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ALKALINE PHOSPHATASE ISOENZYMES FOR THE DIAGNOSIS OF METASTATIC TUMORS AND LYMPHOMAS OF LIVER AND BONE

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Abstract The plasmatic activities of total alkaline phosphatase (ALP) (E.C. 3.1.3.1), high molecular weight-ALP (high Mr-ALP) and bone-ALP isoenzymes, were determined in healthy individuals and in patients with: neoplasia without metastases, hepatic metastases, bone metastases and mixed metastases (hepatic and bone). Variables were individually used to assess incidence of metastases and percentages of false negative and false positive results were calculated. The three values were then used together to assess metastases incidence and sensitivity, specificity, positive predictive value, negative predictive value and predictive capacity were estimated. We conclude that none of the variables *per se* are reliable for the diagnosis of metastases. On the other hand, the three values show high percentages of sensitivity, specificity, positive predictive value, negative predictive value and a high probability (0.93) of accurate diagnosis when applied to a larger population, with similar prevalence values.

Resumen ***Valor diagnóstico de las isoenzimas de la fosfatasa alcalina para tumores metastásicos y linfomas hepáticos y óseos.*** El propósito de este trabajo es investigar la utilidad de la determinación de las isoenzimas de fosfatasa alcalina (E.C. 3.1.3.1) para la detección de metástasis. Para ello se determinaron las actividades de fosfatasa alcalina total, de alto peso molecular y ósea en individuos sanos, en pacientes con neoplasia sin metástasis y en pacientes con metástasis hepáticas, óseas y mixtas (óseas y hepáticas). Para cada variable se calcularon los porcentajes de resultados falsos positivos y falsos negativos generados a partir de los *cut-offs* seleccionados. Luego se emplearon valores combinados de las tres variables y se calcularon su sensibilidad, especificidad, valor predictivo positivo y valor predictivo negativo con respecto al diagnóstico de metástasis. También se utilizó el teorema de Bayes para determinar la probabilidad de realizar un diagnóstico acertado al aplicar los valores combinados a una población mayor que la muestra estudiada y con similares prevalencias. Se concluye que, aisladamente, ninguna de las variables estudiadas es útil para emplearla como indicador de metástasis. En cambio, al emplearse en conjunto los valores de actividad de fosfatasa alcalina total, de alto peso molecular y ósea, se obtuvieron altos porcentajes de sensibilidad, epecificidad, valor predictivo positivo y valor predictivo negativo. Asimismo, de acuerdo a lo anterior, con los tres valores es posible diagnosticar acertadamente la presencia de metástasis en 93 pacientes sobre cien.

Key words: alkaline phosphatase, cancer

Survival of patients with malignant disease may be improved with early treatment because symptoms of bone and hepatic metastases appear later on. Besides, image diagnostic methods are costly and often unavailable for patients in public hospitals. Therefore, availability of simple laboratory techniques for early detection of liver and bone metastases are needed. Many authors have shown that the determination of alkaline phosphatase (ALP) (3.1.3.1) isoenzyme activity, is useful for the diagnosis and clinical evaluation of patients with cancer. It has also been reported that total-ALP is considered an independent predictor of survival for patients with different types of carcinomas[1]. Separation of ALP into its isoenzymes has added considerable value to the mere measurement of total enzyme activity in a clinical setting[2]. A membrane bound high molecular weight form (high Mr-ALP) circulates in plasma of patients with metastatic, infiltrative and cholestatic liver disease. High Mr-ALP is considered a marker that has a positive predictive value higher than total-ALP in detecting liver metastases[3]. Several techniques, such as electrophoresis in different supporting media and HPLC, have been developed to separate the high Mr-ALP[4, 6]. In our laboratory we have also developed a technique that allows the separation of the high Mr-ALP[7, 8]. On the other hand, determination of serum bone-ALP is a useful parameter for monitoring changes of bone formation, and patients with bone metastases show an increased activity of this isoenzyme[9]. It has also been demonstrated that the diagnostic accuracy for bone and hepatic metastases is improved

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by the use of marker combinations[10, 11]. The aim of the present study was to investigate the clinical usefulness of alkaline phosphatase isoenzymes, individually or combined as metastases markers.

