Lipids (2008) 43:79–89
DOI 10.1007/s11745-007-3133-6

ARTICLE

A Reliable Biomarker Derived from Plasmalogens to Evaluate Malignancy and Metastatic Capacity of Human Cancers

Rosina E. Smith · Pablo Lespi · María Di Luca · Claudia Bustos · Fernando A. Marra · María J. T. de Alaniz · Carlos A. Marra

Received: 12 June 2007 / Revised: 25 October 2007 / Accepted: 30 October 2007 / Published online: 29 November 2007

Abstract Antigen tumor markers employed in monitoring therapeutical approaches are limited by their specificity (Sp) and sensitivity (Se). The aim of this study was to investigate the suitability of a lipid tumor marker derived from ether-linked phospholipids and to compare it with others usually assayed in clinical practice. Complex lipids from normal and pathological breast, lung, and prostate tissue were isolated and analyzed by TLC and c-GLC methods. Results were compared as pooled samples, or by means of the averaged percent changes with respect to the composition observed in the normal tissue of the same patient. Sp, Se, negative-predictive (NPV) and positive-predictive values (PPV) were established for conventional markers and for the proposed lipid-derived marker. Results demonstrated that the content of monoenoic fatty acyl chains was significantly increased in total lipids, phosphatidylethanolamine, and especially in ethanolamine-containing ether lipids of neoplastic tissues with respect to their corresponding normal ones. Major changes were observed in the plasmalogen sub-fraction where the ratio monoenoic/saturated fatty acids can distinguish with high Se normal tissues from either benign or neoplastic tissues from breast, lung, or prostate lesions. Analyses of fatty acyl chains from ethanolamine-containing plasmalogens provided a reliable tumor marker that correlated with high Se and linearity with metastases spreading. This fact may be useful in prognosis of the most frequently observed human cancers.

Keywords Ether-lipids · Breast · Lung · Prostate · Tumor markers · Human

R. E. Smith · P. Lespi
Hospital Penna, Bahía Blanca, Pcia. de Buenos Aires, Argentina

P. Lespi · F. A. Marra
Hospital San Martín, La Plata, Argentina

M. Di Luca
Hospital Rossi, Cátedra de Patología. Facultad de Ciencias Médcias de la UNLP, La Plata, Argentina

M. Di Luca · F. A. Marra
Hospital Meléndez, Adrogué, Pcia. de Buenos Aires, La Plata, Argentina

C. Bustos
Hospital Rossi, Servicio de Histopatología, La Plata, Argentina

F. A. Marra
Clínica Vaccarini, Servivio de Medicina General, La Plata, Argentina

M. J. T. de Alaniz · C. A. Marra (✉)
INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata), CONICET-UNLP, Cátedra de Bioquímica. Facultad de Ciencias Médicas de la Universidad Nacional de La Plata, Calles 60 y 120, 1900 La Plata, Argentina
e-mail: camarra@atlas.med.unlp.edu.ar

List of abbreviations

ADAG	Alkyldiacylglycerols
BL	Benign (lesions or tissues)
C	Normal
CEA	Carcinoembryonic antigen
ChoGpl	Choline glycerophospholipids
EtnGpl	Ethanolamine glycerophospholipids
FAs	Fatty acids
FAMEs	Fatty acid methyl esters
GEDE-A	1-*O*-alkyl-2,3-diacyl-*sn*-glycerol
GEDE-E	1-*O*-alkenyl-2,3-diacyl-*sn*-glycerol
NEO	Neoplastic (lesions or tissues)
NPV	Negative-predictive value

PakCho	Plasmanylcholine (1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine)
PakEtn	1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmanylethanolamine)
PlsCho	Plasmenylcholine (1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine)
PlsEtn	1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine)
PtdEtn	Diacyl-*sn*-glycero-3-phosphoethanolamine
PtdCho	Diacyl-*sn*-glycero-3-phosphocholine
PPV	Positive-predictive value
PSA	Prostatic specific antigen
PUFAs	Polyunsaturated fatty acids
Se	Sensitivity
Sp	Specificity

Introduction

Plasmalogens make up approximately 18% of the total phospholipid mass in humans; however, their content varies widely among tissues. The bulk of the 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine or PlsEtn) pool may represent up to 70% of the ethanolamine phospholipids depending on the tissue analyzed [1]. 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmanylethanolamine or PakEtn) and diacyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn) represent the other species present in the ethanolamine glycerophospholipids (EtnGpl) [1]. Concerning neutral lipids, several authors proposed alkyldiacylglycerols (ADAG) as a lipid class marker for malignant tissues. Important contributions in this field were made by Snyder et al. [2, 3], Mangold and Paltauf [4], and Nagan and Zoeller [1] among others. Traditionally, an excess of 1% ADAG in neutral lipids may indicate malignant degeneration, and values observed may be as high as 6% [4]. As a general rule, amounts of PakEtn + PlsEtn over 4% of total phospholipids correlated with neoplastic transformation [4]. However, this reference parameter was obtained comparing lipid compositions from neoplastic and normal tissue samples (of the same type) obtained from donors or normal subjects free from cancer lesions. A meaningful comparison should only be plausible between tumorous and healthy tissue samples of the same patient [4]. Lin et al. [5] performed a study in which hepatocellular carcinomas were compared with the residual hepatic tissue free from tumor cells. They found that ADAG in the neutral lipid fraction exhibited an increased concentration of hexadecyl-glycerol and lower proportions of C18-glyceryl ethers in hepatocellular carcinomas with respect to non-malignant liver tissue. In spite of the fact that higher concentrations of neutral ADAG and cholesterol were found in this kind of tumor, no differences between levels of ether-linked phosphoglycerides, triacylglycerides, and lipid phosphorous were observed [3, 5]. So far, no similar studies have been carried out. Thus, no information concerning PakEtn and PlsEtn content and their fatty acid composition has been reported for normal and pathological samples from the same patient. This may be due to problems with surgical protocols, since the collection of cancer and normal tissues from the same patient is not easy. We explored in detail if there was a compositional marker derived from the fatty acyl pattern of polar and/or neutral lipids and if it correlated with the metastatic dissemination of the most prevalent cancers in our country (and probably in most industrialized nations). Several samples of lung, breast, and prostate cancer tissues were taken and studied in comparison with normal tissues obtained from the same patient in order to investigate which lipid profile (if any) correlated better with histopathological characteristics of cancer tissues and/or predicted metastatic spreading of the primary tumor. Tumor markers usually employed in clinical practice were also determined and compared with the results obtained from lipid analyses.

