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The validation of matrix metalloproteinase-9 mRNA gene expression as a predictor of outcome in patients with metastatic gastric cancer

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Background: The prognostic role of matrix metalloproteinase-9 (MMP-9) in metastatic gastric cancer has not been validated.

Patients and methods: We carried out a molecular analysis in 222 metastatic gastric cancer patients obtained from clinical trials. We assessed the messenger RNA (mRNA) expression of MMP-9, vascular endothelial growth factor

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receptor-A, and epidermal growth factor receptor in a training cohort of 130 patients and conducted an independent validation in 92 patients. Automated RNA extraction from paraffin and RT-quantitative PCR was used. Immunohistochemistry for MMP-9 and diverse immune cell infiltrates was conducted.

Results: In the training cohort, only MMP-9 significantly correlated with patient's survival. At the cut-off with the highest predictive value, 19% of patients had MMP-9 expression above this cut-off and these showed a median survival of 3.6 months compared with 10.5 months ($P = 1.7e^{-6}$) in patients with lower expression. Corresponding 1- and 2-year survivals were 9% and 44% and 0 and 21%, respectively. The application of this cut-off to the validation cohort revealed similar distributions of overall survival according to MMP-9 expression on uni- ($P < 0.001$) and multivariate analyses ($P < 0.001$). No differences in survival according to MMP-9 below best cut-off were found. MMP-9 protein assessed by immunohistochemistry was not prognostic.

Conclusion: MMP-9 mRNA expression above a certain cut-off level is associated with dismal survival.

Key words: esophagogastric cancer, gastric cancer, gastroesophageal cancer, matrix metalloproteinase-9, RNA

introduction

With 737 000 deaths in 2008, gastric cancer represents the second most common cause of cancer deaths worldwide [1]. The disease is often diagnosed in late stages. Indeed, almost 50% of new cases have metastatic or locally unresectable disease and 50%–70% of surgically resected patients relapse within 2 years. In these cases, chemotherapy can extend survival, but 1- and 2-year survival rates rarely exceeded 40% and 10%, respectively, and chemotherapy is generally palliative with no real chance of long-term survival [2, 3].

There is a considerable lack of understanding of the molecular biology of gastric cancer and molecular prognostic factors have not been established yet. Clinical factors such as weight loss and number and location of metastases appeared to influence patient outcome [4] but none of them have been uniformly accepted. Recently, it has been demonstrated that the human epidermal growth factor receptor 2 (HER2) antibody trastuzumab improved outcomes in patients with metastatic gastric cancer who had HER2-positive tumors [5]. This effective treatment can now be applied to ∼15% to 20% of gastric cancer patients and strongly supports the concept that a greater understanding of the biology of gastric cancer may improve treatment selection and outcome of individual patients. Therefore, the identification of additional subsets of patients with distinct molecular profiles is highly warranted.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases that play an important role in various physiological and pathological processes, including invasion and metastases of cancer cells. MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which are produced by both nonmalignant and malignant cells, degrade various components of the extracellular matrix [6]. This enables cell invasion but also liberates ligands of growth factor receptors such as the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR) from the extracellular matrix [7]. Indeed, several reports indicated an important interplay between MMPs, EGFR, VEGFR and related molecules in tumor pathogenesis [8–12]. Specifically, MMP-9 has been shown to play an important role in tumor-induced VEGF-depended angiogenesis and in prepping organs for the formation of distant metastases in a manner dependent upon VEGFR-1 [8, 13, 14].

In the present study, we investigated the association between tumoral VEGF-A, EGFR, and MMP-9 messenger RNA (mRNA) and clinical outcome in metastatic gastric cancer. We identified a subgroup of patients with high MMP-9 levels and dismal survival and validated MMP-9 mRNA threshold expression as a robust and strong prognostic marker in these patients.

methods

study population

A total of 222 patients with metastatic adenocarcinoma of the stomach or esophagogastric junction were evaluated. The training cohort consisted of 130 patients, treated in a first-line phase III trial [15]. The validation cohort consisted of 92 patients, more recently treated within two subsequent first-line phase II trials [16, 17]. Table 1 presents baseline characteristics and treatments of patients in the training and the validation cohorts. One study, the FLOT65+ [17], contained a stratum of operable patients. This was excluded from the analysis. Patients gave informed consent on sample collection and analysis, which was approved by the responsible ethic committee. Standards of the International Conference on Harmonization/ World Health Organization (WHO) Good Clinical Practice were followed.

