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IMMUNOMAGNETIC QUANTIFICATION OF CIRCULATING TUMORAL CELLS IN PATIENTS WITH PROSTATE CÁNCER: CLINICAL AND PATHOLOGICAL CORRELATION

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Summary.- *OBJECTIVES: To detect and enumerate circulating prostatic tumor cells (CTC) in the peripheral blood of patients with prostate cancer (PC) and study the relationship between CTCs and clinical-pathological parameters.*

METHODS: Prospective three-arm study: 26 patients (p) with localised PC (LPC); 24 P with metastatic PC (MPC) and 30 healthy volunteer controls. A single 7.5 ml sample of peripheral blood was retrieved; CTCs were isolated using an immunomagnetic method based on the CellSearch system (Veridex). CTCs were identified as nucleated cells negative for CD45 (leukocytes) and positive for cytokeratins. (8, 18 y 19) The relationship between CTC numbers and PSA levels, Gleason score and TNM classification was studied.

RESULTS: Only 10% of the healthy controls had 1 CTC/7.5 mL, none of the patients with localised PC had more than 3 CTCs (88% ≤ 2 CTCs), and patients with MPC had significantly higher CTC levels [m: 29 (1-178)] compared with the other two groups (P: 0.000). A positive correlation was demonstrated between the CTC count and PSA levels, tumor size, and presen-ce or absence of enlarged lymph nodes. Gleason score was the only parameter that did not show any correlation with CTC levels, and although the number of CTCs was higher in patients with visceral metasta-ses [m: 297 (0-416)] compared with bone metastases patients [m: 68 (9.5-168)], these differences were not significant.

CONCLUSIONS: Immunomagnetic analysis permits CTCs to be enumerated in peripheral blood and could be a possible way to correctly stage and make a reasonable prognosis of metastatic disease.

Keywords: *Circulating. Immunomagnetic enrichment. Metastatic carcinoma. Prostatic carcinoma. Tumoral cells.*

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Resumen.- *OBJETIVOS: Detección y cuantificación de células tumorales prostáticas circulantes (CTC) en sangre periférica de pacientes con cáncer de próstata (CP) y estudiar la relación de las CTCs con los parámetros clínico-patológicos.*

MÉTODOS: Estudio prospectivo con tres brazos: 26 pacientes (p) con CP localizado (CPL); 24p con CP metastático (CPM) y 30 controles voluntarios sanos. Se extrajo una única muestra de 7,5 mL de sangre periférica y se aislaron las CTC según un método inmunomagnético basado en el sistema CellSearch (Veridex). Las CTCs fueron identificadas como células nucleadas negativas para el CD45 (leucocitos) y positivas para las citoqueratinas (8, 18 y 19). Se estudiaron las relaciones del número de CTCs con los niveles de PSA, Gleason y clasificación TNM.

RESULTADOS: Sólo el 10% de controles sanos tenían 1 CTC/7,5 mL, ninguno de los pacientes con CP localizado tuvo más de 3 CTC (88% ≤ 2 CTC) y aquellos con CPM presentaban niveles de CTCs significativamente más altos [m: 29 (1-178)] comparados con los otros dos grupos (P: 0.000). Se demostró una correlación positiva entre el número de CTC y cifras de PSA, con el tamaño del tumor y con la presencia o no de adenopatías. El grado Gleason fue el único parámetro que no mostró correlación con los niveles de CTC y aunque el número de CTC fue mayor en aquellos con metástasis viscerales [m: 297 (0-416)] comparado con los que tenían metástasis óseas [m: 68 (9,5-168)] estas diferencias no fueron significativas.

CONCLUSIONES: El análisis inmunomagnético nos permite cuantificar las CTC en sangre periférica y podría presentar una posibilidad para lograr una estadificación correcta y estimar un pronóstico adecuado de la enfermedad metastática.

