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Elevation of Erythrocyte Zinc- and Free Protoporphyrins with Metastatic Spread in Cancer Patients

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Abstract—*A sensitive fluorometric method for the evaluation of erythrocyte levels of zinc-protoporphyrin (ZPP) and free-protoporphyrin (FPP) in diluted whole blood was used to survey patients with carcinomas in different stages of metastatic dissemination. ZPP levels in patients with primary tumor or with no evidence of metastatic disease were not different from those of normal donors. However, significantly higher ZPP levels were found in patients with carcinomas in correlation with evidence of metastatic disease, irrespective of the histological origin of the tumor. FPP levels were elevated in all stages of malignancy but were found 4 times higher than the normal levels in patients with metastatic malignancies. Similar increases in ZPP and FPP were detected in patients with inflammatory processes, suggesting a common effector of erythropoiesis in these pathological conditions and in metastatic diseases. Simultaneous detection of higher than normal ZPP and FPP levels in the blood of cancer patients may serve as an additional marker of metastatic dissemination in patients without clinical evidence of infectious or autoimmune diseases.*

INTRODUCTION

MINUTE amounts of erythrocyte non-iron protoporphyrins are produced during erythropoiesis. Various pathological conditions are characterized by excessive production of these porphyrins, such as zinc-protoporphyrin-IX (ZPP) in lead intoxication and iron deficiency anemia [1–6], and free protoporphyrin-IX (FPP) which does not contain any metallic ions, in erythropoietic protoporphyria [1, 7–10]. However, in other pathological conditions the increase in non-iron protoporphyrins is less pronounced and their detection is hampered by technical difficulties.

In a recent report we suggested an accurate method for the measurement of low levels of both FPP and ZPP in normal blood samples [11]. The sensitive differential analyses of FPP and ZPP were obtained by the use of a direct fluorometric procedure on minute samples of peripheral blood diluted in buffer. Each protoporphyrin was excited at its specific excitation wavelength, and the resulting fluorescence emission peak was integrated and recorded. Due to the complex absorption of the excitation and fluorescence intensities of ZPP and FPP by the hemoglobin (Hgb) of the intact erythrocyte, the net fluorescence intensity recorded was found to be proportional to the ZPP/Hgb or FPP/Hgb concentration ratios within a wide range of hematocrits (0.06–1.14%) [11].

We report here on the levels of FPP and ZPP measured in patients with various malignancies in relation to the metastatic spread of the disease. Both ZPP and FPP were found to be only minimally elevated in the erythrocytes of patients with no evidence of active disease (NED) or in patients with the primary tumor before initial surgery, without evidence of metastatic spread (primary tumor). In contrast, significantly higher levels were found in patients with metastatic spread, particularly those with liver and bone metastases. The elevation of both FPP and ZPP in the erythrocytes, as recorded in patients with metastatic malignancies, was similar to the increase of these protoporphyrins in non-malignant inflammatory processes.

MATERIALS AND METHODS

Measurements of FPP and ZPP in bloods

Samples of peripheral venous blood from normal donors and the patients were drawn into

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EDTA(K_3) Vacutainers (Vacutainer Systems, Rutherford, N.J.) and kept at 4° C for periods of less than 6 days. Blood samples which were kept under these conditions did not show changes in their ZPP and FPP levels when compared to the levels found immediately after drawing. Samples of whole blood were diluted 1/400 in 4 ml of 10 mM phosphate buffered saline (PBS), pH 7.4, to yield a final average hematocrit of 0.1% (range 0.06–0.14%). Within this hematocrit range the fluorescence intensities of both photoporphyrins reached their maximum readings and remained constant, irrespective of the precise hematocrit of the sample [11]. Analysis was routinely performed on a 1/400 dilution of whole blood in PBS, without lysing the RBC by addition of any detergents, to avoid errors in the FPP determination due to the detergent induced shift of its fluorescene peak [10].

The fluorometric measurements were performed with a sensitive fluorometer (Fluorolog II, Spex Industries, Edison, N.J.) equipped with a Datamate microprocessor. All the emission spectra were automatically corrected for variations in light flux at the various excitation wavelengths by using a standard concentration of rhodamine-B solution as a reference. The direct determination of FPP and ZPP in diluted intact RBC suspensions is based on their excitation by monochromatic sources of 397 and 424 nm, respectively. The fluorescene peaks of both FPP and ZPP with maxima at 624–626 nm and 594–596 nm, respectively, were integrated. The background readings under the integrated peaks were subtracted, so that the net integrals of both FPP and ZPP peaks were used for quantitative analyses.

