21. Blichert-Toft M, Hansen JPH, Hansen OH, Schiødt T. Clinical course of cystosarcoma phyllodes related to histologic appearance. *SGO* 1975, **140**, 929–932.
22. Lindquist KD, Van Heerden JA, Weiland LH, Martin JKJr. Recurrent and metastatic cystosarcoma phyllodes. *Am J Surg* 1982, **144**, 341–343.
23. Hines JR, Murad TM, Beal JM. Prognostic indicators in cystosarcoma phylloides. *Am J Surg* 1987, **153**, 276–280.
24. Zóltowska A, Kozłowski H. Investigations on the transformation of fibroadenoma of the breast into malignant cystosarcoma phyllodes. *Neoplasma* 1969, **16**, 549–556.
25. Kessinger A, Foley JF, Leomn HM, Miller DM. Metastatic cystosarcoma phyllodes: a case report and review of the literature. *J Surg Oncol* 1972, **4**, 131–147.
26. Browder W, McQuitty JTJr, McDonald JC. Malignant cystosarcoma phylloides. Treatment and prognosis. *Am Surg* 1978, **136**, 239–241.
27. Chua CL, Thomas A. Cystosarcoma phyllodes tumors. *SGO* 1988, **166**, 302–306.
28. Murad TM, Hines JR, Beal J, Bauer K. Histopathological and clinical correlations of cystosarcoma phyllodes. *Arch Pathol Lab Med* 1988, **112**, 752–756.
29. Stockdale AD, Leader M. Case report: phyllodes tumour of the breast: response to radiotherapy. *Clin Radiol* 1987, **38**, 287–290.
30. Stephenson HEJr, Gross S, Gumport SL, Meyer HW. Cystosarcoma phyllodes of the breast: a review of the literature with the addition of 15 new cases. *Ann Surg* 1973, **136**, 856–863.
31. Rhodes RH, Frankel KA, Davis RL, Tatter D. Metastatic cystosarcoma phyllodes. A report of 2 cases presenting with neurological symptoms. *Cancer* 1978, **41**, 1179–1187.
32. Oberman HA. Cystosarcoma phyllodes: a clinicopathologic study of hypercellular periductal stromal neoplasms of breast. *Cancer* 1965, **18**, 697–710.
33. Hart WR, Bauer RC, Oberman HA.Cystosarcoma phyllodes: a clinicopathologic study of twenty-six hypercellular periductal stromal tumors of the breast. *Am J Clin Pathol* 1978, **70**, 211–216.
34. Zahner J, Bassler R. The rate of mitosis in cystosarcoma phyllodes (Phyllodes tumor, WHO) of the breast. An analysis of 47 cases. *Gynecol Obstet* 1989, **246**, 153–157.
35. Salvadori B, Cusumano F, Bo RD, *et al.* Surgical treatment of phyllodes tumors of the breast. *Cancer* 1989, **63**, 2532–2536.

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Zn-α_2-glycoprotein Levels in Breast Cancer Cytosols and Correlation with Clinical, Histological and Biochemical Parameters

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Zn-α_2-glycoprotein (Zn-α_2-gp), a protein present at high levels in breast cyst fluid, has been measured in 104 breast tumour cytosols by using an immunoenzymatic assay. Concentrations of Zn-α_2-gp ranged from 0 to 23.5 μg/mg of total soluble protein, with an average value of 2.4 μg/mg. There was no significant correlation between Zn-α_2-gp and menopausal status, tumour size or lymph node involvement, or between this protein and biochemical parameters such as oestrogen receptor, cathepsin D or pS2 levels. However, there was a significant association between Zn-α_2-gp and histological grade of tumours, with higher Zn-α_2-gp levels in well-differentiated tumours (mean 4.6 μg/mg) than in moderately (1.8 μg/mg) or poorly (0.9 μg/mg) differentiated tumours. On the basis of these results, we propose that Zn-α_2-gp may be considered as a biochemical marker of differentiation in breast cancer.
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INTRODUCTION

ZN-α_2-GLYCOPROTEIN (Zn-α_2-gp) is a human protein of unknown biological function, originally described in 1961 by Bürgi and Schmid [1]. The protein consists of a single polypeptide chain with a molecular mass of about 40 kD and displays a marked charge heterogeneity mainly due to variations in the composition of carbohydrate moiety [2].