sample preparation and RNA extraction

Formalin-fixed paraffin-embedded tissue samples obtained before the start of chemotherapy were collected. From each tumor block, a 5-µm section was stained with hematoxylin–eosin and revised by a pathologist and two consecutive 10-µm sections were cut on a standard microtome, placed into individual tubes, and stored at 4°C for ∼1 month until RNA extraction. Fully automated high-throughput RNA extraction has been carried out according to methods previously published [18].

gene expression analysis using quantitative PCR

Expression of *MMP9*, *VEGFA*, *EGFR* and the normalization (housekeeping) gene *RPL37A* was assessed by one-step RT-quantitative PCR (qPCR). SuperScript® III Platinum® One-Step qRT-PCR System with ROX (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions. Experiments were carried out on an ABI PRISM® 7900HT (Applied Biosystems, Darmstadt, Germany) with 30 min at 50°C, 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Relative copy numbers positively correlating with the expression of the genes of interest were calculated by using the $2^{(40-\Delta\Delta CT)}$ method. Each mRNA expression was adjusted with the housekeeping gene.

For assessment of DNA contamination in RNA preparations, a *PAEP* gene-specific qPCR without preceding reverse transcription was carried out using the reagents from the SuperScript III® Platinum® One-Step qRT-PCR System with ROX and Taq DNA Polymerase. In samples with a Cq value <35, the DNase I treatments were repeated to prevent effects on bispecific

Table 1. Patient's characteristics by cohort

	Training cohort ($n = 130$)		Validation cohort ($n = 92$)	
	n	%	*n*	%
Age				
Median (range)	63 (33–86)		69 (29–81)	
Sex				
Female	46	35	27	29
Male	84	65	65	71
Primary tumor site				
Esophagogastric junction	22	17	26	28
Gastric	108	83	66	72
ECOG PS				
Median	1		1	
0–1	114	88	82	89
2–3	15	12	10	11
NK	1	1	0	0
Disease status				
Newly diagnosed	93	72	72	78
Recurrent	37	29	20	22
Origin of sampling				
Primary	106	82	86	94
Metastatic lesion	24	19	6	7
Type of histology according to Lauren				
Intestinal type	62	48	42	46
Diffuse type[a]	64	49	49	53
NK	4	3	1	1
No. of organs involved				
Median	2		2	
1	30	23	14	15
2	45	35	33	36
3	42	32	28	30
4	8	6	13	14
≥5	5	44	4	4
Organs involved				
Liver	67	52	41	45
Lymph nodes	55	42	49	53
Peritoneum	45	35	52	57
Lung	12	9	15	16
Bone	9	7	5	5
Ascites	11	9	0	0
Chemotherapy applied				
5-FU, cisplatin, leucovorin	66	51	0	0
5-FU, oxaliplatin, leucovorin	64	49	29	32
5-FU, oxaliplatin, leucovorin, docetaxel	0	0	63	69

[a]Includes mixed type histology.
ECOG PS, East Cooperative Oncology Group performance status; NK, not known; 5-FU, 5-fluorouracil.

PCR assays. Stratagene human QPCR Reference total RNA (Stratagene, Waldbronn, Germany) was used as positive control for RT-qPCR and human genomic DNA (Roche Diagnostics, Basel, Switzerland) as positive control for qPCR. All PCR assays were carried out in triplicate, and the mean of triplicates was reported. Kinetic RT-PCR was applied for the assessment of mRNA expression using the following TaqMan™-based primer/probe set™-based primer/probe set (Eurogentec, Seraing, Belgium):

