MMP-2, MMP-9 and Activin A Blood Levels in Patients with Breast Cancer or Prostate Cancer Metastatic to the Bone

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Abstract. *Background: The clinical significance of the circulating levels of activin A and matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) was investigated in patients with breast cancer (BC) or prostate cancer (PC) with (M1) or without (M0) bone metastasis. Patients and Methods: MMP-2, MMP-9 and activin A blood concentrations were measured by enzyme immunoassays in 79 cancer patients and in 57 healthy blood donors (HS) who served as a control group. The diagnostic accuracy of these molecules to discriminate between M0 and M1 patients was evaluated by the receiver operating characteristic curve (ROC) and compared to that of tumor markers CA15.3 or prostate-specific antigen (PSA). Results: Activin A and MMP-2 were significantly increased in BC and PC patients as compared to sex-matched HS while MMP-9 levels were more elevated only in the PC patients. Interestingly, in the PC patients, activin A levels were significantly higher than those measured in the BC patients. In this latter group, activin A and CA15.3 but not MMP-2 or MMP-9 were increased in the M1 patients as compared to M0 patients. Furthermore, a significant relationship was also highlighted between activin A concentration and the number of bone metastases and tumor grade, between MMP-9 and tumor grade, and between MMP-2 and CA15.3. ROC curve analysis showed a good diagnostic accuracy for activin A and CA15.3 but a poor accuracy for MMP-2 and MMP-9 in discriminating between M0 and M1 patients. However, CA15.3 retained the best diagnostic accuracy in this respect. In the PC group, only activin A and PSA levels were significantly increased in the M1 patients as compared to the M0 patients. A similar although not statistically significant trend was noted for MMP-9. Interestingly, a significant correlation was observed between PSA and activin A and MMP-9, and between Activin A and Gleason score and the number of skeletal metastases. ROC curve analysis showed a good diagnostic accuracy for activin A, MMP-9 and PSA and a poor diagnostic accuracy for MMP-2 in detecting M1 patients. However, PSA showed the highest diagnostic accuracy. Conclusion: Activin A, MMP-2 and MMP-9 may be regarded as possible therapeutic targets in the treatment of metastatic bone disease. However, their usefulness as additional markers of bone metastasis remains to be better defined.*

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Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are zinc-dependent endopeptidases mainly devoted to the degradation of extracellular matrix proteins (1). Growing experimental evidence suggests that these proteinases may also be implicated in bone remodeling processes associated with various physiological and pathological conditions, including bone metastasis formation (1-3). The mechanisms by which MMP-2 and MMP-9 may facilitate bone metastasization have not yet been fully elucidated. However, recent findings suggest that tumor cells may secrete and/or induce osteoclasts to release into the bone microenvironment several proteolytic enzymes, including MMP-2 and -9, which may degrade bone matrix proteins thus cleaving a number of growth factors pre-incorporated in the bone tissue (3, 4). These factors, in turn, may stimulate tumor cells to release other soluble growth factors, cytokines, hormones and proteolytic enzymes which may further promote cell proliferation, migration and invasion of host tissue, setting up a vicious circle (5).

Table I. *Activin A, MMP-2 and MMP-9 blood concentrations in healthy subjects and cancer patients.*

	Healthy subjects (HS)	Breast cancer patients (BC)	Mean CP/HS ratio	*p*-value*
Activin A (pg/ml)	359.8±177.0 (n=30)	932.3±927.1 (n=35)	2.59	<0.0001
MMP-2 (ng/ml)	781.4±190.7 (n=12)	1564.3±433.0 (n=29)	2.00	<0.0001
MMP-9 (ng/ml)	64.5±39.2• (n=12)	94.5±64.3 (n=32)	1.46	0.2 (n.s.)
	Healthy subjects (HS)	Prostate cancer patients (PC)	Mean CP/HS ratio	*p*-value*
Activin A (pg/ml)	437.3±198.4 (n=27)	1055.6±731.7˚ (n=44)	2.41	<0.0001
MMP-2 (ng/ml)	774.2±217.4 (n=28)	1603.5±437.1 (n=32)	2.07	<0.0001
MMP-9 (ng/ml)	41.3±41.0 (n=26)	102.4±89.6 (n=27)	2.48	0.016

Results are expressed as mean±standard deviation. n=number of evaluable subjects; *Data analysis was computed by the non-parametric Mann-Whitney *U*-test; n.s. not significant; •$p=0.039$ *vs.* male HS and ˚$p=0.027$ *vs.* breast cancer.

