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ORIGINAL ARTICLE

COPPS, a composite score integrating pathological features, PS100 and SDHB losses, predicts the risk of metastasis and progression-free survival in pheochromocytomas/paragangliomas

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Abstract

Current histoprognostic parameters and prognostic scores used in paragangliomas and pheochromocytomas do not adequately predict the risk of metastatic progression and survival. Here, using a series of 147 cases of paraganglioma and pheochromocytoma, we designed and evaluated the potential of a new score, the COPPS (COmposite Pheochromocytoma/paraganglioma Prognostic Score), by taking into consideration three clinico-pathological features (including tumor size, necrosis, and vascular invasion), and the losses of PS100 and SDHB immunostain to predict the risk of metastasis. We compared also the performance of the COPPS with several presently used histoprognostic parameters in risk assessment of these tumors. A PASS score (Pheochromocytoma of the Adrenal gland Scaled Score) ≥ 6 was significantly associated with the occurrence of metastases ($P < 0.0001$) and shorter PFS ($P = 0.013$). In addition, both MCM6 and Ki-67 LI correlated with worse PFS ($P = 0.004$ and $P < 0.0001$, respectively), and MCM6, but not Ki-67, was significantly higher in metastatic group ($P = 0.0004$). Loss of PS100 staining correlated with the occurrence of metastasis ($P < 0.0001$) and shorter PFS ($P < 0.0001$). At a value of greater or equal to 3, the COPPS correlated with shorter PFS ($P < 0.0001$), and predicted reproducibly (weighted Kappa coefficient, 0.863) the occurrence of metastases with a sensitivity of 100.0% and specificity of 94.7%. It thus surpassed those found for either PASS, SDHB, MCM6, or Ki-67 alone. In conclusion, while validation is still necessary in independent confirmatory cohorts, COPPS could be of great potential for the risk assessment of metastasis and progression in paragangliomas and pheochromocytomas.

Keywords Pheochromocytoma · Paraganglioma · Prognosis · Metastasis · MCM6 · PS100 · Ki-67 · SDHB · PASS · COPPS

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Introduction

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare neuroendocrine neoplasms of the chromaffin cells originated from both adrenal medulla and extra-adrenal tissues. The annual incidence rate of adrenal PCC ranges from 0.4 to 9.5 in 1,000,000, but increases to about 1 in 300,000 when includes also PGL [1]. About 30 to 40% of patients with pheochromocytoma and paraganglioma (PPGL) have hereditary predispositions [2–5]. More than 20 sporadic or hereditary susceptibility genes such as mutations of succinate dehydrogenase (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*), *RET*, *VHL*, *NF1*, *FH*, *TMEM 127*, and *MAX* are involved in PPGL [6].

According to WHO 2017 Classification of Tumors of Endocrine Organs, the terms "malignant" or "benign" PPGL are no longer applicable. PPGL are classified as metastatic or not [7]. Approximately 10% of the PCCs are metastatic (5–26% according to the studies), and up to 50% for the *SDHB*-mutant paragangliomas [8, 9]. In half of the cases, metastases are not present during the initial treatment. Without treatment, 5 years survival for the metastatic PPGL is close to 50% [10]. The evaluation of the risks of recurrence and, in particular, metastasis is critical for treatment adaptation and patient follow-up.

To date, only few prognostic markers are available for these tumors. Histological features like tumor necrosis, mitoses over 3/10 high power fields (HPF), high cellularity, capsular, and vascular or adipose tissue invasions are often used as predictive markers of metastatic evolution [11–16]. In 2002, Thompson proposed the pheochromocytoma of the adrenal gland scaled score (PASS) based on 12 histopathological criteria (Supplementary Table S1) to separate benign from malignant PCC [17]. The association of PASS with recurrence and/or the metastatic status was validated independently in a number of studies [11, 12, 17, 18]. However, its application remains controversial as several studies have questioned the predictive value of PASS due to important inter-observer variabilities [19, 20]. Kimura et al. proposed consequently another score named GAPP (Grading system for Adrenal Phaeochromocytoma and Paraganglioma). This latter score is a composite index based on one immunohistochemical (the proliferation marker, Ki-67), one biological (the type of tumor secretion), as well as four histopathological features (histological pattern, cellularity, comedonecrosis, and vascular/capsular invasion). However, despite of some promising preliminary results [21–23], GAPP appeared insufficient to differentiate malignant from non-metastasizing cases in one recent study [24]. Various molecular markers have also been examined. Molecules such as SDHB, MAML3, SNAIL, hTERT, HSP90, STAT3, HuR, COX-2, VEGF, HIF1alpha, and secretogranin [23, 25–31] were shown correlated with PPGL malignancy. Proliferation marker Ki-67 has also been used to predict malignant behaviors of PPGL, despite lack of sensitivity [11] owing to limited number of cells engaging in Ki-67 expressing phase of the cell cycle [12, 15, 16]. Reliable criteria are still missing today to differentiate metastatic from non-metastatic PPGL.

We considered here in addition MCM6, another proliferation marker. MCM (Minichromosome Maintenance) proteins are highly conserved hexameric complex of DNA-binding proteins [32]. They initiate DNA replication during cell cycle, and represent a convergent point of the multiple signaling pathways of cell growth [32–35]. In contrast to Ki-67, MCM proteins are highly expressed in early G1 phase, and are downregulated only later after the cells adopt a terminally differentiated phenotype [35]. Anti-MCMs antibody thus labels more of the actively cycled cells than does anti-Ki-67 antibody [33]. In both lung carcinoma and meningioma, the immunohistochemical LI of MCM6 was reported correlated with histological grade and survival [35, 36]. To our knowledge, the expression of MCM proteins has not been specifically studied in PPGL. In this study, we hypothesized that a higher MCM6 expression may be associated with worse prognosis, and thus may be an interesting prognostic marker, in addition to the classically used clinico-pathological features and Ki-67 LI.

The objective of this study was thus to design a new composite prognosis score using a retrospective mono-centric series of 147 cases in an attempt to better predict the outcomes of PPGL.

Materials and methods

Population, clinical data, and tissues

One hundred and seven consecutive cases of PCC and 40 PGL were included in this retrospective study from the files of the Department of Endocrine Surgery (CHRU de Nancy, France), dating from the 1 March 1998 to the 31 December 2015 (Table 1). The corresponding tissue samples were retrieved from the Biobank of the Department of Pathology (CHRU de Nancy, France). Additionally, seven samples of metastatic PPGL, paired to primary tumors, were retrospectively included.

Main clinical data (age, sex, date of the surgery, hypertension, tumor size and localization, genetic status, and follow-up) were retrospectively collected from the Department of Endocrine Surgery and Department of Endocrinology (CHRU de Nancy, France). Metastases were defined by the occurrence of any tumor in any site distant from the primitive site based on clinical and radiological data confirmed by histopathological examination.

