CLINICAL STUDY

Elevated free IGF2 levels in localized, early-stage breast cancer in women

Ulrick Espelund, Søren Cold¹, Jan Frystyk, Hans Ørskov and Allan Flyvbjerg

Medical Research Laboratories, Clinical Institute and Medical Department M (Diabetes and Endocrinology), Aarhus University Hospital, Nørrebrogade 44, DK-8000 Aarhus C, Denmark and ¹Department R (Oncology), Odense University Hospital, DK-5000 Odense, Denmark

(Correspondence should be addressed to A Flyvbjerg; Email: allan.flyvbjerg@dadlnet.dk)

Abstract

Objective: Epidemiological studies imply an association between circulating IGF1 and breast cancer, whereas the role of IGF2, which also acts on the IGF1 receptor, is less settled. This study investigates the association between IGF2 and breast cancer in patients with localized disease.

Design: The participants were women with well-characterized, early stage, localized breast cancer ($n=43$) and matched healthy women ($n=38$), from whom fasting serum levels of IGF-related peptides were measured.

Results: In patients, mean free IGF2 was increased ($+57\%$, $P<0.001$), in spite of reduced total IGF2 levels (-12% , $P=0.003$) when compared with controls. Similar changes were seen in free IGF1 ($+28\%$, $P=0.004$) and total IGF1 (-16% , $P=NS$). Pro-IGF2 and IGF-binding protein 1 (IGFBP1) were unchanged. IGFBP2 was reduced by 22% in the patients ($P=0.004$). The patients showed reduced IGFBP3 protease activity and accordingly increased levels of intact IGFBP3, whereas total IGFBP3 was unchanged.

Conclusion: Women with localized, early-stage breast cancer show elevated circulating free IGF1 and IGF2, reduced total IGF2 and alterations in IGFBPs. The changes observed despite minimal cancer disease suggest a role for the circulating IGF system in the progression of breast cancer in women.

European Journal of Endocrinology 159 595–601

Introduction

The insulin-like growth factor (IGF) system is involved with normal cell growth, proliferation, differentiation and survival in an endocrine (i.e. hormonal) and paracrine/autocrine manner (1). The system comprises IGF1 and IGF2, mostly bound to specific high-affinity IGF-binding proteins (IGFBPs) in the circulation. On target cells the IGFs bind to membrane-bound receptors of which the IGF1 receptor (IGF1R) is widely distributed throughout the body and thought to be the primary mediator of IGF-related metabolic and growth promoting actions (2). Insulin receptor type A (IR-A) and hybrids of IR and IGF1R are also activated by IGFs and seem to mediate growth promoting effects as well as metabolic effects depending on the ligand (3). The IGF 2 receptor (IGF2R) has high affinity for IGF2 and may scavenge excess growth factor, reducing pericellular IGF2 levels (4).

In vitro studies show mitogenic and anti-apoptotic effects of IGF1 mediated through the IGF1R (1). *In vivo* evidence includes several large prospective studies and in general they suggest a modestly increased breast cancer risk in individuals with serum total IGF1 levels within the upper normal range (5, 6). Some studies have investigated the molar ratio of IGF1 to IGFBP3 (a putative marker of

free IGF1) in breast cancer (7), whereas only a few studies include direct analysis of free IGF1 (6, 8–10).

Analyses of breast tumour tissues have revealed an elevated expression of IGF2 (11). In the transition zone between normal epithelium and tumour tissue, stromal cells and leucocytes express IGF2 that may stimulate angiogenesis (12) and therefore, IGF2 levels in the pericellular environment may be of importance for carcinogenesis. The addition of IGF2 to cancer cells stimulates proliferation (13), whereas transfection of the IGF2R into cancer cells inhibits growth and increases apoptosis (14). In parallel, decreased expression or disrupted ligand binding of the IGF2R has been associated with increased growth potential (15, 16). *In vivo*, loss of heterozygosity in the IGF2R gene correlates with poor histological differentiation (17). However, the amount of clinical studies investigating circulating IGF2 levels in cancer populations is limited.

IGFBPs are expressed by breast tumours depending on oestrogen receptor status, and IGFBP proteases may influence IGF activity in the tumour environment (18). IGFBP3 has undergone the most thorough investigation, showing IGF1 independent anti-proliferative and pro-apoptotic effects *in vitro* (19). However, circulating levels of IGFBP3 have been investigated in

several prospective cohort studies with somewhat unequivocal conclusions (5, 6).

