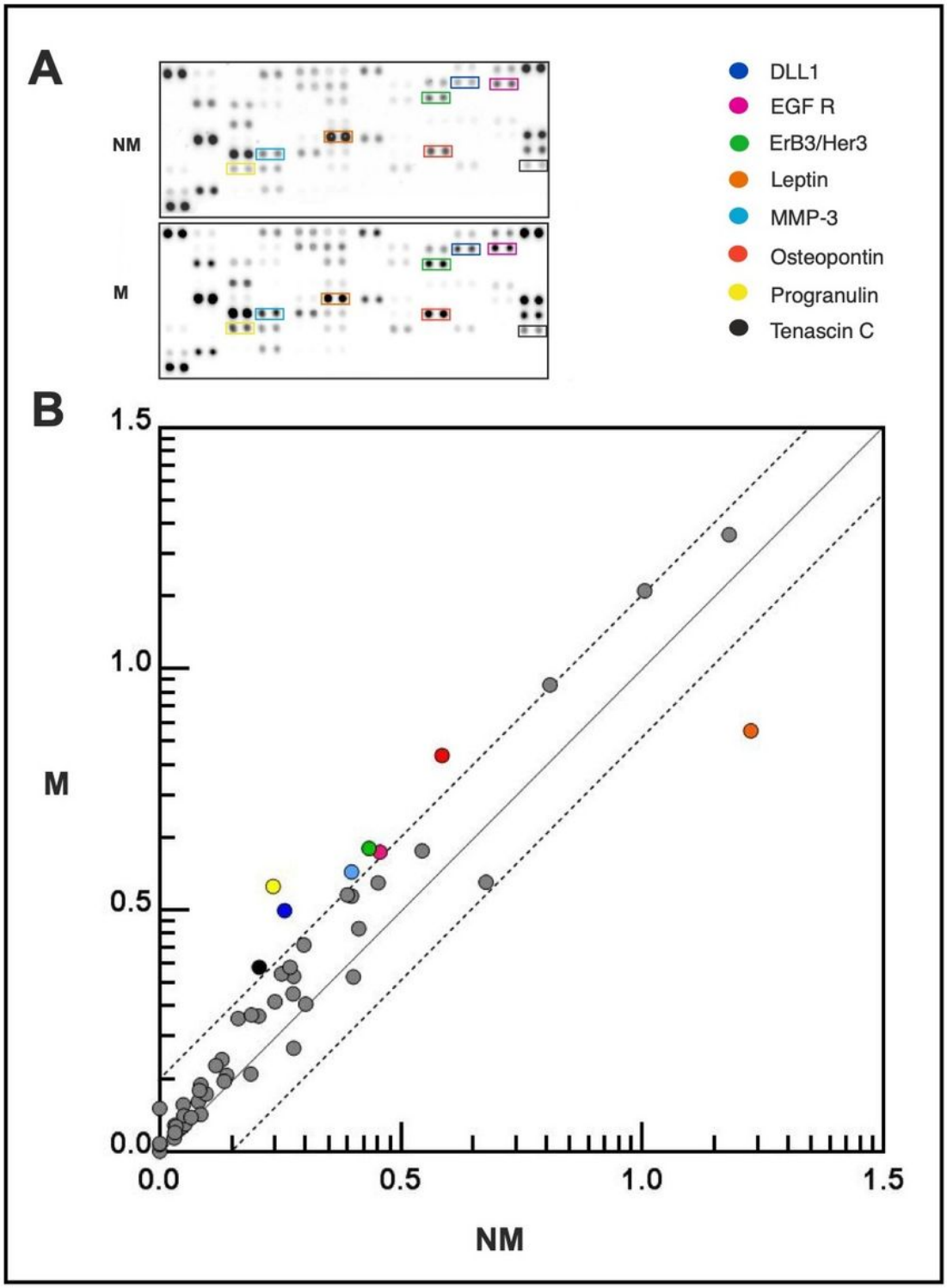

Figure 2

Representative proteins profile from pooled serum samples in primary uveal melanoma. A) Dot blots of 84 cancer-related proteins from non-metastatic (NM) and metastatic (M) groups. B) A threshold of 2-fold deviation was applied and data are presented as a correlation scatter plot for M versus NM. Grey-colored dots are proteins below the cut-off level.