Table 1 Main clinico-pathological characteristics and immunohistochemistry

Clinical data ($n = 147$)	Mean (min. to max.) or proportion		
	Pheochromocytoma	Paraganglioma	Total
Number of cases	107	40	147
Age (years)	50.5 (8–84)	40.4 (9–70)	47.7 (8–84)
Sex ratio (male-to-female)	0.81	0.74	0.79
Hypertension	81.8% (63/77)	47.6% (10/21)	74.5% (73/98)
Tumor size (cm)	4.7 (1–18)	4.9 (1.2–14)	4.7 (1–18)
Metastasis status	1.9% (2/107)	17.5% (7/40)	6.1% (9/147)
Mutation	Proportion (%)		
No mutation	52.4% (22/42)	22.7% (5/22)	42.2% (27/64)
SDHB	7.1% (3/42)	36.4% (8/22)	17.2% (11/64)
SDHD	2.4% (1/42)	27.3% (6/22)	10.9% (7/64)
RET	19% (8/42)	0% (0/22)	12.5% (8/64)
VHL	14.3% (6/42)	9.1% (2/22)	12.5% (8/64)
NF1	4.8% (2/42)	0% (0/22)	3.1% (2/64)
HIF2α	0% (0/42)	4.5% (1/22)	1.5% (1/64)
PASS score ($n = 147$)	Proportion (%)		
PASS score ≥ 4	17.6% (19/107)	32.5%(13/40)	21.7% (32/147)
PASS score ≥ 6	9.3% (10/107)	20% (8/40)	12.2% (18/147)
Microscopic features	Proportion (%)		
Architecture	15% (16/107)	12.5% (5/40)	14.3% (21/147)
Necrosis	6.5% (7/107)	20% (8/40)	10.2% (15/147)
High cellularity	9.3% (10/107)	5% (2/40)	8.1% (12/147)
Cellular monotony	6.5% (7/107)	12.5% (5/40)	8.1% (12/147)
Tumor cell spindling	4.7% (5/107)	2.5% (1/40)	4.1% (6/147)
Mitosis (> 3/10 HPF)	2.8% (3/107)	2.5% (1/40)	2.7% (4/147)
Atypical mitosis	0% (0/107)	0% (0/40)	0% (0/147)
Extension into fat	15% (16/107)	32.5% (13/40)	19.7% (29/147)
Capsular invasion	30.8% (33/107)	75% (30/40)	42.8% (63/147)
Vascular invasion	9.3% (10/107)	20% (8/40)	12.2% (18/147)
Profound nuclear pleomorphism	10.3% (11/107)	17.5% (7/40)	10.2% (15/147)
Nuclear hyperchromasia	23.3% (25/107)	27.5% (11/40)	24.5% (36/147)
Immunohistochemistry	Mean LI ± SD or percent of patients		
MCM6 LI	13.5% ± 14.1	22.3% ± 16.3%	15.5% ± 14.7
Ki-67 LI	1.69% ± 2.46	3.22% ± 3.10%	2.1% ± 2.7
Loss of SDHB	3.7% (4/107)	57.5% (23/40)	20.4% (30/147)
PS100 negativity	3.7% (4/107)	15% (6/40)	6.8% (10/147)
COPPS ≥ 3	8.6% (9/105)	27.8% (10/36)	13.5% (19/141)

COPPS COmposite Pheochromocytoma/paraganglioma Prognostic Score, *HPF* high-power field, *LI* labeling index, *PASS* Pheochromocytoma of the Adrenal gland Scaled Score

Histopathology

The samples were fixed in 4% buffered formalin and paraffin-embedded. Five micrometer paraffin sections were stained by HES (hematoxylin, eosin, saffron). The histological diagnosis was checked by microscopic examination using all HES slides available at the initial diagnosis. Two experienced pathologists blinded to the clinical data reviewed all the cases. PASS score was established for all the cases. Vascular invasion, either lymphatic or venous, was indicated. In cases of disagreement, a consensus was reached by reexamination of all the slides using a multi-headed microscope.

Immunohistochemistry

A representative tissue block was selected for each case. Paraffin sections were immersed in sodium citrate buffer (10 mM, pH 6) for 20 min at 97 °C for dewaxing and antigen retrieval. The following primary antibodies were used: MCM6 (1/400; goat polyclonal, Santa Cruz Biotechnology, Heidelberg, Germany), Ki-67 (ready-to-use; mouse monoclonal, MIB-1, DakoCytomation, Glostrup, Denmark), PS100 (ready-to-use; polyclonal rabbit, DakoCytomation), SDHB (1/200; rabbit polyclonal, Sigma-Aldrich Corp, St Louis, USA).

Immunohistochemistry was performed with the DakoAutostainer Plus (Dako) and Flex+Envision revelation system (Dako). Negative and positive controls were used throughout the experiment.

For Ki-67 and MCM6, the LI was defined as the percentage of cells exhibiting nuclear labeling by counting all the tumor cells on a photographic field at × 200 magnification (0.15 mm^2). For each case, acquisition was performed with DP72 Olympus camera at × 200 magnification, and the LI was

obtained using a semi-automatic image analysis with Olympus Stream image analysis software (Olympus Corporation, Tokyo, Japan).

Staining for PS100 was rated as positive if at least 1% of cells with sustentacular cell morphology within tumor tissue was stained.

Staining for SDHB was rated as positive if the cytoplasm of the tumor cells bore (even weak) granular stain. The normal stromal cells of the fibrovascular network surrounding the Zellballen of tumor tissue were used as internal positive control cells.

Statistical analysis

For quantitative variables, either the Mann Whitney *U* test (two groups) or the Kruskal-Wallis tests with Duns post-analysis (more than two groups) were used because our data did not pass the normality test (Kolmogorov-Smirnov). For qualitative variable, the chi-squared or the Fisher exact tests were used. The Spearman correlation coefficients were calculated to evaluate the correlation between the expression of either MCM6 or Ki-67 and the size of tumor. Survival functions were performed with univariate and multivariate (for variables showing *P* value lesser than 0.10 with univariate analyses) proportion hazard test Cox model, and with Kaplan-Meier method and Log-rank test. Progression-free survival (PFS) was defined as the absence of either local and/or distant (metastatic) relapse. Inter-observer reproducibility was evaluated with the Kappa coefficient for qualitative variables and the weighted Kappa coefficient for ordinal variables. A *P* value lesser than 0.05 was considered as statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corporation, Armonk, NY, USA).