Among the growth factors released during these events, several components of the transforming growth factor β (TGF-β) superfamily seem to play an active role in the regulation of the expression levels of MMP-2 and MMP-9 during bone turnover processes associated with skeletal metastasis (6-9). In this context, recent studies have been focused on a specific member of this family of growth factors, namely activin A. In fact, it has been reported that this multifunctional cytokine appears to be implicated in the regulation of osteoblastic activity and osteoclast differentiation and in the modulation of intracellular MMP-2 and MMP-9 expression levels in osteoblasts, in osteoclasts and in bone metastatic cells (7-18). On the basis of these findings, the present study was undertaken to assess the clinical value of the circulating levels of these molecules as possible markers of bone metastatic spread in patients with breast (BC) or prostate cancer (PC).

Patients and Methods

The present study included 57 registered healthy blood donors (HS) (30 female and 27 male) who served as a control group, 35 patients with histologically proven BC (median age 60, range 36-77 years) and 44 patients with PC (median age 70, range 56-88 years). Both the BC and the PC groups comprised patients with localized disease (M0), and patients with bone metastases (M1) but no clinical evidence of extraskeletal involvement. Bone metastases were diagnosed by skeletal scintigraphy, skeleton x-ray or magnetic resonance imaging, or by a combination of these tests as required. The study was approved by the local ethical committee and carried out in accordance with the Declaration of Helsinki (19).

Plasma MMP-2 and MMP-9 and serum activin A assays. As the measurement of the circulating levels of MMP-2 and MMP-9 in the serum has been reported to be artificially higher compared to the values determined in the plasma (20, 21), blood samples obtained from patients before any surgical and/or clinical treatment were aliquoted either into EDTA-coated tubes for MMP-2 and -9 assay or into polycarbonate tubes for activin A assay. The blood in the polycarbonate tubes was allowed to clot at room temperature. Samples were then centrifuged at 3500 rpm for 15 min (Hereus Omnifuge 2.0 RS, Hereus Sepatech, Osterode, Germany). Serum or plasma aliquots were stored at –80˚C until assayed. MMP-2, MMP-9 and activin A levels were determined by commercially available two-step sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Biotrak MMP-2 and MMP-9 ELISA kits, Amersham Bioscience, Little Chalfont, UK; Activin A Assay kit, Oxford Bio-Innovation Ltd, UK). The reported detection limits were 0.37 ng/ml for MMP-2, 0.6 ng/ml for MMP-9 and <78 pg/ml for activin A.

Serum CA15.3 and PSA. The tumor marker CA15.3 and prostate-specific antigen (PSA) serum levels were determined in the BC and PC patients respectively by an automated commercially available immunoluminometric assay (DiaSorin, Saluggia, Italy). The reference value and the detection limit reported by the manufacturer were 30 U/mL and <0.3 U/ml for CA15.3 and 3.2 ng/ml and <0.009 ng/ml for PSA respectively.

