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Matrix Metalloproteinase-9 (MMP-9) Immunoreactive Protein has Modest Prognostic Value in Locally Advanced Breast Carcinoma Patients Treated with an Adjuvant Antiestrogen Therapy

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Abstract. *Background: Matrix metalloproteinases (MMPs) are involved with tumour invasion and metastasis. Controversial data exists concerning the prognostic value of MMP-9 in breast carcinoma. We examined, here, whether the MMP-9 immunoreactive protein would correlate with the prognosis in breast carcinoma treated with hormonal adjuvant therapy. Materials and Methods: The MMP-9 status was determined immunohistochemically from primary tumour specimens in 168 postmenopausal breast cancer patients with a locally advanced (N+) disease treated with antiestrogen for three years after the primary therapy. Results: A positive immunostaining for MMP-9 was found in 61.3% of 168 primary tumours without any significant correlation to clinical stage, histology or hormone receptor status. MMP-9 immunoreactivity did not correlate with the survival when the entire study population was included in the analysis. There was, however, a compromised disease-free survival in a subgroup of patients presenting with an estrogen receptor-negative and MMP-9-positive tumour. The 5-year disease-free survival was only 37% in those patients, when it was 63% in the patients with a tumour negative for both estrogen receptor and MMP-9. Conclusion: We suggest that the prognostic value of MMP-9 immunoreactivity in the primary tumour is not generally strong in breast carcinoma, but it might correlate with the clinical benefit of an antiestrogen therapy, since MMP-9 positivity seemed to correlate with early recurrence in patients with an estrogen receptor-negative primary tumour.*

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The overall prognosis of breast cancer patients has improved in recent years, partly because of screening mammography and early diagnosis, and the favourable progress in systemic adjuvant treatment (1-3). An individual risk for relapse is related to the biological aggressiveness of the primary tumour, and so there is a great demand for new methods to distinguish various tumour phenotypes and patients at risk of disseminated disease even at early diagnosis. Matrix metalloproteinases (MMPs) are endopeptidases that are involved in the degradation of the extracellular matrix (ECM) as well as tumour cell invasion and metastasis (4,5).

The prognostic value of MMPs has been investigated in several malignancies. MMP-2 overexpression has been correlated with poor survival in breast carcinoma (6,7), especially in node-positive patients (8). Cox *et al.* found MMP-9 immunoreactive protein to be a marker of a poor outcome in operable non-small cell lung carcinoma (9). Data shown by Sienel *et al.* indicated a correlation between immunohistochemically homogenous MMP-9 expression and compromised cancer-related survival also in non-small cell lung cancer patients (10). Foukas *et al.* investigated osteosarcoma resection specimens and demonstrated MMP-9 positivity to be an independent prognostic factor in operated stage IIB osteosacroma patients (11). There are few studies examining the possible prognostic value of MMP-9 in breast cancer patients, but with conflicting results. Remacle *et al.* found that MMP-9 levels in gelatin zymography correlate inversely with numbers of axillary nodal metastases, but do not significantly relate to patients' outcome (12). MMP-9 and MMP-2 immunostaining have also been found to be more intense and diffuse in male than in female breast cancer tissues in an Italian study, and the authors suggest that the phenomenon is related to metastatic behaviour and poor prognosis in male breast cancer patients (13). Ranuncolo *et*

al. further investigated MMP-9 activity in the plasma of patients at breast cancer diagnosis and during the clinical follow-up, and found that the progression of activity predicts the recurrence of the disease. High plasma MMP-9 activity at the primary diagnosis correlated with shortened overall survival in their study (14). Thus, there are several studies linking MMP-9 with an aggressive clinical course in breast carcinoma. However, when Scorilas *et al.* studied the expression of MMP-9 using immunohistochemistry, they found that MMP-9 positivity was significantly associated with a favourable outcome in node-negative breast cancer patients. This association was not seen in node-positive patients (15). Moreover, Van't Veer *et al.* have shown, in studies exploring the gene expression profiles of the tumour tissue in breast cancer patients, that genes involved in cell cycle, signal transduction, angiogenesis, metastasis, as well as in cell invasion – including MMP-9 – were significantly up-regulated in cases with poor prognosis (16).

