$$E(Y_{itv(i)} - Y_{ijv(i)}) = \mu+\alpha_i+\tau_t+\alpha\tau_{it}+\pi_{v(i)}+\epsilon_{itv} - (\mu+\alpha_i+\tau_j+\alpha\tau_{ij}+\pi_{v(i)}+\epsilon_{itv}) = (\tau_t-\tau_j)+(\alpha\tau_{it}-\alpha\tau_{ij}). \quad (2)$$

Hence, with the model as specified in (1) we were able to obtain unbiased estimates of the contrasts with the non-missing data. This is illustrated in Fig. 3. According to the terminology of Little and Rubin [22] all missing data mechanisms are ignorable for the MANOVA model specified in (1).

In Fig. 3 we have graphed the distribution of the scale scores in the two groups at points in time $t = 1$ and $t =$ end. At $t = 1$, the distribution of the scale scores in group I and II were the same. In group I, the scale scores remained constant over time. In group II, the scores increased linearly. Suppose that there was a threshold C such that if the scale score was higher than C, the patient dropped out. Hence, the major part of the distribution group II at $t =$ end would not be observed. If one would compare the means of the observed scales in both groups at $t =$ end, the contrast would be underestimated as well as the rate of the change (dotted line). However, as the rate of the change was assumed to be equal for all patients in one group, the rate of change could be estimated without bias by means of time contrasts within each of the two groups.

Eur J Cancer, Vol. 27, No. 5, pp. 549–552, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Zinc Alpha-2 Glycoprotein Levels in Serum and Breast Fluids: a Potential Marker of Apocrine Activity

N.J. Bundred, W.N. Scott, S.J. Davies, W.R. Miller and R.E. Mansel

Zinc alpha-2 glycoprotein (ZnGP) was measured in human breast microcysts, breast secretions, breast cyst fluid and serum. Detectable amounts of ZnGP were found in all fluids but the highest levels were found in microcysts. Apocrine macrocysts had a higher ZnGP level than flattened macrocysts. In both cysts and secretions levels of ZnGP correlated with those of dehydroepiandrosterone sulphate. Levels were significantly higher in cyst fluids from women who developed further cysts during follow-up compared with those in fluid from women who did not. Concentrations of ZnGP in serum from breast cancer patients were significantly higher than controls but not women with breast cysts. Women with node positive breast cancer had higher serum levels compared with those in node negative patients. Women with more advanced breast cancer had higher serum ZnGP levels than those with earlier disease. ZnGP is a serum and breast marker of apocrine activity and may prove to be a useful prognostic marker in breast cancer.
Eur J Cancer, Vol. 27, No. 5, pp. 549–552, 1991

INTRODUCTION

ZINC ALPHA-2 GLYCOPROTEIN (ZnGP) is a 44 000 Dalton molecular weight protein, first isolated and characterised in 1961 by Burgi and Schmid [1]. It is one of the major component proteins of breast cyst fluid [2] and forms 36% of the total protein content of apocrine sweat [3]. Although a recent immunohistochemical study demonstrated that ZnGP is located in apocrine metaplastic epithelium and apocrine glands [4], little is known about the factors affecting the levels of ZnGP in serum and breast fluids.

Women who have had multiple breast cysts aspirated have an increased risk of subsequent breast cancer [5, 6] and Dixon *et al.* have suggested that it is apocrine breast cysts (defined by a low sodium:potassium ratio) which most often recur [7, 8] and give the highest risk of subsequent breast cancer [9].

The aims of this study were to quantitate ZnGP in breast tissue and to determine if ZnGP levels in serum could be used as a marker of breast disease.

PATIENTS, MATERIALS AND METHODS

130 breast cysts were obtained by needle aspiration from 113 women. In 99 women a single cyst was aspirated and in 14 women multiple cysts were drained. 102 women were premenopausal and 11 were postmenopausal (last menstrual period more than 2 years ago). Sodium and potassium levels in cyst fluid were measured by flame photometry [7] and dehydroepiandrosterone sulphate (DHAS) was measured by radioimmunoassay [7] in 66 cysts. 95 women with breast cysts have been followed up for at least one year and the number of cysts they have developed has been recorded.

