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ORIGINAL PAPER

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Matrix metalloproteinases as diagnostic (MMP-13) and prognostic (MMP-2, MMP-9) markers of prostate cancer

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Abstract Previous studies have detected high levels of matrix metalloproteinases (MMPs) in metastatic prostate cancer. In this study, we recruited 40 patients with prostate cancer (PCa): 20 presented organ-confined carcinoma and 20 had metastatic cancer. We also recruited 40 subjects for control groups, 20 with benign prostate hyperplasia (BPH) and 20 healthy males with similar characteristics. All of the patients were monitored at the beginning (time 0) and after 90 days. We analyzed the plasma concentrations of MMP-2, MMP-9, MMP-13, TIMP-1 and the enzyme activity of MMP-2 and MMP-9,using specific ELISA tests. The plasma concentrations of MMP-2, MMP-9 and MMP-13 were higher in PCa patients with metastasis than in the other groups, and in these patients decreased markedly after therapy began. For MMP-2 and MMP-9, greater differences were observed in enzyme activity than in plasma concentrations. TIMP-1 was reduced in PCa patients with metastasis, even if the intergroup differences were not statistically significant. Our results suggest that the plasma concentration and activity of MMPs, in association with PSA determination, could play a role in diagnosis, monitoring therapy and evaluating malignant progression in PCa.

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Introduction

Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading various components of the extra cellular matrix (ECM) [1, 2]. They are involved in physiological processes occurring during membrane remodeling and repair, and play a crucial role in certain non-malignant and malignant pathologies such as rheumatoid arthritis, aortic aneurysms, myocardial infarctions, septic shock, liver disease, tumor invasion and neoplastic metastasis [3, 4]. The restructuring of normal tissue requires a balanced interaction between MMPs and the tissue inhibitors of metalloproteinases (TIMPs) [5]. Numerous studies on colorectal cancer [6], urothelial cancers [7, 8], renal cell carcinoma [2], breast cancer [9] and prostate cancer [10] have shown that MMPs and their TIMPs are produced by the interstitial monocytes-macrophages and fibroblasts localized in the tumor focus, as well as by neoplastic cells [5], and that their expression is generally correlated with tumor differentiation. In particular, an increase in MMP-9 protein and mRNA concentrations in prostate carcinoma seem to be involved in biochemical events preceding the invasion of the surrounding tissues by the tumor, and play a crucial role in tumor progression [11, 23]. In addition, MMP-13 is an important metalloproteinase involved in the enzymatic cascade, because proMMP-13 is activated by MMP-2 and by MT-MMP-1 (MMP-14), which can also activate proMMP-2. The activated forms of MMP-2 and MMP-13 take part in the activation of proMMP-9 into MMP-9. In this way, MMP-13 is involved in metastatic and non-metastatic tumors (CNS, neck, skin, female genital tract), where molecular expression is stimulated by numerous cytokines, growth factors and tumor promoters acting on tumor cells or

perineoplastic fibroblasts [12]. Studies on MMP-2 expression in prostate cancer have found a correlation between molecular over-expression and enhanced crossing and destruction of the basement membrane and stromal invasion [26]. This was related to the grade of tumor differentiation [10]. By contrast, TIMP-1 values were higher in normal tissues and well differentiated carcinoma tissues, while they were reduced in advanced and anaplastic carcinomas [13]. TIMP-1 seems to have two opposite effects at different concentrations: at between 10 and 100 ng/ml it acts as a growth factor, while at concentrations exceeding 1 μg/ml it inhibits extra cellular matrix proteolytic degradation [4]. At present, PSA in prostate cancer (PCa) diagnosis and follow-up is the universally recognized marker. This glycoprotein, present in different molecular forms, can be determined in serum (normal values 0–4 ng/ml) and has high specificity and diagnostic efficacy (90%) but low sensitivity (54%). It is known that 21–47% of males affected by BPH [14] have PSA values >4 ng/ml and that 38–48% of organ-confined tumors [15] present values in the normal range. On the other hand, low PSA concentrations may be associated with bone metastasis in anaplastic tumors, in percentages varying between 0.5–2% [16]. Other benign pathologies, such as acute retention of urine, prostatitis and prostate infarction, can specifically enhance the levels of this marker [17]. Several studies have found that normal and hypertrophic epithelial prostate cells produce more PSA, inducing the hyper-expression of specific mRNA, than do cancer tissue. Thus, elevated PSA serum values in PCa patients seem to be caused by an altered prostate architecture with ECM degradation, rather than increased synthesis, and in these conditions there is a corresponding increase in the plasma levels of MMPs, which could be used as potential tumor markers [18, 24]. In relation to the findings that during tumor growth various circulating molecules can show structural and quantitative changes, different experimental protocols have been proposed to detect alternative markers to use, in association to PSA, as diagnostic and prognostic tests for PCa evaluation [19]. Since the MMPs/TIMPs ratio is one of the numerous molecular systems that play a crucial role in PCa, the determination of their concentration and plasma activity could be a useful diagnostic and prognostic indicator, as shown by various recent papers [23, 25, 27].