Experimental Procedure

Chemicals

All solvents used were HPLC grade from Carlo Erba, Milan, Italy. Standards for thin-layer chromatography (TLC) and capillary gas-liquid chromatography (c-GLC) were purchased from Nu-Check-Prep., Elysian, MN. 2′,7′-Dichlorofluorescein, boron trifluoride in methanol (14%), phospholipase C from *Bacillus cereus* (type IV, 1,500 U/mg protein), β-mercaptoethanol, sphingomyelin, PtdCho, PtdEth, inorganic salts and other chemicals used in sample preparation and processing were from Sigma Chem. Co., St. Louis, MO.

Patients and Tissue Samples

Lung, breast, and prostate tissues were obtained by surgical procedures and analyzed with the written consent of the patients. This study followed the Helsinki protocol for handling human specimens. Patients were recruited from five public and private health institutions and the survey involved a total of 677 paired samples (C: normal, BL: hyperplastic-displastic, and NEO: malignant tissues from the same patient) collected for 6 years. In most cases samples were obtained surgically. Other samples were

obtained by performing a 3-mm needle disposable punch biopsy using a ultrasound guide. This procedure provides approx. 9 mg of tissue, enough for performing all the analyses. Two portions of tissue from each patient were analyzed, one from a pathological lesion and the other from (assumed) normal tissue. Each portion was divided into two sub-portions. One of them was processed for histopathological examination (PBS with 10% formalin) and the other for lipid analyses. Normal tissue surrounding the lesion was checked for any infiltration of abnormal cells. Suspicious normal samples were excluded from the study together with the corresponding paired (pathological) sample. Each sample was identified with an encrypted code which did not include any information concerning the origin and/or characteristics of the tissues examined. Thus, pathologists, biochemists, and laboratory technicians processed the samples under a blind protocol. At the end of the study, histopathological classification, biochemical determinations, and clinical stage of the samples were revealed and correlated with lipid analyses. For each type of pathological tissue the clinical state of patients and also the criteria for classification of samples (benign or neoplastic) were evaluated following international scores such as those recommended by the American Joint Committee on Cancer Staging, World Health Organization, or Gleason scale [6–8]. Presence and localization of metastases were explored by specific medical studies such as magnetic resonance, X-ray computed tomography, and radioisotopic densitography. Table 1 shows a summary of the main characteristics of patients and samples.

Blood Samples and Biochemical Markers

Morning fasting blood samples were obtained from the antecubital vein on the day of tissue collection. Plasma was separated by centrifugation in the cold at 600 × g (15 min), aliquoted, frozen at −20 °C (no more than one week), and

Table 1 Main characteristics of subjects and samples analyzed

	Tissue		
	Breast	Lung	Prostate
Mean age (years)[a]	61 (53–76)	64 (49–81)	74 (55–86)
n	222	226	229
Sex (n_F; $\%_F$) (n_M; $\%_M$)[b]	(222; 100)	(78; 32.9) (148; 67.1)	(229; 100)
Body weight (kg)[c]	68.4 ± 3.1	75.1 ± 2.0	78.0 ± 4.2
Body mass index (kg/m^2)[c]	26.0 ± 1.5	24.3 ± 1.1	25.8 ± 2.3
Weight loss (kg; %)[d]	(3.1 ± 0.2; 4.5 ± 0.1)	(6.7 ± 0.8; 8.9 ± 0.4)	(4.9 ± 0.5; 6.6 ± 0.3)
Tumor histopathology[b,e]			
Squamous cell carcinoma		(26; 11.5) (54; 23.9)	
Undifferentiated large-cell carcinoma		(3; 1.3) (11; 4.9)	
Adenocarcinoma (ADC)	(148; 66.6)	(14; 6.2) (31; 13.7)	(173; 75.5)
Small cell ADC		(21; 9.3) (26; 11.5)	
Intraductal ADC	(41;18.5)		
Ductal ADC	(90; 40.5)		
Lobulillar ADC	(13; 5.9)		
Medullary ADC	(4; 1.7)		
Extracapsular ADC (C1,C2)			(27; 11.8)
Metastasized ADC (D1,D2)			(146; 63.7)
Fibrous degeneration	(33; 14.9)		
Localized adenoma	(41; 18.5)		(56; 24.5)
Mesenchymal tumor		(6; 2.7) (11; 4.9)	
Hamartoma		(8; 3.5) (15; 6.6)	

At the time of recruitment most patients (71%) were hospitalized for medical tests and/or antitumor treatment (chemotherapy, surgery, radiation, or a combination of these procedures). The meanings of the superscript letters is as follows:

[a] Mean age (range)

[b] ($n_{F:female}$; % female with respect to total sample) ($n_{M:males;}$ % males with respect to total sample)

[c] At the time of sampling, expressed as the mean ± 1SD (standard deviation)

[d] Weight loss (cancer patients) within the preceding 6 months expressed as (mean ± 1SD; percentage with respect to initial weight)

[e] Classification based on the American Joint Committee on Cancer Staging, World Health Organization, or Gleason Score

used for tumor marker determinations. Marker antigens assayed were (cut-off in parenthesis): total PSA (<5 ng/ml), CA-125 (<35 UI/ml), CA-15.3 (<30 UI/ml), CA-19.9 (<25 UI/ml), and CA-27.29 (<38 UI/ml). Determinations were performed according to the procedures recommended by manufacturers of the commercial kits (streptavidin technology of ELISA from Boehringer-Mannheim Immunodiagnostics, Germany, ES-300 BM auto-analyzer), or Abbott Lab., Buenos Aires, Argentina (AXSYM auto-analyzer). CEA (<5 ng/ml) was also determined by enzyme immunoassay (SRL, Fukuoka, Japan). Sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative-predictive values (NPV) were defined and calculated as described in detail by Kuralay et al. [9]. Briefly, for all samples correctly or incorrectly identified by the histological, laboratory, or other complementary studies as being malignant or benign (nonmalignant) were defined as true positive (TP), false positive (FP), true negative (TN), and false negative (FN). The term "positive" was referred to histological by proven malignant sample while benign tissues were referred as "negative" histological findings. Se was defined as TP/(TP + FN), Sp as TN/(TN + FP), PPV as TP/(TP + FP), and NPV as TN/(FN + TN).