Additionally, to identify variables independently associated with "metastasis status," all significant parameters obtained from univariate analyses showing good reproducibility (Kappa coefficient > 0.6) were integrated into binary logistic regression model for multivariate analysis using the "metastasis status" as the dependent variable. All variables with $P < 0.1$ were included in the model and the variables with $P < 0.05$ were retained in the model. Results were shown as regression coefficient, standard error (SE), odds ratios (ORs), and 95% confidence interval (95% CI) for each independent predictor. We assessed model discrimination using ROC (receiver operating characteristic) analysis and model calibration using Nagelkerke R2 statistics [37]. To derive a risk score for the prediction of "metastasis status," we used the method described by Sullivan et al. for converting the coefficients for independent predictors into a simplified risk score system [38]. Specifically, we calculated the number of points assigned to each variable by dividing its OR by the smallest OR of the model. Then, we rounded this ratio to the nearest whole number [39]. Finally, we calculated each subject's risk score by summing up the points of all variables (COPPS, Composite Pheochromocytoma/paraganglioma Prognostic Score). We assessed the optimal cutoff of the COPPS score for the diagnosis of "metastasis status" using receiver operating characteristic (ROC) analysis. These statistical analyses were conducted with the MedCalc package for Windows v16.8.4 (MedCalc Software, Ostend, Belgium) on the basis of a two-sided type I error with an alpha level of 0.05.

Ethics

The experiments reported here were carried out according to the principles of the Declaration of Helsinki and in agreement with the French laws on biomedical research (institutional review board number DC2008-459; CNIL declaration number 1209171). All patients agreed and signed the form of information and non-opposition to the use of tissue samples for the purpose of scientific and medical research.

Results

Clinical data and genetics

The main clinico-pathological characteristics of the 147 consecutive cases of PPGL (107 [72.8%] cases of PCC and 40 [27.2%] PGG) are summarized in Table 1. The mean age of the patients at diagnosis was 47.7 years old. Male to female *sex ratio* was 0.79. The median follow-up duration for the patients after surgery was 3.8 years. Among the 147 patients, 138 (93.9%) were non-metastatic and 9 (6.1%) metastatic (2 PCC and 7 PGL) at various sites: bone (5/9), distant lymph node (2/9), liver (2/9), pulmonary (2/9), peritoneal dissemination (2/9). Multiple anatomical sites were involved in 6 out of 9 metastatic patients (including all the cases with pulmonary and liver involvement). Additionally, 3 cases (2 PCC and 1 PGL) had a local recurrence. Two patients died of disease (1 PCC and 1 PGL).

The genetic background was known in 64 patients (43.5%). Among these, the presence of a single mutation was found in 57.8% (37/64) of the cases, including *SDHB* mutations in 17.2% (11/64) of the cases, followed by *RET* (8/64; 12.5%), *VHL* (8/64; 12.5%), *SDHD* (7/64; 10.9%), and *NF1* (2/64; 3.1%) mutations.

Extra-adrenal primitive localizations of PPGL were significantly associated with occurrence of metastasis ($P = 0.002$) (Table 2). The mean tumor size was 4.6 cm for non-metastatic vs. 7.3 cm for metastatic tumors ($P = 0.007$). Tumor size correlated also with shorter progression-free survival (PFS) ($P = 0.003$) (Table 3). The presence of any *SDHB* mutation was associated with occurrence of metastasis ($P = 0.037$) and shorter PFS ($P = 0.034$).

Table 2 Main variables sorted by metastatic status (chi-squared or Fisher's exact test for qualitative variable; Mann Whitney *U* test for quantitative variable)

Variable	Non-metastatic group	Metastatic group	*P value*
Qualitative variables (*percent of patients*)			
Extra-adrenal localization	23.9% (33/138)	77.8% (7/9)	*0.002**
Multiples synchronous localizations	10.1% (14/138)	0% (0/9)	*0.601*
Incomplete resection	8.7% (12/138)	33.3% (3/9)	*0.072*
SDHB mutation	14.3% (8/56)	60% (3/5)	*0.037**
SDHD mutation	12.5% (7/56)	0% (0/5)	*1.000*
RET mutation	14.3% (8/56)	0% (0/5)	*1.000*
VHL mutation	12.5% (7/56)	20% (1/5)	*0.518*
PASS ≥ 4	18.1% (25/138)	77.8% (7/9)	*0.0004**
PASS ≥ 6	8.7% (12/138)	66.7% (6/9)	*< 0.0001**
Architecture	13% (18/138)	33.3% (3/9)	*0.233*
Necrosis	5.8% (8/138)	77.8% (7/9)	*< 0.001**
High cellularity	7.2% (10/138)	22.2% (2/9)	*0.159*
Cellular monotony	5.8% (8/138)	44.4% (4/9)	*0.001**
Tumor cells spindling	4.3% (6/138)	0.00% (0/9)	*1.000*
Mitosis (> 3/10 HPF)	0.7% (1/138)	22.2% (2/9)	*0.010**
Atypical mitosis	0% (0/138)	0% (0/9)	*NA*
Extension into fat	18.1% (25/138)	44.4% (4/9)	*0.136*
Capsular invasion	39.9% (55/138)	88.9% (8/9)	*0.005**
Vascular invasion	8.7% (12/138) Lymphatic, 6.5% (9/138) Venous, 2.2% (3/138)	66.7% (6/9) Lymphatic, 11.1% (1/9) Venous, 55.6% (5/9)	*< 0.0001**
Profound nuclear pleomorphism	10.9% (15/138)	0.00% (0/9)	*0.599*
Nuclear hyperchromasia	23.9% (33/138)	33.3% (3/9)	*0.813*
Loss of SDHB	18.1% (25/138)	55.6% (5/9)	*0.023**
PS100 negativity	3.6% (5/138)	55.6% (5/9)	*< 0.0001**
Size ≥ 7 cm	14.4% (19/132)	55.6% (5/9)	*0.007**
COPPS ≥ 3	7.6% (10/132)	100% (9/9)	*< 0.0001**
Quantitative variables (*mean*; *min.–max.*)			
Age	48.9 y (8.2–84.4)	29.6 y (8–56.1)	*0.007**
Tumor size	4.6 cm (1–18 cm)	7.3 cm (3.3– 14 cm)	*0.007**
Ki-67 LI	1.9% (0–15.2%)	5.1% (0–20.7%)	*0.233*
MCM6 LI	15.1% (1.14–87%)	37.1% (14.5–98%)	*0.0004**

*Statistically significant ($P < 0.05$)

COPPS COmposite Pheochromocytoma/paraganglioma Prognostic Score, *HPF* high-power field, *LI* labeling index, *NA* not applicable, *PASS* Pheochromocytoma of the Adrenal gland Scaled Score, *y* year

Prognostic value of histopathological criteria

PASS was established by two observers with a good inter-observer reproducibility (weighted Kappa coefficient = 0.689). Considering the different PASS criteria, inter-observer reproducibility (Kappa coefficients) was notably high for necrosis (0.860), mitotic count (0.717), vascular invasion (0.838), and capsular invasion (0.660), and moderate for cellular monotony (0.412) (Fig. 1) (Supplementary Table S2).

PASS was equal to or greater than 4 in 21.7% (32/147) of the cases, and equal to or greater than 6 in 12.2% (18/147) of the cases. A PASS score ≥ 6 was significantly associated with occurrence of metastases ($P < 0.0001$) and shorter PFS ($P = 0.013$).