Materials and methods

We determined the plasma concentrations of MMP-2, MMP-9 and MMP-13 and their inhibitor TIMP-1, and the enzymatic activity of MMP-2 and MMP-9 in PCa organ-confined and metastatic patients, using as control groups non-smokers with benign prostate hyperplasia (BPH) and healthy males with similar general characteristics (age, life habits, weight, etc). The study population consisted of 80 male subjects (age range 68–74 years; mean age 71 years) recruited over 15 months. The study groups were subdivided as follows:

1. Forty patients with PCa. The tumor was organ-confined [PCa (M−)] in 20 subjects (T1–2 N0 M0, Gleason score 5–7), candidates for radical retropubic prostatectomy (RRP); and advanced [PCa (M+)] in the remaining 20 patients (T3–4 N1 M1, Gleason score 6–8) who had begun hormonal treatment using maximal androgenic blockade (MAB, started at time 0).
2. Twenty patients with BPH and obstructive symptoms (LUTS), documented by an elevated International Prostate Symptom Score (IPSS) values of between 16 and 29, who did not respond to α-lytic and/or finasteride treatment and were therefore candidates for endoscopic (TURP) or open sky surgical treatment (prostate adenomectomy).
3. The control group consisted of 20 healthy subjects with similar characteristics to the cancer patients (age, life habits, weight, etc).

A complete clinical check-up was performed on all the subjects (non-smokers) recruited, to exclude concomitant diseases associated with an increase in MMPs plasma levels, such as rheumatoid arthritis, aortic aneurysms, myocardial infarctions, liver disease and other diagnosable tumors. All of the patients (BPH and PCa) were monitored for the following parameters at time 0 and 90 days after treatment. In addition, the healthy subjects were evaluated 3 months after the first checkup (time 0) to confirm the reliability of the data.

Serum PSA determination

PSA was determined using the RIA method. Normal values were considered less than or equal to 4 ng/ml. Blood was collected separately in plastic test tubes containing 2–3 drops of heparin to prepare the plasma for quantitative MMPs and TIMP-1 determination. The tubes were centrifuged at 1,700 *g* for 15 min at 4°C within 30 min of collection. The supernatant was carefully removed, divided into different parts and stored at −20°C until assayed.

ELISA for MMPs and TIMPs

The plasma concentrations of MMP-2, MMP-9, MMP-13 and TIMP-1 were determined using the commercially available EIA method (Biotrack Amersham Life Science) by means of an ELISA sandwich technique which uses two antibodies directed against different epitopes of MMP-2, MMP-9, MMP-13 and TIMP-1. During the initial incubation phase, the MMP-2, MMP-9, MMP-13 and TIMP-1 present in the samples or standards (a