In order to determine the concentrations of the fluorescent protoporphyrins by this method, an internal calibration procedure was used. ZPP and FPP were extracted from eight blood samples with different protoporphyrin levels by an improved ethanol extraction procedure [11, 12] as follows: A sample of 50 µl of the mixed whole blood was lysed in 1 ml of glass distilled water in a glass test tube. An aliquot of 0.2 ml of the lysate was added to 4 ml 95% ethanol (spectroscopic grade), vigorously mixed for 2 min and then centrifuged for 7 min at 7000 rpm. The clear ethanolic phase containing the extracted protoporphyrins was analyzed fluorometrically. For ZPP analysis, the excitation maximum in ethanol was at 415 nm and the net fluorescence peak maximum at 594 nm. FPP in the extract was excited at 400 nm and the fluorescence with the maximum between 624 and 632 nm was measured. In each extract of blood sample used for this calibration procedure, the ZPP and FPP levels were determined by adding small amounts of known concentrations of pure ZPP or FPP (Porphyrin Products, Logan, Utah) dissolved in absolute ethanol. The concentrations of ZPP and FPP found in each blood sample used in this calibration were related to the original hemoglobin (Hgb) level of the relevant sample. These results were found previously to be in high correlation ($r > 0.99$) with the values obtained from direct fluorometric measurements of the 1/400 suspension of the corresponding blood samples [11].

The measurements of Hgb level and hematocrit ratio of the blood samples were performed with a Coulter Counter (Model-S Plus-5, Hialeah, Florida). Serum alkaline phosphatase (AP) was measured with the Technicon SMAC (Technicon Instrument Company, Basingstoke, U.K.).

Statistical analysis of the results: means, standard deviations (S.D.), Student *t*-tests and multivariate analyses of correlations were performed with the use of BMDP Statistical Software programs (Department of Biomathematics, UCLA, California).

Patients

Erythrocyte ZPP and FPP were examined in 56 normal donors (32 males, 24 females), 20–62 years old. Blood samples of 158 patients with various carcinomas were tested and grouped according to their metastatic spread. Twenty-seven patients, 34–75 years old (26 breast, 1 colon), were tested following their course of curative therapies, and at the time of testing had no clinical evidence of active malignancy (NED). Twenty-three patients, 37–91 years old (20 breast, 3 colon), were tested with their primary tumor intact, prior to any therapy, and had also no evidence of metastatic spread (primary tumor). Thirty-three patients, 33–80 years old (10 breast, 4 colon, 4 pancreas, 5 lung, 4 head and neck, 2 unknown primaries, 1 stomach, 1 ovary, 1 gall bladder and 1 liver) had evidence of metastatic spread into various soft-tissues, but not in liver or bones (soft-tissue metastases). Nineteen patients, 40–79 years old, (9 colon, 3 breast, 5 stomach, 1 pancreas and 1 lung) had metastatic spread into the liver with or without other soft-tissue metastases (liver metastases). All patient with bone metastases regardless of the presence of metastases in other sites were grouped together (bone metastases). This group of patients, 32–77 years old, included 30 with carcinomas of the breast, 8 prostate, 6 unknown primaries, 6 lung, 1 kidney, 2 pancreas, 2 cervix and 1 colon carcinoma. Fifty-two patients, 18–82 years old, with no malignancy but with inflammatory processes including various infections (acute pneumonia, cellulitis, pyelonephritis, acute gastroenteritis and acute pancreatitis), or autoimmune diseases (Systemic lupus erythematosus and rheumatoid arthritis), were examined. Since no significant dif-

ferences in the levels of ZPP and FPP were found between these two groups of patients, they were combined. An additional group of 28 patients, 20–79 years old, suffering from other diseases including acute and chronic cardiac failure, pulmonary obstructive diseases with no inflammatory processes, diabetes mellitus, uremia and hypertension, were also examined. The various groups of patients examined did not differ significantly in their age distribution, except for the group of normal donors whose mean age was lower. Nevertheless, in none of the groups tested did the ZPP and FPP levels correlate with the age of the patients.