When considering individually the 12 criteria of the PASS score, the histopathological criteria associated with metastasis status were necrosis ($P < 0.001$), high mitotic index ($P = 0.01$), cellular monotony ($P = 0.001$), capsular invasion ($P = 0.005$), and vascular invasion ($P < 0.0001$) (Table 2). Except for capsular invasion, all these criteria correlated with PFS (Table 3).

Table 3 Progression-free survival (PFS) analyses (log-rank and Cox univariate analyses)

	Log rank	PFS		
		Cox univariate analyses		
	P value	HR	95% CI	*P value*
Clinical data				
Age	–	.970	0.935–1.006	0.100
Sex	0.079	5.804	0.659–51.155	0.113
Extra-adrenal localization	0.218	2.507	0.553–11.372	0.233
Hypertension	0.277	42.610	0.000–6,484,412	0.538
Tumor size	–	1.295	1.091–1.537	0.003*
Tumor size ≥7 cm	0.003*	8.062	1.596–40.729	0.012*
Metastasis	< 0.0001*	21.245	4.040–111.710	< 0.001*
Multiple synchronous localizations	0.211	0.036	0.000–147.593	0.433
Incomplete resection	0.925	1.109	0.130–9.481	0.925
Mutation				
SDHB	0.014*	6.989	1.156–42.250	0.034*
SDHD	0.291	0.036	0.000–714.991	0.510
RET	0.316	0.037	0.000–992.402	0.527
VHL	0.049*	5.029	0.839–30.141	0.077
PASS score				
PASS ≥ 4	0.063	3.760	0.841–16.817	0.083
PASS ≥ 6	0.013*	5.434	1.208–24.447	0.027*
Microscopic features				
Architecture	0.094	3.359	0.746–15.133	0.115
Necrosis	0.001*	8.286	1.828–37.549	0.006*
High cellularity	0.009*	7.036	1.272–38.925	0.025*
Cellular monotony	< 0.0001*	13.405	2.901–61.935	0.001*
Tumor cell spindling	0.597	0.046	0.000–1,476,274.053	0.727
Mitosis (> 3/10) HPF	0.018*	8.595	1.002–73.744	0.050*
Extension into fat	0.738	0.694	0.081–5.961	0.739
Capsular invasion	0.107	3.602	0.685–18.935	0.130
Vascular invasion	0.018*	5.545	1.118–27.498	0.036*
Profound nuclear pleomorphism	0.363	0.041	0.000–1878.181	0.559
Nuclear hyperchromasia	0.682	1.424	0.260–7.783	0.684
Immunochemistry				
MCM6 LI	0.004*	1.077	1.035–1.121	0.0003*
MCMC6 ≥ 30%		7.454	1.498–37.099	0.014*
Ki-67 LI	< 0.0001*	1.622	1.282–2.052	< 0.0001*
Ki-67 ≥ 4%		35.029	4.087–300.246	0.001*
Loss of SDHB	0.379	0.516	0.115–2.313	0.387
PS100 < 1%	< 0.0001*	12.062	4.012–110.566	0.0004*
COPPS	0.001*	1.559	1.210–2.007	0.0001*
COPPS ≥ 3	< 0.0001*	25.59	3.070–213.231	0.003*

*Statistically significant ($P < 0.05$)

CI confidence interval, *COPPS* COmposite Pheochromocytoma/paraganglioma Prognostic Score, *LI* labeling index, *HPF* high-power field, *HR* hazard ratio, *PASS* Pheochromocytoma of the Adrenal gland Scaled Score, *PFS* progression-free survival

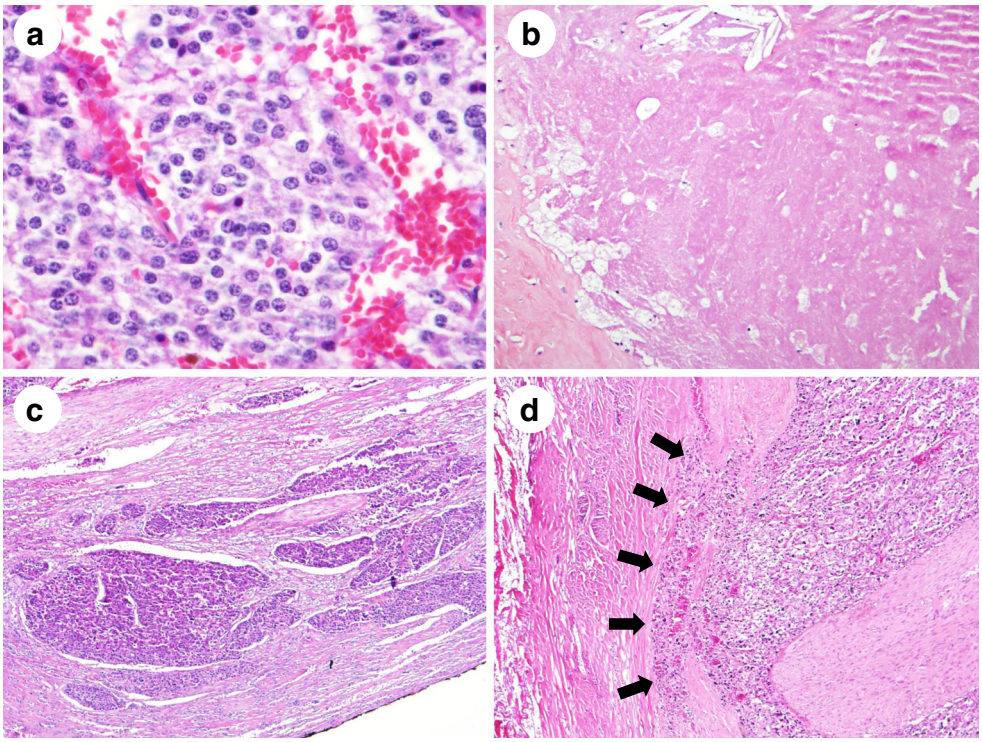

Fig. 1 Prognostic histopathological features in pheochromocytomas and paragangliomas. a Cellular monotony (hematoxylin, eosin, and saffron (HES), × 400). **b** Confluent necrosis (HES, × 40). **c** Vascular invasion (HES, × 40). d Capsular invasion (arrows) (HES, × 100)

MCM6 and Ki-67 expressions

MCM6 LI and Ki-67 LI were analyzed in PPGL by evaluating the proportion of cells showing nuclear staining (Fig. 2). The mean values are detailed in Table 1 and Table 2. LIs were globally greater for MCM6 (mean, 15.5%) than for Ki-67 (2.1%). MCM6 LI was significantly higher in the metastatic group ($P = 0.0004$). The percentage of Ki-67 labeled cells was not significantly greater in the metastatic group ($P = 0.233$). Both MCM6 and Ki-67 LI correlated inversely with PFS ($P = 0.0003$ and $P < 0.0001$, respectively) (Fig. 3). Using the log rank test, the most significant thresholds were ≥ 30% for MCM6 ($P = 0.004$) and ≥ 4% for Ki-67 ($P < 0.0001$). In multivariate Cox regression analysis, including only variables showing P value lesser than 0.10 in univariate analyses (Table 3), no variable remained significant (data not shown).