purified protein kit for the construction of a standard curve) was bound in wells that had been pre-coated with the antibody. This phase lasted 2 h for MMP-2, MMP-13 and TIMP-1 and 1 h for MMP-9. The second incubation phase (1 h for MMP-2 and MMP-13; 2 h for MMP-9 and TIMP-1) showed that the antigen-antibody reaction was mediated by adding a second antibody conjugated with peroxidase. The amount of peroxidase binding to each well was determined by adding a pre-prepared medium (tetramethylbenzidene, TMB). The reaction was blocked by adding a 1 M sulfuric acid solution and color was measured at 450 nm using a microwell reader (Sorin Biomedica) within 10 min of the last phase of the experiment. Concentrations of MMP-2, MMP-9, and MMP-13 in the samples were determined by extrapolation from an adapted standard curve. MMPs and TIMPs were determined in the following ranges: MMP-2 between 1.5 and 24 ng/ml; MMP-9 between 4 and 128 ng/ml; MMP-13 between 0.094 and 3 ng/ml; TIMP-1 between 1 (or 3.13) and 50 ng/ml.

Determination of MMP-2 and MMP-9 activity

MMP-2 and MMP-9 activity was determined using commercial kits (Biotrack Amersham Pharmacia Biotech). The assay uses the pro-form of a detection enzyme that can be changed by capturing active MMP-2 or MMP-9 onto an active enzyme through a single proteolytic event. The natural sequence of activation in the pro-detection enzyme is substituted by an engineered protein with an artificial sequence recognized by specific matrix metalloproteinases. The detection enzyme activated by MMP-2 and MMP-9 can be measured using a specific peptide chromogene medium. Standards and samples were incubated in wells pre-coated with anti-MMP-2 and anti-MMP-9 antibody, respectively. Any MMP-2 or MMP-9 present was bound to the wells. All other compounds in the samples were removed by washing and aspiration. The activity of total MMP-2 or MMP-9 was determined by activating the entire molecule in its proform using p-aminophenylmercury acetate (APMA). Standards consisted of diverse concentrations of proMMP-2 and proMMP-9 which were activated simultaneously in all samples. The active form of MMP-2 was determined by reading the resulting color at 405 nm on a microwell reader (Sorin Biomedica). Concentrations of the active form of MMP-2 and MMP-9 were determined by extrapolation from the appropriate standard curve. The active form of MMP-2 can be determined in a range of between 0.75 and 12 ng/ml with an incubation time of about 1.5–2 h, assay sensitivity being about 0.5 ng/ml. Analogously, MMP-9 activity can be determined in a range between 2 and 32 ng/ml after 2–6 h incubation, the assay sensitivity of this enzyme being 1 ng/ml. Assay sensitivity was reduced by prolonged incubation in both assessments.

Statistics

Calculations were performed using the SPSSv 6.1 software package (SPSS, Chicago, USA). All data are presented as mean ± SEM. Evaluation of the statistical significance was performed using the Student's *t*-test for paired values (pre- to post-treatment) and unpaired values (statistical comparison between different groups). *P* values of less than 0.05 were considered to be significant and are indicated by an asterisk on the graphs; the non-significant values are indicated with NS.

Results

The analyzed parameters (PSA, MMPs, and TIMP-1) were measured at time 0 and after 90 days for the healthy subjects control group. Little statistical variation (only between 2 and 4%) was found in comparing these times.

The plasma concentration data are summarised in Table 1. Plasma MMP-2 concentrations in healthy subjects (controls), BPH patients and patients with prostate adenocarcinoma [PCa (M−) and PCa (M+)] are summarized in Fig. 1. Pretreatment plasma MMP-2 concentrations in PCa (M+) patients were threefold greater than those observed in controls (1,230 ± 54.77 ng/ml vs 471.70 ± 48.96 ng/ml) and higher than those observed in BPH patients (814 ± 80 ng/ml) and in PCa (M−) patients (893 ± 27.39 ng/ml), the ratio for the last two series being about 3:2. Post-treatment, either medical [MAB in PCa (M+)] or surgical [RRP in PCa (M−)], values were reduced, especially in patients with metastatic disease. MMP-2 expression 90 days after treatment was 671 ± 39 ng/ml in PCa (M−) patients;

Table 1 Plasma concentration (PC) of MMP-2, MMP-9, MMP-13, TIMP-1 and enzymatic activity (PA) of MMP-2 and MMP-9 in the study groups. The values are means ± SD