Lipid Analyses

Samples were homogenized on ice with PBS (pH: 7.4, 100 µl per mg wet tissue) using a Potter-Elvejehm glass-Teflon homogenizer. An aliquot of 20 µl was taken for protein measurement [10] and the remainder was treated with Folch reactive [11]. After processing the samples according to Folch methodology, an aliquot of the final extract was taken to determine total lipid content gravimetrically [12] on a XP56-Delta Range Mettler Toledo balance. The evaporated residue was used for quantitative phosphorus determination according to Chen et al. [13]. Another aliquot was analyzed as described by Park et al. [14] in order to separate neutral ether lipids (ADAG) into their components, 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol (GEDE-A), 1-*O*-alkenyl-2,3-diacyl-*sn*-glycerol (GEDE-E), and triacylglycerides (TAG). The remainder of the total lipid extract was separated into various phospholipid subclasses using thin-layer chromatography (TLC) with a biphasic solvent system [15]. TLC was performed in hermetic pre-equilibrated cubes (Sigma Chem. Co., St. Louis, MO) on pre-coated silica gel 60 plates from Merck (Darmstadt, Germany). Phospholipid bands were visualized under ultraviolet light after spraying with 2′,7′-dichlorofluorescein solution and exposure to ammonia vapor. Lipids were identified comparing them with pure standards run in the same plate. In order to separate plasmalogens and DAG components from the PtdCho and PtdEth fractions the spots were scrapped-off and quantitatively eluted with chloroform/methanol (1:2, by vol). Isolated PtdCho and PtdEth were incubated with phospholipase C following the method of Mangold and Totani [16]. The resulting diacylglycerides were then acetylated with acetic anhydride/pyridine (10:5, by vol), and the products were separated by TLC using hexane/diethyl

Table 2 Marker antigens in plasma samples from patients with breast, lung, and prostate tumors

Tissue	Breast		Lung		Prostate	
Histopathology	BL	NEO	BL	NEO	BL	NEO
n	74	148	40	186	56	73
CA-125[a]	160 ± 34	873 ± 202*	83 ± 14	1037 ± 244*	ND	ND
CA-15.3[b]	96 ± 21	919 ± 195*	ND	ND	38 ± 6	318 ± 64*
CA-19.9[c]	ND	ND	31 ± 8	505 ± 191*	ND	ND
CA-27.29[d]	111 ± 29	1006 ± 197*	ND	ND	ND	ND
CEA[e]	40 ± 17	477 ± 165*	46 ± 7	355 ± 106*	13 ± 5	425 ± 113*
PSA[e]	ND	ND	ND	ND	37 ± 9	306 ± 170*

Data correspond to date of tissue collection and they were expressed as the mean of the number of samples indicated as $n \pm 1$ SD. Patients were studied periodically after diagnose to correlate results with tumor progression and/or spreading

ND Non determined

* Significantly different ($P < 0.01$) compared with BL samples

Cut-off values and units for each marker are indicated with a superscript letter as follows:

[a] <35 UI/ml

[b] <30 UI/ml

[c] <25 UI/ml

[d] <38 UI/ml

[e] <5 ng/ml

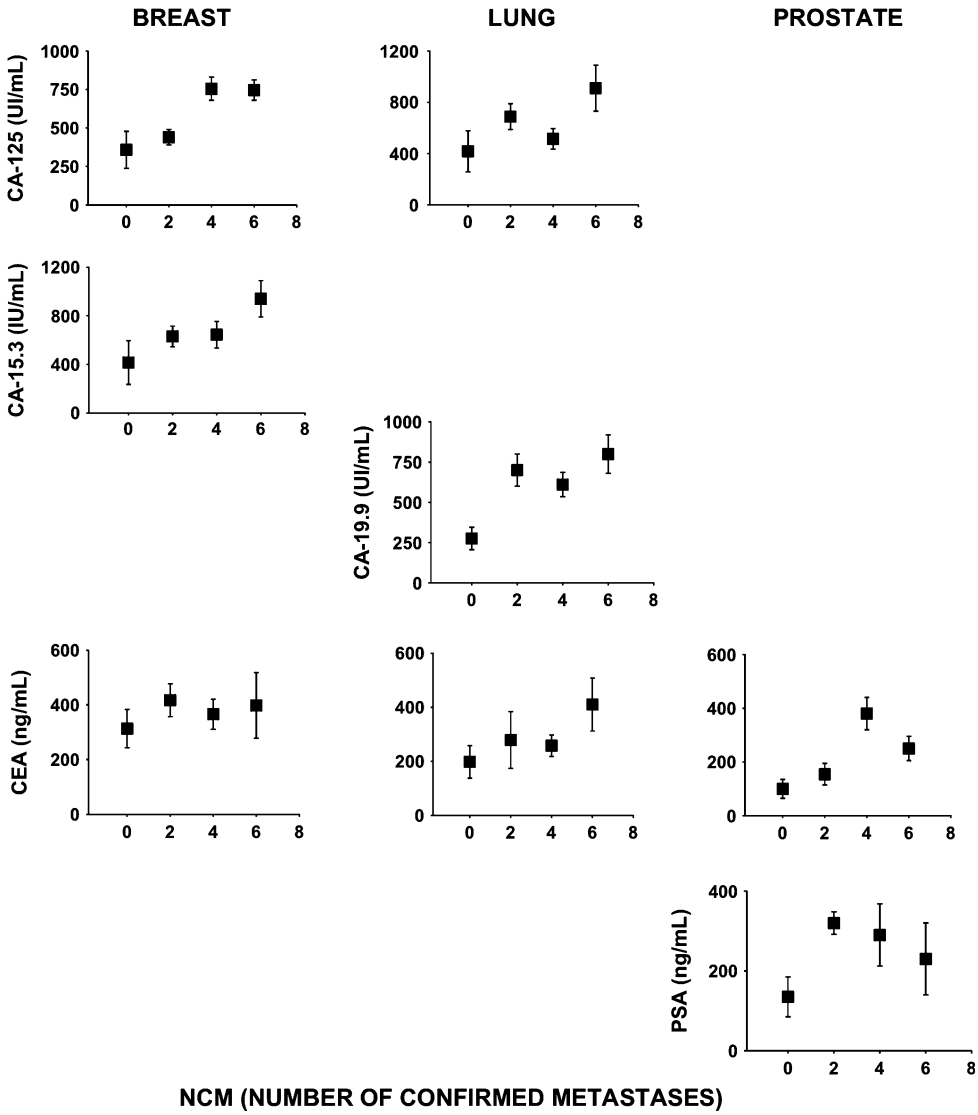

Fig. 1 Correlation between number of confirmed metastases (NCM) and various antigen markers for breast, lung and prostate. Number of neoplastic samples processed was smaller than the total number of patients studied since only those cases with confirmed number of metastases were included (101, 129, and 73 for breast, lung, and prostate, respectively)

ether/acetic acid (90:10:1, by vol) as first solvent system and toluene as second developing solvent [17]. Bands were scrapped-off the plate and transmethylated using F_3B (14% in methanol) under nitrogen atmosphere for 45 min at 80 °C. Monopentadecanoin was added to all the tubes as an internal standard. Fatty acid methyl esters (FAMEs) from the ether lipid species were separated from the aldehyde derivatives by TLC using toluene as a solvent and quantitatively analyzed using a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA) equipped with a terminal computer integrator [18].