23 microcysts were dissected from breast biopsy specimens prior to fixation using a dissecting microscope. Fluid within the microcysts was collected after puncture into calibrated capillary tubes. Breast secretions were obtained from 28 women by a

Correspondence to N.J. Bundred, Department of Surgery, Queen Elizabeth Medical Centre, Birmingham B15 2TH, U.K.
N.J. Bundred is at the Department of Surgery, University of Wales College of Medicine, Cardiff; S.J. Davies is at the Department of Biochemistry, East Glamorgan Hospital, Pontypridd; W.N. Scott and W.R. Miller are at the Royal Infirmary, Edinburgh; R.E. Mansel is at the University Hospital of Manchester, Nell Lane, Manchester, U.K.
Received 19 Nov. 1990; accepted 22 Feb. 1991.

Table 1. Levels (mg/dl) of ZnGP in serum and breast fluids

	Microcysts	Secretions	Macrocysts	Serum
Range	298–1750	30–400	1–135	2.8–7.6
Mean (S.E.)	566 (366)	139 (99)	68 (58)	5.1 (1.1)

standard technique utilising a Sartorius suction cup [10]. 15 women produced a single sample of secretion, 7 produced bilateral breast secretions, 4 produced breast secretions on two separate occasions and 2 produced secretions from one or other breast on 3 occasions.

Serum was collected from a total of 207 individuals. These comprised 30 female and 18 male "normal" controls, 21 women and 6 men with Hidradenitis suppurativa and 30 women with breast nodularity. 69 women with breast cancer and 33 women who had undergone breast cyst aspiration also had serum collected and were followed up for a minimum of 6 months.

Radial immunodiffusion

Rabbit polyclonal antibody to ZnGP and ZnGP antigen was purchased from Hoechst Behring UK (London). ZnGP levels were measured by radial immunodiffusion using barbitone agar plates. The plates were read 24 h after plating with the test samples, using a TG calibrating magnifying viewer (Transidyne General Corporation, Ann Arbor, Michigan) which enables precision measurements of up to 0.1 mm. The lower limits of ZnGP which could be detected was 1.5 mg/dl. Interassay variation was assessed by measuring cyst and serum samples from 16 women on more than one occasion on different plates. A paired t test showed no significant deviation from zero when the samples from each woman were compared and the standard error of difference was less than 2% of the mean.

RESULTS

The range and mean levels of ZnGP in breast secretion, microcysts, aspirated breast macrocysts and serum are shown in Table 1 and Fig. 1. ZnGP levels in microcysts were significantly higher than in the other three fluids ($P < 0.001$). Breast secretion levels of ZnGP were significantly higher than macrocyst or serum ZnGP levels ($P < 0.001$) (Fig. 1). Levels of ZnGP in breast macrocysts were highest in apocrine cysts (defined as those cysts with a sodium:potassium ratio > 3) compared to flattened cysts (with a sodium:potassium ratio equal to or less than 3) ($P < 0.001$, Mann–Whitney U test). ZnGP levels in 66 breast macrocysts correlated significantly with dehydroepiandrosterone sulphate levels ($r = 0.62$, $P < 0.001$). The levels of ZnGP in breast secretions ranged from 29.8–400 mg/dl and again correlated with those of DHAS ($r = 0.81$, $P < 0.001$, Fig. 2).