	Control	BPH		PCa(M−)		PCa(M+)	
Parameters		Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
MMP-2 (PC)	471.7 ± 48.96	814.0 ± 80.00	686.0 ± 31.95	893.0 ± 27.39	671.0 ± 39.00	1,230.0 ± 54.77	790.0 ± 114.11
MMP-2 (PA)	14.30 ± 2.03	15.43 ± 0.87	15.57 ± 0.48	24.20 ± 1.74	18.60 ± 1.08	82.40 ± 21.16	38.60 ± 10.17
MMP-9 (PC)	16.90 ± 1.86	21.75 ± 4.64	13.25 ± 2.69	48.60 ± 13.09	23.40 ± 6.15	108.00 ± 20.04	38.00 ± 12.61
MMP-9 (PA)	12.20 ± 0.33	16.20 ± 2.99	13.20 ± 3.71	30.40 ± 5.31	17.20 ± 2.65	90.00 ± 27.39	30.60 ± 10.48
MMP-13 (PC)	< 0.13	2.20 ± 0.058	1.40 ± 0.49	15.20 ± 1.93	9.00 ± 1.70	28.60 ± 5.05	20.60 ± 4.06
TIMP-1 (PC)	568.50 ± 40.09	450.43 ± 49.53	483.86 ± 68.95	378.00 ± 83.55	475.2 ± 134.15	541.0 ± 103.83	620.0 ± 188.15

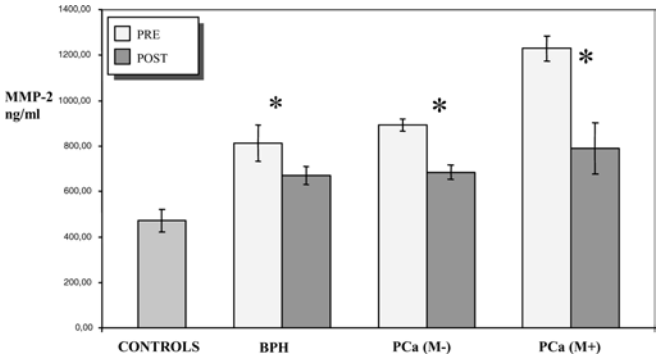

Fig. 1 Plasma MMP-2 values

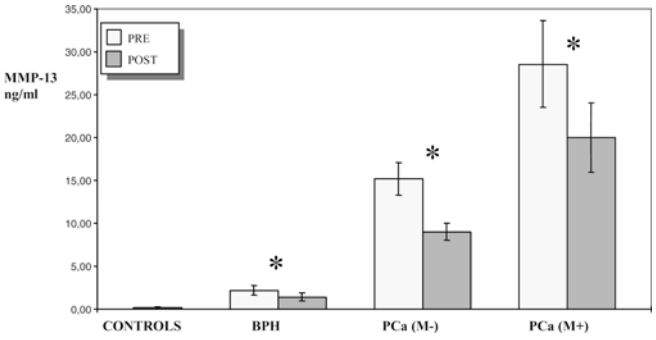

Fig. 3 Plasma MMP-13 values

686 ± 31.95 ng/ml in BPH patients and 790 ± 114.11 ng/ml in PCa (M+) patients. The results were statistically significant compared to pre-treatment and intergroup values, [BPH $P = 0.028$; PCa (M−) $P = 0.001$; PCa (M+) $P = 0.005$].

Figure 2 shows the MMP-9 concentrations for the entire study series. Even if plasma MMP-9 values were determined in tens of ng and MMP-2 in hundreds of ng, both metalloproteinases behaved similarly. Mean values in BPH patients (21.75 ± 4.64 ng/ml) were similar to those observed in control subjects (16.90 ± 1.86 ng/ml), while concentrations in PCa (M−) patients rose to 48.60 ± 13.09 ng/ml, and were much higher (108 ± 20.04 ng/ml) in PCa (M+) patients. Post-treatment plasma MMP-9 concentrations were also considerably decreased, the reduction being greater in PCa (M+) patients (38 ± 12.61 ng/ml) than in organ-confined prostate cancer (23.40 ± 6.15 ng/ml) and BPH patients (13.25 ± 2.69 ng/ml). The results were statistically significant [BPH $P = 0.03$; PCa (M−) $P = 0.028$, PCa (M+) $p = 0.003$].

