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# The Effect of Clinical Covariates on the Diagnostic and Prognostic Value of Soluble Mesothelin and Megakaryocyte Potentiating Factor

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*Background:* Soluble mesothelin (SM) and megakaryocyte potentiating factor (MPF) are serum biomarkers of mesothelioma. This study examined the effect of clinical covariates on biomarkers levels and their diagnostic and prognostic value.

*Methods:* Five hundred ninety-four participants were enrolled in a multicenter study, including 106 patients with mesothelioma and 488 control subjects. Multiple linear regression analyses were used to identify which covariates were independently associated with SM and MPF levels. The effect of these covariates on the diagnostic accuracy was evaluated with receiver operating characteristics curve analysis. In patients with mesothelioma, survival analysis was performed with Cox regression.

*Results:* SM and MPF levels were independently associated with age, glomerular filtration rate (GFR), and BMI in control subjects and with GFR and tumor stage in patients with mesothelioma. The diagnostic accuracy of SM and MPF was significantly affected by the distribution of these covariates in the study population. The patients with mesothelioma were best discriminated from the control subjects with either the youngest age, the highest GFR, or the largest BMI. Furthermore, the control subjects were significantly better differentiated from stage II to IV than from stage I mesothelioma. MPF, not SM, was an independent negative prognostic factor, but only if adjusted for the biomarker-associated covariates.

*Conclusions:* SM and MPF levels were affected by the same clinical covariates, which also had a significant impact on their diagnostic and prognostic value. To improve the interpretation of biomarker results, age, GFR, and BMI should be routinely recorded. Approaches to account for these covariates require further validation, as does the prognostic value of SM and MPF.

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Malignant pleural mesothelioma is a fatal asbestosrelated malignancy.<sup>1</sup> Soluble mesothelin (SM) and megakaryocyte potentiating factor (MPF), two products of the mesothelin gene,<sup>2</sup> are promising serum biomarkers of mesothelioma.<sup>3,4</sup> Both biomarkers might serve as an adjunct in different fields of mesothelioma management, including diagnosis and prognosis. For further validation and clinical application, it is important to elucidate to what extent clinical

covariates affect the biomarker results, and to what extent they should be accounted for.

We and others have shown that SM levels in serum are affected by different covariates, including age, glomerular filtration rate (GFR), and BMI.<sup>5-9</sup> Such associations might influence the diagnostic accuracy (eg, by causing false-positive results). However, the true consequences for the ability of SM to differentiate control subjects from patients with mesothelioma

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are unclear, because the effect of covariates has been evaluated in studies with a "control-subjectsonly" design.<sup>5-9</sup> In addition, the association between covariates and SM levels might affect the evaluation of its prognostic value. Currently, several studies have reported an association with survival in mesothelioma,<sup>10-12</sup> whereas others have failed to do so.<sup>13,14</sup> Although these contradictory data are probably caused by limited sample sizes, none of these studies evaluated whether correcting SM levels for the associated covariates had an impact on the results. MPF has emerged recently as a potential alternative to SM,<sup>4</sup> but neither the effect of covariates, nor its prognostic value, has been evaluated previously.

The aims of this prospective multicenter study were the following: (1) to assess which clinical covariates were associated with SM and MPF levels, (2) to determine whether these covariates had an impact on diagnostic accuracy, and (3) to evaluate whether SM and MPF levels, adjusted for the associated covariates, were prognostic factors of survival in mesothelioma.

# MATERIALS AND METHODS

#### Participants

Between June 2004 and 2010, consecutive individuals were recruited prospectively in a multicenter study. Control subjects included five cohorts: (1) healthy individuals, aged 50 years or

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older, without reported asbestos exposure; (2) individuals with documented asbestos exposure; and patients with (3) a benign non-asbestos-related respiratory condition, (4) lung cancer, and (5) a nonrespiratory epithelial cancer. Cases included patients with histologically proven mesothelioma of the pleura. Patients with cancer had to be treatment naive at inclusion. In the asbestosexposed cohort, the presence of asbestos-related lesions was assessed radiologically. Mesothelioma staging was done with International Mesothelioma Interest Group criteria.<sup>15</sup> The study was approved by the institutional review boards of all participating hospitals (approval no. 2007147), according to the Helsinki recommendations. Written informed consent was obtained from all participants. A large subset of these participants (422 control subjects and 85 case subjects) was previously reported in a study that compared the diagnostic accuracy of SM and MPF.<sup>4</sup>