This glycoprotein is present in high concentrations in cyst fluid from women with gross cystic breast disease [3], which has led to the proposal that Zn-α_2-gp and other major intracystic proteins like apolipoprotein D [4] may contribute to the pathogenesis of the disease. In addition, northern blot analysis of breast tissue specimens has revealed the existence of a certain subgroup of breast tumours which produce significant amounts of Zn-α_2-gp [5]. Similarly, immunohistochemical studies have demonstrated the presence of the protein in about 50% of invasive breast carcinomas [6], which is in good agreement with recent data from our laboratory indicating that Zn-α_2-gp is a major protein component in about 45% of breast secretions from

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patients with breast carcinoma [7]. Interestingly, Bundred *et al.* [8] have reported that women whose tumours show positive staining for Zn-α_2-gp have a poorer prognosis than those negatively stained for this protein. By contrast, Hurlimann and van Melle [9], using the same technique, have found that Zn-α_2-gp expression by breast tumours is weakly associated to a favourable evolution of the disease.

In order to further clarify the potential value of Zn-α_2-gp as a prognostic marker in breast cancer, we have determined the concentration of this protein in cytosol of breast tumour biopsies and we have compared its levels with other well-established prognostic factors in breast carcinoma.

PATIENTS AND METHODS

Patients and tumour tissues

This study was performed on a group of 104 women who had undergone surgery for primary breast carcinoma at Hospital de Jove and Hospital Central (Asturias, Spain) from 1988 to 1991. The patients' characteristics with respect to age, menopausal status and clinical staging of the disease are listed in Table 1. Histological grade was determined according to Bloom and Richardson [10].

Following surgical excision, tumour samples were immediately frozen in liquid nitrogen and stored at −70°C until use. For the biochemical determinations frozen tissues were pulverised and homogenised in 50 mmol/l Tris–HCl buffer (pH 7.4) using a Microdismembrator II (Braun, Germany). The homogenate was centrifuged at 100 000 *g* for 1 h at 4°C and the resulting supernatant (cytosol) used for the assays described below.

Zn-α_2-glycoprotein purification and antiserum production

Zn-α_2-gp was purified from cyst fluid from women with gross cystic breast disease according to the HPLC procedure described previously [7]. Antiserum against the purified protein was produced from New Zealand white rabbits following the method described by Vaitukaitis [11]. The immunised rabbits were bled 6 weeks after inoculation and the resulting serum was dialysed for 24 h at 4°C against 20 mmol/l phosphate buffer, pH 7.2. The material was then fractionated on a diethylaminoethyl (Whatman DE52) column equilibrated and eluted in the same buffer. IgG-containing fractions were collected and stored at −20°C until used.

Table 1. *Characteristics of patients and tumours*

Patients	
Total	104
Age	
Mean	59
Range	27–84
Menopausal status	
Premenopausal	32
Postmenopausal	72
Tumours	
Size	
T_1 (< 2 cm)	23
T_2 (2–5 cm)	55
$T_{3/4}$ (> 5 cm)	26
Nodal status	
N_0	42
N_+	62
Metastasis at time of diagnosis	
M_0	101
M_1	3
Histological grade*	
I	29
II	55
III	13

*Histological grade was not available in seven tumours.

Quantification of Zn-α_2-glycoprotein by enzyme immunoassay

Microtitre plates (Costar, Cambridge, Massachusetts, U.S.A.) were coated for 16 h at 4°C with 100 µl/well of purified Zn-α_2-gp 500 µg/l in phosphate-buffered saline (PBS). After blocking the remaining free adsorption sites with 10% bovine serum albumin in PBS for 1 h at 37°C, the wells were washed six times with 0.1% Tween-20 in PBS. For the assay, antigen solution or tumour cytosol at the suitable dilution in blocking solution (50 µl) and purified IgG from antiserum against Zn-α_2-gp (50 µl diluted 1/1000) were added. After 1 h at room temperature the wells were washed as above and incubated for 1 h with 100 µl of peroxidase-labelled goat antibodies against rabbit IgG (Sigma, St. Louis, Missouri, U.S.A.) diluted 1/1000 in blocking solution. Finally, the plates were washed and the amount of peroxidase fixed to each well was quantified by adding 100 µl of citrate phosphate buffer, pH 5.5 containing H_2O_2 as enzyme substrate and *O*-phenylene-diamine (Sigma) as hydrogen donor; after 10 min at room temperature in the dark, the reaction was stopped by addition of 100 µl of 3 N H_2SO_4. Absorbance was measured at 492 nm in an automatic spectrophotometer (Multiskan; Titertek, MC, Ayrshire, U.K.).