The aim of the present study was to compare the circulating IGF system in women with early stage breast cancer and healthy subjects. To eliminate the effects of disseminated disease on the circulating IGF system, we restricted the study cohort to include well-characterized cancer patients with only small tumours and no metastases or axillary involvement. All participants were studied under fasting conditions to minimize confounding by differences in metabolic state.

Materials and methods

Patient characteristics

A subset of patients and controls were selected from a larger cross-sectional cohort collected at the Department of Oncology R, Odense University Hospital, Denmark. The cohort consisted of patients referred over a period of 4 years. Inclusion criteria were Danish citizenship and a postal address within a certain geographical area. A previous cancer diagnosis was an exclusion criterion. For each cancer patient, one healthy woman from the same geographical region was included, matched by date of birth with the aid of the Danish Central Office of Civil Registration. Fasting blood samples were drawn either prior to surgery (patients) or on an examination day (controls) and stored at -80 °C until analysis. The cohort has been described with clinical examination and anthropometric measures including body composition by bioimpedance measurement (Tetrapolar analyzer, 500 µA, 50 kHz, Animeter, Denmark) according to (20) and questionnaires. Questionnaires were developed specifically for this study and included smoking habits, nutrition, menopausal status, medication and previous medical conditions including previous cancer disease (Table 1). Patient characteristics at surgery were described by tumour size, type and grade (21), oestrogen receptor status (22) and lymph node involvement. From the cohort, we selected cases with minimal disease determined by small tumour size with a histological grading of 2 or less and no lymphatic spread (Table 2). Hormone replacement therapy was taken by 2 of cases and 5 of controls. One patient had diabetes and hypertension, but did not otherwise differ from the group. The controls chosen were not matched directly with the selected cases, but chosen randomly from the same inclusion subperiod. This has introduced a skewness in the study subcohort: women above the age of 50 were offered a screening (mammography) at that time, while women aged 50 or less were not. The result is a larger proportion of small tumours among postmenopausal women than among premenopausal women in the cohort. Selecting cases with small tumours only and at the same time selecting controls

Table 1 Characteristics of breast cancer patients and healthy controls.

	Cancer	Control	P
Number (n)	43	38	
Age	58.6 (56;61)	52.4 (49;56)	0.014*
Premenopausal (n)	10	17	0.06
BMI (kg/m ²)	26.8 (25.1;28.4)	25.5 (24.1;27.0)	
Body composition			
Waist/hip ratio	0.83 (0.81;0.86)	0.82 (0.80;0.84)	
ASD (cm)	22.5 (21.4;23.5)	21.7 (20.7;22.8)	
Fat-free mass (kg)	44.3 (42.8;45.8)	44.9 (43.2;46.6)	
Fat mass (kg)	25.4 (22.3;28.4)	23.9 (20.9;26.8)	0.06
Smoking status (n)			
Daily	20	12	
Sometimes	2	8	
Never	21	18	

ASD, abdominal sagittal diameter. P, P value of t-test or non-parametric test (the latter indicated by asterisk).

randomly introduced a small but significant age difference between cases and controls in the study subcohort (Table 1). Each participant gave informed consent prior to inclusion and the study was approved by the regional ethical committee on human research.

Immunoassays

Measurements were performed in duplicate within the same assay. The only exception was free IGF1 and IGF2, which were assayed in triplicate. Serum total IGF1 and IGF2 were determined after acid ethanol extraction using non-competitive time-resolved monoclonal immunofluorometric assays (TR-IFMAs) as previously described (23). Free IGF1 and IGF2 were separated from bound IGFs by ultrafiltration (24); the detection limit in serum was 0.028 µg/l for free IGF1 and 0.055 µg/l for free IGF2. Pro-IGF2 was determined by acid gel chromatography and subsequent TR-IFMA as previously described (25).

Serum IGFBP1 was determined by ELISA (Medix Biochemica, Kauniainen, Finland). Serum IGFBP2 and IGFBP6 were measured by RIA and serum IGFBP3 by IRMA (Diagnostic System Laboratories Inc., Webster, TX, USA). The average with-in assay and in-between assay coefficient of variations (CVs) for the in-house assays were as follows. Total IGF1 and IGF2: < 5 and

Table 2 Tumour characteristics.