Plasma MMP-13 concentrations in the various study groups are reported in Fig. 3. Pretreatment MMP-13 concentrations in the controls were below the kit minimum detection limit of 0.13 ng/ml, and slightly higher in BPH patients (2.20 ± 0.058 ng/ml). As was observed for MMP-9, the mean MMP-13 level was greater in patients affected by organ-defined carcinoma (15.20 ± 1.93 ng/ml) and in those presenting advanced metastatic cancer (28.6 ± 5.05 ng/ml). Post-treatment plasma MMP-13 concentrations were lower than pretreatment ones in all groups: BPH patients = 1.40 ± 0.49 ng/ml; PCa (M−) patients = 9.00 ± 1.70 ng/ml; PCa (M+) patients = 20.60 ± 4.06 ng/ml. The results were statistically significant [BPH patients $P = 0.003$; PCa (M−) $P = 0.001$; PCa (M+) $P = 0.012$].

TIMP-1 concentrations (Fig. 4) showed a different trend, and, as in the data reported in the literature, this was higher in the controls and patients with metastases. The mean TIMP-1 concentration was 568.5 ± 40.09 ng/ml in the 20 controls, while it was 541 ± 103.83 ng/ml in PCa (M+) patients. The mean TIMP-1 value was lower (378 ± 82.55 ng/ml) in PCa (M−) patients, and slightly higher in BPH patients (450.43 ± 49.53 ng/ml). All plasma TIMP-1 concentrations increased after treatment, being 483.86 ± 68.95 ng/ml in BPH patients, 475.20 ± 134.15 ng/ml in PCa (M−) patients, 620 ± 188.15 ng/ml in PCa (M+) patients, but the rise was not statistically significant [BPH $P = 0.467$; PCa (M−) $P = 0.273$; PCa (M+) $P = 0.498$].

MMP-2 activity is shown in Fig. 5. It mirrored the activity of the corresponding protein. Pretreatment, patients with metastatic cancer had higher mean values (82.40 ± 21.16 ng/ml) than controls (14.30 ± 2.03 ng/ml). BPH patients and controls had overlapping values (15.43 ± 0.87 ng/ml), with negligible intragroup variability. Mean MMP-2 activity was slightly higher in PCa (M−) patients (24.20 ± 1.74 ng/ml), but never reached

