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Assessment of Four Monoclonal Antibodies as Serum Markers in Breast Cancer

J.F.R. Robertson, D. Pearson, M.R. Price, C. Selby, R.A. Badley, J. Pearson, R.W. Blamey and A. Howell

Four monoclonal-antibody-defined serum markers (CA15-3, HMFG1, HMFG2 and NCRC-11) were examined in five groups of subjects: controls, benign breast disease and stage I/II, stage III and metastatic breast cancer. None of the markers were significantly elevated in primary breast cancer (i.e. stage I/II or stage III) compared with controls or patients with benign breast disease. These markers therefore have no role in screening or in the diagnosis of primary breast cancer. CA15-3, HMFG2 and NCRC-11 were significantly increased in the patients with metastatic breast cancer ($P < 0.001$), indicating a potential use in the diagnosis of symptomatic metastases. In patients with metastases, sequential changes in CA15-3 correlated significantly with clinical response to therapy. Thus CA15-3 is a powerful marker of response and in combination with other markers, may provide an objective measurement of response to therapy in patients with advanced breast cancer.
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INTRODUCTION

SINCE THE discovery of carcinoembryonic antigen (CEA) [1] much has been expected of tumour markers in breast cancer. The development of monoclonal antibodies further increased the potential role of tumour markers in diagnosis, treatment and prognosis. The limited clinical usefulness of such markers to date is partly because none are truly cancer-specific while antigen expression is heterogeneous even between different cancers. In this study we evaluated in the same subjects four serum tumour markers identified by monoclonal antibodies raised either to human milk fat globule membrane fractions (HMFG1 and HMFG2) or human breast cancer tissue (CA15-3 and NCRC-11) in the diagnosis of breast cancer and in the measurement of response to therapy in patients with metastatic disease.

The production and immunohistological characterisation of HMFG1 and HMFG2 have been described [2, 3]. In a small study, elevated concentrations of HMFG1 and HMFG2 antigens were detected in the serum of patients with systemic breast cancer compared with normal subjects [4].

Early reports indicated that CA15-3 was raised in patients with breast cancer compared with controls [5–9] and CA15-3 might be useful in monitoring response to therapy in patients with metastatic breast cancer [10, 11]. NCRC-11 is an IgM class monoclonal antibody raised against a human breast cancer metastasis [12]. Such antibodies are not often used in sandwich immunoassays and IgG reagents are usually the antibodies of choice. However, the NCRC-11 antibody is stable when absorbed to its solid phase and can also be radiolabelled to high specific activities ($37–74 \times 10^4$ Bq/μg) with little or no loss in antigen-binding activity. The multivalent nature of an IgM antibody may enhance its activity as capture or tracer antibody. Unlike the other three markers, the immunoreactivity or NCRC-11 in primary breast cancer has been reported to be an independent significant prognostic factor in the diagnosis of primary breast cancer [13]. In a further study Price *et al.* identified NCRC-11 antigen in serum and urine of breast cancer patients [14].

All four monoclonal antibodies identify different epitopes on the same basic antigen. Immunoreactivity of NCRC-11 is similar but not identical with that of HMFG1 and HMFG2 while competitive binding assays suggest that the epitopes are topographically close [15]. The 115D8 monoclonal antibody against CA15-3 identifies a different epitope on the NCRC-11 defined antigen; this epitope is not topographically close to the NCRC-11 epitope [16].

PATIENTS AND METHODS

Patients

We studied five groups of patients.

Systemic breast cancer. 85 consecutive patients with newly diagnosed metastatic breast cancer. The site of initial metastatic disease was bone (33), lung (27), bone and lung (11) and viscera (14). All patients received endocrine therapy. Initial therapy for 14 premenopausal patients was goserelin 3.6 mg per month subcutaneously and tamoxifen 20 mg twice a day. Postmenopausal patients were treated either with tamoxifen ($n = 69$) or with megestrol acetate 160 mg twice a day ($n = 2$). Serum markers were assayed at 0, 2, 4 and 6 months.

Locally advanced primary breast cancer. 60 consecutive patients with histologically confirmed, locally advanced primary breast cancer (i.e. tumour over 5 cm maximum diameter). Since the 5 year survival of patients with locally advanced disease is 25–30%, the majority must have covert metastases at initial presentation. Patients were regarded as having locally advanced disease only if clinical examination and X-rays of chest and skeleton did not detect metastases. All patients had serum marker concentrations measured before any therapy.

