Mapping oligosaccharides of aggressive breast cancer.

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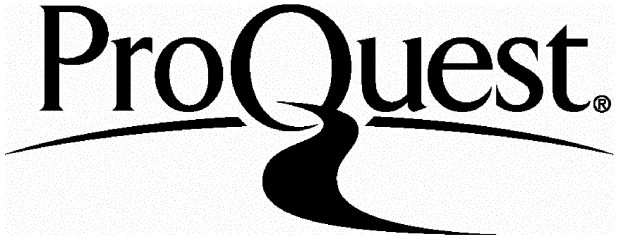

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Abstract.

Virtually all deaths from breast cancer result from the growth of metastases and increasing evidence suggests that cancer cell oligosaccharides may be involved in the metastatic process. We have investigated oligosaccharides as potential biochemical markers for metastasis and survival after breast cancer.

We set out to map oligosaccharides from breast cancers that were rapidly fatal and compare them with cancers that did not form clinical metastases. To do this, it was necessary to use tissues removed from patients who were followed-up for at least 5-10 years. Initially, we developed methods for the release of intact oligosaccharides from archival fixed tissues. The methods proved to be sufficiently sensitive to enable the detection of, typically, 50 different oligosaccharides from single pathology sections.

To seek new markers for the clinical behaviour of breast cancer, oligosaccharides were extracted from 76 specimens, with different clinical outcomes and 5-10 years follow-up. The heterogeneous mixture of oligosaccharides from the different breast cancer specimens was separated using normal-phase high performance liquid chromatography. Pilot studies had previously identified a number of oligosaccharides that appeared related to aggressive breast cancer behaviour. The levels of these in the oligosaccharide pool from the 76 breast cancer specimens were compared, by multivariate analysis, to time to first recurrence and breast cancer death.

Some of the oligosaccharides were found to be independent markers of poor prognosis. The expression of one oligosaccharide correlated with cancer tissue staining using the lectin from the snail *Helix pomatia.* This lectin has previously been shown to detect breast cancers associated with poor prognosis. As a result of this work, further studies will be undertaken, in the first instance, to sequence the oligosaccharide structures which we have found to be associated with aggressive, clinically metastatic, breast cancer.

Table of Contents

List of figures.

List of tables.

List of abbreviations.

2AB	2-aminobenzamide
BHK	baby hamster kidney
BSA	bovine serum albumin
CAM	cell adhesion molecule
CEA	carcinoembryonic antigen
CML	chronic myelogenous leukaemia
CNBr	cyanogen bromide
CNP	1,1,1 - trichloroethane and trichloroethylene
DNA	deoxyribonucleic acid
DOB	date of birth
ecm	extra cellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
FPLC	fast protein liquid chromatography
GAG	glycosaminoglycans
Gal	galactose
GalNAc	n-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
GnT	*N*-acetylglucosaminyltransferase
GPC	gel permeation chromatography
GPI	glycosylphosphoinositol anchor
GTases	glycosyltransferases
GU	glucose unit
H+E	haematoxylin and eosin
HPA	*Helix pomatia* agglutinin
HPLC	high performance liquid chromatography
IHC	immunohistochemical
IS	internal standard
L-PHA	L-phytohaemagglutinin
MALDI/MS	matrix-assisted laser-desorption ionisation / mass spectrometry
MW	molecular weight
MWCO	molecular weight cut-off
NOS	not otherwise specified
NS	not significant
PEEK	poly ether ether ketone
PR	progesterone receptor
RFU	relative fluorescence units
SD	standard deviation (of the mean average)
TBS	tris buffered saline
TNM	tumour node metastasis
UB	unbound
WGA	wheat germ agglutinin

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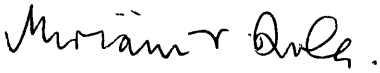
Statement of originality.

I declare that all of the work decribed in this thesis and its composition are my own, unless otherwise stated in the text. This thesis is is presented only to the University of London for consideration for the Degree of Doctor of Philosophy.

Signed: 

Chapter 1

General introduction.

Preface.

Oligosaccharides appear to be important in normal cell behaviour and possibly also in cancer. This thesis briefly describes breast cancer, an outline of different types of oligosaccharides and other studies of glycosylation changes in cancer (chapter 1). We have sought to understand changes in oligosaccharide expression and the clinical behaviour of breast cancer.

1.1 Aims of the study.

The aims of our study were:

1) to develop methods for the extraction and biochemical mapping of oligosaccharides from archival breast cancer specimens (chapter 2),
2) to investigate, in retrospective studies, whether oligosaccharide expression relates to breast cancer behaviour (chapters 3 and 5); and
3) to identify breast cancer oligosaccharides which bind to the lectin HPA (chapters 4 and 5).

This work was conducted in the context of a larger project concerned with the role of oligosaccharides in the development of breast cancer metastases.
This avenue of research may lead to new tools for predicting breast cancer outcome. Potentially, it may also identify targets for new therapies in the treatment of the secondary breast cancer.

1.2 Breast cancer introduction.

Breast cancer is one of the most common malignancies in the Western world and the most common cancer of women in the UK, with currently approximately 35,000 new cases in England and Wales per annum (Imperial Cancer Research Fund, 1998). The lifetime risk of breast cancer increases considerably as a woman grows older, in the USA, for example, the risk of developing breast cancer at age 40 is approximately one in two hundred but by the age of 70 has risen to approximately one in fourteen (Fever *et al.*, 1993). These data illustrate the enormous numbers of women affected by the disease.

1.2.1 Risk factors.

The causes of breast cancer are not known, but there are a number of factors which appear to be associated with an increased risk of developing the disease.

As long ago as the 12th century it was noted that breast cancer occurred more frequently in populations of nuns, characterised by their presumed celibate lifestyle reviewed in Shimkin, (1973). Factors which decrease the numbers of menstrual cycles provide a protective effect against breast cancer such as breast-feeding an infant for more than a year (Yuan *et al.*, 1988) and surgical oophorectomy (Schairer, 1997) may increase the risk of developing breast cancer. Early menarche, late menopause, low parity and relatively late age at first full-time pregnancy are associated with an increased risk of developing breast cancer (MacMahon *et al.*, 1973; Shapiro *et al.*, 1968).

Of the many candidate endogenous hormones, oestrogen and progesterone seem to be implicated in breast cancer (Key and Pike, 1988; Thomas *et al.*, 1997; Toniolo *et al.*, 1995). A number of sources of exogenous hormones exist and population based studies suggest that prolonged use of the oral contraceptive pill correlates with an increased risk of developing breast cancer reviewed in Harris (1996) and Henderson and Bernstein, (1996). Meta-analysis of case-control and cohort studies indicate that certain formulations of hormone replacement therapy taken for prolonged periods of time may increase the likelihood of developing the disease (Faiz and Fentiman, 1998)

Migrant studies have shown that the environment in which an individual lives is an important risk factor in the development of breast cancer (Buell, 1973; Dunn, 1975). Women in North America and Europe have a 4 to 5 fold greater chance of developing breast cancer than those in Asia (Parkin, 1993) and the higher the social class, so the

increased risk of breast cancer (Jacobsen and Lund, 1990; Krieger, 1990).

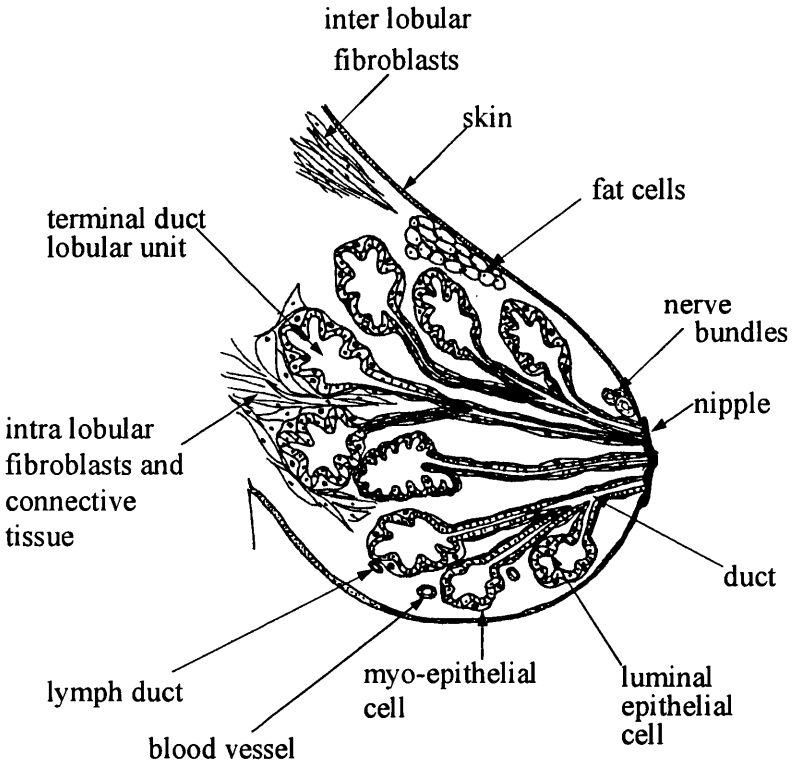
Some familial breast cancers appear to be a due to environmental factors, such as social class, but some are also associated with a genetic predisposition to the disease. It has been estimated that genetic predisposition accounts for approximately 5% of all new breast cancers cases (Ellisen and Haber, 1998). Initially a mutation to the p53 tumour suppressor gene was thought to be important (Malkin *et al.*, 1990), but since then, mutations to a further two genes, BRCA1 (King, 1990) and BRCA2 (Wooster *et al.*, 1995), have been associated with familial breast cancer. Ashkenazi Jewish women with a family history of breast cancer are more likely to have mutated BRCA1 than the general population (Offit *et al.*, 1996; Struewing *et al.*, 1997). Mutated BRCA1 has a high penetrance and carriers are estimated to have a 56% chance of developing the disease by the age of 70 (Struewing *et al.*, 1997). Breast cancers from patients with BRCA1/2 mutations are of a higher grade at presentation than sporadic breast cancers (Breast Cancer Linkage Consortium, 1997).

Of the many other types of breast diseases, atypical hyperplasia is associated with an increased risk of breast cancer (Dupont *et al.*, 1993) and women who have recurrent and extensive cystic disease are also at increased risk reviewed in Bodian, (1993). Patients with diabetes mellitus tend to be of a greater risk of developing breast and endometrial cancer (Weiderpass *et al.*, 1997), this may be related to increased levels of insulin-like growth factor in the breast tissues of these patients (Stoll,1997).

1.2.2 Breast anatomy.

The breast is often considered as a modified sweat gland which comprises 15-20 lobes (Bannister, 1995). Each lobe contains branched ducts, these arise from the lactiferous ducts of the nipple and terminate in secretory lobules called alveoli, or acini (Parks, 1957). The ducts themselves are lined by inner luminal epithelial cells and these are encased by outer myoepithelial cells. The ducts are surrounded by interlobular fibroblasts, connective tissue (stroma) and fat cells. The junctions where the terminal duct lobular units meets the ducts are thought to be the origin of most breast cancers (Wellings *et al.*, 1975). The main anatomical features of the breast are shown in figure 1.1

Figure 1.1 The adult human breast.



The lymphatic drainage of the breast is extensive, the main sites are the axillary lymph nodes and the internal mammary chain, there are also lymphatic vessels which connect with those of the contralateral breast, although no significant volume of lymph has been found to flow through these under normal conditions (Turner-Warwick, 1957). A network of small lymphatic vessels has also been found beneath the skin (Haagensen, 1986).

1.2.3 Pathology of breast cancers.

> "... a tumour is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues, and persists in the same excessive manner after the cessation of the stimuli which have evoked the change." Willis, (1973).

Our limited understanding of what influences breast tumour growth and behaviour *in vivo* means that definitions for breast tumours tend to be descriptive in nature (Walter and Israel, 1987).

1.2.3.1..... Histopathological diagnosis.

A World Health Organisation collaboration between twelve pathologists in ten countries led to an agreement on a uniform system for the histologic classification and grading of breast tumours (Scarff and Torloni, 1981; Scarff and Torloni, 1968). Malignant breast carcinomas were identified as groups of cells exhibiting irregular and atypical epithelia, with hyperchromatic nuclei and frequent mitotic figures. Those breast carcinomas enclosed within a duct or a lobule were classified as *in situ* cancers. Cancers which had infiltrated the surrounding tissues were classified as invasive. For infiltrating carcinomas, the most common are infiltrating ductal and those which are 'not otherwise specified (NOS)' (Rosen, 1996). Other types of invasive breast tumours are classified separately as 'special types' since it was thought they may represent a different prognostic group (Scarff and Torloni, 1968) and subsequently this appears to be the case (Gallagher, 1984). The most common breast cancers are shown in Table 1.1.

1.2.3.2 .....Histopathological grade.

Pathologists have tried to identify breast cancers with different behaviours with reference to the histological grade.

This is determined by:

(i) the extent of tubule formation,

(ii) the hyperchromatism and mitotic activity, and

(iii) the irregularity of size, shape and staining of the nuclei

(Bloom and Richardson, 1957; Scarff and Torloni, 1968).

Each of these factors is awarded between one and three points, the points are summed and divided by three. The values obtained give rise to three grades of breast tumour:

well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3).

Table 1.1 The most common types of breast carcinoma, in symptomatic individuals, modified from Millis, (1984).

Type	*Approximate percentage of all symptomatic breast cancers..*
In situ carcinoma	8
Invasive ductal and NOS	72
Invasive lobular	12
Medullary	3
Mucinous	2
Tubular	2
Rare varieties	1

1.2.4 The natural history of breast cancer.

1.2.4.1 .....The 'curability' of breast cancer.

In the past, breast cancer was thought to be a local disease, which spread by centrifugal permeation from the primary site, first throughout the breast tissue, into the lymphatics, then to the regional axillary lymph nodes and later to distant organs. It was predicted that extensive surgical removal of the affected breast and axilla would result in a cure and this was the basis of the surgical procedures such as the radical mastectomy of Halsted, (1898), reviewed in Haagensen, (1986). Later, this led to even more extreme surgeries such as the super-radical mastectomy of Handley *et al.*, (1956) which involved the removal of the internal mammary lymph node chain. These operations often resulted in local control of the disease, something which had not been achieved in the past, and the effectiveness of radical surgery as a cure for breast cancer became the focus of investigation. The concept of a cure for breast cancer may be described as:

> "... patients can be considered *statistically cured* if their death rate is similar to that of the normal population.... *clinical cure* for an individual refers to the apparent complete eradication of the disease... *personal cure* refers to a patient living symptom-free from breast cancer and dying of other causes..." (Harris and Hellman, 1996).

Amongst others, Brinkley and Haybittle, (1975) reported that breast cancer patients die steadily from metastatic disease for as long as 20 years following their initial surgery. Clearly, extensive surgery and radiotherapy did not cure the majority of patients and it was estimated that only 15-20% of patients have a normal life-expectancy, reviewed in Baum, (1976) and British Medical Journal Editorial, (1972).

1.2.4.2 .....Breast cancer - a systemic disease at presentation ?

Long term follow-up studies, of patients who were axillary lymph node negative at the time of their initial surgery, and who would be predicted to have a favourable outcome, show continued development of distant metastases. For example, Fentiman *et al.*, (1984) showed that 25-35% of patients continued to show metastases up to 20 years after treatment. In addition, isotope labelling experiments showed that patients with 'early' breast cancer (less than 4 cm in diameter) may already have distant bone metastases when their primary cancer is detected (Galasko, 1972). These observations suggest that the majority of breast cancers have already metastasized to distant sites before the primary cancer is detected and that breast cancer should be treated as a

systemic disease.

1.2.4.3 .....Breast cancer - a number of diseases at presentation.

Studies of patients whose cancers were detected by screening mammography have suggested that very small breast cancers, less than 1.5 cm in diameter, may be effectively treated by loco-regional therapies alone (Shapiro, 1989; Tabar *et al.,* 1992). This lends some weight to the hypothesis that breast cancer represents a number of different diseases at presentation, the so-called 'spectrum theory' (Hellman and Harris, 1987).

The 'spectrum theory' of breast cancer proposes that:

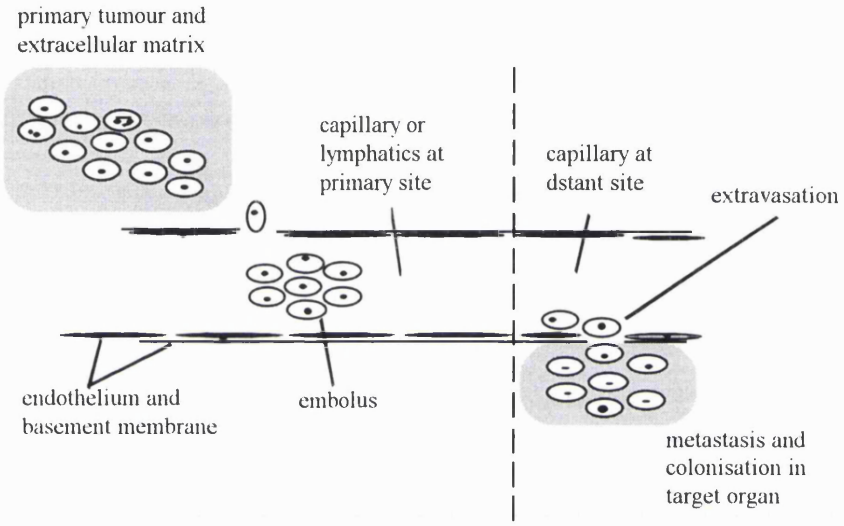
> "axillary lymph node metastases normally precede distant metastases...
> axillary lymph node status is simply a marker of long-term outcome...
> breast cancer is a systemic disease in many, but not all, cases..."
>
> Harris and Hellman, (1996).

Further studies are required to determine if very small tumours, such as those detected by screening mammography, represent a population of cancers which can be cured by loco-regional surgery alone or whether they are simply cancers which have already metastasized but are being detected earlier in their natural history (Hibberd, 1986).

1.2.5 The metastatic nature of breast cancer.

Virtually all deaths from breast cancer result from metastases. A multitude of factors is necessary for the successful development of metastases (Nicolson, 1984). Some of the steps in the metastatic cascade are shown schematically in figure 1.2.

Figure 1.2 A schematic diagram illustrating the steps involved in the distant spread of breast cancer cells.



1.2.5.1 .....Local spread of breast cancer.

Breast cancer cells initially spread by direct infiltration into the surrounding tissues, often in a characteristic 'stellate' manner along bundles of connective tissue or into the surrounding fat (Haagensen, 1986).

Cancer cell infiltration is thought to occur by:

(i) the secretion of enzymes which degrade the extracellular matrix (Evers *et al.*, 1982; Jones and DeClerck, 1982), this is balanced by inhibitory factors such as tissue inhibitors of metalloproteinases (Stetler-Stevenson *et al.*, 1993),

(ii) the expression of cell adhesion molecules which allow cancer cells to bind to the extracellular matrix (Bernstein and Liotta, 1994; Juliano and Varner, 1993) and

(iii) the stimulation of the cancer cells by motility factors (Van Roy and Mareel, 1992).

1.2.5.2 .....Intravasation of cancer cells into lymphatic ducts / blood vessels.

Eventually the mass of cancer cells reaches a lymph duct or blood vessel. The blood

vessel may be part of the normal anatomy of the breast, or may have developed as a result of angiogenesis within the primary tumour (Folkman, 1995; Folkman *et al.*, 1970). The cancer cells enzymatically degrade the basement membrane (Liotta *et al.*, 1986) and penetrate the duct or vessel, this probably happens at a gap in the endothelium (DeBruyn and Cho, 1982). Individual cells or clumps of cells detach from the tumour by a loss of homotypic cell adhesion, (Weiss, 1985), this may be as a result of the down-regulation of cell-adhesion molecules (CAMs) such as E-cadherin (Vleminckx *et al.*, 1991). After detachment from the main bulk of the tumour the cancer cells intravasate the duct or vessel.

1.2.5.3 .....The blood-borne spread of breast cancer.

The concept of blood-borne metastatic spread of breast cancer developed as a result of observations of circulating cancer cells in patients with early operable breast cancers (Engel, 1955) and our understanding of this mechanism of breast cancer dissemination has developed as the natural history of the disease has become better understood. Whilst breast cancer cells may frequently be found in the circulation, animal models suggest that such cells do not necessarily develop into secondary cancers (Fidler, 1970), this seems to be due to the relatively short life-span of circulating cancer cells. Although clumps of tumour cells which have formed aggregates with lymphocytes may have a longer survival in the circulation (Fidler and Bucana, 1977), their survival also relies on their evading immuno-surveillance (Browning, 1995). Another reason for the failure of circulating cancer cells to form metastases is the sheer complexity of the process. Once the cancer cells have entered the circulation, in order to form secondary cancers they need to adhere to the endothelium, extravasate and colonise the organ of metastasis, these events are described in further detail below.

1.2.5.4 .....The 'arrest' of circulating cancer cells.

Cancer cells have been reported to attach to the vasculature at the site of metastasis in particular at the endothelial cell junctions, (Kramer *et al.*, 1982), or sub-endothelial matrix (Nicholson, 1982a). Observations relating to the heterogeneity of the endothelial cells themselves, however, suggest that they may have an important role in metastasis. Endothelial cells of different organs express a heterogeneous mixture of cell surface glycoproteins, reviewed by Pauli *et al.*, (1992). Cancer cell arrest at endothelial cells seems first to take place via lectin-like (carbohydrate-carbohydrate)

interactions then, later, via protein-protein interactions (Saini, 1995).
Parallels have been drawn between the mechanisms used by lymphocytes homing to sites of inflammation and cancer cell 'arrest' at sites of metastasis (Springer and Lasky, 1991). Endothelial cells, when activated by cytokines such as interleukin 1 and tumour necrosis factor, express E-selectin (endothelial leukocyte adhesion molecule, ELAM-1) and increase their expression of intracellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). These cell adhesion molecules have been implicated in the arrest of circulating leukocytes prior to their firmer adhesion to the endothelium via integrins (Springer, 1994).
The structures involved include the oligosaccharides sialyl Lewis x and sialyl Lewis a, but the precise ligands for the CAMs in the main remain uncharacterised (Varki, 1997). It has become apparent that a number of different cancers also express sialyl Lewis x and sialyl Lewis a and that these oligosaccharides can bind to carbohydrate adhesion molecules on selectins on activated endothelial cells *in vitro* (Miller *et al.*, 1996; Takada *et al.*, 1993) and *in vivo* (Dejana *et al.*, 1992; Lauri *et al.*, 1991).
The aim of our research is to identify breast cancer oligosaccharides associated with aggressive clinical behaviour as such oligosaccharides may be important in the initial binding of breast cancer cells to the endothelium at sites of metastasis.

1.2.5.5 .....Extravasation and organ colonisation by cancer cells.
In order to extravasate and colonise the organ of metastasis, the cancer cells need to adhere to the endothelium, breach the basement membrane and invade the tissue. It is thought that tumour cell extravasation at the site of metastasis is mediated by similar mechanisms to those used in the local invasion of cancer and intravasation, as described in sections 1.2.5.1 and 1.2.5.2. A supply of nutrients will be required if the metastasis is to grow to more than 1-3 mm in diameter and it is likely that this will be provided as a result of neoangiogenesis, probably under the influence of a number of growth factors, reviewed by Weiss, 1985)

1.2.5.6 .....Organ-selectivity in the development of distant metastases.
There is an element of selectivity in the sites to which breast cancer cells metastasize, illustrated in table 1.2.
The reason for organ-selectivity in the development of metastases by breast cancer cells is not known. One theory is that metastases develop as a result of the 'mechanical

interaction' between cancer cells and the vasculature at sites of metastasis (Ewing, 1928) reviewed by Fidler, (1991). Whilst this is of some importance, the 'mechanical interaction' theory does not entirely explain the clinical observations of organ-selectivity which have been described in breast cancer (Weiss, 1985). The most probable theory for organ-selectivity in cancer metastasis is that circulating cancer cells will only form secondary cancers if they are in a suitable environment. This idea has been described as the theory of 'seed and soil' of metastasis, the cancer cell is likened to a 'seed' and the target organ the 'soil' (Paget, 1889). Human *in vivo* (Tarin *et al.*, 1984) and animal *in vitro* (Nicholson *et al.*, 1985) experiments support this.

Table 1.2 The most common sites of breast cancer metastasis, detected at post-mortem. Average results of ten studies of 1103 individuals, taken from (Haagensen, 1986)

Site of metastasis	*Approximate frequency (%)*
liver	56
lungs	65
bone	60
brain	15
adrenals	35
skin	21

Observations that endothelial cells from different organs express different cell surface glycoproteins and that these bind with particular oligosaccharides on the surface of the cancer cells, described in section 1.2.5.4, also support the 'seed and soil' theory of metastasis. Since cell surface glycoproteins differ according to the organ from which the endothelial cells originate, it is likely that the cancer-associated oligosaccharide ligands for such glycoproteins also differ in accordance with the sites at which the cells preferentially develop metastases.

1.2.6 Markers of prognosis in breast cancer.

The identification of markers to predict the natural history of individual breast cancers has been the focus of much investigation. Prognostic markers would be useful for the identification of breast cancer patients with:

1) very good or very poor prognosis, to prevent the over-treatment or under-treatment of these patients, and
2) an intermediate category of patients who might benefit from particular types of therapy (Clark, 1994).

An enormous variety of prognostic markers in breast cancer have been described, including clinical, pathological and biochemical (Clark, 1996).

1.2.6.1 .....Clinical

1.2.6.1.1 Stage of disease at presentation.

A number of staging systems have been devised to try to stratify patients according to outcome. All of the systems seek to determine the extent of the disease at presentation and use either clinical or pathological information, or both.

Staging systems include the Manchester system (1940), reviewed by Donegan, (1988), the Columbia classification, devised and reviewed by Haagensen, (1986) and the TNM (tumour, node, metastasis) system (UICC Committee on Clinical Stage Classification and Applied Statistics, 1961). The TNM system has been adapted to make it applicable to any tumour system, but it has been argued that in doing so, it has become so complicated that it is now of limited use (Barr and Baum, 1992; Clark, 1996; Haagensen, 1986). The main draw-back with all the staging systems is that they do not accurately predict the prognosis of patients who fall into an intermediate group of neither very good nor very poor prognosis (Stoll, 1986).

1.2.6.1.2 Demographic aspects.

Young patients, under 35 years of age, and older patients, over 75 years of age, appear to have a poorer prognosis than other groups of patients (Nixon *et al.*, 1994). In the case of patients under 35 years of age, this may be due to 'biologically more malignant' cancers at presentation, whilst in the patients over 75 years of age this may be due to more advanced stage at presentation, comorbidity, and less aggressive systemic therapies (Host and Lund, 1986).

In the United States it has become evident that black women have a worse prognosis

than white women and women from other ethnic groups (Swanson *et al.*, 1993). This may be related to more advanced disease at presentation in this group of women (Nemoto *et al.*, 1980), but some evidence suggests that even when this is taken into account, black women have a poorer prognosis (Pierce *et al.*, 1992).

1.2.6.3 .....Pathological

1.2.6.3.1 Axillary lymph node metastases.

Involvement of axillary lymph nodes with tumour is generally considered to be *the most important prognostic factor* in breast cancer (Berg and Robbins, 1966) and is used as the basis of post-operative patient management, as described below.

Surgical removal of the axillary lymph nodes and their histological examination is required as clinical examination alone is successful in predicting tumour metastases in only approximately 30% of patients (Haagensen, 1971; Wallace and Champion, 1972). In the past, axillary lymph node clearance to remove all of the many (20+) nodes was common surgical practice (Bland *et al*, 1991). More recently, lymph node sampling to obtain 10 to 15 nodes has become more widespread, this generally includes the nodes present in levels 1 and 2 of the axilla (Fisher, 1991). The examination of sentinel lymph nodes for metastatic cancer cells may reduce treatment morbidity and prevent the over-treatment of node negative cancer patients (Veronesi *et al.*, 1997) and is currently being evaluated in comparison to axillary lymph node clearance (Dixon, 1998).

The use of lymph node sampling has become more common place since the risk of recurrence, and speed at which this occurs, rises in direct relation to the number of axillary lymph nodes involved with tumour (Fisher *et al*, 1983). Patients who are lymph node negative have the best prognosis, 80% five year survival rate, those with 1 to 3 nodes have a 60% five year survival rate, 4 to 12 nodes a 30% five year survival and individuals with 13 or more involved lymph nodes have a bleak 16% five year survival rate (Fisher *et al*, 1983)

The poor prognosis of patients with 13 or more involved lymph nodes, has been used to stratify patients into new clinical trials such as high-dose chemotherapy with autologous stem cell transplant (Antman *et al*, 1997).

Other factors which are associated with poor patient prognosis are: extracapsular tumour spread (Fisher *et al*, 1976), fixation of the axillary lymph nodes, involvement of the supraclavicular nodes (Cutler and Myers, 1967) and involvement of the I

nternal mammary chain (Lacour *et al*, 1976).

The importance of micrometastases in the axilla has been studied. Patients with micrometastases (defined as 2mm diameter or less) have been found to have a better prognosis than those with macrometastases (Lane *et al*, 1961; Huvos *et al*, 1971). Axillary lymph node status provides most prognostic information during the first five years after primary surgery, after this time, patients with 1 to 3 positive axillary lymph nodes have almost similar survival patterns as those patients with axillary lymph node negative disease (Fisher *et al.*, 1983). This has led to the suggestion that extent of axillary lymph node infiltration is a reflection of the chronological age of the tumour (Mittra and MacRae, 1991), but this hypothesis does not account for the variability in lymph node involvement between tumours of similar size, type and grade at presentation. Although the axillary lymph node status remains the 'gold standard' of all the prognostic markers, there are still problems associated with its use. In particular, some patients with axillary lymph nodes free of tumour have unexpectedly short survival and other patients with many axillary lymph node metastases may live for long-periods of time despite their apparently poor prognosis (Fentiman *et al.*, 1984).

1.2.6.3.2 Size of tumour at presentation.

A number of studies have shown that tumour size is an important prognostic marker in breast cancer (Adair *et al.*, 1974). Although it was thought that the size of a breast tumour did not provide any additional prognostic information for patients with node-negative breast cancer (Valagussa *et al.*, 1978) and that it was useful mainly in the identification of poor prognosis patients with more than four axillary lymph nodes infiltrated with tumour (Fisher *et al.*, 1969), it has become apparent that the tumour size and number of axillary lymph node metastases are associated with each other (Carter *et al.*, 1989), but lymph node status remains the single prognostic marker of choice (Carter *et al.*, 1989).

1.2.6.3.3 Histopathological type of breast cancer.

The pathology of breast cancer has been described earlier, section 1.2.3.

Some of the special types of breast cancer, if found as a single population of cells, are associated with a good prognosis. These include mucinous, cystic, tubular and juvenile secretory carcinoma, whereas inflammatory carcinoma has been reported to be

associated with poor prognosis, reviewed by Gallagher, (1984).

1.2.6.3.4 Histopathological grade of the cancer.

The grade of a breast cancer can provide useful prognostic information, particularly when taken in conjunction with the axillary lymph node status of the patient (Bloom and Richardson, 1957). There have, however, been problems associated with using grade as a prognostic tool, for example the variability in assessment from one pathologist to another (Gilchrist, 1985).

Some consideration has been given to whether the method used for determining the grade of a breast cancer has been optimised to provide the greatest amount of prognostic information. The most commonly used grading system is a modification of that described by Scarff, Bloom and Richardson which uses *three equally weighed* factors: extent of de-differentiation, pleomorphism and mitotic activity (Bloom and Richardson, 1957; Scarff and Torloni, 1968; National Coordinating Group for Breast Screening Pathology, 1995).

The factors used in determining the modified Bloom and Richardson grade of a breast cancer are shown in table 1.3. The points are summed: 3 to 5 points equals Grade 1 cancer, 6 to 7 points equals Grade 2 cancer and 8 to 9 points equals Grade 3 cancer.

Table 1.3 Factors used in determining the grade of a primary breast cancer (from National Coordinating Group for Breast Screening Pathology, 1995).

	Score of One	***Score of Two***	***Score of Three***
Tubule formation	*Majority of tumour (greater than 75%).*	*Moderate amount (10-75%)*	*Little or none (less than 10%).*
Mitoses	*0-6 mitoses per ten high power fields.*	*7-12 mitoses per ten high power fields.*	*More than 12 mitoses per high power field.*
Pleomorphism, ie. Irregularity of size, shape and staining of nuclei.	*Nuclei small, regular outlines, unitorm nuclear chromatin.*	*Cells larger than normal, visible nucleoli, moderate variation in size and shape.*	*Vesicular nuclei, marked variation in size and shape, occasional large and bizarre forms.*

The importance of mitotic activity as a reflection of proliferative capacity of the cancer cells has been examined. Measures of proliferative activity of breast tumours have been found to provide useful additional prognostic information if used in combination with the grade of the tumour (Russo *et al.*, 1987). This has led to a number of tests for proliferative activity, these include the mitotic index (Baak, 1990), thymidine labelling index (Silvestrini, 1991), S-phase fraction / DNA ploidy (Hedley *et al.*, 1993; Olszewski *et al.*, 1981) and Ki67 monoclonal antibody staining (Gerdes *et al.*, 1983), but none has proved to be sufficiently reliable, reproducible and, with the exception of Ki67, easy to use routinely as prognostic markers.

Recently, patterns of genetic abnormalities in breast cancer have been described in relation to the grade of the primary breast cancer. Breast cancers from patients with mutations in the BRCA1 and BRCA2 genes have been found to be of a higher grade than breast cancers without these mutated genes (Breast Cancer Linkage Consortium, 1997).

1.2.6.3.5 Other tumour characteristics.

Other histological features such as lymphatic and blood vessel invasion (Weigand *et al.*, 1982) and extent of intraductal carcinoma (Clark, 1996) have been described in a number of reports, but the prognostic usefulness of these remains unclear.

1.2.6.4 .....Biochemical.

1.2.6.4.1 Steroid receptors.

Oestrogen receptor (ER) and progesterone receptor (PR) provide useful prognostic information. In ER and PR positive patients there is longer recurrence-free survival post diagnosis than in patients deemed ER and PR negative (Clark and McGuire, 1988). The expression of ER and PR is associated with the proliferative capacity of the tumour (Silvestrini, 1994).

In the past, it was thought that the main clinical value in identifying ER positive patients lay in their responsiveness to hormonal therapies, more recently, however, it has been reported that ER negative patients may also benefit from hormonal treatments (Early Breast Cancer Trialists' Collaborative Group, 1992). This suggests that either: the 'cut-off point' for the categorisation of ER positive and ER negative tumours needs adjustment, reviewed in (Clark, 1996), or that responsiveness to hormonal therapies may be mediated by other cellular mechanisms which are not fully understood

(Benson and Baum, 1993).

1.2.6.4.2 Polypeptide hormone receptors.

In addition to steroid hormones, there are polypeptide hormones in the serum for which there are cell surface receptors, their possible stimulatory effects on epithelial cells has led to the study of their receptors as prognostic markers in breast cancer. Probably the most extensively studied is the epidermal growth factor (EGF) which has been shown to stimulate breast cancer cells *in vitro* (Osborne *et al.*, 1980). The receptor for EGF (EGFR) is over-expressed in breast cancer compared with normal non-malignant breast cells (Fitzpatrick *et al.*, 1984). EGFR levels appear to be related to the proliferative activity of the cells and inversely to the expression of ER (Sainsbury *et al.*, 1985; Toi *et al.*, 1990) and its expression is an independent prognostic marker for early recurrence (Gasparini *et al.*, 1992; Sainsbury *et al.*, 1987). EGFR levels may also be useful in the identification of poor prognosis patients who are axillary lymph node negative and ER negative (Sainsbury *et al.*, 1987).

The HER-2 gene (also known as c-erb B2 / *neu*) encodes for a protein with similar homology to the cytoplasmic domain of EGFR. When the levels of the HER-2 gene product and EGFR are taken in conjunction with one-another, they offer useful information regarding 5 year recurrence-free survival (Osaki *et al.*, 1992).

1.2.6.4.3 Enzymes secreted by breast cancer cells.

A number of enzymes which degrade the extracellular matrix (ecm) are implicated in the intra- and extra-vasation steps in the metastatic cascade, as described in sections 1.2.5.2 and 1.2.5.5. In breast cancer, elevated expression of a number of enzymes has been reported, in particular the plasminogen activators (Duffy *et al.*, 1988; Grondahl-Hansen *et al.*, 1993) and lysosomal enzyme, cathepsin D (Westley and Rochefort, 1980).

Cathepsin D levels are higher in breast cancer than in normal breast tissue (Garcia *et al.*, 1984) and the oligosaccharides attached also appear to be different in breast cancer cells (Capony *et al.*, 1989). This suggests an over-expression as well as an alteration in the processing of the protein during malignancy. Reports regarding the usefulness of cathepsin D as a prognostic marker, are somewhat contradictory, some retrospective (Thorpe *et al.*, 1989) and prospective studies (Pujol *et al.*, 1993) have found elevated levels associated with poor prognosis, but others have failed to do so, for example

Henry *et al.*, (1990). These anomalies may be due to variation in the methods used or confounding effects of stromal cathepsin D (Walker *et al.*, 1994).

1.2.6.4.4 Cell surface molecules as prognostic markers in breast cancer.

Alterations in the cell surface glycoproteins of breast and other cancers have been extensively reviewed, for example by Dennis, (1992); Kim *et al.*, (1996) and Regelson, (1995). A number of the changes observed in breast cancer are described below. A more extensive review of the alterations in glycosylation in breast and other cancers is given in section 1.5.

1.2.6.4.4.1 Cell adhesion molecules.

Changes to the adhesive properties of tumour cells are probably a prerequisite for successful metastasis, both a decrease in homotypic (tumour cell) adhesion and an increase in heterotypic adhesion (such as tumour cell adhesion to endothelium on blood vessels) may be important (Nicholson, 1982b).

Cell adhesion molecules (CAMs) are a large diverse group of glycoproteins, they have intracellular and extracellular domains and may cross the cell membrane one or more times (Kreis and Vale, 1993). Both the protein and the oligosaccharide part of CAMs have been reported to be involved in cell signalling pathways (Keely *et al.*, 1998; Yu *et al.*, 1993). In cancer, therefore, CAMs may have a role in mediating cell proliferation as well as adhesion.

Alterations in the expression of various CAMs have been reported in breast cancer, but few have been evaluated as prognostic markers.

Decreased levels of E-cadherin, associated with poor prognosis breast cancer, has been reported using immunohistochemical (IHC) techniques (Oka *et al.*, 1993; Siitonen *et al.*, 1996). More recently it has been suggested that an enzyme-linked immuno-adsorbent assay, which is a quantitative method, is more reliable than the IHC methods, and that when this is used, E-cadherin is a useful prognostic marker in breast cancer (Maguire *et al.*, 1997). Further studies with longer follow-ups are required to determine whether decreased E-cadherin expression is a prognostic marker in breast cancer.

Decreased expression of the lectin-like cell adhesion molecule galectin-3, which binds the basement membrane glycoprotein laminin, has been reported in breast cancer (Castronovo *et al.*, 1996). Whether the expression of this CAM relates to long-term

outcome, or if it is simply a feature of the histological grade of the tumour (Idikio, 1998) will need to be investigated further if it is to be of any use as a prognostic marker in breast cancer.

1.2.6.4.5 Other alterations in the glycoproteins in breast cancer.

Changes in the glycoproteins expressed by breast cancers, shall be discussed in two parts: those associated with large mucin-like proteins, and those which are found on smaller glycoproteins.

1.2.6.4.5.1 Mucins and carcinoembryonic antigen..

Mucins are characterised by their high molecular weight, over 200,000 daltons, and extensive O-linked glycosylation, which typically accounts for over 50% of their weight. Mucins are described further in section 1.4.2.2.

A number of monoclonal antibodies, directed against human milk fat globule membrane glycoproteins, epithelial membrane antigens and mucins from cancer cell lines were produced and appeared to show promise as a means of identifying malignant breast cells (Burchell, *et al.*, 1983; Ellis *et al.*, 1987; Ellis *et al.*, 1985, Kim *et al.*, 1991). However, the expression of all of the mucin antigens detected by these antibodies was subsequently shown to relate to the grade of the tumours rather than their metastatic capability (Berry *et al.*, 1985, Ellis *et al.*, 1985; Eriksson *et al.*, 1993). At present, mucin antigens appear to be more useful as markers of differentiation rather than prognosis in breast cancer. Carcinoembryonic antigen (CEA) is a large molecular weight transmembrane molecule which was originally detected in serum and cancer cell extracts from patients with colorectal cancer (Gold and Freeman, 1956). Many different antibodies have been produced against CEA but none has been useful in determining prognosis in breast cancer, reviewed by Foster, (1986). Cancer cells shed part of the CEA molecule into the serum, therefore the measurement of serum CEA levels may, in conjunction with other markers, be useful in monitoring response to treatment (Van Dalen *et al.*, 1996).

1.2.6.4.5.2 Smaller glycoproteins.

Cell surface glycoproteins which contain *N*-acetylgalactosamine (GalNAc) , for example, those detected by the lectin *Helix pomatia* agglutinin (HPA) may be particularly useful as prognostic markers in breast cancer, as detailed in section 1.5.3.6.

HPA lectin binding appears to be associated with poor prognosis breast cancer and very closely related to the high incidence of lymph node metastases (Brooks and Leathem, 1991; Noguchi *et al.*, 1993a; Noguchi *et al.*, 1993b). The IHC method for the measurement of HPA lectin-binding is somewhat subjective. In order for the HPA lectin-binding pattern in breast cancer to be used clinically as a prognostic marker, it will be necessary to identify the structures which are detected by the lectin and associated with poor prognosis (Walker, 1993). This approach should enable the development of reliable, validated methods for the detection and measurement of glycoproteins which bind HPA in breast cancer.

Many other prognostic markers have been described for breast cancer and some of these are listed in table 1.4.

Table 1.4 A list of other prognostic markers described in breast cancer.

	Pathological	***Genetic***	***Glycoproteins / Oligosaccharide***	***Enzymes***
Considerable evidence of usefulness.	*axillary lymph node status, grade, proliferation, tumour bed biopsies*		*EGFr pS2 HPA lectin binding*	*uPA tPA Cathepsin D*
Suggestive evidence of usefulness.	*vascular invasion, apoptosis, angiogenesis*	*BRCA1 BRCA2 Bcl-2 HER2/Neu Ataxia telengiactasia*	*CEA CA 15.3 c-erbB-2*	
Little evidence of usefulness, but under experimental scrutiny		*c-myc cyclin D1/E Int-2/FGF-3 nm23*	*Heat shock proteins 27 and 70, P-glycoprotein MUC1 MUC2 PSA Lewis x sugars Tn antigens*	*Thymidine kinase*

1.2.6.5 .....Prognostic markers - the future.

Prognostic markers may be more clinically useful if combined into an index. Several different prognostic indices have been proposed (Haybittle *et al.*, 1982; Stenkvist *et al.*, 1982). The Nottingham prognostic index includes age, tumour size, lymph node stage, tumour grade, combined together as 0.2 x tumour size (cm) + grade (scored 1-3) + lymph node stage (scored 1-3) to give a score from which the prognostic outcome is determined. For an individual with a 2 cm diameter tumour, of grade 2 and with 2 involved lymph nodes the score = 0.2 x (2+2+2) equals 4.4 and such a tumour would be considered to be of an intermediate grade (Haybittle *et al*, 1982).

The Nottingham prognostic index may be of additional use when combined with other biological factors (Galea *et al.*, 1992). The main drawback with biological markers as indicators of prognosis, is that some are purely subjective, for example IHC based techniques, whereas others are so complex that they are not within the scope of the average laboratory. The challenge ahead remains the identification of differences in the biology of poor prognosis breast cancers, and the development of reliable, validated methods for their measurement.

1.3 Glycosylation

1.3.1 The function of oligosaccharides on glycoproteins.

Glycosylation, the covalent addition of mono and oligosaccharides to proteins and lipids, is ubiquitous across the plant and animal kingdoms and glycosylation is the most common post-translational modification found to occur on proteins (Lis and Sharon, 1993).

At the protein level, we know that the attached oligosaccharides have diverse physiological functions such as assisting in the formation of a correctly folded protein (Walsh *et al.*, 1990), the hepato-clearance of serum proteins (Ashwell and Harford, 1982), protection of the protein from enzymatic degradation (Homans *et al.*, 1987), cell-cell recognition, such as sperm-egg recognition Wassarman, (1990), Rademacher *et al.*, (1988) and cellular adhesion Lasky, (1992), Springer, (1990).

The types of oligosaccharide found on proteins vary according to the tissue and the species in question, for example, oligosaccharides on γ–glutamyltranspeptidases vary between liver and kidney, and the species examined (Kobata and Takasaki, 1992). It seems that glycosylation is an essential feature of successful development, for example, particular oligosaccharides are important for both the development and survival of mice *in utero* (Stanley *et al.*, 1996).

Differences in the glycosylation of particular proteins exist with serum proteins such as transferrin and human alpha 1 acid glycoprotein exhibiting minor and more major variations in their oligosaccharide structures, these altered forms may be associated with the function of the protein, but the importance of heterogeneity in glycosylation is not yet fully understood (Rudd and Dwek, 1997).

1.4 Glycosylation of proteins and lipids.

Cellular glycoconjugates fall into four main groups: glycoproteins, glycolipids, glycosaminoglycans (GAGs) and glycophosphoinositol (GPI) anchors. Glycoproteins are found intracellularly, on the cell membrane and in the sera (Lis and Sharon, 1993), whilst glycolipids are located predominantly on cell membranes (Hakomori, 1986). GAGs form some of the extracellular matrix connective tissue components and have predominantly a structural role. GPI anchors (Low *et al.,* 1986) are exclusively membrane bound, both GPIs and inositolphosphoglycans, from non-protein bound GPIs, have recently been implicated in cell signalling Merida *et al.,* (1990); Rademacher *et al.,* (1994). The structure of oligosaccharides varies according to the glycoconjugate to which they are attached, for example in GAGs the oligosaccharides are formed from many repeating di- and tri- saccharides and when attached to the peptide backbone help to form rigid molecules in keeping with the GAG's structural role (Hascall *et al.,* 1991).

1.4.1 The types of monosaccharides found in humans.

Seven monosaccharides are commonly found on the oligosaccharides linked to glycoproteins and glycolipids and are shown in figure 1.3. In addition, neuraminic acid may be substituted in triplicate with N-acetyl or N-glycolyl neuraminic acid, either on the 4, 7, 8 or 9 hydroxyl group (Harduin-Lepers *et al.,* 1995), this gives rise to considerable diversity in the structures which may be formed when different monosaccharides link together. Even greater diversity is created when monosaccharides link to one another, since they may do so via alpha or beta bonds, illustrated in figure 1.4.