The Spearman test revealed a positive correlation between MCM6 and Ki-67 LI, with a *rho* coefficient measured at *rho* = 0.487 ($P < 0.001$) (Supplementary Table S3). However, only MCM6 LI correlated with tumor size (*rho* = 0.228; $P = 0.007$), but not Ki-67 LI (*rho* = 0.002; $P = 0.983$). We also compared Ki-67 and MCM6 LI between metastases and paired primary tumors: a significant correlation was found between metastases and paired primary tumors for MCM6 (*rho* = 0.94; $P = 0.02$; $n = 6$); this correlation was positive. However, no significant correlation was found for Ki-67 (*rho* = 0.70; $P = 0.23$; $n = 5$).

Prognostic value of loss of PS100 staining

In majority of the cases, positive PS100 staining was detectable within tumors, outlining sustentacular cells (Fig. 2). In 8% (10/147) of the cases, staining for PS100 was negative. This loss of PS100 labeling was significantly correlated with the occurrence of metastasis ($P < 0.0001$), and with a shorter PFS ($P < 0.0001$) (Fig. 3). The 5 metastatic cases showing PS100 losses were localized to the adrenal gland (2/5) or to the extra-adrenal retroperitoneum (3/5). PS100 labeling was also evaluated in seven paired metastatic tissues, which showed a perfect concordance of PS100 immunostaining with the primary tumor (Kappa correlation coefficient, 1).

SDHB expression

Staining for SDHB was negative in 20.4% (30/147) of the cases. The loss of SDHB labeling was strongly correlated with the presence of any single mutation in either *SDHB* or *SDHD* ($P < 0.0001$; kappa coefficient, 0.784 [95% CI 0.623–0.945) and also significantly associated with the occurrence of metastases (18.1% vs. 55.6%; *$P = 0.023$*); this labeling loss, however, was not significantly related to PFS (log-rank, *$P = 0.379$*). In the seven metastatic samples, the concordance to the paired primary tumor SDHB status was perfect (Kappa correlation coefficient, 1).

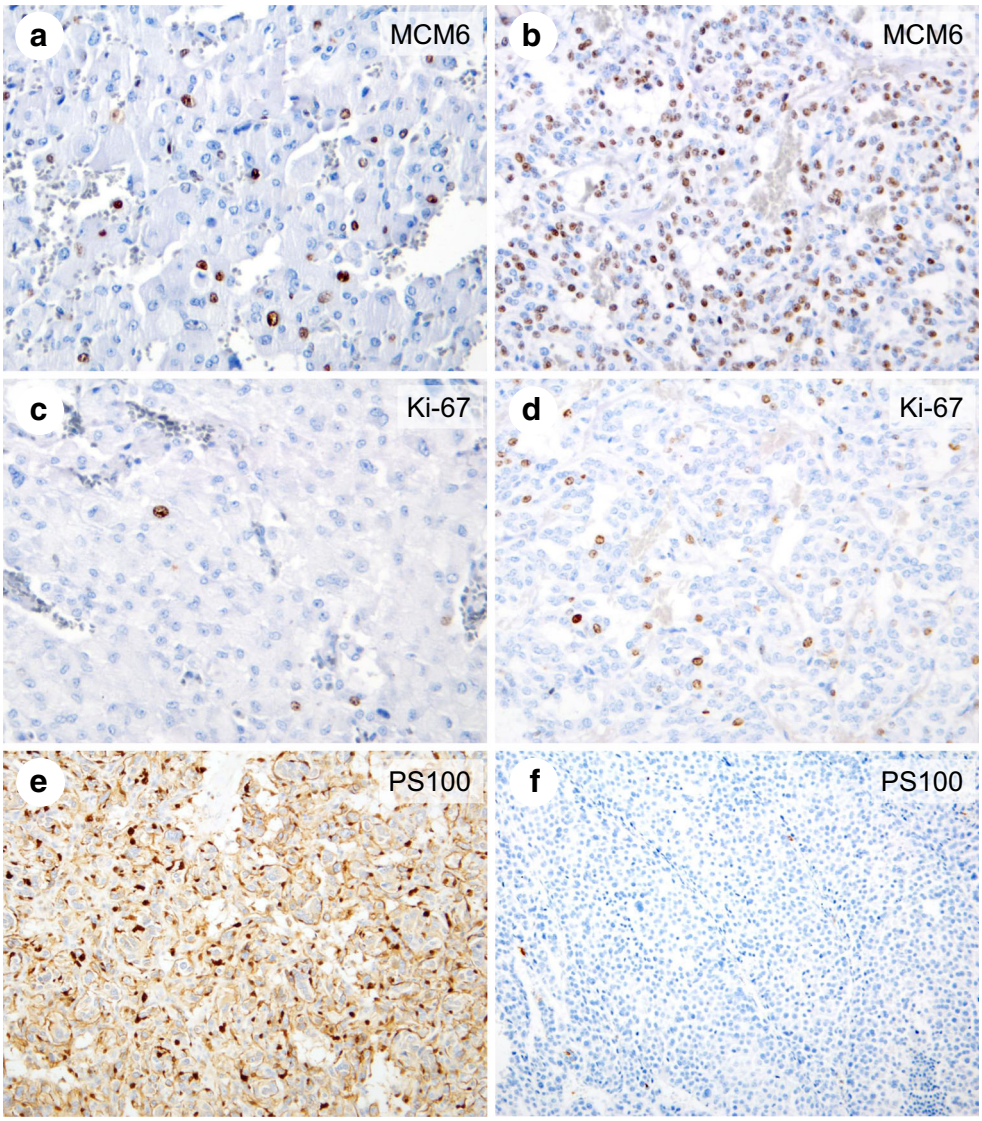

Fig. 2 Immunohistochemical markers. **a** Low MCM6 labeling index (LI) (13%) in a non-metastatic pheochromocytoma (PCC) (× 200). **b** High MCM6 LI (57%) in a metastatic PCC (× 200). **c** Very low Ki-67 LI (2%) in a non-metastatic PCC (× 200; same case as **a**). **d** Moderate Ki-67 LI (11%) in a metastatic PCC (× 200; same case as **b**). **e** PS100 immunolabeling in a non-metastatic PCC, showing the presence of numerous sustentacular cells (× 200). **f** Loss of PS100 in a metastatic PCC (× 100)

Multivariate analysis and composite pheochromocytoma/paraganglioma prognostic score