Correspondence to J.F.R. Robertson.
J.F.R. Robertson and R.W. Blamey are at the Professorial Unit of Surgery, D. Pearson is at the Unit of Medical Physics and C. Selby is at the Unit of Clinical Chemistry, City Hospital; J. Pearson is at the Unit of Community Medicine, University Hospital; and M.R. Price is at the Cancer Research Campaign Laboratories, Nottingham; R.A. Badley is at the Unilever Research Laboratory, Sharnbrook, Bedford; and A. Howell is at the Department of Medical Oncology, Christie Hospital, Manchester, U.K.

Primary operable breast cancer. 100 consecutive patients with histologically confirmed primary operable breast cancer (clinically stage I and II) had serum marker concentrations measured before surgery.

Benign breast disease. Pre-surgery blood samples were obtained from 100 consecutive women presenting with benign breast disease confirmed by excision biopsy and histology.

Controls. 56 women with no evidence of breast disease. 25 women were either normal healthy females or were patients with benign conditions admitted for minor surgical procedures; all had no history of breast disease and normal breasts on clinical examination. Blood samples were obtained before any surgical procedure. Blood samples were also obtained from 31 women who attended one session of the breast screening programme in Nottingham; all women had no history of breast problems and no mammographic evidence of breast disease.

The mean ages (S.D.) of the five groups of patients were: controls 48.8 (14.1), benign breast disease 43.1 (12.1), stage I/II breast cancer 52.9 (8.9), stage III breast cancer 63.4 (11.5) and systemic breast cancer 60.5 (12.2) years (analysis of variance $P < 0.0001$). There was no significant difference between patients with stage III and systemic breast cancer; both these groups were significantly older than controls, patients with benign breast disease and patients with stage I/II breast cancer. Of the last three groups patients with stage I/II breast cancer were significantly older than patients with benign breast disease.

Assessment of response

Clinical response. Patients with metastatic breast cancer were assessed by UICC criteria [17] before starting anticancer therapy and after 2, 4 and 6 months' therapy or between these times if clinically indicated. To qualify for complete (CR) or partial response (PR) or static disease (SD) the minimum duration taken was 6 months as recommended by the British Breast Group [18]. Patients with a life expectancy of less than 3 months at presentation and patients with systemic disease unassessable by UICC criteria were excluded ($n = 20$) from the analysis of response. Assessment of response in all patients was externally reviewed (A.H.). Patients with static disease for at least 6 months on endocrine therapy have similar survival to patients with responding disease, both groups surviving significantly longer than patients with progressive disease by 6 months [19, 20]. In analysing the correlation between biochemical markers after therapy and clinical response we combined CR, PR and SD into a "non-progressive" disease group and compared this with the group of patients showing progression.

Biochemical response. Biochemical response to therapy in patients with metastatic breast cancer was assessed in the same manner for all serum markers studied in this unit. Namely, any change in marker while the patient is on therapy is related to the pretreatment value. A cut-off for each marker of mean + 2S.D. of the controls was calculated. Patients who never showed an elevation of the marker above this level were regarded as biochemically unassessable for that marker. Patients who started with an initially high value which fell to below the cut-off or patients with an initial value above the cut-off which subsequently decreased by more than the interassay coefficient of variation (CV) for that marker were regarded as showing a decreasing marker level (scored −2), indicative of biochemical response. Patients with an initial pretreatment value below the cut-off which subsequently rose above the cut-off or patients with an initial value above the cut-off which subsequently increased above the interassay (CV) were regarded as showing an increasing marker level (scored +2), indicative of biochemical progression. Patients with levels which started and remained above the cut-off but which moved by less than the interassay CV were regarded as biochemically stable and scored +1.

Statistical analyses. Data were analysed with analysis of variance and Scheffe range-testing to compare marker values for stage of disease. χ^2 analysis with Yates' correction where appropriate and Fisher's exact test were used to compare frequencies.

Serum markers

Venous blood was withdrawn and allowed to clot. After centrifugation, serum was removed and aliquots were stored initially at −20°C and subsequently moved to −70°C. All samples were assayed blind of clinical information and thawed once only. Marker concentrations were measured in duplicate.