Figure 1.3 Monosaccharides associated with human glycoproteins and glycolipids

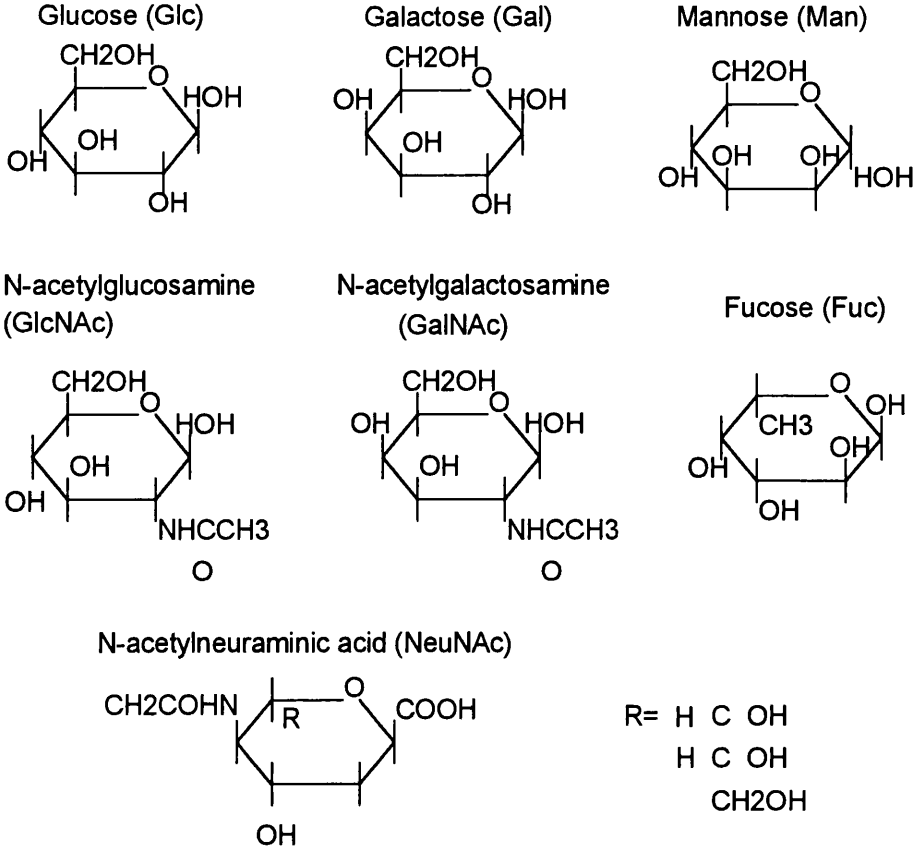
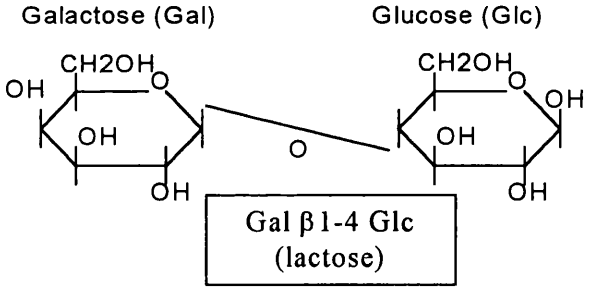
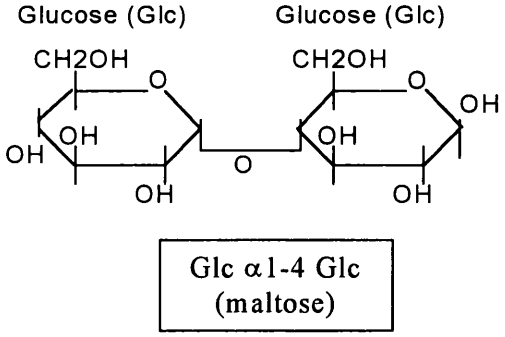


Figure 1.4 Monosaccharide binding via alpha and beta bonds.





1.4.2 Glycosylation of proteins

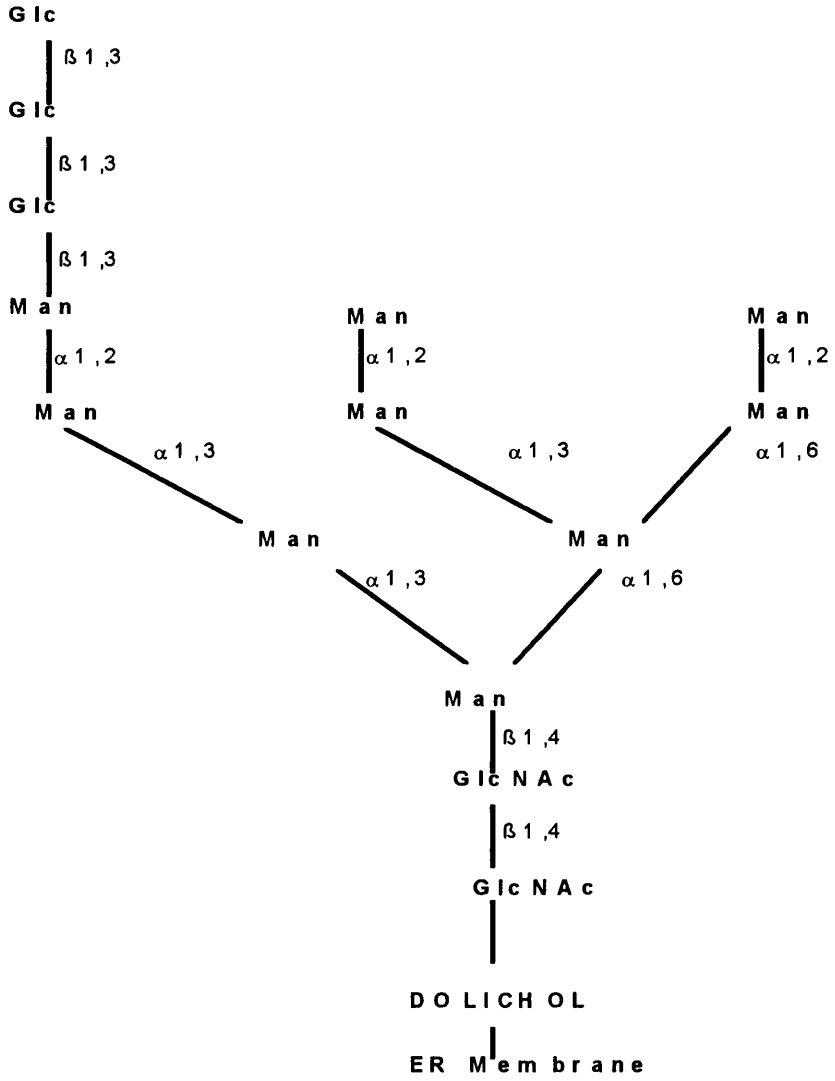
The glycosylation of proteins is described by the type of linkage between the oligosaccharide and the protein, there are two main types of linkage N- and O-linked. In N-linked structures the oligosaccharides are attached to the protein via nitrogen on asparagine and in the O-linked structures the oligosaccharides are attached via oxygen on serine or threonine of the protein backbone.

1.4.2.1 .....The formation of N-linked oligosaccharides.

1.4.2.1.1 Production of lipid intermediate.

N-linked oligosaccharide formation starts in the endoplasmic reticulum. A dolichol molecule is anchored to the endoplasmic reticulum (ER) membrane and glycosyltransferase enzymes add monosaccharide nucleotides in a specific manner resulting in a large lipid-bound oligosaccharide intermediate Hubbard and Ivatt (1981), Kobata and Takasaki (1992), Lis and Sharon (1993), figure 1.5.
The lipid-bound oligosaccharide is transferred to the nitrogen of asparagine (Asn) on the growing polypeptide chain by dolichylpyrophosphoryloligosaccharide: polypeptide oligosaccharyltransferase. It has been found that the asparagine molecule must be in the sequence Asn---X---Thr/Ser where X may be any amino-acid other than proline (Voet and Voet, 1995). N-linked glycosylation does not always take place, even when the correct amino-acid sequence occurs. This may be due to a variety of reasons, for example, competition of substrate, transport of nucleotide donors to the ER, presence of glycosyltransferases and inaccessibility of enzymes to the growing protein backbone (Schachter, 1991). The observation that oligosaccharides often protrude from the polypeptide chain into the surrounding space, lends weight to this (Homans, 1994).

Figure 1.5 The glucosylated oligosaccharide lipid intermediate, from Kobata and Takasaki, (1992).



1.4.2.2 .....Trimming the lipid intermediate and formation of final oligosaccharide.

After attachment to the nascent polypeptide chain, the oligosaccharide is trimmed by a series of glycosidases in the Golgi. First, the glycosidases remove the glucose, then the mannose molecules. Depending on how many mannose (Man) molecules are removed, different types of N-linked oligosaccharides are produced. If none, or only some are removed, an oligomannose type oligosaccharide results. When, however, the two Man molecules from the antennae of the Manα1-3 branch are removed, the addition of GlcNAc results in a hybrid type oligosaccharide. Finally, the removal of

the remaining two Man molecules, from the antennae of the Manα1-6 branch, results in a complex type oligosaccharide being formed (Kobata and Takasaki, 1992), the types of oligosaccharides produced are illustrated in figure 1.6.

A common core of Man3GlcNAc2 is observed in all N-linked oligosaccharides (Kobata and Takasaki, 1992). The action of sequential glycosyltransferases in the trans Golgi results in a wide range of extensions to the oligosaccharide arms, figure 1.7. The posttranslationally modified protein is then transported via vesicles to the cell surface (Zubay, 1993).

1.4.2.3 .....O-linked oligosaccharides.

All O-linked oligosaccharides are characterised by an alpha bond of an oxygen molecule, from a monosaccharide, to serine or threonine on a polypeptide backbone. Many different types of O-linked oligosaccharide exist (Hounsell *et al.*, 1996) and a variety of functions have been described (Fukuda, 1994).

O-linked oligosaccharides vary in size, ranging from simple monosaccharides to larger branched structures. Examples of O-linked oligosaccharides include GalNAc linked to serine or threonine, often extended with poly-lactosamine branches; core GlcNAc only, linked to serine or threonine (Holt and Hart, 1986; Holt *et al.*, 1987) and xylose linked to serine or threonine found in proteoglycans. The most well known O-linked oligosaccharides are the blood group antigens, such as the ABH system, the Lewis system and Thomsen-Freidenreich antigens found on erythrocytes (King, 1994).

Our understanding of the biosynthesis of O-linked oligosaccharides has been greatly influenced by the study of mucins. Mucins are carbohydrate-rich cell membrane associated molecules which can also be found in mucus seretions. Mucins usually contain regions of extended peptide repeats, often duplicated; these regions, called tandem repeats, may be enriched in serine, threonine and proline and are frequently the site of O-linked oligosaccharides (Gendler *et al.*, 1991). Apomucins, ie the deglycosylated structures, may be several hundred kDa in weight, but when glycosylated the molecular weight may rise to several million daltons (Kim *et al.*, 1996).

Figure 1.6 Types of N-linked oligosaccharides

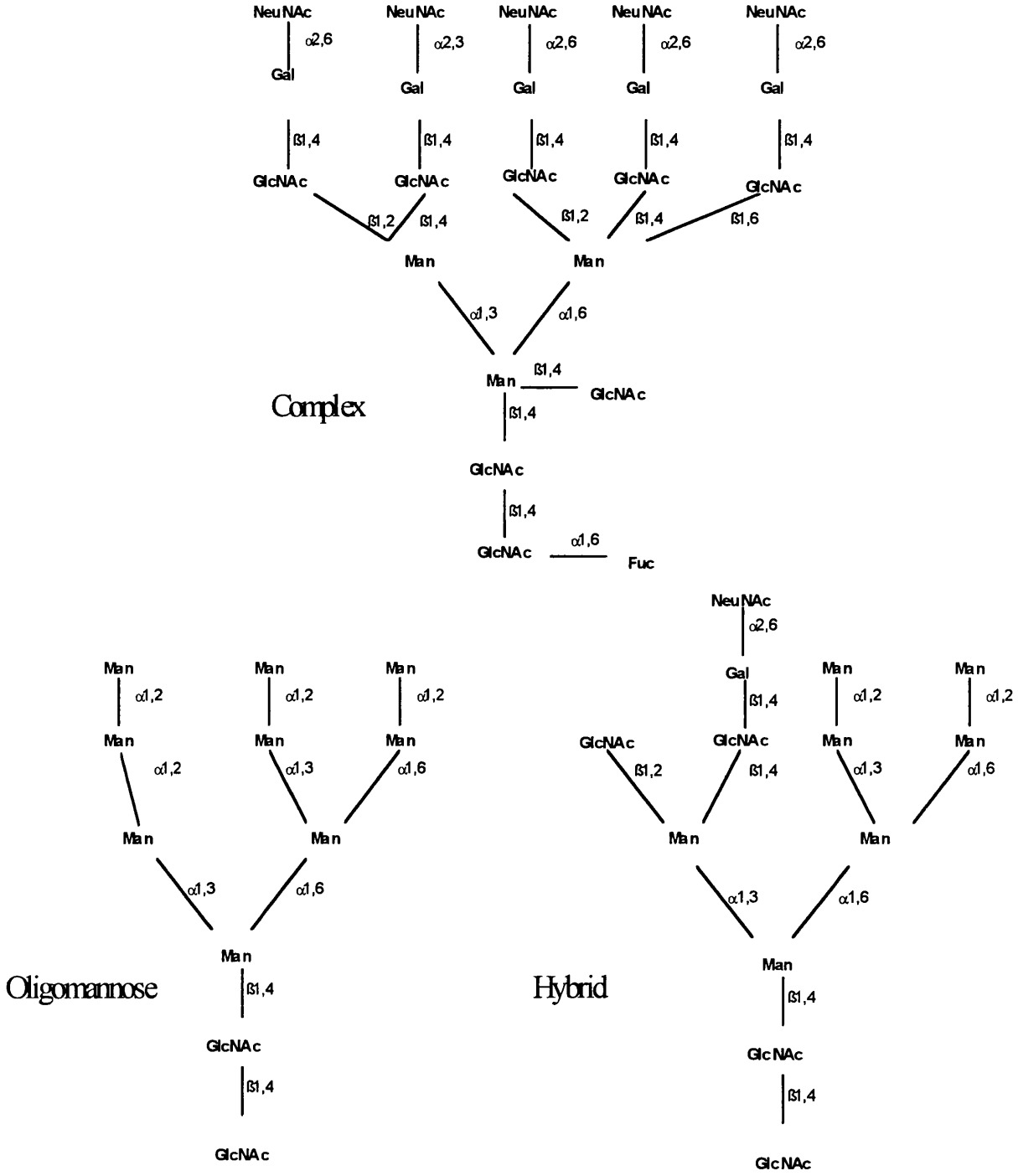
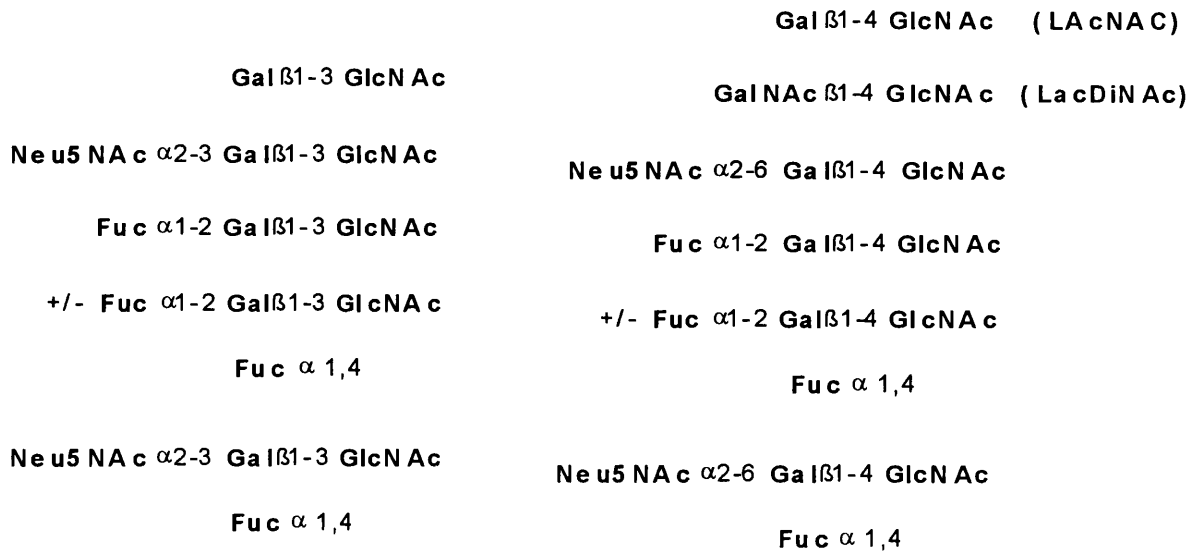


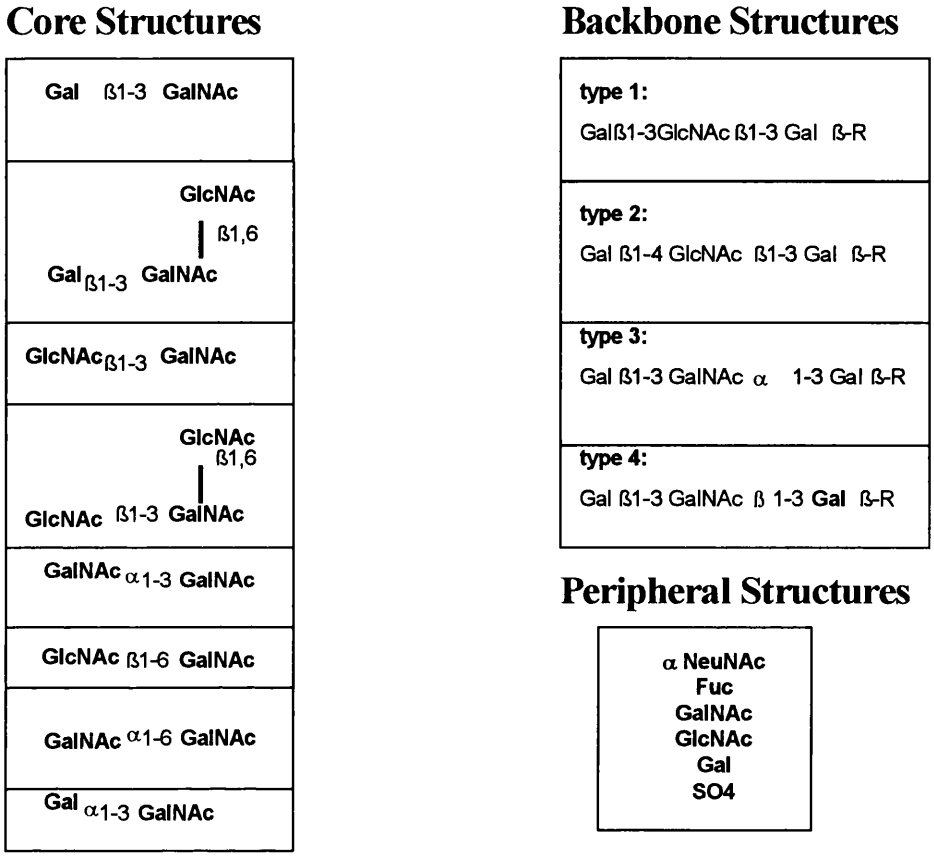
Figure 1.7 The main types of extensions found in N-linked oligosaccharides.



1.4.2.3.1 Formation of O-linked oligosaccharides.

In contrast to N-linked oligosaccharides, O-linked oligosaccharides are not produced *en bloc* and transferred to the growing peptide backbone, instead they are generally thought to be formed by the sequential action of glycosyltransferases and their appropriate nucleotide donors in the trans Golgi (Hanover *et al.*, 1982) there is, however, some evidence that they may also be formed in the endoplasmic reticulum (Strous, 1979). N and O-linked oligosaccharides exhibit different core structures but have in common some of the extensions and branches, shown in figure 1.8

Figure 1.8 Types of O-linked oligosaccharides found on glycoproteins and mucins.



1.4.3 Glycosylation of lipids.

Our work has focussed on the oligosaccharides attached to glycoproteins, but the oligosaccharide portion of glycolipids have been implicated in both cellular signalling and recognition (Zubay, 1993).

1.4.4 Enzymes of glycosylation and glycosidases.

The enzymes responsible for construction of oligosaccharides are the glycosyltransferases (GTases) and the glycosidases.

1.4.4.1 .....The structure of glycosyltransferases.

The GTases involved in oligosaccharide manufacture are themselves glycoproteins and are found attached to the ER and Golgi membranes (Schachter, 1991). Amino acid sequences, predicted from cDNA structures, suggest that GTases are transmembrane molecules with a cytoplasmic tail, a trans-membrane signal-anchor

region, a stem region and a catalytic domain (Paulson and Colley, 1989).
All the *N*-acetylglucosaminyltransferases (GnT) genes cloned since Paulson and Colley proposed the basic structure show similar basic domains (Taniguchi and Ihara, 1995).

1.4.4.2 .....Diversity, distribution and function of glycosyltransferases.
The diversity of complex oligosaccharides and the specificity of the GTases for their nucleotide donors, suggests that there should be at least 100 GTases to enable the construction of the different complex oligosaccharides found *in vivo* (Schacter, 1994). Many of the cDNAs of GTases involved in the biosynthesis of N-linked oligosaccharides have now been cloned. At the end of 1985 four GTases had been cloned but by the end of 1994 this figure had risen to 130 (Field and Wainwright, 1995). The galactosyl-, fucosyl- and sialyltransferases are the most intensely studied, presumably because they are responsible for the addition of terminal monosaccharides, which may then be exposed by virtue of being located on the outside of the oligosaccharide molecule. The cloning of GTase genes has revealed that they are conserved evolutionarily and that in some cases several enzymes with the same function exist (Harduin-Lepers *et al.*, 1995).
The genes for the addition of GlcNAc to Man in N-linked oligosaccharides have been found in most of the tissues of the human body and the genes for GnT-I, II, III and V have been cloned (Taniguchi and Ihara, 1995). In the case of the GnT-I gene knock-out mice (Ioffe and Stanley, 1994), the absence of this gene was fatal to mice *in utero* between 9.5 and 10.5 days. The murine embryos were reported to be developmentally retarded, showed impared vascularisation and neural tube formation. It is predicted that knock-out mice will provide valuable information regarding the importance of particular types of oligosaccharide linkages during embryonic development and other cellular functions.

1.4.4.3 .....The mode of operation of glycosyltransferases.
GTases act in competition with one another, for example, multiple polypeptide GalNAc transferases exist to form the core for O-linked oligosaccharides, but each enzyme may act on the same substrate and produce the same product (Marth, 1996). Which of the enzymes is successful appears to depend on the levels of the enzymes, the levels of the appropriate nucelotide donors and the presentation and accessibility

of the oligosaccharide to the enzyme's active site (Brockhausen *et al.*, 1996). Enzyme levels are both tissue and functionally regulated at the transcription level (Paulson and Colley, 1989), but some evidence exists that protein kinases either directly or indirectly regulate GTases (Ju *et al.*, 1995).

1.5 Altered glycosylation in cancer.

1.5.1 Background on oligosaccharide changes in cancer.

Some key studies initially linked oligosaccharides and malignancy.

Using lectins, "glycoproteins of non-immune origin which bind sugars" (Goldstein *et al.*, 1980). Aub et al reported that malignant cells tend to clump together more than their non-malignant counterparts (Aub *et al.*, 1965). Such clumping was found to be due to a non identified 'cell surface marker' subsequently shown to be the oligosaccharides which are attached to proteins and lipids. Further work suggested that Aub's observations were not 'transformation dependant' and appeared to occur in normal as well as transformed cells (Sivak and Wolman, 1972).

Changes in cell surface glycosylation in neoplasia was established by Warren *et al.*, (1972) who used metabolic labelling to compare the oligosaccharides of cell membrane proteins in normal and virally transformed baby hamster kidney (BHK) cells. An increase in the size of glycopeptides was attributed to fucose and sialic acid residues and was observed in cells which were actively growing. The observation was thought to be associated with a novel growth dependent enzyme (Warren *et al.*, 1972). Work by Ogata et al (1976), however, using transformed BHK cells, showed that the changes were more probably due to the presence of large branched oligosaccharides, particularly galactose and N-acetylglucosamine sugars rather than sialic acid (Ogata *et al.*, 1976).

Large numbers of monoclonal antibodies to carcinoma and developmental associated antigens were generated using hybridoma technology in the late 1970s. Using these monoclonal antibodies, it was shown that many of these antigens were oligosaccharides, some of which were found on both glycoproteins and glycolipids. Some antigens, usually developmentally regulated, were also found in carcinomas as oncofetal antigens (Feizi, 1985).

1.5.2 Approaches used to monitor changes in glycosylation and cancer.

Altered glycosylation in cancer has been monitored using a variety of histochemical, biochemical and molecular biological approaches. Oligosaccharide structures in normal and malignant tissues have been compared using lectins or antibodies as probes (Brooks and Leathem, 1991; Burchell *et al.*, 1987), other studies have investigated the effect of transformation (Yamashita *et al.*, 1984) and / or lectin resistance on cellular glycosylation (Altevogt *et al.*, 1983; Takano *et al.*, 1994). The oligosaccharides found

on metastatic cell lines have been investigated by comparing lectin binding patterns (Reese and Chow, 1992; Schumacher *et al.*, 1994a), and the levels of the glycosyltransferases responsible for the construction of oligosaccharides have been monitored by measuring enzyme levels in cell lysates (Easton *et al.*, 1991; Harvey *et al.*, 1992). More elaborate studies have used glycosylation inhibitors to determine whether the oligosaccharide changes found in cancer are associated with N or O linked structures (Devine *et al.*, 1991) and whether such changes are associated with the *in vivo* behaviour of cancer cells (Dennis *et al.*, 1987).

1.5.3 Changes in oligosaccharides in a range of malignancies.

Changes to the oligosaccharide chains found in breast and other cancers are usually associated with glycoproteins (Dennis, 1992; Kobata and Takasaki, 1992) or glycolipids (Hakomori, 1985). Glycoproteins and glycolipids, however, share some common monosaccharide sequences, in particular in the outer arms of their oligosaccharide chains, described in section 1.3, therefore, tumour associated alterations to the outer arm(s) of oligosaccharide chains may be observed on both glycoproteins and the glycolipids.

1.5.3.1 .....Thomsen-Friedenreich antigens.

Hubener in 1926 and Thomsen in 1927 noted that erythrocytes have a tendency to agglutinate *in vitro* (Hubener, 1926; Thomsen, 1927). The phenomenon was linked to the presence of bacterial contaminants by Friedenreich in 1930 (Friedenreich, 1930), in particular, the action of bacterial sialidases (Anstee and Lisowska, 1990). These observations led to the antigen revealed being termed the Thomsen-Friedenreich, or T, antigen, which is now known to comprise
Galß1-3GalNAc linked α1-3 to serine or threonine (Dahr *et al.*, 1975; Vaith and Uhlenbruck, 1978). T-antigen may be found as the core disaccharide in other O-linked oligosaccharide chains (Thomas and Winzler, 1969) or otherwise may be terminated with a sialic acid residue to form sialyl-T. The Thomsen-Friedenreich antigens are a family of antigens which include sialyl-T, Tn and sialyl-Tn.

1.5.3.1.1 Tn antigen: GalNAc α1-3 Ser/Thr

Tn, the precursor molecule of the Thomsen-Friedenreich (T) antigen and MN / A blood

group antigens, forms the linkage between O-linked oligosaccharides and serine or threonine amino acids on a protein backbone (Springer and Desai, 1975). Circulating anti-Tn antibodies found in normal healthy individuals (Desai *et al.*, 1987) have been reported to bind to human breast cancers (Springer *et al.*, 1975, 1984). Other studies, using murine monoclonal antibodies raised against the Tn epitope, have failed to find the same patterns of expression (Reed, 1994; Schmitt, 1995), this may be due to the antibodies used, since some recognise the Tn antigen when present as a single monosaccharide attached to the protein backbone, whereas others only bind successfully when the antigen is presented in a clustered form (King, 1994). Whilst the usefulness of Tn in predicting prognosis in breast cancer is still unclear, there is some evidence that it may be a good indicator of outcome in uterine cancer (Hirao *et al.*, 1993).

The presence of Tn in breast cancer tissues suggests that it is concealed by other monosaccharides in normal and benign breast (Springer, 1988) and its localisation to mainly foetal and cancer tissue indicates that it may be considered an oncofoetal antigen (Barr *et al.*, 1989). Alternatively, the expression of Tn may simply be due to the 'over-glycosylation' of amino acids, perhaps not normally found to be glycosylated (Springer, 1988b).

1.5.3.1.2 STn antigen : NeuNAc α2-6GalNAcα-O-Ser/Thr

Tn antigen is usually extended to form O-linked structures but in some cases this does not occur and a sialyl-Tn (STn) structure is formed instead. Following the addition of sialic acid to Tn no further monosaccharides can be added (Kobata and Takasaki, 1992) and the oligosaccharide chain cannot be elongated any further.

Over-expression of STn has been reported in a variety of colorectal tissue disorders (Itzkowitz *et al.*, 1989) and STn expression correlates with the over-expression of α2,6-sialyltransferase-I activity in colon cancer tissue (Yang *et al.*, 1994). It has been suggested that changes in the glycosylation of the Tn family occurs as an early event in the development of a malignancy, since STn is found predominantly in carcinomas with a large *in situ* component (Cho *et al.*, 1994) and the enzyme for the formation of STn, α2,6-sialyltransferase-I, is not found to be elevated in breast cancer cells lines (Brockhausen *et al.*, 1995).

The presence of STn in breast cancer may not be associated with outcome (Miles *et al.*,

1994) as it appears to be in colorectal cancer (Itzkowitz *et al.*, 1990; Itzkowitz *et al.*, 1989). This apparent anomaly, between breast and colon cancer STn expression, may be due to the fact that STn is found mainly on mucin molecules (Itzkowitz *et al.*, 1992) themselves over expressed in colorectal cancer (Yuan, 1992). Since mucins are found in abundance throughout the gastrointestinal tract, bladder, uterus and in other tissues (Devine and McKenzie, 1992), it might be expected that the changes observed in colorectal cancer are common to other malignancies. Preliminary investigations in gastric carcinoma suggest that this may be the case (Kakeji *et al.*, 1995; Yamashita *et al.*, 1995).

1.5.3.1.3 ST and T (TF) antigen: Galß1-3GalNAcα1-3Ser/Thr

In breast cancer the non-apical expression of T-antigen has been associated with lymph node metastases (Mustac *et al.*, 1996) and may correlate with the grade and degree of differentiation of the cancer; well differentiated carcinomas expressing more antigen than poorly differentiated cancers (Beham *et al.*, 1985; Newman *et al.*, 1979). Increased expression of T-antigen has been observed in other malignancies, for example, transitional cell carcinomas (Limas and Lange, 1986), ovarian (Ghazizadeh *et al.*, 1990) and colorectal cancers (Boland *et al.*, 1982; Itzkowitz *et al.*, 1989; Orntoft *et al.*, 1985). In colorectal cancer, increased expression of T-antigen was accompanied by an increased risk of liver metastases (Cao *et al.*, 1995) and in gastric cancer a similar expression correlated with the occurrence of lymph node metastases (Yamashita *et al.*, 1995). The analysis of O-linked oligosaccharides from colorectal mucins (Campbell *et al.*, 1995) has shown that T-antigen is usually masked by other monosaccharides but becomes exposed on mucin molecules in malignancy. Alternatively the T-antigen may become mono- or di-sialylated in breast cancer and myelogenous leukaemia cells (Fukuda *et al.*, 1986; Hull *et al.*, 1989), both these phenomena may be due to the 'inappropriate localisation' of glycosyltransferases in malignant cells (Hull and Caraway, 1988).

1.5.3.2 .....Blood-group and Lewis Antigens .

The ABO blood group system was discovered by Karl Landsteiner in 1902. Since then more than 600 erythrocyte antigens have been described (Schenkel-Brunner, 1995). ABH blood-group antigens are oligosaccharides associated with membrane glycolipids

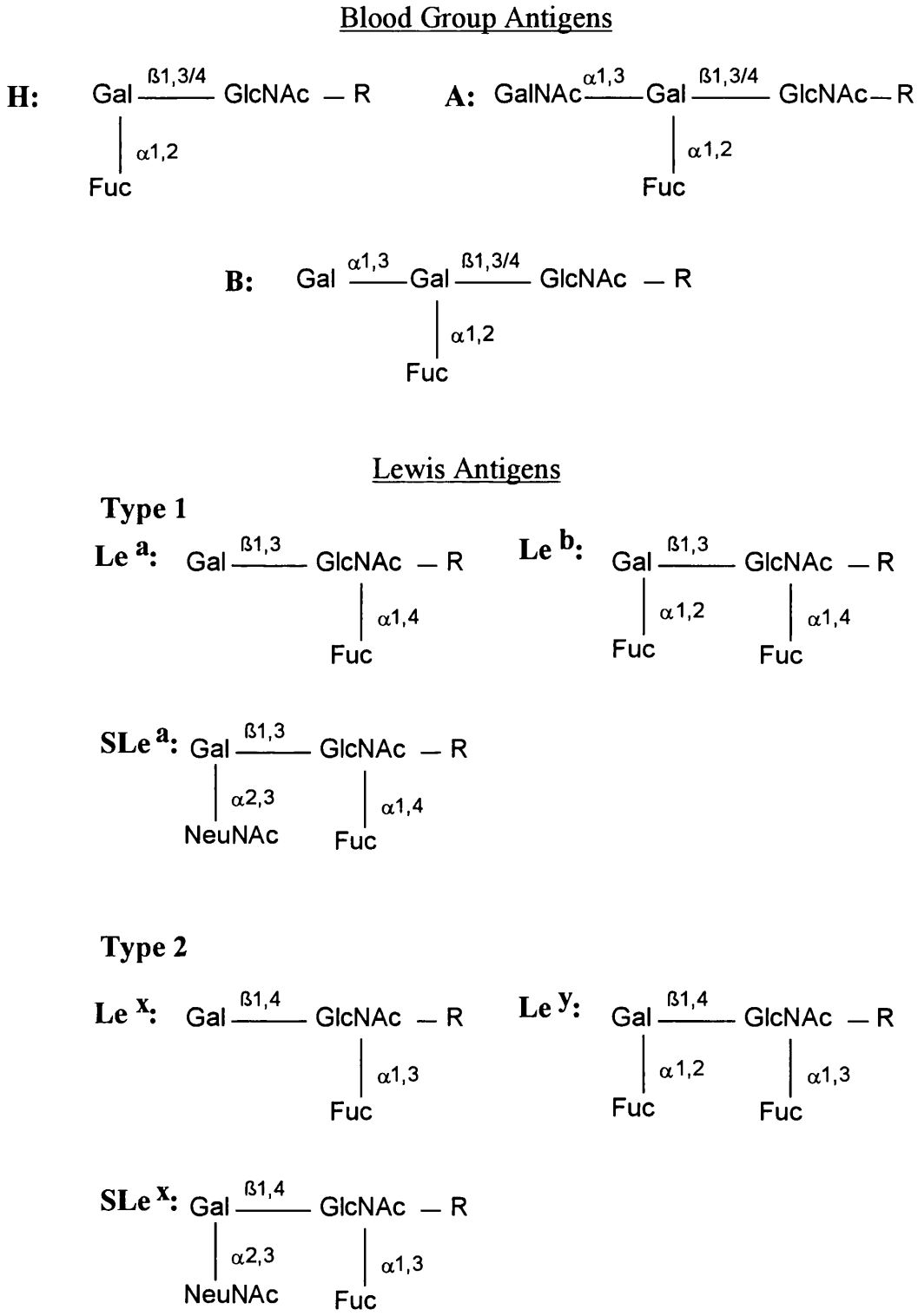
of erythrocytes, they are also found on the epithelial cells of glandular tissues (Coon and Weinstein, 1986), in secretions such as mucus and saliva and on glycoproteins, reviewed by King, (1994).

Lewis (Le) antigens are another series of oligosaccharide blood-group antigens. In common with the ABH antigens, Lewis antigens are usually associated with glycosphingolipids (Hakomori, 1986). The Lewis antigens fall into two main classes, type 1 of which Le^a and Le^b are the most common and type 2, of which Le^x and Le^y are the most common, and may be found in sialylated, dimeric or trimeric forms (Hakomori, 1986). The structures of the most common ABH blood-group and Lewis antigens are shown in figure 1.9.

1.5.3.2.1 ABH blood-group antigens.

The distribution of different blood groups amongst breast cancer patients has been evaluated but no particular blood group is clearly associated with an increased risk of breast cancer (Constantini *et al.*, 1990). In both benign breast disease and breast cancer a total or partial loss of A and B blood-group antigens has been reported (Idikio and Manickavel, 1993; Strauchen *et al.*, 1980; Ura *et al.*, 1992; Vowden *et al.*, 1986) and often at the same time the precursor to these antigens, H, has appeared elevated (Lee *et al.*, 1985). Similar patterns of ABH antigen expression have been reported in other cancers, for example, colorectal (Wiley *et al.*, 1981; Yonezawa *et al.*, 1982), gastric (Murata *et al.*, 1992), non small-cell lung carcinoma (Lee *et al.*, 1991) and transitional cell carcinoma of the bladder (Lange *et al.*, 1978).

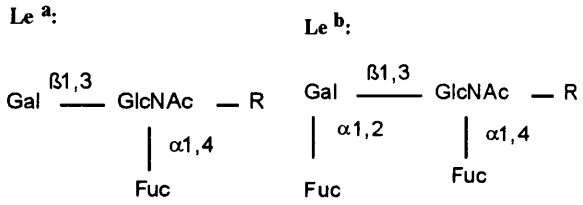
Figure 1.9 Structures of the most common blood-group and Lewis antigens.



The decreased expression of blood-group antigens A and B in breast cancer does not appear to correlate with prognosis (Lee *et al.*, 1985), but loss of expression is associated with the size of the tumour, and therefore prognosis, in non small-cell lung

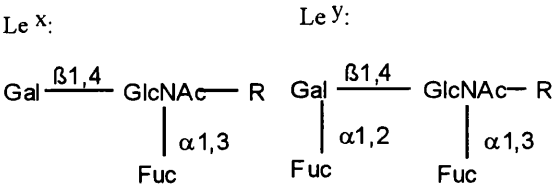
cancer (Lee *et al.*, 1991). The loss of blood-group antigen H has been related to the invasive front of the cancer cells in squamous cell carcinoma (Bryne *et al.*, 1990) and transitional cell carcinoma (Lange *et al.*, 1978). Blood-group antigen expression has been correlated with the degree of differentiation of a cell (Pann and Kuhns, 1972), the inappropriate localisation of blood-group antigens may therefore be associated with the dedifferentiated phenotype often observed in cancers. Overall, the observations suggest that in cancer tissues there appears to be a loss of and / or inappropriate expression of the usual tissue associated blood-group antigens.

1.5.3.2.2 Le^a and Le^b antigens.



In breast cancer, the Le^a, Le^b and sialyl Le^a antigens are often found in decreased amounts when compared to the surrounding normal tissue (Narita *et al.*, 1993; Ura *et al.*, 1992). The loss of expression of Le^b in breast cancer correlates with the grade of the lesion but not with the lymph node status (Idikio and Manickavel, 1993; Idikio and Manickavel, 1991). Le^a and sialyl Le^a antigens are also found on the MUC-1 mucin molecule in colon (Baeckstrom *et al.*, 1993) and pancreatic (Ho *et al.*, 1995) cancer cell lines, but whether the levels of these type 1 antigens are also decreased compared to normal non-malignant cell lines is not yet known.

1.5.3.2.3 Le^x and Le^y antigens.

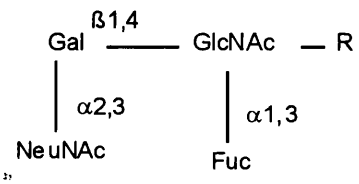


The pattern of Le^x expression during murine embryonic development (Solter and Knowles, 1978), coupled with its presence in cancer tissues, has led to the suggestion that it is an oncofetal antigen (Feizi, 1985). Le^x has been found in several solid tissue carcinomas, for example, breast (Brooks and Leathem, 1995b), colorectal (Shi *et al.*, 1984), renal (Cordon-Cardo *et al.*, 1989) and transitional cell carcinomas (Shirahama *et al.*, 1992). Le^x expression is not a prognostic indicator in breast cancer but does appear

to be associated with the invasive edge of the tumour (Brooks and Leathem, 1995b). The difucosylated Le^x structure, Le^y, is found in colorectal, liver and uterine cancer and appears to be a marker of the degree of dedifferention of these cancers (Abe *et al.*, 1986; Umezaki *et al.*, 1995; Wakabayashi *et al.*, 1995). These observations lend weight to reports which suggest that Le^x and related structures are oncofetal antigens (Feizi, 1985).

1.5.3.2.4 *Sialyl Le^x.*

SLe^x:



The possibility that tumour cells may use some of the same cell adhesion molecules as immune cells to enable them to metastasize by passing through the basement membrane, entering the circulatory system and passing into tissues (Nicolson, 1984; Ruiz and Gunthert, 1996), as described in section 1.1, has prompted studies into the expression of sialyl Le^x in cancer tissues. An *in vitro* study to investigate the adhesion of a colon cancer cell line to stimulated human umbilical cord endothelial cells has shown that adhesion may be inhibited by pre-treating the cancer cells with antibodies to sialyl Le^x, these results provide some evidence for a functional role for sialyl Le^x in adhesion to endothelial cells (Dejana *et al.*, 1992). Immunohistochemical studies have identified the presence of sialyl Le^x in a variety of carcinomas, for example gastric, colon, bladder (Fukushima *et al.*, 1984) and colon carcinoma glycoproteins (Itzkowitz *et al.*, 1986). More recent reports suggest that the presence of sialyl Le^x correlates with occurrence of metastases (Ono *et al.*, 1996) and patient survival (Nakamori *et al.*, 1993) indicating that sialyl Le^x expression on cancer cells may be important in one, or several, of the steps in the metastatic cascade.

1.5.3.3 .....Polylactosamine and Increased ß1-6 Branching.

1.5.3.3.1 Polylactosamine extensions

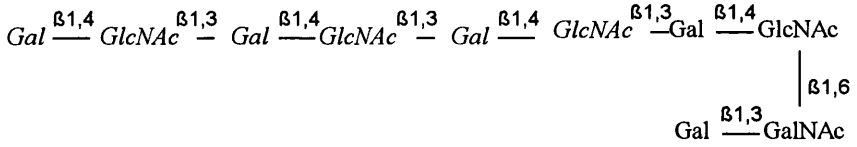
Polylactosamine units, Galß1-4GlcNAc, were first recognised in their sulphated form as keratan sulphate (Bhavanandan and Meyer, 1966). They have been found on N and O linked oligosaccharides of glycoproteins (Carlsson *et al.*, 1986; Yamashita *et al.*, 1984), lipids (Watanabe *et al.*, 1979) and mucins (Fukuda *et al.*, 1986). Polylactosamine extensions are repeating units of $(Galß1\text{-}4GlcNAcß1\text{-}3)_n$, where '*n*' is more than two (Fukuda *et al.*, 1984). Such repeating units may be linear or branched, fucosylated and sialylated (Fukuda, 1994) and sialic acid is usually attached in an α2-3 configuration to the terminal Gal molecule (Yamashita *et al.*, 1984). In N-linked oligosaccharides, polylactosamine extensions preferentially form on the GlcNAcß1-6Man (Yamashita *et al.*, 1984) branch of the α2-6 Man antenna in the tri-mannosyl core, this *in vitro,* appears to be due to enzyme specificity (Do and Cummings, 1993). In O-linked oligosaccharides, polylactosamine extensions form on 'type 2' core structures: GlcNAcß1-6GalNAc (Piller *et al.*, 1988). The polylactosamine units found in N and O -linked oligosaccharides are illustrated in figure 1.10. Polylactosamine extensions, by virtue of their fucosylation, may express the Le^x and Le^y determinants (Kannagi *et al.*, 1986; Spooncer *et al.*, 1984) and blood group antigens (Watanabe *et al.*, 1979).

1.5.3.3.2 Polylactosamine extensions and malignant transformation.

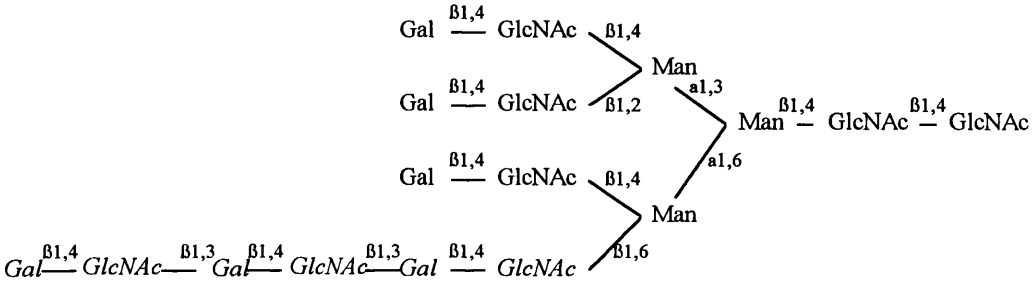
One frequent observation following malignant transformation is the acquisition of large N-linked oligosaccharides (section 1.4.1). Baby hamster kidney cells transformed with polyoma virus or Rous Sarcoma virus and NIH 3T3 fibroblasts transformed with the N-ras oncogene, each show an increase in the amount of linear and branched polylactosamine extensions on their N-linked oligosaccharides (Easton *et al.*, 1991; Pierce and Arango, 1986; Yamashita *et al.*, 1984). These observations appear to be in part responsible for the increased size of N-linked oligosaccharides found following malignant transformation. Increased amounts of polylactosamine extensions in transformed rodent cells have been associated with raised levels of GlcNAc-transferase V (Easton *et al.*, 1991; Pierce and Arango, 1986; Yousefi *et al.*, 1991), this enzyme forms the GlcNAcß1-6Man linkage, to which polylactosamine structures are attached in N-linked oligosaccharides.

Figure 1.10 An example of the polylactosamine extensions described in N-linked and O-linked oligosaccharides, modified from Fukuda, (1994).

Poly-N-acetyllactosamine residues (italics) on an O-linked oligosaccharide:



Poly-N-acetyllactosamine residues (italics) on an N-linked oligosaccharide:



Residues may be sialylated, fucosylated and/or branched as detailed in the text.