RESULTS

ZPP and FPP in normal donors and states of disease

Figures 1 and 2, and Table 1 show the ZPP and FPP levels in normal donors and in various groups of patients tested. FPP levels were similar for males and females in both normal donors and patients. ZPP was slightly, but not significantly, higher for females in the group of normal donors and in most of the other groups tested. Therefore data of ZPP or FPP levels for both sexes were merged together in each group. The ZPP level of normal donors as measured by the direct fluorometric procedure (2.70 ± 0.70 μg/g Hgb) was found to be within a narrow range. This enabled the detection of minute but still significant changes in the ZPP levels in patients with various diseases. In patients with carcinoma, ZPP was found to correlate well with the type and state of the metastatic spread. In these patients, ZPP level did not depend on the histologic type and origin of the tumor (data not shown). In patients with NED and with primary tumors, ZPP was not significantly higher than in normal controls. In soft-tissue metastases and liver metastases, ZPP was found to be significantly elevated ($P < 0.01$). Similar but even more significant elevation was found in patients with proved metastatic spread to the bones($P < 0.001$) (Table 1).

FPP was found to be present in only minute quantities in normal erythrocytes. The mean FPP level in normal donors was 0.20 μg/g Hgb, i.e., about one order of magnitude lower than that of ZPP (Table 1). The higher quantum yield of the fluorescence of FPP in conjunction with the sensitivity of the direct fluorometric procedure, provided for the accurate determination of this protoporphyrin as well as the detection of alterations in the various subgroups of patients with malignancies. The mean FPP level was found to be 2-fold higher in the groups of patients with NED and primary tumors, and by about 4-fold in patients with metastatic diseases (Fig. 1, Table 1).

The level of both ZPP and FPP in patients with

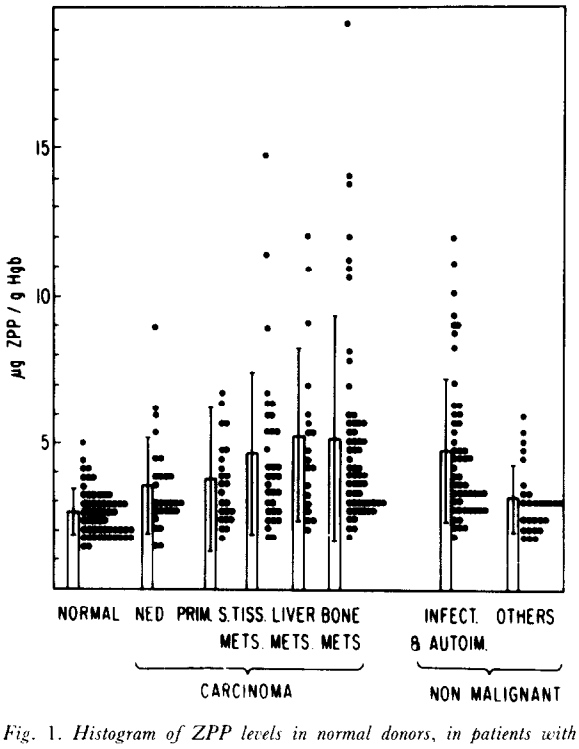

Fig. 1. Histogram of ZPP levels in normal donors, in patients with carcinoma grouped according to the site of metastatic spread, in patients with inflammatory processes, and in patients with other non-inflammatory diseases. S.TISS.METS. = *soft tissue metastases,* PRIM. = *primary tumor,* INFLAM = *inflammatory processes. Column and bar beside each histogram represent mean* ± 1 *S.D.*