To further clarify the role of MMP-9 in the progression of locally advanced breast carcinoma, we examined here the prognostic value of MMP-9 immunoreactive protein in primary tumours of postmenopausal patients suffering from a node-positive breast cancer treated with antiestrogen as an adjuvant therapy.

Materials and Methods

The present series was based on 168 node-positive, postmenopausal breast cancer patients receiving an antiestrogen adjuvant therapy for three years; either tamoxifen 20mg daily (54% of the patients) or toremifene 40 mg daily (46% of the patients). The patients were treated at Oulu University Hospital, Finland, during the years 1992-1999. Also patients with unknown or negative estrogen (47) or progesterone (82) receptors in their primary tumours were included in the early 90's. The average age of the patients was 64 years (range 48-87 years). Postmenopausal status was verified by measuring the follicle stimulating hormone (FSH >30 IU) in patients with less than one year elapsing since menopause or hysterectomy patients before the breast carcinoma diagnosis. Ninety-five patients underwent mastectomy and axillary evacuation, whereas segmential resection of the breast with axillary dissection was preferred in 62 cases. Postoperative irradiation covering the ipsilateral axilla, the supraclavicular region and the chest wall on the side of the tumour, was given in 158 cases. Ten patients did not receive radiotherapy, either because of patient choice or on account of retarded wound healing. Patients with a contraindication for the use of antiestrogens, those presenting primarily with distant metastases or patients receiving adjuvant chemotherapy were excluded. Fifty-six patients relapsed within the follow-up (range 7-111 months).

The stage of the disease was determined according to the TNM classification of tumours issued by the International Union Against Cancer (UICC) and the WHO classification for the characterisation of tumour histopathology. Estrogen and progesterone receptor status were analysed by radioimmunoassay (71%) or immunohistochemistry (29%).

Immunohistochemistry. MMP-9 expression was determined immunohistochemically from formalin-fixed, paraffin-embedded primary tumour specimens (4mm) by using the streptavidin-biotin peroxidase immunostaining as described previously (17). Paraffin sections were incubated at 37°C for at least 4 hours before dewaxing in a histological clearing agent (Histo-Clear®, National Diagnostic, Atlanta, GA, USA), and then hydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase/methanol incubation for 20 minutes, followed by an incubation in 10% goat serum for 15 minutes to prevent any non-specific binding. The specimens were incubated with a primary (10 μg/ml) antibody for MMP-9 (CA-4001; Diabor Ltd, Oulu, Finland) in a humidity chamber at room temperature for 24 hours. The antibody recognises the aminoterminal end of latent MMP-9, both as a free enzyme and as a complex with a tissue inhibitor of metalloproteinase. A biotinylated anti-mouse IgG served as a secondary antibody with an incubation time of 15 minutes (Histostain-bulk kit®, Zymed, San Francisco, CA, USA) and, after that, the peroxidase was introduced with a streptavidin conjugate (Histostain-bulk kit®). Romulin AEC chromogen substrate (Biocare Medical, CA, USA) was then used to visualise the immunohistochemical reaction. The sections were counterstained with hematoxylin, dehydrated and mounted in Pertex® (Histo-lab, Gothenburg, Sweden). For the negative controls, the primary antibody was replaced either by mouse non-immune serum or phosphate-buffered solution (PBS). Known sections positive for MMP-9 were included in PBS between every step of the staining process.

Evaluation of MMP-9 staining. The staining result was analysed by three individual observers including an experienced pathologist, all blinded to clinical data. The cytoplasmic staining was scored in neoplastic cells. The staining was interpreted as negative (-) in sections where there were no positive cells, or less than 1% of all malignant cells showed a positive staining, while 2-50% of the tumour cells appearing as positive indicated a moderate overexpression (+) of MMP-9 protein. Sections presenting more than 50% of the neoplastic cells as positive were considered to have an extensive (++) MMP-9 overexpression in the tumour.

Statistical analysis. Statistical significance was tested using standard tests: Student's *t*-test, Mann-Whitney, Pearson and Chi-square tests. Survival was defined as the time between the primary surgery and the last clinical follow-up visit or death. Survival analysis was carried out regarding disease relapse or breast cancer-related death as an end-point. Patients dying for any other reasons than breast cancer were censored from the survival data at the date of death. Survival curves (Kaplan-Meier) were compared using the log rank, Breslow or Tarone-Ware tests and $p<0.05$ was considered as statistically significant. The effect of MMP-9 immunoreactivity on survival was analysed in different prognostic subgroups of the patients. Cox regression analysis and stepwise regression analysis were used to find significant predictors of survival.