Other biochemical assays

Protein concentration in breast tumour cytosol was determined by the Bradford technique [12] using bovine gamma globulin as standard. Oestrogen receptor (ER) analysis was performed using a commercially available kit from Du Pont Company (Billerica, Massachusetts, U.S.A.). Cathepsin D and pS2 protein were determined in breast tumour cytosols by immunoradiometric assays using kits from CIS Bio-International (Gif-sur-Yvette, France).

Statistical analysis

For analysis of data, patients or breast tumour cytosols were subdivided into two groups based on different clinical or biochemical parameters. Mann–Whitney U-test was performed to assess differences between the different groups. Relationships between Zn-α_2-gp levels and tumour histological grade were evaluated with F-tests (ANOVA) followed by the Newman–Keuls test. Associations between variables were assessed by the Spearman rank correlation test. A multivariate polychotomous logistic regression model (BMDPRR) was used to evaluate simultaneously the influence of more than one factor on the levels of Zn-α_2-gp in breast tumour cytosols. Significance was established at the $P < 0.05$ level. Data were expressed as mean ± S.E.M.

RESULTS

Distribution of Zn-α_2-gp in breast tumour cytosols and correlation with clinical parameters

The clinical characteristics of the 104 women included in this study are shown in Table 1. Zn-α_2-gp concentration in the corresponding breast tumour cytosols was measured by an enzyme-linked immunoassay in which free Zn-α_2-gp present in

tissue specimens competes with purified Zn-α_2-gp fixed to a solid phase for binding to a limited amount of antibody. The antiserum specificity was confirmed by western blot of serum and breast cyst fluid. As indicated in Fig. 1, Zn-α_2-gp concentration varied between 0 and 23.5 μg/mg cytosol protein with an average value of 2.4 μg/mg. There was no correlation between the absolute values of Zn-α_2-gp and total cytosolic protein indicating that differences were not due to variations in the amount of protein extracted from the different tumour tissues.

In order to establish the possible correlation between Zn-α_2-gp and disease status, clinical staging was determined in the 104 women included in the study and the concentration of Zn-α_2-gp was measured in each of the different groups. Fig. 1 shows the distribution of Zn-α_2-gp in relation to tumour size. The mean concentration of Zn-α_2-gp was lower in T_1 tumours than in those belonging to the other groups. However, these differences were not significant. Similarly, although Zn-α_2-gp levels were lower in axillary node-positive patients than in node-negative women (Fig. 2), and in premenopausal patients than in postmenopausal women (Fig. 3), these differences were not statistically significant.

Finally, we examined the possible relationship between Zn-α_2-gp and histological grade of tumours. As shown in Fig. 4, the average content of Zn-α_2-gp was higher in well-differentiated tumours than in those moderately or poorly differentiated. Statistical analysis revealed that these differences were significant (grade I vs. grade II: $P < 0.05$; grade I vs. grade III: $P < 0.01$). Multivariate analysis adjusted for potential confounders confirmed a significant association between Zn-α_2-gp levels in breast tumour cytosols and differentiation grade of tumours.