#### Clinical Characteristics

From all participants, data on sex, race (white or other vs black), age, BMI (kg/m<sup>2</sup>), serum creatinine levels (mg/dL), and smoking history (pack-years) were collected at inclusion. Creatinine was measured by the rate-blanked compensated Jaffé method on a Modular P analyzer (Roche Diagnostics). These values, together with sex, race, and age, were included in the Chronic Kidney Disease Epidemiology Collaboration equation to estimate the GFR (mL/min/1.73 m<sup>2</sup>) of each participant.<sup>16</sup> A GFR above 90 mL/min/1.73 m<sup>2</sup> is considered normal,<sup>17</sup> whereas a lower value represents a decrease in renal function. In patients with mesothelioma, performance status (PS),<sup>18</sup> serum C-reactive protein (CRP) (normal range, 0-0.5 mg/dL), WBC count (normal range, 4-10 10<sup>3</sup>/µL), hemoglobin (normal range, 13.3-17.7 g/dL), and platelet count (normal range, 140-409 10<sup>3</sup>/µL) values were recorded at inclusion.

#### Biomarker Assays

Serum SM (nmol/L) and MPF levels (ng/mL) were measured with the Mesomark (Cis bio International, Gif sur Yvette, France) and Human MPF enzyme-linked immunosorbent assays (Medical and Biologic Laboratories), respectively, according to the manufacturer's instructions.<sup>19,20</sup> Analyses were performed blinded to the coded sample data.

#### Statistical Analysis

Continuous variables were reported as median with 25th to 75th percentile values, and compared using the Kruskal-Wallis and Mann-Whitney U tests. Correlation analysis was performed with the Spearman rank test. To identify the covariates that were independently associated with SM and MPF levels, multiple linear regression analysis was applied in control subjects and cases separately. The effect of the associated clinical covariates on the diagnostic accuracy of SM and MPF was evaluated with receiver operating characteristics curve analysis. For each continuous covariate separately, all control subjects were stratified according to the covariate quartiles, after which the resulting groups of control subjects were differentiated from all patients with mesothelioma. For tumor stage, all control subjects were differentiated from the patients with epithelioid stage I and stage II to IV mesothelioma, respectively. The differences in area under the curve (AUC) were evaluated with the method of Hanley and McNeil.<sup>21</sup> Survival analysis was performed with univariate and multivariate Cox proportional hazards regression. Covariates with a P value < .10 in the univariate analysis were included in a multivariate analysis, together with the biomarker-associated covariates. The SM and MPF thresholds that best differentiated those with favorable from those with poor prognoses were searched both manually and with an algorithm of maximization of hazard

ratio (HR).<sup>22</sup> Overall survival was computed as the number of days from inclusion until time of death. Patients who were alive on December 1, 2010, were censored on that date. All hypothesis tests were performed two-sided at the 5% significance level. Statistical analyses were done with SPSS, version 17 (SPSS Inc) and SAS, version 9.2 (SAS Institute Inc).

#### Results

### Participant Characteristics

A total of 594 individuals were included: 106 patients with histologically proven malignant pleural mesothelioma and 488 control subjects including 101 healthy and 215 asbestos-exposed individuals, 78 patients with a benign non-asbestos-related respiratory disease, 69 with a lung cancer, and 25 with a nonrespiratory epithelial cancer (Table 1). All participants were white or other, and none was black. In the cohort of asbestos-exposed individuals, 71 had pleural plaques, 39 diffuse pleural thickening, and 16 other lesions, mainly asbestosis, whereas 89 had no radiologically obvious asbestos-related lesions. From those with a benign respiratory disease, 49 patients had asthma or COPD, and 29 had a pleural effusion. The cohort with nonrespiratory cancer consisted of six breast, four gynecologic, and 15 GI carcinomas. In the patients with mesothelioma, 91 had an epithelioid, eight a biphasic, and seven a sarcomatoid histology. Twentyfour, 24, 36, and 22 patients had a tumor in stage I, II, III, and IV, respectively. Ninety patients had a PS of 0 to 1, and 16 a PS of 2 to 4. Additional characteristics, including age, creatinine levels, GFR, and BMI, are displayed in Table 1.