Results

The clinical characteristics of the tumours are presented in Table I. The major proportion of the primary tumours were 20-50 millimetres in size (50%) and the histology most often (75%) represented a ductal carcinoma as well as a moderate

Table I. *MMP-9 immunoreactivity in relation to other tumour characteristics.*

	No. of patients (%)	MMP-9 positivity (%) (moderate+ extensive)	*p* value
Tumour size			
T1	72 (43)	43	NS
T2	84 (50)	52	
T3	9 (5)	6	
T4	2 (1)	2	
Unknown	1 (1)		
Axillary nodal status			
1-3 positive nodes	108 (64)	69	NS
≥4 positive nodes	30 (18)	20	
Number not specified	30 (18)		
Clinical stage			
II	153 (91)	65	NS
III	14 (8)	77	
Unknown	1 (1)		
Histology			
Ductal	126 (75)	65	NS
Lobular	35 (20)	66	
Other	5 (3)	60	
Unknown	2 (1)	100	
Histological grade			
I	20 (12)	74	NS
II	63 (38)	72	
III	51 (30)	56	
Unknown	34 (20)	65	
ER status			
Positive	122 (72)	65	NS
Negative	33 (20)	67	
Unknown	14 (8)	67	
PR status			
Positive	86 (51)	64	NS
Negative	68 (41)	67	
Unknown	14 (8)	67	

ER, estrogen receptor; PR, progesterone receptor

Table II. *Cox univariate and multivariate analysis of disease-free survival.*

Factor	Univ. analysis			Multiv. analysis		
	p-value	HR	(95% CI)	*p*-value	HR	(95% CI)
MMP-9 status	0.482					
Negative						
Positive		0.8	0.5-1.4			
Tumour size	0.000			0.005		
T1						
T2	0.000	3.51	1.8-6.9	0.000	4.41	1.9-10.1
T3	0.000	9.80	3.7-25.6	0.396	2.10	0.4-11.6
T4	0.125	4.97	0.6-38.6	0.954	1.08	0.1-13.1
Nodal metast.	0.021					
1-3						
≥4	0.015	2.14	1.2-3.9			
Clinical stage	0.000			0.007		
II						
III		4.22	2.1-8.5		7.73	1.7-34.5
Histol. Grade	0.170					
I						
II	0.100	2.75	0.8-9.1			
III	0.060	3.21	1.0-10.8			
ER status	0.000			0.005		
Positive						
Negative		3.02	1.7-5.3		2.48	1.3-4.7
PR status	0.029					
Positive						
Negative		1.84	1.1-3.2			

ER, estrogen receptor; PR, progesterone receptor

differentiation grade (38%). Receptor positivity predominated; estrogen and progesterone receptors were positive in 72% and 51% of the cases, respectively. The majority of the patients did not have any extensive tumour load in the axilla; 1-3 positive nodes only were detected in 64% of the cases. Most patients represented clinical stage II (91%). MMP-9 immunoreactivity did not significantly relate to the histological type, clinical stage or hormone receptor status of the primary tumour (Table I).

Disease-free survival (DFS). Primary tumour size ($p<0.0005$), clinical stage ($p<0.0005$) and estrogen receptor status ($p<0.0005$) were statistically powerful predictors of the disease-free survival in a univariate analysis and showed a strong prognostic power also in a multivariate analysis (Table II). There were no statistically significant differences in terms of DFS in subgroups of patients with MMP-9-negative or -positive tumours if studied in the entire study population.