Tumour size (mm)	11 (9;12)
Histological type (n)	
Lobular	11
Ductal grade 1	18
Ductal grade 2	14
Ductal grade 3	0
Tumour ER status	41 of 43 positive
Lymph nodes (n)	
Examined nodes per patient	16 (14;18)
With cancer spread	0

Tumour ER status, oestrogen receptor status.

10%; free IGF1: 17 and 20%; free IGF2: 15 and 16%; Pro-IGF2: 5% and 10%. Commercial assays were used for determination of IGFBP1, -2, -3 and -6, and according to the manufacturer with-in and in-between assay CVs below 7 and 6%, respectively.

Western ligand blotting (WLB) and western immunoblotting (WIB)

SDS-PAGE and ligand blot analyses were performed according to the method of Hossenlopp *et al.* (26), as previously described (27). Intact IGFBP3 appears as a 38–42 kDa doublet in both WLB and WIB. IGFBP4 appears as a single 24 kDa band. The *in vivo* proteolysis of IGFBP3 was estimated by WIB and expressed as the percentage of immunoreactive IGFBP3 appearing in smaller molecular mass bands with sizes of 30, 20 and 16 kDa, respectively (28). With-in and in-between assay variation coefficients were 8 and 12%, respectively.

Statistical analysis

All variables were natural-log-transformed and tested using the Shapiro-Wilk *u* test for normality. For normal-distributed variables, groups were compared using the two-tailed Student's unpaired *t*-test, otherwise by Wilcoxon rank-sum test. Linear regression analysis was performed to adjust for age, menopausal status and smoking habits. Correlation analysis was performed to assess relationships between variables. A *P* value of 0.05 or less was considered statistically significant. *P* values above 0.10 are reported as non-significant (NS). Numbers are reported as mean and 95% confidence intervals of the mean or alternatively as the relative difference of the mean between groups. Data were analysed using Intercooled Stata 8.2 for Windows.

Results

Women with localized breast cancer showed changes in most components of the IGF system when compared with healthy controls (Table 3). Free IGF2 was increased in spite of reduced total IGF2 levels, values averaging 157% (*P* < 0.001) and 88% (*P* = 0.003) of control levels respectively. Similar changes were seen in free and total IGF1 with levels averaging 128% (*P* = 0.004) and 84% (*P* = NS) of controls respectively (Fig. 1).

Pro-IGF2 was similar in patients and controls comprising ~20% of total IGF2 levels (Table 3). IGFBP1 levels were unchanged in cancer patients, whereas IGFBP2 levels were reduced to 78% (*P* = 0.004; Table 3). The difference in immunoreactive IGFBP3 levels was NS in the adjusted analysis, whereas levels measured by WLB (intact IGFBP3) were elevated to 109% in cancer patients relative to controls (*P* < 0.001). Accordingly, IGFBP3 proteolysis was reduced (*P* = 0.01; Table 3). IGFBP3 WLB and IGFBP3 proteolysis were inversely correlated (*R* = −0.42, *P* < 0.001). No differences were observed for IGFBP4 WLB, IGFBP6 or IGF1:IGFBP3 ratio. Neither IGF levels nor IGFBP levels correlated with tumour characteristics (i.e. histology, tumour size).

Discussion

In this cross-sectional study, we observed several abnormalities in the serum concentrations of total and free IGFs as well as IGFBPs in women with localized, early-stage breast cancer. Most notably, free IGF2 was increased in spite of reduced total IGF2 levels in breast cancer patients, and the same pattern was evident for IGF1.

Table 3 Results for insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs).

	Cancer	Control	Crude P	Adj. P
IGF1				
Total	108 (99;117)	128 (116;140)	0.02	NS
Free	0.63 (0.53;0.74)	0.49 (0.39;0.59)	0.04	0.004
IGF2				
Total	731 (692;771)	828 (786;871)	0.002	0.003
Free	1.68 (1.51;1.85)	1.07 (0.92;1.23)	<0.001*	<0.001
Pro-IGF2	167 (145;189)	181 (162;200)	NS	NS
Pro/total	23	22	NS	NS
IGFBP1	7.1 (5.6;8.5)	6.2 (4.9;7.5)	NS	NS
IGFBP2	473 (405;540)	604 (525;684)	0.013*	0.004
IGFBP3				
Immunoreactive	3203 (3067;3339)	3474 (3322;3625)	0.02	0.09
WLB	789 (750;828)	722 (679;765)	0.03*	<0.001
Protease activity	39 (36;42)	43 (40;47)	0.07	0.01
IGFBP4 WLB	48 (45;52)	47 (43;50)	NS	NS
IGFBP6	235 (209;260)	197 (174;219)	0.06*	0.15
IGF1:IGFBP3	0.034 (0.031;0.036)	0.037 (0.034;0.039)	NS	NS