# **Biomarker** Levels

Each of the control cohorts had significantly lower SM and MPF levels than the patients with mesothelioma (SM, P < .001; MPF, P < .001) (Table 1). Biomarker levels of control subjects with a lung or nonrespiratory cancer did not differ significantly from those without a malignancy (SM, P = .54; MPF, P = .22). Patients with stage I mesothelioma had significantly lower biomarker levels than those with stages II to IV (SM, P < .001; MPF, P < .001). The former group had a median SM and MPF level of 1.52 nmol/L (1.25-2.07 nmol/L) and 10.90 ng/mL (7.02-17.02 ng/mL), respectively. The latter had a median SM and MPF level of 2.83 nmol/L (1.73-7.73 nmol/L) and 60.75 ng/mL (26.54-126.50 ng/mL), respectively. Biomarker levels did not differ significantly according to mesothelioma histology (SM, P = .11; MPF, P = .14).

#### Covariates and Biomarker Levels

In control subjects, SM and MPF levels were positively correlated with age (SM, r = 0.33, P < .001; MPF, r = 0.30, P < .001) and negatively with GFR (SM, r = -0.26, P < .001; MPF, r = -0.23, P < .001)and BMI (SM, r = -0.15, P < .01; MPF, r = -0.10, P < .05). In the multiple linear regression analysis, all three covariates were independently associated with SM and MPF, with an adjusted squared multiple correlation coefficient  $(R^2)$  of 12.7% and 10.0%, respectively. Neither sex nor smoking history displayed a significant association (e-Table 1). In patients with mesothelioma, SM and MPF correlated with GFR (SM, r = -0.20, P < .05; MPF, r = -0.18, P < .05),but not with age or BMI (e-Table 1). In addition, MPF, not SM, correlated positively with platelet count (r = 0.21, P < .05) and CRP (r = 0.27, P < .01). In the multiple linear regression analysis, however, only GFR and tumor stage (I vs II-IV) were independently associated with SM and MPF, with an adjusted  $R^2$  of 14.2% and 13.0%, respectively. Neither age and BMI, nor sex, smoking history, tumor histology, CRP, or any of the blood count parameters were significant (e-Table 1). Similar to GFR, creatinine levels correlated with SM and MPF in control subjects (SM, r = 0.17, P < .001; MPF, r = 0.14, P < .01) and in patients with mesothelioma (SM, r = 0.24, P < .05; MPF, r = 0.19, P < .05). When incorporating creatinine instead of GFR in the multiple linear regression models, this covariate also displayed an independent association with the biomarker levels (data not shown). Because

Table 1—Participant Characteristics

Characteristic Healthy		Asbestos-Exposed	Benign Respiratory Disease	Nonrespiratory Cancer	Malignant Pleural Mesothelioma	
No. (female)	101 (21)	215 (18)	78 (24)	69 (18)	25 (12)	106 (11)
SM, nmol/L	0.95 (0.72-1.12)	0.96 (0.74-1.37)	1.07(0.77 - 1.55)	1.03 (0.73-1.35)	1.06 (0.80-1.48)	2.31(1.45-5.04)
MPF, ng/mL	6.16 (5.24-7.78)	6.68 (5.28-8.92)	7.42 (5.26-9.97)	7.12 (6.32-9.80)	7.80 (5.64-11.78)	18.20 (9.41-47.25)
Age, y	56.2(53.5-59.9)	55.7 (51.9-66.1)	62.1 (49.0-70.9)	65.4(58.6-71.0)	66.7 (56.7-74.6)	65.0 (59.4-73.3)
Creatinine, mg/dL	0.99 (0.89-1.12)	1.02 (0.91-1.16)	0.97(0.81 - 1.25)	0.90 (0.80-1.10)	0.88 (0.73-1.07)	0.92 (0.75-1.13)
GFR, mL/min/1.73 m <sup>2</sup>	79.2 (69.1-88.8)	79.5 (66.2-90.8)	81.1 (56.3-94.4)	83.8 (67.0-91.4)	78.0 (59.2-91.5)	86.1 (65.1-96.6)
BMI, kg/m²	26.2 (23.7-28.7)	26.7 (24.6-30.0)	24.8 (22.6-27.7)	$24.2\ (22.0-27.7)$	$25.4\ (21.9-27.1)$	$24.8\ (23.1-27.1)$

Data are presented as median (25th-75th percentile) unless otherwise indicated. GFR = glomerular filtration rate; MPF = megakaryocyte potentiating factor; SM = soluble mesothelin.