Graphic Software and Statistical Treatment of Data

Data were reported as the mean ± 1 standard deviation (SD). Statistical significance was tested by student *t*-test or by ANOVA (analysis of variance) plus Bonferroni test. Correlation and regression analyses and data plotting were performed with the aid of Systat (version 12.0 for Windows) from SPSS Science (Chicago, IL), Sigma Scientific Graphing Software (version 8.0) from Sigma Chem. Co. (St. Louis, MO), and/or GB-STAT Professional Statistics Program (version 4.0) from Dynamic Microsystems Inc. (Silver Springs). Multivariable regression analyses were performed as described by Kleinbaum and Kupper [19].

Results and Discussion

Patients and Tumor Markers

Our study was carried out with a representative (characteristic) population of neoplastic samples, composed of a typical distribution of cancer types (Table 1). Histopathological data were in agreement with previous

studies performed in Western countries [20]. Tumor marker levels (Table 2) were also in accordance with data previously reported for each kind of tumor [21–24]. These values of tumor markers are often useful for monitoring the evolution of patients; however, some of them may not be helpful in detecting recurrence. For example, we found that CA-125, CA-19.9, CA-27.29, CEA, and total PSA remained unaltered in a significant proportion of cases in which large spreading of primary tumors was detected by other tests such as radioisotopic and/or X-ray scanning (data not shown). Total PSA was the antigen that showed the best correlation with clinical status. Notwithstanding, it was within normal values in 12% patients with prostate cancer detected by digital rectal and scanner exams, and then corroborated by histological studies after needle punch biopsy. Moreover, spreading of primary prostatic or breast tumors with bone metastases was not correlated with a significant raise in any of the antigen markers tested in 22 and 31% of the samples analyzed, respectively. In addition, approximately 25% of patients with metastatic dissemination of primary lung cancer (especially in central nervous system and liver) showed no significant changes with respect to the starting level of the antigenic markers.

Sp and Se of tumor markers are topics under continuous evaluation. The ideal marker would be a molecule which only appears in patients with a specific malignancy, and it would correlate directly with stage and response to treatment. Up till now, no tumor marker has met this requirement. Thus, establishment of a biochemical index for either diagnosis of cancer, or management of patients with neoplastic diseases remains as one of the important goals to be achieved in cancer research. It is accepted that measurement of one antigen concentration in plasma is not recommended as a useful diagnostic tool in malignancy.

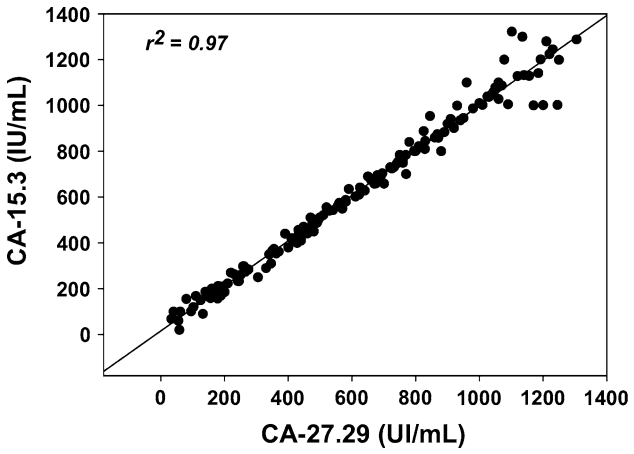

Fig. 2 Linear correlation between plasma levels of antigens CA-15.3 and CA-27.29 was determined as described in "Experimental Procedure" for 148 patients with breast cancer

In this regard, multivariable regression analyses of data in relation to tumor marker concentrations and clinical status of patients (summarized in Figs. 1 and 2, and in Tables 2 and 3) led us to these conclusions: (1) high levels of conventional markers did not directly correlate ($r^2 < 0.80$) with both stage and /or spreading of the primary tumor. (2) simultaneous determinations of two or more antigenic tumor markers did not improve PPV and NPV values ($0.58 < r^2 < 0.73$), and (iii) specifically, tumor markers CA-15.3 and CA-27.29 were directly proportional between them ($r^2 = 0.97$). Thus, simultaneous determinations of these two antigens do not offer advantages over the measurement of each marker alone. These statements confirm previous suggestions and findings made by other researchers [25–30] and answered the question raised by Klee et al [31]. It is important to remark that even the association of two or three of these markers (CA-125 + CA-15.3 + CEA; CA-125 + CA-19.9 + CEA; or PSA + CEA for breast, lung, and prostate cancers, respectively) could not significantly improve PPV and NPV values compared with prognosis or spreading predictions based on determinations of the primary marker alone (Table 3).

The optimal interpretation of tumor markers requires knowledge about both methodological limitations and the course of the disease in a particular patient. Even in those cases where these conditions were met, the correlation with clinical status was poor. For that reason we explored the usefulness of other tumor markers derived from ether lipid

Table 3 Sensitivity (Se), specificity (Sp), and negative- and positive-predictive values (NPV; PPV) of marker antigen levels tumors from human breast, Lung, and Prostate

	Tissue	Parameters			
		Se	Sp	NPV	PPV
CA-125	Breast	84	90	90	95
	Lung	89	92	96	89
CA-15.3	Breast	66	91	88	93
CA-19.9	Lung	87	96	94	95
CA-27.29	Breast	64	91	89	93
CEA	Breast	46	80	68	76
	Lung	53	75	77	72
	Prostate	38	89	65	70
PSA	Prostate	93	92	100	94
CA-125 + CA-15.3 + CEA	Breast	91	90	92	95
CA-125 + CA-19.9 + CEA	Lung	99	97	98	97
PSA + CEA	Prostate	100	93	100	96

Values were calculated as indicated in the experimental part. For simplicity only three types of associations between markers were shown. Data were calculated on the day of tissue collection. Patients were studied periodically after diagnosis to correlate results with tumor progression and/or spreading

Table 4 Absolute amounts of lipids of normal (C), benign (BL), and neoplastic (NEO) samples from human breast, lung, and prostate tissues