Palabras clave: *Células tumorales circulantes. Análisis inmunomagnético. Adenocarcinoma de próstata. Carcinoma metastático.*

INTRODUCTION

Metastases are the principal cause of morbidity and mortality in patients with prostate cancer (PC). The term metastasis covers a complex process of events: the neoplastic cells from the primary tumour cross the basement membrane, penetrate lymph and blood vessels and are dispersed in distant tissues. The detection of circulating tumour cells (CTC) in peripheral blood may have major prognostic and therapeutic implications (1). Preliminary experiences in determining CTC levels were attributed to Ashworth (2) who, in 1869, reported a case of a patient diagnosed with cancer who had cells in the blood which were similar to those in the original tumour. Although their presence could appear to imply advancing disease, it has not been possible to establish their true biological significance because they are small in number and it is difficult to isolate and identify them using habitual procedures. In recent years there has been a great interest in detecting circulating tumour cells in solid tumours in both peripheral blood and in bone marrow. This is partly due to the arrival of immunohistochemical techniques, especially reverse transcriptase polymerase chain reaction (RT-PCR). However, the results of different studies conducted with RT-PCR have not shown a clear association with PC disease staging (3).

A new system called CellSearchTM of Veridex has been developed that identifies and enumerates CTCs in peripheral blood by means of immunomagnetic analysis (4) and it has recently been approved by the FDA to determine CTC levels in patients with cancer of the breast, colon and prostate (5-7). This method has already been used to isolate CTCs in patients with hormone-refractory metastatic prostate cancer in previous studies and it has been demonstrated that CTC levels play a prognostic role with regard to disease survival (7-10).

The principal objective of this study is to detect and enumerate CTC levels in patients with localised and metastatic PC before commencing treatment, and to investigate its relationship with PSA levels, Gleason score and TNM staging.

MATERIALS AND METHOD

Study design

The trial was designed as a prospective diagnostic study. It was conducted at our hospital between May 2006 and November 2008. The investigation protocol and informed consent were approved by the institutional review board and all patients were given a written informed consent. There were three groups with 30 patients in each group. One group had localised PC (LPC) (T1-T2, TNM 2002 classification), another group had metastatic PC (MPC)(T2-4 N1-2 M1a, b, c), and the third group consisted of 30 healthy volunteers under the age of 40, without a family history of PC and with a prostate specific antigen (PSA) of less than 1 ng/mL in peripheral blood, assuming that PC incidence under these circumstances would be very low.

The diagnosis of PC was established by means of histological analysis of a prostate biopsy in patients with MPC or with a sample from the radical prostatectomy in patients with LPC. Exclusion criteria consisted of patients diagnosed with another tumour, patients on androgen deprivation therapy, and patients who had undergone a prostate biopsy within 15 days of inclusion in the study.