Among the variables that were significant in univariate analysis, five were independently associated with "Metastasis status" phenotype, namely, "'Focal or confluent necrosis" (OR = 125.23; 95% CI 2.77 to 5664.27), "PS100 loss" (OR = 37.79; 95% CI 1.22 to 1172.61), "Vascular invasion" (OR = 36.61; 95% CI 1.36 to 983.63), "SDHB loss" (OR = 41.84; 95% CI 1.62 to 1080.46), and "'Tumor size > 7 cm" (OR = 26.20; 95% CI 1.06 to 645.15) (Table 4). The logistic regression model revealed a rather good overall model calibration with good discrimination capacity characterized by a Nagelkerke R2 of 0.80 and an AUROC of 0.991 (95% CI 0.958 to 1.000), respectively (Fig. 4). Using the five independent predictors for the "Metastasis status," we derived the COPPS score (Table 1). As anticipated, we found the COPPS score significantly higher in the "Metastasis positive" subgroup (median = 8, IQR 5.5 to 8) than that in "Metastasis negative" subgroup (median = 0, IQR 0 to 1.5). In ROC analysis, the COPPS score had an AUROC of 0.981 (95% CI 0.942 to 0.996; $P < 0.0001$). In comparison, AUROC was 0.897 for PASS (optimal threshold, PASS ≥ 6; sensitivity = 66.7%, specificity = 91.3%), 0.851 for MCM6 LI (LI ≥ 30%; sensitivity = 44.4%, specificity = 89.1%), and 0.619 for Ki-67 (LI ≥ 4%; sensitivity = 44.4%, specificity = 89.9%) (Fig. 4).

The optimal threshold for COPPS to predict successfully (with a sensitivity of 100% and specificity of 94.7%) a "Metastasis status" phenotype was found to be ≥ 3 (95% CI > 3 to > 5). In both PCC and PGG subgroups, a COPPS ≥ 3 was significantly associated with the occurrence of metastases ($P = 0.005$ and $P < 0.0001$, respectively). Additionally, with both the log rank test ($P < 0.0001$) and Cox univariate analysis ($P = 0.003$; HR = 25.59 [95% CI 3.070–213.231]), a COPPS

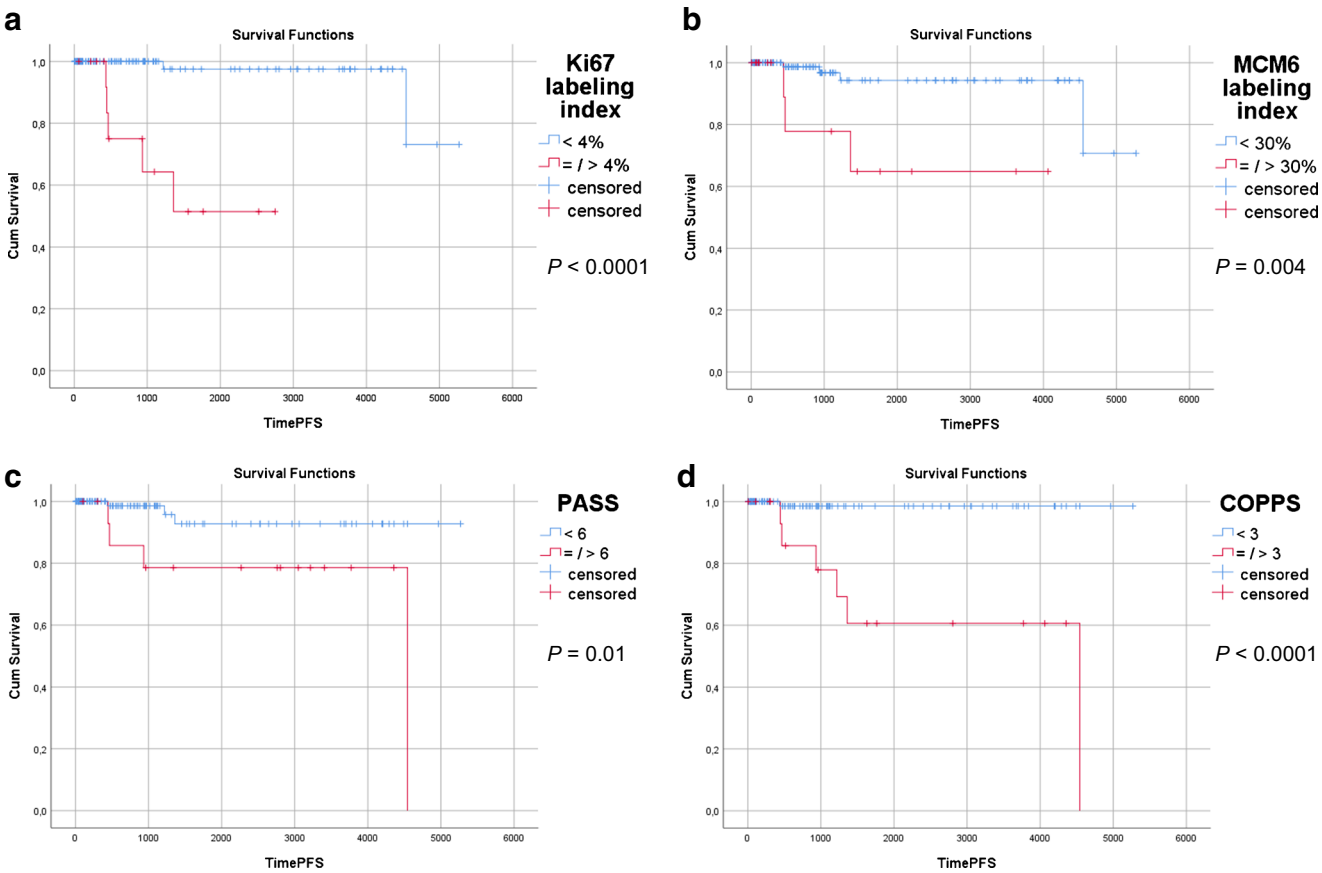

Fig. 3 Kaplan-Meier curves for progression-free survival (log-rank tests). **a** Ki-67 labeling index < 4% vs. ≥ 4% ($P < 0.0001$). **b** MCM6 labeling index < 30% vs. ≥ 30% ($P = 0.004$). **c** PASS (Pheochromocytoma of the Adrenal Gland Scaled Score) < 6 vs. ≥ 6 ($P = 0.01$). **d** COPPS (COmposite Pheochromocytoma/paraganglioma Prognostic Score) < 3 vs. ≥ 3 ($P < 0.0001$)

Table 4 Predictors of the "Metastasis status" in univariate and multivariate analysis in the whole cohort and Composite Pheochromocytoma/Paraganglioma Prognostic Score (COPPS); cutoff for a high risk of metastasis: COPPS ≥ 3