CA15-3. ELSA kits (CIS, High Wycombe) were used to measure CA15-3. This kit incorporates two monoclonal antibodies, 115D8 [21] and DF3 [22]. Intra-assay variation was estimated with sera containing low (mean 7.8 U/ml), medium (mean 30 U/ml) and high values (mean 723 U/ml) of CA15-3: the CVs were 13.2, 5.0 and 3.1% respectively. The interassay CV estimated with the medium value of CA15-3 was 9.2%.

HMFG1 and HMFG2. HMFG1 and HMFG2 were measured with chemiluminescence sandwich assays. Solid phases consisted of antibody on magnetic beads (Dyno) and detector antibodies were horseradish peroxidase (HRP) conjugates. Light generation used an enhanced chemiluminescent luminol reaction [23]. Six standards were used to calibrate the assays which were in duplicate. White spots were formed on Polaroid film from the magnetically pelleted beads with attached enzyme. This assay, originally developed semi-quantitatively, was made quantitative by measuring spot diameters in a Quantimet image analyser. 50 μl standard, control or sample and 50 μl antibody-bead dilution were added to each well of a microtitre plate which was then shaken at room temperature for 30 min. The magnetic beads were localised at one side of the well for 2 min with a magnet while the liquid was aspirated. The magnet was removed and 50 μl diluted antibody–HRP conjugate was added to each well, followed by incubation at room temperature for 30 min. The beads were again localised on the side for 2 min, the fluid was aspirated and the wells were washed twice with 150–200 μl phosphate-buffered saline, Tween and methylate, making sure the beads were resuspended. 100 μl substrate solution containing luminol was added to each well. The beads were localised on the bottom for 2 min and Polaroid film was exposed to the chemiluminescent reaction for 5 min. The film was developed for 1 min. The diameter of the light spot was used to plot standard curves and calculate antigen concentrations in the samples. The inter-assay variation was assessed at two concentrations for both HMFG1 and HMFG2 and as expected for this type of assay was higher than for a fully optimised immunoradiometric assay such as that for CA15-3. The inter-assay CV for HMFG1 ranged from 12.1% to 18.2% and for HMFG2 from 15.1% to 17.0%.

NCRC-11. The serum NCRC-11 assay was a two site immunoradiographic sandwich assay which had been slightly modified from that previously described [14]. The intra-assay CV ranged

from 5.05% to 10.8% for three samples of different NCRC-11 concentrations: means 6.1 (2.5), 112.3 (49.7) and 349 (139) μ/ml. The inter-assay CV remained constant between 39.9% and 44.3%.

RESULTS

Markers in serum

Patients with non-malignant disease (i.e. control and benign breast disease groups, $n = 156$) were divided into two groups above and below 60 years (mean 41.8 [10.8] and 65.1 [5.4]) to establish if the serum marker concentrations were age-related. There was no significant difference between the two groups for any of the four markers (μ/ml): CA15-3 15.6 (7.9) and 16.2 (7.1), NCRC-11 38.6 (52.5) and 44.5 (43.6), HMFG1 29.8 (76.1) and 15.2 (43.1) and HMFG2 137.7 (146.9) and 122.7 (79.5), respectively.

Scatterplots of each of the four markers in the five patient groups are shown in Figs 1–4. Three of the markers were significantly raised in the serum of breast cancer patients (analysis of variance: CA15-3 and HMFG2, $P < 0.0001$; and NCRC-11, $P < 0.006$). Multiple range testing between the five patient groups for each of these markers showed that elevation was confined only to the systemic disease group: CA15-3 and HMFG2 were significantly increased over all the other groups

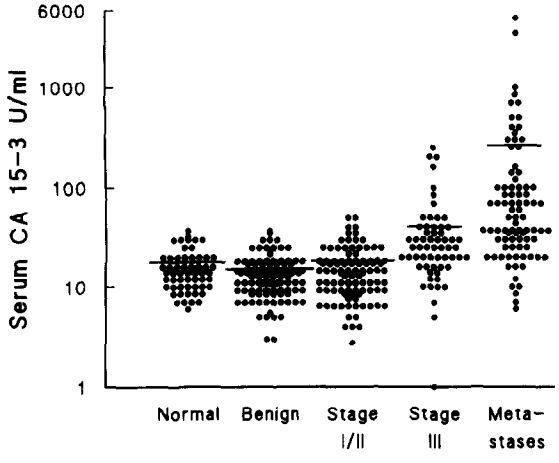

Fig. 1. Serum CA15-3 in controls and in breast cancer patients.