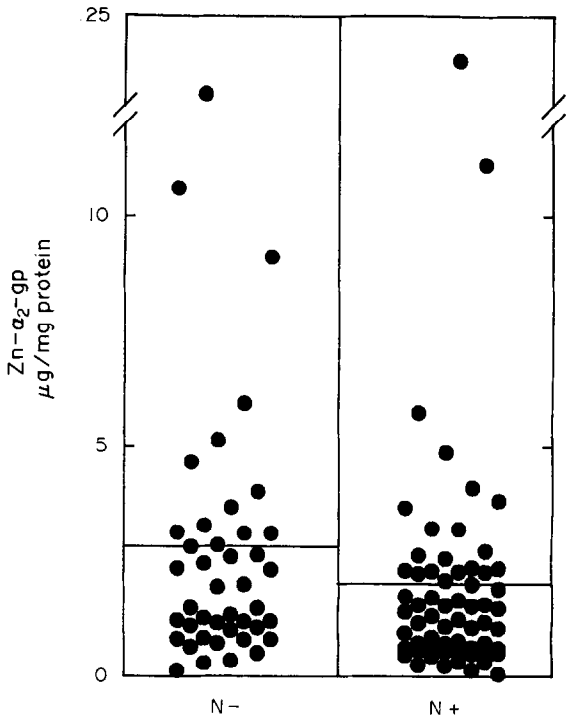

Fig. 2. Zn-α_2-gp concentration in breast tumour cytosols in relation to axillary node (N) status. Mean values in each group are indicated by a horizontal bar (N−: 2.8 ± 0.6 μg/mg; N+: 2.1 ± 0.4 μg/mg).

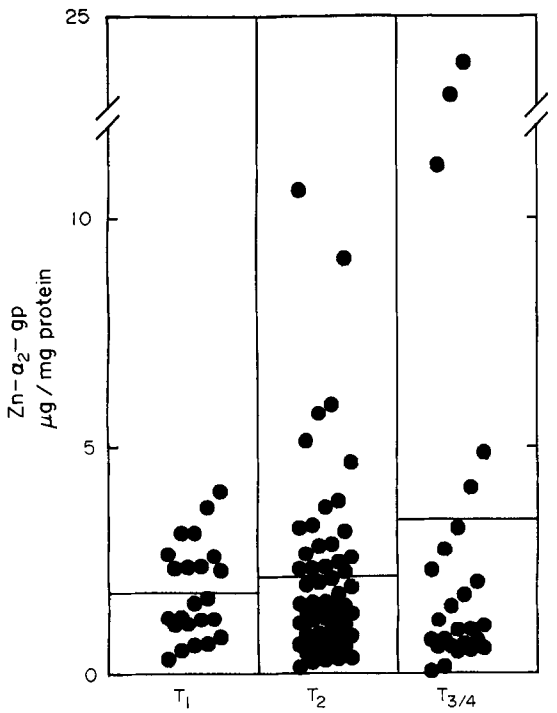

Fig. 1. Zn-α_2-gp concentration in breast tumour cytosols in relation to tumour size. Mean values in each group are indicated by a horizontal bar (T_1: 1.8 ± 0.2 μg/mg; T_2: 2.1 ± 0.3 μg/mg; $T_{3/4}$: 3.4 ± 1.2 μg/mg).

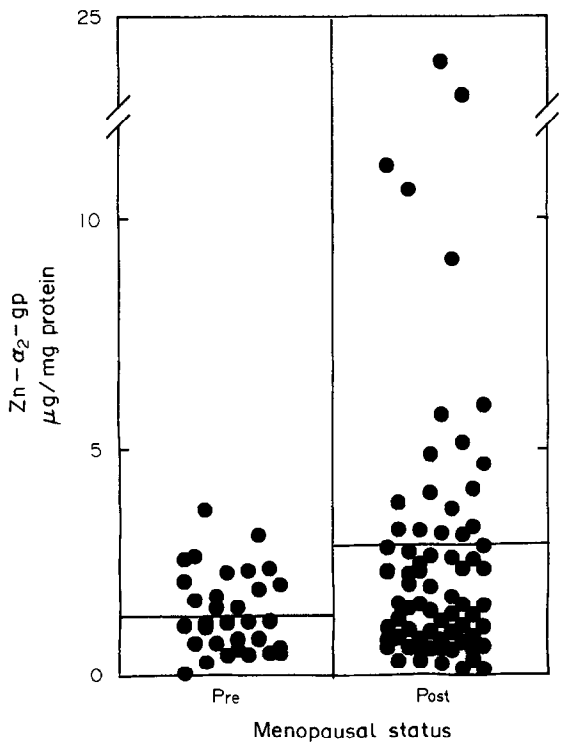

Fig. 3. Zn-α_2-gp concentration in breast tumour cytosols in relation to menopausal status. Mean values in each group are indicated by a horizontal bar (premenopausal: 1.4 ± 0.1 μg/mg; postmenopausal 2.8 ± 0.5 μg/mg).