1.5.3.3.3 Polylactosamine extensions and ß1-6 branching in animal models of metastasis.

Using lectin resistant cell line mutants, Dennis and Laferte selected wheat germ agglutinin (WGA) and leukoagglutinin (L-PHA) resistant mutants of the lymphoma MDAY-D2 cell line (Dennis and Laferte, 1987). An MDAY-D2 mutant enriched in polylactosamine extensions and exhibiting increased GlcNAcß1-6 branching was highly metastatic to the lungs when injected subcutaneously into syngeneic mice (Dennis *et al.*, 1987). Mutants which lacked GlcNAc-transferase V activity did not metastasize (Dennis, 1986). A non-metastatic murine mammary carcinoma cell line, SP1, transfected with neomycin resistance and T24H-ras oncogene and selected for increased expression of GlcNAcß1-6 branching, acquired a metastatic phenotype when tested in the same murine model (Dennis *et al.*, 1989; Dennis *et al.*, 1987). Similarly, human colon carcinoma cell lines whose N-linked oligosaccharides contain branched polylactosamine antennae, with sialyl Le^x repeating units, were found to be metastatic

in nude mice (Saitoh *et al.*, 1992).

To understand possible roles for oligosaccharides containing polylactosamine extensions and GlcNAcß1-6 branching in the metastatic pathway, an immortalised, non malignant, lung epithelial cell line, Mv1Lu, was transfected with a vector system which increased the expression of GlcNAc-transferase V. The transfectants showed a loss of contact-inhibition of cell growth, a reduction in the Mv1Lu cell's adhesiveness to extracellular matrix proteins (Dennis, 1995) and increased incidence of metastasis in nude mice (Demetriou *et al.*, 1995).

Rak and Miller examined GlcNAcß1-6 branching of oligosaccharides on glycoproteins from murine breast cancer cell lines with different patterns of behaviour *in vitro*; in contrast to Dennis *et al*, they did not find that GlcNAcß1-6 branching was required for formation of metastases, but did note that GlcNAcß1-6 branching may occur during the development of a malignant phenotype, the precise role of GlcNAcß1-6 branching on cancer cells remains unclear (Rak and Miller, 1993).

1.5.3.3.4 Polylactosamine extensions and ß1-6 branching in human cancers.

Leukocytes from patients with chronic myelogenous leukaemia (CML) and acute myeloid leukaemia (AML) show increased expression of polylactosamine extensions containing sialyl-Le^x (Fukuda *et al.*, 1985), these patients also show increased levels and activity of core 2 (ß1-6 GlcNAc) transferase, the enzyme involved in the formation of the initial branch that polylactosamine extensions attach to, in O-linked oligosaccharides (Brockhausen *et al.*, 1991). Human breast and colon cancer tissues have been examined for their ability to bind L-PHA lectin, both breast and colon cancer exhibit increased GlcNAcß1-6 branching, correlating with the pathological grade of the lesion (Fernandes *et al.*, 1991). Biochemical analysis of N-linked oligosaccharides from the membranes of three human breast cancers and their lymph node metastases has also demonstrated an increase in GlcNAcß1-6 branching and polylactosamine extensions in the cancerous epithelium compared with normal tissue (Hiraizumi *et al.*, 1992). The same pattern has been observed in three oesophageal squamous carcinomas (Hiraizumi *et al.*, 1990). A direct association between the expression of polylactosamine extensions / GlcNAcß1-6 branching in primary human tumours and occurrence of local or distant metastasis has yet to be reported.

1.5.3.4 .....Sialylation and cancer

There are many different types of sialic acid, as described in section 1.4.1 and in cancer, changes occur in the amount, type, distribution and bonding of sialic acids to adjacent molecules (Bhavanandan and Furukawa, 1995).

An increase in the amount of *N*-acetylneuraminic acid (Neu5Ac) on oligosaccharide chains has been reported in colorectal cancer (Dall'Olio and Trere, 1993). Neu5Ac containing oligosaccharides seem to be associated with the metastatic spread of colon cancer cells in studies using nude mice, this may be due to the overexpression of sialyl Le^x (Saitoh *et al.*, 1992), described in section 1.4.3.3.2.4.

Some sialic acids may be termed 'oncodevelopmental antigens' since they are often inappropriately expressed on human tumours. *N*-glycolylneuraminic acid (Neu5Gc), for example, is found on gangliosides during embryonic development, minimally in adult tissues, but overexpressed in malignancy (Hirabayashi *et al.*, 1987). Neu5Gc has also been found on N-linked oligosaccharide chains in a metastatic clone of the MDAY-D2 lymphoma cell line (Takano *et al.*, 1994), on O-linked oligosaccharides, such as those attached to MUC-1 protein in the MCF-7 human breast cancer cell line (Devine *et al.*, 1991), on gangliosides of human melanomas (Furukawa *et al.*, 1989) and on human colorectal cancer tissues (Higashi *et al.*, 1985).

The attachment of sialic acid to its neighbouring monosaccharide may alter in malignancy and result in the presence of unusual structures. It is most common to find Neu5Ac attached to adjacent monosaccharides via an α2,3 or an α2,6 bond. In some cases, such as in the neural cell adhesion molecule, however, the attachment is an α2,8 bond, this type of bonding is developmentally regulated (Sunshine *et al.*, 1987) and the resulting structures called polysialic acids, reviewed in (Troy, 1995). Polysialic acids have been found in lung cancer (Michalides *et al.*, 1994; Nilsson, 1992), leukaemia and breast cancer cell lines (Martersteck *et al.*, 1996).

Alterations in the sialic acids found on the surface of cancer cells might lead to changes in their adhesive properties, by virtue of the resulting exposure of sub-terminal monosaccharides, and / or the inappropriate exposure of peptides; these changes may be important in the process of cellular dissemination and may influence the ability of cancer cells to adhere to endothelia at distant sites for the formation of metastases (Altevogt *et al.*, 1983; Yogeeswaran and Salk, 1980).

1.5.3.5 .....Glycolipid associated changes

Often the expression of tumour associated oligosaccharide antigens occurs on both glycolipids and glycoproteins, for example, the inappropriate expression of blood-group antigens in gastric cancer (Hakomori *et al.*, 1977; Wang *et al.*, 1983), increased expression of sialyl-Le^x in a variety of cancers (Fukushima *et al.*, 1984), dimeric Le^x in small-cell lung cancer (Holmes *et al.*, 1985), Le^y in gastric cancer (Abe *et al.*, 1983) and poly-N-acetyllactosamine repeats in colon and hepatocellular cancers (Miyake *et al.*, 1989). Few reports have described changes in the glycosylation of glycolipids in breast cancer (Mariani-Constantini *et al.*, 1984). The prognostic significance of changes in the oligosaccharides on glycolipids in breast cancer remains unclear (Perrone *et al.*, 1993) but changes to the glycolipids found on carcinoma cells in lung and gastrointestinal tumours appear to be of value in predicting 5 year survival rates (Hakomori, 1991; Miyake *et al.*, 1992).

1.5.3.6 .....Oligosaccharides which bind HPA lectin

In an immunohistochemical study of normal breast and breast cancer tissues, Leathem *et al* used a variety of lectins, with different monosaccharide specificities, to compare patterns of sugar expression *in vivo* (Leathem *et al.*, 1983).

Of a panel of lectins tested, that from the albumin gland of the snail, *Helix pomatia* (HPA), showed 'the strongest and most precise localisation' to the luminal epithelial cells in normal breast tissue, as well as binding strongly to most (24/26) of the cancers tested. The observed localisation of staining led to an investigation of the pattern of binding of HPA to breast cancers, described below.

1.5.3.6.1 HPA binding oligosaccharides in breast and other cancers.

A retrospective study of 179 breast cancers which had been followed-up for 15-20 years showed a significant association between HPA binding in premenopausal patients, occurrence of lymph node metastases, time to first recurrence and overall survival (Leathem and Brooks, 1987). It was postulated that there may be a glycoprotein which exhibits altered glycosylation, is associated with metastatic spread and may be under hormonal control. Fenlon *et al* in a similar study evaluated the staining pattern in 100 patients and observed the same relationship between HPA binding and poor survival (Fenlon *et al.*, 1987).

The series was increased to 373 pre- and post- menopausal breast cancers with a maximum of 24 years follow-up and revealed that there was a significant association between breast cancers which stained with HPA and the occurrence of axillary lymph node metastases (Brooks and Leathem, 1991). Using multivariant analysis, neither tumour size, grade, S-phase fraction nor age were associated with HPA binding. The presence of lymph node spread was strongly associated with HPA binding.

Other groups have confirmed the association between HPA binding and poor prognosis in breast (Fukutomi *et al.*, 1989; Noguchi *et al.*, 1993a; Noguchi *et al.*, 1993b; Thomas *et al.*, 1993), colorectal (Ikeda *et al.*, 1994; Schumacher *et al.*, 1994b), oesophageal (Yoshida *et al.*, 1993), prostate (Shiraishi *et al.*, 1992) and gastric cancers (Kakeji *et al.*, 1991).

Two reports, those of Galea *et al* 1991 and Gusterson *et al* 1993, found no association between HPA binding and lymph node status in their cohorts of breast cancer patients. These differences could be due to the use of a direct method of immunohistochemistry using peroxidase conjugated lectin, rather than an in-direct method using native non-conjugated lectin. HPA lectin appears to have a different binding site when conjugated to peroxidase (Brooks *et al.*, 1996). Processing conditions and fixation of the specimens also appears to be important in the presentation of the ligand to the lectin (Schumacher *et al.*, 1995).

1.5.3.6.2 HPA binding oligosaccharides and formation of metastases.

Lectin immunohistochemistry data described earlier resulted in the development of the hypothesis that 'HPA lectin detects oligosaccharides associated with metastases and poor survival in a variety of solid tumours'.

Data from two animal models of metastasis support this hypothesis: when the HPA positive LOX human melanoma cell line is injected into the tail vein of nude mice, the cells form multiple pulmonary metastases, in contrast to the cell lines which do not bind HPA (Kjonniksen *et al.*, 1994); in addition, the strongly HPA positive HT29 human colon cancer cell line forms pulmonary metastases within 19 days when implanted subcutaneously into severe combined immunodefficient mice (Schumacher *et al.*, 1994a).

This suggests that the oligosaccharides which bind HPA may be associated with the formation of metastases, even if only in immunocompromised mice.

1.5.3.6.3 HPA binding ligands.

The ligands for HPA which are present in metastatic breast cancers have yet to be biochemically characterised. The processing methods used to prepare paraffin-wax embedded tissue for immunohistochemistry are generally assumed to remove the lipids (Lillie, 1965) and therefore it is likely that the HPA binding ligands described are glycoprotein rather than glycolipid in origin.

It has been proposed that HPA recognises predominantly the Tn antigen (Springer, 1989). In an immunohistochemical study using serial sections of breast cancers stained with either HPA lectin or two anti-Tn antibodies BRIC III and BRIC 66, common patterns of staining were observed. However, the anti-Tn antibodies stained only about 30% of the cancers, whereas HPA stained 85% of the cancers. The Tn antigen appears to represent only one part of the HPA binding ligand (Brooks and Leathem, 1995).

1.5.4 Alterations in the oligosaccharides in breast cancer.

Lectin-binding studies have indicated that changes in expression of *N*-acetylgalactosamine containing oligosaccharides correlate with patient outcome in breast cancer (Brooks and Leathem, 1991; Fukutomi *et al.*, 1989; Noguchi *et al.*, 1993a), such immunohistochemical studies have the drawback of providing limited information regarding the oligosaccharide structures involved. Other studies of a biochemical nature have sought to identify glycosylation changes in breast cancer but the conclusions drawn have been based on observations of only a few individuals, without clinical follow-up (Hiraizumi *et al.*, 1992). Thus, whilst glycosylation changes are known to occur in breast cancer, they need to be more fully characterised using clinically relevant models of the disease, which would enable correlation of oligosaccharide expression with other known prognostic factors for breast cancer.

1.6 The work described in this thesis.

The work described here is in four parts:

1) The development of methods for the release, separation and analysis of oligosaccharides from frozen and archival breast cancer specimens. This part of the study was essential and, in practice, was undertaken before the clinical studies (chapter 2).

2) A pilot study (chapter 3) to compare the oligosaccharides from breast cancers of nine patients with long-term, and ten patients, with short-term survival. The cancers were dissected on the paraffin-wax blocks prior to oligosaccharide release and separation.

3) An evaluation of oligosaccharides released from breast cell lines grown *in vitro*, these were selected by affinity chromatography using the lectin HPA. This study was performed in order to identify any differences in the HPA binding oligosaccharides and as a means of focussing on oligosaccharides which may be associated with metastasis (chapter 4)

4) Differences were observed between the oligosaccharides extracted from the long and short-term survival breast cancers (chapter 3) and from the HPA binding cell lines (chapter 4). This enabled us to generate hypotheses regarding oligosaccharide expression and metastatic / poor prognosis breast cancer, which was then tested on a series of 76 breast cancer specimens with between five and ten years follow-up (chapter 5).

Chapter 2

Method development – the release of oligosaccharides from frozen and archival breast cancer specimens.

2.1 Introduction.

The natural history of breast cancer means that it is desirable to use measurable end points such as time to first distant recurrence and / or death when comparing biochemical changes associated with aggressive disease. To relate these end-points to the cancer cells, however, we needed to be able to extract intact oligosaccharides from tissues stored for long periods of time, such as formalin-fixed paraffin-wax embedded specimens. Proteins and oligosaccharides in fixed tissues are insoluble and it was necessary to develop methods to release oligosaccharides from such tissues. The chemical hydrazine was used for this purpose since it had been found to completely solubilise proteins from fixed tissues and, unlike enzymatic methods for oligosaccharide release, it was not necessary to solubilise the proteins by denaturing in detergents first. To check that the solubilisation had not affected the oligosaccharides we compared the oligosaccharides extracted from breast cancer specimens which had been stored either frozen or embedded in paraffin-wax blocks.

2.2 Materials and methods used in experiments to establish conditions for oligosaccharide release.

2.2.1 Specimens used.

We initially determined conditions for the release of oligosaccharides using a frozen breast cancer specimen and then the feasibility of oligosaccharide release from formalin-fixed paraffin-wax embedded tissues. Three breast cancer specimens were used, the specimens were available both as frozen and paraffin-wax embedded. The details of the specimens used are shown in table 2.1. The reproducibility of oligosaccharide release was evaluated using preparations of porcine liver as a homogeneous tissue.

Table 2.1 Features of the breast cancer specimens used.

Tissue Bank Reference Number	*Histological Diagnosis*	*Grade*	*Size (cm)*
1176	infiltrating ductal	III	9x8x3.8
1182	infiltrating ductal	II	2x2x1
1199	infiltrating NOS	III	4x4x4

2.2.2 Storage of specimens.

Prior to storage, all specimens were trimmed with a scalpel to remove extraneous fat and fibrous tissue and then cut into two pieces of equal size. One piece was frozen to -70°C and the other was fixed in 10% $^{v}/_{v}$ formol saline for a minimum of 24 hours then processed to paraffin-wax.

2.2.3 Processing of specimens to paraffin-wax blocks.

The specimens fixed in 10% $^{v}/_{v}$ formol saline were embedded in wax, using the procedure normally adopted in our laboratory, by passing the specimens through 70% $^{v}/_{v}$ ethanol in water for 2 hours, 100% ethanol for 2 hours, CNP as a non-hazardous alternative to xylene, for 2 hours, and paraffin-wax at 60°C for 18 hours. The entire procedure was undertaken using a Shandon Citadel Tissue Processing Machine.

2.2.4 Preparation of specimens for oligosaccharide release.

2.2.4.1 Removal of lipids.

This project focusses on the oligosaccharides present on glycoproteins of breast cancer specimens. Therefore we sought to avoid the potential release of oligosaccharides, acyl and acetyl groups from glycolipids (Heinze *et al.*, 1984). To this end, lipids from the frozen tissue specimens were extracted into chloroform and acetone. For the frozen tissues, specimens 0.05 - 0.1 g weight, were removed from the -70°C freezer, allowed to thaw and crudely chopped into pieces of approximately 2-3 mm^3. The pieces were placed into separate containers (plastic Tissue-Tek) and into a Soxhlet thimble for the extraction of lipids. 250 ml chloroform was poured into a 500 ml round-

bottomed flask, the Soxhlet thimble attached and the apparatus placed onto an electromantle set to reflux at 60°C for five hours at a rate of five refluxes per hour. After the extraction, the Soxhlet apparatus was dismantled, the containers opened and the tissue removed. To remove residual lipids, the specimens were homogenised in cold acetone as described by Wing *et al* for the preparation of proteins from brain specimens (Wing *et al.*, 1992). 15 ml of -20°C acetone was added and the material minced for 5 minutes using a Jankle and Kunkel homogeniser, the homogenate was poured into a funnel and the insoluble material (proteins) collected on Whatman 52M filter paper, the proteins were then collected on a spatula and re-homogenised a further two times. Paraffin-wax embedded tissues were not delipidified since lipids have been reported to be extracted into the solvents used during the processing of tissue to paraffin-wax blocks (Lillie, 1965).

2.2.4.2 Removal of wax.

In the case of the archived specimens it was necessary to remove the paraffin-wax fully before the oligosaccharide extraction procedure. To provide sufficient protein for the release of oligosaccharides from the tissues, fifty sections 5 μm thick were cut from the paraffin-wax block of each specimen and placed into glass universal containers. The sections were dewaxed by washing with three cycles each 15 ml of: CNP, then 99.98% ethanol, 70% $^{v}/_{v}$ ethanol in water and then HPLC grade water. During each of the washing steps the specimens were allowed to soak for 20 minutes and the tissue collected on an Anodisc 47, 0.1 μm pore size filter. After removal of lipids and paraffin-wax, the protein preparations were transferred to 5 ml (Chromocol) reactor vials and stored at -20°C prior to lyophilisation.

2.2.4.3 Lyophilisation of proteins prior to oligosaccharide release.

The preparations were freeze-dried so that oligosaccharide extraction could be performed under anhydrous conditions using hydrazine (Takasaki *et al.*, 1982). The preparations were transferred to the chamber of an Edwards Modulyo freeze-drier connected to an Edwards EM2 pump and lyophilised for 48 hours.

2.2.5 Oligosaccharide release.

2 mg freeze-dried weight of material from each breast cancer specimen was used in the oligosaccharide release experiments. Oligosaccharides were extracted using semi-automated hydrazinolysis in a GlycoPrep 1000 (Merry *et al.*, 1992; Patel *et al.*, 1993). The conditions used to release the oligosaccharides were: 200 µl anhydrous hydrazine and 18 hours incubation at 95°C. These conditions were used after a number of different hydrazinolysis incubation times had been evaluated, as detailed in the results, appendix 2.4. Fetuin from fetal calf serum (Sigma) has been reported to contain both N (Green *et al.*, 1988) and O-linked oligosaccharides (Edge and Spiro, 1987; Spiro and Bhoyroo, 1974) and was used as a standard to check that oligosaccharide release was proceeding satisfactorily. In practice every sixth oligosaccharide release in the GlycoPrep 1000 was performed on a 2 mg sample of freeze-dried fetuin. After oligosaccharide release and re-*N*-acetylation, the samples were transferred immediately for drying, without heating, on a Heto VM2 centrifugal evaporator connected to an Edwards EM2 pump.

2.2.6 Removal of peptides after oligosaccharide release.

To remove peptides produced during oligosaccharide release, the samples were purified by spotting onto paper and running descending paper chromatography using a mobile phase in which peptides, but not oligosaccharides, are soluble (Takasaki *et al.* 1982,). Paper chromatography was set-up as follows: the oligosaccharide pool was reconstituted in 10 µl of water and spotted onto one end of a sheet of Whatman 3MM paper 3 cm wide 51 cm long. To ensure all the oligosaccharides had been adequately transferred onto the paper, the procedure was repeated four times, each time the oligosaccharides were allowed to air-dry on the paper. When all the oligosaccharides had been transferred to the paper, the paper was placed into a descending chromatography tank with a reservoir containing 4/1/1 butan-1-ol/ ethanol/ water v/v/v, as the mobile phase. Descending paper chromatography was run for 48 hours, after-which the paper was removed from the tank and allowed to air-dry in a fume cupboard. The origin,

containing the oligosaccharide pool, was cut away from the rest of the paper and placed into a syringe fitted with a 0.2 μm nylon syringe-top filter (Whatman). The paper was 'wetted-out' by soaking for 10 minutes in 0.5 ml methanol. The methanol was extracted into a plastic tube by centrifuging for 10 minutes at 3000 rpm in a Centaur bench-top centrifuge. To elute the oligosaccharides, 1 ml HPLC grade water was added and the contents soaked for 10 minutes. The syringe containing the paper and the plastic tube were centrifuged as before and the oligosaccharides collected. The procedure was repeated using a further 1 ml of HPLC grade water. The oligosaccharide pool was then transferred from the centrifuge tube to two1.5 ml Eppendorf tubes and dried, without heating, using a Heto VM2 centrifugal evaporator connected to an Edwards EM2 pump.

2.2.7 Labelling oligosaccharides.

The prepared oligosaccharides were labelled either with tritium, Oxford GlycoSciences Kit K-400, based on the method of McClean, (1973) or with the fluorescent compound 2-aminobenzamide (2-AB) from Oxford GlycoSciences (Bigge *et al.*, 1994; Patel *et al.*, 1994). Free non-conjugated label was removed by spotting the labelled oligosaccharide pool onto strips of paper and running them in a descending chromatography tank for 1.5 hours as detailed in section 2.2.6.

2.2.8 Analysis of oligosaccharides.

Initially a gel permeation chromatography (GPC) column was used to evaluate the oligosaccharides released from glycoprotein preparations from frozen breast cancer specimens. Later, high performance liquid chromatography (HPLC) apparatus became available and the oligosaccharides released, from preparations of glycoproteins from frozen and paraffin-wax embedded specimens, were compared by their separation on anion-exchange and hydrophillic interaction chromatography columns.

2.2.8.1 Gel permeation chromatography (GPC).

2.2.8.1.1 Desialylation of oligosaccharides..

It was necessary to desialylate oligosaccharides prior to GPC on BioGel P4

since oligosaccharides with a net negative charge (for example, sialylated) elute in the void volume of the column. Oligosaccharides with a hydrodynamic volume of more than 16 glucose units also elute in the void volume of the column but smaller, asialo-, oligosaccharides are separated on the column matrix (Kobata *et al.*, 1987; Nishigaki *et al.*, 1978).
A sialidase from *Arthrobacter ureafaciens* was used for the desialylation of the oligosaccharide pool since it cleaves sialic acid attached to adjacent monosaccharides in the α 2,6 and α 2,3 positions (Uchida, 1979).
Each tritium labelled, dried, oligosaccharide pool was mixed with 0.2U sialidase (Oxford Glycosystems) in a 100 mmol / l sodium acetate buffer pH 5.0 and the mixture incubated at 37°C for 18 hours. Afterwards, the incubation buffer, free sialic acid and enzyme were removed by passing the contents through a mixed bed column of 0.2 ml each of BioRad resins: AG1, Chelex, AG50, AG3, QAE, housed in a 10 ml Polyprep column (BioRad). The mixed-bed column was washed with four column volumes of water prior to loading the incubation mixture, the oligosaccharides were then eluted in four column volumes of water, collected and dried by centrifugal evaporation (Parekh *et al.*, 1989).

2.2.8.1.2 Separation of asialo oligosaccharides: GPC chromatography.

A BioGel P4 column, 10 x 480 mm, void volume 7ml, fitted to a GlycoMap instrument (Oxford Glycosciences) was used for the GPC separation of oligosaccharides. The column was connected to a refractive index detector and solid or liquid scintillation Berthold radioactivity detector. Later, a fluorescence detector was fitted to the instrument. Water was used as the eluant and the column maintained at 55°C at all times. The flow rate was 30 ml / minute for the first 11ml and 160 ml / minute thereafter. The oligosaccharides eluting from the column were each assigned a hydrodynamic volume with reference to a calibration standard of a dextran ladder (Oxford GlycoSciences), the data handling software supplied with the GlycoMap was used for this purpose. A 20 µl aliquot of 'dextran ladder' from a stock solution of 7.5 mg /ml was used, the dextran ladder was unlabelled and detected by an in-line refractive index detector. The dextran ladder was

run through the GPC column either on the run immediately preceeding the sample, or otherwise at the same time as the sample, by mixing together with the labelled oligosaccharide pool released from the breast cancer specimen.

2.2.8.1.3 HPLC separations.

HPLC separations were performed using columns fitted to Gilson 305 and 306 pumps with an in-line manometric module, high pressure mixing and PEEK (poly ether ether ketone) Rheodyne injection valve. In all cases a 10 µl loop was used. The 2-AB labelled oligosaccharides were detected using an in-line Gilson 121 filter fluorimeter λ Ex. 330nm, λ Em. 420nm. Data were collected into a Kontron MT2 data handling system on a Dell 486 Personal Computer. Oligosaccharide peaks were integrated manually from trough to trough and with a minimum peak area accepted of 15 m volts. Each peak area was expressed as a percentage of the total oligosaccharide pool.

2.2.8.1.3.1 Anion-exchange chromatography.

Sialylated oligosaccharides were separated by weak anion-exchange chromatography on a GlycoSep C column (Oxford GlycoSciences) using the methods described by Guile, G. *et al* (1994). The GlycoSep C column matrix comprises 5 µm polymer coated divinylbenzene resin beads, column dimensions were 4.6 x 100 mm. To achieve separation of the sialylated oligosaccharides, a gradient was formed between 20% ($^{v}/_{v}$) acetonitrile in water and 20% acetonitrile with 80% 250 mmol / l ammonium formate ($^{v}/_{v}$) pH 4.5, with a flow rate of 0.4 ml / min as shown in table A2. The samples were loaded onto the column in water. The column was calibrated at the beginning of each day, using 2-AB labelled oligosaccharides released from fetuin. The day-to-day coefficient of variation (c.v.) in retention times of the monosialylated N-linked oligosaccharides from fetuin was found to be less than 5%.

Table 2.2 Conditions used for the separation of oligosaccharides by anion-exchange chromatography.

Time (minutes)	*Description*	*20% acetonitrile*	*20% acetonitrile, 80% 250 mmol / l ammonium formate, pH 4.5*
0 - 5.0	isocratic	100	0
5.1 - 35.0	gradient	0	100
35.1 - 40.0	isocratic	0	100
40.1 - 45.0	re-equilibration	100	0

2.2.8.1.3.2 Hydrophilic-interaction chromatography.

One of the neutral oligosaccharides present in the oligosaccharide pool from both the frozen and the paraffin-wax embedded breast cancer specimens was prepared for further analysis as follows: Fractions were taken after GPC, dried by centrifugal evaporation and the oligosaccharides separated using hydrophilic-interaction chromatography on a GlycoSep C column.

To obtain separation, first the column was equilibrated in 80% acetonitrile: 20% 100 mmol / l ammonium formate ($^{V}/_{V}$) pH 4.5 then a gradient was formed with 20 mmol / l ammonium formate pH 4.5, as shown in table 2.3. Separation of asialo oligosaccharides on this column was first checked by calibrating the column with 2-AB labelled dextran ladder, then the oligosaccharides released from the breast cancer specimens were loaded onto the column in an acetonitrile / water mixture (70:30 $^{V}/_{V}$ ratio).

Table 2.3 Conditions used for the separation of oligosaccharides by hydrophilic interaction chromatography.

Time (minutes)	*Description*	*80% acetonitrile: 20% 100mmol/l ammonium formate pH 4.5*	*20mmol/l ammonium formate pH 4.5*
0-5	Isocratic	100	0
5.1-55	Gradient	65	35
55.1-60	Reequilibration	100	0

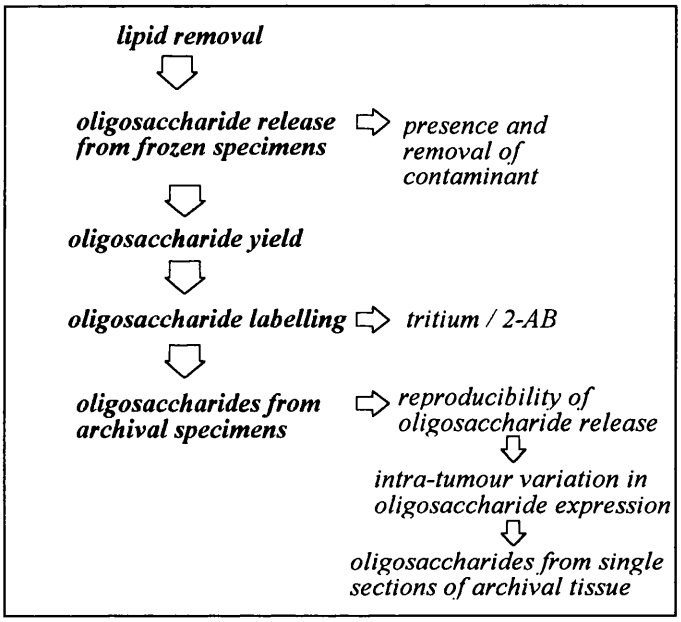
2.2.8.2 Matrix-assisted laser-desorption ionisation mass spectrometry (MALDI/MS).

MALDI/MS was used to determine whether two, apparently identical, oligosaccharides released from the same breast cancer specimens, stored frozen or paraffin-wax embedded, had the same mass value. A MALDI/MS machine (Finnegan MAT) at the Glycobiology Institute, University of Oxford, was used. The analysis was performed with the assistance of Dr. David Harvey. The procedure was as follows: a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile was prepared (the matrix). 2-AB labelled oligosaccharides were taken up in 2 µl of water, dropped onto a stainless steel target and allowed to air dry. 1 µl of matrix was added to the target and crystals formed. A biantennary, galactosylated core fucosylated, bisected N-linked oligosaccharide (NGA2FB, MW 1689.6 Da, Oxford Glycosciences) was added to the oligosaccharide / matrix mixture on the target as an internal standard. Ethanol was then added to the target, to recrystallise the matrix / oligosaccharide / internal standard mixture. The target was placed into the chamber of the mass spectrometer and the laser was fired. The laser power and position were adjusted in order to obtain a spectrum for the positive ions produced. The procedure was repeated five times to obtain repeat spectra of the oligosaccharide and internal standard. The mass value of the unknown oligosaccharide was calculated with reference to the mass value of the internal standard. The 2-AB label contributed 120 Da, the positive (sodium) ions contributed 23 Da to the overall mass value of the oligosaccharide and internal standard.

2.3 Results of experiments to release oligosaccharides from breast cancer specimens.

The results of method development experiments for the extraction of oligosaccharides from breast cancer specimens are shown below. The steps involved are shown in figure 2.1.

Figure 2.1 Flow diagram to illustrate the development of methods for the extraction of oligosaccharides from breast cancer specimens.



2.3.1 Introduction.

Most methods for the release of intact oligosaccharides from glycoproteins have been established to study glycosylation of single glycoproteins such as human chorionic gonadotrophin (Mizuochi *et al.*, 1983) and tissue plasminogen activator (Parekh *et al.*, 1989). In breast cancer, changes in glycosylation appear associated with several, rather than single, glycoproteins (Brooks and Leathem, 1995a; Streets *et al.*, 1996), therefore, we chose to use a mixture of breast cancer glycoproteins to determine suitable methods for the extraction of intact oligosaccharides.

Oligosaccharides can be released from proteins using enzymatic and / or chemical methods. Enzymatic methods typically use either Peptide *N* glycosidase F for the

release of N-linked structures (Plummer and Tarentino, 1991) or O-glycosidase for the release of O-linked structures (Glasgow et al.,1977 *et al.,* 1990).

The size and bulkiness of oligosaccharides means it is often necessary for the protein to be denatured if enzymes are to be used to cleave the oligosaccharide-protein bond (Nuck *et al.*, 1990). Chemical methods offer the possibility of solubilising the glycoproteins during oligosaccharide extraction, thereby eliminating the need to denature the proteins before oligosaccharide release. Chemical methods of oligosaccharide release include hydrazinolysis for N-linked structures (Takasaki *et al.*, 1982) and β-elimination, in alkali conditions, for O-linked structures (Fukuda, 1989). We investigated the use of anhydrous hydrazine for the release of oligosaccharides from freeze-dried preparations of frozen breast cancer glycoproteins. Hydrazine had previously been used for the release of N-linked oligosaccharides from glycoproteins Takasaki *et al.*, (1982) and more recently, had been reported to be of value for the release of both N-linked and O-linked structures (Korrel *et al.*, 1985; Patel *et al.*, 1993). In addition, a GlycoPrep 1000 machine, for automated hydrazinolysis, offered the possibility of reproducible and easy oligosaccharide release (Merry *et al.*, 1992). The release of oligosaccharides using hydrazine had been reported to result in less desialylation of the oligosaccharide pool than enzymatic methods (when conducted in the presence of sodium dodecyl sulphate, Hermentin *et al.*, 1992). To enable a comparison to be made between the oligosaccharides extracted from different breast cancer specimens it was necessary to determine hydrazinolysis conditions which would ensure non-selective, reproducible and reliable release of oligosaccharides from one breast cancer specimen to the next.

2.3.1.1 Specimen used in oligosaccharide release experiments.

To determine suitable methods for oligosaccharide release, experiments were initially undertaken using a single breast cancer specimen from the Breast Cancer Research Group tissue bank. The breast cancer specimen was selected primarily as its large size would allow multiple experiments to be conducted, in addition, it was available both frozen at -70'C and as formalin-fixed paraffin-wax-embedded blocks of tissue. The breast cancer specimen was a modified Bloom and Richardson grade

III infiltrating ductal carcinoma, with extensive necrosis and lymphatic permeation, 9 x 8 x 3.8 cm in size, reference number 1176.

2.3.2 The removal of lipids prior to oligosaccharide extraction.

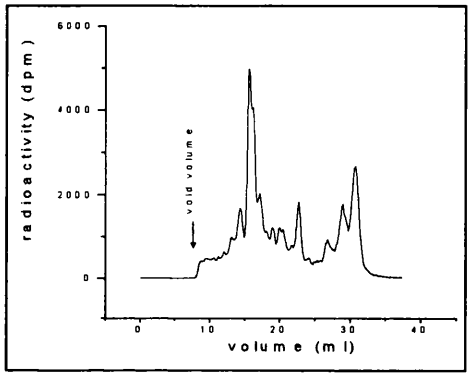
Breast cancer specimens usually contain fatty tissue. Fat cells are enriched in glycolipids and anhydrous hydrazine may be used to release acyl and acetyl groups from glycolipids (Heinze *et al.*, 1984). To prevent the release of these structures for this study, of protein glycosylation in breast cancer, it was necessary to remove as much of the glycolipid component from the specimens as possible. A modification of the method of Wing *et al.*, (1992) was used to remove the glycolipids from the breast cancer specimens. Delipidification was achieved by homogenising the tissue in cold (-20°C) acetone for 5 minutes three times and an additional chloroform extraction step, with the specimen housed in a Soxhelt thimble, for 5 hours and five refluxes of chloroform per hour.

2.3.3 Oligosaccharide release from frozen breast cancer specimens.

Anhydrous hydrazine was used to release oligosaccharides from 1mg of freeze-dried protein preparation from breast cancer specimen number 1176. The hydrazinolysis and re-*N*-acetylation steps were undertaken in the GlycoPrep 1000 as detailed in section 2.2.5. After their release, the oligosaccharides were labelled with tritium and desialylated using neuraminidase to allow their analysis on a BioGel P4 column. It was necessary to remove a contaminant from the oligosaccharides released by the GlycoPrep 1000, prior to the oligosaccharide labelling step. This is detailed further in section 2.4. The removal of the contaminant was achieved by spotting the entire oligosaccharide pool onto paper and running in a descending chromatography tank for 48 hours with butan-1-ol : ethanol : water (4:1:1 v/v/v) as the mobile phase (Takasaki *et al.*, 1982). Figure 2.2 shows the asialo oligosaccharide pool extracted from breast cancer specimen 1176, using the techniques above and separated on a BioGel P4 chromatography column.

The results showed that the method of hydrazinolysis and re-N-acetylation could be successfully used to extract a wide range of different sized oligosaccharides from a mixture of glycoproteins prepared from a frozen breast cancer specimen.

Figure 2.2 Oligosaccharides released from a frozen breast cancer specimen.

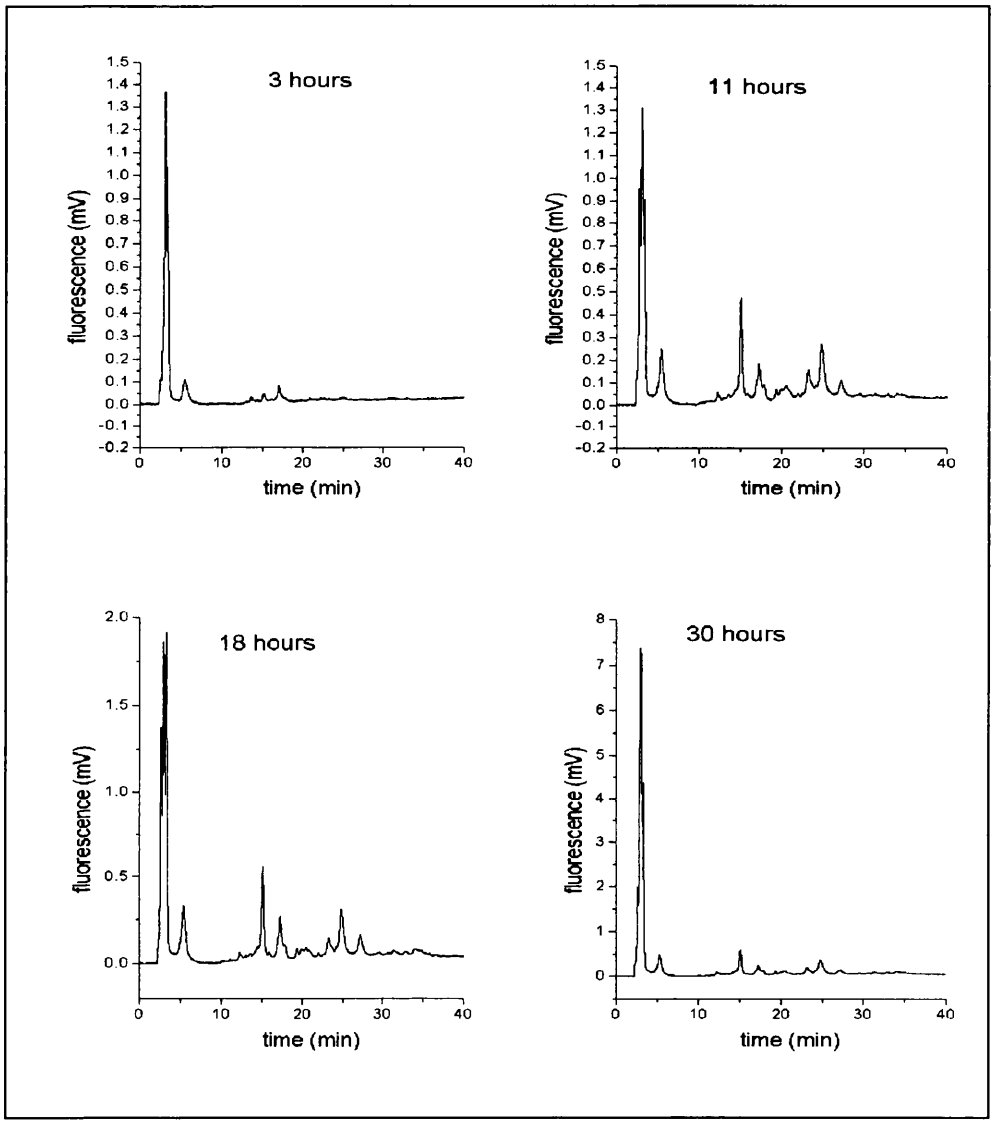


2.3.4 Oligosaccharide yield.

The length of the incubation time in hydrazine has been reported to be of importance in ensuring adequate extraction of oligosaccharides from proteins (Takasaki *et al.*, 1982) but extended hydrazinolysis has been reported to damage O-linked oligosaccharides. To identify suitable hydrazinolysis incubation times for the release of oligosaccharides from mixtures of proteins prepared from frozen breast cancer specimens, 1mg freeze-dried proteins from specimen number 1176 were taken and four time courses spanning 3, 11, 18 and 30 hours were evaluated. After oligosaccharide release the structures were separated by anion-exchange chromatography as shown in figure 2.3. The 30 hours incubation resulted in the greatest overall yield of oligosaccharides, but the structures were predominantly neutral, suggesting that sialylated oligosaccharides had become desialylated during the prolonged incubation time.

The 18 hours hydrazinolysis incubation time resulted in the greatest yield of a wide range of different size and charged oligosaccharide structures. In subsequent oligosaccharide release experiments 18 hours incubation time was selected. Using these conditions the neutral and sialylated oligosaccharides extracted from different breast cancers could be compared in a qualitative manner.

Figure 2.3 Anion-exchange separation of oligosaccharides released from breast cancer specimen 1176 using different hydrazinolysis incubation times, indicated.



2.3.5 Oligosaccharide labelling.

Oligosaccharides released were labelled to allow their detection after separation through chromatographic media. Initially we used tritium linked to the non-reducing terminus of the oligosaccharides (McClean *et al.*, 1973) but there were drawbacks with the technique, such as the hazardous nature of the labelling process, the cost and the relatively poor sensitivity of detection (μMol). As an alternative to tritium we used the fluorescent label 2-aminobenzamide (2-AB) which has been shown to label oligosaccharides in a non-selective manner and using a comparable method to that used for tritiation of oligosaccharides (Bigge *et al.*, 1995). In our evaluation, 2-AB

appeared to be suitable for the labelling of oligosaccharides released from breast cancer specimens, section 2.5. In addition, the 2-AB label allowed oligosaccharides to be detected with greater sensitivity than tritium (sub picoMol) and was compatible with a number of fluorescent detectors available for use with HPLC equipment.

2.3.6 Oligosaccharide release from archived (paraffin-wax embedded) breast cancer specimens.

Any correlation between oligosaccharide expression and breast cancer progression is best sought using archival tissues since these are stored for extended long periods (often decades) and extended patient follow-up can be obtained from the medical records. Long-term follow-up information, to determine patient outcome, is particularly important in breast cancer owing to the long natural history of the disease, discussed further in section 1.1.4.

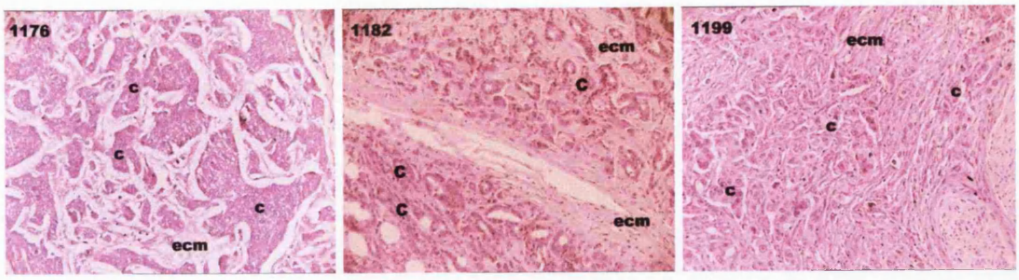
Formalin-fixed paraffin-wax-embedded tissues are routinely prepared from surgical specimens for diagnostic purposes, any surplus tissue is stored in archives and offers a useful resource for retrospective research studies. We aimed to extract oligosaccharides from formalin-fixed paraffin-wax-embedded breast cancers and determine whether or not the structures are damaged during processing and storage.

2.3.6.1 Specimens used for oligosaccharide release.

Three breast cancer specimens were used for oligosaccharide release. The specimens selected were available both frozen (at -70°C) and formalin-fixed paraffin-wax embedded. Figure 2.4 shows the histology of the specimens used stained with haematoxylin and eosin.

To examine the feasibility of releasing intact oligosaccharides from formalin-fixed paraffin-wax embedded breast cancers, and to compare the types of oligosaccharides extracted, freeze-dried protein preparations were made from each of the specimens shown in figure A2.4. Oligosaccharides were released using anhydrous hydrazine and labelled with 2AB using the methods, developed for frozen breast cancer specimens, detailed in sections 2.2.5, 2.2.6 and 2.3.3.

Figure 2.4 Breast cancer specimens used for oligosaccharide release. Each specimen cut to 5μm thickness and stained with haemoxylin and eosin. The cancer cells (c) and connective tissue (ecm) are indicated.



2.3.6.2 Sialylated oligosaccharides extracted from frozen and paraffin-wax embedded breast cancer specimens.

The oligosaccharides from frozen and paraffin-wax embedded breast cancers were compared by separation on an anion-exchange chromatography column, figure 2.5. Under the conditions used, sialylated structures separate primarily according to the number of acidic residues attached, larger more negatively charged species elute from the column with increased molarity ammonium formate. Sulphated and phosphorylated oligosaccharides may also be separated under these conditions (Guile *et al.*, 1994).

2.3.6.3 Neutral oligosaccharides extracted from frozen and paraffin-wax embedded breast cancer specimens.

To compare the uncharged, neutral oligosaccharides, the samples were passed down a BioGel P4 gel permeation chromatography column fitted to a GlycoMap 2000. The oligosaccharide elution profiles obtained for the breast cancers, stored frozen and paraffin-wax embedded, are shown in figure 2.6.

The results showed that oligosaccharides could be extracted from paraffin-wax embedded archival breast cancer tissues and that the structures present in the paraffin-wax embedded tissue were also found in the equivalent frozen specimens.

There were, however, differences in the relative amounts of the oligosaccharides present. The source of variation in the oligosaccharide profiles was evaluated by determining the intra- tumour variation in oligosaccharide expression (detailed in section 2.6) and examination of oligosaccharides released from a more homogeneous non-diseased tissue (detailed in section 2.7).

Figure 2.5 Separation of oligosaccharides from frozen and paraffin-wax embedded breast cancer specimens using anion-exchange chromatography.