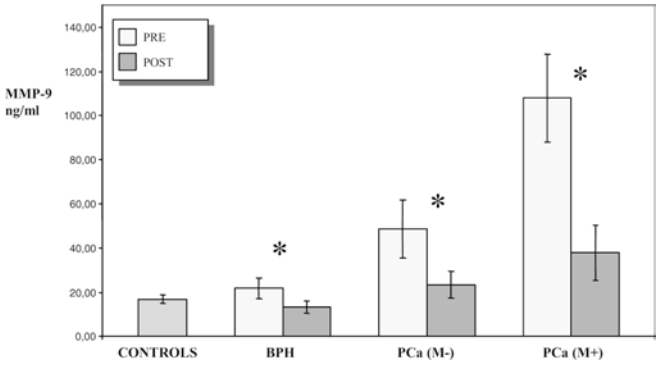

Fig. 2 Plasma MMP-9 values

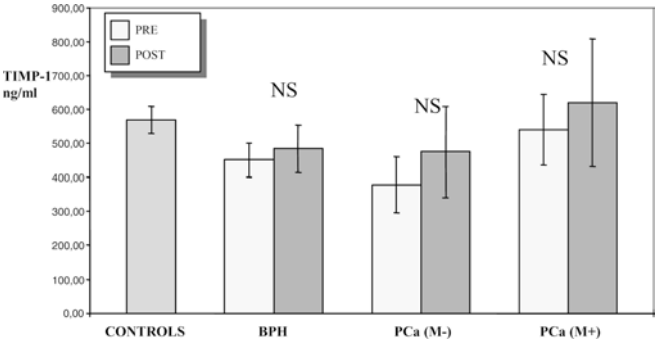

Fig. 4 Plasma TIMP-1 values

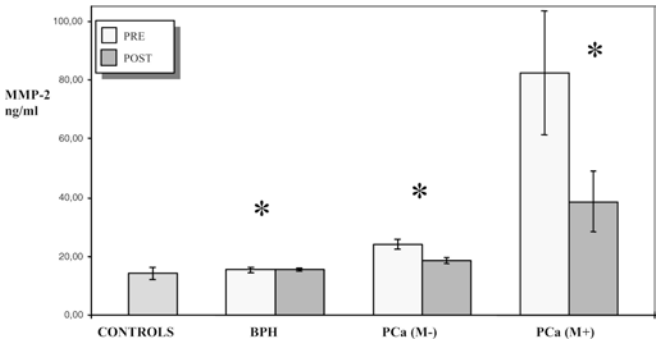

Fig. 5 Plasma MMP-2 activity

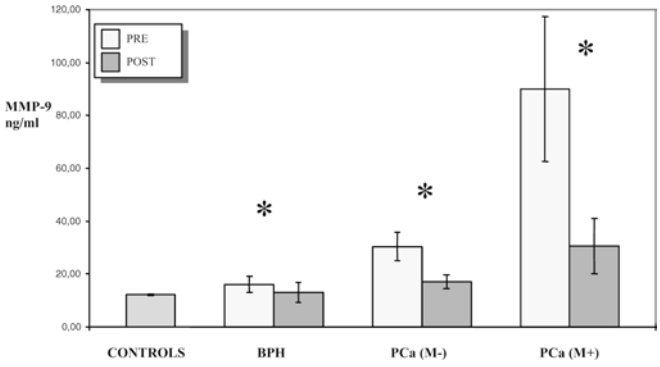

Fig. 6 Plasma MMP-9 activity

the values observed in PCa (M+) patients. Post-treatment values were almost unchanged in BPH patients (15.57 ± 0.48 ng/ml) and in PCa (M−) patients (18.60 ± 1.08 ng/ml). Conversely, the elevated MMP-2 activity concentrations observed in PCa (M+) patients prior to treatment dropped to 38.60 ± 10.17 ng/ml 90 days after treatment. Thus the results obtained for MMP-2 activity were only significant in PCa (M−) ($P = 0.001$) and especially in PCa (M+) patients ($P = 0.022$). No statistically significant difference was observed between BPH patients and healthy subjects ($P = 0.788$).

Plasma MMP-9 activity followed the same trend as MMP-2 and was elevated in PCa (M+) patients (90.0 ± 27.39 ng/ml) (Fig. 6). MMP activity presented an absolutely minimum concentration in controls (12.20 ± 0.33 ng/ml), was low in BPH patients (16.20 ± 2.99 ng/ml) and slightly higher in PCa (M−) (30.40 ± 5.31 ng/ml). As was observed for MMP-2 activity, post-treatment MMP-9 activity was slightly reduced in all groups, except in PCa (M+) patients where it decreased markedly from pretreatment values to a mean post-treatment value of 30.6 ± 10.48 ng/ml. Mean post-treatment MMP-9 activity was 13.20 ± 3.71 ng/ml in BPH patients and 17.20 ± 2.65 ng/ml in PCa (M−). As observed for MMP-2, no statistically significant difference was seen in MMP-9 activity values in BPH patients ($P = 0.119$). However, statistically significant differences were observed in PCa (M−) ($P = 0.011$), and in this case also primarily in PCa (M+) patients ($P = 0.025$).