Table II. *Activin A, MMP-2, MMP-9, CA15.3 and PSA circulating levels in patients with (M1) or without (M0) bone metastases.*

Parameter	No metastasis (M0)	Bone metastasis (M1)	M1/M0 mean ratio	*p*-value*
Breast cancer				
Activin A (pg/ml)	604.9±217.6 (n=19)	1322.6±1261.7 (n=16)	2.19	$p=0.047$
MMP-2 (ng/ml)	1699.6±347.9 (n=17)	1532.5±482.4 (n=12)	0.90	$p=0.14$ (n.s.)
MMP-9 (ng/ml)	104.5±74.4 (n=17)	83.1±50.6 (n=15)	0.79	$p=0.52$ (n.s.)
CA15.3 (U/ml)	17.9±8.1 (n=18)	82.6±123.9 (n=16)	4.6	$p<0.0001$
Prostate cancer				
Activin A (pg/ml)	705.9±311.1 (n=17)	1315.3±831.0 (n=27)	1.86	$p=0.0038$
MMP-2 (ng/ml)	1525.5±382.5 (n=16)	1681.4±481.5 (n=16)	1.1	$p=0.48$ (n.s)
MMP-9 (ng/ml)	87.03±103.0 (n=11)	113.05±80.9 (n=16)	1.3	$p=0.084$ (n.s)
PSA (ng/ml)	5.5±5.6 (n=15)	463.7±1207.5 (n=28)	84.3	$p<0.0001$

Results are expressed as mean±SD; n=number of evaluable subjects. Due to missing samples the number of patients in each group may vary. *Mann-Whitney *U*-test; n.s.=not significant.

Statistical analysis. The statistical analysis was carried out by the Medcalc 7.4 statistical software package (MEDCALC version 7.4, Mariakerke, Belgium). Because of the uneven distribution of the data, as assessed by the D'Agostino-Pearson test for normal distribution, data analysis was performed where required by the non-parametric Mann-Whitney *U*-test and the Kruskall-Wallis test. The diagnostic sensitivity and the specificity of activin A, MMP-2, MMP-9, CA15.3 and PSA to discriminate between M0 and M1 patients were determined by the receiver operating characteristic curve (ROC) analysis (22). The significance of the difference between the areas under the ROC curves (AUC) was assessed according to Hanley and McNeil (23). *P*-values ≤0.05 were considered statistically significant.

Results

Activin A and MMP-2 blood levels were markedly increased in the BC and PC patients as compared to sex-matched HS ($p<0.0001$) while statistically significant higher levels of MMP-9 were observed only in the PC patients ($p=0.016$) (Table I). Interestingly, a slight, statistically significant increase in activin A concentration was observed in PC patients as compared to BC patients ($p=0.027$). In this latter group, activin A and CA15.3 were more elevated in M1 patients than in M0 patients (2.2- and 4.6-fold, respectively; $p=0.047$ and $p<0.0001$) while no significant difference was observed for MMP-2 and MMP-9 ($p=0.14$ and $p=0.52$, respectively) (Table II). Interestingly, in these patients a significant relationship was highlighted between activin A and the number of bone metastases ($p=0.007$) and tumor grade ($p=0.046$), between MMP-9 and tumor grade ($p=0.013$), and between MMP-2 and CA15.3 ($p=0.0088$) (Table III). ROC curve analysis showed a fair and good diagnostic performance for activin A ($p=0.031$) and CA15.3 ($p=0.001$), respectively, and a poor accuracy for MMP-2 ($p=0.13$) and MMP-9 ($p=0.51$) in discriminating between the M0 and M1 patients (Table IV). However, CA15.3 showed the largest AUC which was significantly higher than that of activin A ($p=0.036$), MMP-2 ($p=0.01$) or MMP-9 ($p=0.002$) (Figure 1). Conversely, the combination of CA15.3 with activin A, or MMP-2, or MMP-9 did not result in a diagnostic accuracy better than that of CA15.3 alone (data not shown). In the PC group, activin A and PSA but not MMP-2 blood levels were significantly increased in the M1 patients as compared to the M0 patients ($p=0.0038$; $p<0.0001$, and $p=0.48$ respectively) (Table II). A trend toward higher MMP-9 plasma concentrations was also noted in the M1 patients ($p=0.084$) (Table II). Moreover, in these patients, activin A serum levels