Frozen *Paraffin-wax embedded*

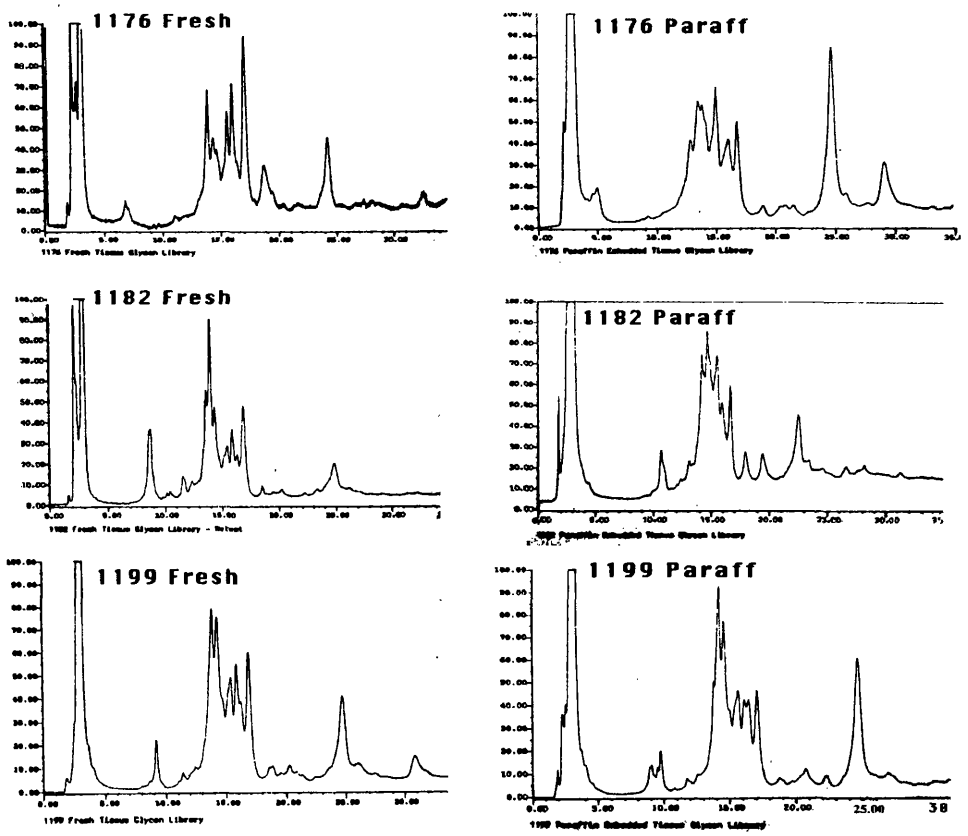
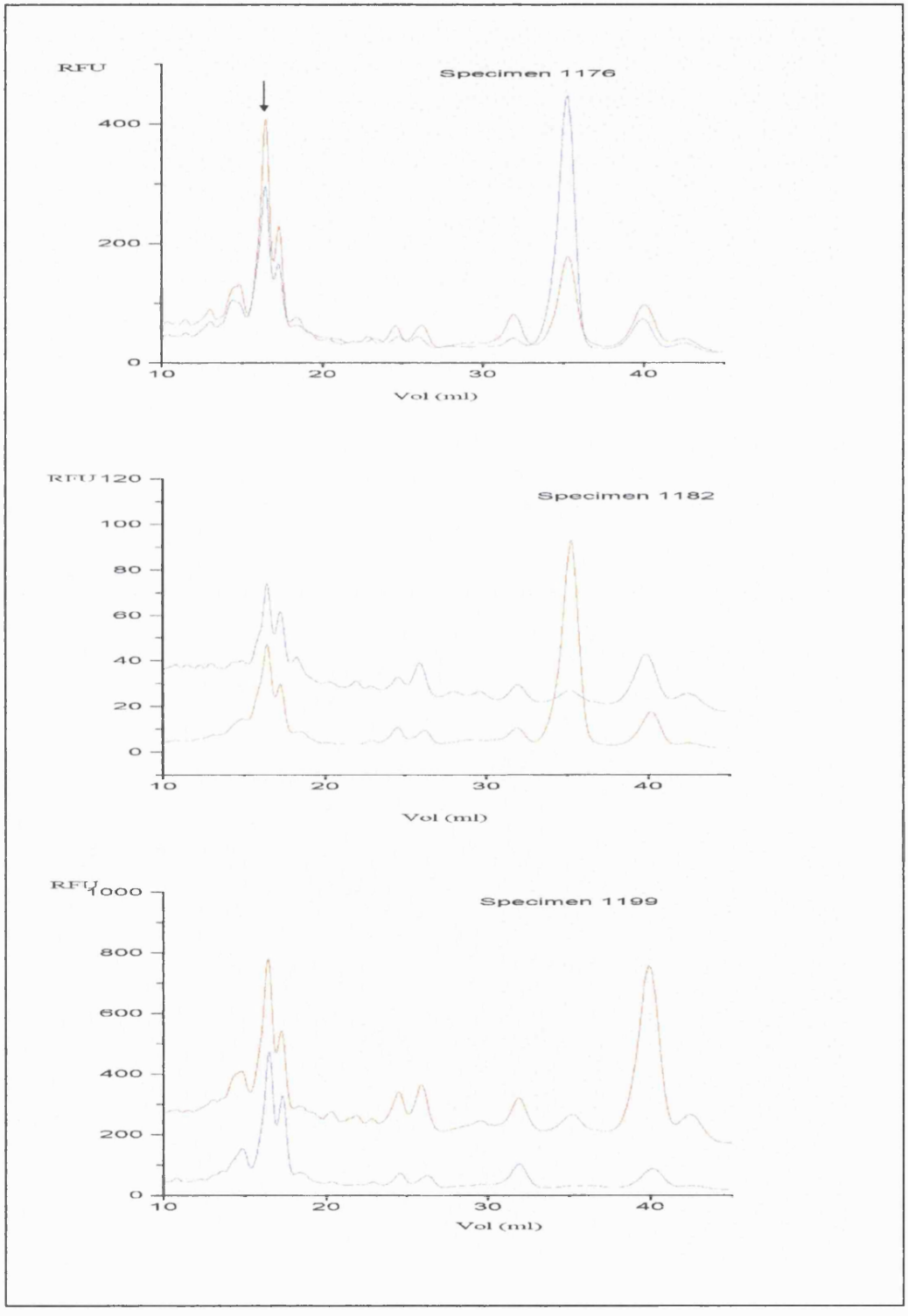


Figure 2.6 Neutral oligosaccharides extracted from three frozen (red) and paraffin-wax embedded (blue) breast cancers, separated using a BioGel P4 column. Arrow indicates 11.6 glucose unit oligosaccharide purified for further analysis. RFU = relative fluorescence units.



2.3.6.4 Analysis of an oligosaccharide, extracted from both frozen and paraffin-wax embedded tissue, using MALDI/MS.

To check that the oligosaccharides extracted from the frozen and paraffin-wax embedded breast cancer tissues were the same molecular weight, one of the oligosaccharides present in the oligosaccharide pool from specimen 1176 (11.6 glucose units in size) was purified and analysed by MALDI/MS. An example of the MALDI/MS spectra obtained for the oligosaccharides is shown in figure 2.7.
The analysis by MALDI/MS showed that the mass value of the oligosaccharide, corrected with an internal standard, was 1930.7. This value was the same as that expected for an asialo, biantennary, galactosylated, core fucosylated 2-AB labelled N-linked oligosaccharide. No significant differences were identified between the mass values obtained for the oligosaccharide purified from the frozen and the paraffin-wax-embedded breast cancer tissue, as shown in table 2.4. MALDI/ MS analysis of an oligosaccharide purified from the same frozen and paraffin-wax embedded breast cancer showed that the oligosaccharide was the same molecular weight. The MALDI/MS, and gel permeation chromatography results, were suggestive that the oligosaccharides extracted from the frozen and paraffin-wax embedded specimens were structurally identical.

Figure 2.7 Mass spectra of an oligosaccharide, purified from frozen (A) and paraffin-wax embedded (B) breast cancer tissue, obtained using MALDI/MS. The oligosaccharide of interest (arrow) and internal standard (IS) are shown.

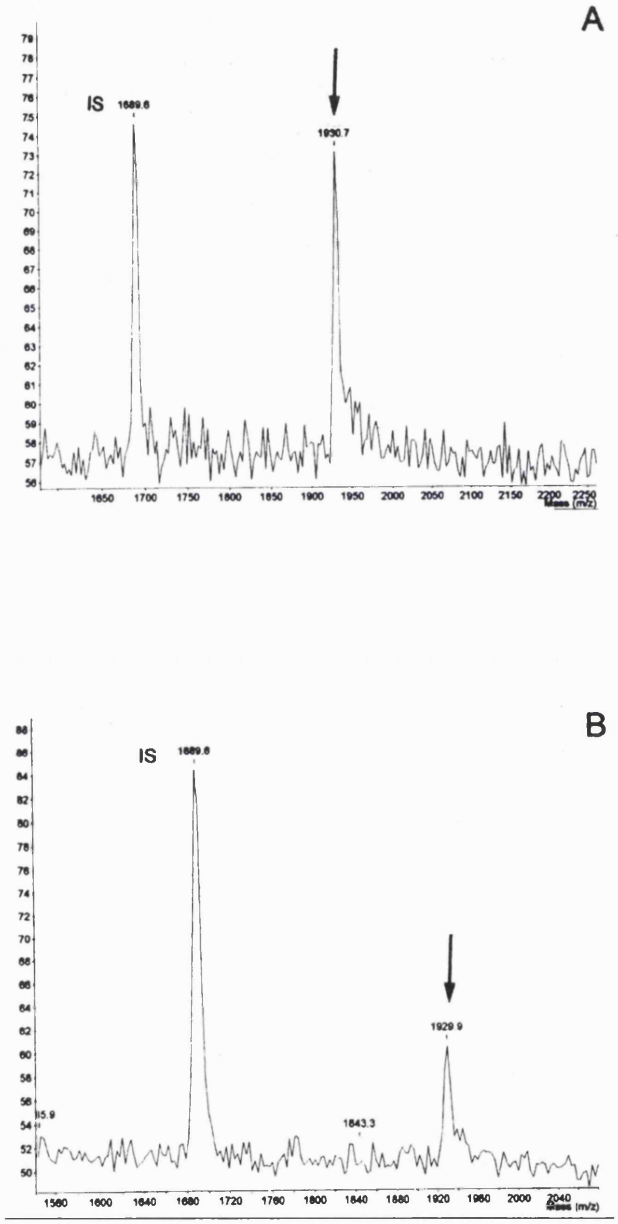


Table 2.4 MALDI/MS mass values, from six spectra, of an oligosaccharide purified from frozen / paraffin-wax embedded breast cancer tissue, reference number 1176.

	Mass Value (m/z)					
Frozen						
result =	1929.2	1929.2	1930.4	1930.8	1931.5	1931.9
mean =	1930.5					
sd =	0.95					
Paraffin-wax embedded						
result =	1930.1	1933.8	1933.8	1933.8	1934.2	1934.2
mean =	1931.5					
sd =	2.3					
Student t Test						
t test =	-0.406					
P=	0.7					

2.4 The removal of a contaminant obtained after oligosaccharide release from breast cancer specimens

2.4.1 Introduction.

The presence of a contaminant following the hydrazinolysis step to release oligosaccharides might be due to ineffective action of the cellulose columns fitted to the GlycoPrep 1000 machine. Cellulose columns were fitted to the GlycoPrep 1000 in order to remove peptides and other small molecules following hydrazinolysis and re-*N*-acetylation based on the method described by Wing *et al* (1992). In brief, oligosaccharides and peptides, from the hydrazinolysis reaction, are adsorbed onto the cellulose column, peptides are washed off the column using a buffer system in which oligosaccharides are not soluble, oligosaccharides remain on the column and are later eluted in water. To improve this 'clean-up' step and try to remove the material, two techniques were evaluated following oligosaccharide release in the GlycoPrep 1000, gel filtration and descending paper chromatography.

2.4.2 The use of gel filtration for the removal of the contaminating material.

Oligosaccharides were released from breast cancer specimen 1176 using the conditions detailed in section 2.2.5. The oligosaccharide pool was passed down a BioGel P4 gel permeation chromatography column, fitted to a GlycoMap 1000 and material larger than 2.5 glucose units collected, dried and labelled with tritium. After labelling, the oligosaccharides were desialylated as described before and re-run through the BioGel P4 column, the results obtained are shown in figure 2.8.

2.4.3 Results of gel filtration 'clean-up' step.

Partial removal of the contaminating material was achieved using gel permeation separation prior to labelling the oligosaccharides with tritium, but the technique was time-consuming and a speedier, more effective method was required.

2.4.4 The use of paper chromatography for removal of the contaminant.

Oligosaccharides were extracted from specimen 1176 using the conditions detailed in section 2.2.5. After oligosaccharide release, the contents of the GlycoPrep 1000 collection vial were dried and spotted onto Whatman 3M paper. The paper was placed in a descending chromatography tank with butan-1-ol, ethanol, water, in proportions 4:1:1, as the mobile phase and allowed to run for 24 or 48 hours. After the time period had elapsed, the paper was removed from the tank, allowed to air dry and the oligosaccharides, which remain at the origin, eluted from the paper. The oligosaccharides were labelled with tritium and analysed by separation on a BioGel P4 chromatography column as shown in figure 2.9.

2.4.5 Results of paper chromatography 'clean-up' step.

The results show that the contaminant was considerably reduced by the addition of the descending paper chromatography step. There appeared to be a greater yield of oligosaccharides in the sample which had been run on descending paper chromatography for 48 hours prior to labelling. There did seem to be some difference between the relative amounts of the different oligosaccharides collected, but it was considered that this might reflect intra tumour variability in oligosaccharide expression and is described further in section 2.6.

In subsequent experiments the oligosaccharide pool was subjected to 48 hours descending paper chromatography before labelling.

Figure 2.8 Oligosaccharides from breast cancer specimen 1176, run through a BioGel P4 chromatography column prior to labelling oligosaccharides larger than 2.5 glucose units in size (2.5 GU).

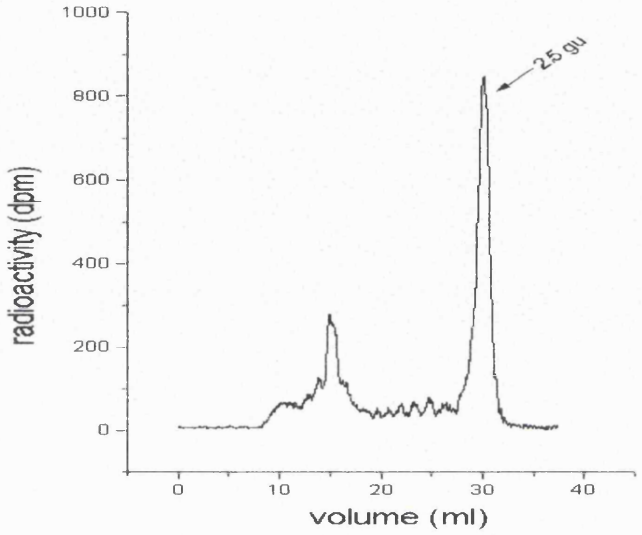
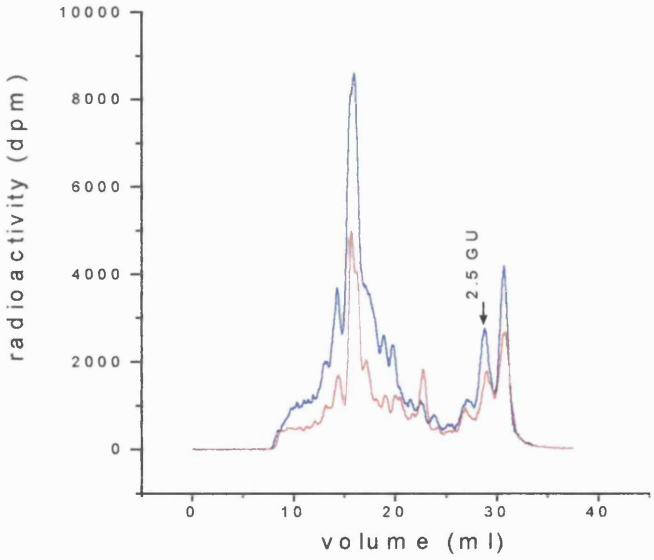


Figure 2.9 Oligosaccharides from breast cancer specimen 1176, separated on a BioGel P4 chromatography column. The oligosaccharides had been subjected to descending paper chromatography for 24 (red) or 48 (blue) hours. The 2.5 glucose units material is indicated by the arrow.

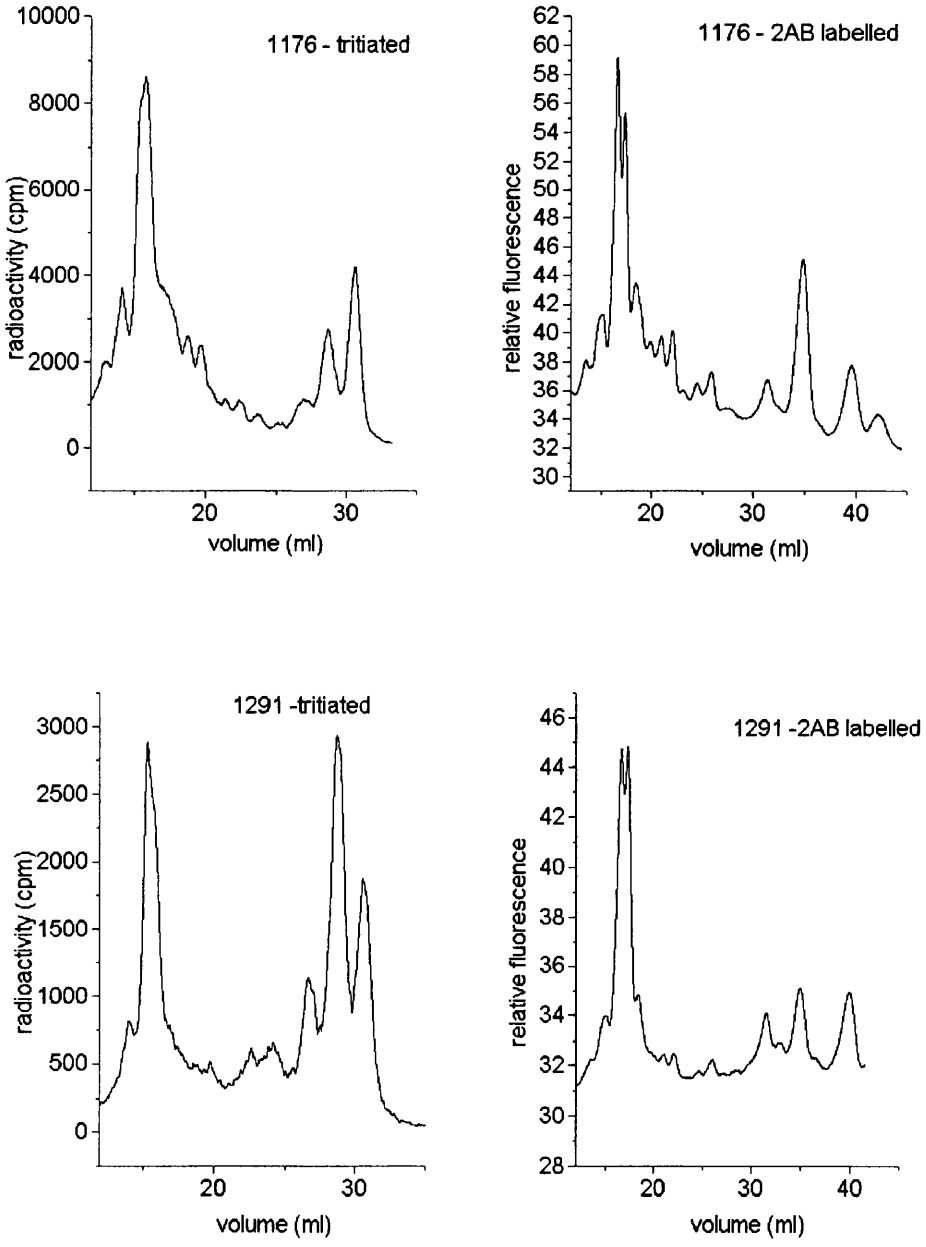


2.5 *A comparison of breast cancer oligosaccharides labelled with tritium and 2-aminobenzamide.*

Oligosaccharides were released from two breast cancer specimens using the methods described in section 2.2.5. The oligosaccharide pool was divided into two equal portions, one portion was labelled with tritium, the other with 2AB. The oligosaccharides were passed through a BioGel P4 chromatography column and the results are shown in figure 2.10. There did not appear to be any selectivity of labelling when the 2AB labelled oligosaccharides were compared to those labelled with tritium.

The 2AB label offered several clear advantages over tritium. The 2AB label is slightly retarded on the BioGel P4 column and this resulted in improved resolution of separation of the oligosaccharides which were labelled with 2AB compared with tritium. 2AB has a small net negative charge (Bigge *et al.*, 1995) but the label remained compatible with weak anion-exchange chromatography HPLC columns for separation of charged oligosaccharides. The availability of HPLC fluorescence detectors meant that HPLC separation of charged oligosaccharides could be undertaken using 2AB labelled structures. The 2AB label could be more sensitively detected than tritium, allowing smaller amounts of breast cancer tissue to be used for oligosaccharide analysis.

Figure 2.10 Oligosaccharides from breast cancer specimens 1176 and 1271 labelled with tritium and 2AB and separated on a BioGel P4 chromatography column.

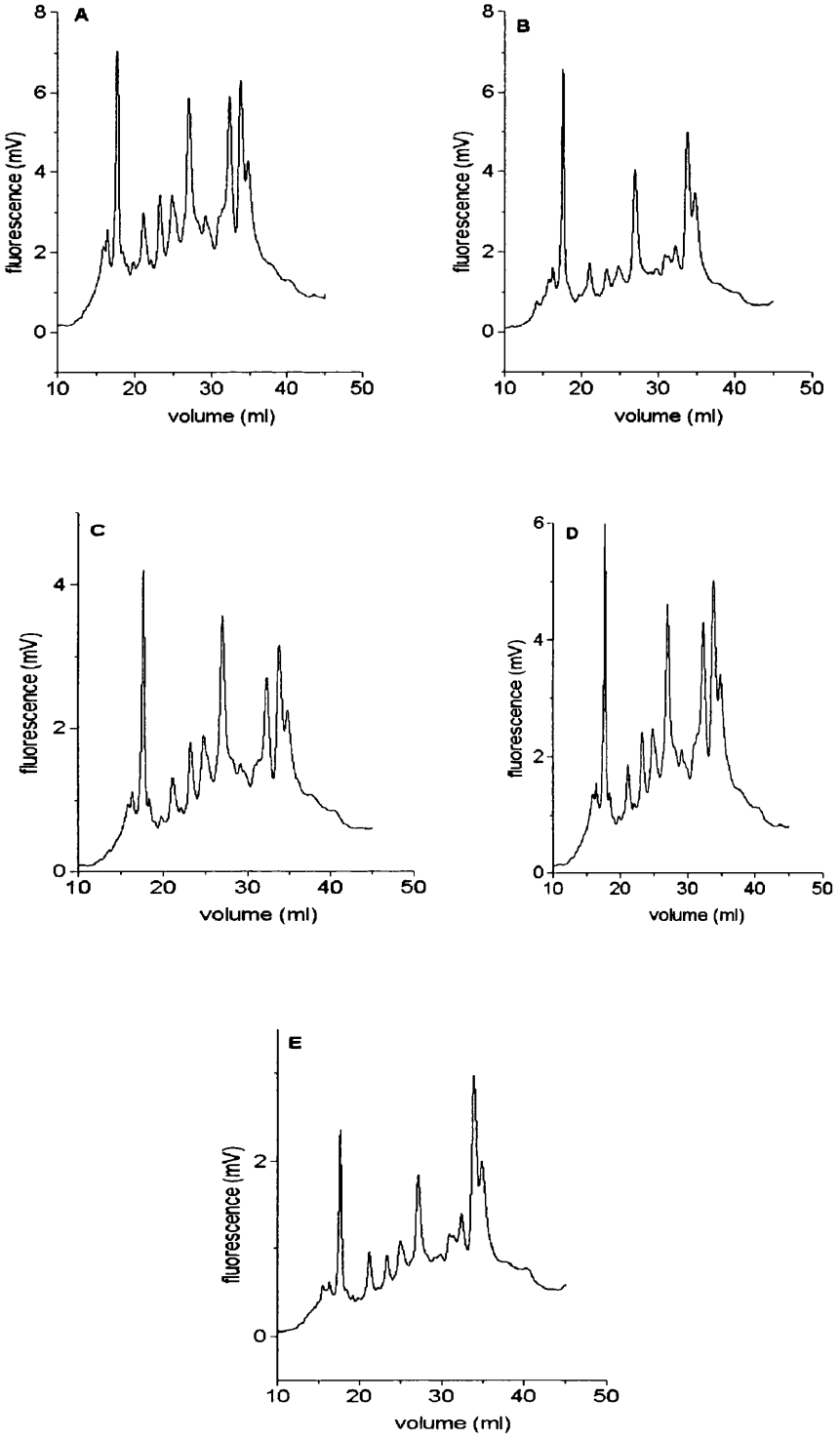


2.6 Intra-tumour variation in oligosaccharide expression – an evaluation.

To investigate the intra-tumour variation in the expression of sialylated oligosaccharides, breast cancer specimen 1176, for which five paraffin-wax embedded blocks were available, was used for multiple oligosaccharide release experiments. The oligosaccharides were extracted using anhydrous hydrazine, labelled with 2AB and separated using a GlycoSep C anion-exchange chromatography column, the results are shown in figure 2.11.

The results of multiple oligosaccharide release experiments, using samples taken across a single large advanced infiltrating breast cancer, indicated that there was some variation in oligosaccharide expression across the specimen. Oligosaccharides from paraffin-wax blocks A, C and D were similar, in terms of the types and relative amounts of different oligosaccharides released. In blocks B and E there appeared to be a change in the distribution of the oligosaccharides released, some of the more predominant oligosaccharides in oligosaccharides released from paraffin-wax embedded tissue of blocks B and E were lesser component in blocks A, C and D.
In conclusion, the relative amounts of oligosaccharides released varied across the tumour but there were no differences detected between the types of oligosaccharide structures which had been extracted.

Figure 2.11 Oligosaccharides released from five sites (A-E) across breast cancer specimen 1176 and separated on a GlycoSep C anion-exchange chromatography column.



2.7 The reproducibility of oligosaccharide release from paraffin-wax embedded tissue.

A histologically homogeneous tissue (porcine liver) was used to compare the relative amounts of sialylated oligosaccharides extracted from tissue stored either frozen or paraffin-wax embedded. To compare the amounts and retention times of the different sialylated oligosaccharides from the porcine liver specimens, six hydrazinolysis experiments were undertaken. The oligosaccharides were released using methods detailed in section 2.2.5 and separated on a GlycoSep C anion-exchange chromatography column as detailed in section 2.8.1.3.1.
The results obtained are shown in figure 2.12. The different sialylated oligosaccharide retention times and the relative amounts of the different oligosaccharides released were compared and are shown in figures 2.13 and 2.14 respectively.
In conclusion, the separation of oligosaccharides from frozen and paraffin-wax embedded porcine liver indicated that there was no significant difference between either the amounts, or types, as measured by elution times, of sialylated oligosaccharides released.

Figure 2.12 Oligosaccharides released from frozen and paraffin-wax embedded porcine liver and separated on an anion-exchange chromatography column.

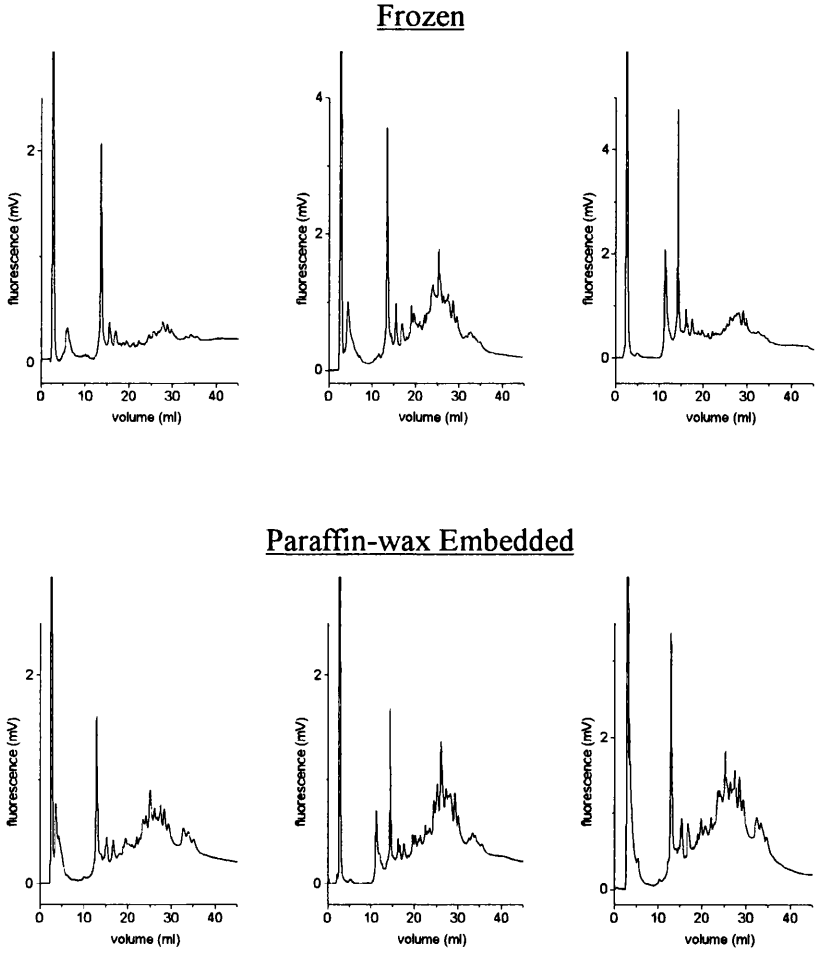


Figure 2.13 Peak elution times of sialylated oligosaccharides from porcine liver tissues separated by anion-exchange chromatography. Mean values +/- SD, *t* test values not significant, *P* values stated. Red = frozen, blue = paraffin-wax-embedded.

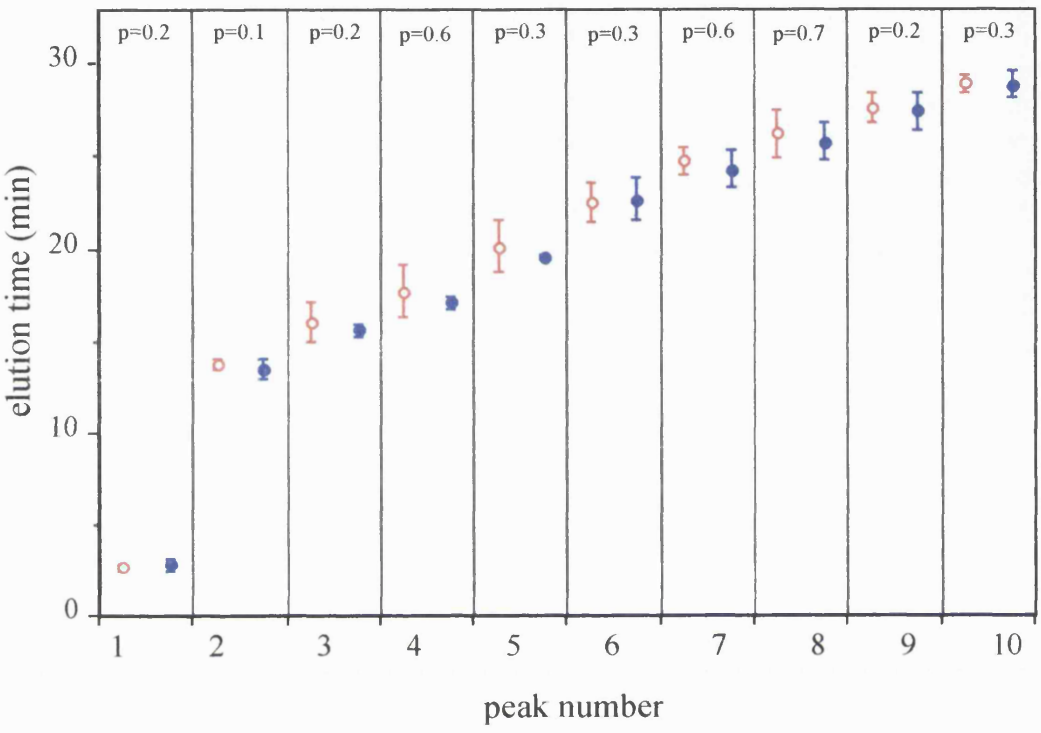
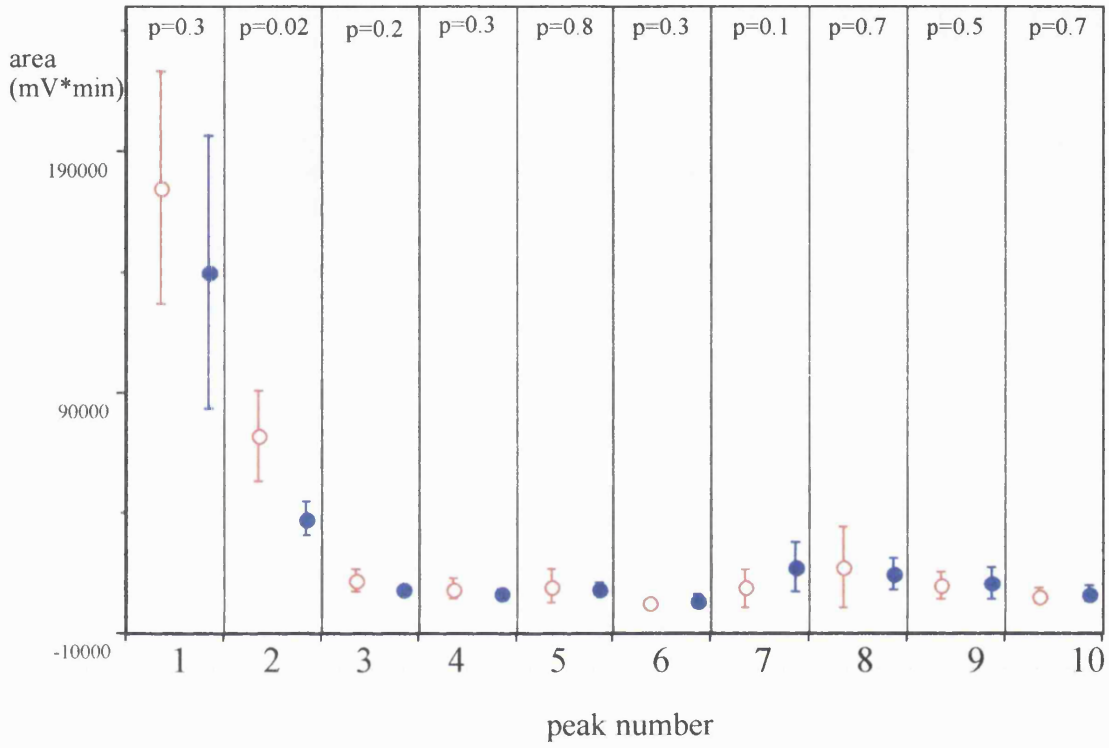


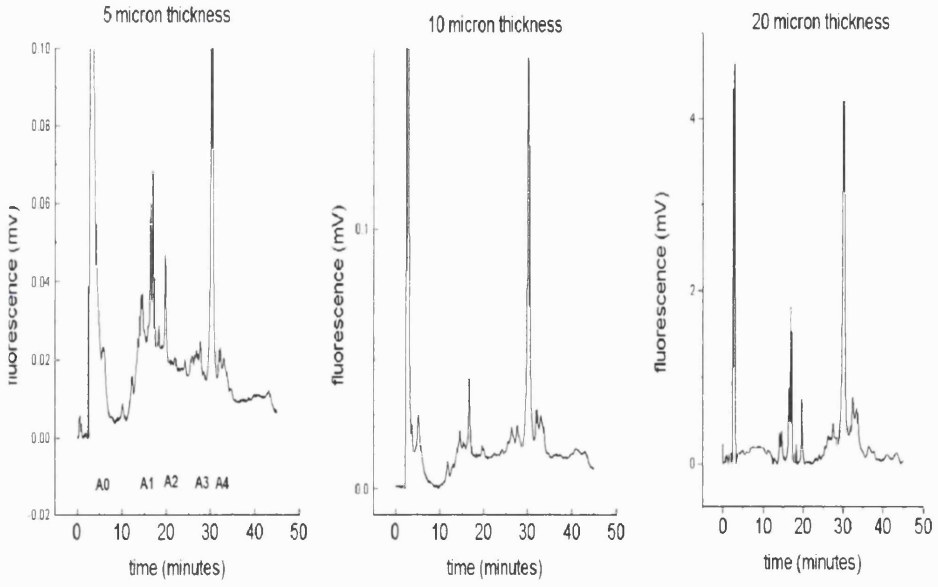
Figure 2.14 Peak areas of sialylated oligosaccharides from porcine liver tissues separated by anion-exchange chromatography. Mean values +/- SD, *t* test values not significant, *P* values stated. Red = frozen, blue = paraffin-wax-embedded.



2.8 Release of oligosaccharides from single sections of tissue 10μm thick.

To enable oligosaccharides to be extracted and analysed from a series of archived breast cancer specimens it was necessary to speed up the methods for microdissection of the cancer cells. We evaluated the feasibility of oligosaccharide release from single sections taken from breast cancer specimen number 1176 as the sensitivity and resolution had been so improved.
Three different thickness sections were used for oligosaccharide release, 5μm, 10μm and 20μm. The sections were mounted on acid-washed glass slides and areas other than malignant epithelium dissected away with a steel scalpel blade. The sections were dewaxed as described in section 2.4.2 and the protein preparations used for oligosaccharide release and labelled using the methods described in sections 2.2.5 to 2.2.7. The released oligosaccharides were separated by anion-exchange chromatography on a GlycoSep C column using the methods detailed in section 2.2.8.1.3.1. The results obtained are shown in figure 2.15.

Figure 2.15 Oligosaccharides released from different thickness sections of breast cancer specimen number 1176, separated by anion-exchange chromatography .



Oligosaccharides were released in sufficient quantity that sialylated oligosaccharide profiles could be obtained from single 5μm, 10μm, and 20μm thickness sections. In subsequent experiments 10μm thick sections were used owing to ease of handling, in particular the 10 μm thick sections were the easiest to cut, to float on the water bath and to mount on the slides.

2.9 Conclusions.

2.9.1 Oligosaccharide release from frozen breast cancers.

Using anhydrous hydrazine and mixtures of glycoproteins from breast cancer specimens, a variety of different sized oligosaccharides was successfully released. After oligosaccharide release it was necessary to purify the oligosaccharide pool and this was achieved using a descending paper chromatography step in butan-1-ol, ethanol, water (4:1:1 v/v/v) for 48 hours.

2.9.2 Oligosaccharides extracted from paraffin-wax embedded specimens.

In addition to the release of oligosaccharide from frozen breast cancer specimens, we have shown that intact oligosaccharides may be released from paraffin-wax embedded archival tissues specimens (Dwek *et al.*, 1996). The analysis of oligosaccharides from such specimens showed that asialo, monoantennary, biantennary, triantennary and tetraantennary sialylated structures could be extracted. Differences were observed in the relative amounts of oligosaccharides extracted from the specimens stored in the two different ways. On analysis, these differences appeared to be due to intra-tumour variation in glycosylation. The oligosaccharides released from healthy tissue, from porcine liver, frozen and paraffin-wax embedded, showed no significant differences between either the types or relative amounts of oligosaccharides extracted.
MALDI/MS was used to analyse the oligosaccharides from the frozen and paraffin-wax embedded breast cancers further. The results indicated that the oligosaccharides from the frozen and the paraffin-wax embedded breast cancers, which have the same hydrodynamic volume, also have the same mass values.
As a further application of the technique, we have shown that oligosaccharides can be released in sufficient quantities from single 10μm thick sections of breast cancer tissue to allow analysis using normal-phase HPLC.
Our studies illustrated that whilst fixing tissues in formalin and embedding them in

paraffin-wax may cross link the proteins, such processing does not affect the oligosaccharides which can later be extracted from the paraffin-wax embedded tissues.

It is now feasible to release intact oligosaccharides, structurally map them and investigate whether oligosaccharides are over-expressed in aggressive breast cancers, using tissue from patients followed-up for extended periods of time.

Chapter 3.

Pilot study of oligosaccharides from nineteen long and short-term survival breast cancer patients.

3..1 Introduction.

We have developed methods for the release of oligosaccharides from archived, paraffm-wax embedded, breast cancer specimens as detailed in chapter 2. Initially we aimed to evaluate oligosaccharide expression in a pilot study of a small number of breast cancer specimens of patients with known outcome. We extracted oligosaccharides from malignant tissue of the breast, separated them, and sought to determine if their expression related to the occurrence of distant metastases. Various reports have suggested a link between oligosaccharide expression and formation of metastases in breast cancer, described in section 1.4 and in particular as measured by HPA lectin binding to tissue sections (Brooks and Leathem, 1991) and increased β1-6 GlcNAc branching in an animal model of metastasis (Dennis *et al.*, 1987). In this study, oligosaccharides extracted from primary breast cancers from two groups of patients were compared: patients without apparently aggressive disease versus patients with aggressive disease as measured by the development of secondary cancers at distant sites.

3.2 Materials and methods used in the study.

3.2.1 Specimens used.

Specimens were collected from the Pathology archives from two groups of patients treated at the Middlesex and University College Hospitals, London, between 1979 and 1981. The patients were matched for similar clinical features, pathology and treatment but had either long or short-term survival after presentation. In the short-term survival group, the primary cancers progressed to form distant metastases within five years and all the patients died within seven years of primary surgery. In the long-term survival group, there was no sign of cancer recurrence despite fifteen years follow-up. The clinical and histopathological features of the specimens used are shown summarised in tables 3.1 and 3.2, the individual patient details are shown in appendix 1.

Table 3.1 Histopathological features of the breast cancer specimens used in the pilot study.

Description	*Short-term survivors*	*Long-term survivors*
Histopathological type:		
Infiltrating ductal cancer	9	8
Infiltrating ductal and lobular cancer	-	1
Infiltrating lobular cancer	1	-
Grade:		
I	1	1
II	1	3
III	5	3
Axillary lymph node involvement:		
Lymph node negative	2	5
Lymph node positive	8	4

3.2.2 Patient follow-up to monitor disease progression.

Disease progression was monitored by patient follow-up, this was achieved by examining the patient's medical records and recording relevant information. Patients typically attended the hospital every three months for the first year post-operatively, every six months for the following year and then yearly until ten years had elapsed. Patients with metastases may have attended at other times if the disease was symptomatic. Metastatic spread was recorded only if it had been identified clinically and by further investigation, for example, X-ray in the case of metastases to lung or bone. Cases where a patient had died from metastatic disease were confirmed by death certificate from the Office of Population Census and Surveys. In the case of patients who had been discharged with more than ten years disease free survival, their General Practitioner was contacted to confirm that the patient was still alive with no clinical symptom or sign of local or distant recurrence.

Table 3.2 Clinical features of the breast cancer specimens used.

Description	*Short-term survivors*	*Long-term survivors*
age at presentation in years (mean)	64	58
range =	53 - 76	53 - 68
size of tumour in cm (mean)	2.9	2
range =	1 - 6	1 - 6
wide local excision	-	1
Mastectomy	10	8
follow-up information:		
no sign of recurrence in 15 years	-	9
widespread metastases within 5 years	10	-

3.2.3 Macrodissection of the specimens.

Breast tumour tissue contains a heterogeneous mixture of cancer, normal cells and reactive tissue in different amounts. We removed as much of the non-tumour areas as possible in order to limit sample-to-sample variation. In each case, a 5μm thick section was cut from the paraffin-wax embedded breast cancer specimen and mounted onto a glass slide. The section was dewaxed by passing through xylene and graded alcohols to water then stained with haematoxylin and eosin to identify the different cell types. The specimens were dissected on the paraffin-wax blocks by comparing the haematoxylin and eosin stained section with the paraffin-wax block and carefully removing areas of non-malignant cells, necrosis or lymphocyte infiltration. A number of specimens were stained with haematoxylin and eosin after macrodissection to confirm that the areas of non-malignant cells had been effectively removed by the procedure. A size 22 scalpel was used for the macrodissection step.

3.2.4 Preparation of cancer specimens for oligosaccharide release.

Glycoproteins were prepared from a pool of twenty 5 μm thick sections cut from each of the macro-dissected paraffin-wax embedded tissues, using the methods detailed in section 2.2.4.

3.2.5 Oligosaccharide release and labelling.

Oligosaccharides were released using the methods detailed in section 2.2.5, but because only two specimens could be processed at the same time in the GlycoPrep 1000, the oligosaccharide release procedure was performed manually on batches of six specimens. 2 mg of freeze-dried fetuin from fetal calf serum (Sigma) was included to check that oligosaccharide release was proceeding in a predictable manner. The freeze-dried weight of breast cancer glycoproteins was not determined accurately since the single milligram quantities had previously been determined on other breast cancer specimens, to check solubilisation in hydrazine. In addition, there were difficulties associated with weighing such small quantities of lyophilised proteins since freeze-dried material takes up atmospheric moisture and this makes such measurements both difficult and inaccurate. By reference to previous preparations from breast cancer specimens of a similar size, it was predicted that a maximum of 2 mg of glycoprotein had been prepared from each of the specimens. To release the oligosaccharides 800 µl of anhydrous hydrazine (Oxford Glycosciences) was added. The volume of hydrazine used was selected to ensure that there was an excess of hydrazine present. The hydrazinolysis reaction was allowed to proceed under a blanket of argon at 95^{o}C for 18 hours. Other steps from the method of Takasaki et al., (1982) were followed. Residual peptides were removed as detailed in section 2.2.6 and the oligosaccharides were labelled with 2-AB as detailed in section 2.2.7.

3.2.6 Analysis of the oligosaccharides.

The oligosaccharide pool from each of the breast cancer specimens was reconstituted in 20 µl of HPLC grade water.

3.2.6.1.....Sialylated oligosaccharides.

A 10 µl aliquot of the oligosaccharide pool was separated by anion- exchange chromatography on a GlycoSep C column as detailed in section 2.2.8.

3.2.6.2.....Neutral oligosaccharides.

The remaining 10 µl aliquot of the oligosaccharide pool was mixed with 20 µl of dextran ladder and separated by gel permeation chromatography (GPC) on a BioGel P4 column, using the instrument and conditions described in section 2.2.8.

3.2.6.3.....Separation of monosaccharides using a Carbo Pak PA100 column.

The GPC fractions corresponding to less than 3 glucose units hydrodynamic volume were collected from one of the long-term and one of the short-term survival patients. The fractions were dried by centrifugal evaporation and the monosaccharide structures analysed by separation on a CarboPak PA100 column (Dionex). The analysis was performed by Miss Heidi Lacey as follows: column flow rate was set to 0.5 ml / min and the column was washed with 20 column volumes of 1 mol / l sodium hydroxide. The column was equilibrated in 160 mmol / l sodium hydroxide and the unknown monosaccharide loaded onto the column in the same buffer. The elution position of the unknown monosaccharides from the GPC separations were compared with the elution positions of 2-AB labelled GalNAc, GlcNAc, Glc and Gal.

3.2.7 Analysis of the results.

The peaks on the chromatograms corresponding to fluorescently labelled oligosaccharides were integrated as detailed in section 2.2.8.1.3. The proportion of neutral to sialylated oligosaccharides, and the diversity of sialylated oligosaccharides were compared using the Student *t*-test (non-paired test). 'Significant' differences were recorded for values of P which were less than 0.05, 'suggestively significant' values were recorded for values of P which were less than 0.1 but greater than 0.05. Values of P which were greater than 0.1 were recorded as 'not significantly different'.