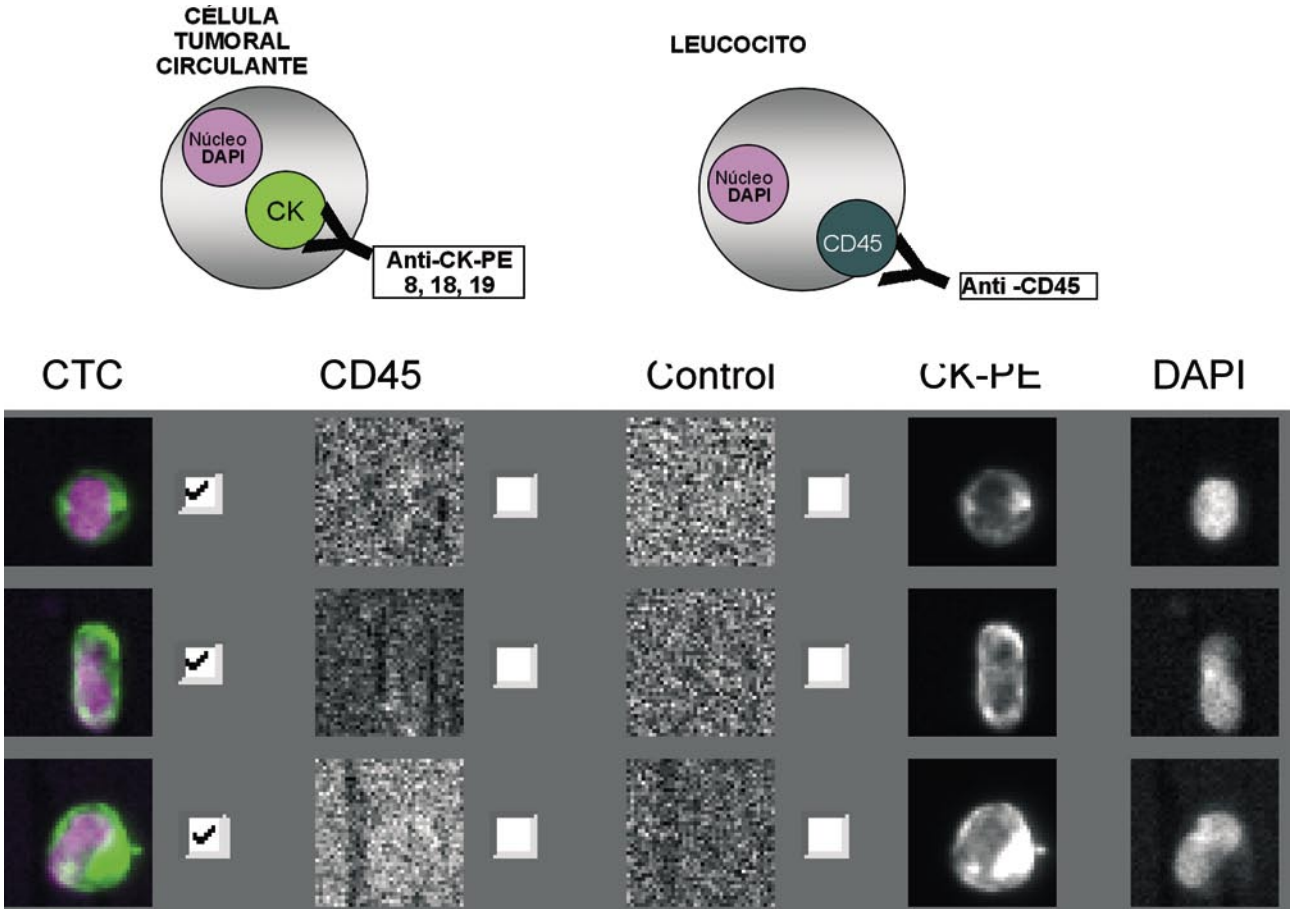

FIGURE 1. Inmunophenotype of circulating tumour cells (DAPI+, CK-PE+, CD45-) of leukocytes (DAPI+, CD45+, CK-PE-). The image to the right of the cells shows what the CTCs look like under the fluorescent microscope.

In accordance with UICC recommendations, all patients with PC underwent digital rectal examination and general blood test including PSA (Tandem E, Hybritech Inc, San Diego, CA, USA) and transrectal ultrasound (Siemens Sonoline G-50). A bone scan and abdominal CT or NMR were performed to determine the presence of disseminated metastases.