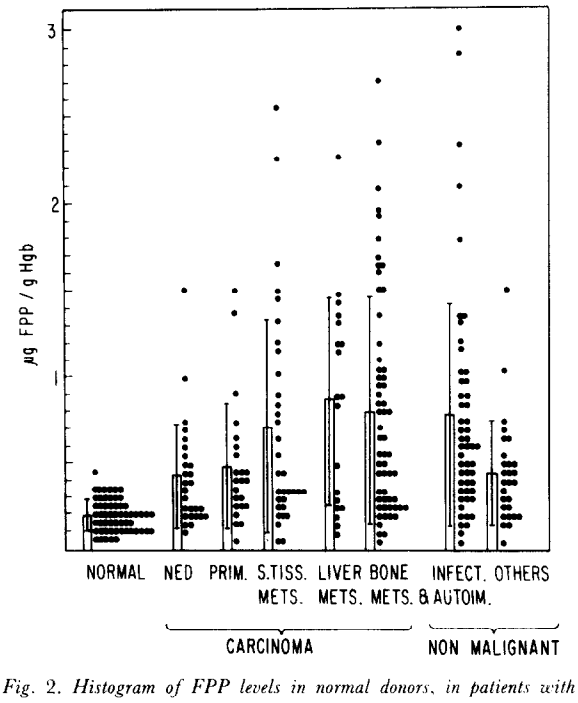

Fig. 2. Histogram of FPP levels in normal donors, in patients with carcinoma grouped according to the site of metastatic spread, in patients with inflammatory processes, and in patients with other non-inflammatory diseases. S.TISS.METS. = *soft tissue metastases,* PRIM. = *primary tumor,* INFLAM = *inflammatory processes. Column and bar beside each histogram represent mean* ± 1 *S.D.*

Table 1. ZPP, FPP, Hgb and AP in normal donors and patients

	n	Hgb (gr%) Mean±S.D.	ZPP (μg/g Hgb) Mean±S.D. (range)	FPP (μg/g Hgb) Mean±S.D. (range)	AP (units/l) Mean±S.D. (range)
Normal donors					
Males	32	N.D.	2.46±0.61 (1.64–4.36)	0.20±0.08 (0.05–0.37)	
Females	24	N.D.	3.09±0.91 (1.64–5.10)	0.18±0.12 (0.04–0.45)	
Males + females	56	N.D.	2.70±0.79 (1.64–5.10)	0.20±0.10 (0.04–0.45)	(30–90)‡
Carcinoma					
NED	27	13.4±1.2	3.56±1.63 (1.42–8.98)	0.43±0.30* (0.11–1.48)	80±24* (30–134)
Primary	23	12.4±1.3*	3.75±1.45 (1.86–6.72)	0.48±0.36* (0.07–1.48)	119±88** (57–440)
Soft tissue metastases	33	11.8±1.9**	4.65±2.76** (1.16–14.69)	0.72±0.62** (0.06–2.58)	104±58** (44–374)
Liver metastases	19	11.6±1.4**	5.22±2.86** (2.07–12.10)	0.84±0.60** (0.11–2.22)	381±251*** (90–912)
Bone metastases	56	11.6±1.5***	5.12±3.49*** (1.89–19.28)	0.81±0.66*** (0.06–2.65)	243±342*** (23–2300)
Non-malignant diseases					
Inflammatory processes	52	12.7±1.8	4.69±2.42** (1.84–11.74)	0.79±0.65** (0.06–2.98)	114±50** (51–278)
Other diseases	28	13.5±1.8	3.10±1.16 (1.82–6.33)	0.45±0.31* (0.06–1.55)	84±18* (50–127)

Significance levels relative to normal donors: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. N.D.: not determined (Significance for Hgb and AP was calculated using the values published for average population of normal donors). ‡Values from literature [15].

bacterial infections and autoimmune disease were similar to the levels in metastatic malignancies. In contrast, the findings of some significant FPP elevation with no significant ZPP elevation in patients with non-inflammatory, non-malignant diseases were similar to those observed in patients with NED and primary tumors. The coefficients of correlation between FPP and ZPP in normal donors and patients with non-inflammatary diseases were low ($r < 0.35$), but in all other groups examined it reached higher values ($0.61 < r < 0.81$), indicating a simultaneous correlated increase in both forms of protoporphyrins in the various states of disease examined.

Although Hgb levels were found to decrease significantly in patients with metastatic spread, there was a poor correlation between Hgb and the protoporphyrins in all the groups of patients examined ($-0.02 < r < -0.51$), indicating that the changes in Hgb levels were probably independent of the increase in the level of either ZPP or FPP.

Serum AP was not determined in the group of normal donors. Analysis of the results was therefore related to the values for normal controls, as indicated by the SMAC manufacturer and on literature data [15]. Only results of serum AP determined concurrently with the protoporphyrin measurements were included in this comparison. As expected, highly significant elevation of AP was found in patients with metastatic cancer, especially in cases with bone and liver metastases. In non-malignant diseases, serum AP was less elevated and was not significantly different from values obtained in patients with NED or primary tumor. Though serum AP was elevated concomitantly with the elevation in ZPP and FPP in metastatic diseases, no significant correlation was found between these parameters in all the groups of patients examined.