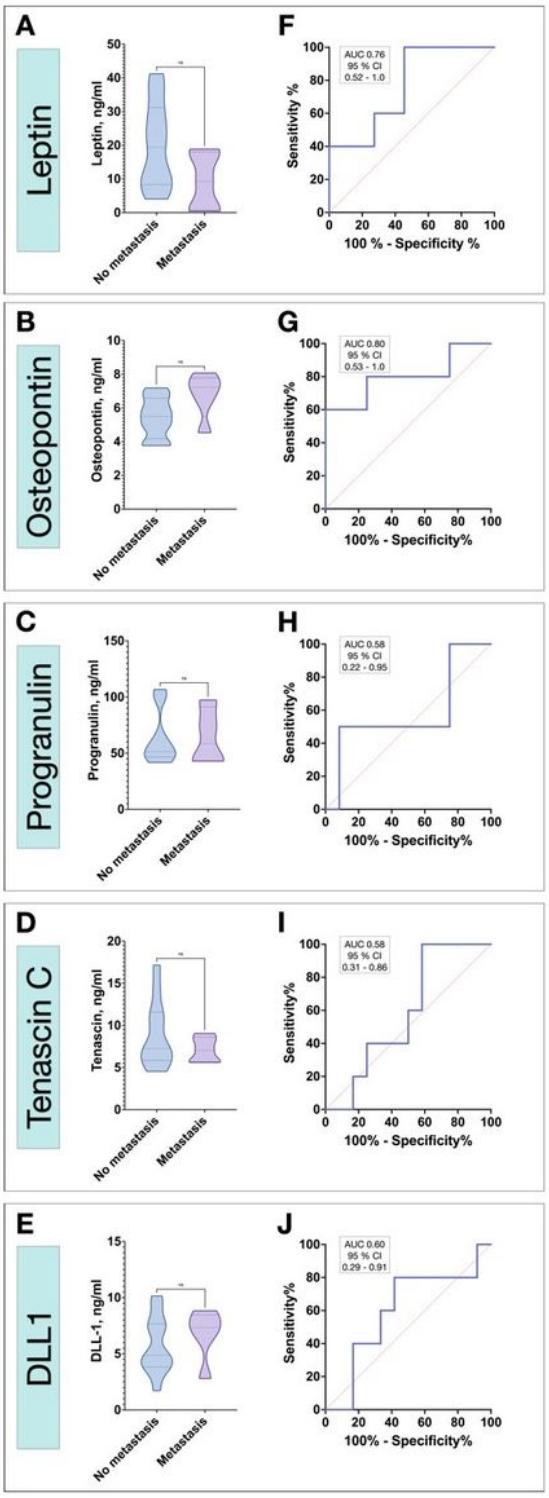

Figure 3

Violin plots and receiver operating characteristics of candidate proteins examined in the training cohort. A) Serum Leptin concentrations in individual samples from patients that later developed metastases versus patients that did not (median 9.3 ng/ml versus 19.4 ng/ml, Mann-Whitney U p=0.11). B) Osteopontin (7.2 ng/ml versus 5.5 ng/ml, Mann-Whitney U p=0.06). C) Progranulin (58.6 versus 51.3 ng/ml, p=0.68), D) Tenascin C (7.0 versus 7.3 ng/ml, p=0.65) and E) DLL1 (7.3 versus 4.9 ng/ml, p=0.58).

In receiver operating characteristics with equal emphasis on sensitivity and specificity for metastasis, prognostically meaningful cutoffs were identified for F) Leptin (area under the curve, AUC 0.76, 95 % CI 0.52 to 1.0), and for G) Osteopontin (AUC 0.80, 95 % CI 0.53 to 1.0). No meaningful cutoffs could be found for H) Progranulin (AUC 0.58, 95 % CI 0.22 to 0.95), I) Tenascin C (AUC 0.58, 95 % CI 0.31 to 0.86) or for J) DLL1 (AUC 0.60, 95 % CI 0.29 to 0.91).