Isolation and enumeration of CTCs

Each patient had two samples of 7.5 ml of peripheral blood taken from the same venopuncture site (15 days after prostate biopsy in the CPM group and before of prostatectomy in CPL group), and the samples were collected in CellSave vacuum collection tubes (Immunicon, Huntingdon Valley, Pa). The samples were maintained at room temperature and were processed within 72 hours of being taken. The first sample was to rule out epithelial cells that could result from the venopuncture procedure itself and it was also used for the general blood analysis. The second sample was centrifuged at 2000 rpm for 10 minutes and was used to determine CTC levels using the CellSearch System (Veridex). This is a semiautomatic system that uses the CellSearch Epithelial Cell Kit (Immunicon) to isolate the cells with ferrofluid and marks them with antibodies directed against the epithelial cell adhesion molecule (anti-EpCAM). The circulating epithelial cells are isolated by means of magnetic fields and are then stained with fluorescent nucleic acid dye 4.2-diamidino-2-phenylindole dihydrochloride (DAPI). To distinguish the epithelial cells from the leukocytes, leukocyte-specific (CD45-allophycocyan) and epithelial-specific [cytokeratins 8, 18, 19-phycoerythrin (CK-PE)] monoclonal fluorescent-stained antibodies were used. The CTC were identified and counted using the CellSpotter analyser, which is a semiautomatic fluorescence microscope with specific cell-recognition software. CTCs were defined as objects with a nucleated (DAPI+), cell-like appearance (oval with a diameter greater than 3 mm), with cytokeratin positive staining (CK-PE 8, 18, 19+), lacking expression for CD-45 (Figure 1). Circulating tumour cell levels were enumerated as the number of CTCs / 7.5 ml of peripheral blood.

Statistical analysis

The median was used to study CTC levels their relationship with regional enlarged lymph nodes (N), applying a statistical significance of 0.05. Variables are summarised in medians and interquartile ranges (P_{25}-P_{75}).

To assess relationships between CTC levels and PSA levels, Gleason score and PC TNM staging, Kendall's Tau and Spearman's Rho correlation coefficients were used, depending on the case.

In all hypothetical contrasts, the null hypothesis was rejected where there was a type I error or α error of less than 0.05.

The statistical analysis was performed using the SPSS 15.0 programme.

RESULTS

A total of 80 patients were recruited at our hospital between May 2006 and November 2008. 4 patients of LPC group were denied because there was a mistake in the blood processing (coagulated, insufficient sample), and 6 patients of the MPC group were rejected because they have been diagnosed of the other tumour during the study. The demographic data and PSA levels are shown in Table I, and Table II shows the clinical and pathological data of patients diagnosed with localised adenocarcinoma of the prostate (LPC) and metastatic adenocarcinoma of the prostate (MPC).

All patients with LPC were treated with open, laparoscopic or robotic prostatectomy. The patients with MPC were commenced on hormone block therapy after they were included in the study. During the course of the study, three patients in the MPC group died (12.5%) (2 died due to disease progression and one from sepsis one week after diagnosis).

In the LPC group, three patients who were initially diagnosed as T≤2 were later classified as T3 [2T3a and 1T3b] (10% were under-staged) when the prostatectomy resection was analysed, one of these patients with LPC T3a was in the highest CTC range in this group (3 CTC), and in the other two, no CTCs were obtained in peripheral blood.

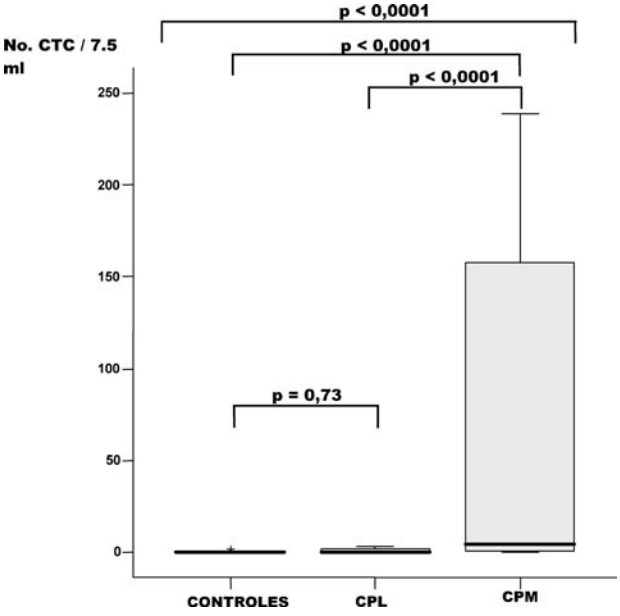

FIGURE 2. Comparison of CTC levels between the different groups studied. Statistically significant differences were observed between the 3 groups (p< 0.0001), although no significant differences were found between the control group and the group with localised adenocarcinoma of the prostate (p: 0.73).