The frequencies of elevation in the levels of either FPP or ZPP, or of both, are presented in Fig. 3, Fig. 4, and Table 2. In more than 50% of the patients with carcinomatous metastases, both FPP

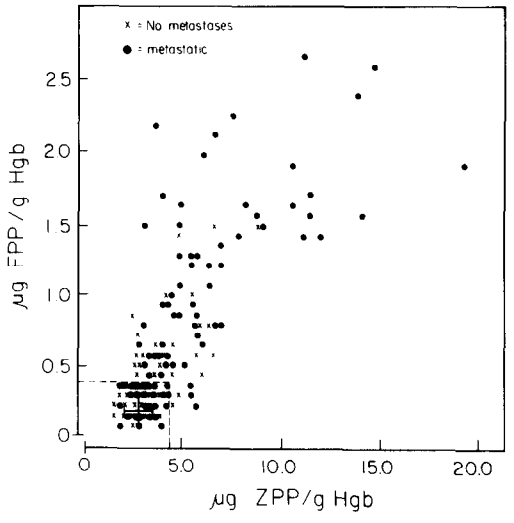

Fig. 3. Correlation of ZPP and FPP levels in malignancy. The mean values ± 1 S.D. are presented by the bars at the lower left. The rectangle at the lower left corner delimits 2 S.D. above the mean values of normal donors. NED and primary tumors (x). Patients with all types of metastatic spread (o).

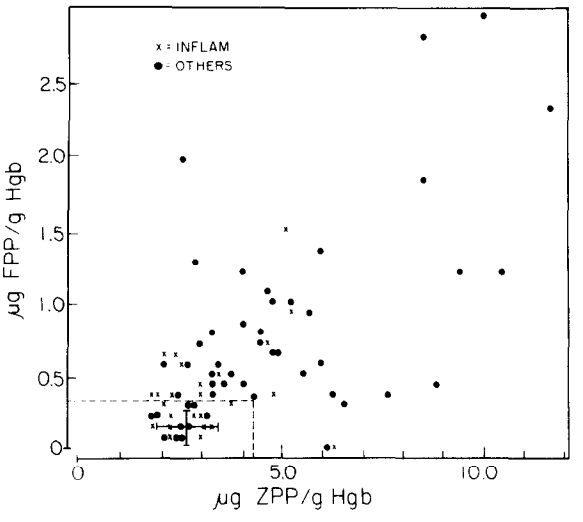

Fig. 4. Correlation of ZPP and FPP in non-malignant diseases. The normal mean values ± 1 S.D. are presented by the bars at the lower left. The rectangle at the lower left corner delimits 2 S.D. above the mean values of normal donors. Patients with inflammatory processes (o). Patients with non-inflammatory diseases (x).

and ZPP were elevated by more than 2 S.D. above the mean of the normal donors. In abut 61% of the patients with soft tissue metastases, the level of at least one of these protoporphyrins was elevated by more than 2 S.D., compared with 73% and 65% in patients with liver and bone metastases, respectively. Similar elevation of at least one of the protoporphyrins examined was also found in patients with inflammatory processes. Only in 5% of the normal controls, at least one of the erythrocyte protoprophyrins examined was elevated, while in patients with NED or primary tumor, high levels were recorded in about 40% of the cases, similar to the results obtained from patients with non-inflammatory diseases.

DISCUSSION AND CONCLUSIONS

Extreme elevations of erythrocyte ZPP and FPP are common in lead intoxication [1–4, 6, 14, 15] and in cases of severe iron deficiency anemia [1, 5, 16–19]. Elevation of erythrocyte FPP was described in erythropoietic protoporphyrias [7–10]. Most of the analytical methods used in these studies were not sensitive enough to detect minor variations in the protoporphyrins levels in other pathological conditions.