Figure 7 shows the mean PSA concentrations for the entire study series: 87.6 ± 28.8 ng/ml in PCa (M+) patients, 6.2 ± 2.4 ng/ml in PCa (M−) patients, 3.2 ± 0.38 ng/ml in BPH patients and 2.23 ± 0.6 ng/ml in controls. As expected, the PSA evaluated after 90 days of treatment was markedly reduced in patients with PCa (M−) treated with RRP, with values of 0.09 ± 0.03 ng/ml, while the patients with metastatic diseases PCa (M+) had values of 5.6 ± 3.3 ng/ml, which shows the efficiency of the treatment used. The reduction post-TURP or prostate adenomectomy in patients with progressive BPH was markedly less, and the PSA serum values oscillated between 1.3 ± 0.8 ng/ml.

The results obtained confirm the sensitivity and reproducibility of the commercial kits used.

Discussion

Plasma MMP activity (MMP-2, MMP-9) and protein concentration (MMP-2, MMP-9, MMP-13) were easily determined using the ELISA method and were all more markedly increased in metastatic prostate cancer patients. The study results revealed that MMP-2 and MMP-9 plasma concentrations, but mainly enzymatic activity, were greatly increased in PCa patients, especially in the presence of metastatic disease, compared to all the other study groups (healthy subjects and BPH patients), and showed that these values underwent an important decrease which was related to the treatment performed. The differences in concentrations in enzymatic activity were, indeed, more pronounced than in the plasma protein levels. However, it is not clear what mechanism determines this condition. It is probably due to an unidentified biological interaction in a biochemical cascade. For example, not all of the MMP-2 proenzyme produced is activated; there are other factors that influence proMMP-9 and proMMP-2 activation, such as an enzymatic cascade in which there is an amplification of the activation phases in the lower parts. MMP-2 is located in the upper part, while MMP-9 is in the lower, so it is mostly activated.

In addition to their biological meaning, our findings confirm the direct correlation between MMP-2/MMP-9 expression-activity and the potential spread of the tumor in relation to the stage of cancer invasion. Plasma concentrations and the enzyme activity of these MMPs could thus furnish useful information on tumor staging and on the potential or acquired trend to tumor progression. Thus, they could be used, especially the plasma MMP activity, in association with PSA to monitor the therapeutic efficacy in advanced prostate adenocarcinoma patients. Data from the literature supports this hypothesis and suggests that MMPs could be used as biochemical progression markers to detect invasive and metastatic tumor potential and to highlight the possibility of detecting these markers in the urine, as proposed by Moses et al. [21].