3.3 Results: specimens used for oligosaccharide release.

The H+E stained breast cancer specimens after dissection are shown in figure 3.1. The cancer specimens in the two patient groups: long-term survival and short-term were similar when the extent of connective tissue and the dedifferentiation of the cancer cells were compared.

3.4 Results: sialylated oligosaccharides.

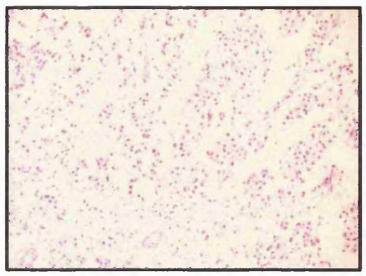
To compare the sialylated structures present in the specimens from the long-term and short-term breast cancer survivors, the oligosaccharides were separated by anion-exchange chromatography on a GlycoSep C column, the profiles obtained are shown in figure 3.2.

3.4.1 The percentage of sialylated oligosaccharides.

Each of the peaks corresponding to fluorescently labelled oligosaccharide were integrated as described in section 2.2.8.1.3. The integration area values obtained for individual peaks were added together to give a value for the 'total oligosaccharide pool'. This 'total oligosaccharide pool' value was set to 100%, from this, the relative amounts of different oligosaccharide peaks, such as the neutral and sialylated, were calculated. All the sialylated oligosaccharides, expressed as a percentage of the total oligosaccharide pool, were compared for the long-term survivor and short-term survivor specimens. The results are shown in figure 3.3.

Figure 3.1
Breast cancer specimens from short-term survival patients, stained with H+E.

—
scale bar =
50 micron



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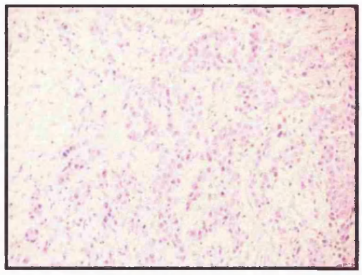

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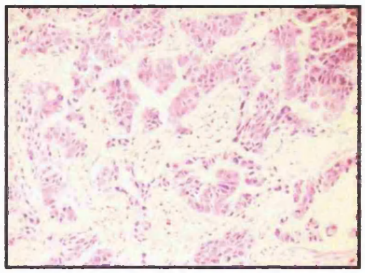

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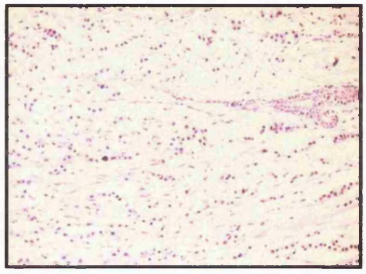

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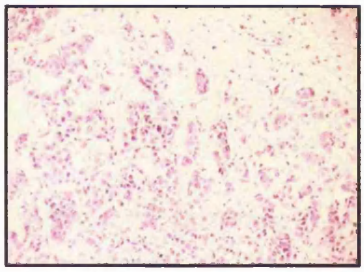

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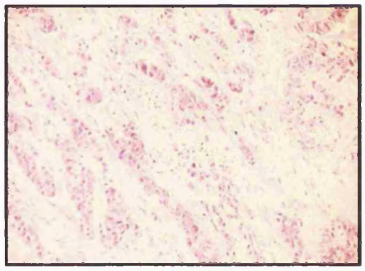

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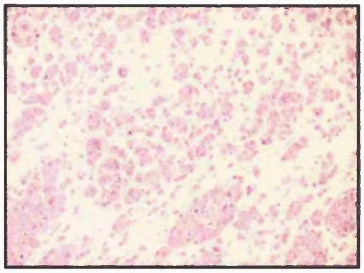

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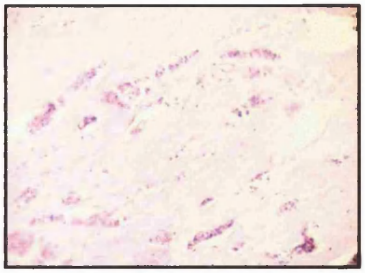

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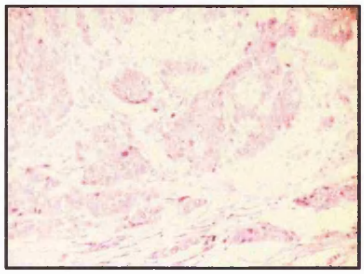

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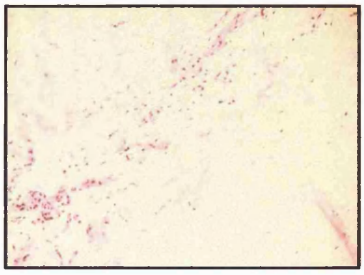

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Figure 3.1 continued
Breast cancer specimens from long-term survival patients, stained with haematoxylin and eosin.

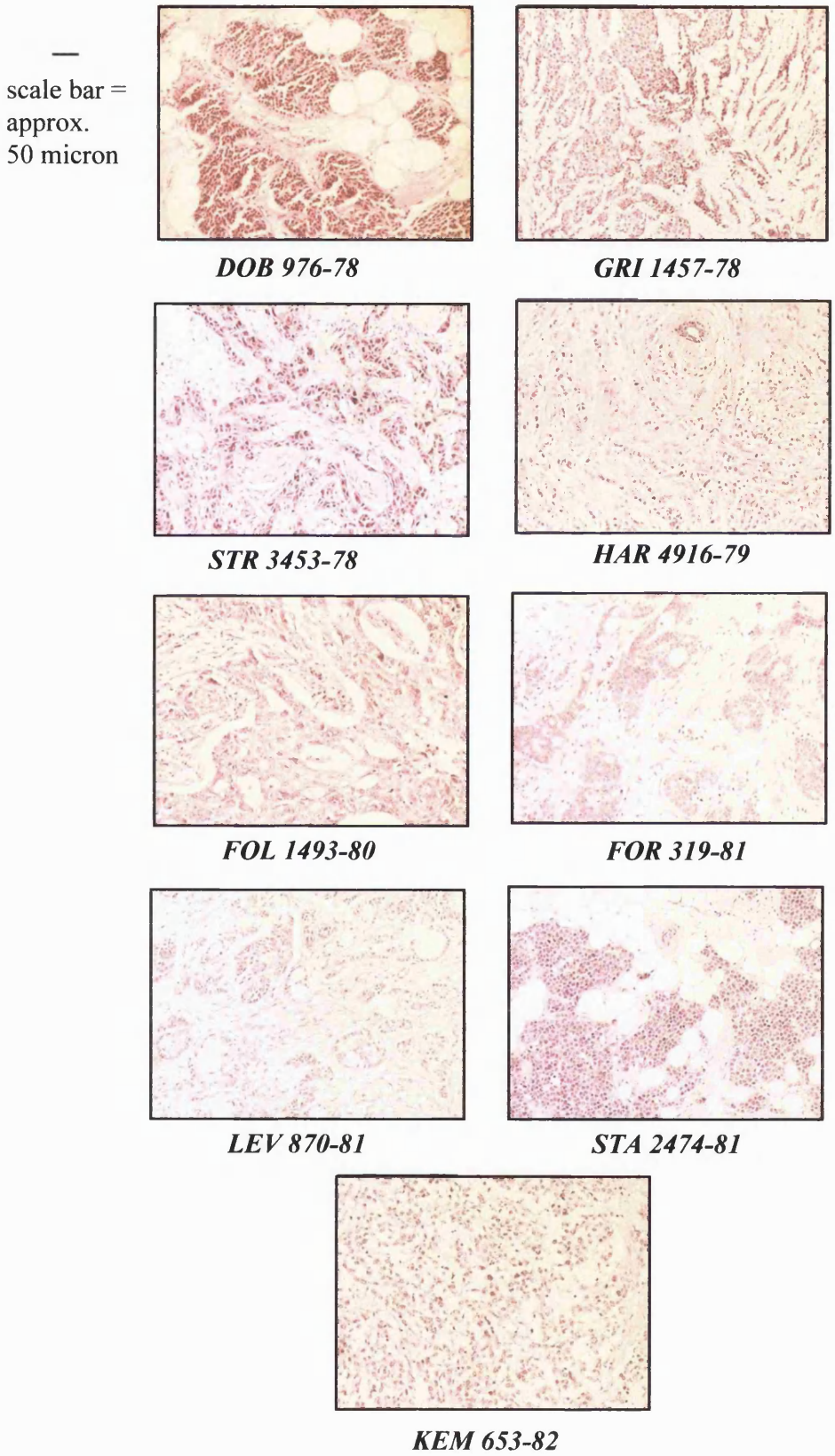


Figure 3.2
Oligosaccharides from long-term (blue) and short-term (red) breast cancer survivors, separated by anion-exchange chromatography.
Elution position of asialo (A0), monosialylated (A1), bisialylated (A2), trisialylated (A3) and tetrasialylated (A4) oligosaccharides indicated.

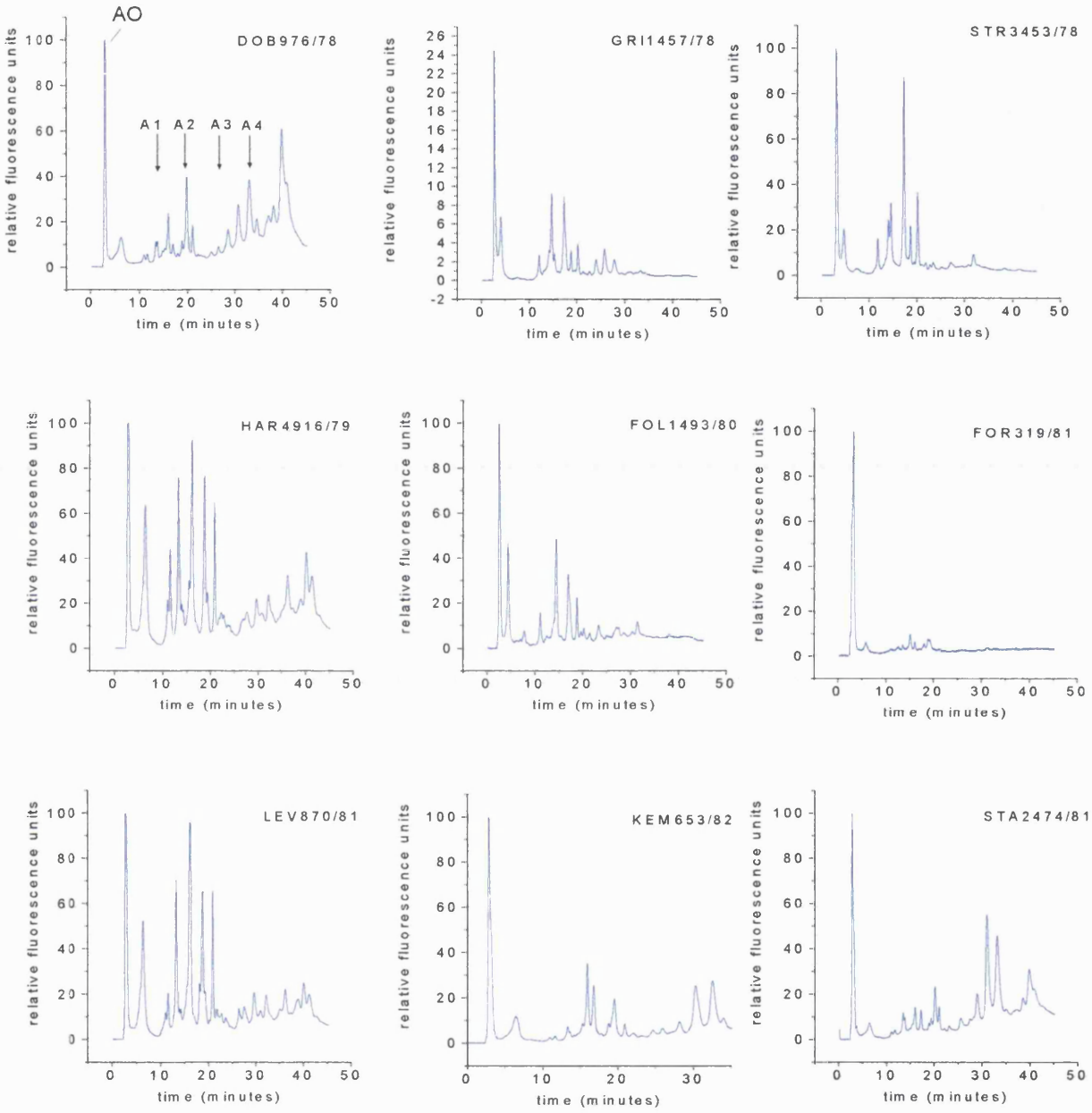
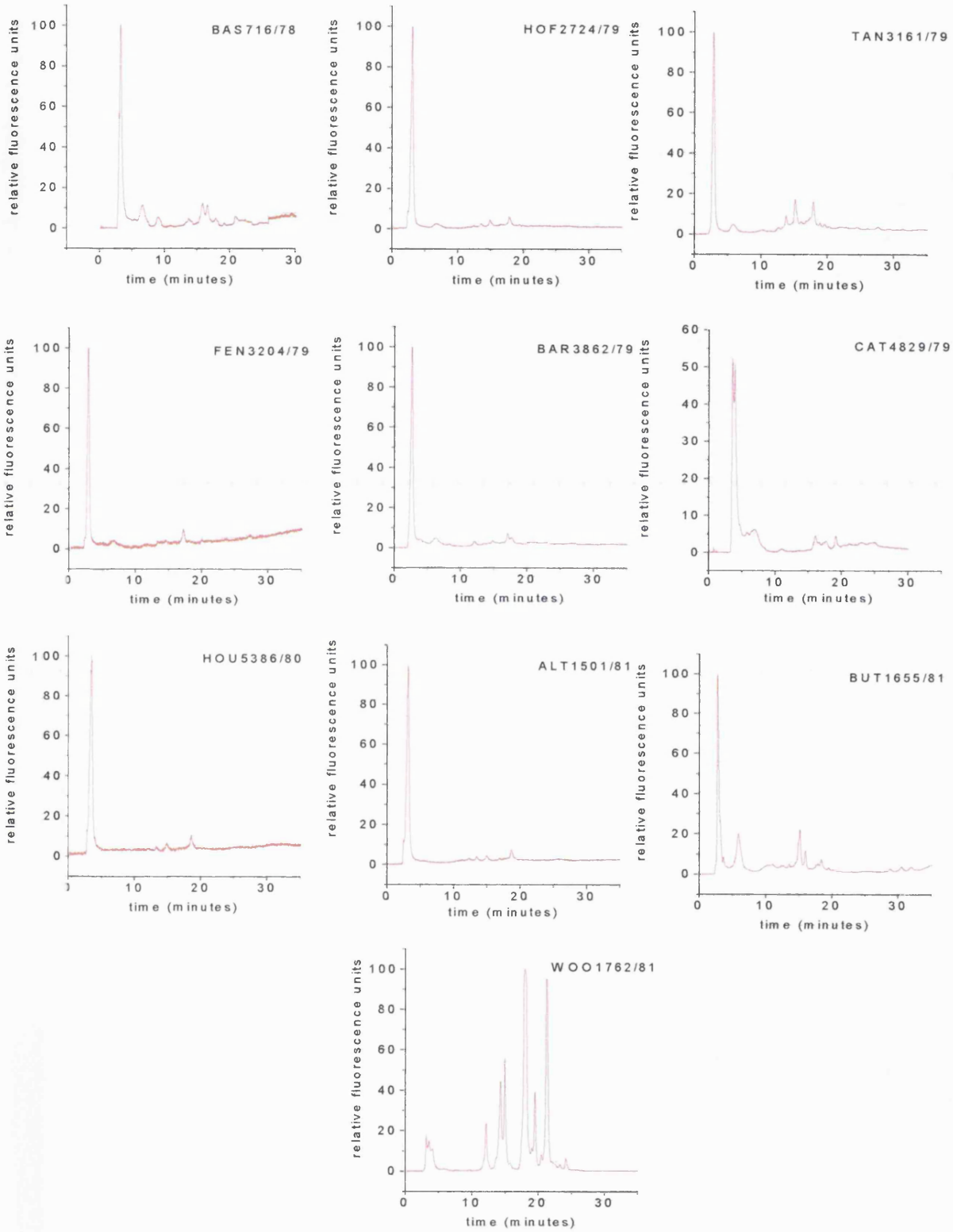


Figure 2.2 continued



3.4.2 The relationship between neutral and sialylated oligosaccharide expression.

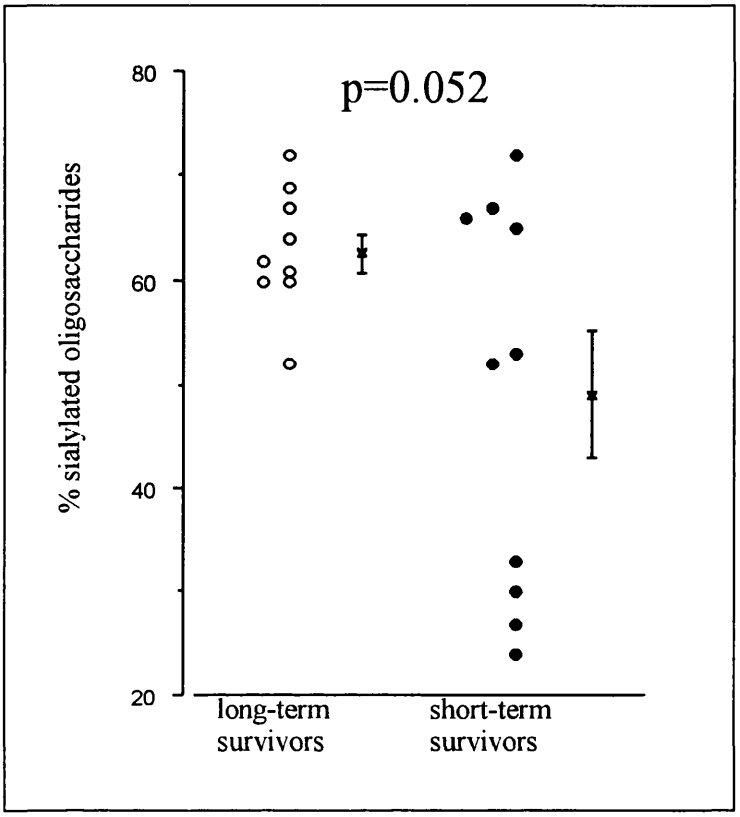
It was thought that the decrease in the relative amounts of sialylated oligosaccharides observed in the short-term survival patients might be due to over-expression of neutral oligosaccharides which would normally be sialylated. This possibility was evaluated by comparing the ratio of the percentage neutral to sialylated oligosaccharides for the long-term and short-term survivor specimens. No significant difference between the ratio values was observed. The trend towards significantly decreased amounts of sialylated oligosaccharides in the short-term survival patients, section 3.4.1, was, therefore, independent of the amount of neutral oligosaccharides present and was not simply due to a failure of sialylation of neutral oligosaccharides.

3.4.3 The number of different sialylated oligosaccharides present.

The oligosaccharides extracted from the different breast cancer specimens were assessed further by comparing the number of sialylated species separated on anion-exchange chromatography, as shown in figure 3.4.

Sialylated oligosaccharides elute from the anion-exchange column according to their size, the number of acidic residues attached and depending on the anomericity of the bond between the sialic acid and adjacent monosaccharide (α2-3, α2-6 or α2-8) (Guile *et al.*, 1994). The specimens from the short-term survival breast cancer patients showed a statistically significant decrease in diversity of charged oligosaccharide structures compared with the specimens from the long-term survivor patients, Student t-test $p=0.006$.

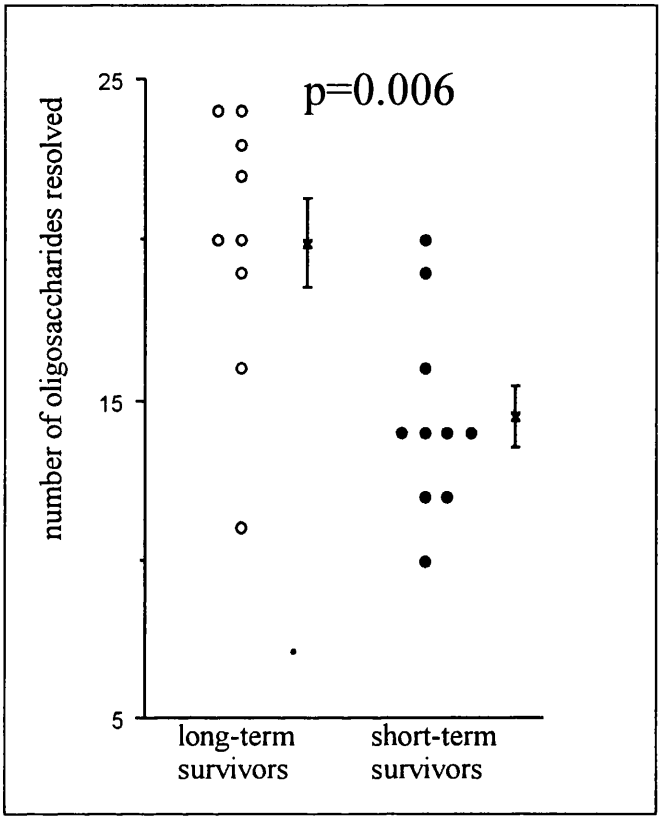
Figure 3.3 Sialylated oligosaccharides from long-term and short-term breast cancer survivors. Mean values +/- standard error and Student *t* test P value indicated.



In each of the populations the distribution of the relative quantities of oligosaccharides were found to show a normal distribution about the mean and a paired Student t-test was used to compare the relative amounts of sialylated oligosaccharides in the two populations.

There was a trend towards significantly decreased amounts of sialylated oligosaccharides in the short-term survival group ($p = 0.052$). This was due to four of the ten short-term survivor specimens having much reduced levels of sialylated oligosaccharides compared with the specimens from the long-term survivors. No apparent difference in the histological or clinical features, such as extent of lymph node metastases, was identified to account for the reduced levels of sialylated structures in the four short-term survival specimens.

Figure 3.4. Numbers of sialylated oligosaccharides separated on anion-exchange chromatography from breast cancers of short-term and long-term survivors. Mean values +/- standard error and Student *t* test P value indicated.



There was an overlap between the number of oligosaccharides resolved on anion-exchange chromatography from the short-term and long-term survivor specimens. In the long-term survivor specimens, there were two specimens for which few sialylated structures were resolved, in the short-term survivors there were two specimens for which many sialylated oligosaccharides were resolved on anion-exchange chromatography. The reason why this variation occurs is not obvious, examination of the H+E stained section does not provide any obvious explanation(s), nor do the clinical features that were recorded for the specimens.

3.5 Results : neutral oligosaccharides.

Where possible, the neutral oligosaccharides from a number of the specimens were evaluated by separation on a BioGel P4 gel permeation chromatography column. Only a few of the samples could be run in this way, since some of the specimens had insufficient oligosaccharide present for detection following gel

permeation separation. Of those samples run, the analysis indicated an absence of larger oligosaccharides in the short-term survival specimens, figure 3.5. Also, an increase in the relative quantities of small neutral glycans was observed in the short-term survival specimens. One of the structures over expressed in the short-term survival specimens, figure 3.6, was analysed further on a CarboPak PA100 column in monosaccharide mode. The structure over-expressed in the short-term survival specimens was found to elute at the same time as GalNAc, figure 3.7.

Figure 3.5 Neutral oligosaccharides from long-term survival (blue) and short-term survival (red) cancer specimens, separated on a BioGel P4 column fitted to a GlycoMap 2000. Elution positions of hydrolysate of dextran in glucose units (GU) shown.

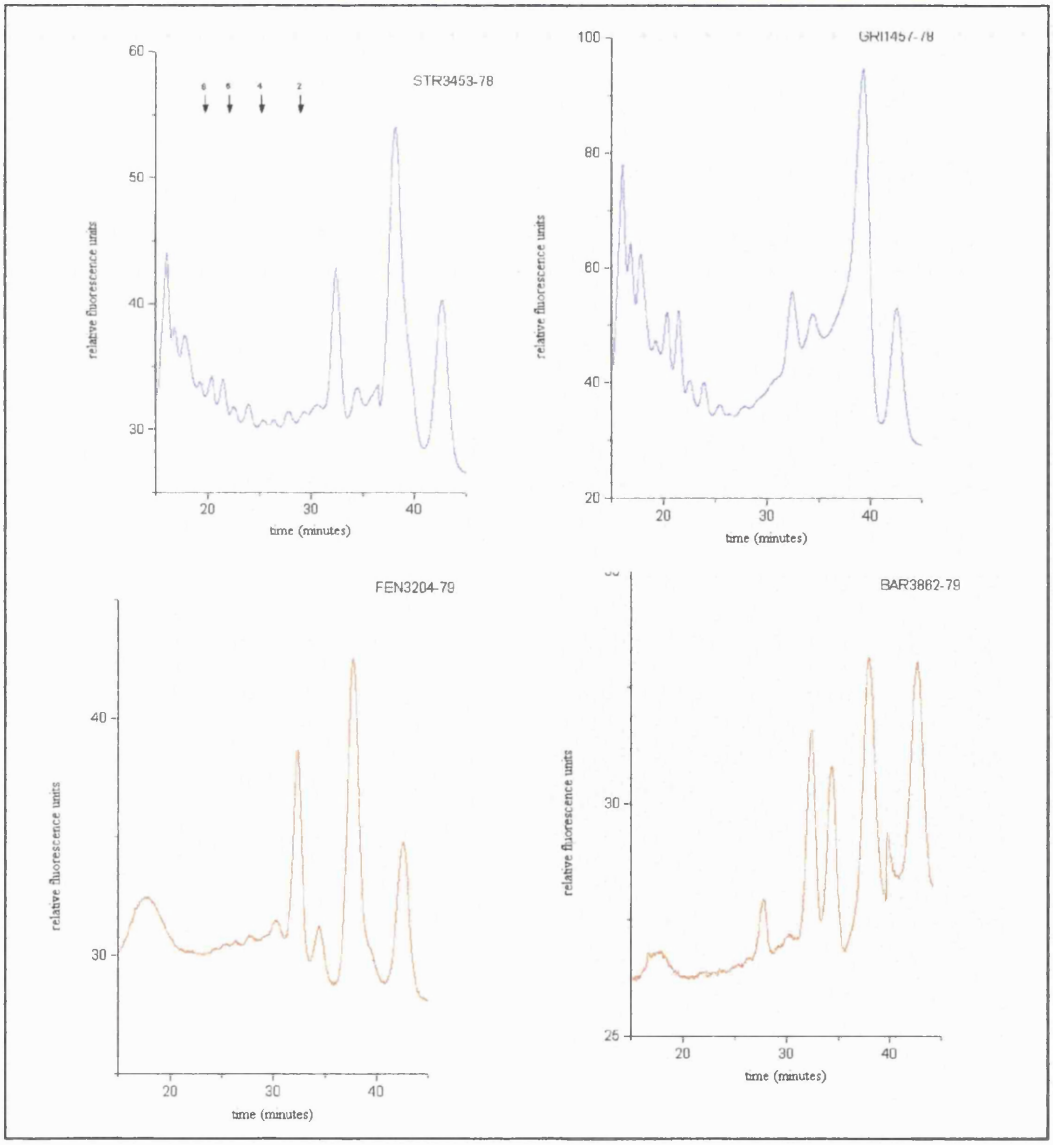


Figure 3.6 BioGel P4 separation of oligosaccharides from breast cancer specimens of a long-term survival (blue) and a short-term survival (red) breast cancer patient. The peak over-expressed in the short-term survival patient is shown by the arrow.

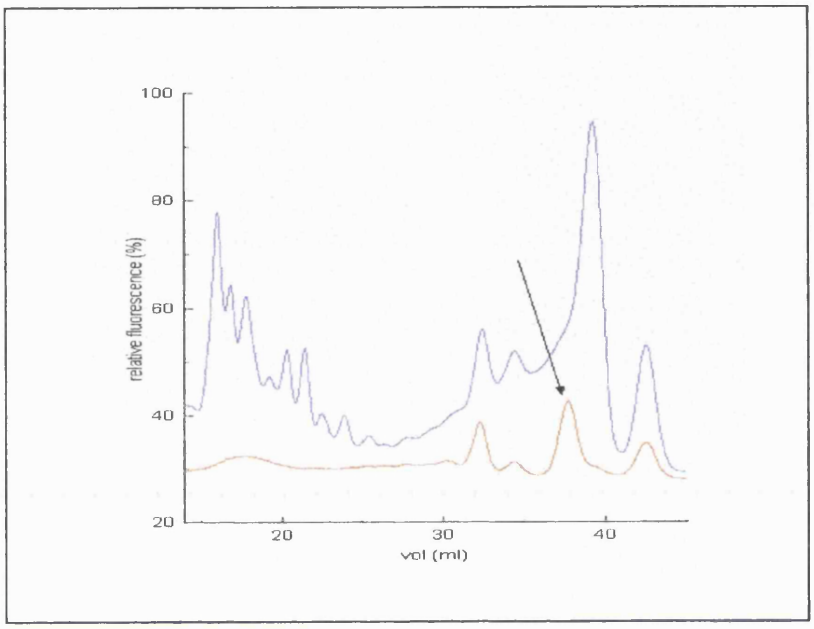
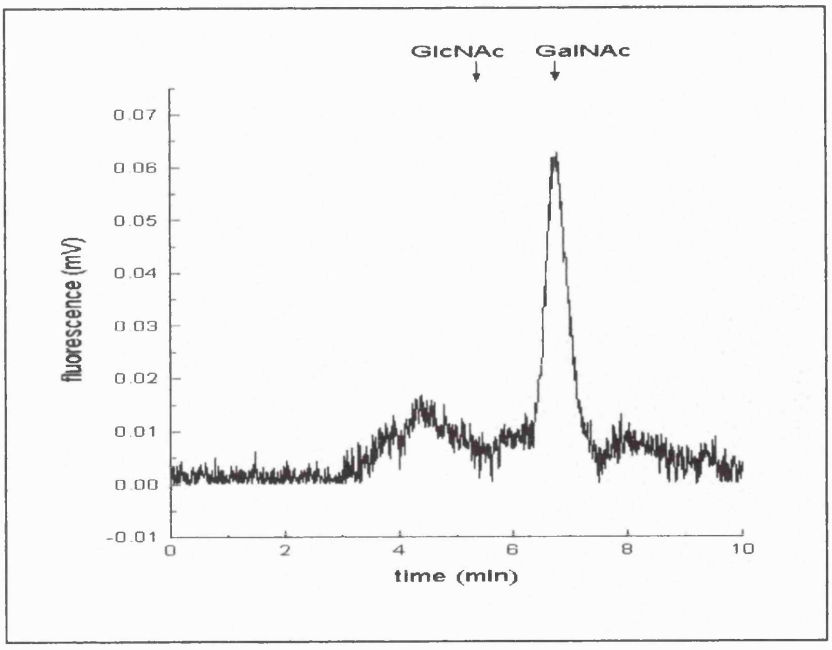


Figure 3.7 Analysis of a monosaccharide over-expressed in the short-term survival breast cancer specimen on a CarboPak PA100 column. The elution positions of 2AB-labelled GlcNAc and GalNAc are indicated.



3.6 Discussion of pilot study.

We have used techniques, developed for the extraction of oligosaccharides from archival breast cancer specimens, to compare oligosaccharides from primary breast cancers of patients who did / did not develop clinical metastases. The primary breast cancers were from post-menopausal patients presenting in the clinic with similar grade and stage disease, who underwent similar surgical and post-operative therapies.

Various differences have been identified in the oligosaccharides extracted from the 'metastatic' compared with the 'non-metastatic' breast cancers. In particular, a reduction in the amount of sialylated oligosaccharides, as a percentage of the total oligosaccharide pool was found in the short-term survival specimens. This does not appear to be due to a failure of sialylation of neutral oligosaccharides, since the relative quantities of neutral glycans were not increased in the specimens from the short-term survival compared with the long-term survival patients.

Also, a significant reduction in the number of different charged oligosaccharide structures was observed in the specimens from the short-term compared with long-term survival patients ($p=0.006$). Reduced numbers of neutral oligosaccharide structures were also seen in the short-term, compared with the long-term, survival patients. Finally, the short-term survival patients showed an over-expression of the monosaccharide GalNAc.

The cellular events which might lead to the production of different glycoforms in the breast cancers with different clinical behaviours have not been investigated in this study. The results, of decreased sialylated glycans and decreased numbers of sialylated and neutral structures in the short-term survival specimens, both suggest that an overall failure of glycosylation may be occurring. Possible reasons for the differences in the oligosaccharides extracted from the short-term survivor specimens include the inappropriate expression of glycosyltransferase enzymes in the Golgi, reduced levels of donor sugars and faster turnover of cellular proteins. Various reports suggest that there are differences in the levels and activities of glycosyltransferases in cancer cells, for example, increased levels and activity of sialyltransferase (CMP-sialic acid Galβ1-3GalNAc α3-sialyltransferase) in some breast cancer cell lines resulting in the over-expression of shortened O-linked oligosaccharides (Brockhausen *et al.*, 1995). To date, however, there have not

been any reports of cancer-associated changes in glycosyltransferases which would explain the increased expression of GalNAc observed in poor prognosis breast cancers, but as the mode of operation of the enzymes of glycosylation becomes better understood, mechanisms which lead to increased GalNAc expression on proteins may become apparent.

The results of this study correlate with some reports of altered glycosylation in breast cancer. Brockhausen *et al* have reported decreased branching and reduced diversity of sialylated O-linked oligosaccharides in breast cancer cell lines, as noted above (Brockhausen et al., 1995), but this has not been reported to be associated with the formation of metastases. Such associations may need to be verified by clinical studies similar to this one. The increased level of GalNAc extracted from the specimens of the short-term survival patients also correlates with reports of increased Tn antigen expression (Springer, 1989) and HPA lectin binding (Brooks and Leathem, 1995a; Brooks and Leathem, 1991) in poor prognosis breast cancers. We did not find any association between increased β1-6GlcNAc branching and polylactosamine expression in the metastatic breast cancers, despite reports of its importance in the formation of metastases in an animal model (Dennis *et al.*, 1987).

The formation of metastases is undoubtedly a multistep event that is dependent on many factors, as described in section 1.2.6. Nevertheless, the differences observed between the oligosaccharides extracted from the metastatic compared with non-metastatic breast cancers suggest that particular changes in glycosylation of proteins are associated with, and may be advantageous for, the successful development of metastases. There are various ways in which changes in glycosylation may affect the function and recognition of secreted and cell surface proteins, discussed in section 1.3.1. Some changes in glycosylation of proteins might confer advantages to a cell during the process of metastasis, for example by affecting cell adhesion, section 1.2.5.4, or changing the immune recognition of cancer cells, section 6.6. To understand better which oligosaccharides are important in primary breast cancer and their possible role in the metastatic process, a larger series of specimens needed to be examined and multivariant analysis performed. This has been addressed in a larger retrospective study of 76 patients as described in chapter 5.

Chapter 4

Oligosaccharides associated with poor prognosis determined by Helix pomatia agglutinin (HPA) lectin binding to breast cancer cell lines.

4.1 Introduction

A number of reports suggest that HPA lectin-binding is a useful indicator for the prediction of aggressive metastatic cancer, discussed further in section 1.5.3.6., but the cancer associated oligosaccharides to which HPA binds have yet to be identified (Walker, 1993).

It is likely that the HPA binding oligosaccharides of interest, so far, are associated with glycoproteins, rather than glycolipids, since the predictive value of HPA for poor prognosis breast cancer has been determined using paraffin-wax embedded tissues (Brooks and Leathem, 1991) and such tissues are largely free from lipids. HPA lectin affinity chromatography experiments, using glycoproteins extracted from breast cancers, have revealed that many different glycoproteins bind to the lectin (Brooks and Leathem, 1995; Streets *et al.*, 1996).

We aimed to identify HPA binding oligosaccharides on glycoproteins from breast cancer cells and relate the oligosaccharide expression to the lectin binding pattern of the same samples when stained, using an indirect immunohistochemical method (Leathem and Brooks, 1987). Initially, we used human breast cell lines which exhibit a spectrum of HPA binding patterns (Schumacher *et al.*, 1995), later we compared the oligosaccharides from the cell lines with those extracted from an HPA binding human breast cancer specimen.

4.2 Materials and methods used in the identification of oligosaccharides which bind to Helix pomatia agglutinin (HPA) lectin.

4.2.1 Cells grown in vitro and human breast cancer specimen used.

4.2.1.1 .....Human breast cells grown in vitro.

The cells grown *in vitro* were originally established from human breast epithelia. Four human breast cell lines were evaluated, HBL100 derived from milk, BT549, BT20 and MCF7 derived from metastatic breast cancer cells. The cell lines were selected since, when formalin-fixed and paraffin-wax embedded, they exhibit a range of HPA lectin-binding patterns. HBL100

cells do not bind HPA lectin, BT549 and BT20 bind very weakly and MCF7 cells binds the lectin strongly (Schumacher et al., 1995), the cells used are shown in table 4.1.

Table 4.1 Human breast epithelial cells grown *in vitro*

Description	*Source from which cell line was established.*	*HPA lectin-binding pattern. From Schumacher (1995)*
HBL100	Human milk.	none (-)
BT20	Breast cancer	weak (+)
BT549	Breast cancer.	weak (+)
MCF7	Pleural effusion, ie. cells from metastases of a primary breast cancer.	strong (++++)

The cells were cultured and donated by Dr. E. Adam and Prof. U. Schumacher, Department of Human Morphology and Anatomy, University of Southampton, using the media recommended on the data sheet supplied with the cells from the EACCC / ATCCC. In each case, one $75cm^3$ flask of confluent cells was provided.

4.2.1.2 .....HPA lectin-binding human breast cancer specimen .

A human breast cancer specimen was selected which had been shown to stain intensely with HPA lectin (Leathem and Brooks, 1987). The specimen, reference number 1303, was a grade III multifocal infiltrating ductal carcinoma.

4.2.2 Lectin affinity chromatography of proteins prepared from cell lines.

4.2.2.1 .....Sample preparation.

Sample preparation was carried out with help from Mr. Andrew Streets. The cells were washed three times with 10 ml of 5 mmol / l Tris-HCl and 0.15 mmol / l phenylmethylsulfonylfluoride, pH 7.4. After washing, 10 ml of fresh buffer was added to each flask and the cells removed with a cell scraper. The cells were lysed in a Parr cell distribution bomb apparatus (nitrogen

bomb) which was maintained at 700 psi for 15 min (apparatus loaned by the Department of Medicine, UCL). Conditions to achieve cell lysis had previously been determined. To remove any gross cellular debris the cell lysate was centrifuged at 2,000 g for 20 min in a Centaur-2 MSE centrifuge. The supernatant was collected and filtered through a 0.2 μm syringe top filter. Lipids were extracted from the cell lysates by phase-separation using one part of chloroform to two parts methanol (Wessel and Flugge, 1984). Free sugars and salts were removed by passing the preparations through a Sephadex HR10/10 'desalting' column fitted to a Pharmacia FPLC machine with in-line UV detector and λ 254 nm filter. The HR10/10 column retains material of less than 10,000 Da in size and larger proteins elute in the void volume. The buffer used was 20 mmol / l Tris-HCl, 50 mmol / l NaCl, pH 7.6, the flow rate was set to 3 ml / min and fractions of 1 ml volume were collected. The elution of the proteins from the cell lysate preparations was monitored by their absorbance in the UV. In practice, the proteins were collected in two 1 ml fractions. The fraction with the greatest amount of protein, as measured by optical density at λ 254 nm, was used in the lectin-affinity chromatography experiments.

4.2.2.2.....HPA lectin affinity chromatography.

The experiments were conducted by Mr. Andrew Streets. First the HPA-agarose beads (Sigma) were 'fined' as follows: 6 ml of beads were suspended in 20 mmol / l Tris-HCl, 50 mmol / l NaCl, pH 7.6 and then transferred to a 10 ml measuring cylinder. After 30 min had elapsed the (cloudy) supernatant was removed. The procedure was repeated a further two times. After the beads had been 'fined', four HPA-agarose affinity chromatography columns were prepared each containing 1 ml bed volume of beads. Each of the columns was washed with 20 column volumes of 20 mmol / l Tris-HCl, 50 mmol / l NaCl pH 7.6 buffer. The proteins prepared from the cell lines, as detailed in 4.2.2.1, were loaded onto the HPA-agarose. In each case, the void volume, 0.8 ml, was allowed to flow, drip-wise, through the column. The column was stoppered for 40 min to allow interaction between the proteins and the beads, this time was determined in

previous experiments. After 40 min had elapsed, the column was unstoppered and unbound / weakly bound proteins eluted from the column with five column volumes of buffer. 1ml fractions were collected. To elute the bound glycoproteins, 1 ml of freshly prepared 0.25 mol / l GlcNAc in 20 mmol / l Tris-HCl, 50 mmol / l NaCl pH 7.6 buffer was added to the column. The column was stoppered and left at 4°C overnight. The following day the HPA lectin-binding glycoproteins were eluted from the column using 5 column volumes of freshly prepared 0.25 mol / l GlcNAc in 20 mmol / l Tris-HCl, 50 mmol / l NaCl pH 7.6 buffer. Again, 1 ml fractions were collected.

4.2.2.3.....Dot-blot to confirm the presence of HPA lectin-binding proteins.

To confirm the presence of HPA lectin-binding glycoproteins, following the affinity chromatography experiments, a dot blot was performed. A 5 µl aliquot from each of the fractions was taken for this purpose, spotted onto a piece of nitrocellulose membrane and allowed to air dry. The unreacted sites on the nitrocellulose membrane were blocked using a 1% $^{w}/_{v}$ solution of bovine serum albumin (BSA, Sigma) in Tris buffered saline with 0.05% $^{v}/_{v}$ Tween 20, pH 7.6 (TBS-Tween). The BSA had previously been checked for non-reaction with HPA lectin. The blocking step was performed for 30 min with agitation, afterwhich the nitrocellulose was washed three times with the TBS-Tween solution to remove the BSA solution. The nitrocellulose membrane was then incubated overnight in a solution of 1 µg/ml HPA lectin.peroxidase label (Sigma) in TBS-Tween buffer. The next day, the nitrocellulose membrane was washed three times in TBS-Tween and six times with TBS to remove the lectin solution and then the blot was developed with diaminobenzidine and hydrogen peroxide for 10 min.
The dot blot confirmed the presence of HPA lectin-binding glycoproteins, as shown in figure 4.1.

4.2.2.4.....Dialysis prior to oligosaccharide release.

The fractions containing the HPA lectin-binding glycoproteins were collected and transferred to dialysis cassettes MWCO 10 kDa (Pierce). The samples

were dialysed at 4°C for 48 hours, with stirring and four changes of 500 ml HPLC grade water. After dialysis, the glycoproteins were transferred to 5 ml reactor vials (Chromocol) and freeze-dried for a minimum of 48 hours as detailed in section 2.2.4.3.

4.2.3 Preparation of peptides from an HPA binding breast cancer specimen.

The experiments to release peptides from an HPA lectin-binding breast cancer specimen, reference number 1303, detailed in section 4.2.1.2, were conducted with help from Miss Stephanie Slinn. 50 sections, each 15 μm thick, were cut from the paraffin-wax embedded block. The sections were placed into a glass universal container and dewaxed using the method described in section 2.2.4.2 and lyophilised as detailed in section 2.2.4.3. Cyanogen bromide (CNBr) was used to cleave the lyophilised proteins into peptide fragments (Gross and Witkop, 1962) CNBr cleavage of proteins has been reported not to damage the oligosaccharides attached (Brooks *et al.*, 1998).

48.1 mg of lyophilised protein was transferred to a round bottomed flask and a saturated solution of CNBr (Sigma), approximately 0.25 g in 25 ml of 70 % $^{V}/_{V}$ formic acid, was added. A nitrogen blanket was applied and the round bottomed flask corked with glass wool then incubated at 60°C for 2 hrs and then at 37°C for a further 60 hrs. At the end of the incubation period the reaction mixture was diluted to 7 % $^{V}/_{V}$ formic acid solution and filtered through nylon 140 μm mesh, the material was then centrifuged at 2,000 g for 20 min in a Centaur MSE centrifuge. The supernatent was collected and freeze-dried for 48 hours to remove residual CNBr. The peptide preparation was then solubilised into water and transferred to a dialysis cassette, MWCO 10 kDa (Pierce), and dialysed at 4°C with stirring, for 48 hours to remove any remaining CNBr. Four changes of water were made, each 500 ml. After dialysis, the peptides were transferred to two 5 ml reactor vials (Chromocol) and freeze-dried for 48 hours. One of the reactor vials, containing 30 mg of freeze-dried peptides, was used for oligosaccharide release as shown below. The other was stored at -20°C for later use.

4.2.4 Oligosaccharide release and labelling.

The oligosaccharides were released, from the cell line preparations using 200 µl of hydrazine and from the peptide preparation using 2.5 ml of hydrazine, then worked-up and labelled using the methods detailed in section 2.2.5.

4.2.5 Analysis of oligosaccharides released.

4.2.5.1 .....Anion-exchange chromatography.

Oligosaccharides were separated by weak anion-exchange chromatography column using the column and conditions detailed in section 2.2.8.1.3.1.

4.2.5.2 .....Reverse-phase chromatography.

In order to evaluate them further, the oligosaccharides were separated by reverse-phase chromatography. The fractions of interest from the anion-exchange chromatography separations were taken and dried by centrifugal evaporation. A porous graphitised carbon column, 4.6 mm diameter, 100 mm in length, was used for this purpose (GlycoSep H, Oxford Glycosciences). The oligosaccharides were loaded onto the column in 10 µl of 70:30 acetonitrile:water solution ($^{V}/_{V}$) and separation was undertaken using the conditions shown in table 4.2.

Table 4.2 Conditions used for the separation of oligosaccharides by reverse-phase chromatography

Time (minutes)	*Description*	*5% acetonitrile with 0.1% trifluoracetic acid*	*acetonitrile*
0 - 10	isocratic	100	0
10.1 - 30	gradient	80	20
30.1 - 50	gradient	60	40
50.1 - 60	re-equilibration	100	0

flow rate = 0.3 ml / min

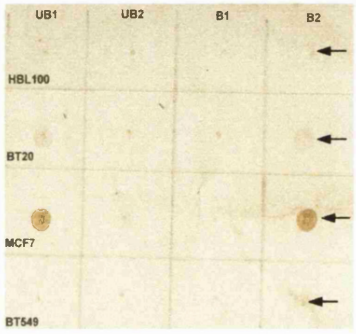
4.2.5.3 .....Normal-phase chromatography.