Using the sensitive direct fluorometry procedure [11] we were able to measure the levels of both FPP and ZPP in the blood samples from normal donors and to detect minor changes of their concentrations in states of disease which do not involve massive increase of the erythrocyte protoporphyrins. Recently, significant changes in the level of ZPP were reported in patients with iron deficiency anemia, as well as in the peripheral blood from patients with infectious diseases, by

*Table 2. Elevation of ZPP FPP and AP by more than 2 S.D. over the normal means in carcinomas and non-malignant diseases**

	ZPP	FPP	ZPP and/or FPP	AP
Normal controls	2/57(4%)	1/57(2%)	3/57(5%)	N.D.
NED	6/27(26%)	11/27(41%)	12/27(44%)	7/27(26%)
Primary tumor	7/23(30%)	9(23(39%)	10/23(43%)	10/20(50%)
Soft tissues metastases	15/33(45%)	17/33(52%)	20/33(61%)	15/32(47%)
Liver metastases	12/19(63%)	12/19(63%)	14/19(73%)	19/19(100%)
Bone metastases	30/55(55%)	36/55(65%)	36/55(65%)	49/53(92%)
Inflammatory processes	23/54(44%)	34/54(63%)	38/52(73%)	25/51(49%)
Other diseases	5/28(18%)	13/28(46%)	14/28(50%)	8/28(29%)

*Only patients whose AP was measured concurrently with the respective ZPP and FPP evaluation are included. AP levels of more than 90 units/l were considered as elevated. N.D.: not determined.

using a front face fluorometric ZPP meter [19]. These findings were confirmed with the direct fluorometric method used in this work [11]. We found ZPP level in the erythrocytes of normal donors to be 2.7±0.8 μg/g Hgb and the FPP level was found to be 0.2±0.1 μg/g Hgb. In patients with carcinoma both ZPP and FPP were significantly elevated depending upon the state of metastatic spread of their disease rather than upon the histological origin of the carcinoma. In contrast to ZPP, FPP level was significantly elevated even in patients with no evidence of metastatic disease (NED and primary tumor). In patients with inflammatory processes both ZPP and FPP were elevated almost to the same extent as in patients with bone and liver metastases. These findings suggest that hemopoiesis could be similarly influenced by metastatic spread and non-malignant inflammation.

Significant elevations in the total erythrocyte zinc concentrations were recently reported in patients with metastatic cancer [20]. Although ZPP was found to be significantly elevated in metastatic diseases, it could account for less than 1% of the total elevation of zinc level observed in the erythrocytes of these patients. Therefore, the elevation of ZPP in metastatic carcinoma is probably not associated with the increase of total zinc in the erythrocytes of these patients.

Analysis of the frequencies of the abnormal elevation of ZPP and FPP in various states of metastatic spread (Fig. 3 and Table 2) shows such increase in about 70% of the patient with liver or bone metastases, compared to 40% in patients with carcinoma with no evidence of metastatic spread (NED and primary tumor), and in less than 5% of the normal donors. At present, it is unknown whether those NED and primary tumor patients with elevated erythrocyte protoporphyrins may have as yet clinically undetected metastatic spread. Further follow-up of these patients over the next few years may provide an answer to this question. Nevertheless, the present data suggest that determinations of ZPP and FPP levels in the blood of patients with carcinoma could serve as an additional test to assist in the diagnosis of suspected metastatic dissemination, provided that the presence of other diseases which involve elevated non-iron erythrocyte protoporphyrins, can be ruled out. Serum AP which is currently used as a sensitive diagnostic tool for the detection of metastatic spread to bones or liver, was found to be elevated in most of the patients with bone and liver metastases, but it was also found to be higher than the normal values in about 50% of the patients with NED, primary tumor and non-malignant inflammatory processes. Therefore, the combination of the analysis of both ZPP and FPP, together with the determination of other independent parameters such as serum AP, could improve the ability to diagnose metastases in patients with various carcinomas.

The reason for the elevation of ZPP and FPP levels in metastatic malignancies is still not clear. Since no significant correlation was found between the Hgb level and ZPP or FPP, the slight anemia concurrent with metastatic spread does not seem to explain the increase in the erythrocyte protoporphyrins. The possibility that a depletion of total blood iron could account for the observed elevation, can be ruled out, as no significant alterations in this parameter were found in these patients [20]. Decrease of plasma transferrin and increase of plasma ferritin have been observed in inflammation [19, 21, 22]. This imbalance results in a partial blocking of the iron supply from the reticuloendothelial system to the hemopoietic system and the erythron. Further investigation is necessary to clarify whether the relative deficiency of iron available for hemopoiesis is involved in the elevation of FPP and ZPP in carcinoma with metastatic spread.

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