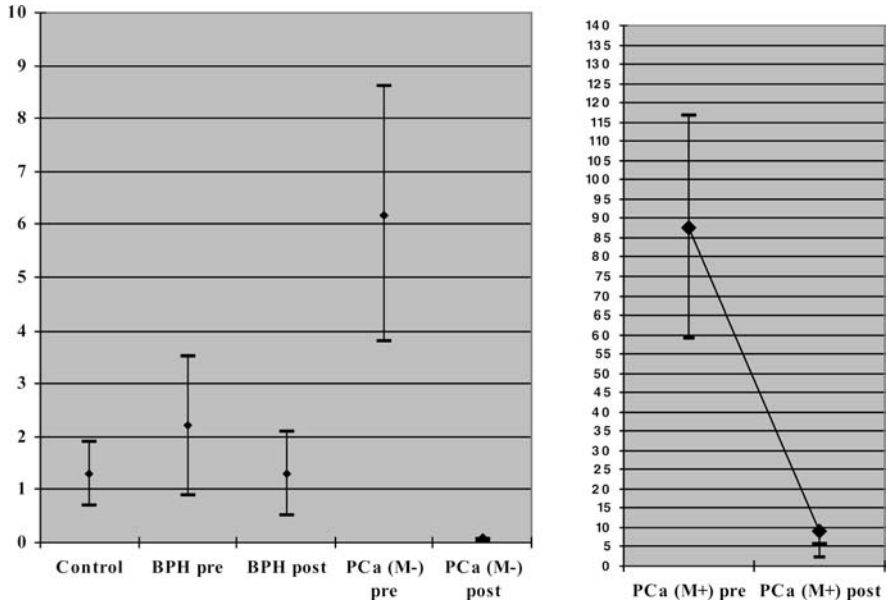

Fig. 7 PSA serum levels

Interesting results have also been obtained on MMP-13 plasma concentrations; MMP-13 is the most carcinoma-specific metalloproteinase. Indeed, plasma values of this marker were markedly reduced in healthy subjects and those with BPH, while in patients with prostate carcinoma the plasma levels recorded were increased of seven to eightfold in PCa (M−) patients and of 14–15-fold in PCa (M+). There was a clear doubling of values in metastatic patients compared to subjects with organ-confined disease. However, no statistically meaningful, reliable reduction, comparable to basic levels, was found in post-treatment MMP-13 concentrations in organ-confined and metastatic carcinomas.

The determination of plasmatic MMP-13 values could be used, in association with PSA, as a diagnostic but not as a prognostic marker, primarily in patients with borderline PSA levels and multiple negative prostate biopsies in which PCa is clinically and biochemically suspected but not histologically confirmed.

In these patients, the increase in plasma concentrations of MMP-13 could represent a strong indicator of PCa, suggesting an increase in the number of samples and biopsies performed in relation to the high probability of discovering a masked carcinoma. In addition, the presence of higher plasma concentrations of MMPs in organ-confined PCa, could allow the identification of carcinomas with elevated metastatic potential that should be treated with radical surgery rather than through conventional external radiotherapy or interstitial brachytherapy [22, 23, 24].

In light of our results, MMPs could represent interesting biomolecular diagnostic indicators (MMP-13) and prognostic markers (MMP-2, MMP-9) of tumor progression, besides being promising targets for molecular anti-cancer therapy for blocking tumor proliferation by inhibiting ECM degradation, which inhibits the spread of tumors.

Contrarily, plasma levels of TIMP-1 were reduced in PCa patients with metastatic cancer and showed a modest increase after the beginning of treatment. For this marker, intergroup differences were not statistically significant. The role played by TIMP-1 in prostate cancer is not well defined, probably because of its double, seemingly opposite, effects on tumor progression reported by other authors [4]: TIMP-1 acts as a powerful antagonist of MMPs and inhibits important stages of tumor progression, and at the same time enhances the growth of malignant cells. High concentrations of TIMP-1 (up to 1 μg/ml) seem to determine the former, while lower concentrations (10–100 ng/ml) seem to stimulate the growth of malignant cells. Because of this contradictory behavior, we do not suggest using plasma TIMP-1 levels as markers of prostate cancer.

In conclusion, we believe that the determination of metalloproteinase (MMP-2, MMP-9) concentration and activity in the plasma could be a useful prognostic support marker, associated with PSA, in the follow-up of advanced or metastatic prostate cancer patients. This could be especially meaningful for anaplastic diseases, in which PSA levels sometimes do not correspond to the real clinical tumor progression. In addition, an increase in metalloproteinase plasma concentrations (MMP-13) could lead, always correlated to serum PSA values, to repeat prostate biopsies when a strong suspicion of neoplastic disease is present and for determining the best treatment at the intra-capsular stage, in relation to the power of progression of the diagnosed tumor (MMP-2, MMP-9). The evaluation of high plasma MMP concentrations and enzyme activities should always be used after the exclusion of pathologies, such as rheumatoid arthritis, aortic aneurysms, myocardial infarctions, liver disease or other neoplasms responsible for increases in MMP blood levels.

Technical recommendations

Plasma should be carefully collected and placed in test tubes containing heparin, and not the zinc chelating agent EDTA, which can falsify the results of activity measurements since MMPs are zinc dependent. Blood samples should be stored at −20°C until the moment of immuno-enzymatic determination, and sharp changes in temperature need to be avoided, especially when determining enzyme activity. We also recommend that serum determination should not be carried out because it has been widely reported by other authors [20, 21, 22, 23, 24, 25, 26, 27] that the process of coagulation influences the results, especially for MMP-2, MMP-9, and MMP-13 protein determination. The same is true for TIMP-1, whose serum levels were five to sevenfold higher than those observed in the plasma, probably because this inhibitor is released during coagulation.

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