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GFR is a better estimate of renal function than is creatinine,<sup>16</sup> the former was used in further analyses.

# Covariates and Diagnostic Accuracy

All 488 control subjects were stratified according to the quartiles of age, GFR, and BMI. For each covariate, this resulted in four groups of 122 individuals, which were differentiated from the 106 patients with mesothelioma. The resulting diagnostic accuracies of SM and MPF varied substantially across the different control groups, and improved steadily when either age decreased or GFR or BMI increased (Table 2). AUCs ranged from 0.79 to 0.92 for SM, and from 0.79 to 0.88 for MPF. For age and GFR, the difference in AUC was only significant between the 25% control subjects with the lowest and the highest covariates values, respectively (Fig 1, Table 2). For BMI, the difference in AUC between these two control groups only reached significance for SM (P < .05), although a similar trend was observed for MPF (P = .26). Sensitivities for mesothelioma, derived at 95% specificity, ranged from 36% to 69% for SM, and from 43% to 68% for MPF, across the stratified control groups. Similarly, the diagnostic thresholds, derived at 95% specificity, ranged from 1.67 to 3.34 nmol/L for SM, and from 11.16 to 22.54 ng/mL for MPF. The application of these thresholds across the different control groups resulted in large trade-offs in specificity. For example, in the 25% of control subjects with the highest GFR, the 11.16 ng/mL threshold yielded a 95% specificity for MPF. In the 25% of control subjects with the lowest GFR, the specificity of this threshold dropped to 21%. When differentiating all 488 control subjects from the 22 patients with epithelioid stage I mesothelioma and the 69 with epithelioid stage II to IV mesothelioma, SM and MPF had significantly higher AUCs in the latter (Table 2). Noteworthy, SM had a much lower sensitivity (23%) for stage I mesothelioma than did MPF (46%), although their AUCs were very similar.

# Covariates and Survival Analysis

From the 106 patients with mesothelioma, nine received no active treatment, 19 underwent a combined modality treatment (neoadjuvant chemotherapy, extrapleural pneumonectomy with or without radiation therapy), and 78 received cisplatin and/or pemetrexed-based chemotherapy. Survival analysis focused on the last group of patients, of whom 50 (64%) died. In the univariate analysis, tumor stage (I vs II-IV) and

		SM	MPF		
Design/Covariate	AUC (95% CI)	Sensitivity at 95% Specificity, % (Threshold, nmol/L)	AUC (95% CI)	Sensitivity at 95% Specificity, % (Threshold, ng/mL)	
Control subjects, stratified by the quartiles of each covariate, are differentiated from all 106 patients with mesothelioma					
Age, <sup>a</sup> y		00 (1 51)	0.07 (0.02,0.02)		
18.1-52.7	0.91 (0.88-0.95)	69 (1.71)	0.87 (0.82-0.92)	67 (11.92)	
52.8-58.1	$0.88 (0.84 - 0.92)^{b}$	67(1.77)	0.85 (0.80-0.91) <sup>b</sup>	67 (11.70)	
58.2-67.1	$0.86 (0.81 - 0.91)^{b}$	59 (2.02)	$0.84 (0.79 - 0.90)^{b}$	66 (13.28)	
67.2-89.1	$0.79 \ (0.73 - 0.85)^{\circ}$	39(2.99)	$0.78 \ (0.71 - 0.84)^{\circ}$	50(18.36)	
GFR, <sup>a</sup> mL/min/1.73 m <sup>2</sup>					
4.9-66.5	0.80(0.75 - 0.86)	36 (3.34)	0.79(0.73 - 0.85)	43 (22.54)	
66.6-79.7	0.87 (0.83-0.92) <sup>b</sup>	60(2.00)	$0.84 (0.79 - 0.90)^{b}$	66 (12.74)	
79.8-90.4	0.88 (0.84-0.92) <sup>b</sup>	67(1.78)	$0.85 (0.80-0.91)^{b}$	67 (11.92)	
90.5-135.1	$0.92 (0.88 - 0.95)^{\circ}$	69(1.71)	0.88 (0.83-0.93)d	68 (11.16)	
BMI, <sup>a</sup> kg/m <sup>2</sup>					
17.8-23.7	0.84 (0.78-0.89)	47 (2.51)	0.82 (0.76-0.87)	60 (15.5)	
23.8-26.1	0.86 (0.81-0.91)b	41 (2.81)	$0.84 (0.78 - 0.89)^{b}$	59 (15.62)	
26.2-28.7	$0.87 (0.83 - 0.92)^{b}$	65(1.88)	0.84 (0.79-0.90) <sup>b</sup>	66 (12.46)	
28.8-39.7	$0.90(0.86-0.94)^{d}$	69(1.67)	0.86 (0.81-0.91)b	67 (12.06)	
Epithelioid patients with mesothelioma, stratified by tumor stage, are differentiated from all 488 control subjects	× ,	· · /		× /	
Stage I $(n = 22)$	0.78 (0.69-0.86)	23(2.12)	0.76 (0.65-0.86)	46 (13.42)	
Stage II-IV $(n = 69)$	$0.90 (0.86-0.94)^{d}$	69(2.12)	$0.89(0.84-0.94)^{d}$	74 (13.42)	