EGFR probe CCTTGCCGCAAAGTGTGTAACGGAAT
Forward primer CGCAAGTGTAAGAAGTGCGAA
Reverse primer CGTAGCATTTATGGAGAGTGAGTCT
VEGF-A probe CACCATGCAGATTATGCGGATCAAACCT
Forward primer GCCCACTGAGGAGTCCAACA
Reverse primer TCCTATGTGCTGGCCTTGGT
MMP-9 probe CAGGCAGCTGGCAGAGGAATACCTGTAC
Forward primer CCCTGGAGACCTGAGAACCA
Reverse primer CCACCCGAGTGTAACCATAGC

immunohistochemistry

Immunohistochemistry was carried out in the training cohort only. Sections (2- to 3-μm thickness) were fixed in acetone and immunostained by indirect immunoperoxidase method (DAKO, Glostrup, Denmark) as recommended by the manufacturer. Monoclonal antibodies against the T cell marker CD8 (C8/144B), the macrophage marker CD68 (PG-M1), the natural killer cell marker CD56 (123C3), and the mast cell tryptase (AA1) and a polyclonal antibody against MMP-9 were used. The sections were examined by a pathologist and an additional investigator trained in the histopathology of stomach cancer. For scoring, a semiquantitative approach on a scale of 1+ to 3+ (scattered or mild 1+, moderate 2+, and strong 3+) was used. The immunohistochemical expression of MMP-9 was assessed separately in the tumoral tissue and nontumoral (stroma) cells.

statistics

The clinical trials analyzed here used identical definitions for clinical outcomes. Responses were classified according to the WHO criteria. Progression-free survival (PFS) was measured from the date of assignment until disease progression or death of any cause. Overall survival (OS) was measured from date of assignment until death of any cause. Time-to-event curves were calculated by the Kaplan–Meier method and the log-rank test was applied. The cut-offs with the highest predictive values for OS were estimated using the JMP 9.0.0 software (SAS Institute Inc., Cary, NC). The Cox regression model was used for the multivariate analysis. All *P* values were two-sided with *P* values <0.05 indicating statistical significance.

results

the training cohort

In the training cohort, we measured the mRNA expression of three genes: *VEGFA, EGFR,* and *MMP-9*. Then, for each we looked for the expression cut-off with the highest predictive value for OS (best cut-off). The identified best cut-offs were 1616, 219, and 777 for VEGF-A, EGFR, and MMP-9, respectively, where the unit is a relative copy number as described above. OS according to best cut-off is shown in Figure 1A–C. VEGF-A showed a statistical trend ($P = 0.064$) toward longer OS for patients with low-VEGF-A expression (Figure 1A), while EGFR did not result in any survival differences (Figure 1B). In contrast, MMP-9 strongly separated patients' survival, with median OS being 3.6 versus 10.5 months ($P < 0.001$), favoring the group with low MMP-9 mRNA levels (Figure 1C). Overall, 24 of 130 (19%) patients had MMP-9 mRNA values more than or equal to the best cut-off and 106 (82%) patients had values less than the best cut-off. For patients with high versus low MMP-9 expression, 1- and