Units are $\mu\text{g/l}$ except for western ligand blots (Arbitrary units/ mm^2), protease activity (percent) and pro-/total IGF2 (percent). Asterisk (*) indicates a non-parametric statistical test. Crude P: *P* value of *t*-test or non-parametric test (the latter indicated by asterisk). Adj.P: *P* value of linear regression analysis with adjustment for age, menopausal status and smoking habit.

Figure 1 Breast cancer patients (BC) versus healthy subjects (HS). Box represent approximated 25th and 75th percentile with the median indicated as a horizontal line. Whiskers indicate adjacent values and squares (■) indicate single observations outside the adjacent values interval. The asterisk (☆) indicates statistical significance. All units are in ng/L .

Only a few studies have investigated circulating IGF2 levels in breast cancer patients. We have identified three published cross-sectional studies investigating IGF2 in breast cancer patients (8, 29, 30). None of these studies found differences in total IGF2 between cancer patients and controls. One potential explanation of why we were able to demonstrate a significant difference in our study may be due to a large degree of homogeneity among our cancer patients. In addition, the early-stage and localized nature of breast disease among cases in our study might be an explanation as well. The negative findings of case-control studies are contrasted with previous findings from our group. In a prospective study,

the risk of developing oestrogen-receptor-positive breast cancer increased with rising levels of total IGF2 at baseline, prior to the development of any tumour (31). There are several similarities in the present study: both populations were Danish and consist of both pre- and postmenopausal women. Furthermore, most cancers were oestrogen receptor positive. On the basis of these two studies IGF2 appears to be involved with the development and progression of breast cancer since both studies show an elevated level of IGF2. The shift from elevated total IGF2 (before the formation of a tumour) towards low total IGF2 and elevated free IGF2 (with the presence of a small tumour) is not clear, but is

likely to reflect changes in IGFBPs. The impact of tumour tissue on the circulating IGF system may be elevated free IGF2 levels despite normal or reduced total IGF2 levels.

Total IGF1 levels were reduced in cancer patients, but this was NS after adjusting for age. A prospective study (6) and a meta-analysis (5) found elevated total IGF1 to be a risk factor among premenopausal women, whereas circulating IGF1 was not a risk factor in postmenopausal women. The variation in total IGF1 levels between subjects depends upon age and IGFBP levels. As a consequence diverging results may be obtained from study to study. It has required large studies and meta-analyses to conclude on the possible link between IGF1 levels and development of cancer. Hence, we have limited power to demonstrate differences in total IGF1 most likely due to the limited study size.

We found elevated free IGF1 levels in patients. A large prospective study (6) and other studies (8, 10) did not find any association between breast cancer and free IGF1. The mentioned studies have all used a commercially available assay, which is believed to determine free plus readily dissociable IGF1. However, we know from previous comparisons that there are genuine differences between the two methods, and that they do not necessarily show superimposable results (32). The ratio of total IGF1 to IGFBP3 has been suggested as a surrogate marker of free IGF1. In our study, there were marked differences regarding free IGF1, but no differences in IGF1:IGFBP3 ratio (Table 3). Thus, the present data confirm that IGF1:IGFBP3 ratio is not a good marker of free IGF1 (32).