Tissues	Breast			Lung			Prostate		
Histopathology	C	BL	NEO	C	BL	NEO	C	BL	NEO
n	222	74	148	226	40	186	229	56	173
GEDE-A	0.5 ± 0.1	1.9 ± 0.3*	4.6 ± 0.5*,**	1.3 ± 0.2	1.8 ± 0.5	2.8 ± 0.3*	0.6 ± 0.1	0.9 ± 0.2	1.7 ± 0.3*,**
GEDE-E	0.7 ± 0.1	1.6 ± 0.4	3.3 ± 0.8*	0.7 ± 0.1	0.9 ± 0.2	1.3 ± 0.3*	0.5 ± 0.1	0.8 ± 0.2	2.2 ± 0.7*
PakEtn	0.7 ± 0.1	1.6 ± 0.2*	3.9 ± 0.4*,**	4.6 ± 0.3	6.6 ± 0.6	8.9 ± 0.5*	0.8 ± 0.2	0.8 ± 0.3	1.5 ± 0.4*,**
PlsEtn	1.1 ± 0.2	2.4 ± 0.3	5.8 ± 0.4*,**	11.5 ± 2.4	19.8 ± 3.0	24.1 ± 2.9*	3.3 ± 0.4	6.0 ± 1.8*	12.5 ± 3.0*
PakCho	0.5 ± 0.1	0.6 ± 0.1	2.1 ± 0.3*,**	1.0 ± 0.2	1.5 ± 0.4	2.9 ± 0.3*	0.6 ± 0.1	0.5 ± 0.3	1.6 ± 0.2*,**
PlsCho	0.8 ± 0.2	1.2 ± 0.3	3.5 ± 0.7*	2.3 ± 0.3	3.3 ± 0.7	4.4 ± 0.4*	0.8 ± 0.2	1.1 ± 0.3	3.6 ± 0.3*,**

Results were expressed as μg of each lipid subclass per mg protein, and they correspond to the mean ± SD of the number of samples indicated as *n*

Statistically different ($P < 0.01$) *compared with C, **compared with BL

compositional analysis of biopsy samples to perform histopathological studies.

Lipids and Fatty Acyl Composition of Ether Lipids as Tumor Markers

As a first approach in searching for a new lipid marker for human tumor tissues, we followed the strategy employed in this kind of investigation, that is, to compare pooled results between normal and malignant tissues classified according to their histopathological criteria and obtained from different patients. Table 4 shows data obtained from the analyses of neutral and polar ether lipid subclasses among normal, BL and NEO samples from breast, lung, and prostate human tissues. Results corresponded to absolute amounts of lipids (μg per mg tissue protein). They were pooled and averaged as a function of their histopathological classification on the day of collection. In agreement with previous studies from other researchers we found that tumor tissues were significantly enriched in GEDE-A and GEDE-E [4, 32–34]. Interestingly, concerning lung, no differences were found between normal and BL samples. Similar conclusions can be obtained examining the data obtained from PakEtn, PlsEtn, PakCho and PlsCho. Notwithstanding, the EtnGpl sub-fraction exhibited higher Se than ChoGpl since changes observed in ethanolamine-containing plasmalogens were more pronounced than those of choline. Drastic modification of data interpretation occurred when the results were separately compared. We calculated the amount of each lipid subclass with respect to the content of the same lipid in normal sample from the same patient. Table 5 shows the mean values obtained after these calculations. Data were expressed as ratios of the absolute amount of lipids (μg/mg protein). In the tumors studied, we found significant differences between normal, BL, and NEO samples. Moreover, differences observed among groups were even more evident than those observed

Table 5 Mean values of ratios between the content of ether lipids in benign (BL) or neoplastic (NEO) and normal (C) samples in human breast, lung, and prostate tissues

Tissues	Breast		Lung		Prostate	
Histopathology	BL	NEO	BL	NEO	BL	NEO
n	74	148	40	186	56	173
GEDE-A	4.2 ± 0.2*	10.1 ± 0.3*,**	1.9 ± 0.1*	3.9 ± 0.2*,**	1.5 ± 0.1*	4.7 ± 0.1*,**
GEDE-E	3.5 ± 0.4*	5.8 ± 0.2*,**	1.8 ± 0.2*	3.2 ± 0.1*,**	2.4 ± 0.2*	4.8 ± 0.2*,**
PakEtn	4.1 ± 0.1*	7.0 ± 0.2*,**	2.0 ± 0.2*	4.5 ± 0.2*,**	1.9 ± 0.1*	5.6 ± 0.1*,**
PlsEtn	2.7 ± 0.1*	6.9 ± 0.1*,**	3.1 ± 0.2*	6.3 ± 0.3*,**	3.5 ± 0.2*	7.8 ± 0.1*,**
PakCho	1.8 ± 0.1*	4.7 ± 0.2*,**	1.5 ± 0.1*	3.9 ± 0.1*,**	2.1 ± 0.1*	3.9 ± 0.2*,**
PlsCho	2.0 ± 0.2*	5.4 ± 0.1*,**	2.0 ± 0.2*	4.2 ± 0.2*,**	3.0 ± 0.2*	5.1 ± 0.2*,**

Results were expressed as the mean of ratios between the absolute amount (μg/mg protein) of each lipid subclass in BL or NEO samples and the content measured in normal tissues from the same patient ± 1SD of the number of analyses indicated as *n*. SD of normal samples was between 8 and 19% of the mean value depending on the group of samples analyzed

Statistically significant ($P < 0.01$) *compared with control, **compared with BL

in the previous analyses (Table 4). Thus, using this type of analysis all the groups were statistically different from each other.

As reported by other authors [4, 32–34], pooled samples had a lower proportion of PakEtn or PakCho than PlsEtn or PlsCho. We calculated the relative proportion of GEDE-A to GEDE-E, and the PakEtn/PlsEtn and PakCho/PlsCho ratios. The content of GEDE-A compared to GEDE-E decreased in NEO samples from the three tissues studied, while the proportion between plasmanyl- and plasmenyl-derived compounds (in both PE and PC) increased in BL and in NEO sample tissues with respect to the normal ones. An important conclusion was that changes in EtnGpl were more pronounced than those in ChoGpl. Analytical ratios shown in Table 6 would be useful for the classification of samples according to their histopathological characteristics. However, there were two major problems with these calculations: (a) except in the case of plasmalogen ratios, no differences were found between BL and normal samples, and (b) they did not reflect the fact that individual samples may not differ from the mean values obtained for the corresponding histopathological group and, consequently, they would be misclassified. Considering PakEtn/PlsEtn and PakCho/PlsCho ratios as the most discriminating biomarkers, 31.4, 26.2 and 12.5% of NEO samples from breast, lung and prostate (respectively) were classified as normal samples when compared with the means shown in Table 4. However, they became malignant when comparisons were made against those values obtained from normal tissue from the same patient. Using the same strategy of comparison, BL samples from breast, lung and prostate were 25.0, 20.1 and 11.3% misclassified. These records were similar to those obtained using the conventional primary antigenic tumor markers as discriminators.