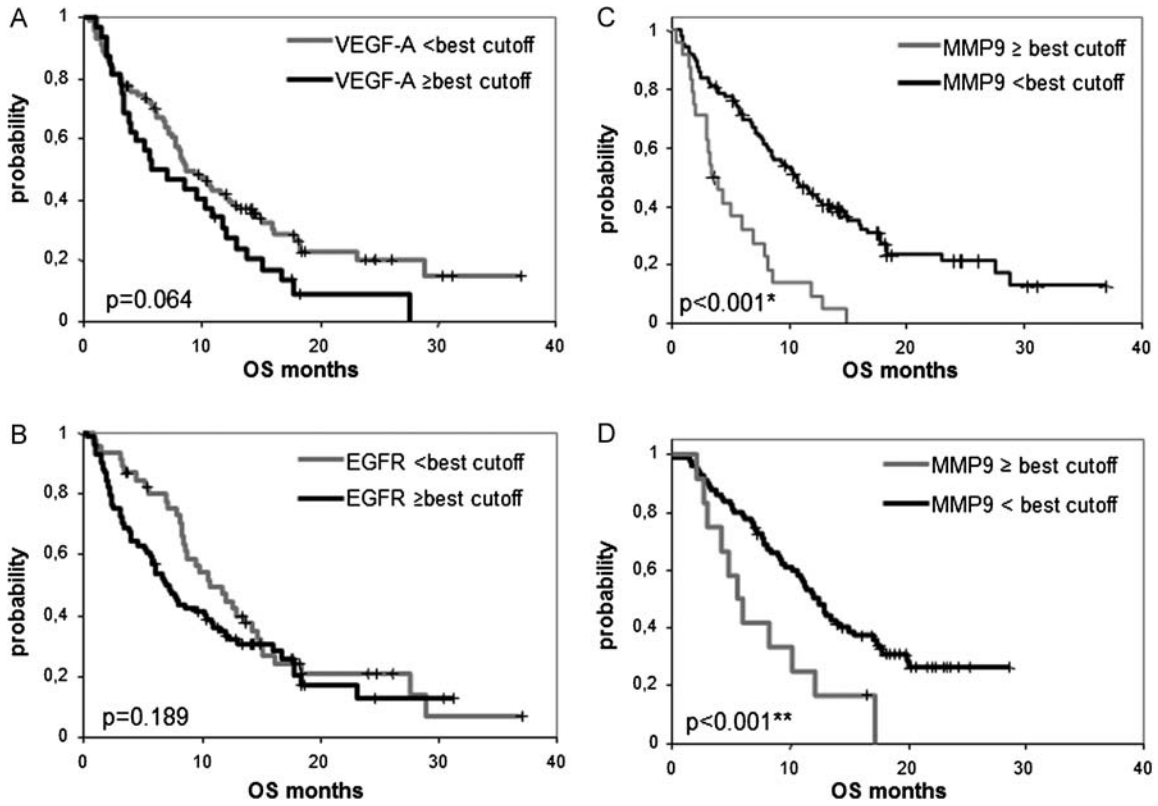

Figure 1. Overall survival (OS) according to the best cut-off expression of (A) vascular endothelial growth factor (VEGF)-A, (B) epidermal growth factor receptor (EGFR), and (C) matrix metalloproteinase-9 (MMP-9) messenger RNA in the training cohort. (D) OS according to the prespecified cut-off expression of MMP-9 in the validation cohort. *$P = 1.7e^{-6}$, **$P = 0.000765$.

2-year survival rates were 9% and 44% and 0% and 21%, respectively. MMP-9 mRNA expression at this cut-off also significantly predicted differences in PFS (2.1 versus 4.8 months, P <0.001) and response rates (complete + partial response: 8% versus 30%, $P = 0.022$).

the validation cohort

Compared with the training cohort, the validation cohort patients were older, with a larger proportion of patients receiving three-drug combinations (Table 1), and with better outcome. The adjusted hazard ratio (HR) for survival was 1.4 favoring the validation cohort ($P = 0.042$).

The application of the prespecified cut-off to the validation cohort showed that 12 (13%) patients were assigned to the high risk with a median OS of 5.5 months, and 80 (87%) patients assigned to the low risk with a median OS of 11.9 months (P <0.001), confirming that the test was able to stratify patients into different prognostic groups in an independent cohort (Figure 1D). The corresponding 1- and 2-year survival rates were 25% and 50% and 0% and 26% for patients with high and low risk, respectively.

pooled cohort

In order to generate a robust data cohort for further analyses, we pooled the training and validation cohorts ($n = 222$). The application of the prespecified cut-off resulted in the Kaplan–Meier survival curve shown in Figure 2A. Overall, 16% of the patients had MMP-9 mRNA levels more than or equal to the cut-off and this significantly predicted all outcome parameters, i.e. overall response rates (14% versus 38%, $P = 0.035$), median PFS (2.16 versus 5.2 months, P <0.001), and OS (4.3 versus 11.3 months, P <0.001).

In the pooled cohort, a significant association between MMP-9 mRNA levels and OS was still detectable when patients were alternatively analyzed according to the median distribution of MMP-9 mRNA expression (≥median versus <median, data not shown) or when the patients with the highest quartile of MMP-9 mRNA expression were compared with the rest (Figure 2B). However, the patients with the second, third, and lowest quartiles of MMP-9 expression did not have different OS (Figure 2B), indicating a nonlinear threshold association of MMP-9 and survival.

Similar distributions of OS according to MMP-9 expression were also observed in the subgroup analysis based on tumor histology (intestinal versus diffuse type; Figure 2C and D).