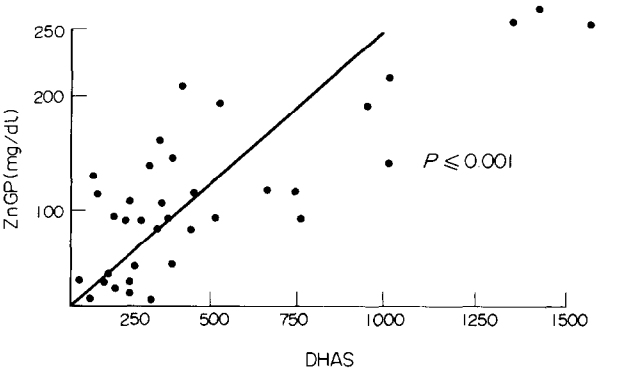

Fig. 2. Correlation between DHAS and ZnGP in nipple secretions. DHAS and ZnGP levels in breast secretions are closely related.

Serum levels of ZnGP in normal individuals and patients with either Hidradenitis suppurativa, benign breast disease, breast cysts or breast cancer are shown in Fig. 3. Concentrations of ZnGP in serum from normal women were not related to age or menopausal status (data not shown). There was no difference in the mean serum levels of ZnGP from "normal" men and women, patients with Hidradenitis suppurativa and patients with benign breast disease. However women who had undergone breast cyst aspiration had significantly higher serum ZnGP levels (range 3.5–7.6 mg/dl mean 5.1) than the 105 "control" patients who were either normal or who had Hidradenitis suppurativa or breast nodularity (range 2.8–7.7 mg/dl mean 4.4 mg/dl)

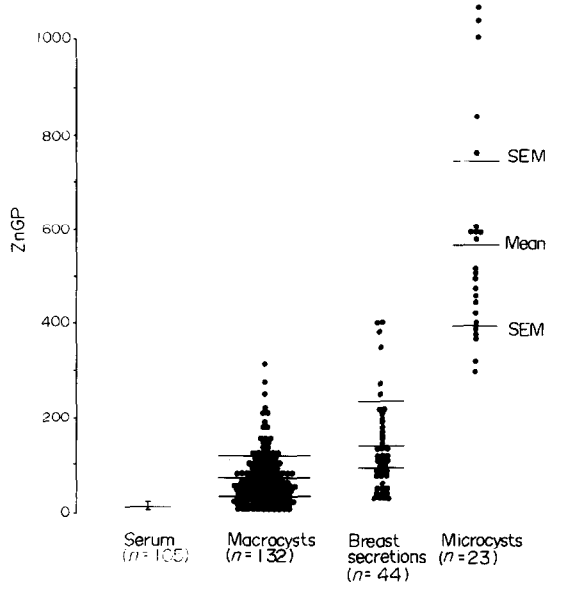

Fig. 1. ZnGP levels in serum and breast fluids. ZnGP levels are highest in microcysts, compared to breast secretions, compared to macrocysts and serum ($P < 0.001$).

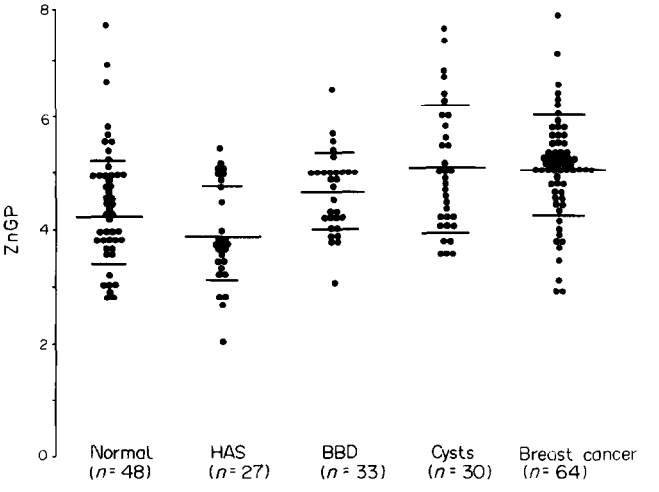

Fig. 3. ZnGP levels in the serum of normal women and men, patients with hidradenitis suppurativa (HAS), benign breast disease (BBD), breast cysts and breast cancer. Levels are significantly higher in women with breast cysts or breast cancer compared to normal women and women with BBD.