Table 2-Effect of Covariate Distribution on the Diagnostic Accuracy of SM and MPF

For each continuous covariate, each of the four stratified subgroups included 122 individuals. AUC = area under the curve. See Table 1 for expansion of other abbreviations.

<sup>a</sup>Minimum-maximum range

For each covariate, the AUC of the first subgroup was compared with the others (bnot significant, P < .001, P < .05).

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FIGURE 1. Receiver operating characteristics (ROC) curves of SM and MPF for differentiating 106 patients with mesothelioma from control subjects, stratified according to their age or GFR. A, SM and MPF better differentiated the cases from the 25% youngest control subjects than from the 25% oldest control subjects. B, SM and MPF better differentiated the cases from the 25% of control subjects with the highest GFR than from the 25% of control subjects with the lowest GFR. GFR = glomerular filtration rate; MPF = megakaryocyte potentiating factor; SM = soluble mesothelin.

continuous CRP and MPF levels were significant prognostic factors of survival, whereas histology (epithelioid vs sarcomatoid-biphasic) and PS (0-1 vs 2-4) had a *P* value between .05 and .10 (Table 3). In addition to these variables, age, GFR, and BMI were also added to the multivariate analysis, to account for their effect on MPF. In this setting, MPF, CRP, PS, tumor stage and histology were all independent predictors of survival (Table 3). Tumor stage displayed the highest HR (3.0652, 95% CI, 1.0631-8.8344). Patients with stage II to IV mesothelioma consequently had a 206.5% increase in risk of death, compared with those with stage I. In contrast, the continuous MPF levels had an HR of only 1.0024 (95% CI, 1.0001-1.0047). A 1-ng/mL increase in MPF consequently corresponded to a 0.2% increase in risk of death, whereas a 50-ng/mL increase yielded a 10.5% increase in risk of death. Importantly, if the multivariate analysis was not adjusted for age, GFR, and BMI, MPF was not significant (P = .11). SM had no prognostic value, even when corrected for the associated covariates. A prognostic threshold for SM and MPF was not found.

# DISCUSSION

SM and MPF are among the best available serum biomarkers of mesothelioma, and might serve as an adjunct in the different fields of mesothelioma management. This study evaluated to what extent clinical covariates affect SM and MPF results and should be accounted for in the further validation and clinical application of both biomarkers.

We reported recently that SM and MPF are highly correlated, and have an equivalent diagnostic accuracy.<sup>4</sup>