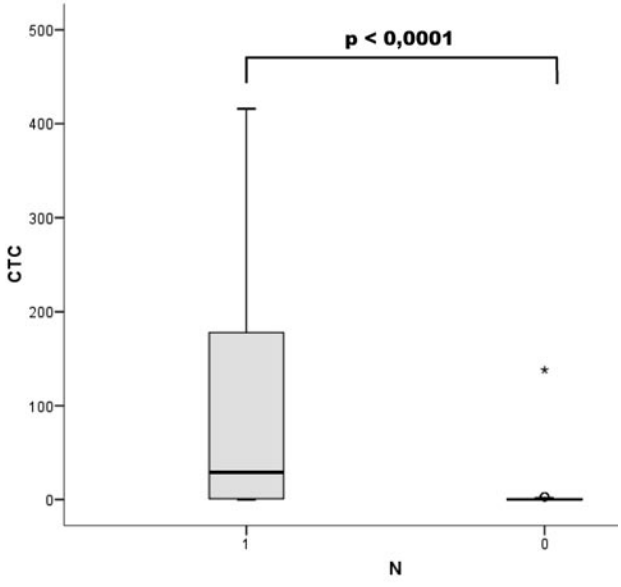

FIGURE 3. Comparison of CTC levels between patients with (N1) or without regional enlarged lymph nodes (N0). CTC levels were significantly higher in patients with regional enlarged lymph nodes [median: 29 (50-226)] (P< 0.0001), compared with patients who had no evidence of enlarged lymph nodes [median: 0.0 (0.0-1)] (P< 0.0001).

TABLE I. DEMOGRAPHIC DATA AND PSA LEVELS OF DIFFERENT GROUPS.

	CONTROL	**LPC**	**MPC**
Number	30	26	24
Age	32(29-38)	64(54-77)	73(53-91)
PSA (ng/mL)	0.49(0.2-1.1)	4.87 (2.16-14.6)	485 (41.73-2435)

In 7 patients who were clinically diagnosed with PC, and had evidence of bone metastases (in the MPC group), it was not possible to obtain histology due to general health complications.

CTC levels (expressed as the number of CTCs / 7.5 ml) are summarised in Table III.

In the control group of healthy volunteers, one CTC / 7.5 mL was found in 10% of subjects, and no patients diagnosed with localised PC had more than 3 CTC (11.5%, 3/26). In fact, 88.4% had ≤ 2 CTC. In the MPC group, the median was 29 CTC / 7.5 mL, and 71% (17/24) had ≥ 2 CTC. Applying a cut-off point of >= 5 CTC, the percentage fell to 58% and applying a cut-off point of ≥ 3 CTC there was hardly any variation, at 66.6% (16/24).

The differences with regard to the number of CTCs between the three groups under study (healthy

TABLE II. GLEASON SCORE AND STAGE ACCORDING TO THE TNM 2002 CLASSIFICATION.

		LPC (%)	**MPC (%)**
GLEASON	<=7	25 (96)	7 (41.1)
	8	0	4 (23.5)
	9-10	1 (4)	5 (29.4)
T	T2a	11 (42.3)	0
	T2b	3 (11.5)	0
	T2c	8 (30.7)	1 (4.1)
	T3a	3 (11.5)	6 (25)
	T3b	1 (4.5)	9 (37.5)
	T4	0	3 (12.5)
N	Nx	0	0
	N0	26 (100)	2 (8.3)
	N1	0	22 (91.6)
M	M1a	0	3 (12.5)
	M1b	0	18 (75)
	M1c	0	3 (12.5)

control, LPC and MPC), were of statistical significance (P< 0.0001). However, there were no differences in CTC levels between the healthy volunteers and the LPC group (P: 0.738). CTC levels were significantly higher in patients with metastatic PC [median: 29 (1-178)] compared with patients with localised PC [median: 0.0 (0.0-1)] (P< 0.0001) and healthy control subjects [median: 0.0 (0.0-0.0)] (P< 0.0001) (Figure 2).