As a second approach to find a more reliable lipid tumor marker, analyses of fatty acyl composition of each ether lipid subclass isolated from normal and pathological samples were carried out. Significant changes in fatty acyl composition were easily observed directly on the chromatograms even without ulterior calculations of peak areas (data not shown). This pattern represented a "finger print" and it was a constant finding in the samples analyzed. The main changes observed involved the proportion of monoenoic to saturated fatty acids and also the relative amount of PUFAs to saturated fatty acids. For these reasons, the absolute amount (nmoles per mg protein) of fatty acyl chains in each lipid subclass from normal, BL and NEO samples was calculated and used to obtain several analytical ratios. A few selected (statistically significant) ratios were presented in Table 7. They were expressed as the percent change calculated by comparing the absolute content of fatty acyl chains of BL or NEO samples with the

Table 6 Ratio between ether-lipid subclasses in normal (C), benign (BL), and neoplastic (NEO) human tissues

Tissue	Breast			Lung			Prostate		
Histopathology	C	BL	NEO	C	BL	NEO	C	BL	NEO
n	222	74	148	226	40	186	229	56	173
GEDE-A ± GEDE-E/ Total plasmalogens	0.24 ± 0.05[a]	0.39 ± 0.06[a]	0.47 ± 0.08[b]	0.08 ± 0.01[a]	0.11 ± 0.01[a]	0.18 ± 0.03[b]	0.20 ± 0.04[a]	0.19 ± 0.02[a]	0.21 ± 0.03[a]
GEDE-A/ GEDE-E	5.01 ± 0.42[a]	6.33 ± 0.22[a]	2.70 ± 0.22[b]	1.91 ± 0.21[a]	2.02 ± 0.16[a]	1.30 ± 0.11[b]	1.22 ± 0.11[a]	0.91 ± 0.19[a]	0.78 ± 0.03[a]
PakEtn/ PlsEtn	0.58 ± 0.07[a]	0.92 ± 0.10[b]	1.64 ± 0.06[c]	0.33 ± 0.03[a]	0.57 ± 0.04[b]	0.91 ± 0.06[c]	0.21 ± 0.04[a]	0.45 ± 0.02[b]	0.74 ± 0.01[c]
PakCho/ PlsCho	0.23 ± 0.01[a]	0.44 ± 0.03[b]	0.67 ± 0.05[c]	0.40 ± 0.03[a]	0.82 ± 0.06[b]	1.11 ± 0.08[c]	0.63 ± 0.08[a]	0.87 ± 0.05[b]	1.15 ± 0.04[c]

Ratios were calculated from the absolute amounts (μg/mg protein) of each lipid subclass. They were expressed as the mean of the number of samples indicated as $n \pm 1SD$

Lipids were determined chromatographically using internal standards or phosphorous measurements

Different superscript letters within each row -of the same kind of tissue- are significantly different at $P < 0.01$ or lesser

Table 7 Analytical markers of fatty acyl composition of lipids from benign (BL) and neoplastic (NEO) samples of human breast, lung and prostate

Histopathology	Breast		Lung		Prostate	
	BL	NEO	BL	NEO	BL	NEO
n	74	148	40	186	56	173
Total lipids						
Σ Monoethylenic/Σ Saturated	66 ± 7*	144 ± 17*,**	39 ± 6*	127 ± 15*,**	51 ± 4*	185 ± 19*,**
18:2n − 6 + 18:3n − 3/20:4 (n − 6)	54 ± 8*	196 ± 21*,**	27 ± 5*	222 ± 17*,**	36 ± 5*	193 ± 12*,**
18:2n − 6/Σ PUFAs	43 ± 5*	232 ± 25*,**	31 ± 7*	204 ± 21*,**	49 ± 4*	209 ± 15*,**
Σ (n − 3)/Σ (n − 6)	−21 ± 2*	−39 ± 4*,**	11 ± 3	−40 ± 5*, **	−8 ± 2	−53 ± 4*, **
EtnGpl						
Σ Monoethylenic/Σ Saturated	58 ± 3*	195 ± 13*,**	30 ± 3*	193 ± 13*,**	65 ± 7*	303 ± 25*,**
18:2n − 6 + 18:3n − 3/20:4 (n − 6)	49 ± 4*	107 ± 14*, **	36 ± 4*	131 ± 19*,**	57 ± 6*	148 ± 17*,**
18:2n − 6/Σ PUFAs	33 ± 5*	121 ± 27*,**	27 ± 2*	97 ± 6*,**	38 ± 4*	106 ± 12*,**
Σ (n − 3)/Σ (n − 6)	−7 ± 2	−11 ± 3	4 ± 1	−13 ± 2	−5 ± 1	−16 ± 4
PlsEtn						
Σ Monoethylenic/Σ Saturated	74 ± 5*	303 ± 18*,**	28 ± 2*	298 ± 21*,**	76 ± 8*	355 ± 20*,**
18:2n − 6 + 18:3n − 3/20:4 (n − 6)	31 ± 3*	90 ± 6*, **	19 ± 2	49 ± 3*,**	27 ± 3*	67 ± 5*,**
18:2n − 6/Σ PUFAs	35 ± 4*	76 ± 7*,**	15 ± 3	34 ± 5*,**	19 ± 3	46 ± 6*,**
Σ (n − 3)/Σ (n − 6)	−6 ± 1	−5 ± 1	11 ± 2	10 ± 3	−7 ± 1	−12 ± 3

Data were calculated as percent change with respect to normal tissue of each individual sample

total lipids unfractionates (neutral plus polar) lipids, *EtnGpl* ethanolamine glycerophospholipids (PtdEtn + PakEtn + PlsEtn), *PlsEtn* plasmalogen fraction of ethanolamine phospholipids

Calculations were averaged and expressed as the mean ± 1SM. *n* Indicates the number of samples within each category

Statistical differences ($P < 0.01$) were noted as * for BL or NEO vs. control, and ** for NEO vs. BL

amount measured in the normal tissue of the same patient. Similar calculations were made concerning the fatty acyl composition of GEDE-A, GEDE-E, and DAG. However, in those cases the statistical differences, though significant, were of lesser extension. Thus, we focused our attention in fatty acyl composition of PakEtn and PlsEtn sub-fractions. Ratios allowed the differentiation not only of NEO samples from normal tissue, but also of BL from NEO samples with high Se and Sp. These statistical differences almost disappeared when ratios were calculated in pooled samples since values of SD were notoriously increased. This fact can be attributed to a marked variability observed in the baseline contents of fatty acyl chains among patients under different nutritional conditions, concomitant pathologies, therapeutical approaches, hormonal status, gender, age, and other factors which are not easily controlled. In agreement with this, reports from other laboratories have attempted to characterize plasma fatty acyl profile in patients undergoing benign and/or neoplastic illness with a variable success [35–37].