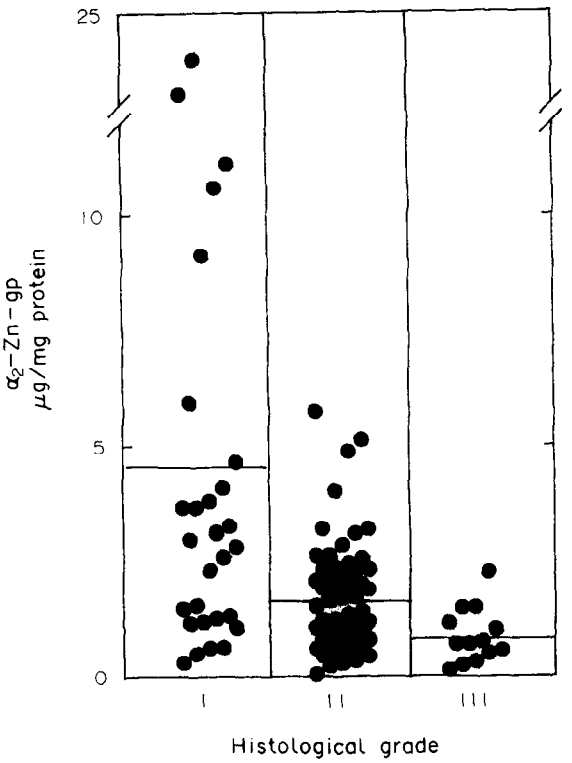

Fig. 4. Zn-α_2-gp concentration in breast tumour cytosols in relation to histological grade. Mean values in each group are indicated by a horizontal bar (grade I: 4.6 ± 1.1 μg/mg; II: 1.8 ± 0.2 μg/mg; III: 0.9 ± 0.2 μg/mg).

Correlation between Zn-α_2-gp and other biochemical markers

Patients were subdivided into two groups according to their status of ER, cathepsin D and pS2 protein. Tumours were considered ER positive (ER+) if they contained more than 10 fmol/mg of cytosolic protein. The distribution of Zn-α_2-gp concentrations in relation to the steroid receptor status is shown in Table 2. The mean concentration of Zn-α_2-gp in ER+ tumours was higher than in those ER negative (ER−). However, these differences were not statistically significant.

In relation to the cathepsin D levels, the value of 40 pmol/mg protein was chosen as the corresponding cut-off, as indicated by Granata *et al.* [13]. As Table 2 shows, the mean concentration of Zn-α_2-gp was slightly higher in those tumours whose cathepsin D levels were below 40 pmol/mg, although again these differences were not statistically significant.

Table 2. Zn-α_2-glycoprotein concentrations in tumour cytosols classified in groups according to other biochemical parameters

Biochemical parameter	No.	Mean ± S.E.M.*	Range*
ER+	74	2.6 ± 0.4	0.1–23.5
ER−	30	2.3 ± 0.5	0–11.1
Cathepsin D < 40 pmol/mg	65	2.6 ± 0.4	0.1–22.3
Cathepsin D > 40 pmol/mg	39	2.0 ± 0.6	0–23.5
pS2 protein > 11 ng/mg	37	3.1 ± 0.8	0–23.5
pS2 protein < 11 ng/mg	67	2.0 ± 0.2	0.2–11.1

*Values are expressed in μg/mg of total protein.
ER = oestrogen receptor.

Finally, we examined the possible correlation between Zn-α_2-gp and pS2 protein. In this case, and according to Foekens *et al.* [14], breast tumours were considered as pS2 positive if they contained more than 11 ng/mg protein. As indicated in Table 2, high concentrations of Zn-α_2-gp were found more frequently in the subclass of pS2-positive tumours, however, as in the other biochemical parameters considered above, the observed differences were not significant at the $P < 0.05$ level.

Statistical analyses of potential associations between Zn-α_2-gp and ER, cathepsin D or pS2 were performed by determination of the corresponding Spearman rank correlation coefficients. The results obtained indicated that there was no overall correlation between Zn-α_2-gp and any of the other markers studied. Multivariate analysis confirmed the absence of association between Zn-α_2-gp levels in breast tumour cytosols and all biochemical markers analysed in the same cytosols.