There is a contrast between *in vitro* findings of a strong link between the IGF system and cancer and the somewhat conflicting *in vivo* data. The use of total IGF measurements may be troublesome because of the complex *in vivo* interaction between the IGFs and their IGFBPs. Total IGF levels are influenced by the binding capacity in serum, which may impede total IGF measurements in reflecting IGF activity, whereas free IGFs determined by ultrafiltration is a measure of unbound IGF available for interaction with IGF and insulin receptors (24). Our data document the pitfall of using total IGF measurements alone. We found unchanged total IGF1 levels and a decrease in total IGF2, which could be interpreted as a reduced activity of the IGF system, if there was no other contributing data. The increased free IGF1 and IGF2 levels contrast with the above-mentioned interpretation based on total IGFs, and overall our findings indicate that the bioactivity of the IGF system is increased rather than decreased in non-disseminated breast cancer patients (i.e. no metastases). In concert with this view, the elevated levels of free IGF1 and IGF2 may by increased feedback inhibition of the pituitary GH secretion explain the reduced total IGF levels that we speculate are secondary to a diminished synthesis of the major IGF carrier, the GH dependent IGFBP3.

We did not include patients with cancer catabolism, large tumour burdens or disseminated disease, and all participants were sampled under fasting conditions. This eliminated the risk of introducing differences in the IGF system caused by systemic effects of the neoplasias, for instance liver metastases and cancer-related cachexia, which both may affect the circulating IGF system. If carcinomatous tissue has a direct effect on the IGF system, we would expect an association between tumour size and/or volume and changes in IGF-related peptides. This was not observed, possibly due to the small tumour size and range of sizes in the present study. One study found a correlation between circulating free IGF2 and breast tumour size, but surprisingly cancer patients showed lower levels of free IGF2 than matched controls, and noteworthy, these findings only became evident after correcting for the changes in body fluid composition in the post-operative state (10).

The prospective studies as well as the present study, with marked changes in the circulating IGF levels despite the localized and early nature of the breast cancers included, support the first scenario. Since tumours were small, the differences observed in breast cancer patients may have existed before the development of a tumour. We investigated possible associations between tumour characteristics (histology, tumour size) and levels of IGFs and IGFBPs but found no significant correlations, which opposes the second scenario. However, the present study remains cross sectional, which is a major limitation towards elucidating possible causal relations.

Changes in the IGF-binding capacity caused by IGFBP proteolysis by tumour tissue may result in discrete changes in total IGF levels and marked increases in free IGF levels. For instance following major surgery, an elevated enzymatic degradation of IGFBP3 may explain that levels of total IGF1 are markedly reduced whereas levels of free IGF1 are relatively increased (28). In the present study, the levels of immunoreactive IGFBP3 were unchanged among cancer patients after adjustment. We also evaluated the amount of intact IGFBP3 by WLB and proteolysis assay. This revealed an *increased* amount of intact IGFBP3 and reduced proteolysis among breast cancer patients. Thus, the change in IGFBP3 binding capacity is not likely to explain the observed changes in free and total IGF level. Other case-control studies have found reduced immunoreactive IGFBP3 levels (7, 29). The large prospective studies have yielded unequivocal results. Both increased (5, 31) and unaltered (6) risk were reported for patients with the highest levels of IGFBP3. In a cohort of both pre- and postmenopausal women proteolytic activity has been found unaltered in early-stage breast cancer, increasing with disease in later stages (8). Our data contribute to the conflicting data regarding IGFBP3 and breast cancer.

Cancer patients showed lower levels of serum IGFBP2 than controls. This binding protein is an important determinant of free IGF2 levels; low IGFBP2 levels may

increase the free fraction of IGF2 (33), a hypothesis supported by our data. The *IGFBP2* gene is over-expressed in a number of tumours, but its role is unclear (34). We and others have investigated IGFBP2 expression in different breast cancer cell lines and found that the expression may be a marker of anti-oestrogen resistance but not cell survival (35).

Several mechanisms may link the development of breast cancer to the observed changes in IGF1 and IGF2: First, of all, elevated levels of free IGFs may increase IGF1R and induce IR activation, leading to cell proliferation and reduced apoptosis. Secondly, the presence of carcinomatous tissue may alter the IGF system in ways that increase free IGFs (e.g. tumour IGF production or IGFBP proteolysis). Hopefully, future studies will clarify the changes in the IGF system at the tissue level. In conclusion, the elevated levels of circulating free IGF2 found in women with breast cancer may reflect an increased bioactivity of this axis. It is well established that tumour cells produce IGF2 that act locally, enhancing growth promoting and anti-apoptotic signalling. The clear changes that were observed despite minimal cancer disease suggest a role for the IGF system in the progression of breast cancer in women.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

The study was supported by grants from The Danish National Research Council, the Eva and Henry Frønkels Foundation, the Novo Nordisk Foundation and Clinical Institute at Aarhus University Hospital.