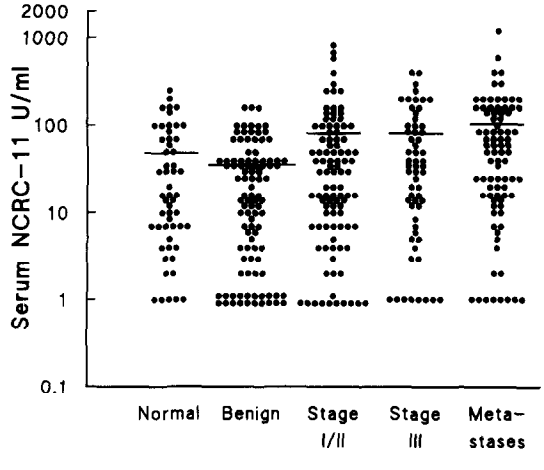

Fig. 2. Serum NCRC-11 in controls and in breast cancer patients. Note log scale.

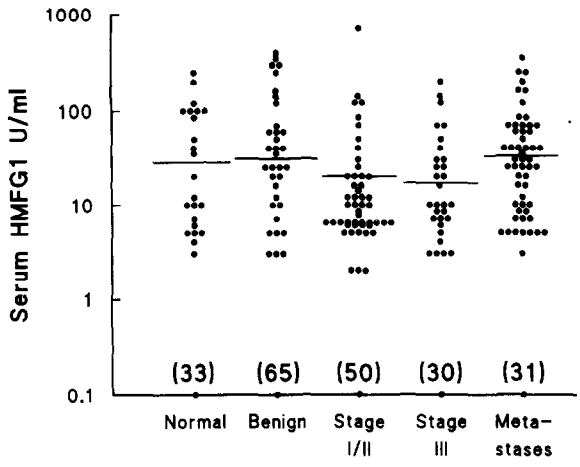

Fig. 3. Serum HMFG1 in controls and in breast cancer patients.

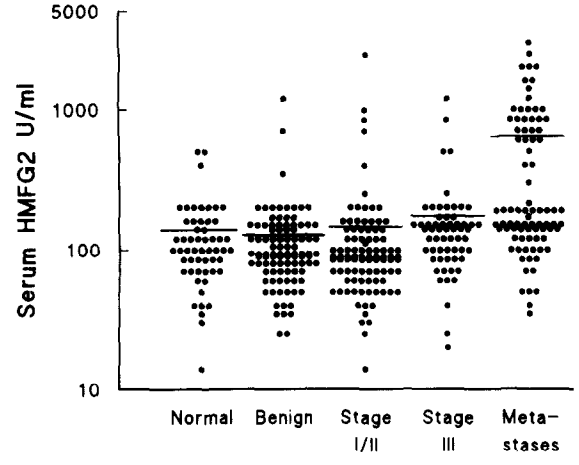

Fig. 4. Serum HMFG2 in controls and in breast cancer patients.

while NCRC-11 was elevated in the systemic disease group compared with the benign disease group only. HMFG1 was not increased in the serum of any of the groups of patients compared with controls.

The four markers were further examined to calculate the percentage of patients in each group with raised levels. The concentration initially regarded as elevated for each marker was the mean +2 S.D. of the controls. There was no difference between the groups for serum HMFG1, confirming the analysis of variance result that HMFG1 is not elevated in the serum of patients with breast cancer (Table 1). CA15-3 was increased in 8%, 33% and 68% of patients with stage I/II, III and IV disease, respectively, compared with under 3% of patients without breast cancer. HMFG2 was elevated in 5%, 7% and 38% of patients with stages I/II, III and IV disease, respectively, compared with 4% of controls. NCRC-11 was raised in 10%, 17% and 19% of patients with stage I/II, III and IV disease, respectively, compared with 4% of controls.

There was no significant difference between the dominant site of initial metastatic disease and either the mean serum CA15-3 or NCRC-11 concentration (Table 2). However, there were significant differences between the sites of initial metastatic disease and the mean serum concentration of both HMFG1 and HMFG2: the mean concentration was highest for both markers in the group of patients with hepatic metastases and lowest in patients with lung metastases. In the group of patients with

Table 1. Number (%) of patients with marker levels above mean +2 S.D. of controls