Overall survival (OS). Comparable to DFS, primary tumour size ($p<0.0005$), clinical stage ($p<0.0005$) and estrogen

Table III. *Cox univariate and multivariate analysis of overall survival.*

Factor	Univ. analysis			Multiv. analysis		
	p-value	HR	(95% CI)	*p*-value	HR	(95% CI)
MMP-9 status	0.805					
Negative		1.08	0.5-2.0			
Positive						
Tumour size	0.000			0.043		
T1						
T2	0.001	4.5	1.8-10.8	0.004	4.7	1.6-14.0
T3	0.000	13.2	4.4-39.8	0.368	2.9	0.2-28.8
T4	0.026	11.1	1.3-92.7	0.531	2.5	0.1-47.7
Nodal metast.	0.074					
1-3						
≥4		1.98	1.0-4.0			
Clinical stage	0.000					
II						
III		7.72	2.2-10.0	0.066	7.2	0.9-58.5
Histol. Grade	0.187					
I						
II	0.138	3.03	0.7-13.1			
III	0.070	3.89	0.9-16.9			
ER status	0.000			0.003		
Positive						
Negative		3.5	1.9-6.7		3.1	1.5-6.4
PR status	0.055					
Positive						
Negative		1.9	1.0-3.5			

ER, estrogen receptor; PR, progesterone receptor

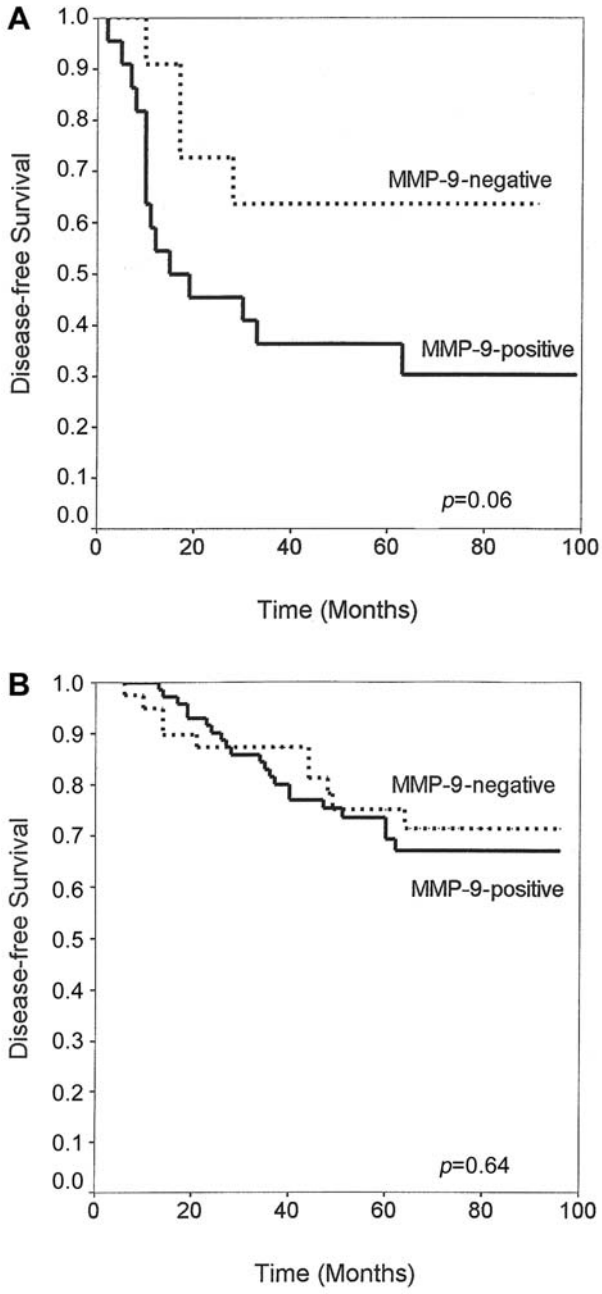

Figure 1. *A. Disease-free survival of estrogen receptor-negative patients in relation to MMP-9; - - - - = MMP-9-negative; ——— = MMP-9-positive. B. Disease-free survival of estrogen receptor-positive patients in relation to MMP-9; - - - - = MMP-9-negative; ——— = MMP-9-positive.*

receptor status ($p<0.0005$) were significant predictors of overall survival in a univariate analysis. Estrogen receptor was shown to be the strongest predictor of overall survival in a multivariate analysis ($p<0.005$) along with the primary tumour size ($p<0.05$) (Table III). MMP-9 immunoreactivity was not associated with differences in the overall survival rates of the patients.