We compared the baseline criteria of the patients who overexpressed MMP-9 with those of the remaining patients in order to figure out whether MMP-9 overexpression can be linked to certain clinical features. Interestingly, patients with MMP-9 overexpression did not differ from the remaining patients in terms of all important baseline criteria, including age, sex, primary tumor site, East Cooperative Oncology Group performance status, histology according to Lauren, and the type and the median number of organs involved in metastatic disease (supplemental Table S1, available at *Annals of Oncology*

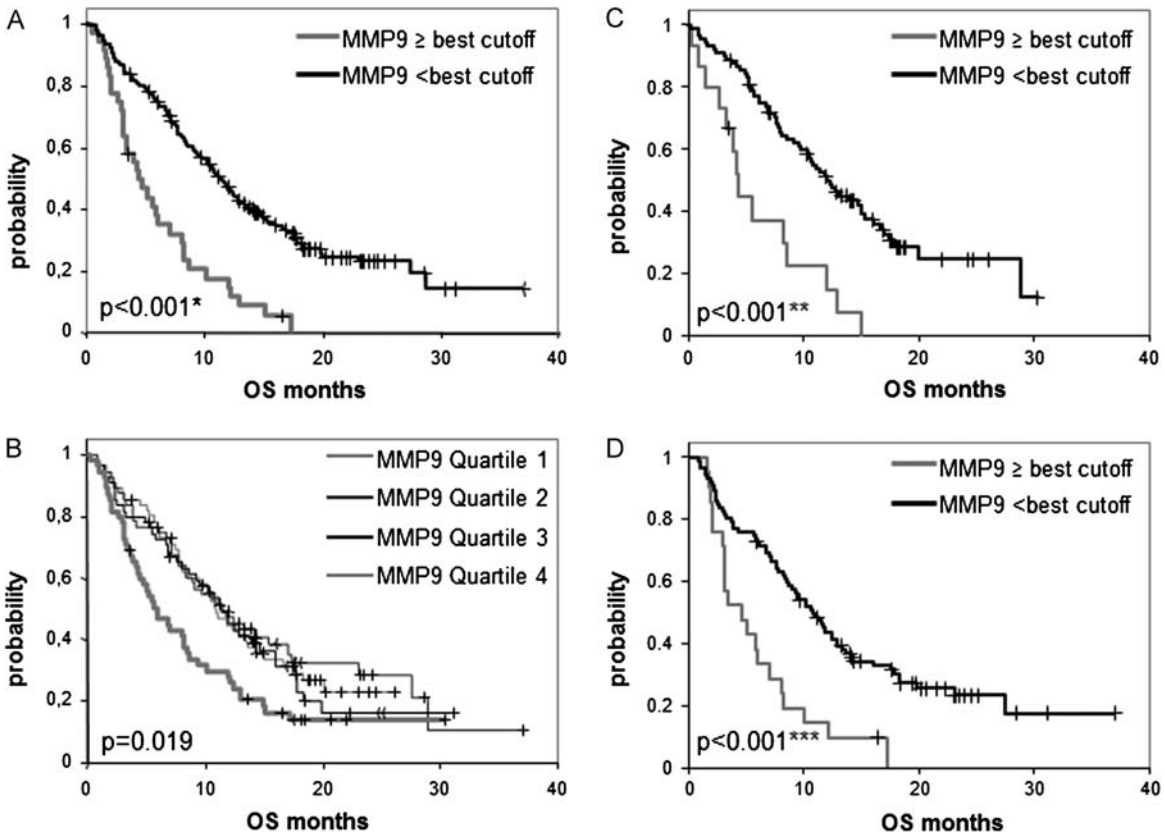

Figure 2. Distributions of overall survival (OS) in the pooled cohort. (A) OS according to the matrix metalloproteinase-9 (MMP-9) messenger RNA (mRNA) expression at the best cut-off in the total population. (B) OS according to the quartile distribution of MMP-9 mRNA expression in the total population. (C) OS according to MMP-9 at the best cut-off in the intestinal type subgroup. (D) OS according to MMP-9 expression at the best cut-off in the diffuse type subgroup. $^{*}P = 3.7e^{-8}$, $^{**}P = 7.3e^{-5}$, $^{***}P = 0.000104$.