Table III. *Activin A, MMP-2 and MMP-9 distribution in patients with breast cancer or prostate cancer according to some clinicobiological parameters of progression.*

	Breast Cancer		
Parameter	MMP-2 (ng/ml)	MMP-9 (ng/ml)	Activin A (pg/ml)
Tumor grade			
G1	1810.0±187.0 (n=9)	141.5±58.6 (n=9)	662.9±507.5 (n=7)
G2	1464.1±636.1 (n=13)	103.9±62.8 (n=13)	692.8±340.2 (n=17)
G3	1581.5±492.8 (n=9)	70.4±56.8 (n=9)	1576.4±1424.3 (n=11)
	p=0.28 (n.s.)˚	p=0.013˚	p=0.046˚
No. of metastases			
M0	1699.7±347.5 (n=15)	104.5±74.7 (n=15)	603.7±217.9 (n=19)
M>1-4	1180.5±249.4 (n=6)	105.0±62.4 (n=6)	701.1±434.4 (n=9)
M>4	1509.7±576.3 (n=7)	66.7±33.1 (n=7)	2121.4±1551.6 (n=7)
	p=0.3(n.s.)˚	p=0.5 (n.s.)˚	p=0.007˚
CA15.3			
<24.9 U/ml•	1831.0±439.4 (n=14)	90.2±64.4 (n=17)	871.4±961.3 (n=21)
>24.9 U/ml	1348.0±529.4 (n=15)	99.3±66.01 (n=15)	927.5±745.9 (n=13)
	p=0.0088*	p=0.5 (n.s.)*	p=0.4(n.s.)*
	Prostate Cancer		
Parameter	MMP-2 (ng/ml)	MMP-9 (ng/ml)	Activin A (pg/ml)
Gleason score			
2-4	1616.0±314 (n=8)	51.5±22.4 (n=8)	718.9±432.4 (n=9)
5-7	1589.5±401 (n=12)	133.9±108.2 (n=12)	868.7±292.8 (n=23)
8-10	1513.7±473 (n=6)	70.4±56.8 (n=6)	1630.0±1069.5 (n=10)
	p=0.72 (n.s.)˚	p=0.23 (n.s.)˚	p=0.029˚
No. of metastases			
M0	1657.9±423.3 (n=11)	87.1±103 (n=11)	700.6±314.1 (n=17)
M1-4	1514.2±527.9 (n=6)	114.7±36.2 (n=6)	1247.1.0±803.8 (n=7)
M>4	1535.3±346.7 (n=10)	112.4±39.8 (n=10)	1295.0±859.7 (n=20)
	p=0.46 (n.s.)˚	p=0.77 (n.s.˚)	p=0.022˚
PSA			
<16.9 ng/ml•	1532.9±365.3 (n=18)	70.8±77.5 (n=11)	868.5±687.6 (n=20)
>16.9 ng/ml	1686.5±536.0 (n=13)	127.4±83.1 (n=15)	1277.4±745.9 (n=23)
	p=0.7 (n.s.)*	p=0.021*	p=0.0051*

Data are expressed as mean±SD; n= number of evaluable subjects. Due to some missing samples the number of patients in each group may vary. *Mann-Whitney *U*-test; ˚ Kruskall-Wallis test; n.s.= not significant; •cut-off value determined by ROC curve.

positively correlated with PSA (p=0.005), Gleason score (p=0.029) and number of bone lesions (p=0.022), while MMP-9 plasma concentrations were significantly associated with PSA only (p=0.021) (Table III). In contrast, no correlation was observed between MMP-2 and the clinicobiological parameters considered (Table III). ROC curve analysis showed a good diagnostic accuracy for activin A, MMP-9 and PSA but a poor diagnostic performance for MMP-2 in discriminating between the M1 and M0 patients (Table IV). PSA showed the largest AUC which was significantly higher than the AUCs of activin A (p=0.0012), MMP-2 (p=0.006) and MMP-9 (p=0.004) (Figure 1). Again, the combination of PSA with any of the other markers did not result in an improved diagnostic performance in detecting patients with bone metastasis (data not reported).