Recurrence of breast carcinoma in hormone receptor-negative cases according to MMP-9 status. An average disease-free survival time in patients with an estrogen receptor (ER)-negative and MMP-9-positive tumour was 42 months (95%, confidence interval (CI): 25-59 months), while patients with both ER- and MMP-9-negative tumours had a more favourable outcome with an average survival time of 64 months (95%, CI: 44-95 months). Five-year disease-free survival rate was 63% in patients with ER-positive and

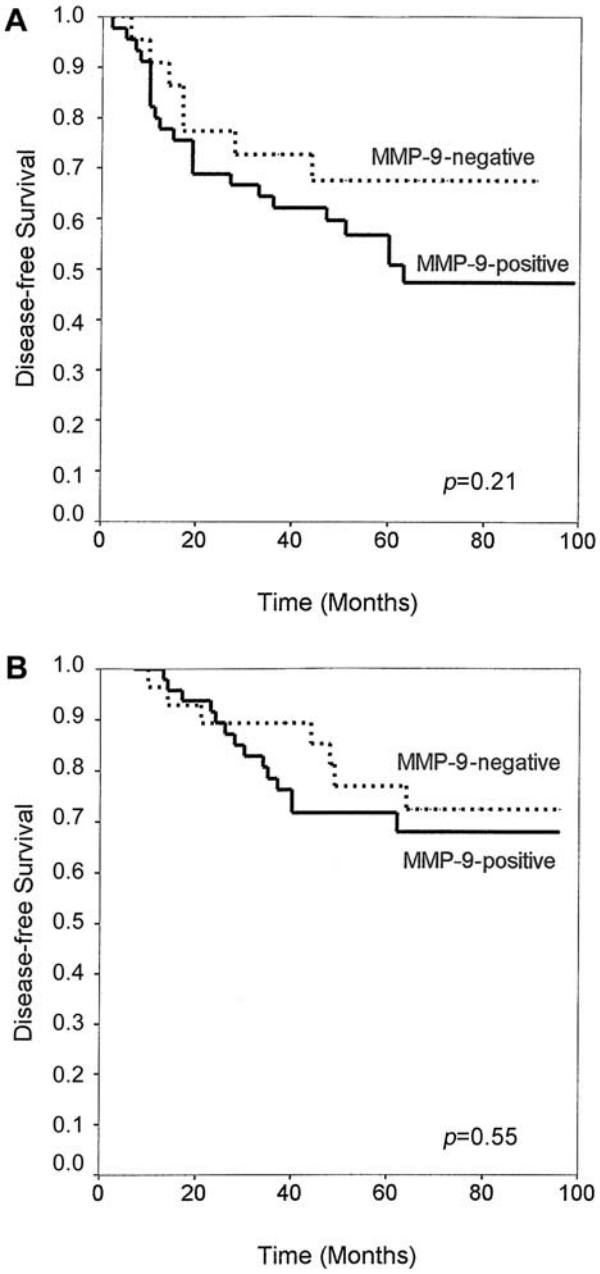

Figure 2. *A. Disease-free survival of progesterone receptor-negative patients in relation to MMP-9; - - - - = MMP-9-negative; —— = MMP-9-positive. B. Disease-free survival of progesterone receptor-positive patients in relation to MMP-9; - - - - = MMP-9-negative; —— = MMP-9-positive.*

MMP-9-negative tumour, while only 37% of patients with ER-negative but MMP-9-positive tumour were free of recurrence after 5-year surveillance (by Breslow test *p*-value=0.06) (Figure 1A).

Seven patients with progesterone receptor (PR)-negative and MMP-9-positive primary tumour relapsed during the follow-up, while there was 22 recurrences in the patient group presenting with a primary tumour negative for both PR and MMP-9, but the difference was not statistically significant ($p=0.21$) due to the small number of the receptor-negative cases in this material (Figure 2A.) MMP-9 status did not correlate with the disease-free survival in the hormone receptor-positive cases (Figure 1B, 2B).

Survival in relation to primary tumour size, nodal status and MMP-9 positivity. Most primary tumours were 20-50 millimetres in size, and among this population, MMP-9-positive immunostaining was associated with a shortened disease-free survival, but the result did not reach statistical significance ($p=0.081$). Both disease-free and overall survival were also analysed according to tumour burden in the axilla and MMP-9 immunostaining. The MMP-9 overexpression did not correlate with survival in the subgroups of patients with 1-3 or ≥4 positive axillary lymph nodes.