From the data reported in Table 7 we calculated Se, Sp, NPV, and PPV for the different lipid markers studied. The best results were obtained for Σ monoenoic/Σ saturated fatty acids in PlsEtn showing major changes with minor dispersion together with an excellent correlation with clinical status of patients. Se and Sp were between 96 and 100%, and NPV and PPV values were both over 97%. Figure 3 shows correlation studies between the lipid marker and the spreading capacity of primary tumors. Interestingly, in all of the tumors studied we found a linear correlation, ($0.96 < r^2 < 0.98$) that agreed with the clinical prognosis of each kind of damage. It seems that this lipid marker does not depend on the nature of the tumor, but it clearly reflects the mestastatic potential of the primary cancer even when an accurate quantification of this condition is so difficult to obtain. Extrapolation of the regression lines to zero metastases yielded values of percent change (Σ monoenoic/Σsaturated) PlsEtn FAs which would correspond to a tumor with a hypothetically null spreading capacity. These values were similar to those obtained for BL samples (Table 7). Thus, the proposed marker has an excellent correlation with clinical status of the patient, especially number of metastases. Probably, it may be limited to the follow-up of the patient and to distinguish between BL and NEO samples in conflicting histopathological examinations in which complementary studies would be required. A population study at large scale may confirm the utility of the marker in prognosis, but our results are promising. We demonstrated that this highly sensitive marker is, at least, equally or even better than the existing clinical chemistry tumor markers.

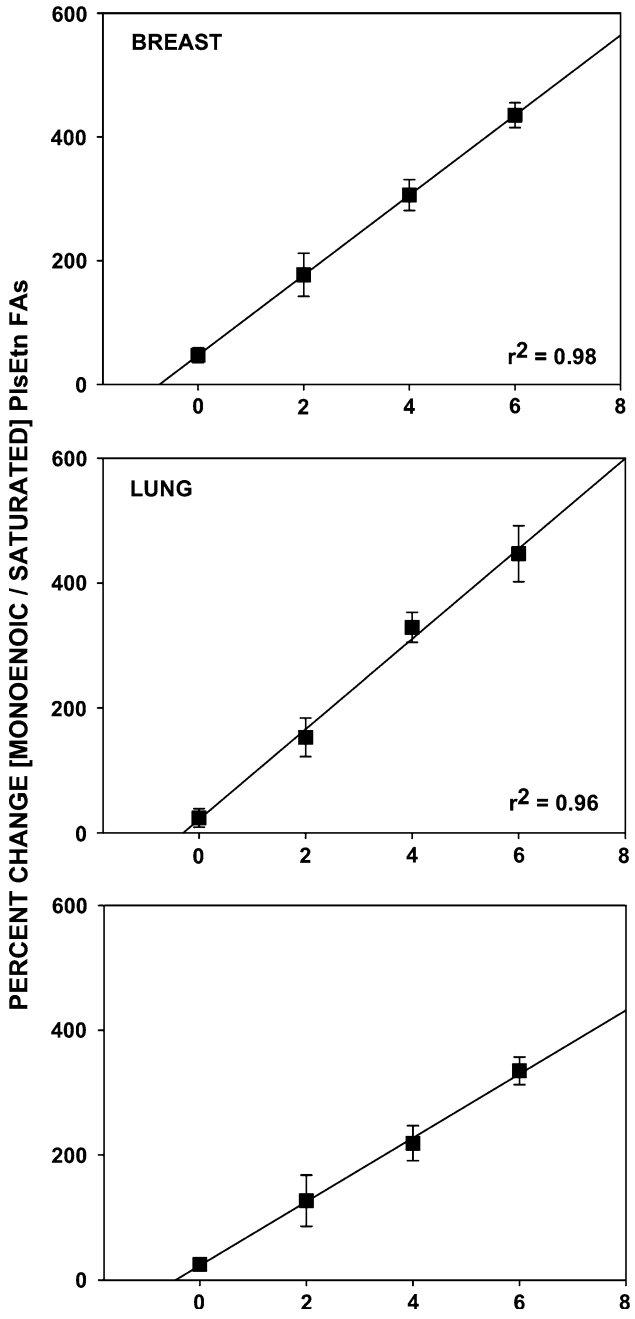

Fig. 3 Correlations between the relative content of monoenoic fatty acyl chains in PlsEtn and number of confirmed metastases (NCM) in 101 breast, 129 lung, and 73 prostate neoplastic samples. Calculations were done upon the percent change of the amount of monoenoic fatty acyl chains in PlsEtn of neoplastic samples with respect to normal tissue of the same patient. We included only those patients in which complementary studies confirmed the presence and number of metastases. Linear regression coefficients were calculated electronically using the software mentioned in "Experimental Procedure"

Surprisingly, the early findings reported in this field on lipid biochemistry have not been further developed for decades. So, we expect that our contribution may stimulate future research in this area.

Conclusions

To the best of our knowledge, this is the first time that a biochemical marker derived from composition studies of ethanolamine-containing plasmalogens provides a reliable index capable of distinguishing between BL and NEO tissues, and it correlates linearly with metastases spreading in vivo. Lipid analyses were performed with minor amounts of tissue and may be carried out with a relatively low number of complex instruments. Samples could be obtained during punch needle biopsy for histopathological examination. Results displayed high Se and excellent clinical correlation, and they would have interesting applications in prognosis of the most frequently observed human cancers.

Acknowledgments We are very grateful to the surgeons who contributed to this study. The authors also thank Elsa Claverie and Norma Cristalli for excellent technical assistance. Partially supported by grants from CIC, CONICET and UNLP, Argentina.