To evaluate the oligosaccharides further, fractions containing the structures of interest from the reverse-phase separation were collected and dried by centrifugal evaporation. The oligosaccharides were then separated on a GlycoSep N column (Oxford Glycosciences) containing 5 μm amide derivatised silica beads, the column was 4.6 x 250 mm in size and flow rate was set to 0.4 ml/min. Retention on the column is based on hydrophilicity and is related to the hydrodynamic volume and size of the oligosaccharides (Guile *et al.*, 1996). The column was housed in an oven at 30°C and the oligosaccharides loaded onto the column in 70:30 acetonitrile:water $^{v}/_{v}$ then eluted from the column using the buffers and conditions shown in table 4.3.

Table 4.3 Conditions used for the separation of oligosaccharides by normal-phase chromatography

Time (minutes)	*Description*	*acetonitrile*	*250 mmol/l ammonium formate pH 4.4*
0	isocratic	0	20
0 – 132	gradient	47	53
132 – 135	gradient	0	100
135 – 142	isocratic	0	100
142 – 145	re-equilibration	0	20
145 – 180	re-equilibration	0	20

Figure 4.1 Dot blot analysis of fractions obtained from HPA-lectin affinity chromatography of cell line glycoproteins. Unbound fractions (UB) and bound fractions (B) indicated. The arrows show the fractions taken for further analysis.



The separation of the oligosaccharides extracted from the HPA lectin-binding proteins by anion-exchange chromatography is shown in figure 4.2.
The oligosaccharides from the MCF7, strongly HPA binding, cell line showed increased expression of a monosialylated oligosaccharide compared with the other, weakly HPA binding, cell lines. The fractions corresponding to the monosialylated oligosaccharide peak were collected from the anion-exchange separations, dried, and passed through a reverse-phase GlycoSep H chromatography column.
The reverse-phase separation, of the peak initially detected by anion-exchange chromatography, revealed that numerous monosialylated oligosaccharides coelute on anion-exchange chromatography, figure 4.3.
Evaluation of the oligosaccharides from HPA binding glycoproteins of breast cell lines showed that charged oligosaccharides are present in increased levels in the strong HPA binding cell line (MCF7) and, in particular, a monosialylated structure appears to be over-expressed The monosialylated oligosaccharide found in elevated levels in the MCF7 cell line might represent an important HPA binding ligand, of the many, present in primary human breast cancers.

Figure 4.2 Oligosaccharides released from HPA binding glycoproteins from HBL100, BT20 and MCF7 cell line preparations, separated by anion-exchange chromatography. Elution postion of standard oligosaccharides are shown. The red arrow indicates the oligosaccharide peak over expressed in MCF7 cells.

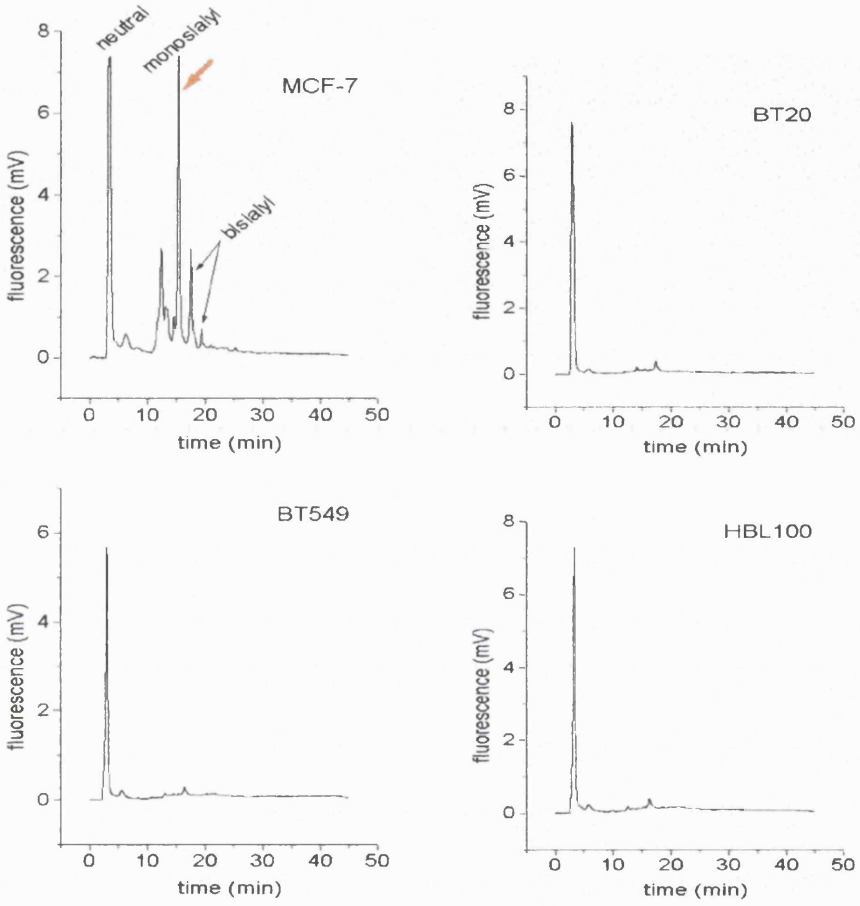
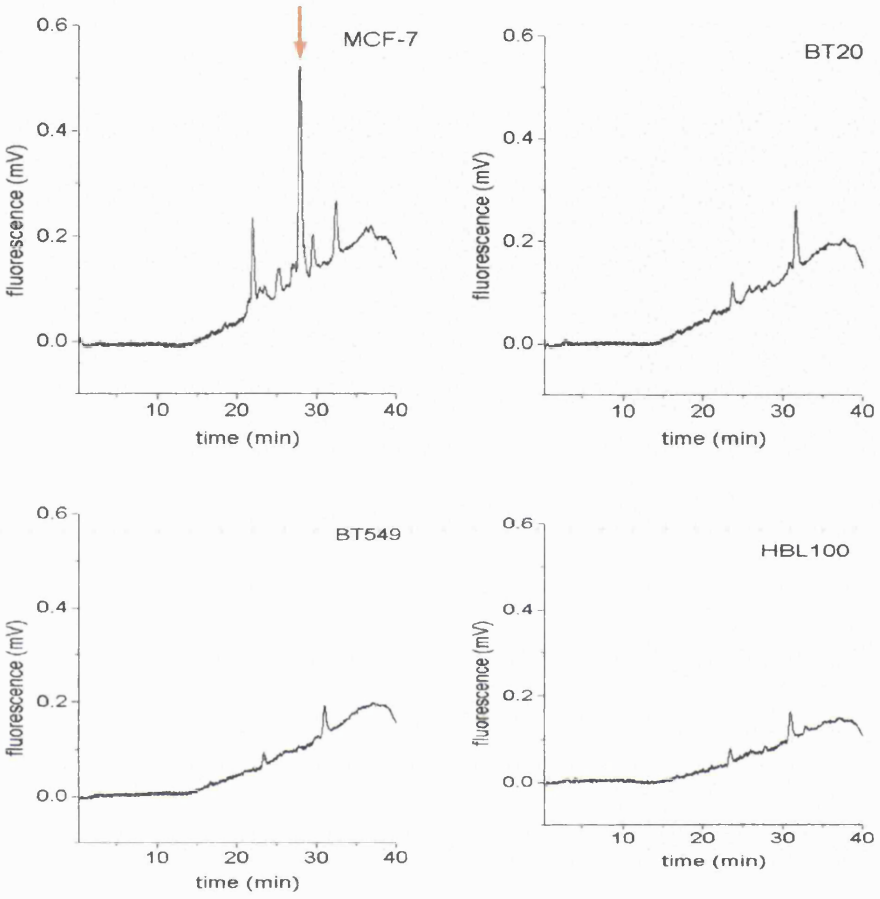


Figure 4.3 Monosialylated oligosaccharides from HPA binding proteins of human breast cell lines separated by reverse-phase chromatography. The red arrow indicates the oligosaccharide overexpressed in the MCF7 cell line.



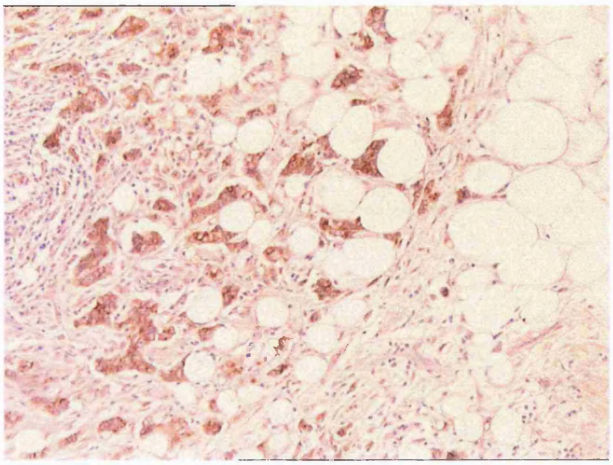
4.4 Results : oligosaccharides released from a breast cancer specimen.

We wanted to determine if the monosialylated oligosaccharide over-expressed on the HPA binding glycoproteins from the MCF-7 cells was also present in a human breast cancer specimen and, if so, to characterise the structure further.

4.4.1 Breast cancer specimen.

A primary breast cancer which stained intensely with HPA lectin by the indirect histochemical method (Brooks and Leathem, 1991) was selected from the archival tissue bank. The breast cancer specimen was a grade III, infiltrating ductal carcinoma, and is shown in figure 4.4.

Figure 4.4 Infiltrating ductal carcinoma of the breast, reference number 1303, stained with HPA (brown) and counter-stained with haematoxylin (blue) and eosin (pink), magnification approximately x200.

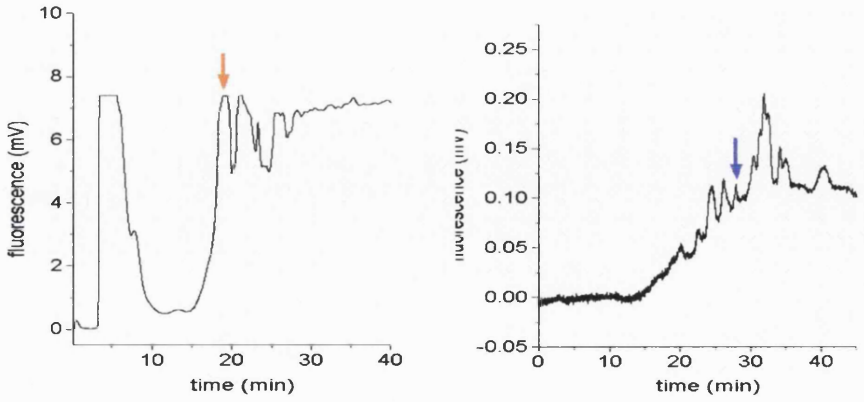


4.4.2 Oligosaccharide analysis.

Peptides and oligosaccharides were released from breast cancer specimen reference number 1303 as detailed in sections 4.2.3 and 4.2.4. The 2-AB labelled oligosaccharide pool was first separated by anion-exchange and then by reverse-phase separation, in the same way as the oligosaccharides released from the cell line preparations. The separation of the oligosaccharides obtained is shown in figure 4.5.

Figure 4.5 Oligosaccharides extracted from glycopeptides of breast cancer specimen number 1303.

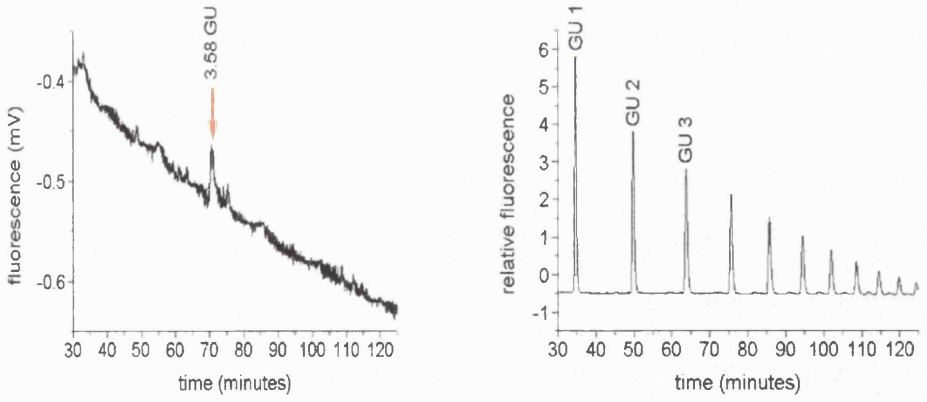
Anion-exchange separation. *Reverse-phase separation.*



In figure 4.5 the red arrow indicated the structure of interest which was then separated by reverse-phase chromatography. On analysis of the monosialylated oligosaccharides by reverse-phase chromatography, there was an oligosaccharide with the same elution position as the one overexpressed in the MCF7 cell line preparations, the oligosaccharide of interest, is shown by the blue arrow on the figure.

To further analyse this oligosaccharide, fractions were collected, dried and passed through a GlycoSep N normal-phase chromatography column. The hydrodynamic volume of the oligosaccharide of interest was compared to that of 2-AB labelled hydrolysate of dextran. In this way, the monosialylated oligosaccharide was found to have a hydrodynamic volume of 3.58 glucose units, as shown in figure 4.6.

Figure 4.6 Separation of a monosialylated oligosaccharide from breast cancer specimen number 1303 (left-hand panel) and a hydrolysate of 2-AB labelled dextran (right-hand panel) by normal phase chromatography.



4.5 Discussion of work to identify HPA binding oligosaccharides.

An *in vitro* model of normal and breast cancer cells was used in an attempt to identify differences between the oligosaccharides present in weak and strong HPA lectin-binding cell populations. Lectin affinity chromatography was used to collect cellular glycoproteins which bind HPA. On extracting and mapping the oligosaccharides from such glycoproteins, it was noted that the cells which bind the lectin strongly (MCF7) contain glycoproteins with proportionally more sialylated oligosaccharides than those cells which only bind the lectin weakly (BT20 / BT549) or not at all (HBL100). In addition, there appears to be a monosialylated oligosaccharide present on the glycoproteins from the MCF7 cell preparation which is absent in the other cell line preparations. Examination of an HPA lectin-binding primary breast cancer also revealed the presence of the monosialylated oligosaccharide, as well as many other oligosaccharides. Further analysis of the monosialylated oligosaccharide showed that the material had a hydrodynamic volume of 3.58 glucose units.

Whilst the results suggest a dramatic difference between the oligosaccharides present in the strong and the weak HPA lectin-binding cell lines, there remain numerous considerations. Firstly, it is possible that the oligosaccharides which bind the lectin strongly on immunohistochemistry are different from those which bind under the conditions used for affinity chromatography. In addition, in the cell line preparations we have used glycoproteins rather than glycopeptides for the affinity chromatography experiments and it is possible that the oligosaccharide over-expressed in the MCF7 cells was not an HPA lectin-binding oligosaccharide, but rather, associated with another (HPA binding) oligosaccharide on the same glycoprotein. Nevertheless, the presence of the 3.58 GU oligosaccharide in the breast cancer cell preparation would suggest that we have detected an interesting HPA binding structure.

We now need to investigate whether the 3.58 GU monosialylated structure is present in other primary breast cancers and, if so, whether its expression relates to the HPA lectin-binding pattern of breast cancer tissue sections (chapter 5).

Chapter 5

Oligosaccharide expression and long-term outcome: a study of 76 breast cancer specimens with 5-10 years follow-up.

5.1 Introduction.

The purposes of this larger retrospective study were:

i. to investigate if the differences in sialylated oligosaccharides in the breast cancer specimens associated with short-term survival (chapter 3) remained important;
ii. to determine if oligosaccharides, associated with poor prognosis, as detected by HPA binding to cells grown *in vitro* (chapter 4) remained important;
iii. to determine if the oligosaccharide in (ii) above, related to the immunohistochemical HPA lectin staining pattern of the specimens;
iv. to evaluate whether there were any other gross differences in the oligosaccharides which were associated with aggressive breast cancer; and
v. to determine if expression of the oligosaccharides, above, related to other prognostic markers as examined by multivariate analysis of the results.

5.2 The cohort of patients studied.

The breast cancer cases used in this study were identified from a database of patients treated by Mr. A. Wilson; a Consultant Surgeon at the Whittington Hospital London, between 1987 and 1991, post-operative radiotherapy and chemotherapy was provided at the Middlesex Hospital, London. A total of 96 patients were identified on Mr. A. Wilson's database and of these 76 cases were used in the final analyses as described below.

5.3 Materials and methods used in the study.

5.3.1 Selection of specimens.

The cases were selected from the database if they were between 1.0 and 3.0 cm in size, of no special histological type and approximately equal numbers of cases with and without axillary lymph node metastases.

Dr. S. Ramchandra (Pathologist) arranged for the histology reports for the first 79 cases on the database list to be copied from the archival records and allowed access to the corresponding tumour specimens, a total of 76 cases were used in the final study. The histopathological features of the specimens are shown in table 5.1. Haematoxylin and eosin (H+E) stained sections prepared by the Pathology Department for the original diagnosis were used by Dr. Anthony Leathem (Pathologist) for the identification of the breast cancer cells and for the determination of the Bloom and Richardson grade for each of the specimens.

Table 5.1 Histopathological features of the specimens used in main study.

Description	*Number*
Histopathological type:	
infiltrating ductal carcinoma	71
infiltrating ductal and lobular carcinoma	3
infiltrating lobular carcinoma	1
tubular	1
Grade:	
I	7
II	32
III	34
not graded	3
Axillary lymph node negative	42
Axillary lymph node positive	33
Axillary lymph nodes not sampled	1

5.3.2 Patient follow-up.

Patients were followed-up to at least May 1997, the clinical and histopathological information recorded is shown in Appendix 2. The data obtained was entered onto a FileMaker Pro computer database. A summary of the clinical and post-operative treatment and the follow-up information is shown in table 5.2. A total of three cases were withdrawn from the study: two of the individuals had previously presented with ductal carcinoma *in situ* and one patient had neurological symptoms,

the cause of which was undetermined. One patient could not be followed-up died in Africa, the cause of death was thought to be syphilis.

A number of patients for whom only censored data was available were included in the study. The censored data included: patients who died of other diseases (n=3), or other cancers (n=2), or who emigrated (n=2). In all cases, the last attendance at the Breast Clinic or attendance at their General Practitioner with no sign of recurrence was taken as the final recurrence-free follow-up date.

Table 5.2 Summary of the clinical and follow-up information obtained. Mean average values given.

Description	*Patients with no sign of recurrence*	*Patients with any recurrence*
Number of patients:	37	39
Age at presentation (years) =	55	59
Size of tumour (cm) =	2.0	2.3
Primary surgical procedure:		
Exision biopsy:	17	10
Wide local excision:	16	16
Mastectomy:	4	13
Post operative treatment:		
Radiotherapy:	19	20
Tamoxifen	30	33
Chemotherapy	5	12
Follow-up information:		
Mean follow-up (months)	85	-
Mean time to first recurrence (months)	-	41

5.3.3 HPA lectin-binding.

HPA lectin binding pattern of the breast cancers was determined using the indirect IHC method of Leathem and Brooks, (1987), this was undertaken by Mrs Ann Titcomb. The HPA lectin-binding pattern was then scored according to the criteria

described (Leathem and Brooks, 1987) by Dr. A. Leathem. Five cases were not included in the HPA staining, two because there were insufficient cancer cells to assess lectin binding pattern, two whose cellular morphology and staining pattern was not sufficiently good, and one case which had damaged cells that had the appearance of infarction.

5.3.4 Microdissection of specimens prior to oligosaccharide release and mapping.

A 5 μm and a 10 μm thick serial section was cut from each of the paraffin-wax embedded breast cancer specimens. The 10 μm thick section was mounted onto an acid washed glass slide, whilst the 5 μm thick sections were mounted onto standard glass slides. The following steps were performed by Miss Heidi Lacey: For each specimen, the 5 μm and 10 μm sections were dewaxed by passing through 1,1,1-trichloroethane and trichlorethylene (CNP) then graded alcohols to water.
The 5 μm section was stained with H+E, examined under a pre-cleaned stage of a light microscope, and compared with the corresponding unstained 10 μm thick section. The areas of the 10 μm thick section which did not contain tumour cells were carefully removed using the tip of a scalpel. In practice, clumps of cells adjacent to the tumour cells, such as lymphocytes, adipose cells and necrosis could be effectively removed. Cells which infiltrated the tumour, such as lymphocytes and some connective tissue were more difficult to remove but this was attempted as far as was practicable.

5.3.5 Specimen preparation.

The 10 μm microdissected specimens mounted onto glass slides were cut into shards using a glass cutter and placed into 5 ml Chromocol reactor vials. The specimens were then placed into the chamber of an Edwards Modulyo freeze-drier fitted to an Edwards EM2 pump and freeze-dried for a minimum of 48 hours.

5.3.6 Oligosaccharide release and labelling.

The oligosaccharides were released manually in batches of nine specimens and one standard protein (fetuin from fetal calf serum) as described in sections 2.2.5 and 2.2.6. After their release, the oligosaccharides were labelled as detailed in section 2.2.7. A number of microdissected specimens where the *cancer* cells were removed and breast connective tissue / fat cells remained, were used as 'control' cases. The results indicated that few oligosaccharides were released from these cell types.

5.3.7 Analysis of the oligosaccharides using normal-phase chromatography.

Normal-phase HPLC was used to separate the oligosaccharides released from this large group of breast cancer specimens. Using the normal-phase HPLC system, monosaccharides and oligosaccharides, asialo and sialylated structures are separated in a single 3 hour run (Guile *et al.*, 1996). The analysis was performed by Miss Heidi Lacey as follows: The oligosaccharides, released from each of the specimens and the fetuin standards, were reconstituted in 20µl of 70:30 acetonitrile: water buffer $^{v}/_{v}$. A 5 µl aliquot was then loaded onto the normal phase chromatography column and eluted using the conditions detailed in section 4.2.5.3. In this study, the oligosaccharides were detected on elution from the column by an in-line Jasco FP920 fluorescence detector connected to a Borwin data handling package.

5.3.8 Calibration of the normal phase chromatography column.

Each day, before running the oligosaccharides from the breast cancer specimens, the column was calibrated with a 2-AB labelled dextran ladder, as shown in figure 5.1. The day-to-day variation was excellent with a coefficient of variation of 3% at GU 1 and 0.4% at GU 10. A standard curve was produced by plotting the dextran ladder GU values against their elution times, figure 5.2. A Lorentzian curve was found to fit the data. To determine where standard sialylated oligosaccharides eluted from the column, known 2-AB labelled sialylated N-linked oligosaccharides were passed through the column and their GU values determined by reading from the standard curve: the monosialylated structure had a GU value of 7.37; disialylated structure 8.37 and trisialylated structures were 8.58, 9.04, 9.57 and 9.95, according to the branches on which the sialic acid was attached.

Figure 5.1 Calibration of normal-phase HPLC column using dextran ladder. The glucose unit (GU) values (1-10) are shown.

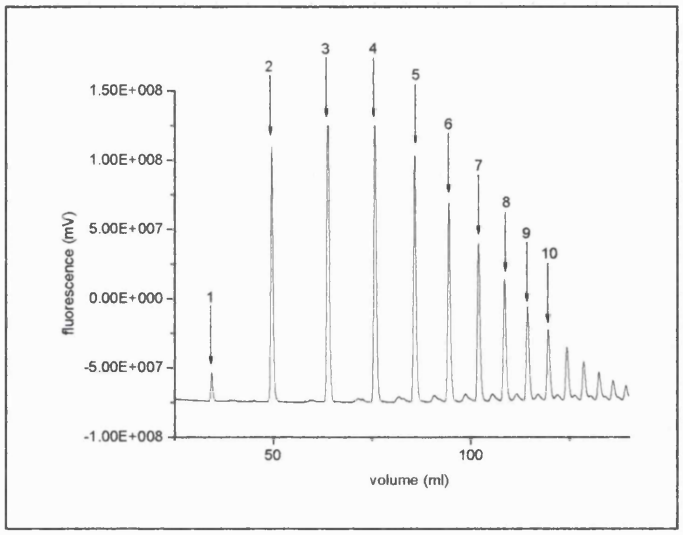
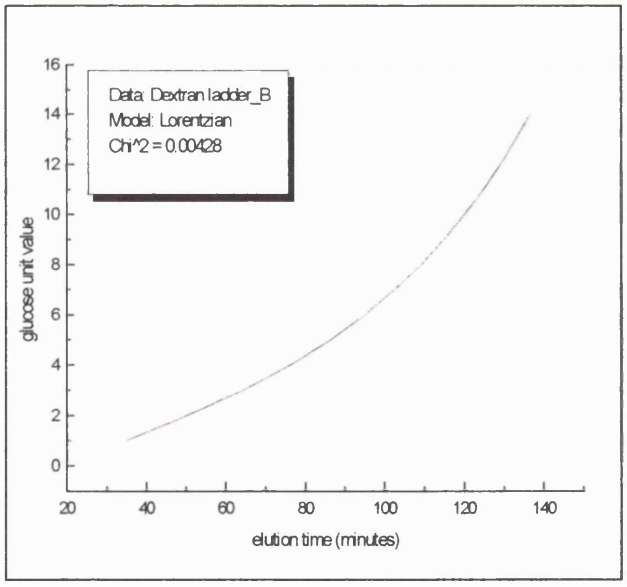


Figure 5.2 Standard curve produced by plotting the elution time of incremental glucose oligomers eluted from the normal-phase HPLC column. The black line shows the standard curve produced, the red line shows the curve fitted to the data.



5.3.9 Evaluation of the breast cancer oligosaccharides.

The elution times of all the breast cancer oligosaccharides were read from the standard curve and a GU value generated for each of the oligosaccharides extracted. The area under the peak for each oligosaccharide was determined, by integration, and tabulated with its appropriate GU value. Each of the GU values was then expressed as a percentage of the total oligosaccharide pool.

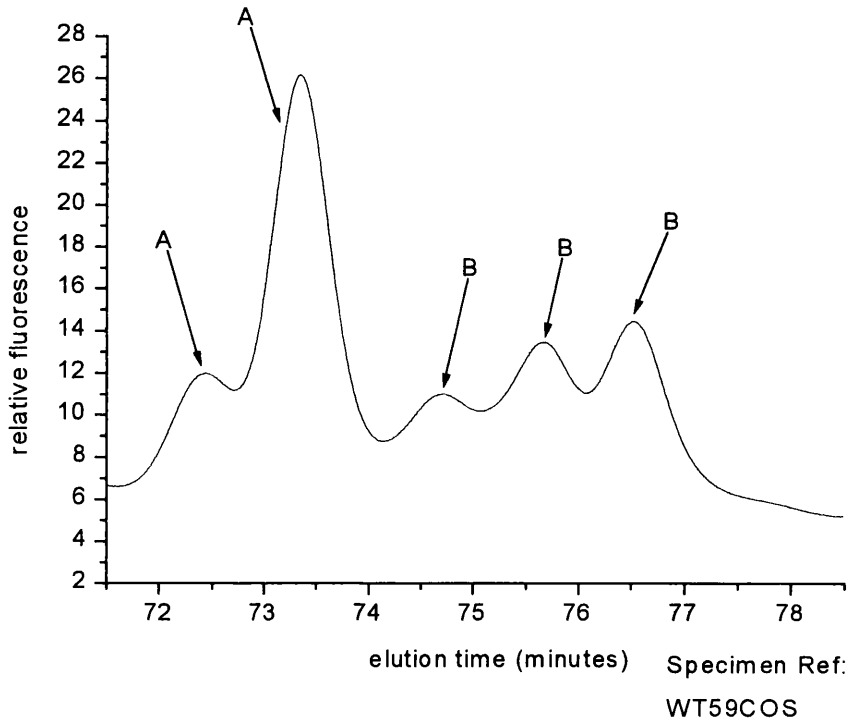
5.3.9.1.....Sialylated oligosaccharides

We wanted to determine if the levels of sialylated oligosaccharides were reduced in the cancers of patients who subsequently developed metastases, as appeared to be the case in the pilot study, chapter 3. To do this we measured the relative amounts of the oligosaccharides with the same GU values as the standard sialylated oligosaccharides which had previously been run, described in section 5.3.8.

5.3.9.2.....Oligosaccharide associated with HPA lectin-binding.

The oligosaccharide over-expressed in the HPA lectin-binding cell lines, detailed in chapter 4, was found to have a GU value of 3.58 when separated by normal-phase HPLC. When the oligosacccharides from these breast cancer specimens were separated by normal-phase HPLC, there were five different structures all eluting around 3.58 GU, the oligosaccharides were grouped as shown in figure 5.3.
In summary, there were oligosaccharides with:
1) mean GU values 3.57 and 3.67 in size: designated oligosaccharides **A**
2) mean GU values 3.77, 3.86 and 3.96 in size: designated oligosaccharides **B**
To compare the levels of the oligosaccharides extracted from the different breast cancer specimens, the relative amounts were determined as a percentage of the entire oligosaccharide pool.

Figure 5.3 An example of breast cancer oligosaccharides separated by normal-phase HPLC eluting at approximately 3.58GU. The arrows show oligosaccharides A and B.



5.3.9.3.....The predominant oligosaccharides

A predominant 'cluster' of oligosaccharides was found in the oligosaccharide pool from the different breast cancer specimens. Two main 'clusters' of oligosaccharides were observed as shown in figure 5.4.

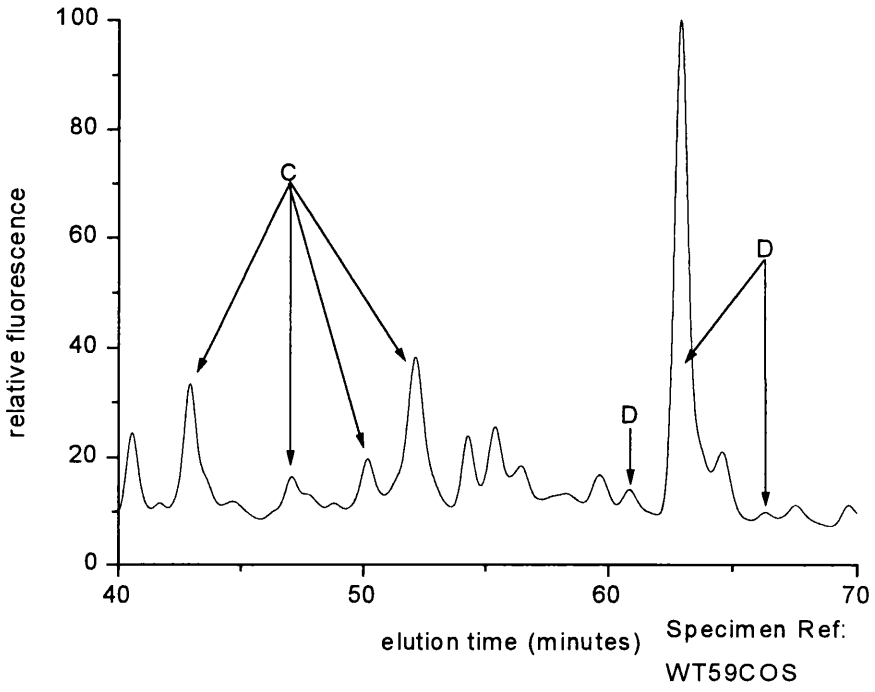
In summary there were oligosaccharides which were predominantly

1) 1.37, 1.55, 1.83 or 2.17 GU in size: designated oligosaccharides **C**,

2) 2.75, 2.88 or 3.05 GU in size: designated oligosaccharides **D**,

To compare the levels of the oligosaccharides extracted from the different breast cancer specimens, the relative amounts were determined as a percentage of the entire oligosaccharide pool.

Figure 5.4 An example of breast cancer oligosaccharides separated by normal-phase HPLC and eluting at approximately 2 GU in size. The letters show the oligosaccharides C and D.



5.3.10 Analysis of the results.

The oligosaccharide expression was compared from one patient to the next. The patients were categorised according to whether or not they developed recurrences, or whether they had died from breast cancer. The Student *t*-test was then used to evaluate if the differences in oligosaccharide expression for the different groups of breast cancer patients was statistically significant (as described in section 3.2.7). The relationship between the expression of particular oligosaccharides and disease-free survival was evaluated using the log-rank test and by drawing life-tables according to the method of Kaplan-Meier. The statistical analyses were conducted by Dr. J. Woodside (UCL) using the SPSS data handling package.

5.4 Results : expression of sialylated oligosaccharides.

There was no statistically significant difference between the amounts of the oligosaccharides with the same GU values as the standard N-link sialylated oligosaccharides, in the cancers from patients who developed recurrences compared with those that did not. The results are summarised in table 5.3 and in full in appendix 3.

Table 5.3 Relative amount of oligosaccharides of same GU size as standard N-link oligosaccharides. Values given are a percentage of total oligosaccharide pool.

Patient group	*7.37 GU mono sialylated*	*8.37 GU di sialylated*	*8.58 GU tri sialylated*	*9.57 GU tri sialylated*	*9.95 GU tri sialylated*
No Sign of Recurrence					
Mean	1.27	1.42	2.68	1.35	0.76
95% CI	-1.29 - 3.84	-1.5 - 4.34	-4.76 - 10.13	-4.85 - 7.55	-0.76 - 2.28
Any Recurrence					
mean	1.41	2.14	1.92	0.32	1.40
95% CI	-0.41 - 3.23	-0.32 - 4.60	-0.12 - 3.97	-0.24 - 0.88	-3.56 - 6.36

5.5 Discussion of results of sialylated stuctures.

In this study we did not detect any difference in the oligosaccharides with the same GU values as standard mono, bi and tri sialylated N-linked structures.

Although changes in sialylation in breast cancer have been reported by ourselves (Dwek *et al.*, 1998) and others (Brockhausen *et al.*, 1995) we have not detected any differences in this study. This may be because we have only evaluated sialylated oligosaccharide expression by comparing the breast cancer structures with reference to their elution position at the same GU value as standard N-linked oligosaccharides. There are many other sialylated oligosaccharides in addition to the ones we have evaluated (see section 1.4.1), thus, we have not considered changes in sialylation in their entirety. An approach for future work would be to

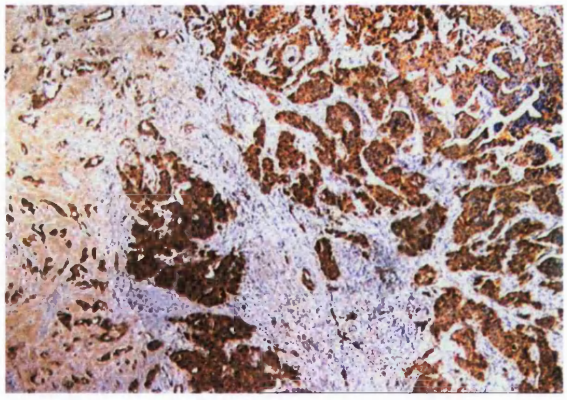
conduct exoglycosidase digests, using enzymes with different specificities to cleave off different sialic acid residues from the oligosaccharide pool, and compare the oligosaccharide profiles before and after exoglycosidase treatment (Rudd *et al.*, 1997). Issues relating to which changes in sialylation are associated with aggressive breast cancer remain unresolved.

5.6 Results : oligosaccharides associated with HPA binding.

5.6.1 Oligosaccharide expression and HPA histological staining.

The relative amounts of oligosaccharides **A** and **B** were compared to the HPA staining pattern of the cancers, a range of HPA staining patterns are shown in appendix 4. An example of an HPA binding breast cancer is shown in figure 5.5.

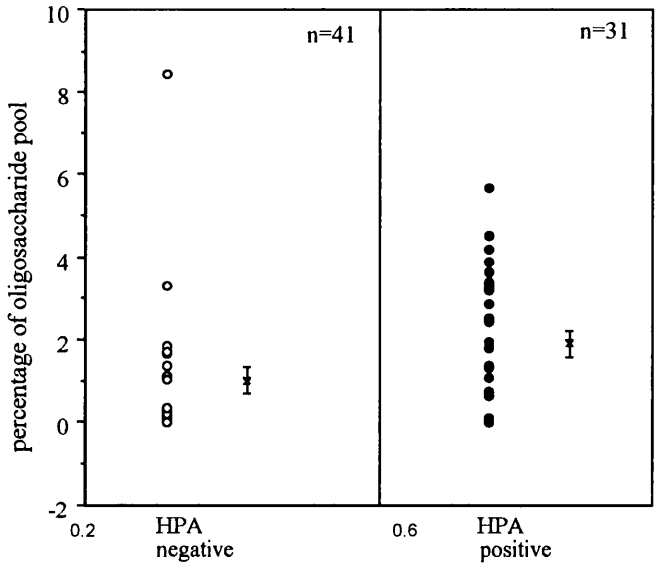
Figure 5.5 Infiltrating ductal carcinoma of the breast, reference number 2696-86, nests of cancer cells stained with HPA (brown) and tissue counter-stained with haematoxylin (blue), magnification approximately x200.



The different oligosaccharide elution profiles obtained and the quantity of oligosaccharides **A** and **B** in each of the cancers are shown in appendices 5 and 6. The expression of oligosaccharide **A** appeared to be more closely related to HPA lectin-binding than oligosaccharide **B,** since there was more oligosaccharide **A** in the HPA stainers compared with non-stainers. The differences between the amount of oligosaccharide **A** in the HPA stainers and non-stainers almost reached statistical significance (Student *t*-test, p=0.054), the results are illustrated in figure 5.6.

Figure 5.6

Oligosaccharide 'A' and HPA lectin-binding to tissue sections. Mean values +/- standard error.



5.6.2 Oligosaccharides A and B and the development of metastases.

We investigated if the expression of oligosaccharides **A** or **B** related to the metastatic capability of the breast cancers, as measured by the time to first recurrence and death from breast cancer.

The relative amounts of oligosaccharides **A** and **B** extracted from each of the breast cancer specimens was categorised as either 'low' or 'high' if they fell above or below the median value (for **A**, median = 0.51%, for **B** median = 1.325%). Survival curves were drawn and compared with survival curves using data on lymph node status and HPA tissue staining status. The results obtained are shown in figures 5.7 and 5.8. The *p* values for statistical significance were determined using the log-rank test.

As the levels of oligosaccharide **A** increased and the levels of oligosaccharide **B** decreased, so the poorer the prognosis, but the levels of the two oligosaccharides, **A** and **B**, did not appear to be related.

Figure 5.7 Survival curves illustrating the levels of oligosaccharides **A** and **B**, the lymph node status, HPA staining status and recurrence-free survival.

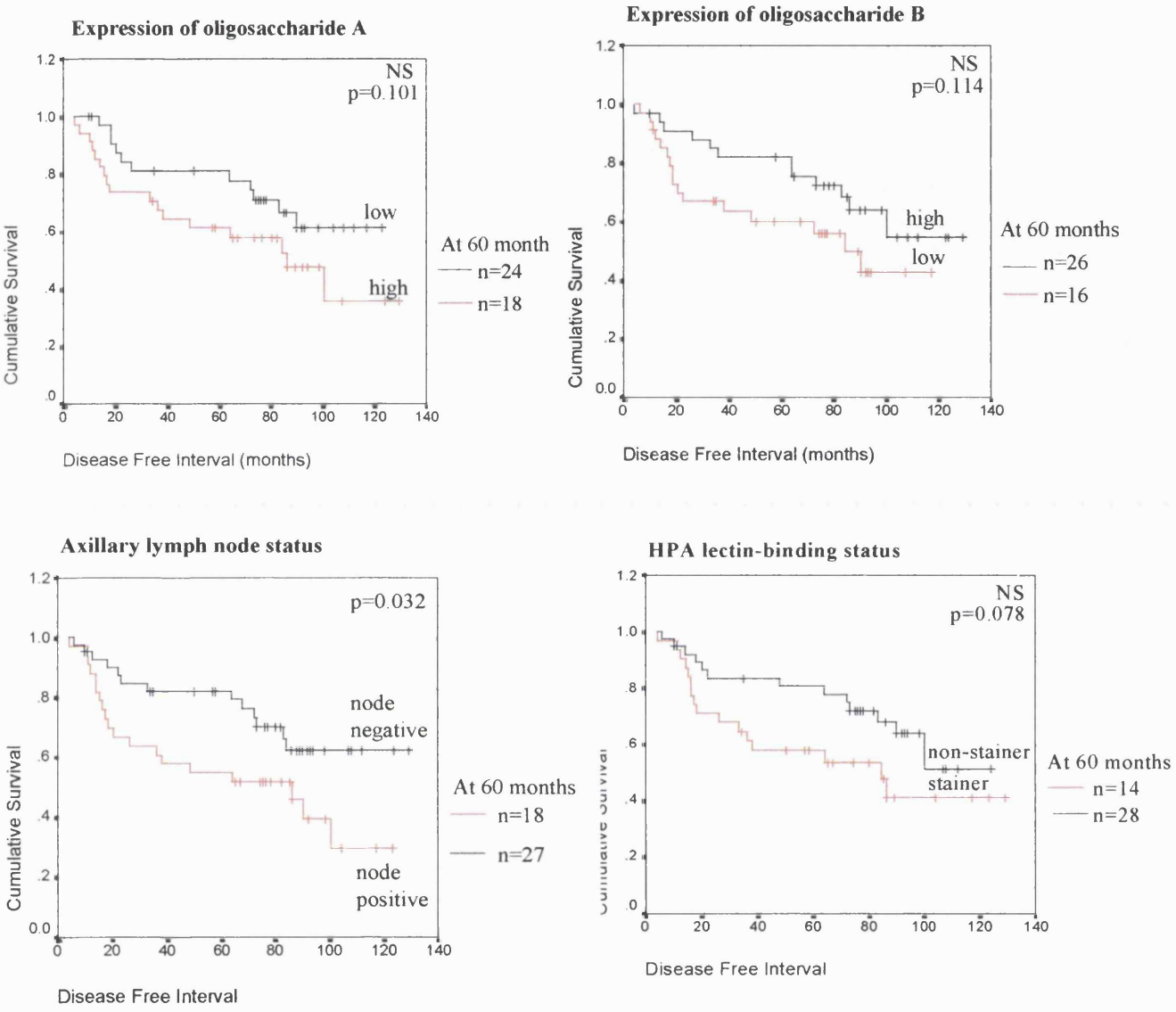
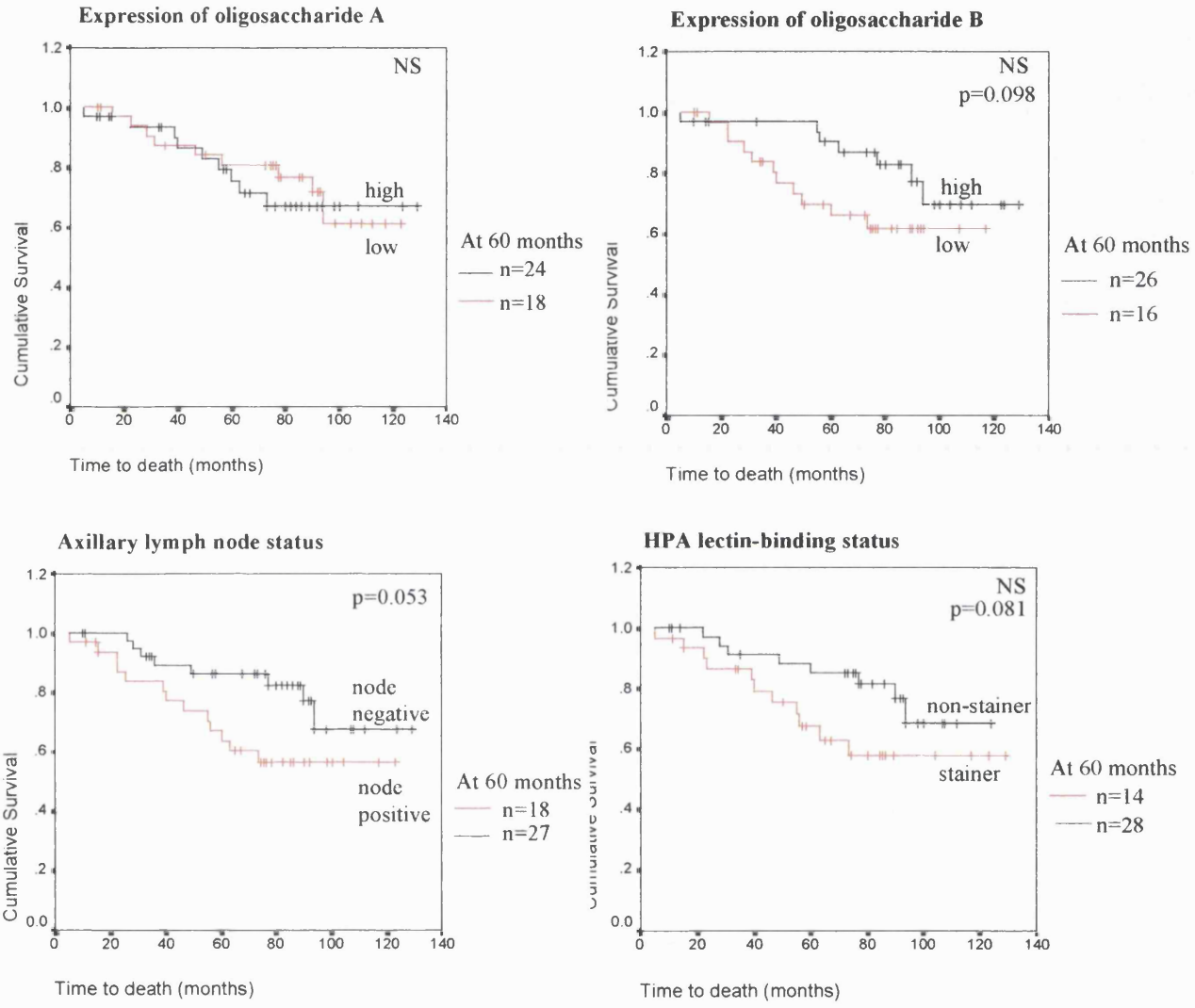


Figure 5.8 Survival curves illustrating the levels of oligosaccharides **A** and **B**, the lymph node status, HPA staining status and death from breast cancer. NS= not significant



5.7 Discussion of the results of oligosaccharides associated with HPA binding.

We have shown that an oligosaccharide over-expressed in the MCF7, HPA binding cell line, chapter 4, is also present in greater amounts in breast cancer which are HPA stainers compared with non-stainers. The association between HPA staining and the expression of the 3.57 GU value oligosaccharide was found to approach statistical significance.

This is the first time, to our knowledge, that IHC lectin staining has been correlated with biochemical mapping of the oligosaccharide structures. The generation of full sequence data is now warranted.

The survival analysis showed that neither of the oligosaccharides **A** or **B** were significantly associated with recurrence-free survival or death from breast cancer although the results were approaching statistical significance. The results from the HPA staining and lymph node status life-table analysis suggest that the limited number of patients in this study may be one factor which has influenced the relatively weak associations between the different prognostic factors and the recurrence-free survival / death from breast cancer.