Material and Methods

Population: A total of 144 individuals were studied, 66 men and 78 women, with an average age of 54 years: 58 were healthy individuals and 86 had primary cancer diagnosed by clinical methods. Primary cancers were: head and neck[3], colon[6], uterus[7], esophagus[5], stomach[4], liver[4], Hodgkin lymphoma[5], no Hodgkin lymphoma[5], breast[12], myeloma[4], ovary[5], pancreas[4], skin[6], prostate[5], lung[5], chondrosarcoma[3] and testis[3]. Five experimental groups were considered:

Group I: 58 healthy individuals.

Group II: 31 patients with neoplasia, without evidence of bone or hepatic metastases.

Group III: 26 patients with hepatic metastases.

Group IV: 14 patients with bone metastases.

Group V: 15 patients with mixed metastases (bone and hepatic metastases).

Diagnostic of metastases

Evidence of bone metastases was obtained by total bone scintillography with Tc99 and X-rays of the affected bones. Patients with clinically diagnosed bone metastases were defined as bone metastases positive[12]. Hepatic metastases was assessed by abdominal echography, abdominal axial computed tomography and biopsy. Patients with clinically diagnosed hepatic metastases were considered as hepatic metastases positive[13].

Determination of alkaline phosphatase

Blood samples were obtained by venous puncture and were centrifuged at 10,000 xg during 10 min to separate cells. Plasma was centrifuged again at 10,000xg during 10 min to eliminate remaining debris. A 100 µL aliquot of the supernatant was separated for total-ALP activity determination. The rest of the supernatant was centrifuged at 100,000 xg during 2 hours. Soluble alkaline phosphatase was determined in the supernatant and the pellet was suspended in Tris-HCI buffer pH 7.3 to determine high molecular weight ALP (high Mr-AlP)[7]. An aliquot of the 100,000 xg supernatant was used to determine bone-ALP activity with wheat germ lectin[14]. Alkaline phosphatase activity was measured by using p-nitrophenylphosphate substrate in diethanolamine buffer at 37 °C[15]. Total-ALP activity is expressed in Units per liter of sample (U/L), high Mr-ALP and bone-ALP values are expressed as percentages of total-ALP.

Descriptive statistics

We calculated mean, median, standard deviation and percentiles. The comparison among mean values of activity of total-ALP, high Mr-ALP and bone-ALP from the experimental groups was carried out by ANOVA and Duncan's test for a level of significance of $p < 0.05$. The no parametric test of Kruskal-Wallis was used to evaluate the hypothesis of equal prospective values for each variable among the studied groups. The average values of activity of different ALP isoenzymes were compared among the studied groups. When the statistical hypothesis of equality among groups was rejected ($p < 0.05$), non parametric paired comparisons among average values were carried out in order to detect differences between groups. Intervals for each variable and the frequency of cases for each interval of values were calculated. This was directed to establish the usefulness of determination of ALP activity in the diagnosis of metastases. A cut-off was also selected for each variable, keeping in mind the percentage of false positive and false negative results for each cut-off value[16]. Values of total-ALP, high Mr-ALP and bone-ALP were used together to classify the patients. Their usefulness as indicators of metastases was assessed calculating: sensitivity, specificity, positive predictive values and negative predictive values. The sensitivity is the ratio between the true positives and the sum of true positives and false negatives. The specificity is the ratio between the true negatives and the sum of true negatives and false positives. The positive predictive value is the proportion of patients with metastases detected as such. The negative predictive value is the proportion of patients without metastases diagnosed as such. These values are prevalence-dependent. The Bayes theorem was applied to estimate the three values predictive capacity over a wider universe than that of the studied sample[16].