Discussion

The results showed that the patients with breast or prostate cancer have increased circulating levels of activin A, MMP-2 and MMP-9 as compared to the sex-matched control group. These data confirm, in part, previous observations from other

Table IV. *Sensitivity, specificity and diagnostic accuracy of activin A, MMP-2, MMP-9, CA15.3 and PSA in the detection of patients with bone metastases.*

	AUC (95% CI)	Cut-off value[§]	Sensitivity (95%CI)	Specificity (95%CI)	+LR	–LR	PPV	NPV
Breast cancer								
Activin A	0.69±0.1 (0.48-0.84) p=0.031	570 pg/ml	75.0 (47.6-92.6)	63.2 (38.4-83.6)	2.04	0.40	63.2	75.0
MMP-2	0.68±0.1 (0.47-0.83) p=0.13 (n.s.)	1195.6 ng/ml	41.7 (15.3-72.2)	100 (80.3-100)	–	0.58	100	70.8
MMP-9	0.51±0.1 (0.31-0.71) p=0.68 (n.s.)	133.2 ng/ml	93.3 (68.0-98.9)	29.4 (10.4-55.9)	1.32	0.23	53.8	83.4
CA15.3	0.92±0.05 (0.78-0.99) p=0.0001	24.9 U/ml	81.2 (54.3-95.7)	88.9 (65.2-98.3)	7.31	0.21	86.6	84.2
Prostate cancer								
Activin A	0.77±0.07 (0.61-0.89) p=0.0002	740 pg/ml	85.7 (63.6-96.8)	73.7 (48.8-90.8)	3.26	0.19	78.3	82.4
MMP-2	0.57±0.11 (0.39-0.78) p=0.48 (n.s.)	1482 ng/ml	62.5 (35.5-87.4)	56.2 (29.9-80.2)	1.43	0.67	58.8	60.0
MMP-9	0.70±0.1 (0.49-0.86) p=0.049	49.9 ng/ml	87.5 (61.6-98.1)	63.6 (30.9-88.8)	2.41	0.20	77.8	77.8
PSA	0.95±0.04 (0.82-0.99) p=0.0001	16.9 ng/ml	81.8 (58.1-94.4.)	100 (76.7-100)	–	0.19	100	77.8

[§]Cut-off limits determined by ROC curve analysis; AUC: area under the ROC curve (95% confidence interval); +LR=positive likelihood ratio; –LR=negative likelihood ratio; PPV=positive predictive value; NPV=negative predictive value; (n.s.)=not significant.

studies and further support the hypothesis of an active role of these molecules in tumor progression (24-34). On the other hand, our investigations failed to highlight any significant difference in the plasma levels of MMP-2 or MMP-9 between M0 and M1 patients either in breast cancer or prostate cancer. However, in the PC group, MMP-9 levels bordered on statistical significance. These findings fit well with the data from some investigations (35, 36) but, at the same time, are in contrast with those from others (28, 31-34). The discrepancies in these results may in part, be explained by the different criteria for selection of patients. Unlike other investigations, our study included only patients with bone metastasis and no clinically evident extraskeletal involvement. Additionally, the different assay methods used and the number of subjects included may also account for these conflicting results. Interestingly, our data have further highlighted the significantly higher circulating levels of activin A in the PC patients compared to the BC patients. These findings and the strong correlation observed between the serum concentrations of this cytokine and the number of bone metastases and PSA levels support the hypothesis of an active role of activin A in the modulation of the osteoblastic activity which, in prostate cancer, is known to be predominant and may account for the osteoblastic reactions induced by tumor cells (8, 37-40). Additionally, the trend towards higher levels of MMP-9 noted in the M1 patients and the positive correlation between this proteinase and PSA concentrations further support the concept of a preferential involvement of MMP-9 in facilitating bone metastasis formation in prostate cancer (3-5, 8, 9, 18, 33, 34, 36, 39). These data were suggestive of a potential clinical use of these molecules as additional indicators of bone metastatic spread in prostate cancer. However, ROC analysis performed to assess this hypothesis showed that neither activin A nor MMP-9 showed a diagnostic accuracy superior to the classical tumor marker PSA in detecting patients with metastatic bone disease, nor did their combination result in an improved diagnostic efficiency. In summary, the present study suggests that activin A and MMP-9 may have a preferential role in facilitating bone metastasis formation in prostate cancer, while a direct role of MMP-2 in this pathogenesis in PC and BC remains questionable. Therefore, activin A and MMP-9 may be regarded as possible novel therapeutic targets in the treatment