Table 2. Multivariate Cox regression analysis for overall survival (pooled cohort)

Parameter	Hazard ratio	95% CI (±)	*P* value
MMP-9 (≥ versus <best cut-off)	2.89	0.42	<0.001[a]
Sex (male versus female)	1.19	0.37	0.362
Age (≥65 versus <65)	1.42	0.35	0.047
ECOG PS (>0 versus 0)	1.57	0.39	0.019
Disease status (newly diagnosed versus recurrent)	0.99	0.41	0.948
Stomach versus esophagogastric junction	1.11	0.42	0.613
No. of affected organs[b]	1.01	0.17	0.893
Liver metastasis (yes versus no)	1.31	0.38	0.172
Peritoneal involvement (yes versus no)	1.28	0.43	0.261
Histology (intestinal versus diffuse[c])	0.71	0.30	0.023

[a] $P = 1.16732e^{-6}$.
[b] Continuous parameter.
[c] Includes mixed type histology.
CI, confidence interval; ECOG PS, East Cooperative Oncology Group performance status; MMP-9, matrix metalloproteinase-9.

online). The only notable point was a slight trend toward a lower proportion of patients having one metastatic site only in the overexpression group (8% versus 22%, $P = 0.159$).

In the multivariate Cox regression model, the prognostic value of MMP-9 remained highly significant and was independent from clinical variables. This could be demonstrated for the training (data not shown), the validation (data not shown), and the pooled cohorts (Table 2). Further statistical adjustments using logistic regression showed that the association of MMP-9 expression with survival was also independent from potential confounding factors such as whether the evaluated samples were obtained by biopsy or by surgical resection and whether they were taken from metastatic lesions or primary tumors (HR for OS 2.48 favoring patients with MMP-9 expression below best cut-off, P <0.001).

To assess selection bias, we compared the patients we analyzed with the patients who were not analyzed in the training and the validation cohorts. There were no significant differences in baseline characteristics, PFS, or OS between the two groups (data not shown).

immunohistochemistry

MMP-9 immunoreactive protein was expressed in different cell types, including granulocytes, macrophages, stromal fibroblasts, and tumor cells. The intensity of the staining was highest in granulocytes and macrophages while the tumor cells were negative or showed a weak staining. Serial sections showed that the strongest and most consistent MMP-9 staining was found

in the areas where macrophages were present. No significant association between MMP-9 protein expression as assessed by immunohistochemistry and OS was found, neither when tumor cells or stromal cells were analyzed separately nor when a combined analysis was carried out.

We analyzed whether the overexpression of MMP-9 was associated with immune cell infiltrates that are implicated in the regulation or production of MMPs, such as the presence of macrophages, natural killer cells, mast cells, and CD8+ T cells in or around the tumor. In line with previous reports, the tumors were strongly infiltrated by CD8+ T cells and macrophages, which were detected within tumor cell islets, in peritumoral stroma, or both. Natural killer cells were not seen. Few mast cells were detected in the peritumoral stroma. None of these cell types showed a significant correlation to MMP-9 mRNA levels or to patient survival (data not shown).

discussion

The expression of several members of the MMP family has been shown to be associated with unfavorable clinical course in various cancer types. However, the prognostic value of MMPs has not been systematically validated and results have been conflicting in some studies [19]. In terms of gastric cancer, most of existing data refer to curatively resected patients. For instance, high levels of tissue MMP-2 and/or MMP-9, as determined by quantitative gelatin zymography, were significantly associated with poor survival in patients who underwent surgical resection [20, 21]. In a more recent study, gastric cancer specimens from 286 patients who underwent surgical resection were investigated by immunohistochemistry for MMP-9 expression (positive or negative). MMP-9 was significantly correlated with depth of invasion, lymph node, and distant metastasis as well as short PFS and OS [22]. In another immunohistochemistry study, neither MMP-2 nor MMP-9 was found to be independently prognostic in a series of 315 consecutive patients operated for gastric cancer [23]. In two earlier studies of surgically treated patients, the imbalance between the expression of MMP-9 protein and its inhibitor was shown to be associated with survival, but a clear correlation between MMP-9 itself and survival was not observed [23–25]. The conflicting results are most likely related to methodological aspects and to the heterogeneity of the patient populations evaluated in the respective studies.