Results

Table 1 shows that total-ALP activity was significantly higher ($p < 0.05$) in plasma of patients with hepatic and bone metastases (groups III and IV). Groups I and II (healthy and without metastases) showed the lowest values of total-ALP activity ($p < 0.05$). Group V (mixed metastases) showed a total-ALP activity significantly different ($p < 0.05$) to the other group. Fig. 1 shows that percentile 25 of group III is separated from percentile 90 of group II, while percentile 25 of groups IV and V (bone metastases and

TABLE 1.– *Activity of total-ALP (U/L), high Mr-ALP (%) and bone-ALP (%) in plasma. Group I: Healthy individuals. Group II: Patients with neoplasia without metastases. Group III: Patients with hepatic metastases. Group IV: Patients with bone metastases . Group V: Patients with mixed metastases. Mean ± SEM. Number of cases between parentheses. * Statistically significant differences (p < 0.05)*

ALP isoenzymes	Group I n = 58	Group II n = 31	Group III n = 26	Group IV n = 14	Group V n = 15
Total-ALP U/L	122.0 ± 4.6	138 ± 11	815 ± 146*	659 ± 212*	369 ± 65
High Mr-ALP (%)	0.98 ± 0.6	1.6 ± 0.3	4.6 ± 0.6*	1.2 ± 0.2	4.0 ± 0.8*
Bone-ALP (%)	63 ± 2	52 ± 3	33 ± 4	79 ± 4.1*	39.0 ± 3.5

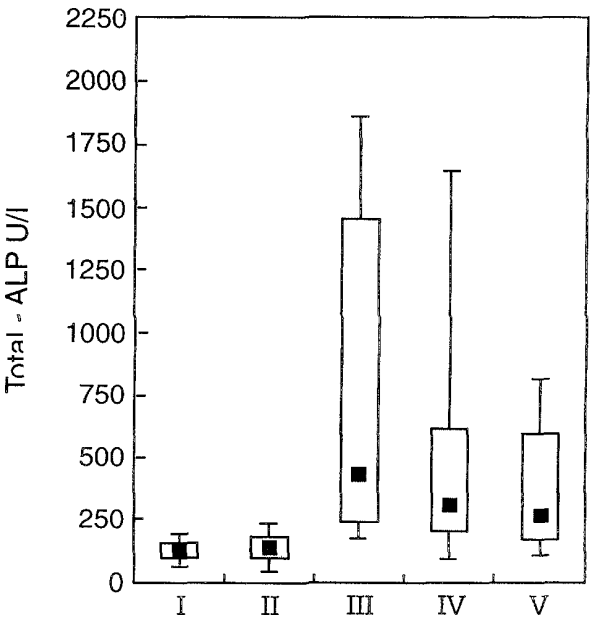

Fig. 1.– Distribution of total-ALP activity in plasma. I: healthy individuals. II: patients with neoplasia without metastases. III: patients with hepatic metastases. IV patients with bone metastases. V: patients with mixed metastases. ■ Median. Box: 50% of observed values, percentiles 25 to 75. Under and upper lines: percentiles 10 and 90 respectively.

mixed metastases) overlapped with percentile 90 of group II. It is important to point out that none of the patients with bone or hepatic metastases showed total ALP values lower than 100 U/L. The total-ALP activity that produced the lowest percentage of false result (250 U/L) was chosen as cut-off value. But this cut-off produced 25% and 50% of false negative results among patients with bone or hepatic metastases and mixed metastases, respectively. Table 1 indicates that high Mr-ALP activity is significantly higher ($p < 0.05$) in patients with hepatic and mixed metastases (groups III and V) and there were no significant differences among the remaining groups. Fig. 2 shows that percentile 25 from patients with hepatic metastases contacted with percentile 90 of patients without metastases (group II). A cut-off value of high Mr-ALP activity of 2.4% was chosen. It produced 22% and 38% of false negative results among patients with hepatic and mixed metastases respectively and 16% of false positive results. It is important to note that the high Mr-ALP values for all patients with bone metastases (group IV) were lower than the cut-off (Fig. 2). Table 1 indicates that patients with bone metastases show the highest bone-ALP activity ($p < 0.05$), whereas the lowest one corres-ponds to patients with hepatic and mixed