Predictors	Metastasis status, absent ($n = 138$)	Metastasis status, present* ($n = 9$)	Univariate analysis†, P value	Multivariate analysis‡, P value	Regression coef. (SE)	Odds ratio (95% CI)	COPPS score
Focal or confluent necrosis (percentage, 95% CI)	5.8 (1.9 to 9.8)	78 (44 to 100)	8.21×10^{-7}	0.013	4.83 (1.94)	125.23 (2.77 to 5664.27)	5
PS100 loss (percentage, 95% CI)	3.6 (0.5 to 6.8)	56 (15 to 96)	5.27×10^{-5}	0.038	3.63 (1.75)	37.79 (1.22 to 1172.61)	2
Vascular invasion (percentage, 95% CI)	8.7 (3.9 to 13.5)	67 (28 to 100)	9.82×10^{-5}	0.032	3.60 (1.68)	36.61 (1.36 to 983.63)	1
SDHB loss (percentage, 95% CI)	18.1 (11.6 to 24.6)	56 (15 to 96)	1.78×10^{-2}	0.024	3.73 (1.66)	41.84 (1.62 to 1080.46)	1
Tumor size > 7 cm (percentage, 95% CI)	14.4 (8.3 to 20.5)	56 (15 to 96)	7.54×10^{-3}	0.046	3.27 (1.63)	26.20 (1.06 to 645.15)	1
Capsular invasion (percentage, 95% CI)	39.9 (31.6 to 48.1)	89 (63 to 100)	5.07×10^{-3}	Not retained	Not retained	Not retained	Not retained
Increased mitotic figures (percentage, 95% CI)	0.7 (0 to 2.2)	22 (0 to 56)	9.74×10^{-3}	Not retained	Not retained	Not retained	Not retained
MCM6 > 30% (percentage, 95% CI)	10.9 (5.6 to 16.1)	44 (4 to 85)	1.68×10^{-2}	Not retained	Not retained	Not retained	Not retained

*Percentages were rounded to the nearest percentage point given the low sample size; †Fisher's exact test; ‡Multivariate logistic regression analysis using the stepwise method

SE standard error, *95% CI* 95% confidence interval, *COPPS* COmposite Pheochromocytoma/paraganglioma Prognostic Score

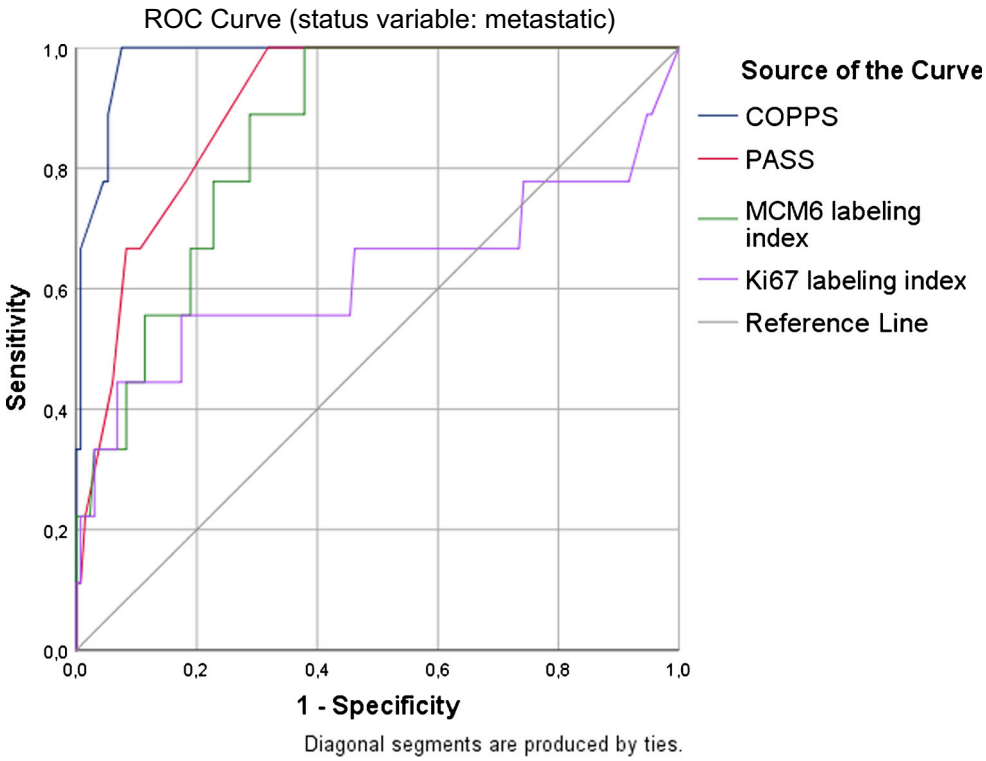

Fig. 4 Receiver operating characteristic (ROC) curves (status variable: metastatic) for Ki-67, MCM6, PASS (Pheochromocytoma of the Adrenal gland Scaled Score), and COPPS (COmposite Pheochromocytoma/paraganglioma Prognostic Score)

≥3 was significantly associated with a shorter PFS; this correlation to survival was significant both in the PCC ($P = 0.001$) and PGG ($P = 0.026$) subgroups.

The reproducibility of COPPS was found excellent (weighted Kappa, 0.863) (Supplementary Table S2).

Discussion

Clinical features, tumor size, and genetics

The occurrence of metastasis in PPGL is infrequent and difficult to predict. The development of prognosis marker is critical, notably to adapt to efficient treatments during follow-ups of the patients. In our cohort here, we found low metastatic rate (6.1%, 9/147) occurring mainly in young patients. Similar low incidence of metastatic progression [8] in young patients [18] has also been reported. In our study, we further noted that larger tumor size, measured on surgical specimens, correlated with a more probable metastatic status ($P = 0.007$) and a shorter PFS ($P = 0.003$). This, however, contrasts some earlier observations. In fact, contradictory findings have been reported previously on the correlation between tumor size and occurrence of metastasis, with some noted significant positive correlations [12, 15, 40], while others failed to observe the presence of such correlations [11, 17, 18, 20]. Although it remains unclear the reasons underlying these different observation, our data suggested that genetic background may influence the risk to metastatic progression. In the 64 patients with whom we were able to perform the genetic testing, we found a correlation between the presence of *SDHB* mutation and the occurrence of metastasis, in agreement with previous data reported in the literature [1, 41–43]. On the contrary, *SDHD*, *RET*, and *VHL* mutations were not correlated to the prognosis.

Histopathological criteria

Histopathological criteria have been included in prognostic predictions to define more precisely the risk of metastasis and recurrence. To date, in daily practice, the most widely used score based on these types of criteria is the PASS [17], a score based solely on morphological histopathological criteria. This score has been the subject of several studies; however, its value as prognostic marker remains unclear. In some cases, it is recommended as a prognostic factor [18, 44], but in other cases its use is not favored due either to a lack of reproducibility [19] or to an absence of correlation with the occurrence of metastasis [20]. In the present study, we found that a PASS threshold of ≥6 was more significantly associated with prognosis than when a PASS ≥4. This result is similar to the study by *Strong* et al., which suggests an increase in the cutoff of PASS score to 6 to better differentiate benign from malignant lesions [11].