Tab	le 3—Survi	val Analysis i	n 78 I	Patients	With	Mesothelioma
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		Categories	Univariate Analysis		Multivariate Analysisª	
Covariates	(P25-P75)		HR (95% CI)	P Value	HR (95% CI)	P Value
SM, nmol/L	2.43 (1.44-6.04)	Continuous	1.0121 (0.9906-1.0340)	.27		
MPF, ng/mL	19.26 (9.15-58.13)	Continuous	1.0023 (1.0003-1.0044)	.03b	1.0024 (1.0001-1.0047)	$.04^{b}$
CRP, mg/dL	3.50 (0.89-8.25)	Continuous	1.0165 (1.0050-1.0282)	$.01^{b}$	1.0159 (1.0036-1.0283)	$.01^{b}$
WBC count, 103/µL	8.42 (6.26-10.37)	Continuous	1.0121 (0.9209-1.1125)	.80		
Hemoglobin, g/dL	13.1 (12.0-14.1)	Continuous	0.9455 (0.8152-1.0967)	.46		
Platelet count, 10 <sup>3</sup> /µL	333 (272-418)	Continuous	1.0006 (0.9986-1.0027)	.52		
Tumor stage		> I (n = 59)	3.4544 (1.3674-8.7270)	$.01^{b}$	3.0652 (1.0631-8.8344)	$.04^{b}$
Tumor histology		Sarcomatoid-biphasic $(n = 8)$	2.0341 (0.9081-4.5566)	.08	2.9061 (1.1637-7.2528)	.02 <sup>b</sup>
PS		> 1 (n = 67)	$2.0360\ (0.8951\text{-}4.6311)$	.09	$2.6788\ (1.0364 - 6.9291)$	$.04^{b}$

CRP = C-reactive protein; HR = hazard ratio; P25-P75 = 25th and 75th percentile; PS = performance status. See Table 1 for expansion of other abbreviations.

 $^{a}$ Age, GFR, and BMI were added to the multivariate analysis (data not shown) to account for their effect on MPF.  $^{b}P$  values below .05 are significant.

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In addition, the present study found that the serum levels of both biomarkers were independently associated with the same covariates: age, GFR, and BMI in control subjects, and tumor stage and GFR in cases. In control subjects, these associations have been reported previously, but not all jointly, for SM.6-8 The similar molecular masses of SM (40 kDa) and MPF (31 kDa)<sup>2</sup> explain the inverse association with GFR, because such low-molecular weight proteins can accumulate in the blood when the renal function decreases.6 SM and MPF levels also increased with age. Although older individuals typically have a decreased GFR, the outcome of the multiple linear regression models suggested that age also affected SM and MPF levels irrespective of renal function. Compared with age and GFR, BMI showed the weakest correlation with the biomarker levels. The rationale behind its inverse relation with the biomarker levels is unclear, but it has also been described for prostate-specific antigen (PSA).<sup>23</sup> The positive association of SM and MPF with tumor stage is in agreement with previous reports, and reflects the correlation with tumor burden.<sup>3,4</sup> Biomarker levels did not differ according to histology, although the expression of mesothelin is typically limited to epithelioid mesothelioma cells.<sup>2</sup> This was likely due to the small number of patients with nonepithelioid mesothelioma in our series.

The diagnostic accuracy of SM and MPF was significantly affected by the distribution of the associated covariates in the study population. SM and MPF had either a high or a poor diagnostic accuracy, depending solely on the age, GFR, and BMI of the control subjects, or the tumor stage of the patients with mesothelioma. To improve the interpretation of future validation studies, these covariates should therefore be recorded routinely, and control subjects and cases should be matched for age, GFR, and BMI. The finding that MPF was twice as sensitive as SM for stage I mesothelioma should be interpreted with caution because it was based on a limited number of patients and both AUCs were virtually identical. Pending further validation, the low sensitivity of both biomarkers for stage I mesothelioma should nevertheless be kept in mind when propagating SM and MPF too vigorously as markers of early detection.

Some additional remarks require consideration when interpreting our exploratory analyses. First, control subjects were stratified according to the covariate quartiles, irrespective of their medical condition, although a number of these control subjects had a malignancy that could also overexpress mesothelin (eg, lung, ovarian, and pancreatic cancer).<sup>2</sup> The consequences for our analyses, however, were limited because the biomarker levels of these individuals were not significantly higher than those of the control subjects without malignant disease. Second, in contrast to the control subjects, patients with mesothelioma were not stratified according to the covariate quartiles, to ensure an appropriate comparison of the diagnostic accuracy across the stratified control groups. Stratification of the patients with mesothelioma would have led to a biased evaluation because of the small number of cases and the unbalanced tumor stage distribution in the resulting subgroups.