When CTC levels were compared with the different clinical-pathological parameters, significant differences were observed in the number of CTCs between those who presented regional enlarged lymph nodes and those who did not (N) (P= 0.01) (Figure 3), good positive correlation was demonstrated between CTC levels and PSA levels (Rho=0.59, p<0.0001) and metastases (Tau=0.56, p<0.0001), respectively (Figure 4). Although the number of CTCs was progressively higher in subgroups M1a (non-regional enlarged lymph nodes), M1b (bone metastases) and M1c (visceral involvement) there were no significant differences (p=0.319) (Figure 5). A slight positive correlation was also demonstrated between the number of CTCs and tumour size (T)(Tau=0.45, p<0.0001) (Figure 4). The Gleason score was the only parameter that did not show any correlation (Tau= 0.23, p=0.07).

DISCUSSION

It is very important to detect the presence of occult metastatic cells in patients diagnosed of tumour, because early dissemination of tumour cells is one of the principal causes of relapse and of death from cancer (11-13). Previous studies have suggested that the presence of CTCs in patients with metastases originating from primaries in the colon (6, 14), breast (5, 15) and hormone-resistant prostate cancer (8, 10,16, 17) is associated with progression-free survival, and overall shorter survival.

Among the different techniques that have been used to isolate and characterise CTCs, appears the RT-PCR (18), but there have been contradictory results. Thus, some studies have managed to significantly correlate RT-PCR levels for PSA in peripheral blood, for both clinical and pathological PC staging (19-23), but other studies have not been able to corroborate these findings (3, 24, 25). Such a lack of homogeneity in findings may be due to the complexity of processing the sample, which requires many different steps and then entails the problem of quantifying results.

With the immunomagnetic method, the possibility of counting and characterising CTC in patients with PC is now a clinical reality (5-10). The system is semiautomatic and suitable for clinical laboratory use, and is therefore perfectly reproducible. The CellSearch analysis system was designed to detect epithelial cells in peripheral blood. It has been reported that the chromosomal abnormalities obtained in CTCs isolated in patients with epithelial carcinoma with MTS are equivalent to those found in the primary tumour cells (26), which shows that these circulating cells were derived from the tumour. In another recent study about hormone-resistant PC, Shaffer et al. (9) demonstrated that these CTCs were truly neoplastic and can be studied to determine their protein level as well as chromosome changes using fluorescent in situ hybridisation techniques.

TABLE III. CTC LEVELS FOR THE THREE GROUPS

CTC / 7,5 mL	**CONTROL**	**LPC**	**MPC**
Minimum	0	0	0
Maximum	1	3	919
p25	0.0	0.0	1.0
p50	0.0	0.0	29
p75	0.25	1.0	178

CTC levels for the three groups, expressed as absolute values (minimum and maximum number of CTCs), medians (p50) and interquartile ranges (p25-p75).