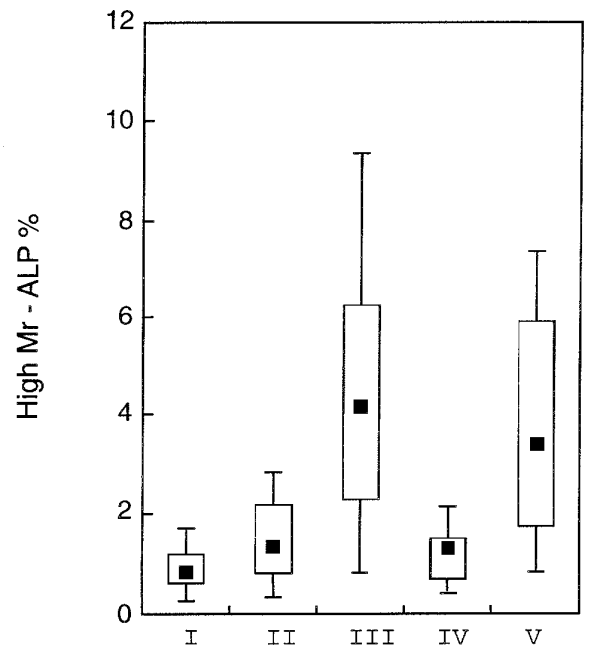

Fig. 2.– Distribution of high Mr-ALP activity in plasma. I: healthy individuals. II: patients with neoplasia without metastasis. III: patients with hepatic metastasis. IV patients with bone metastasis. V: patients with mixed metastases. ■ Median. Box: 50% of observed values, percentiles 25 to 75. Under and upper lines: percentiles 10 and 90 respectively.

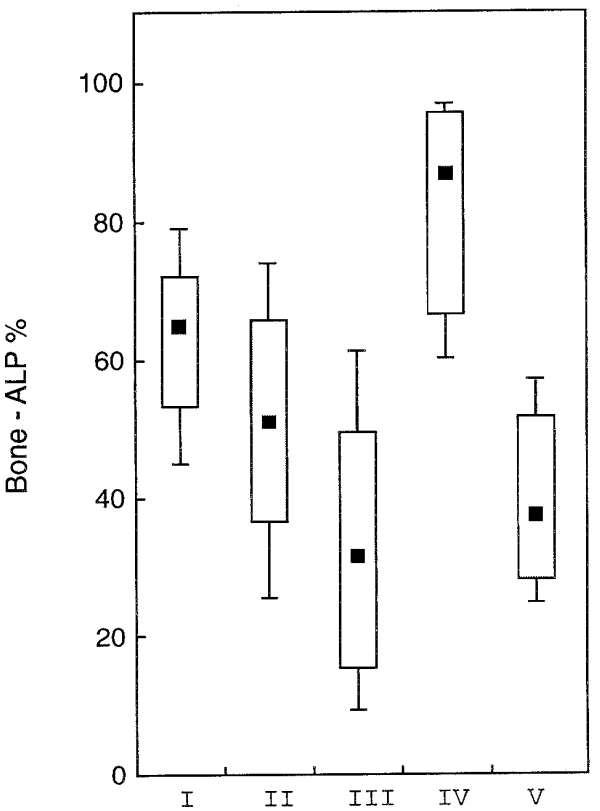

Fig. 3.– Distribution of bone-ALP in plasma. I: healthy individuals. II: patients with neoplasia without metastases. III: patients with hepatic metastases. IV patients with bone metastases. V: patients with mixed metastases. ■ Median. Box: 50% of observed values, percentiles 25 to 75. Under and upper lines: percentiles 10 and 90 respectively.

TABLE 2.– *Combined values of the variables for the detection of metastasis*

Total-ALP U/L	High Mr-ALP %	Bone-ALP %	Diagnosis
≤ 100			No risk of metastases
100-250	< 2.4	< 70	" " "
> 100	≥ 2.4	< 60	High risk of hepatic or mixed metastases
100-250	< 2.4	≥ 70	High risk of bone metastases
> 250	< 2.4	> 60	" " "

metastases. Fig. 3 shows that there was overlapping among bone-ALP activity distributions from the five studied groups. A selected cutt-off value of 60% would allow to distinguish bone metastases from hepatic and mixed metastases, since 85% of patients with hepatic metastases and 94% of those with mixed metastases, showed bone-ALP values lower than the cut-off. On the other hand, this cut-off produced 12% of false negative results.

As shown in Table 2 the results described above indicate that, considering the three values, it is possible to classify the patients as follows.

Without risk of metastases (total-ALP ≤ 100 or total-ALP ≥ 100, high Mr-ALP < 2.4 and bone-ALP < 70). The sensitivity was 83%, specificity 87%, positive predictive value 94% and negative predictive value 80%.