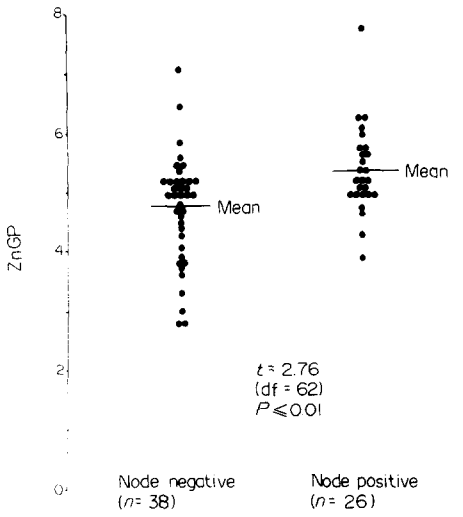

Fig. 4. Serum ZnGP levels in breast cancer patients with early breast cancer. Patients who had one or more positive axillary lymph node had significantly higher ZnGP levels.

(t = 3.37, df 136, $P < 0.001$). Levels of ZnGP in the serum of the 22 women who did not develop any further cysts at 6 months follow-up (range 3.5–6.3 mg/dl, mean 4.7 mg/dl) were significantly lower than the levels in the 11 females who developed new cysts (range 4.2–7.6, mean 5.7 mg/dl). There was no significant difference in serum ZnGP levels between women who had high potassium cysts and those with high sodium cysts. However, the cyst fluid ZnGP level was significantly higher in women who developed further cysts compared to those who did not (P = 0.013, Mann-Whitney U test).

Levels of serum ZnGP ranged from 2.8–8.0 mg/dl, (mean 5.1) in women with operable breast cancer these being higher than levels in normal women (t = 4.2, $P < 0.001$) but not significantly different from women who had undergone cyst aspiration (Fig. 3). The mean serum ZnGP level of women with advanced breast cancer (T4 and T3 tumours) was significantly higher than that of women with early breast cancer (T1 and T2 tumours) (t = 2.31, $P < 0.02$). Amongst the 64 women who underwent surgical treatment for their breast cancer the 26 women who had positive axillary nodes (mean 5.4 mg/dl) had significantly higher serum ZnGP levels compared to the 38 women with negative axillary nodes (mean 4.7 mg/dl) (t = 2.76, $P < 0.01$, Fig. 4).

DISCUSSION

ZnGP is a major component protein in breast cyst fluid [2] and apocrine sweat [3]. Its distribution within the breast has been determined by immunohistochemical techniques; few cells stain in normal breast tissue, whereas apocrine glands in the axilla and apocrine epithelium within the breast stain uniformly [4]. The findings in the present study that (1) ZnGP levels in cyst fluid and breast secretion parallel levels of another known marker of apocrine activity (dehydroepiandrosterone sulphate) in the same fluids, (2) amongst breast fluids, those derived from microcysts which are reported to be lined by apocrine epithelium [11] had the highest ZnGP levels and (3) apocrine macrocysts (defined by a low sodium:potassium ratio) had higher ZnGP levels than flattened macrocysts, provide further confirmatory evidence of the correlation between ZnGP levels and apocrine activity.

Levels of ZnGP in both breast fluids and serum in women with and without breast disease have also been examined to determine the potential clinical relevance. Women with apocrine cysts are thought to be more prone to new cyst formation [8]. It is pertinent that women with high ZnGP levels in cyst fluid or serum were more likely to need further cysts aspirated. Nevertheless, the findings that levels of ZnGP in sodium cysts were still on average 10 times that of serum implies that all cysts have an apocrine derivation and retain some apocrine characteristics which is in keeping with the theory that both cyst types originate from a single "apocrine" microcyst population [11].

The levels of serum ZnGP found in this study are similar to those reported by Becker *et al.* in 1969 [12]. The higher levels of ZnGP in the serum of women with breast cysts compared to women with a range of other benign breast conditions provides evidence of a difference in apocrine activity between these women. Since the elevated levels of another cyst protein (Gross cystic disease fluid protein 15) in plasma have been claimed to be associated with a five times increased risk of subsequently developing breast cancer [13] we intend to follow-up the women with high serum ZnGP levels to determine if they have a similar increased risk.