Marker (U/ml)	Non-malignant (NM)	Stage I/II	Stage III	Non-systemic (NM+I/II+III)	Systemic disease
CA15-3					
≤33	152 (97.4)	92 (92)	40 (66.7)	284 (89.9)	27 (31.8)
>33	4 (2.6)	8 (8)	20 (33.3)	32 (10.1)	58 (68.2)
HMFG1					
≤130	146 (94.2)	97 (98)	58 (96.7)	301 (95.9)	79 (92.9)
>130	9 (5.8)	2 (2)	2 (3.3)	13 (4.1)	6 (7.1)
HMFG2					
≤340	149 (96.1)	94 (94.9)	56 (93.3)	299 (95.2)	53 (62.4)
>340	6 (3.9)	5 (5.1)	4n (6.7)	15 (4.8)	32 (37.6)
NCRC-11					
<180	148 (96.1)	90 (90)	50 (83.3)	288 (91.7)	69 (81.2)
>180	6 (3.9)	10 (10)	10 (16.7)	26 (8.3)	16 (18.8)

bone metastases (with or without lung metastases) the mean HMFG1 and HMFG2 concentrations were between those for the groups of patients with hepatic and lung metastases.

Response to endocrine therapy by serum marker measurements

The change regarded as significant for predicting outcome was a change above the CV for a particular marker: for CA15-3, over ±10%; for HMFG1 and HMFG2, over ±20%; and for NCRC-11, over ±40%. Clinical response was compared to changes in the four markers at 2, 4 and 6 months. Only changes in serum CA15-3 showed a significant correlation with clinical response at 6 months (Table 3). Changes in serum HMFG1, HMFG2 and NCRC-11 showed no correlation at these times with clinical response (data not shown).

DISCUSSION

The consistent finding throughout this study, that significant elevation of serum markers was confined only to the group of patients with systemic breast cancer, may be important in the construction of an appropriate tumour model. It may also reflect either a proportional relation between serum concentration and increasing tumour bulk or a relation with the amount of tumour products that have easy access to the systemic circulation.

Hilkens *et al.* [24] reported mean serum concentrations of 115D8 in control subjects, stage I, stage II, stage III and stage IV breast cancer patients as 2.9, 4.3, 4.2, 5.3 and 14.2 U/ml, respectively. A study by Kufe *et al.* [22] on DF3 showed a significant elevation in mean serum concentration between normal subjects (82 U/ml) and patients with systemic breast cancer (1497 [S.D. 2984] U/ml); there were no patients with stage I–III breast cancer for comparison. A study of serum CA15-3 reported mean concentrations of 10.0 (4.3) U/ml for controls and 10.6 (8.3) (stage I), 13.9 (10.2) (stage II), 26.7 (27.6) (stage III) and 78.7 (115.9) (stage IV) for breast cancer patients [6]; CA15-3 values for stage IV and recurrent breast cancer were significantly higher than for stages I–III. Kerin *et al.* [9] reported that CA15-3 was evaluated in the serum of patients with stage III as well as in patients with stage IV disease. We have been unable to confirm this later finding, not only with respect to CA15-3 but also for the other markers.

HMFG1 and HMFG2 antigens have been detected in the serum of both normal subjects and patients with breast cancer. Both antigens were significantly elevated in the serum of patients with systemic breast cancer [4]. There was an increased frequency of raised serum levels of HMFG1 and HMFG2 in patients with metastatic breast cancer: 30% of patients with systemic disease showed higher levels of HMFG1 compared with 6% of normal subjects; for HMFG2 the figures were 53% and 16.6%, respectively. In our study HMFG2 was significantly increased in the serum of patients with systemic breast cancer but HMFG1 was not. The differences between the HMFG1 results in our study and the previous report [4] could be due to the small numbers of samples analysed by Burchell and colleagues or

Table 2. Mean (S.D.) pre-treatment serum marker concentration by site of initial metastases

Marker (U/ml)	Bone (n = 33)	Lung (n = 27)	Bone and lung (n = 11)	Viscera (n = 14)	P*
CA15-3	275.5 (696.5)	74.5 (134.1)	187.0 (172.9)	550.1 (1560.4)	0.3100
HMFG1	49.0 (88.4)	12.5 (21.4)	23.6 (26.7)	69.5 (81.6)	0.0463
HMFG2	561.3 (650.5)	266.2 (274.9)	601.0 (638.4)	1630.8 (2599.6)	0.0060
NCRC-11	105.4 (153.4)	75.5 (78.6)	111.5 (100.4)	182.2 (342.0)	0.3431

*Analysis of variance.