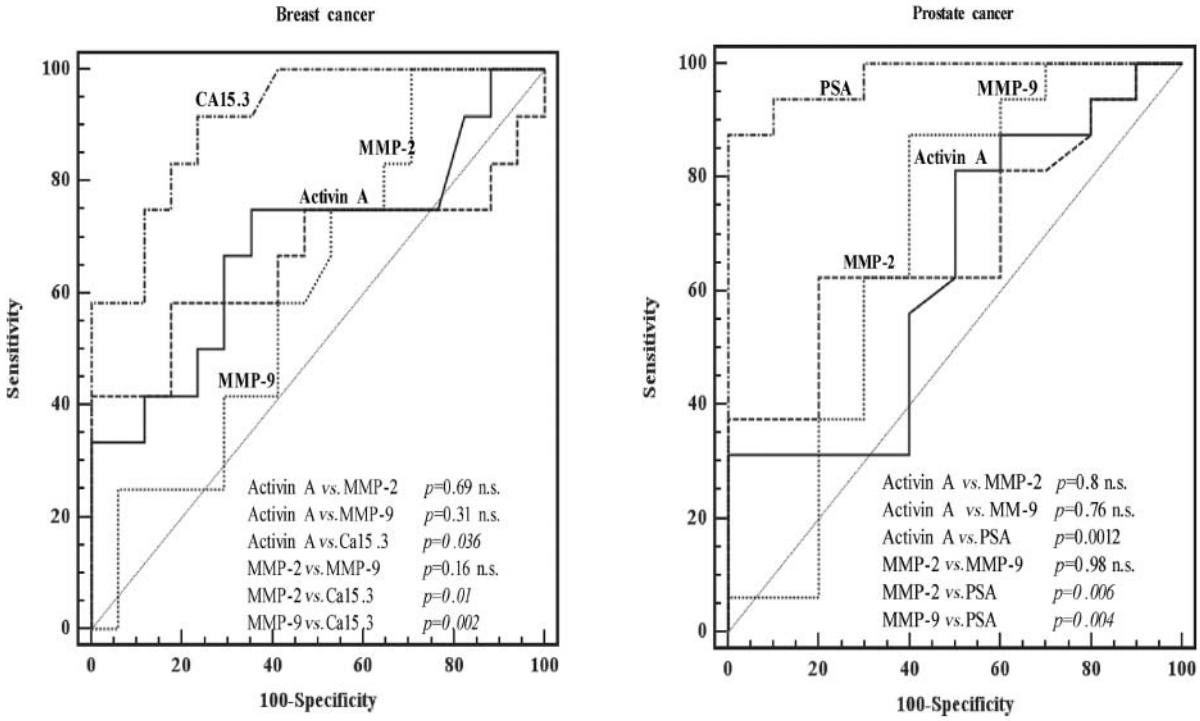

Figure 1. *ROC curves for activin A, MMP-2 and MMP-9 in patients with bone metastases from breast or prostate cancer. Pairwise comparison with CA15.3 or PSA.*

of metatastic bone disease. However, the clinical relevance of all these molecules as additional markers of skeletal metastasis needs to be better defined. Further studies with a larger number of subjects are warranted by these findings.

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