In a recent immunohistochemical study of ZnGP staining in breast carcinoma, only 55% of tumours showed staining [14] and even allowing for tumour heterogeneity, it would be surprising if serum levels of ZnGP were raised in all women with breast cancer. Nevertheless, levels of ZnGP were significantly higher in women with breast cancer compared to women with "normal breast" or benign breast disease and the highest levels were seen in women with positive axillary nodes or advanced disease suggesting ZnGP may be a marker of disease "load" in breast cancer.

We have already reported that tumours with ZnGP staining had a shorter disease free interval and poorer survival compared to women whose tumours showed no staining [14] and the finding of a raised ZnGP in the serum of women with breast cancer implies that their tumours have apocrine characteristics. Evidence is accumulating that apocrine tumours do not respond to anti-oestrogen hormone therapy [15] and the presence of high serum ZnGP levels is being examined in women undergoing hormonal manipulation to determine if such women are less likely to have hormonal responses. In conclusion, ZnGP is a marker of apocrine activity in the breast and may prove useful as a prognostic marker in benign and malignant breast disease.

1. Burgi W, Schmid K. Preparation and properties of zinc alpha-2 glycoprotein of normal human plasma. *J Biol Chem* 1961, **236**, 1066–1074.
2. Haagensen DG, Mazoujian G, Dilley WG, Pedersen CE, Kister SJ, Wells SA. Breast gross cystic disease fluid analysis 1. Isolation and radioimmunoassay for a major component protein. *J Natl Cancer Inst* 1979, **62**, 239–247.
3. Jirka M. An alpha-2-globulin component present in sweat, saliva, tears, human milk, colostrum and Cerumen. *FEBS Lett* 1968, **1**, 77–80.
4. Bundred NJ, Miller WR, Walker RA. Immunohistochemistry of a breast cyst protein zinc alpha-2-glycoprotein. *Histopathology* 1987, **11**, 603–610.
5. Roberts MM, Jones V, Elton RA, Fortt RW, Williams S, Gravelle IH. Risk of breast cancer in women with history of benign disease of the breast. *Br Med J* 1984, **288**, 275–278.
6. Haagensen CD. *Diseases of the Breast*, 3rd ed. London, WB Saunders, 1986, 47.
7. Miller WR, Dixon JM, Scott WN, Forrest APM. Classification of human breast cysts according to electrolyte and androgen conjugate composition. *Clin Oncol* 1983, **9**, 227–232.

8. Dixon JM, Scott WN, Miller WR. Natural history of cystic disease. The importance of cyst type. *Br J Surg* 1985, **70**, 190–192.
9. Dixon JM, Lumsden AB, Miller WR. The relationship of cyst type to risk factors for breast cancer and the subsequent development of breast cancer in patients with breast cystic disease. *Eur J Cancer Clin Oncol* 1985, **21**, 1047–1050.
10. Sartorius O. Breast fluid cells help in early cancer detection. *J Am Med Assoc* 1973, **224**, 823.
11. Dixon JM, Scott WN, Miller WR. An analysis of the content and morphology of human breast microcysts. *Eur J Surg Oncol* 1985, **11**, 151–154.
12. Becker W, Schwick HG, Stomko K. Immunologic determinations of proteins found in low concentrations in human serum. *Clin Chem* 1969, **15**, 649–660.
13. Haagensen DG Jr, Mazoujian G, Holder WR, Kister SJ, Wells SA Jr. Evaluation of a breast cyst fluid protein detectable in the plasma of breast carcinoma patients. *Ann Surg* 1977, **185**, 279–285.
14. Bundred NJ, Walker RA, Everington D, *et al.* Is apocrine differentiation in breast carcinoma of prognostic significance? *Br J Cancer* 1990, **62**, 113–117.
15. Bundred NJ, Stewart HJ, Sturgeon C, *et al.* Apocrine differentiation: relationship to receptor status, prognosis and hormonal response in breast cancer. *Eur J Cancer*, 1990 **26**, 1145–1147.