When considered separately, the different criteria were used to obtain the PASS score. The presence of necrosis appeared to be the most relevant parameter correlated to metastasis [11–16, 18]; it is followed by the mitotic index [11, 12, 16, 18], vascular invasion, and capsular invasion [12, 14, 16,

18], high cellularity [11, 14], and the invasion of fat [13, 14]. In our study, cellular monotony was found also of great prognostic value with moderate reproducibility (Kappa = 0.412). With the exception of capsular invasion, all these criteria correlated with survival (PFS). We considered also two other parameters used, "profound nuclear pleomorphism" and "nuclear hyperchromasia"; in our cohort they both showed no correlation with prognosis, and had rather low reproducibility (Kappa = 0.248 and 0.183). Their uses as parts of the criteria for prognostic evaluation were thus excluded from the COPPS.

Proliferation markers

Ki-67 is a proliferative marker that has been studied in PPGL. It was shown to be predictive of the occurrence of metastases in several studies, but with a low sensitivity, ranging from 35 to 50% according to the studies [11, 12, 15, 16]. In this current study, Ki-67 correlated indeed with PFS ($P < 0.0001$), but not with the occurrence of metastasis ($P = 0.233$). The most significant threshold in our study was ≥ 4%, while, in previous studies, the use of various thresholds was recommended, ranging from 2 to 5% [11, 12, 15, 16].

Among other proliferation markers, mini-chromosome maintenance (MCM) proteins and key proteins in the initiation of DNA synthesis and DNA replication [45] were shown previously associated with histological grades in various neoplastic processes [36, 46–54]. For example, higher expression of MCM2 and MCM5 correlated with shorter survival in breast and urothelial cancers [53, 54]. Among the MCM proteins, MCM6 correlated with shorter survival in mantle cell lymphoma [50]. We recently also reported an inverse correlation with overall survival in lung cancer [36]. Our study in meningioma demonstrated as well the expression of MCM6 correlating strongly with high histological grades and a short progression-free survival [35]. In PPGL, this study here is the first to assess the prognostic value of MCM6; in these tumors it indeed correlates with both PFS ($P = 0.0003$) and the occurrence of metastasis ($P < 0.0001$). Its prognostic value surpasses thus that of the classical Ki-67 LI (ROC under-curve area, 0.851 for MCM6 vs. 0.619 for Ki-67). Nevertheless, the sensitivity and specificity of MCM6 are not sufficient enough to be used alone to predict the risk of metastasis.

Loss of PS100

Another interesting result of our study concerns the prognosis value of the loss of sustentacular cells. The absence of these cells is frequently cited as a metastatic prognostic marker [12, 15, 17, 55, 56]. In our study, 5 out of the 9 metastatic cases (56%) showed a loss of sustentacular cells, with a significant difference between the non-metastatic group and the metastatic group. But, like other immunohistochemical markers, this marker alone is not sensitive enough for use independently without other criteria.

Loss of SDHB

Loss of SDHB expression can be detected immunochemically with high sensitivity and specificity [22, 57–59]. In our present study as well as in previous studies, this loss is strongly correlated to mutations of SDHs (SDHB, SDHC, or SDHD). Despite so, 5 cases showing SDHB loss of expression in our cohort have no detectable mutations. This may stem from the fact that SDH mutations can escape detection by the DNA sequencing and MLPA methods, or that *SDH*s genes may be epigenetically silenced. F. H van Nederveen and colleagues thus recommended that these patients be followed as patients with hereditary syndrome [58].

Given the significant association of SDHB loss with metastasis occurrence in both univariate and multivariate analyses ($P = 0.023$ and $P = 0.024$, respectively), it was included in the COPPS. The excellent reproducibility of SDHB immunohistochemistry rating (Kappa correlation coefficient, 0.871) also advocates its use as a routine marker, both for prognostic and genetic purposes.

Establishment of the COPPS, a new prognostic score

To date, no prognostic parameter is reliable enough to evaluate the risk of recurrence or of metastasis. However, metastatic risk assessment is an important part of the patient care; it complements clinical, biological, and radiological surveillance of the patient, and can potentially serve as an indication for adjuvant treatment. The PASS, in our experience, is strongly correlated with PFS and the metastatic status, with an overall good reproducibility. However, its sensitivity (66.7%) in our study was not sufficient enough to predict the risk of metastasis.

We have designed here the COPPS, a new composite score that takes into account a number of pathological (tumor size, necrosis, vascular invasion) and immunohistochemical (PS100, SDHB) elements associated to metastatic status. In this scoring system, we have excluded cell monotony because of its relatively low reproducibility (Kappa correlation coefficient, 0.412). Our data here show that this score correlates strongly with PFS and the metastatic status ($P < 0.0001$), and has excellent sensitivity (100.0%) with high specificity (94.7%). COPPS is, to our best knowledge, the first score to reach such prognostic performances, and is more reproducible than PASS. Despite this, confirmations of the prognostic power of the COPPS will have to be performed in other cohorts to validate our results here, since like all retrospective studies, our cohort is subjected to unaccounted bias, as well as certain limitations. These include the low number of metastatic events studied despite that a relatively large number of cases are

included. PPGL studies are quite difficult to design because these tumors are rare, with a low rate of events. This is why a very large number of patients and a long clinical follow-up are required.

Conclusion

In conclusion, we have designed a new composite score, the COPPS, which allowed a better prediction of the risk of metastasis and progression with high sensitivity and reproducibility in our series. It is an easy-to-use score that integrates a gross pathological feature (tumor size) with several well-defined histopathological criteria, and two immunohistochemical markers (PS100, SDHB). In the present cohort, its sensitivity surpasses largely that of the PASS. This new score system could potentially be a powerful and reliable tool to revamp the follow-ups of patients as a function of their tumor progression. Further studies are needed to confirm these results, and to evaluate its inter-laboratory reproducibility.

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Contributions G.G. designed the study, developed the methodology, performed the statistical analyses, and wrote the manuscript. M.A. and H.B.-V. designed the study, developed the methodology, collected the data, and wrote the manuscript. Ch.P. collected and analyzed the data and wrote the manuscript. L.B. designed the study, developed the methodology, and collected the data. G.W. designed the study, developed the methodology, and collected the data. S.-F.B.-H. wrote and edited the manuscript. A.M., Ce.P., and C.N. collected and analyzed the data. S.L. developed the methodology and collected the data. J.-M.V. developed the methodology and edited the manuscript. A.O. performed the statistical analyses and wrote the manuscript. All authors have reviewed and approved the manuscript.

Compliance with ethical standards

The experiments reported here were carried out according to the Declaration of Helsinki principles and in agreement with the French laws on biomedical research (institutional review board n°DC2008-459; CNIL declaration n°1209171).

Conflict of interest The authors declare that they have no conflict of interest.

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