Despite these limitations, our findings allow us to speculate on how the effect of these covariates on SM and MPF can be accounted for in diagnostic practice. In theory, biomarker levels could be adjusted for the associated covariates by means of a multiple linear regression equation. However, in agreement with previous reports about SM,6-9 age, GFR, and BMI displayed a weak association with the biomarker levels, as illustrated by the low adjusted  $R^2$ of the presented regression models. This indicated that these covariates explained only a very limited proportion of the between-individual biomarker variation. Such a regression equation would consequently have a very limited predictive power, and is of no use in clinical practice. A first, potentially more suited, approach to account for biomarker-associated covariates is to challenge the use of a single SM and MPF threshold for all individuals. Currently, there is no agreement on the diagnostic thresholds of both biomarkers, because the reported "optimal" thresholds range widely. Our findings can explain this variety because we demonstrated that, for the same specificity, the threshold values varied up to factor two, depending on the covariate distribution in the control subjects. The application of covariate-specific biomarker reference intervals, instead of a single threshold, might therefore improve the diagnostic use of SM and MPF. A well-known example of such an approach is the use of age-specific reference intervals of PSA,<sup>24</sup> although current guidelines do not concur on their clinical usefulness.<sup>25,26</sup> SM and MPF reference intervals also have to account for GFR and BMI, resulting in a more laborious development.<sup>27</sup> A second, more simple, approach is to a priori restrict the use of SM and MPF to individuals who have normal values of the associated covariates. After all, the most significant changes in diagnostic accuracy were observed in the extremes of the covariate distribution. The main challenge here is to establish at which age, GFR, or BMI, the measurement of SM and MPF is no longer recommended. The development of both approaches requires the measurement of SM and MPF in a large numbers of control subjects with no malignant disease, who display a sufficiently wide distribution in each of the associated

covariates. Our series were not appropriate for such purpose, either in terms of number or in covariate distribution.

Before embarking on such a laborious endeavor, it is important that we acknowledge the limitations of both of these approaches. Age-specific SM and MPF reference intervals, for example, have the potential to increase the sensitivity for mesothelioma in younger patients, and improve the specificity in older patients. As a consequence, significant malignancies might be missed in the latter, a concern that also exists with the age-specific PSA reference intervals.<sup>25</sup> Similarly, restricting the use of SM and MPF to individuals with normal covariate values will predominantly exclude older patients, because they are also susceptible to a decreased renal function. These limitations might reduce the clinical application of SM and MPF, because patients at risk of mesothelioma are typically middle aged or elderly. Therefore, once both approaches are established, large-scale prospective evaluation is needed to determine whether the benefits of accounting for the biomarker-associated covariates outweigh its limitations.

The effect of the covariates on SM and MPF levels also had an impact on the results of the survival analyses. In agreement with the literature, mesothelioma stage, histology, PS, and CRP levels were independent prognostic factors in mesothelioma. For SM, the absence of prognostic value was in agreement with the findings of some previous studies,<sup>13,14</sup> whereas others did report a significant association.<sup>10-12</sup> In our series, MPF was an independent negative prognostic factor, but only if adjusted for the effect of age, GFR, and BMI. The prognostic impact of MPF was relatively low, compared with other factors such as tumor stage, histology, and PS. No prognostic MPF threshold was found, likely because of the relatively low number of patients and events. This low power also implied that the absence of prognostic significance for SM should be interpreted with caution. The role of SM and MPF as markers of outcome consequently requires further validation in larger study populations, ideally with adjustment for the biomarker-associated covariates. It remains to be seen, however, whether these biomarkers can provide prognostic value beyond the currently available clinical factors.

Further research should also focus on the effect of other covariates on SM and MPF levels. Several factors, including BP,<sup>19</sup> small nucleotide polymorphisms,<sup>28</sup> and methylation of the mesothelin gene,<sup>29</sup> have been suggested to affect SM levels, but lack extensive validation. To determine their significance for SM and MPF levels, it will be important to elucidate whether these putative effects act independently of age, GFR, and BMI.

### CONCLUSIONS

In conclusion, SM and MPF levels were affected by the same clinical covariates, which also had a significant impact on their diagnostic and prognostic value. To improve the interpretation of biomarker results, age, GFR, and BMI should be recorded routinely. Approaches to account for these covariates require further validation, as does the prognostic value of SM and MPF.

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