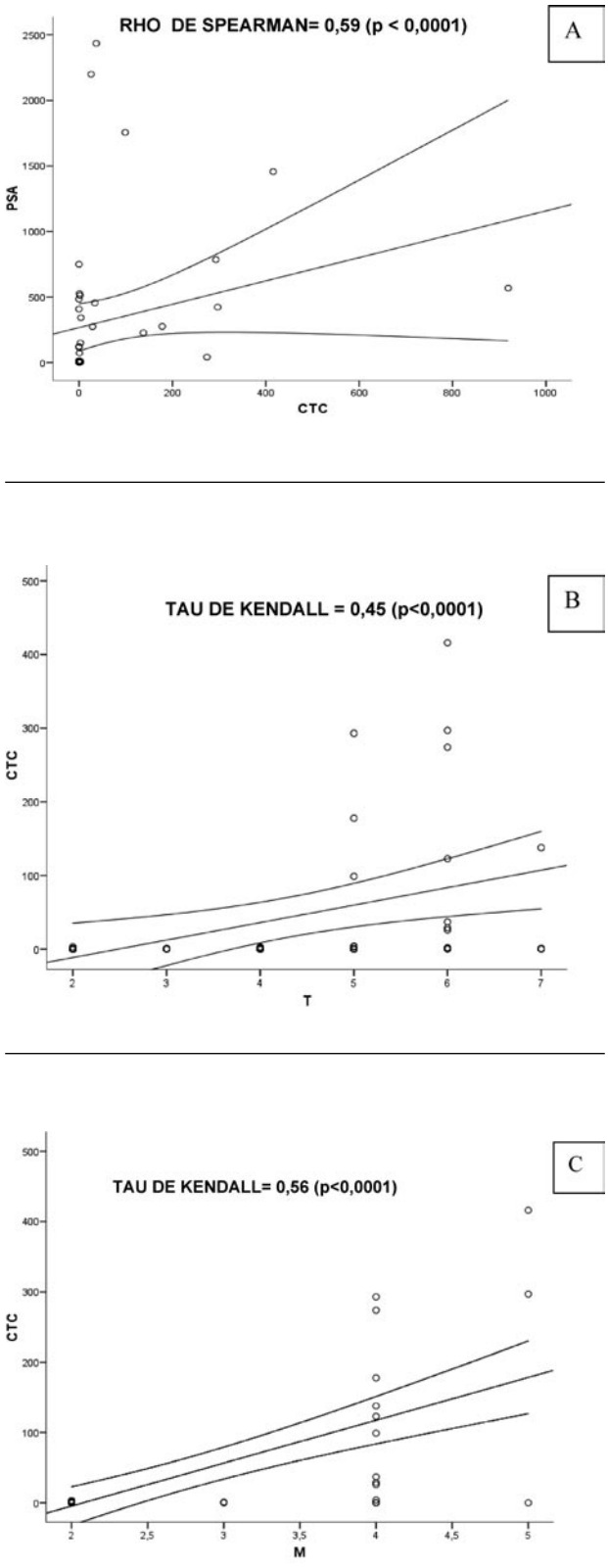

FIGURE 4. Study of the correlation between CTC levels and tumour burden markers. A: Association between CTC and PSA (Spearman's Rho=0.59, p<0.0001); B: CTC and tumour size (T) (Kendall's Tau=0.45, p<0.0001); C: CTC and metastases (Kendall's Tau=0.56, p<0.0001).

The number of CTCs required to consider that a sample is positive in patients with metastatic PC is yet to be determined. In a multi-centre double-blind prospective study, Cristofanilli et al. (5) established a cut-off point of 5 CTCs/7.5 mL in patients with metastatic breast cancer, demonstrating that these CTC levels prior to commencing therapy, or even more importantly, afterwards, during follow-up, were useful in predicting progression-free survival and overall survival. Moreno et al. (7) used a cut-off point in metastatic PC patients with 2 or more CTCs, and also demonstrated that the number of CTCs did not vary during the course of the day. Chen et al. (8) used the same cut-off value in patients with advanced PC, because when they used a cut-off value of 5 CTCs, the rate of patients with CTCs fell from 62% to 42%. In other more recent studies in which a positive result was considered as isolating 5 or more CTCs, the percentages ranged between 57 and 65% (9, 10). There is only one study, conducted by Moreno et al. (7), that has analysed the number of CTCs in patients with localised PC, and it obtained a highly variable number of CTCs (1-16, mean 5.9 CTCs). The principal disadvantages were that there were a very small number of patients -10-, the PSA range varied from 0.2 to 22.6 ng/mL and there was no anatomical-pathological confirmation of staging.