Finally, the expression of oligosaccharide **A** was only weakly associated with HPA staining. Whether this is because of the relatively small numbers of individuals in the study, or because oligosaccharide **A** is only one, of several potential HPA binding ligands, is an area for further investigation.

5.8 Results: the predominant oligosaccharides.

5.8.1 Description of results.

In the patients who had no sign of recurrence, there was a predominant cluster of oligosaccharide peaks (**C**) which were of a smaller GU value than the predominant cluster of oligosaccharides (**D**) extracted from the patients who had developed recurrences. The profiles obtained and the amounts of the oligosaccharides extracted are shown in appendices 7 and 8 respectively.

5.8.2 Amount of oligosaccharides C and D and the development of metastases.

The amount of oligosaccharide **C**, was compared in the different patients grouped according to presence or absence of recurrence, and site of recurrence as shown in figure 5.9. There was more of oligosaccharide **C** extracted from cancers of poor prognosis patients. The result was statistically significant when evaluated using a non-paired Student's independent sample *t*-test. The most significant differences were found when the patients with any recurrence were compared with those who had no sign of recurrence ($p=0.001$) but the results were also statistically significant when patients who developed distant recurrences ($p=0.0012$), died from breast cancer ($p=0.002$) and those who developed local recurrences ($p=0.004$) were compared with patients with no sign of recurrence.

In addition, there was also a different cluster of predominant oligosaccharides (**D**) present in the patients who developed recurrences.

The levels of oligosaccharide **D** have been compared for the patients who did, and did not, develop recurrences and the results are shown in figure 5.10. There were no significant differences in the relative amounts of oligosaccharide **D** extracted from the breast cancer specimens from patients with / without any recurrences, but the difference in the patients who died from breast cancer, compared to those with no sign of recurrence, almost reached statistical significance (Student *t*-test, $p=0.079$).

Figure 5.9 Levels of oligosaccharide C in breast cancer specimens. The site of recurrence and the mean values +/- standard error are given.

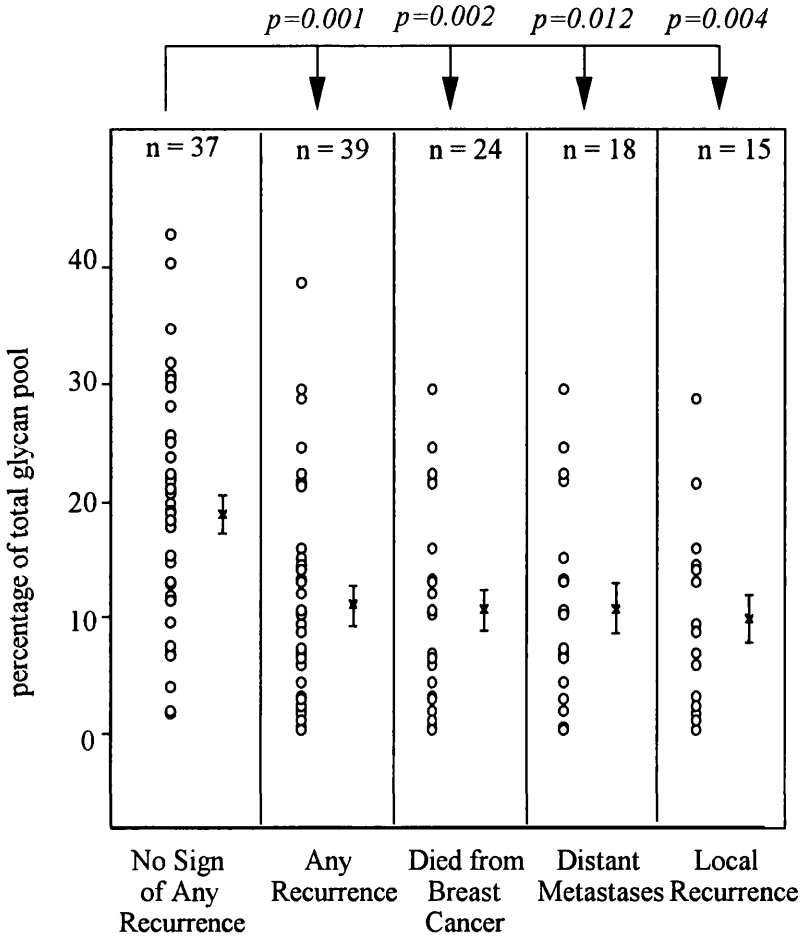
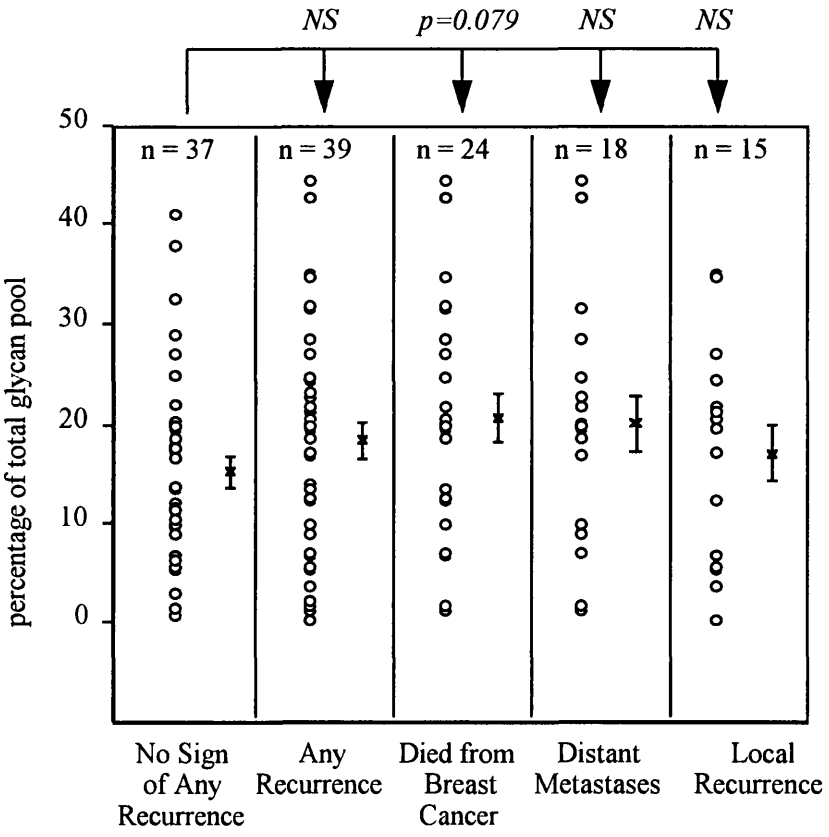


Figure 5.10 Levels of oligosaccharide **D** in breast cancer specimens. The site of recurrence and the mean values +/- standard error are given.



5.8.3 The relationship between the oligosaccharides C and D.

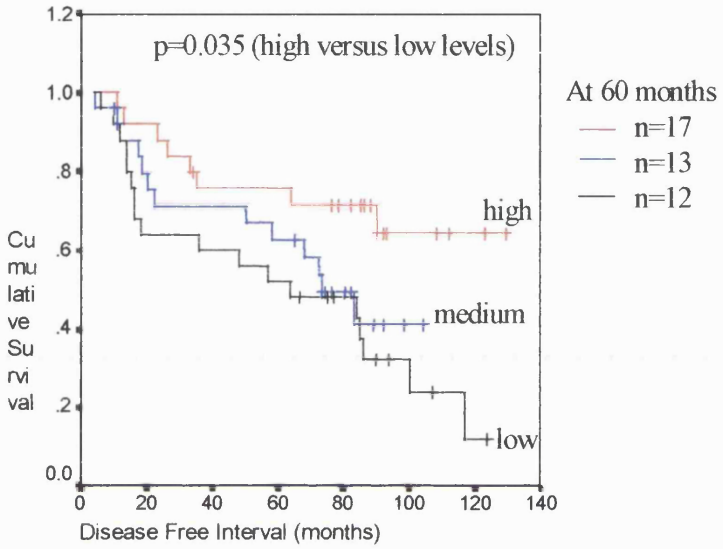
The possibility that elevated levels of oligosaccharide **D** were associated with decreased levels of oligosaccharide **C** was considered, but when the relative amounts of these oligosaccharides were plotted no association was observed.

5.8.4 Oligosaccharides C and D as prognostic markers in breast cancer.

We compared the survival patterns of patients with different levels of oligosaccharides **C** and **D** by drawing Kaplan Meier survival curves and comparing the results obtained. To do so, the relative amounts of oligosaccharides **C** and **D** extracted from each of the breast cancer specimens were divided into tertiles, each corresponding to either 'low', 'medium' or 'high' levels of expression. The results obtained were compared using the log-rank test.

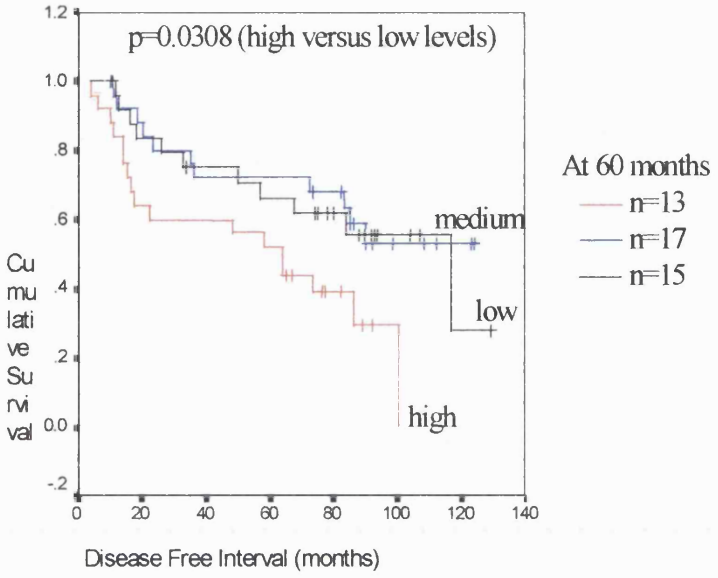
A statistically significant shorter recurrence-free survival was observed in the patients who had low and medium levels of oligosaccharide **C**, compared with those who had high levels, the results obtained are shown in figure 5.11.

Figure 5.11 The expression of oligosaccharides **C** and recurrence-free survival. Vertical lines indicate censored data.



Conversely, a faster time to first recurrence was observed in the patients with high levels of oligosaccharide **D**, figure 5.12.

Figure 5.12 The expression of oligosaccharide **D** and recurrence-free survival. Vertical lines indicate censored data.



The relative amounts of oligosaccharides **C** and **D** were compared in the same way, but with death from breast cancer as the end-point for the analysis. The results obtained are shown in figures 5.13 and 5.14.

Figure 5.13 Oligosaccharide **C** and time to death from breast cancer.

Vertical lines indicate censored data. NS=not statistically significant.

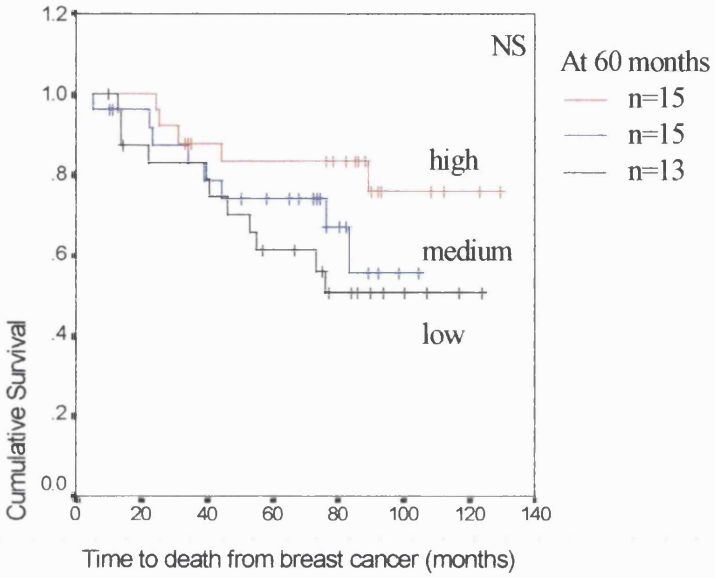
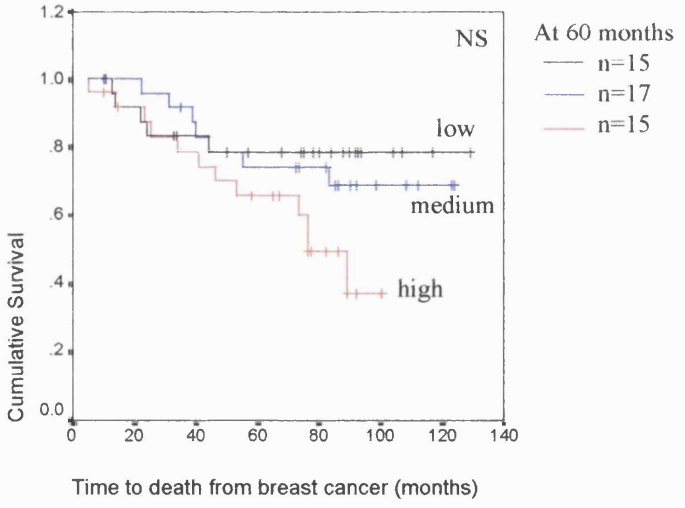


Figure 5.14 Oligosaccharide **D** and time to death from breast cancer.

Vertical lines indicate censored data. NS= not statistically significant.



There was no statistically significant association between the low, medium or high levels of expression of either of the two groups of oligosaccharides and death from breast cancer.

5.9 Discussion of results of predominant oligosaccharides C and D.

We saw a difference in a cluster of oligosaccharides (**C**) which appeared to be significantly decreased in patients with shorter recurrence-free survival, but not death from breast cancer. These changes perhaps reflect the expression of oligosaccharides associated with cellular proliferation or, alternatively, response to treatment, as opposed to oligosaccharides which are involved in metastasis. Oligosaccharides **D**, when present in elevated levels, appeared to be associated with both a shorter recurrence-free survival and earlier death from breast cancer, although the latter failed to reach statistical significance.

The observations which have been made here were based on the predominant oligosaccharides but other oligosaccharides may be found to be better prognostic indicators if examined in the same way as oligosaccharides **A**, **B**, **C** and **D**.

5.10 Oligosaccharide expression and other clinical features.

A number of clinical and pathological features which have previously been reported as prognostic markers in breast cancer were compared with the expression of the oligosaccharides described above.

The comparisons were conducted in two ways. Firstly by taking the factor of interest (for example tumour grade) and comparing whether it was related to the expression of the oligosaccharides in an independent manner, and secondly, by taking the factors of interest and comparing them to oligosaccharide expression in a multi-variate analysis using a linear regression model. The results of the univariate analysis is shown in table 5.4.

Table 5.4 A comparison between pathological / clinical factors which influence outcome after breast cancer and the expression of different groups of oligosaccharides, A, B, C and D. *P* values given.
NS = not significant, NT = not tested

	Statistical test	*A*	*B*	*C*	*D*
Grade	one-way ANOVA	NS	NS	p=0.010	NS
Lymph node status	Student *t*-test	NS	NS	p=0.046	p=0.030
Number of nodes	Chi-Square test	NS	NS	NS	NS
HPA binding	Student *t*-test	0.029	NS	NT	NT
Size of tumour	Chi-Square test	NT	NT	NS	NS
Age at presentation	Chi-Square test	NS	NS	NS	NS
Radiotherapy	Student *t*-test	NS	NS	NS	NS
Chemotherapy	Student *t*-test	NS	NS	NS	NS

The effect of Tamoxifen treatment was not evaluated since nearly all the patients received Tamoxifen post-operatively.

The independent tests suggested that tumour grade and lymph node status were significantly associated with the expression of oligosaccharides **C** and **D**, but when these factors were included in the multivariate analysis, not surprisingly, this no longer remained the case.

In the multivariate analysis, oligosaccharides **A** and **B** related to HPA staining, but not to any of the other features examined. HPA staining was significantly associated with lymph node status (Chi-square test : p=0.001).

In the multivariate analysis oligosaccharides **C** and **D** were found to be independent variables not associated with any of the other factors examined.

Chapter 6

General Discussion.

6.1 Oligosaccharide extraction and mapping.

During the course of this project we have developed methods for the extraction of oligosaccharides from formalin-fixed, paraffin-wax embedded breast cancer specimens which have been stored in archives for many years (Dwek *et al*, 1996). We have adapted our methods throughout, and used new techniques and equipment as it has become available, for the labelling (Bigge *et al.*, 1995), separation (Guile *et al.*, 1996; Guile *et al.*, 1994) and detection of oligosaccharides. The methods we have developed have enabled the extraction of oligosaccharides from a single section of paraffin-wax embedded breast cancer tissue, with sufficient sensitivity for the detection of typically 50 or more structures when separated by normal-phase HPLC.

These methods have been an essential part of our clinical studies and their development has enabled us to generate oligosaccharide 'fingerprints' from small, as well as larger, breast cancer specimens. We have, for the first time, presented data on the oligosaccharides extracted from breast cancers of patients for whom extended follow-up information has been obtained.

6.2 Hypothesis generation.

Two studies were conducted which were used to generate hypotheses relating to oligosaccharide expression in aggressive, metastatic breast cancer.

6.2.1 A 'pilot study' of long and short-term survival breast cancer patients.

We extracted oligosaccharides from nineteen breast cancer specimens which had been stored in archives and followed-up for fifteen years or until the patients died from breast cancer, which ever was the earlier. We observed differences in the oligosaccharides extracted, with reduced sialylation being a feature of the short-term survival cases. Not all of the cases, however, showed the same pattern of reduced sialylation and to understand the reasons for this we needed to examine more specimens. We sought to determine if the patient-to-patient variation in oligosaccharide expression observed reflected differences such as tumour

infiltration by other cell types, or alternatively whether it reflected features of the cancer cells themselves, such as their differentiation. We tried to limit the former by microdissection of single sections and tested the latter by evaluating a larger series of breast cancer specimens.

6.2.2 Oligosaccharides associated with HPA lectin-binding.

In the second study, cells lines grown *in vitro* which exhibited a range of different HPA lectin binding patterns were evaluated in an attempt to identify oligosaccharide(s) associated with metastasis. HPA lectin-binding glycoproteins were collected by affinity chromatography and their oligosaccharides released. A difference between the levels of a mono-sialylated oligosaccharide was found, with an increased expression of this structure in the strongly HPA binding cell-line. The same structure also appeared to be present in oligosaccharides released from peptides of an HPA lectin-binding breast cancer. The oligosaccharide had a GU value of 3.58 when separated by normal-phase HPLC.

We examined whether the expression of the 3.58 GU oligosaccharide related to HPA lectin staining of tissue sections by IHC and recurrence-free survival after breast cancer. This was addressed by comparing the relative amounts of the 3.58 GU material extracted from a larger series of breast cancer specimens.

In summary, two main hypotheses were generated during our early studies:

1) that a reduction in sialylated oligosaccharides is associated with poor prognosis breast cancer and
2) that an oligosaccharide of 3.58 GU in size is present in increased levels in HPA lectin binding tissue sections.

6.3 Hypothesis Testing.

The extraction and separation of oligosaccharides from 76 different breast cancer specimens enabled the hypotheses, developed above, to be tested.

6.3.1 Sialylation and aggressive breast cancer.

We attempted to evaluate differences in sialylation in relation to recurrence-free survival and death from breast cancer by examining the levels of oligosaccharides with the same GU values as standard N-linked sialylated structures. Using this approach, we found no significant difference between the amount of N-linked sialylated oligosaccharides extracted from the cancers of patients who did, and those that did not, develop recurrences. This suggests that the differences in sialylation observed in the pilot study may be associated with O-linked oligosaccharides rather than N-linked structures, but that has not been addressed in this study.

A number of IHC based studies report that sialylated oligosaccharides may be associated with poor prognosis, for example STn in colorectal and gastric cancer (Carneiro *et al.*, 1994; Itzkowitz *et al.*, 1990; Itzkowitz *et al.*, 1992), but this has not been found in breast cancer (Miles *et al.*, 1994). Alternatively, the differences in sialylated oligosaccharides which were observed in the pilot study may be associated with 'unusual' N-link oligosaccharide structures, such as α2-8 linked sialic acid. Such structures have been reported in leukaemia cells and the human metastatic breast cancer cell line, MCF7 (Martersteck *et al.*, 1996). Another possibility is that the sialylated oligosaccharides contain N-glycolneuraminic acid rather than N-acetylneuraminic acid. This type of neuraminic acid has been found on breast cancer cell line mucin proteins (Devine *et al.*, 1991) and on gangliosides of breast cancers (Marquina *et al.*, 1996).

The pilot study results are substantiated by those of Harvey *et al.*, (1992) who evaluated metastatic cell lines and found that increased sialylation was a feature of well differentiated carcinomas.

The large volume of literature relating to the role of sialylated oligosaccharides in the metastatic process, section 1.5.3.4, suggests that further evaluation of the sialylated oligosaccharides extracted from this series of breast cancers would be

worthwhile. Clearly, however, a different approach is required, it may, for example, be useful to pass the breast cancer oligosaccharides through the neutral-phase HPLC column and compare their profiles before and after neuraminidase digestion of the entire oligosaccharide pool (Guile *et al.*, 1996). This would also serve to identify which oligosaccharides, on elution from the normal-phase HPLC column, are of interest and would be the first step in the sequencing of the structures involved.

6.3.2 Oligosaccharides associated with HPA lectin binding on tissue sections.

We have for the first time, to our knowledge, correlated IHC staining with the biochemical extraction / mapping of oligosaccharides. In doing so, we have identified an oligosaccharide which appears to be associated with HPA lectin-staining. This oligosaccharide was originally observed in an HPA binding (MCF7) cell line and we have now found that the oligosaccharide is over-expressed in excised breast cancers which stain with HPA. These results indicate that the breast cancer cell line model proposed by Schumacher *et al.*, (1995) for HPA binding is relevant in terms of the oligosaccharides present. It would now be interesting to determine the levels of the 3.58 GU material in cell lines such as those which have been reported to bind HPA and are metastatic in nude (Kjonniksen *et al.*, 1994) and severe immunodefficient mice (Schumacher *et al.*, 1994b). It would also be useful to evaluate the glycopeptides and glycoproteins to which the 3.58 GU oligosaccharide is attached, since cancer-associated oligosaccharides have been reported to be present in a clustered form on proteins, reviewed by (King, 1994) and the way in which oligosaccharides are presented to lectin binding site(s) are probably important for successful binding, reviewed by Lis and Sharon, (1986). In our current study, we have not structurally characterised the 3.58 GU oligosaccharide, but such experiments would now seem warranted, particularly since a number of reports indicate that HPA lectin-binding may be an important means of identifying metastatic breast (Brooks and Leathem, 1991; Noguchi *et al.*, 1993b) and other cancers (Ikeda *et al.*, 1994; Kakeji *et al.*, 1991; Schumacher *et al.*, 1994a). It has been suggested that before HPA lectin-binding can be used in a diagnostic test for poor prognosis cancer, improved methods for the evaluation of the ligands that bind the lectin are required (Walker, 1993). We have found an

oligosaccharide which may represent part of the HPA binding ligand and need to investigate this material further. It seems likely that tests based on the measurement of levels of this material might become useful as future diagnostic tools for predicting outcome after cancer.

6.3.3 The predominant breast cancer oligosaccharides (C and D).

A number of predominant oligosaccharides became apparent on examination of the normal phase separations (Miss H. Lacey June 1998). The levels of two of these (**C** and **D**) appeared related to aggressive breast cancer, as measured by the levels of the oligosaccharides and the time to first recurrence, which were significantly correlated with one another. The levels of one of the predominant oligosaccharides (**D**) was associated with an increased likelihood of dying from breast cancer, although the results failed to reach statistical significance. In addition to the oligosaccharides associated with HPA lectin-binding, the structural characterisation of other 'predominant' oligosaccharides (**C** and **D**) is also justified.

6.4 Oligosaccharide expression and prognosis.

There is a need for improved markers which will enable clinicians to determine outcome, section 1.2.6.

The 3.58 GU oligosaccharide levels were associated with HPA lectin binding and appeared to be a marker of aggressive breast cancer, as evidenced by the shorter disease-free survival of patients with high levels, compared with those with low levels, of the oligosaccharide, section 5.6.2. In the same way, the predominant oligosaccharides may also reflect the aggressiveness of breast cancer and be useful as a reflection of longer-term outcome, section 5.8.4.

It is also possible that two of the oligosaccharides which we have found to be over-expressed in poor prognosis breast cancers, namely structure '**D**' which we found to be associated with poor long-term outcome and the 3.58 GU structure, may be taken in conjunction and usefully provide a combined indicator, or index, for both short and long-term prognosis.

One of the advantages in the approach we have taken is that our methods allow reproducible, non-subjective evaluation of the oligosaccharides present. This is

clearly an advantage over other subjective methods for the measurement of prognostic markers, particularly IHC based systems. The development of new methods, after sequencing the oligosaccharides, may result in a new-generation of validated prognostic tests for breast cancer outcome.

6.5 Additional future studies.

A number of studies lead from this which may further our understanding of the glycobiology of breast cancer. For example, if the amount of protein used for oligosaccharide release were quantified, an understanding of whether breast cancer cells are over or under glycosylated would be gained. This might usefully be related to the levels of the glycosyltransferases, as described in section 6.6. Another way in which over / under glycosylation could be investigated would be to relate the quantity of oligosaccharides released with the amount of tissue or number of cells from which the oligosaccharides were extracted. In this way, a comparison between the absolute levels of the oligosaccharides in different cancers would be obtained.

In these studies we have focussed on the identification of oligosaccharides that may be associated with an aggressive breast cancer phenotype, this has been examined by HPLC separation of the oligosaccharides extracted.

Characterisation of the oligosaccharides which we have found to be associated with aggressive breast cancer is now an important requirement in a project which is concerned with understanding the role of oligosaccharides in metastasis. One way in which this might be performed is by the use of exoglycosidase enzymes to cleave individual monosaccharide residues away from the oligosaccharides, such experiments will enable the reconstruction of the oligosaccharide structures.

Measuring levels of the oligosaccharides in breast cancer metastasis specimens is now a requirement, to enable us to understand whether the oligosaccharides are maintained after a cancer cell has formed metastases. If the oligosaccharide structures are present in breast cancer metastases, this may be suggestive of a functional role of oligosaccharides in the metastatic process.

6.6 Oligosaccharides as therapeutic targets.

There are a number of ways in which abberant oligosaccharide expression may be used as targets for new breast cancer therapies.

A group of compounds termed 'carbohydrate processing inhibitors' (CPI), which inhibit glycosyltransferases reponsible for the construction of oligosaccharides, have been proposed as potential cancer therapies (Goss *et al.*, 1995). Appropriate CPIs will, however, only become apparent as our understanding of which oligosaccharides are important in the development of metastatic breast cancer develop. It would seem that despite a number of early reports of altered glycosylation in breast cancer (Burchell *et al.*, 1987; Dennis *et al.*, 1987), further studies have failed to find any association between the oligosaccharide changes described and prognosis (Rak and Miller, 1993; Wilkinson *et al.*, 1984). It is, therefore, important in our understanding of glycosylation changes in breast cancer, to undertake retrospective studies to subsequently determine the oligosaccharides which appear to be associated with poor prognosis. From this information we may then determine the glycosyltransferases which are involved, and develop *useful* CPIs. Finally, early clinical trials of one CPI, swainsonine, in advanced cancer patients and has shown that it is reasonably tolerated (Goss *et al.*, 1997).

Another approach to targeting oligosaccharides which are associated with metastatic breast cancer, is to use the oligosaccharides as immunogens. There is some evidence that cytotoxic T lymphocytes can bind lectins, and therefore recognise oligosaccharides (Kimura *et al.*, 1979) reviewed by Parish *et al.*, (1992) and that antibodies can also bind carbohydrates, some of which are over-expressed in cancer (Longenecker *et al.*, 1994). A number of studies suggest that oligosaccharides on gangliosides may be useful targets for melanoma immunotherapy, reviewed by Hellstrom and Hellstrom, (1985). It is possible that the oligosaccharides which we have found to be over expressed in poor prognosis breast cancer may also form the basis of targets for immunotherapy, in a similar way as has been decribed for other molecules (Zbar *et al.*, 1998).

At present, however, we are investigating whether patients who survive for long-periods of time after breast cancer have humoral immunity to the oligosaccharide structures which we have been found to be associated with poor prognosis. It has long been reported that glycoproteins are shed from cancer cell surfaces, reviewed

by Weiss, (1985). In a number of cancers, gangliosides are found in elevated levels in the serum; the oligosaccharides attached may be immunogenic at low levels but immunosuppressive at elevated concentrations, reviewed by Rebbaa et al., (1995) clearly, the immune recognition of cancer is a complex matter, reviewed by Sikora and James, (1991).

Our current work may enable us to understand apparent anomalies between groups of breast cancer patients who, according to the conventional staging and prognostic markers described in section 1.2.6, would be predicted to have similar outcomes, but in whom the clinical course of the disease follows markedly different patterns.

6.7 Conclusions.

We have found a number of oligosaccharides which are over-expressed in poor prognosis breast cancer. Further studies are now required to structurally characterise the oligosaccharides. An understanding of the role of oligosaccharides in the development of metastases will be a focus of future work. The results of studies such as ours may potentially lead to new therapeutic approaches, perhaps as a result of our increased understanding of the immune response to cancer-associated carbohydrate antigens.

Appendix 1

Details of treatment and clinical follow-up of patients used in pilot study.

Patient identifier	BAS
Middlesex / University College Hospital number	M/1840065
Histology number	716/78
Date of birth	02/07/1924
Age at presentation	53
Operation type	L mastectomy, axillary clearance
Disease spread at operation	LN
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	polyglonal cell carcinoma
Size of tumour (mm)	25
Bloom and Richardson grade	III
Lymph node involvement / total examined	15/16
Disease free survival with no recurrences (months)	< 12
Site of first recurrence	unknown
Sites of subsequent recurrences	unknown

Patient identifier	DOB
Middlesex / University College Hospital number	U/FV7446 & M/188268
Histology number	976/78
Date of birth	23/05/1921
Age at presentation	56
Operation type	L mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	Tamoxifen
Histological diagnosis	invasive ductal carcinoma
Size of tumour (mm)	15
Bloom and Richardson grade	II
Lymph node involvement / total examined	0/21
Disease free survival with no recurrences (months)	120
Site of first recurrence	contralateral breast
Sites of subsequent recurrences	N/A

Patient identifier	GRI
Middlesex / University College Hospital number	M/189709
Histology number	1457/78
Date of birth	21/04/1922
Age at presentation	55
Operation type	R Mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	20
Bloom and Richardson grade	II
Lymph node involvement / total examined	3/11
Disease free survival with no recurrences (months)	192
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	FEN
Middlesex / University College Hospital number	M/B28189
Histology number	3204/78
Date of birth	1913
Age at presentation	65
Operation type	L mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	ductal infiltrating carcinoma of the breast
Size of tumour (mm)	40
Bloom and Richardson grade	III
Lymph node involvement / total examined	3/7
Disease free survival with no recurrences (months)	< 12
Site of first recurrence	unknown
Sites of subsequent recurrences	brain

Patient identifier	STR
Middlesex / University College Hospital number	M/R54536
Histology number	3453/78
Date of birth	29/09/1919
Age at presentation	58
Operation type	L mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	15
Bloom and Richardson grade	II
Lymph node involvement / total examined	0/7
Disease free survival with no recurrences (months)	193
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	HOF
Middlesex / University College Hospital number	M/205862
Histology number	2724/79
Date of birth	21/08/1914
Age at presentation	64
Operation type	L mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	25
Bloom and Richardson grade	III
Lymph node involvement / total examined	2/22
Disease free survival with no recurrences (months)	66
Site of first recurrence	unknown
Sites of subsequent recurrences	unknown

Patient identifier	TAN
Middlesex / University College Hospital number	M/210343
Histology number	3161/79
Date of birth	17/10/1914
Age at presentation	64
Operation type	R mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating lobular and ductal carcinoma
Size of tumour (mm)	unknown
Bloom and Richardson grade	II
Lymph node involvement / total examined	1/9
Disease free survival with no recurrences (months)	77
Site of first recurrence	unknown
Sites of subsequent recurrences	lungs

Patient identifier	BAR
Middlesex / University College Hospital number	M/217761
Histology number	3862/79
Date of birth	19/08/1919
Age at presentation	60
Operation type	L mastactomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating polyglonal cell carcinoma
Size of tumour (mm)	25
Bloom and Richardson grade	II
Lymph node involvement / total examined	4/5
Disease free survival with no recurrences (months)	49
Site of first recurrence	lungs
Sites of subsequent recurrences	bones

Patient identifier	CAT
Middlesex / University College Hospital number	M/223078
Histology number	4829/79
Date of birth	15/05/1922
Age at presentation	57
Operation type	R mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	Tamoxifen
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	60
Bloom and Richardson grade	III
Lymph node involvement / total examined	0/4
Disease free survival with no recurrences	< 12
Site of first recurrence	skin
Sites of subsequent recurrences	liver and lungs

Patient identifier	HAR
Middlesex / University College Hospital number	M/219388
Histology number	4916/79
Date of birth	15/10/1913
Age at presentation	55
Operation type	R mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating lobular carcinoma
Size of tumour (mm)	20
Bloom and Richardson grade	N/A
Lymph node involvement / total examined	4/10
Disease free survival with no recurrences (months)	183
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	FOL
Middlesex / University College Hospital number	M/257309
Histology number	1493/80
Date of birth	06/08/1926
Age at presentation	53
Operation type	R mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	polyglonal cell carcinoma
Size of tumour (mm)	50
Bloom and Richardson grade	III
Lymph node involvement / total examined	0/12
Disease free survival with no recurrences (months)	169
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	HOU
Middlesex / University College Hospital number	M/116637
Histology number	5386/80
Date of birth	03/11/1920
Age at presentation	60
Operation type	L mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	mammary carcinoma
Size of tumour (mm)	55
Bloom and Richardson grade	II
Lymph node involvement / total examined	2/5
Disease free survival with no recurrences (months)	9
Site of first recurrence	unknown
Sites of subsequent recurrences	brain

Patient identifier	FOR
Middlesex / University College Hospital number	U/EW4533
Histology number	319/81
Date of birth	08/07/1913
Age at presentation	68
Operation type	L mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	unknown
Bloom and Richardson grade	unknown
Lymph node involvement / total examined	0/9
Disease free survival with no recurrences (months)	180
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	LEV
Middlesex / University College Hospital number	M/253780
Histology number	870/81
Date of birth	27/02/1981
Age at presentation	53
Operation type	R mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	polyglonal cell adenocarcinoma
Size of tumour (mm)	20
Bloom and Richardson grade	II
Lymph node involvement / total examined	0/12
Disease free survival with no recurrences (months)	156
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	ALT
Middlesex / University College Hospital number	U/EK7617
Histology number	1501/81
Date of birth	28/02/1911
Age at presentation	72
Operation type	R mastectomy
Disease spread at operation	unknown
Post operative radiotherapy (y/n)	Y
Systemic therapy	N
Histological diagnosis	
Size of tumour (mm)	
Bloom and Richardson grade	
Lymph node involvement / total examined	
Disease free survival with no recurrences (months)	< 12
Site of first recurrence	bone
Sites of subsequent recurrences	brain
Other information	family history of breast cancer

Patient identifier	BUT
Middlesex / University College Hospital number	U/AB3024
Histology number	1655/81
Date of birth	16/07/1908
Age at presentation	73
Operation type	L mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	Y
Systemic therapy	Tamoxifen post operatively, later - chemotherapy
Histological diagnosis	
Size of tumour (mm)	20
Bloom and Richardson grade	
Lymph node involvement / total examined	6/7
Disease free survival with no recurrences (months)	< 12
Site of first recurrence	bone and liver
Sites of subsequent recurrences	no further recurrences

Patient identifier	WOO
Middlesex / University College Hospital number	U/EW8248
Histology number	1762/81
Date of birth	02/04/1905
Age at presentation	76
Operation type	R mastectomy, axillary clearance
Disease spread at operation	localised to breast
Post operative radiotherapy (y/n)	N
Systemic therapy	Tamoxifen for recurrences
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	
Bloom and Richardson grade	
Lymph node involvement / total examined	0/2
Disease free survival with no recurrences (months)	< 16
Site of first recurrence	contralateral breast
Sites of subsequent recurrences	skin and bone

Patient identifier	STA
Middlesex / University College Hospital number	M/396122 & 91144744
Histology number	2474/81
Date of birth	20/03/23
Age at presentation	58
Operation type	L lumpectomy
Disease spread at operation	unknown
Post operative radiotherapy (y/n)	Y
Systemic therapy	N
Histological diagnosis	infiltrating ductal carcinoma
Size of tumour (mm)	20
Bloom and Richardson grade	
Lymph node involvement / total examined	not examined
Disease free survival with no recurrences (months)	181
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Patient identifier	KEM
Middlesex / University College Hospital number	M/273224
Histology number	653/82
Date of birth	04/03/1915
Age at presentation	66
Operation type	R mastectomy, axillary clearance
Disease spread at operation	axillary lymph nodes
Post operative radiotherapy (y/n)	N
Systemic therapy	N
Histological diagnosis	polyglonal cell carcinoma
Size of tumour (mm)	60-80
Bloom and Richardson grade	III
Lymph node involvement / total examined	1/13
Disease free survival with no recurrences (months)	133
Site of first recurrence	N/A
Sites of subsequent recurrences	N/A

Appendix 2 Clinical information obtained for patients used in larger study.

BREAST CANCER RESEARCH GROUP

Database

General Patient Information

Surname :

Forename :

DOB :

Age at Presentation :

Hospital :

Hospital No :

Hospital No (if second) :

NHS Number:

Patient's Address :

GP Name :

GP Address and Phone No :

Occupation of Patient :

Occupation of Partner :

Religion :

Ethnic Group :

Date of First Presentation :

Referred By :

Location of Notes :

Notes Collected : ○ Yes - From clinic ○ Some - From clinic ○ Some - other
○ Yes - From notes ○ Some - From notes ○ No

Pilot Study : ○ Yes ○ No

General Patient Information (Cont.)

Menopausal Status : _____ **Age at Menopause :**

Menarche :

HRT : _____ **Total Time on HRT :**

Oral Contraceptive : _____

Blood Group : _____

Medical History

History of Childhood Diseases :

Diseases :

- ☐ Tuberculosis
- ☐ Ovarian Cancer
- ☐ Malignant Melanoma
- ☐ Other Carcinoma
- ☐ Bowel Surgery
- ☐ Autoimmunity
- ☐ Diabetes Mellitus
- ☐ Thyroid Disease
- ☐ Previous Benign Breast Disease
- ☐ Heart Disease
- ☐ Arthritis
- ☐ Other...

Other Diseases :

No of Children/ Pregnancies :

Age at Birth of First Child :

Age at Birth of Last Child :

Were Children Breast Fed? : _____

Hysterectomy : _____

Smoker? : _____

Amount Smoked : _____

Alcohol : _____

Units per Week : _____

Family History

Family History of Breast Cancer :

Affected Relative(s) : **Maternal :** ☐ Mother ☐ Sister ☐ Grandmother ☐ Aunt ☐ Cousin

Paternal : ☐ Grandmother ☐ Aunt ☐ Cousin

Other Familial Cancers :

Urine Study

Urine study :

Date of Collection :	Sample No :	Disease status :	Number of months Post-Op	pH :	LMP :

Clinical Information

Date of investigations:

Assessment : **Mammography :** **Ultrasound :**

Size (cm): **Size (cm) :** **Size (cm):**

Cytology :

Site :

MRI : **If yes, results :**

Biopsy : **If yes :** **Diagnosis :**

Diagnosis :

Comments:

Benign Breast Diseases

Type of Benign Breast Disease :

☐ Fibroadenoma	☐ Fibroadenosis	☐ Hyperplasia
☐ Mastalgia (cyclical)	☐ Haematoma	☐ Not stated
☐ Mastalgia (non-cyclical)	☐ Breast augmentation	☐ Not applicable
☐ Polycystic Disease	☐ Dust ectasia	☐ Other...
☐ Cysts	☐ Abcess	
☐ Fibrocystic change	☐ Fibrous Mastopathology	
☐ Intraduct Lesion	☐ Periductal mastitis	
☐ Lipoma	☐ Nipple discharge	
☐ Breast Reduction	☐ Benign Breast Disease NST	

Treatment :

Operation : **Operation Date :**

If yes, Operation type :

Consultant :

Histopathology

Histology number :

Date :

Comments :

Follow-up

Date	Status	Treatment

CANCER

Pre-operative Treatment : Neo - adjuvant

Radiotherapy : ☐ Yes ☐ No ☐ Not Stated ☐ Not Applicable ☐ Other...

Consultant :

Start Date : **End Date :**

Radiotherapy to :

☐ Breast	☐ Supraclavicular fossa
☐ Axilla	☐ Chest wall
☐ Internal Mammary	☐ Other...

Total Dose :

Comments1 :

Chemotherapy : ☐ Yes ☐ No ☐ Not Stated ☐ Not Applicable

Consultant :

Start Date : **End Date :**

Regimen :

Comments :

Endocrine : ☐ Yes ☐ No ☐ Not Stated ☐ Not Applicable

Consultant :

Start Date : **End Date :**

Regimen :

Comments :

Further Comments:

Operation

Consultant :

Operation Date :

Operation Type : **Axilla:**

Bed Biopsy performed : **If yes, Result :**

Further Excision :

Date :

Type of Excision :

Further Comments :

Histopathology

Invasive : ☐ Ductal ☐ Medullary ☐ Lobular ☐ Tubular or Cribrioform ☐ Mucoid or colloid ☐ Other ☐ Not Specified

Histology Number:

Report Date :

Histology Block :

Lymph Node Block :

Specify Type :

Non-Invasive / In Situ : ☐ Ductal ☐ Lobular ☐ Paget's Disease

DCIS Grade :

Size (cm) :

Vascular Invasion :

Microinvasion :

Microcalcification :

Excision :

Distance From Nearest Margin (mm) :

ER Receptor Status : ○ Positive ○ Negative ○ Not Stated

Axillary Node Status :

Total No :

No Positive :

Sentinel Node Biopsy :

Grade : ○ 1 ○ 2 ○ 3 ○ Not Stated ○ Not assessable

Stage : T N M

Clinical Stage :

Further Comments :

Post-Operative Treatment

Radiotherapy : ☐ Yes ☐ No ☐ Not stated ☐ Not applicable

Consultant :

Start Date : **End Date :**

Radiotherapy to :

☐ Breast	☐ Supraclavicular fossa
☐ Axilla	☐ Chest Wall
☐ Internal mammary	☐ Other...

Total Dose : **No of Fractions:**

Boost to Tumour Bed : ☐ **If yes, Dose :** **No of Fractions:**

Comments :

Chemotherapy : ☐ Yes ☐ No ☐ Not stated ☐ Not applicable

Consultant :

Start Date : **End Date :**

Regimen :

Comments :

Endocrine : ☐ Yes ☐ No ☐ Not stated ☐ Not applicable

Consultant :

Start Date : **End Date :**

Regimen :

Comments :

Radiation Menopause : ☐ Yes ☐ No ☐ Not stated ☐ Not applicable

Consultant :

Start Date : **End Date :**

Oopherectomy : ☐ Yes ☐ No ☐ Not stated ☐ Not applicable

Randomized Trial : ☐ Yes ☐ No ☐ Not stated

Other Treatment :

Follow - up Chronology

Date	Status	Site of Recurrence (if any)

Summary and Patients Results

Last Follow up date :	
Last Follow up status :	
Recurrence :	○ Yes ○ No ○ Not applicable
Time to First Recurrence (months) :	
Disease Free interval (months) :	

Reason for Operation :	

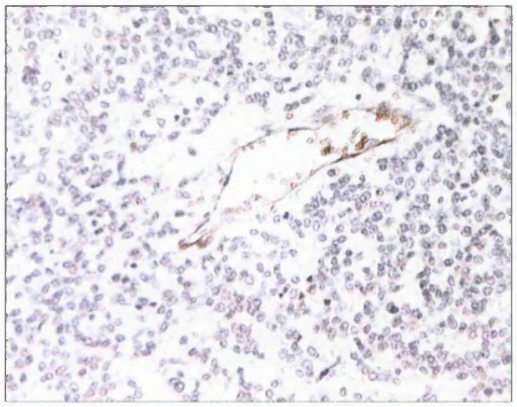
Appendix 3

The relative amount of oligosaccharides with the same glucose unit values as standard N-linked oligosaccharides.