Eur J Cancer, Vol. 27, No. 5, pp. 552–556, 1991.
Printed in Great Britain
0277–5379/91 $3.00 + 0.00

Specialist Interest Articles

Aggressive Chemotherapy for Acute Leukaemia Frequently Causes Intestinal Protein Leakage

Simon Daenen, Frits A.J. Muskiet, Jan Marrink and M. Ruud Halie

Cytostatic drugs are known to produce disturbances in intestinal absorption of carbohydrates. To further explore the gastrointestinal (GI) toxicity of cytostatic therapy, 37 patients with acute leukaemia were investigated during and/or after remission induction courses by the use of the differential sugar absorption test (DSAT) and the intestinal clearance of alpha-1-antitrypsin (Cl_{AAT}). The ratio of the lactulose to the mannitol excretion in the urine was found abnormal in 44% of the tests. The Cl_{AAT} was increased in 74% of tests. The test results differed considerably from patient to patient and depended on the chemotherapy course; correlation between the tests was low, probably indicating that unrelated pathophysiological processes were measured. After haematological regeneration, abnormal test results normalised. It is concluded that aggressive chemotherapy not only causes a reduction in the absorption of sugars, but commonly also protein leakage. These GI side-effects are reversible, and the application of both tests in combination provides a practical and reproducible method for investigation of GI toxicity in patients treated with cytostatic drugs.
Eur J Cancer, Vol. 27, No. 5, pp. 552–556, 1991

INTRODUCTION

TREATMENT WITH cytostatic drugs can lead to moderate to severe gastrointestinal (GI) side-effects [1–3]. Some of these, such as nausea, vomiting and diarrhoea, are easily recognised and the clinical situation mostly suggests a direct relation with the cytostatic therapy. Impairment of the intestinal mucosa is less well explored, especially when aggressive anti-leukaemia type chemotherapy is administered. Invasive methods of investigation are not applicable when the patient is deeply pancytopenic. Breath hydrogen tests, as used by Hyams *et al.* [4], are of limited value when antibiotics are given prophylactically, or otherwise. Other investigators [5] used the xylose absorption test and found a trend towards an increasing incidence of life-threatening infections when xylose absorption was decreased. Parrilli *et al.* [6] investigated early changes in intestinal permeability to lactulose; they concluded that this test was more sensitive than other absorption tests, or even biopsy.

We studied the differential absorption of sugars (differential sugar absorption test, DSAT) and the intestinal clearance of alpha-1-antitrypsin (Cl_{AAT}) in 37 evaluable patients treated with aggressive chemotherapy for acute leukaemia. Until now, DSAT is used mainly for diagnosis and follow up of mucosal damage in coeliac disease [7–9]. Abnormal DSAT is based on a reduction of the active absorption via the aqueous pores of a monosaccharide, mostly mannitol [10], and/or an increase of the permeability via an intercellular pathway of a disaccharide, mostly lactulose. When the number of cells and the absorptive surface are reduced by the cytostatic drugs, absorption of mannitol is expected to decrease; when the tight junctions of the cells in the mucosal lining are damaged or subendothelial structures lie bare altogether, passive diffusion of lactulose can be expected to increase.

The anti-enzyme AAT is normally secreted in small amounts in the intestinal lumen. Since it is not degraded by intestinal

Correspondence to S. Daenen.
S. Daenen and M.R. Halie are at the Division of Haematology, Department of Internal Medicine; F.A.J. Muskiet is at the Central Laboratory for Clinical Chemistry; and J. Marrink is at the Laboratory for Immunochemistry, University and University Hospital of Groningen, Oostersingel 57, 9713 EZ Groningen, the Netherlands.
Revised 29 Nov. 1990; accepted 28 Jan. 1991.