In our study, no healthy volunteers had more than one CTC, like Moreno et al. (7), who found that in patients with localised PC, none exceeded a level of more than 3 CTCs, and when this cut-off level was applied to patients with MPC, the percentage fell slightly to 66% (71% with more than 2 CTC). Further follow-up with our patients may clarify this point.

In any case, it is clear that in view of the lack of studies on this subject, further analyses and prospective studies with a larger number of patients are required in order to determine a suitable cut-off point.

Although the CTC level is considered as an important prognostic biomarker in metastatic disease survival, its relation with different parameters to inform on disease spread is not of clear clinical significance. Chen et al. (8) demonstrated a positive correlation between CTC levels and PSA levels, alkaline phosphatase and low haemoglobin level. However, the Gleason score (p = 0.351) and presence of bone or visceral metastases (p=0.085) did not predict the presence of CTCs. Shaffer et al. (9) demonstrated that despite a strong association between the CTC level and survival, when their relationship was studied with PSA level, and with the number of bone metastases (bone scan index), there was only slight relation. In our study we found a significant association between

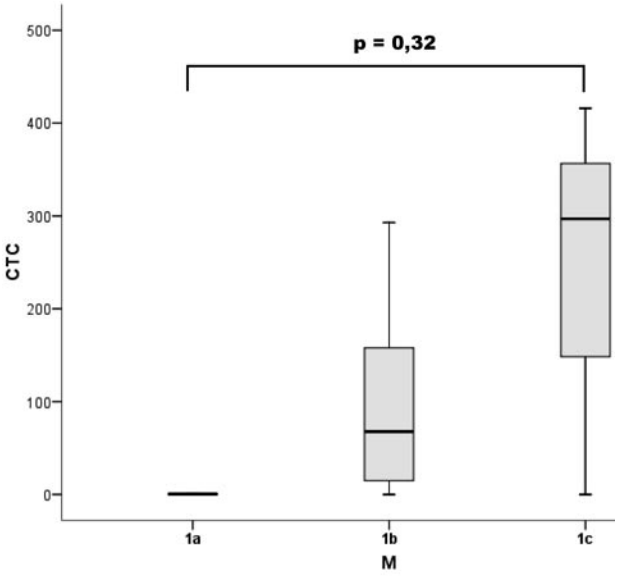

FIGURE 5. Comparison of CTC levels between patients with metastases (M). CTC levels were higher in patients with visceral metastases(M1c) [median: 297 (0-416)], compared with patients with bone metastases (M1b) [median: 68 (9.5-168)] and with non-regional enlarged lymph nodes (M1a) [median: 0.5 (0-1)], however, these differences were not significant (p=0.32).

CTC levels and PSA, tumour size and the presence of enlarged lymph nodes and metastases. The strongest correlation was established between PSA (Rho=0.59) and metastases (Thau=0.56). No significant relationship was found between the Gleason score and CTC numbers, and despite finding differences between CTC count and the location of bone or visceral metastases (the CTC level was higher in visceral MTS), they were not significant differences. A higher number of patients could possibly make these differences significant, but this, together with the other parameters analysed above, will have to be clarified in later studies.

Although the results are promising, the isolation and enumeration of tumour cells in peripheral blood does not consider the biological potential of these CTCs. The different distribution with regard to CTC levels in patients with metastatic PC that varies from 0 to 919 CTC / 7.5 mL (29% of patients with MPC had ≤ 2 CTC) despite evidence of disseminated disease, appears to confirm the heterogeneity of the biological behaviour of prostate tumours. The different dissemination routes of metastatic tumour disease could be related to this findings, and also, blood circulation penetration is only one step in the metastatic dissemination process and it may not be the most important event in the metastatic process in PC.

CONCLUSIONS

The immunomagnetic analysis system is a good method for detecting and enumerating circulating prostatic tumours cells in peripheral blood.

It was demonstrated a positive association of the CTC levels with all tumour burden markers studied except Gleason grade, therefore its determining in patients with PC could provide a possibility for correctly staging, estimating the prognosis, commencing early therapy, and assessing the response to different therapies.

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