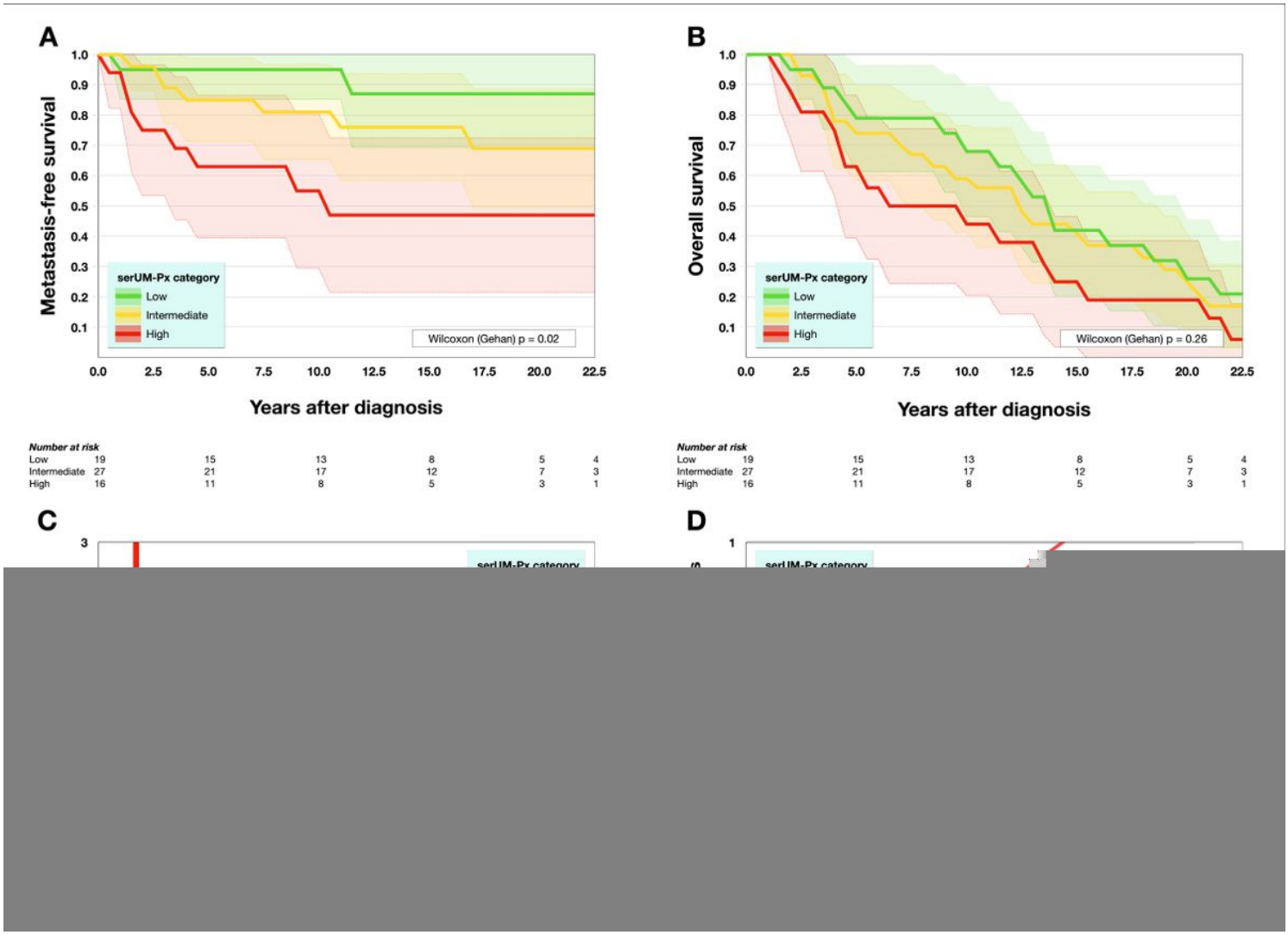

Figure 4

Survival analyses of the validation cohort (*n*=62). A) Patients had shorter metastasis-free survival with increasing serUM-Px metastatic risk category (Wilcoxon (Gehan) p = 0.02). B) There were no significant differences in overall survival between the metastatic risk categories (Wilcoxon (Gehan) p = 0.26). C) Metastases appeared up to 16 years after diagnosis, with 65 % (11 of 17), 76 % (13 of 17), and 94 % (16 of 17) of metastases occurring during the first five, ten, and 15 years after diagnosis, respectively. D) In multivariate Cox regression with tumor diameter and serUM-Px metastatic risk category as covariates, serUM-Px was an independent predictor of metastasis (hazard ratio 2.2, 95 % CI 1.1 to 4.7).