References

1. Nagan N, Zoeller RA (2001) Plasmalogens: biosynthesis and functions. Prog Lipid Res 40:199–229
2. Snyder F (1999) The ether lipid trail: a historical perspective. Biochim Biophys Acta 1436:265–278
3. Snyder F, Lee TC, Wykle RL (2002) Ether-linked lipids and their bioactive species. In: Vance DE, Vance JE (eds) Biochemistry of lipids, lipoproteins and membranes. Elsevier Science BV, Amsterdam, pp 233–262
4. Mangold HK, Paltauf F (1983) Ether lipids as chemical indicators in neoplasms. In: Mangold HK, Paltauf F (eds) Ether lipids. Biochemical and biomedical aspects. Academic, London, pp 248–251
5. Lin HJ, Ho FCS, Lee CLH (1978) Abnormal distribution of O-alkyl groups in the neutral glycerolipids from human hepatocellular carcinomas. Cancer Res 38:946–949
6. Mostofi FK, Davis CJ Jr, Sesterhenn IA (1992) Pathology of carcinoma of the prostate. Cancer 70:235–253
7. Mostofi FK, Murphy GP, Mettlin C (1995) Pathology review in an early prostate cancer detection program: results from the American Cancer Society-National Prostate Cancer Detection Project. Prostate 27:7–12
8. Travis WD, Colby TV, Corrin B (1999) Histological typing of lung and pleural tumors. World Health Organization International Histological Classification of Tumors, 3rd edn. Springer, Berlin
9. Kuralay F, Tokgöz Z, Cömlekci A (2000) Diagnostic usefulness of tumor marker levels in pleural effusions of malignant and benign origin. Clin Chim Acta 300:43–55
10. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
11. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
12. Marra CA, Alaniz MJT de (2000) Calcium deficiency modifies polyunsaturated fatty acid metabolism in growing rats. Lipids 35:983–990

13. Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758
14. Park JH, Park EJ, Kim KS, Yeo YK (1995) Changes in ether-linked phospholipids in rat kidney by dietary α-Linolenic acid in Vivo. Lipids 30:541–546
15. Neskovic NM, Kostic DM (1968) Quantitative analysis of rat liver phospholipids by a two-step thin-layer chromatographic procedure. J Chromatogr 35:297–300
16. Mangold HK, Totani N (1983) Procedures for the analysis of ether lipids. In: Mangold HK, Paltauf F (eds) Ether Lipids. Biochemical and biomedical aspects. Academic, London, pp 377–387
17. Yeo YK, Philbrick DJ, Holub BJ (1989) Altered acyl chain compositions of alkylacyl, alkenylacyl, and diacyl subclasses of choline and ethanolamine glycerophospholipids in rat heart by dietary fish oil. Biochim Biophys Acta 1001:25–30
18. Marra CA, Alaniz MJT de (1989) Influence of testosterone administration on the biosynthesis of unsaturated fatty acids in male and female rats. Lipids 24:1014–1019
19. Kleinbaum DG, Kupper LL (1997) Applied regression analysis and other multivariable methods, (3rd edn.) Duxbury Press, MA
20. Boring CC, Squires TS, Tong T, Montgomery S (1994) Cancer statistics. Cancer J Clin 44:7–26
21. Giai M, Roagna R, Ponzone R, Biglia N (1996) TPS and CA 15.3 serum values as a guide for treating and monitoring breast cancer patients. Anticancer Res 16:875–881
22. De La Lande B, Hacene K, Florias JL, Alatrakchi N, Pichon MF (2002) Prognostic value of CA 15.3 kinetics for metastatic breast cancer. Int J Biol Markers 17:231–238
23. Sawabata N, Okada M, Higashiyama K (2006) Diagnostic strategy based on preoperative serum CEA levels in clinical stage IA NSCLC. J Clin Oncol 24:17011
24. ÓNistrian AM, Schwartz MK, Greenberg EJ, Smith CA, Schwartz DC (1991) Evaluation of CAM26, CAM29, CA 15–3 and CEA as circulating tumor markers in breast cancer patients. Tumor Biol 12:82–90
25. Daoud E, Bodor G (1991) CA-125 concentrations in malignant and nonmalignant disease. Clin Chem 37:1968–1974
26. Correale M, Abbate I, Gargano G (1992) Analytical and clinical evaluation of a new tumor marker in breast cancer: CA 27.29. Int J Biol Markers 7:43–46
27. Devine PL, Walsh MD, McGuckin MA (1995) Prostate-specific antigen (PSA) and cancer-associated serum antigen (CASA) in distinguishing benign and malignant prostate disease. Int J Biol Markers 10:221–225
28. Rodríguez de Paterna L, Arnaiz F, Esternoz J, Ortuno B, Lanzos E (1995) Study of serum tumor markers CEA, CA 15.3 and CA 27.29 as diagnostic parameters in patients with breast carcinoma. Int J Biol Markers 10:24–29
29. Pamies RJ, Crawford DR (1996) Tumor markers: an update. Med Clin North Am 80:185–199
30. Roulston JE (2002) Screening with tumor markers: critical issues. Mol Biotechnol 20:153–62
31. Klee GG, Schreiber WE (2004) MUC1 gene-derived glycoprotein assays for monitoring breast cancer (CA 15–3, CA 27.29, BR): are they measuring the same antigen? Arch Pathol Lab Med 128:1131–1135
32. Snyder F, Blank ML, Morris HP (1969) Occurrence and nature of O-alkyl and O-alk-1-enyl moieties of glycerol in lipids of Morris transplanted hepatomas and normal rat liver. Biochim Biophys Acta 176:502–510
33. Snyder F, Wood R (1969) Alkyl and alk-1-enyl ethers of glycerol in lipids from normal and neoplastic human tissues. Cancer Res 29:251–257
34. Howard BV, Morris HP, Bailey JM (1972) Ether-lipids, α-glycerol phosphate dehydrogenase, and growth rate in tumors and cultured cells. Cancer Res 32:533–1538
35. Yang YJ, Lee SH, Hong SJ, Chung BC (1999) Comparison of fatty acid profiles in the serum of patients with prostate cancer and benign prostatic hyperplasia. Clin Biochem 32:405–409
36. Aronson WJ, Glaspy JA, Reddy ST, Reese D, Heber D, Bagga D (2001) Modulation of Omega-3/Omega-6 polyunsaturated ratios with dietary fish oils in men with prostate cancer. Urology 58:283–288
37. Zuijdgeest-Van Leeuwen SD, Van Der Heijden MS, Rietveld T (2002) fatty acid composition of plasma lipids in patients with pancreatic, lung and oesophageal cancer in comparison with healthy subjects. Clin Nutr 21:225–230