Acknowledgements

Karen Mathiesen, Kirsten Nyborg, Ninna Rosenquist and Susanne Sørensen are thanked for skilled technical assistance.

References

- 1 Khandwala HM, McCutcheon IE, Flyvbjerg A & Friend KE. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocrine Reviews* 2000 **3** 215–244.
- 2 De Meyts P & Whittaker J. Structural biology of insulin and IGF1 receptors: implications for drug design. *Nature Reviews. Drug Discovery* 2002 **10** 769–783.
- 3 Denley A, Wallace JC, Cosgrove LJ & Forbes BE. The insulin receptor isoform exon 11-(IR-A) in cancer and other diseases: a review. *Hormone and Metabolic Research* 2003 **11–12** 778–785.
- 4 Ellis MJ, Leav BA, Yang Z, Rasmussen A, Pearce A, Zweibel JA, Lippman ME & Cullen KJ. Affinity for the insulin-like growth factor-II (IGF-II) receptor inhibits autocrine IGF-II activity in MCF-7 breast cancer cells. *Molecular Endocrinology* 1996 **3** 286–297.
- 5 Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet S & Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* 2004 **9418** 1346–1353.
- 6 Schernhammer ES, Holly JM, Pollak MN & Hankinson SE. Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. *Cancer Epidemiology, Biomarkers and Prevention* 2005 **3** 699–704.
- 7 Bruning PF, Van Doorn J, Bonfrer JM, Van Noord PA, Korse CM, Linders TC & Hart AA. Insulin-like growth-factor-binding protein 3 is decreased in early-stage operable pre-menopausal breast cancer. *International Journal of Cancer* 1995 **3** 266–270.
- 8 Helle SI, Geisler S, Aas T, Paulsen T, Holly JM & Lønning PE. Plasma insulin-like growth factor binding protein-3 proteolysis is increased in primary breast cancer. *British Journal of Cancer* 2001 **1** 74–77.
- 9 Li BD, Khosravi MJ, Berkel HJ, Diamandi A, Dayton MA, Smith M & Yu H. Free insulin-like growth factor-I and breast cancer risk. *International Journal of Cancer* 2001 **5** 736–739.
- 10 Singer CF, Mogg M, Koestler W, Pacher M, Marton E, Kubista E & Schreiber M. Insulin-like growth factor (IGF)-I and IGF-II serum concentrations in patients with benign and malignant breast lesions: free IGF-II is correlated with breast cancer size. *Clinical Cancer Research* 2004 **12** 4003–4009.
- 11 Yee D, Cullen KJ, Paik S, Perdue JE, Hampton B, Schwartz A, Lippman ME & Rosen N. Insulin-like growth factor II mRNA expression in human breast cancer. *Cancer Research* 1988 **23** 6691–6696.
- 12 Heffelfinger SC, Miller MA, Yassin R & Gear R. Angiogenic growth factors in preinvasive breast disease. *Clinical Cancer Research* 1999 **10** 2867–2876.
- 13 Karey KP & Sirbasku DA. Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17 β -estradiol. *Cancer Research* 1988 **14** 4083–4092.
- 14 Souza RE, Wang S, Thakar M, Smolinski KN, Yin J, Zou TT, Kong D, Abraham JM, Toretzky JA & Meltzer SJ. Expression of the wild-type insulin-like growth factor II receptor gene suppresses growth and causes death in colorectal carcinoma cells. *Oncogene* 1999 **28** 4063–4068.
- 15 Byrd JC, Devi GR, de Souza AT, Jirtle RL & MacDonald RG. Disruption of ligand binding to the insulin-like growth factor II/mannose 6-phosphate receptor by cancer-associated missense mutations. *Journal of Biological Chemistry* 1999 **34** 24408–24416.
- 16 O'Gorman DB, Costello M, Weiss J, Firth SM & Scott CD. Decreased insulin-like growth factor-II/mannose 6-phosphate receptor expression enhances tumorigenicity in JEG-3 cells. *Cancer Research* 1999 **22** 5692–5694.
- 17 Chappell SA, Walsh T, Walker RA & Shaw JA. Loss of heterozygosity at the mannose 6-phosphate insulin-like growth factor 2 receptor gene correlates with poor differentiation in early breast carcinomas. *British Journal of Cancer* 1997 **12** 1558–1561.
- 18 Perks CM & Holly JM. IGFBPs and breast cancer. *Breast Disease* 2003 **17** 91–104.
- 19 Baxter RC, Butt AJ, Schedlich LJ & Martin JL. Antiproliferative and pro-apoptotic activities of insulin-like growth factor-binding protein-3. *Growth Hormone and IGF Research* 2000 **10** (Suppl A) S10–S11.
- 20 Heitmann BL. Prediction of body water and fat in adult Danes from measurement of electrical impedance. A validation study. *International Journal of Obesity* 1990 **9** 789–802.
- 21 Elston CW & Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991 **5** 403–410.
- 22 Talman ML, Rasmussen BB, Andersen J & Christensen IJ. Estrogen Receptor analyses in the Danish Breast Cancer Cooperative Group. History, methods, prognosis and clinical implications. *Acta Oncologica* 2008 **4** 789–794.
- 23 Frystyk J, Dinesen B & Ørskov H. Non-competitive time-resolved immunofluorometric assays for determination of human insulin-like growth factor I and II. *Growth Regulation* 1995 **4** 169–176.