DISCUSSION

An important objective in breast cancer research is the finding of biochemical markers for establishing prognosis and monitoring the response to hormonal therapy. In this regard, proteins present in cyst fluid from women with cystic breast disease have received considerable attention as possible markers of certain subtypes of breast cancer. These studies have been mainly focused on two proteins denominated GCDFP-24 (recently identified as apolipoprotein D) and GCDFP-15. However, at present no data are available for the breast tumour cytosol concentrations of Zn-α_2-gp, one of the major components of breast cyst fluids. In this work we have determined Zn-α_2-gp levels in 104 breast tumour biopsies by using a highly sensitive enzyme-linked immunoassay. The concentration of Zn-α_2-gp showed a wide variability, ranging from non-detectable levels to high values representing about 2% of the tumour's soluble protein. The percentage of tumours producing appreciable amounts of Zn-α_2-gp (arbitrarily chosen as higher than 1 μg/mg of cytosol protein) was similar to the one observed by immunohistochemical techniques [8, 9]. According to these data, we can conclude that a significant percentage of breast carcinomas retain the ability to produce this protein during malignant transformation.

Due to the short follow-up of patients whose tumour Zn-α_2-gp levels were measured, we tried to evaluate the prognostic value of Zn-α_2-gp by studying the possible correlations with other well-defined prognostic factors in breast cancer. These analyses revealed the absence of significant associations between Zn-α_2-gp levels and different patient and tumour characteristics including menopausal status, tumour size or axillary node status. Similarly, we did not detect significant relationships between Zn-α_2-gp and different biochemical markers, such as ER status, cathepsin D or pS2 protein, which are widely used in breast cancer management.

By contrast, Zn-α_2-gp was significantly associated with histological grade of tumours. Thus, higher levels of Zn-α_2-gp were found in histopathologically well-differentiated tumours than in those moderately or poorly differentiated. According to this finding, it is tempting to speculate that Zn-α_2-gp may be a marker of good prognosis in breast cancer. Several lines of evidence from this and other laboratories provide additional support to this proposal.

Thus, northern blot analysis performed with a cDNA coding for Zn-α_2-gp has shown that this gene is expressed at higher levels in benign than in malignant tumours [5]. In addition, this glycoprotein is a major component in a subtype of breast

secretions usually associated with normal or benign conditions [7]. Finally, results obtained by Silva *et al.* [15] in relation to the breast tumour cytosol levels of the other major cyst fluid proteins (apolipoprotein D and GCDFP-15), have revealed the same trend to that found in this work for Zn-α_2-gp. Both proteins have mean values that decrease consecutively from the highest levels detected in benign tissues and grade I carcinomas to the lowest values observed in grade III carcinomas. Similarly, studies from Lea *et al.* [16] and Soreide *et al.* [17] have indicated that the presence of progesterone-binding cyst protein (apolipoprotein D) in a breast tumour may be a marker of high grade of differentiation and low metastatic potential.

Taken together, these results point to the existence of a subset of breast tumours which possess the degree of differentiation required to produce appreciable amounts of proteins such as Zn-α_2-gp, apolipoprotein D and GCDFP-15, which are synthesised and secreted by normal breast tissue [5, 7]. Taking into account that the differentiation grade is considered of prognostic significance, we propose that production of Zn-α_2-gp and other cyst proteins by breast tumours may be a reliable marker of good prognosis in breast cancer.

It is noteworthy that this proposal does not agree with immunohistochemical data from Bundred *et al.* [8] indicating that apocrine differentiation assessed by Zn-α_2-gp staining in breast tumours appears to be of poor prognostic significance. It can be argued that methodological aspects could contribute to explaining these differences, since our results and those obtained for the other cyst proteins by different groups were derived from quantitative measurements on tumour cytosols, while results from Bundred *et al.* were based on immunohistochemical analyses. However, recent data from Hurlimann and VanMelle [9], using the same immunohistochemical procedure, seem to rule out this explanation. Thus, according to their results, Zn-α_2-gp was detected in lesions with a favourable evolution. Therefore, alternative explanations including the use of antibodies of different affinity, variations in the populations studied or existence of unknown factors of potential influence in Zn-α_2-gp production by breast tumours should be required to clarify this point.

In addition to its potential value as a prognostic factor in breast cancer, determination of Zn-α_2-gp levels in breast tumours could be of importance in identifying a subset of tumours with a specific pattern of hormonal regulation. In relation to this, it is remarkable that Zn-α_2-gp is one of the rare proteins which is induced by androgens in breast cancer cells [18]. Therefore, the presence of appreciable amounts of Zn-α_2-gp in breast tumours might point to a role for androgens in the development of this specific category of breast carcinomas. It is also worthwhile mentioning that several studies performed by Secreto *et al.* [19, 20] have provided evidence for the role of androgenic hormones in the development of a subgroup of breast cancer. According to this, identification of androgen-responsive breast tumours could contribute to selection of a subgroup of patients requiring endocrine therapy with anti-androgens instead of the more widely used anti-oestrogen treatment.