Discussion

There are convincing preclinical data implicating the remarkable role of the matrix metalloproteinases in cancer progression (4,5). In breast carcinoma, the immunoreactive protein for MMP-2 is identified as a prognostic factor in several studies (6,7), while MMP-9 in this regard has been less frequently investigated.

The aim of this study was, foremost, to examine the possible prognostic value of MMP-9 in node-positive postmenopausal breast cancer patients treated with an adjuvant antiestrogen therapy. We showed here a tendency for compromised disease-free survival in a subgroup of hormone receptor-negative patients expressing MMP-9 immunopositivity in their primary tumour, while MMP-9 immunopositivity did not predict survival in patients with a hormone receptor-positive primary tumour. Ranuncolo *et al.* (14) reported that patients with decreasing plasma MMP-9 activity during adjuvant therapy had better survival compared to patients with lack of response whose plasma activity increased. All the patients in this study had been treated with an adjuvant antiestrogen therapy due to axillary node involvement, which is now known to be effective in patients with a hormone receptor-positive primary tumour. Bearing that in mind, it is logical that, in this study, MMP-9 seemed to be associated with unfavourable prognosis only in patients negative for hormone receptors, while in other patients no such association was identified.

Well-established prognostic factors such as tumour size, clinical stage and estrogen receptor status correlated strongly with survival also in this study population. Scorilas *et al.* (15)

found MMP-9 positivity to be an indicator of a favourable prognosis in node-negative patients, but this was not seen in node-positive patients, which is comparable to our results.

MMP-9 positivity was seen here in 103 cases (61.3%), the proportion being slighty higher than that in a study by Scorilas *et al.* (52%) (15). In the present study, the disease was more advanced, which might, in theory, be reflected in the percentage of positive cases. However, the clinical stage did not significantly correlate here to MMP-9 immunoreactivity. The criteria for MMP-9 positivity and the rate of cases with positive immunostaining vary in different tumours. Choe *et al.* investigated MMP-9 overexpression by a gelatine zymography method in 20 glioblastoma multiforme patients and found latent form MMP-9 in 90%, and active form in 50% of tumours in autopsy specimens (18). MMP-9 immunopositivity was seen in 52% of tumour specimens in 169 resectable non-small cell lung carcinoma patients in a study presented by Cox *et al.* (9).

Our results suggest that MMP-9 positivity relates to a compromised disease-free survival in hormone receptor-negative breast carcinoma, while MMP-9 immunoreactivity did not affect survival in patients presenting with an estrogen receptor-positive tumour. Analogous to the present data, Talvensaari-Mattila *et al.* (7) found a statistically significant correlation between MMP-2 positivity and significantly shortened survival in a patient group with a hormone receptor-negative breast carcinoma. The result can be further interpreted in the light of preclinical data regarding a connection between estrogen-mediated signals in a cell and MMP expression. Razandi *et al.* have investigated breast cancer cells *in vitro* and found estradiol signal transduction resulting in EGFR transactivation along with activation of both MMP-9 and MMP-2 (18). Letrozole, an aromatase inhibitor, has shown to be a potent *in vitro* inhibitor of cell proliferation and to decrease both MMP-9 and MMP-2 expression (19). Wolczynski *et al.* investigated raloxifene, an antiestrogenic and breast cancer preventive compound, in breast cancer cells *in vitro*, and they found it to exert a dose-dependent effect on collagen metabolism and MMP expression (20). The authors conclude that these mechanisms may explain raloxifene's role in the prevention of breast cancer development.

New prognostic factors, such as gene-expression profiling, are needed in the individual risk evaluation of breast cancer patients to improve selection of patients for adjuvant therapies. In this study population, MMP-9 immunoreactivity did not have significant prognostic power in the whole population consisting mostly of receptor-positive cases. In the early 90's, however, also some receptor-negative cases were included in antiestrogen adjuvant treatment and, among them, a tendency for a shortened disease-free survival was seen in patients with MMP-9-positive primary tumours. The difference in 5-year disease-free survival rate was 26%, which would be clinically important if confirmed in larger material. In future studies, it would be interesting to find out whether aromatase inhibitors could prevent the risk effect of MMP-9 positivity similarly, or in some groups even better, than do the antiestrogens.

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