Our study adds several important points to the existing data. First, our study evaluates metastatic disease, while the majority of previous works focused on operable gastric cancer. Second, it identifies and validates clear and fully quantitative cut-offs for MMP-9 in metastatic gastric cancer, which are linked to highly relevant differences in survival. In contrast, none of the previous works contained validation cohorts, and cut-offs were not reported because the methods used were semiquantitative. And third, our study demonstrates, for the first time, that the association between MMP-9 and outcomes is nonlinear.

It appeared that MMP-9 negatively correlated with survival only when a certain threshold of MMP-9 expression was met (overexpression). While patients with the highest quartile of MMP-9 expression significantly had worse OS, no between-group differences with regard to OS were found among the second, third, and lowest quartiles. One explanation for this phenomenon could lie in the MMP/tissue inhibitors of MMP (TIMPs) balance theory. Usually, up-regulation of MMPs is assumed to be associated with similar up-regulation of TIMPs and, particularly, altered balance between MMPs and TIMPs has been found to associate with numerous malignant and nonmalignant pathological conditions [26, 27]. In our case of metastatic gastric cancer, one may speculate that a certain threshold amount of MMP-9 levels was required to overcome the physiological capacity for TIMPs up-regulation, resulting in a negative effect on patient's survival.

The magnitude of the effect on OS was clinically highly relevant. The median OS was more than twice as high in patients without as in those with MMP-9 overexpression. Patients with MMP-9 overexpression had a 9%–25% chance (versus 44%–50%) of surviving 1 year and no chance (versus 21%–25%) of surviving 2 years, considering the training and validation cohorts, respectively. MMP-9 expression was not associated with any baseline clinical or pathological characteristics reflecting a unique role in influencing prognosis, i.e. independent from other known prognostic variables.

One important observation in our study is that immunohistochemistry was not as useful as RT-qPCR in detecting the prognostic role of MMP-9. Highly accurate and reproducible measurement of MMP-9 using immunohistochemistry was difficult because conventional immunohistochemistry methods are semiquantitative and not uniformly standardized. For instance, in our immunohistochemical studies, it was nearly impossible to distinguish positive from negative MMP-9 tumor samples because all samples contained positive cells, either in the tumor or in the surrounding stroma. Furthermore, adequate quantification of the intensity of the staining was difficult and did not allow for the determination of intensity thresholds.

The important question is where these results fit into the evolving field of metastatic gastric cancer therapy. Overall, 16% of the patients showed MMP-9 overexpression. This group, which is small but still significant, did not seem to benefit from chemotherapy, where treatment results were quite similar to those achieved with best supportive care alone [28]. Therefore, it seems to be justified to exclude this group of patients from conventional chemotherapy trials, in order to include them into those utilizing specific targeting agents.

A number of rationally designed MMP inhibitors have shown promise in preclinical investigations. However, these have carried out poorly in clinical trials. The failure of these drugs was largely due to toxicity (particularly musculoskeletal toxicity) and failure to show expected results. Notably, all studies lacked a sufficient biomarker in which they did not stratify patients for MMP expression. In addition, at least in terms of gastric cancer, the studies were not fully negative. One phase III trial randomized 369 patients with inoperable stomach cancer to receive either the broad spectrum MMP inhibitor marimastat or placebo [29] as a maintenance treatment with OS as the primary end point. The study indeed missed its primary objective but the HR (1.23) and the *P* value ($P = 0.07$) were clearly in favor of the patient group treated with marimastat, indicating that MMP inhibition may have activity in a subgroup of gastric cancer patients. Other new

MMP inhibitors, e.g. the tetracycline analog COL-3, are currently under investigation [30]. Future trials utilizing new drugs that interact with the MMP pathway should pay attention to the particularities associated with MMP-9 expression in gastric cancer. For instance, the poor prognostic group patients could be selected for such trials since these patients may be more likely to benefit from MMP-9 inhibition. Due to the short survival expected in this group, MMP-9 inhibitors could be used in an early setting, e.g. in addition to a well-tolerated first-line chemotherapy.

Further research should focus on defining new prognostic cut-offs for patients receiving neoadjuvant chemotherapy for operable stages and on determining factors, cell types, or interactions that are primarily associated with MMP-9 overexpression.

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disclosure

The authors declare no conflicts of interest.

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