Further studies and clinical follow-up of women whose breast tumours were analysed in this work are in progress to establish the precise role of Zn-α_2-gp as a biochemical marker of good prognosis in breast cancer, as well as to determine its value as an indicator of androgen-responsive tumours.

1. Burgi W, Schmid K. Preparation and properties of Zn-α_2-glycoprotein of normal human plasma. *J Biol Chem* 1961, **236**, 1066–1074.
2. Kamboh MI, Ferrell RE. Genetic studies of low-abundance human plasma proteins. I. Microheterogeneity of zinc-α_2-glycoprotein in biological fluids. *Biochem Genet* 1986, **24**, 849–857.
3. Haagensen DE, Mazoujian G. Biochemistry and immunohistochemistry of fluid proteins of the breast in gross cystic disase. In Haagensen CD, ed. *Diseases of the Breast*, 3rd edition. Philadelphia, W.B. Saunders Co., 1986, 474–500.
4. Balbín M, Freije JMP, Fueyo A, Sánchez LM, López-Otín C. Apolipoprotein D is the major protein component in cyst fluid from women with human brest gross cystic disease. *Biochem J* 1990, **271**, 803–807.
5. Freije JP, Fueyo A, Uría J, López-Otín C. Human Zn-α_2-glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissues. *FEBS Lett* 1991, **290**, 247–249.
6. Bundred NJ, Miller WR, Walker RA. An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, zinc alpha$_2$ glycoprotein. *Histopathology* 1987, **11**, 603–610.
7. Sánchez LM, Vizoso F, Díez-Itza I, López-Otín C. Identification of the major protein components of breast fluid from women with benign and malignant breast diseases. *Cancer Res* 1992, **52**, 95–100.
8. Bundred NJ, Walker RA, Everington D, White GK, Stewart HJ, Miller WR. Is apocrine differentiation in breast carcinoma of prognostic significance? *Br J Cancer* 1990, **62**, 113–117.
9. Hurlimann J, van Melle G. Prognostic value of serum proteins synthesized by breast carcinoma cells. *Am J Clin Pathol* 1991, **95**, 835–843.
10. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. *Br J Cancer* 1957, **11**, 359–377.
11. Vaitukaitis JL. Production of antisera with small doses of immunogen: multiple intradermal injections. *Methods Enzymol* 1981, **73**, 46–52.
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
13. Granata G, Coradini D, Cappelletti V, DiFronzo G. Prognostic relevance of cathepsin D versus oestrogen receptors in node negative breast cancers. *Eur J Cancer* 1991, **27**, 970–972.
14. Foekens JA, Rio MC, Seguin P, *et al.* Prediction of relapse and survival in breast cancer patients by pS2 protein status. *Cancer Res* 1990, **50**, 3832–3837.
15. Silva JS, Cox CE, Wells SA, *et al.* Biochemical correlates of morphologic differentiation in human breast cancer. *Surgery* 1982, **92**, 443–449.
16. Lea OA, Kvinnsland S, Thorsen T. Progesterone-binding cyst protein in human breast tumor cytosol. *Cancer Res* 1987, **47**, 6189–6192.
17. Soreide JA, Lea OA, Anda O, Skarstein A, Varhaug JE, Kvinnsland S. Progesterone-binding cyst protein (PBCP) in operable breast cancer: correlations with prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Anticancer Res* 1991, **11**, 601–606.
18. Chalbos D, Haagensen D, Parish T, Rochefort H. Identification and androgen regulation of two proteins released by $T_{47}D$ human breast cancer cells. *Cancer Res* 1987, **47**, 2787–2792.
19. Secreto G, Toniolo P, Pisani P, *et al.* Androgens and breast cancer in premenopausal women. *Cancer Res* 1989, **49**, 471–476.
20. Secreto G, Toniolo P, Berrino F, *et al.* Serum and urinary androgens and risk of breast cancer in postmenopausal women. *Cancer Res* 1991, **51**, 2572–2576.

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