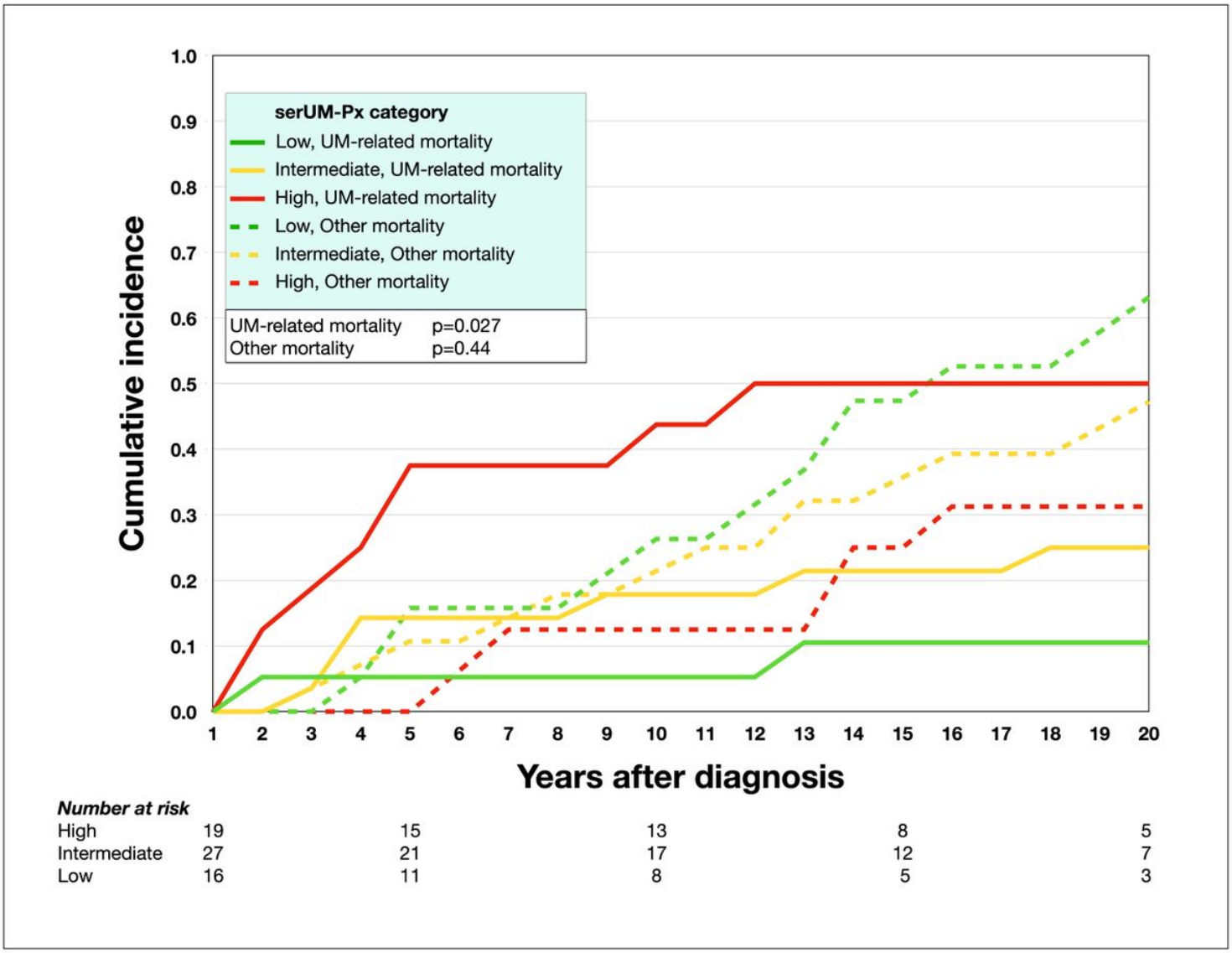

Figure 5

Cumulative incidence of UM related mortality in competing risk analysis. Patients had significantly greater incidence of UM-related mortality with increasing serUM-Px metastatic risk category (Gray's test for equality p=0.027). There was no significant difference in the incidence of death from other causes.

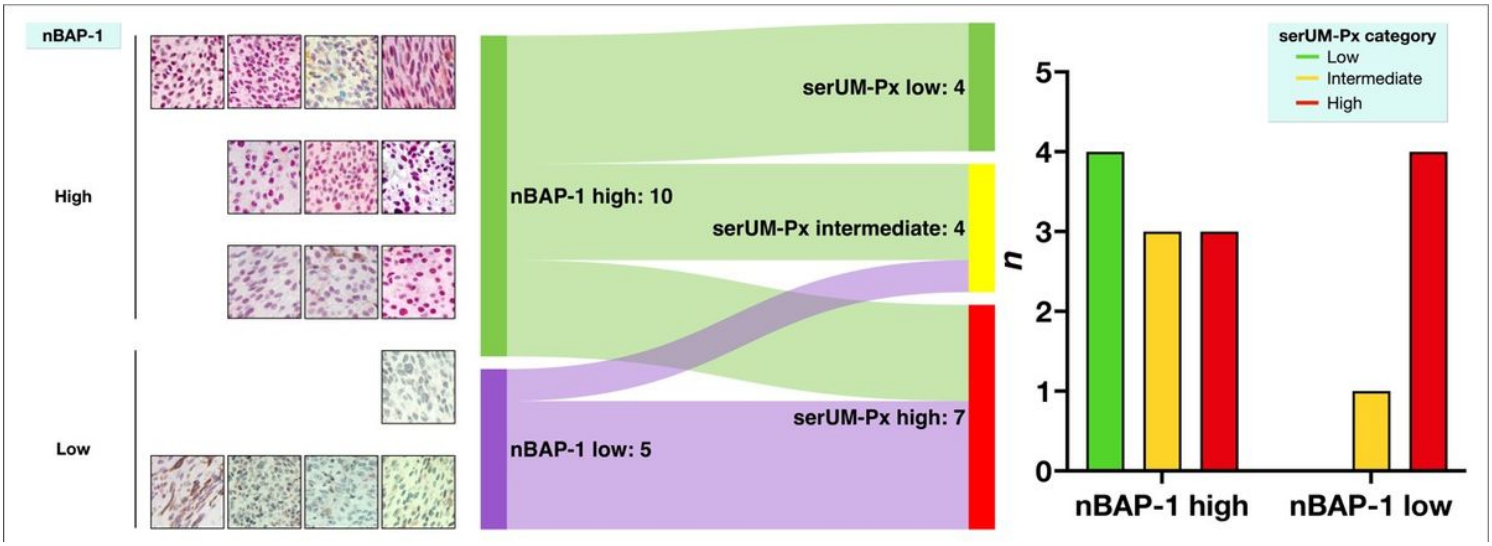

Figure 6

Nuclear BAP-1 expression (nBAP-1) versus serUM-Px. Of 15 available enucleations, five (33 %) had low nuclear BAP-1 expression (associated with high metastatic risk) and ten (67 %) had high expression (associated with low metastatic risk). The relationship between primary tumor BAP-1 expression and serUM-Px metastatic risk category was not significant (Kruskal-Wallis p=0.056).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Rawdata.xlsx