- 24 Frystyk J, Skjærbaek C, Dinesen B & Ørskov H. Free insulin-like growth factors (IGF-I and IGF-II) in human serum. *FEBS Letters* 1994 **2** 185–191.
- 25 Espelund U, Bruun JM, Richelsen B, Flyvbjerg A & Frystyk J. Pro- and mature IGF-II during diet-induced weight loss in obese subjects. *European Journal of Endocrinology* 2005 **6** 861–869.
- 26 Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S & Binoux M. Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analytical Biochemistry* 1986 **1** 138–143.
- 27 Flyvbjerg A, Kessler U, Dorka B, Funk B, Ørskov H & Kiess W. Transient increase in renal insulin-like growth factor binding proteins during initial kidney hypertrophy in experimental diabetes in rats. *Diabetologia* 1992 **6** 589–593.
- 28 Skjærbaek C, Frystyk J, Ørskov H, Kissmeyer-Nielsen P, Jensen MB, Laurberg S, Møller N & Flyvbjerg A. Differential changes in free and total insulin-like growth factor I after major, elective abdominal surgery: the possible role of insulin-like growth factor-binding protein-3 proteolysis. *Journal of Clinical Endocrinology and Metabolism* 1998 **7** 2445–2449.
- 29 Kaulsay KK, Ng EH, Ji CY, Ho GH, Aw TC & Lee KO. Serum IGF-binding protein-6 and prostate specific antigen in breast cancer. *European Journal of Endocrinology* 1999 **2** 164–168.
- 30 Yu H, Jin F, Shu XO, Li BD, Dai Q, Cheng JR, Berkel HJ & Zheng W. Insulin-like growth factors and breast cancer risk in Chinese women. *Cancer Epidemiology, Biomarkers and Prevention* 2002 **8** 705–712.
- 31 Grønbaek H, Flyvbjerg A, Mellembjær L, Tjønneland A, Christensen J, Sørensen HT & Overvad K. Serum insulin-like growth factors, insulin-like growth factor binding proteins, and breast cancer risk in postmenopausal women. *Cancer Epidemiology, Biomarkers and Prevention* 2004 **11** 1759–1764.
- 32 Frystyk J. Free insulin-like growth factors – measurements and relationships to growth hormone secretion and glucose homeostasis. *Growth Hormone and IGF Research* 2004 **5** 337–375.
- 33 Frystyk J, Skjærbaek C, Vestbo E, Fisker S & Ørskov H. Circulating levels of free insulin-like growth factors in obese subjects: the impact of type 2 diabetes. *Diabetes/Metabolism Research and Reviews* 1999 **5** 314–322.
- 34 Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, Weber MM & Wolf E. Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter? *Cancer Research* 2001 **24** 8601–8610.
- 35 Juncker-Jensen A, Lykkesfeldt AE, Worm J, Ralfkiaer U, Espelund U & Jepsen JS. Insulin-like growth factor binding protein 2 is a marker for antiestrogen resistant human breast cancer cell lines but is not a major growth regulator. *Growth Hormone and IGF Research* 2006 **4** 224–239.

Received 22 July 2008

Accepted 29 July 2008