Table 3. CA15-3 levels and clinical response

Time*	CA15-3 score †	
	−2	+1 or +2
At 2 months		
Response	9	4
Static	3	5
Progression	4	24
At 4 months		
Response	9	2
Static	4	1
Progression	2	22
At 6 months		
Response	9	3
Static	5	2
Progression	3	19

*No. measured/no. with level over 33 U/ml: 2 months, 63/49; 4 months, 57/40; and 6 months, 58/41.

† χ^2 test, response + static vs. progression: 2 months, $P = 0.004$; 4 months, $P < 0.0001$; and 6 months, $P = 0.0004$.

perhaps due to assay differences. Both studies used sandwich assays but Burchell *et al.* used microtitre wells and radiolabels in contast to the magnetic beads and enzymes we used. These studies need to be repeated on a different group of patients to confirm or refute these findings.

Price *et al.* (14) reported, in a small number of patients, that the frequency of elevated NCRC-11 values was greater in patients with stage IV breast cancer than in normal subjects. In our study NCRC-11 was increased in the serum of patients with breast cancer but significant elevation was found only in the group of patients with systemic disease compared with patients with benign breast disease. There are potential problems in interpreting the NCRC-11 data in our study due to the large interassay CV.

None of these four serum markers appear to have a role in breast screening or in the differential diagnosis of primary breast cancer. The frequency of elevated concentrations in each group did increase from non-malignant control group through clinically primary breast cancer to metastatic disease (Table 1). CA15-3, HMFG2 and NCRC-11 may be useful markers for the diagnosis of secondary breast cancer. Lowering the cut-offs for CA15-3 (and also for HMFG2 and NCRC-11) increased the sensitivity of a raised level indicating systemic breast cancer but decreased the specificity. Raising the cut-offs of the markers decreased the sensitivity of diagnosing systemic breast cancer but increased the specificity (data not shown).

There is no agreement between studies as to whether the site of metastatic disease is associated with elevated serum marker levels. Although several studies have reported that increased serum CEA in patients with secondary breast cancer is associated with the site of metastases [25–27], there is no agreement between these studies as to which sites of diseases are associated with raised levels. There was no correlation between the site of initial metastases and either serum CA15-3 or NCRC-11 levels. However, there was an association between the mean serum concentration of HMFG1 and HMFG2 and the sites of initial metastatic disease. This finding has not been previously reported for either HMFG1 or HMFG2.

CA15-3 and CEA have been compared as markers for monitoring the effect of therapy in patients with metastatic disease: CA15-3 was more useful than CEA and CA15-3 alone was as useful as both in combination [11]. Sacks *et al.* [10], reporting on 4 patients, suggested that in monitoring the effect of therapy in systemic breast cancer, CA15-3 in combination with mammary serum antigen (MSA) gave better results than CA15-3 or MSA alone. Kerin *et al.* [9] reported that in 2 patients with progressing disease there was a marked rise in serum CA15-3. In our study of 65 patients, changes in serum CA15-3 at 2, 4 and 6 months were signficantly correlated with response to therapy (Table 3). CA15-3 is thus a powerful marker of response to endocrine therapy in patients with systemic breast cancer. CA15-3 (and CEA) has also been reported useful: monitoring the response to chemotherapy in patients with advanced breast cancer [28].

In this investigation, only CA15-3 proved useful as a marker of response even though all antibodies detect the same antigen. We do not know why this is so. The different antibodies vary in class, subclass and affinity for their respective epitopes which may reside in the protein core or oligosaccharide side-chains (or both). Indeed, there may be variability in the number of repeats of each of the epitopes defined by different antibodies. A major factor contributing to the differing results with the four antibodies is almost certainly the design of the assay. There are many important factors in assay construction which will influence the performance of an immunoassay and the optimisation of these factors will have major contributions to the results obtained.

There was no correlation between changes in either serum HMFG1 or HMFG2 and response. Also sequential serum NCRC-11 measurements were of no value in measuring response to therapy. These results, however, should be interpreted with caution since these three assays were still in the process of being developed and, unlike that for CA15-3, were not commercially available. In particular there was the problem of the large interassay CV for NCRC-11. We are doing a small pilot study measuring serial NCRC-11 samples within a single assay in an attempt to assess the potential value of NCRC-11 in monitoring therapy in patients with advanced breast cancer. Further work is in progress to develop more robust and quantitative assays for NCRC-11, HMFG1 and HMFG2, and to determine whether the large CVs within the control populations can be reduced.

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