High risk of hepatic or mixed metastases (total-ALP > 100, high Mr-ALP ≥ 2.4 and bone-ALP < 60. For the diagnosis of hepatic metastases the sensitivity was 93%, specificity 91%, positive predictive value 93% and negative predictive value 97%. For the diagnosis of mixed metastases the sensitivity was 63%, specificity 91%, positive predictive value 83% and negative predictive value 85%.

High risk of bone metastases (total-ALP ≥ 100, high Mr-ALP < 2.4 and bone-ALP ≥ 70 or total-ALP > 250, high Mr-ALP < 2.4 and bone ALP > 60). The sensitivity was 87%, specificity 94%, positive predictive value 93% and negative predictive value 97%. On the other hand, according to Bayes theorem, the three values together have 93% of probability of defining metastases.

Discussion

Our results demonstrate that none of our patients with total-ALP values lower than 100 U/L presented metastases. On the contrary, patients with metastases (groups III, IV and V) show a significantly increased total-ALP activity. This finding is in agreement with data from other authors. It has been demonstrated that total-ALP is highly sensitive to detect hepatic metastases[3] and patients with osteosarcoma and metastases show higher total-ALP activity than those with localized tumors[17]. Moro et al.[18] used the total-ALP activity in plasma as marker of bone metastases in breast cancer. Our patients with hepatic or bone metastases show the highest total-ALP activity and there are no significant differences of total-ALP activity between them. According to our results, total-ALP could not differentiate between hepatic and bone metastases and it is necessary to take into account bone-ALP values that, in bone metastases, always exceed 60%. Our results also show that the patients with hepatic and mixed metastases present the highest values of high Mr-ALP activity. It has been demonstrated that high Mr-ALP is more reliable than other isoenzymes to detect hepatic metastases, since in this fraction contains biliary tract cells and hepatocyte membranes released to the blood as a result of cellular damage[1]. According to our results, high Mr-ALP allows to distinguish between hepatic and bone metastases, but cannot differentiate between hepatic and mixed metastases.

On the other hand, our results show that bone-ALP activity is significantly lower in plasma of patients with hepatic and mixed metastases than in plasma of patients with bone or without metastases. It has been demonstrated that bone-ALP and high Mr-ALP do not increase simultaneously, on the contrary, a massive increase of one of them is usually accompanied by a reduction of the other[19]. Similarly we found that patients with mixed metastases (group V) show low values of bone-ALP activity, high values of high Mr-ALP activity and a total-ALP activity higher than those corresponding to healthy individuals and patients without metastases (groups I and II). Therefore, we assume that the increase of total-ALP activity in plasma from patients with mixed metastases is mainly attributable to high Mr-ALP. Our results also show that any of the three studied variables taken alone give high percentages of false results. Therefore, it should not be used as the unique criterion to diagnose metastases.

By considering the three values together (total-ALP, high Mr-ALP and bone-ALP), our results show that high values of bone-ALP and total-ALP allows differentiate bone metastases. We also found that high values of total-

ALP accompanied by low activity of high Mr-ALP and bone-ALP indicate that a patient with neoplasia does not have hepatic or bone metastases. On the contrary, high values of total-ALP and high Mr-ALP accompanied with low values of bone-ALP show a high risk of hepatic or mixed metastases. Definition of high risk is important since, when a patient with neoplasia shows a high value of high Mr-ALP, the existence of hepatic or mixed metastases is possible, but no bone metastases. Similarly, in a patient with bone metastases, it is possible to infer the presence of hepatic metastases. We also demonstrate that, the application of the three values show high percentages of sensiitivity, specificity, positive predictive value, negative predictive value and high probability of accurate diagnosis (93% according Bayes theorem).

The proposed method is highly sensitive, simple and it may be carried out in routine laboratories in whatever hospitals. The methods of image diagnosis as scintillography, axial computed tomography and ecography are costly and only available in hospitals of high complexity. Therefore, these methods are often unavailable for patients in the majority of our public hospitals.

We can conclude that none of the studied variables are reliable *per se* for the diagnosis of metastases. On the contrary, the three values taken together reach higher diagnostic usefulness than individual values. Consequently, it would be interesting to expand their application to a greater number of patients, to confirm their qualities.

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