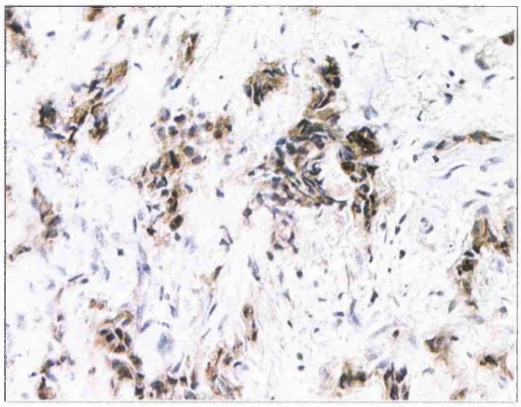
Patient / run code	status at follow-up	Sugar A1 % 7.37 GU	Sugar A2 %8.37 GU	Sugar A3 %8.58 GU	SugarA3 %9.04 GU	Sugar A3 %9.57GU	SugarA3 %9.95GU
	patient deceased		1.99				0.72
AS7 011	patient deceased	1.68		2.76			
AS4 003	loco regional and distant recurrence	0.80		1.68			0.36
AS7 021	patient deceased	2.16		3.05		0.12	
AS7 012	distant recurrence			2.71			
WT 43	patient deceased			2.42			
AS4 013	loco regional recurrence	1.21					0.39
AUTO 002	patient deceased	1.12	1.49			0.15	0.06
AS7 027	patient deceased						1.53
AS4 012	loco regional and distant recurrence	1.35		3.07			6.09
AS7 025	loco regional recurrence	1.99		2.24			
AS7 019	patient deceased			0.87			
AS4 008	loco regional and distant recurrence						
WT 33	loco regional recurrence						0.38
WT 46	patient deceased	2.53	4.26			0.14	0.61
AS7 016	distant recurrence		3.07				2.56
AS4 016	loco regional recurrence	2.50		2.44			
AUTO 004	patient deceased		2.10				0.45
AS4 011	loco regional recurrence						0.39
AUTO 005	patient deceased	0.28	1.49				
WT 29	patient deceased			2.99			
WT 37	patient deceased	0.89	2.14			0.46	1.35
AS4 015	patient deceased		2.44				0.12
AS4 005	patient deceased	0.01					
WT 13	loco regional recurrence	0.06	0.03				
WT 44	patient deceased		1.07/0.19				10.22
AS7 004	patient deceased		1.33			0.14	0.24
WT 27	patient deceased			2.23			
WT 47	patient deceased		1.06	0.02			0.30
NICKI 40	patient deceased		1.92				0.46
AUTO2 005	loco regional recurrence			1.02		0.88	
AS4 014	loco regional recurrence						
WT 23	patient deceased						
AS4 010	patient deceased						
AS4 002	distant recurrence			0.55			
AS7 014	loco regional recurrence			0.50			0.08
AUTO 008	patient deceased	2.32	4.49			0.35	0.43
AS3 005	patient deceased			0.95			
AS3 004	patient deceased	0.85		1.92			1.20
AS4 019	patient deceased	2.82		3.17			
	mean	1.41	2.14	1.92	-	0.32	1.40
	sd	0.91	1.23	1.02	-	0.28	2.48
	2sd	1.82	2.46	2.04	-	0.56	4.96
	mean-2sd	-0.41	-0.32	-0.12	-	-0.24	-3.56
	mean+2sd	3.23	4.60	3.97	-	0.88	6.36

Patient / run code	status at follow-up	Sugar A1 % 7.37 GU	Sugar A2 %8.37 GU	Sugar A3 %8.58 GU	SugarA3 %9.04 GU	Sugar A3 %9.57GU	SugarA3 %9.95GU
AS7 006	no sign of recurrence			3.52			0.12
	no sign of recurrence			2.47			
AS7 013	no sign of recurrence	0.28	0.07	1.20			0.18
AS7 007	no sign of recurrence	1.53		2.11			0.38
AS 005	no sign of recurrence						
AUTO2 007	no sign of recurrence	5.29		15.63		0.03	
AS3 003	no sign of recurrence	0.66	0.67				
AS7 023	no sign of recurrence			2.63			0.11
WT 059	no sign of recurrence			2.11			1.64
AS4 006	no sign of recurrence	3.29	5.42			0.01	0.83
AS7 003	no sign of recurrence	0.34				0.80	
WT 4	no sign of recurrence			0.88			
AUTO 003	no sign of recurrence		1.28	0.01		11.45	1.46
AS4 018	no sign of recurrence						
AS7 005	no sign of recurrence	0.82	1.27				0.22
AS7 009	no sign of recurrence						
AS7 017	no sign of recurrence		0.24				
WT 060	no sign of recurrence	2.54	2.63		0.07	0.07	0.11
AS7 022	no sign of recurrence	0.30	0.53			1.56	0.64
AUTO 006	no sign of recurrence	0.08					
AUTO2 002	no sign of recurrence	0.96		1.30		0.09	
WT 41	no sign of recurrence						0.19
WT 50	no sign of recurrence						
AS7 008	no sign of recurrence	0.38	1.14			0.26	1.14
AUTO 007	no sign of recurrence	1.34		1.30			0.32
AUTO2 004	no sign of recurrence						
WT 003	no sign of recurrence	0.64		2.26		2.02	
WT 30	no sign of recurrence	0.12	0.85			0.16	1.74
WT 36	no sign of recurrence	2.26	3.11			0.50	2.12
AS4 004	no sign of recurrence	0.58	0.12			0.14	
AUTO2 006	no sign of recurrence	0.79	0.70				
WT 55	no sign of recurrence		1.87				
AS7 024	no sign of recurrence					0.46	2.25
WT 31	no sign of recurrence	2.26		3.39			
AS7 030	no sign of recurrence	1.84		1.13			0.08
AS4 007	no sign of recurrence	0.47		0.32			0.17
	mean	1.27	1.42	2.68	-	1.35	0.76
	sd	1.28	1.46	3.72	-	3.10	0.76
	2sd	2.57	2.92	7.45	-	6.20	1.52
	man-2sd	-1.29	-1.50	-4.76	-	-4.85	-0.76
	mean+2sd	3.84	4.34	10.13	-	7.55	2.28

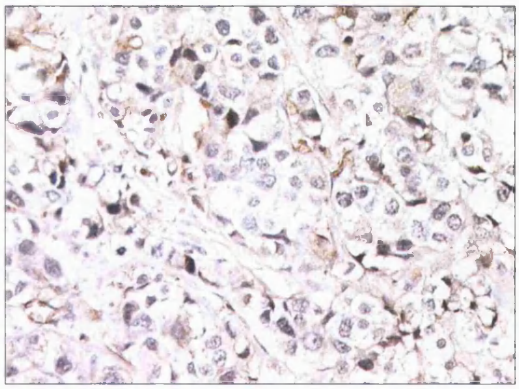
Appendix 4.
A range of breast cancer specimens stained with HPA (brown) and counter-stained with haematoxylin (blue).



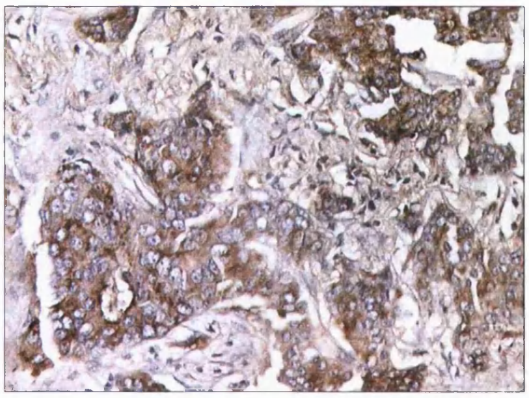
Specimen 1844/90
Cancer cells negative, blood vessel positive



Specimen 3903/91(1)
Cancer cells ++ cytoplasmic



Specimen 8537/87
Cancer cells +, cytoplasmic



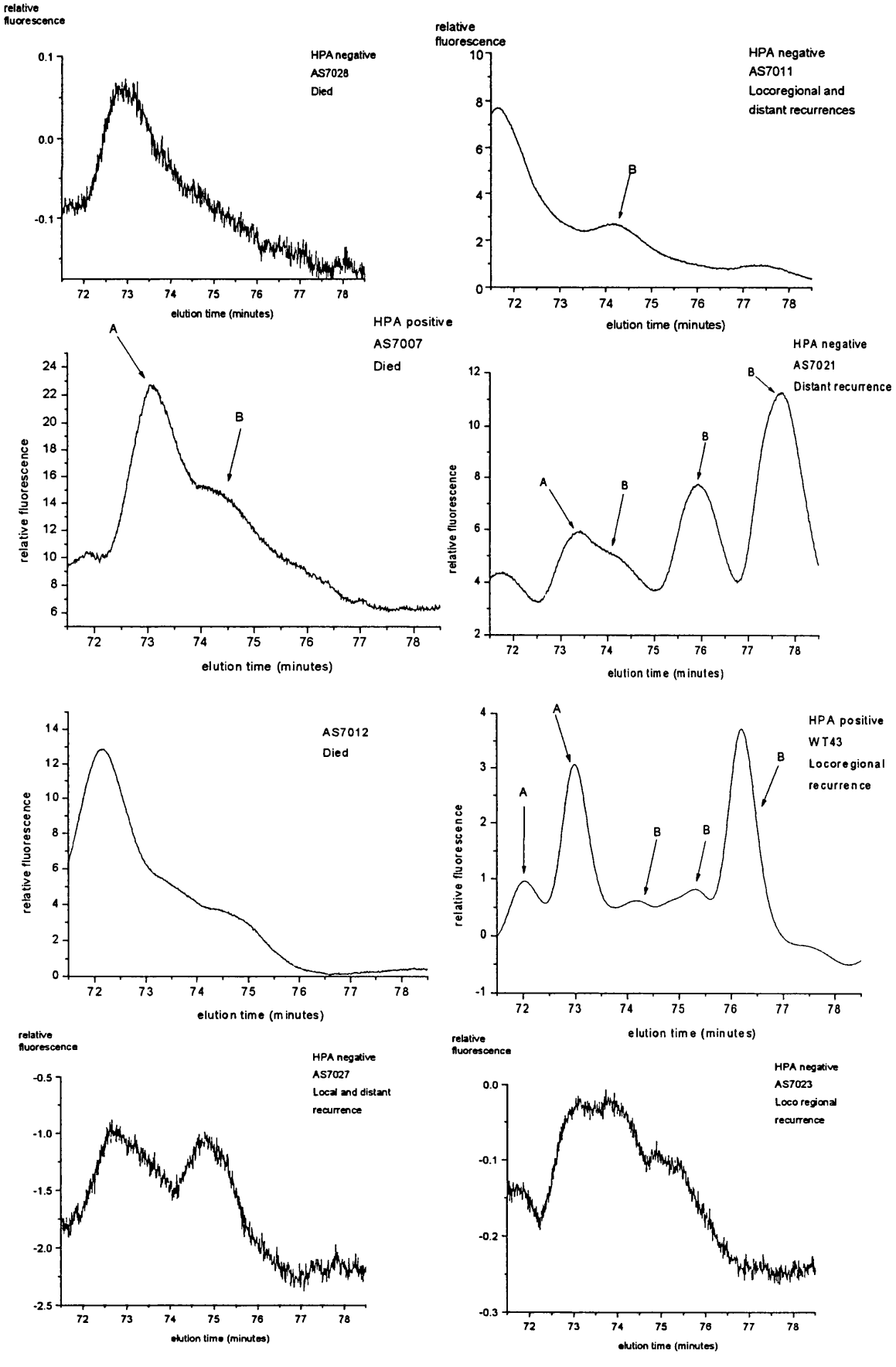
Specimen 6178/90
Cancer cells +++, cytoplasmic

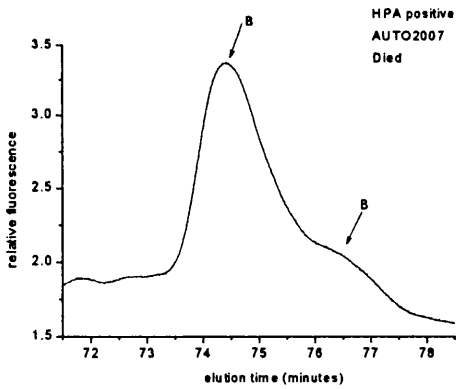
—

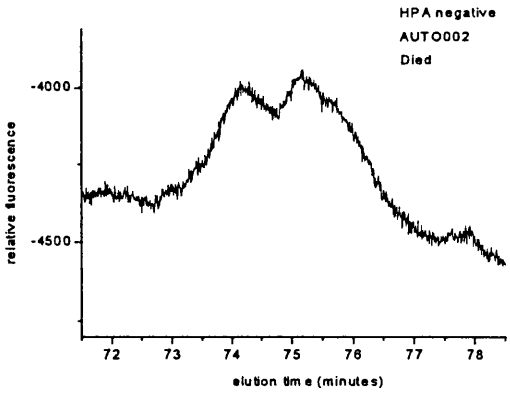
scale bar = approximately 30 micron

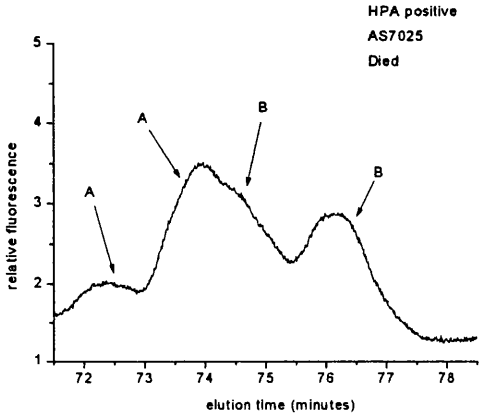
Appendix 5

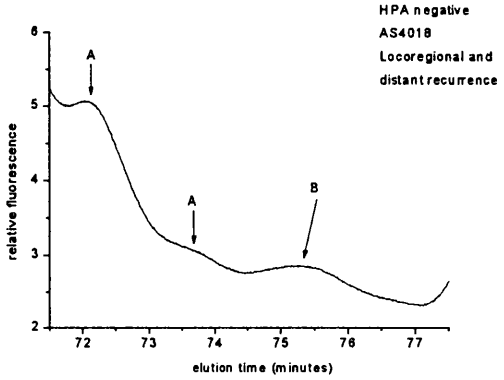
Normal-phase HPLC separation of oligosaccharides A and B.

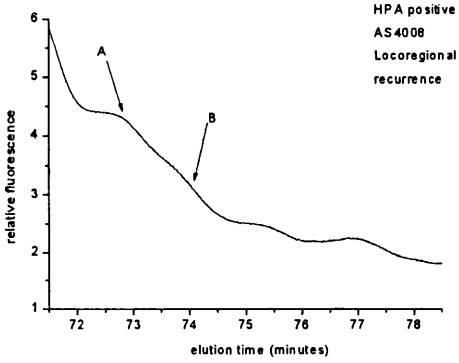


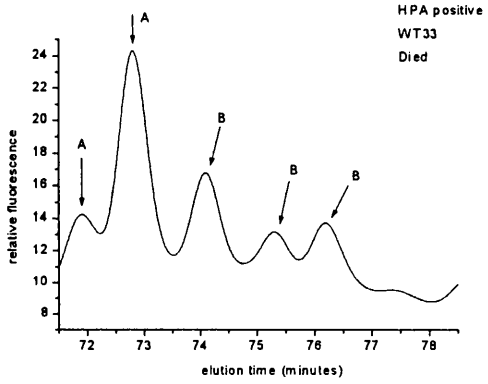

HPA positive
AUTO2007
Died
relative fluorescence
elution time (minutes)

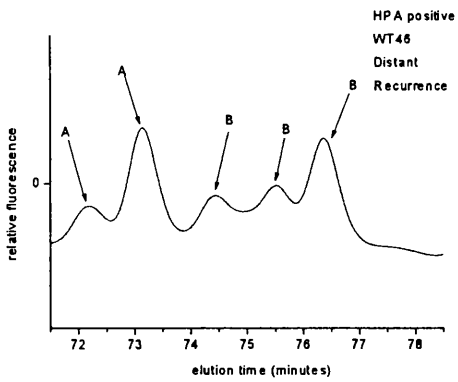

HPA negative
AUTO002
Died
relative fluorescence
elution time (minutes)

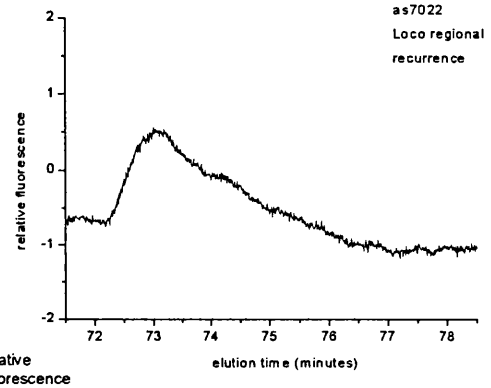

HPA positive
AS7025
Died
relative fluorescence
elution time (minutes)

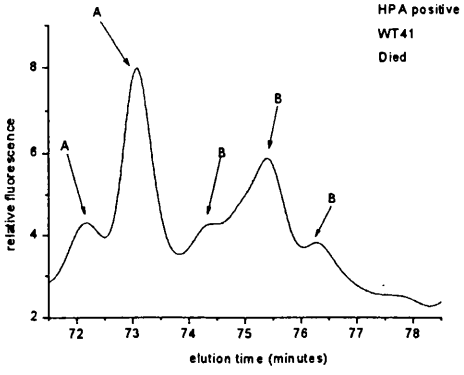

HPA negative
AS4018
Locoregional and
distant recurrence
relative fluorescence
elution time (minutes)

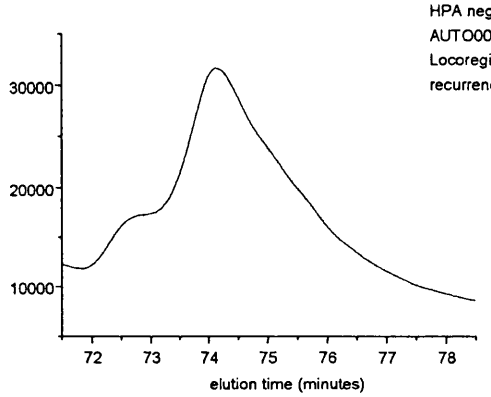

HPA positive
AS4008
Locoregional
recurrence
relative fluorescence
elution time (minutes)

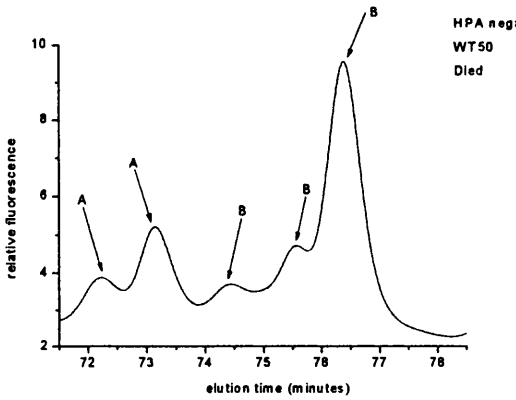

HPA positive
WT33
Died
relative fluorescence
elution time (minutes)

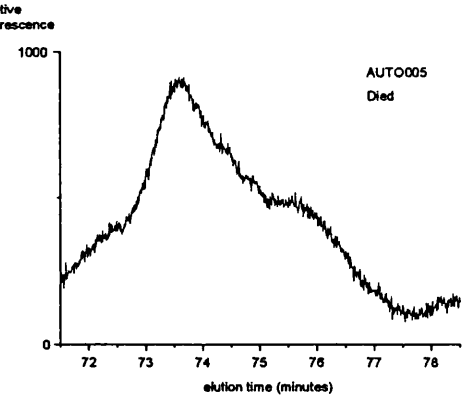

HPA positive
WT46
Distant
Recurrence
relative fluorescence
elution time (minutes)

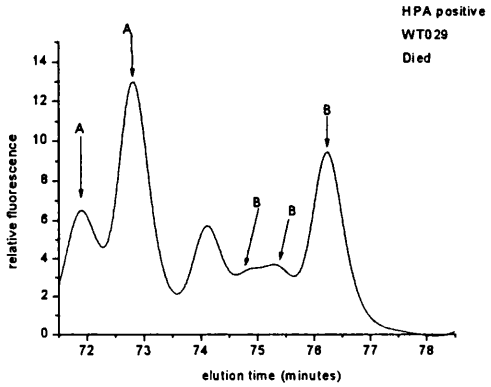

as7022
Loco regional
recurrence
relative fluorescence
elution time (minutes)

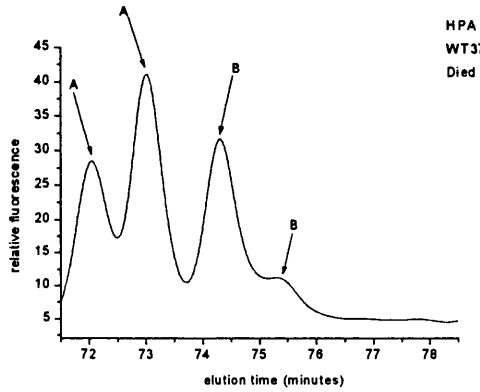

HPA positive
WT41
Died
relative fluorescence
elution time (minutes)

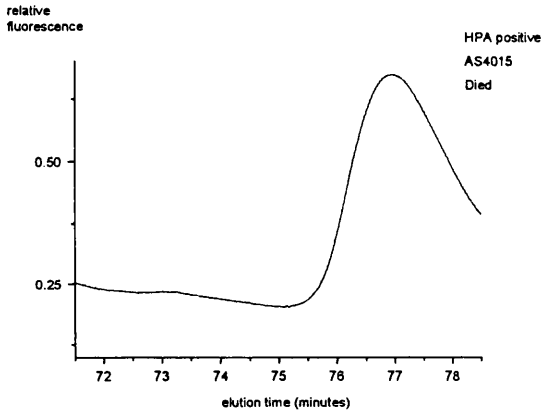

HPA negative
AUTO004
Locoregional
recurrence
relative fluorescence
elution time (minutes)

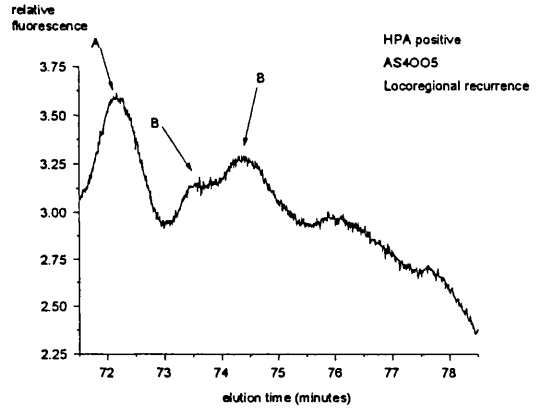

HPA negative
WT50
Died
relative fluorescence
elution time (minutes)

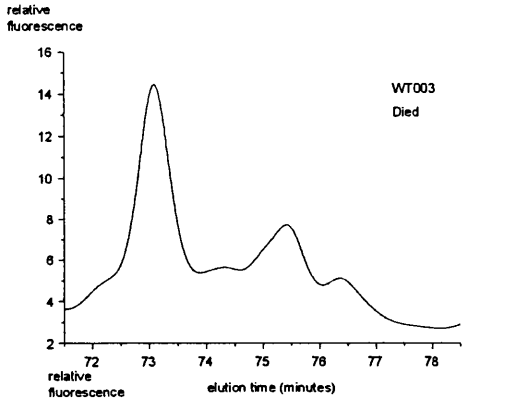

relative fluorescence
AUTO005
Died
elution time (minutes)

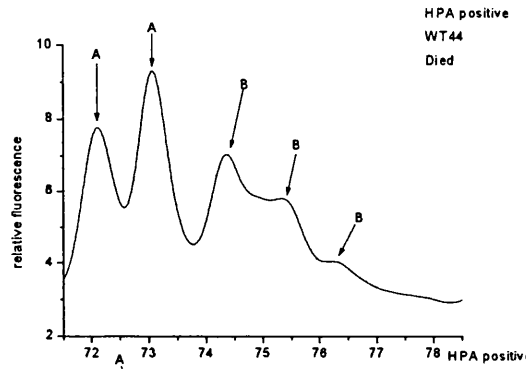

HPA positive
WT029
Died
relative fluorescence
elution time (minutes)

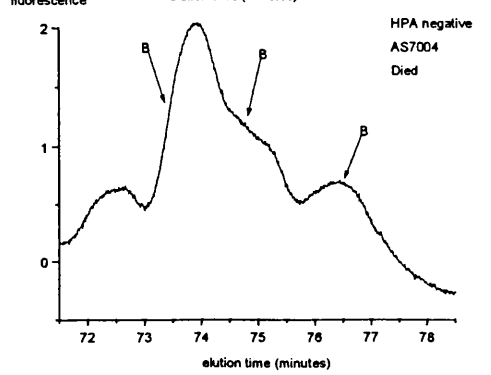

HPA negative
WT37
Died
relative fluorescence
elution time (minutes)

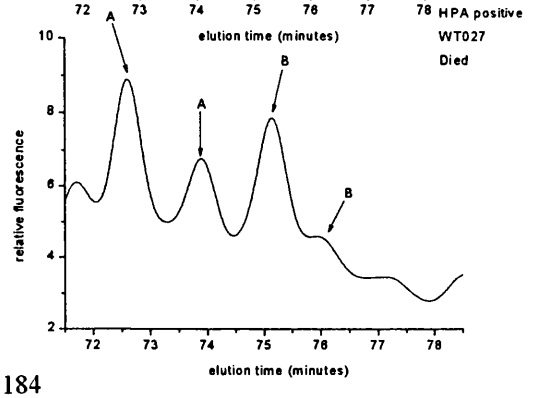

relative fluorescence
HPA positive
AS4015
Died
elution time (minutes)

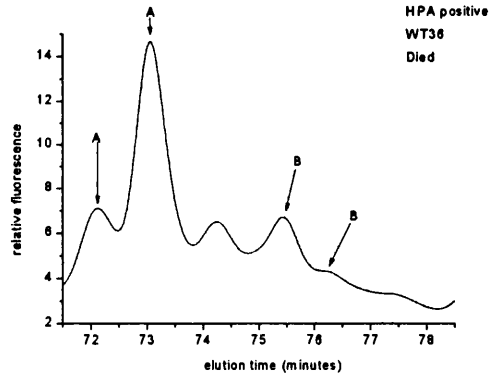

relative fluorescence
HPA positive
AS4005
Locoregional recurrence
elution time (minutes)

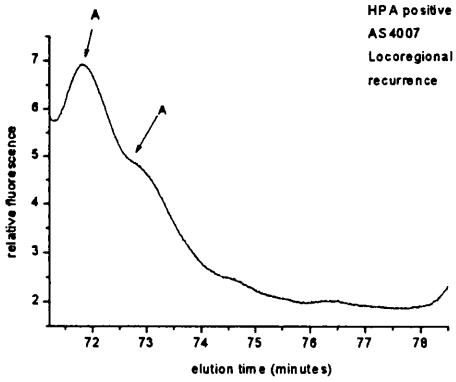

relative fluorescence
WT003
Died
elution time (minutes)

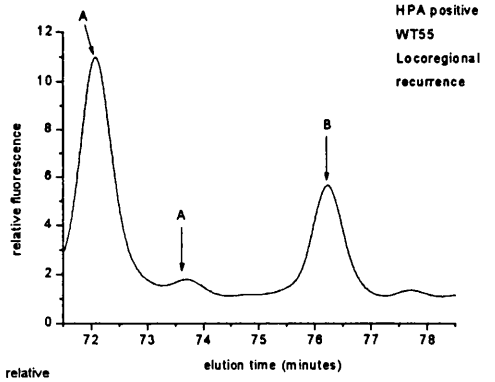

HPA positive
WT44
Died
relative fluorescence
elution time (minutes)

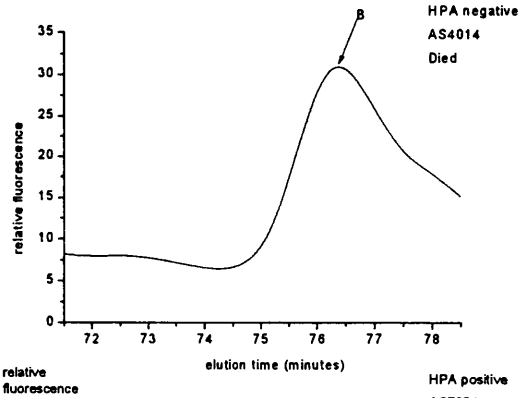

relative fluorescence
HPA negative
AS7004
Died
elution time (minutes)

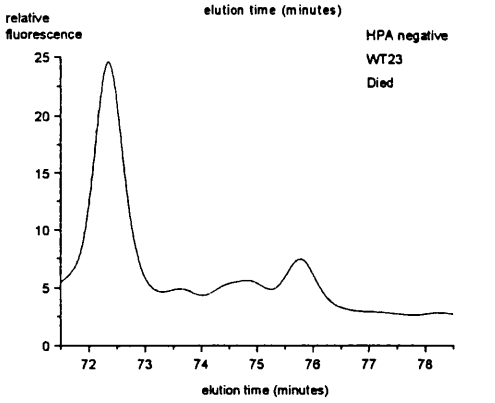

HPA positive
WT027
Died
relative fluorescence
elution time (minutes)

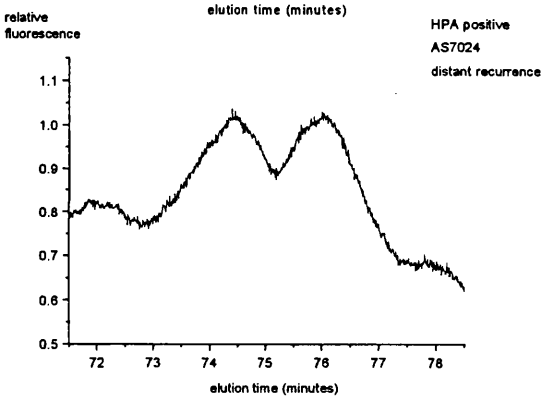

HPA positive
WT36
Died
relative fluorescence
elution time (minutes)

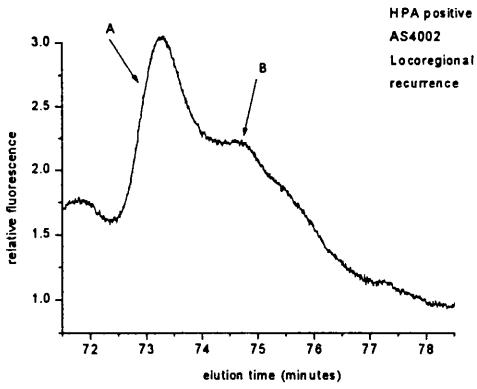

HPA positive
AS4007
Locoregional
recurrence
relative fluorescence
elution time (minutes)

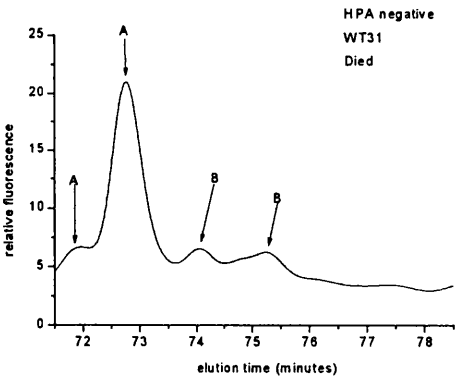

HPA positive
WT55
Locoregional
recurrence
relative fluorescence
elution time (minutes)

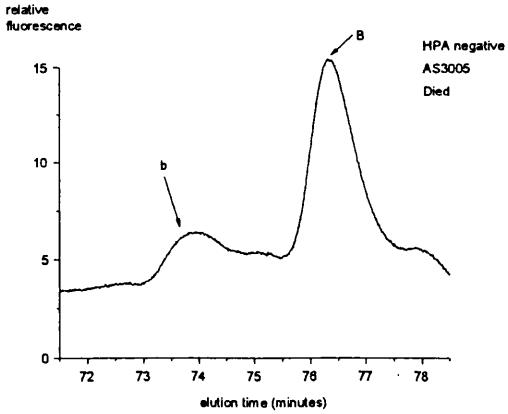

HPA negative
AS4014
Died
relative fluorescence
elution time (minutes)

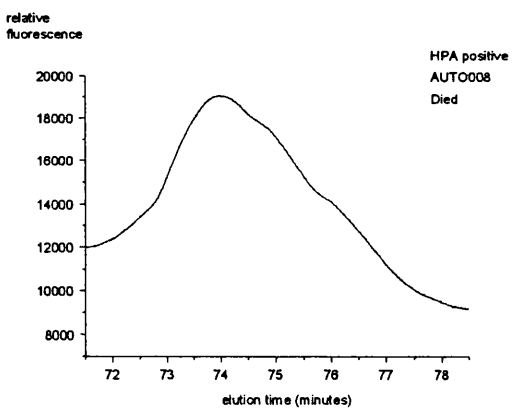

relative fluorescence
HPA negative
WT23
Died
elution time (minutes)

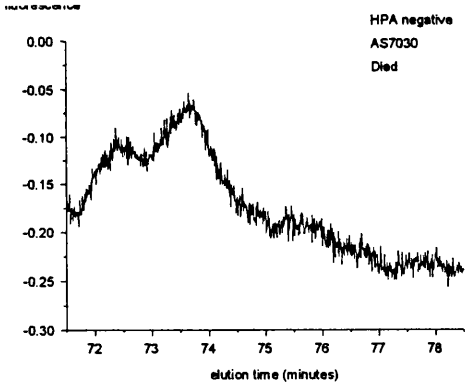

relative fluorescence
HPA positive
AS7024
distant recurrence
elution time (minutes)

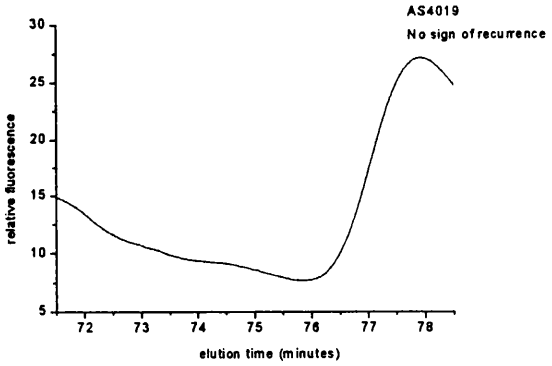

HPA positive
AS4002
Locoregional
recurrence
relative fluorescence
elution time (minutes)

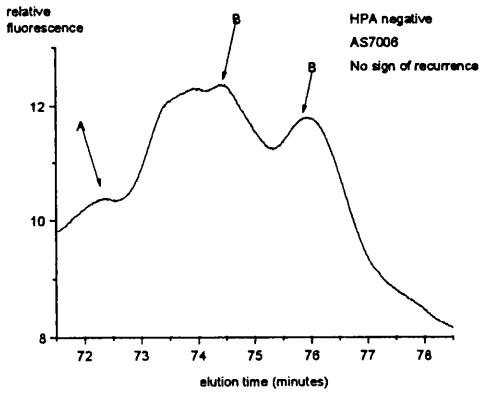

HPA negative
WT31
Died
relative fluorescence
elution time (minutes)

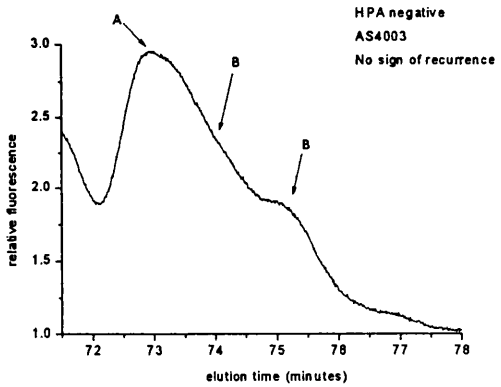

relative fluorescence
HPA negative
AS3005
Died
elution time (minutes)

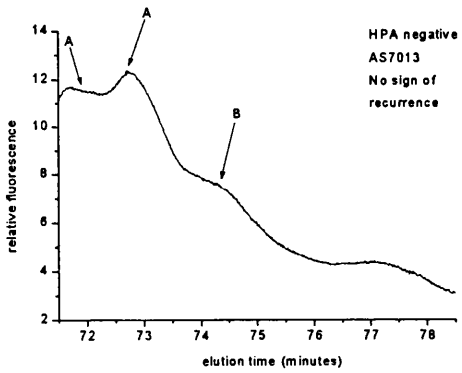

relative fluorescence
HPA positive
AUTO008
Died
elution time (minutes)

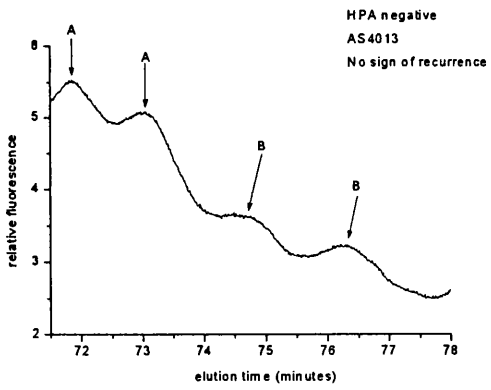

HPA negative
AS7030
Died
elution time (minutes)

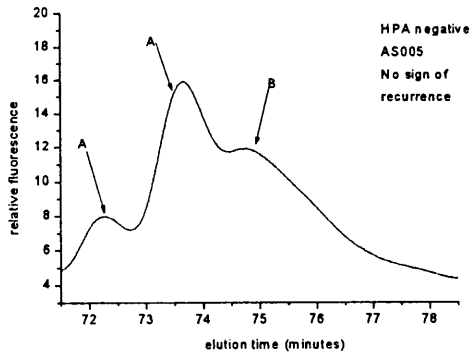

AS4019
No sign of recurrence
relative fluorescence
elution time (minutes)

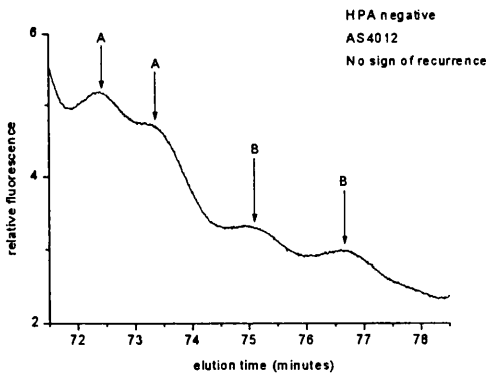

relative fluorescence
HPA negative
AS7006
No sign of recurrence
A
B
elution time (minutes)

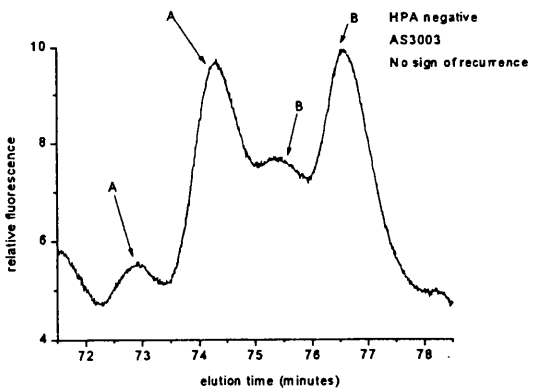

HPA negative
AS4003
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

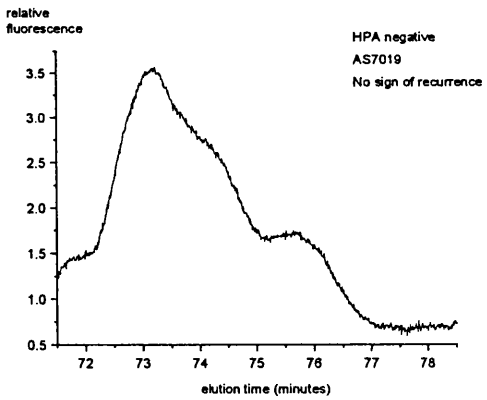

HPA negative
AS7013
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

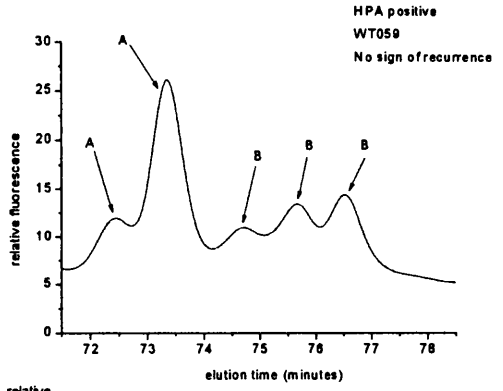

HPA negative
AS4013
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

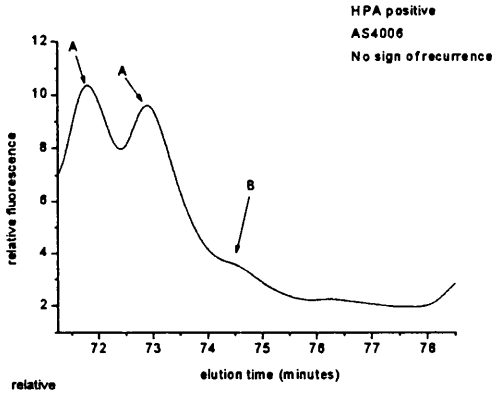

HPA negative
AS005
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

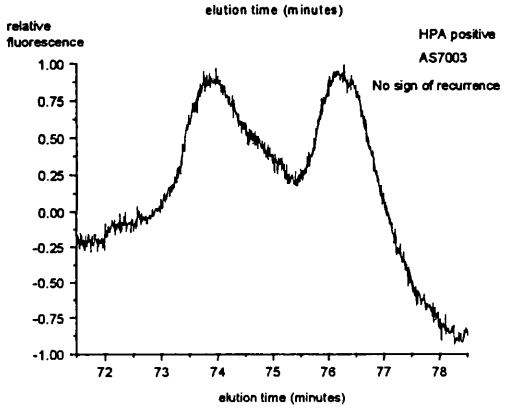

HPA negative
AS4012
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

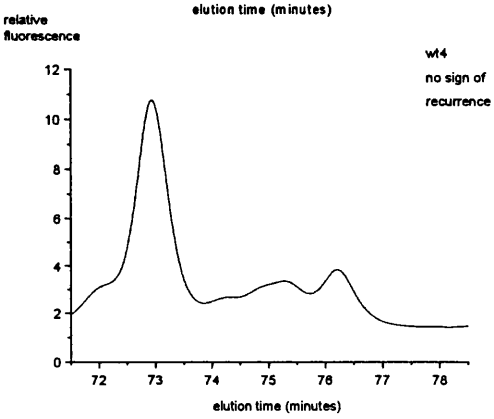

HPA negative
AS3003
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

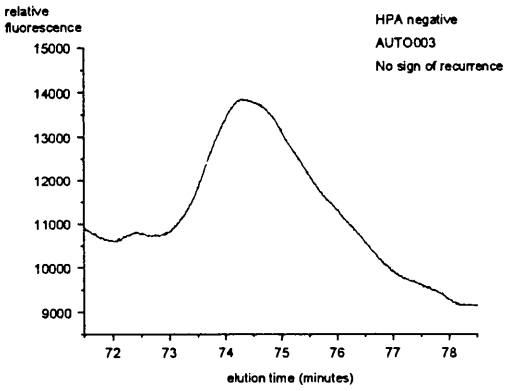

relative fluorescence
HPA negative
AS7019
No sign of recurrence
elution time (minutes)

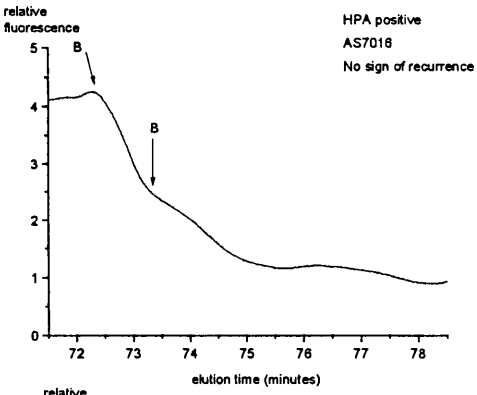

HPA positive
WT059
No sign of recurrence
relative fluorescence
elution time (minutes)

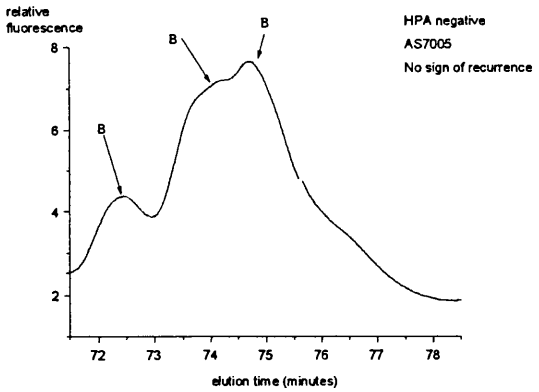

HPA positive
AS4006
No sign of recurrence
relative fluorescence
elution time (minutes)

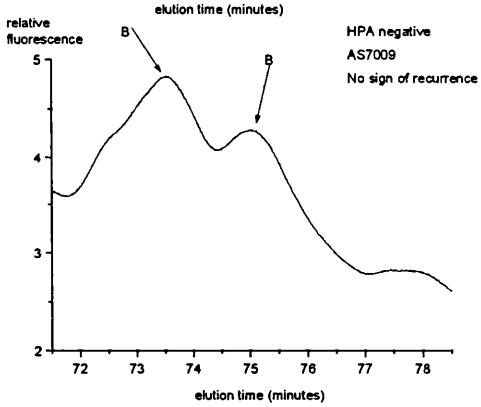

relative fluorescence
HPA positive
AS7003
No sign of recurrence
elution time (minutes)

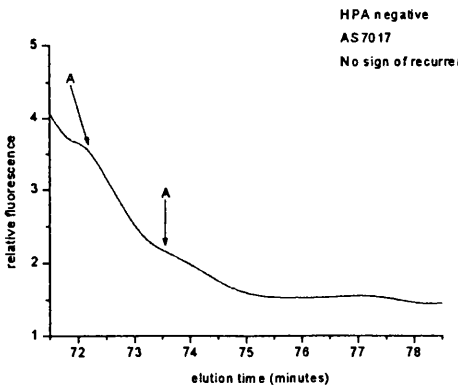

relative fluorescence
wt4
no sign of recurrence
elution time (minutes)

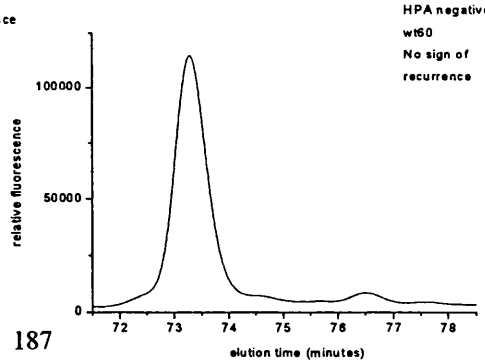

relative fluorescence
HPA negative
AUTO003
No sign of recurrence
elution time (minutes)

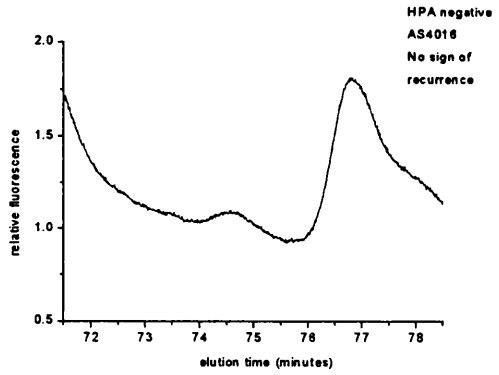

relative fluorescence
HPA positive
AS7016
No sign of recurrence
elution time (minutes)

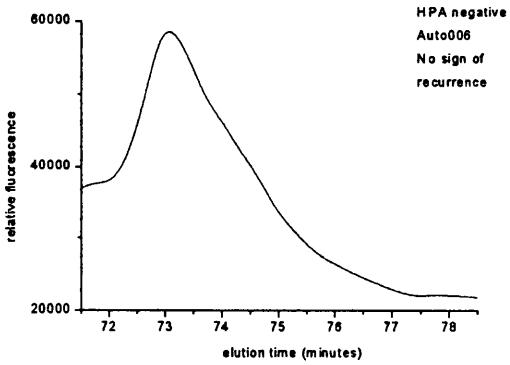

relative fluorescence
HPA negative
AS7005
No sign of recurrence
elution time (minutes)

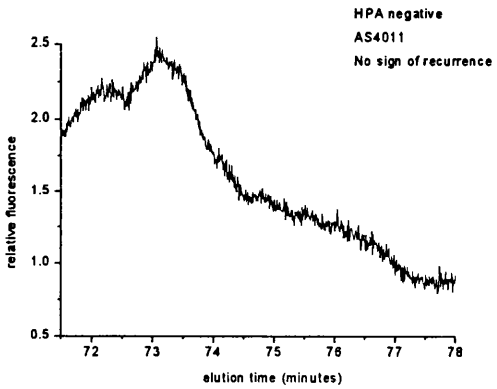

relative fluorescence
HPA negative
AS7009
No sign of recurrence
elution time (minutes)

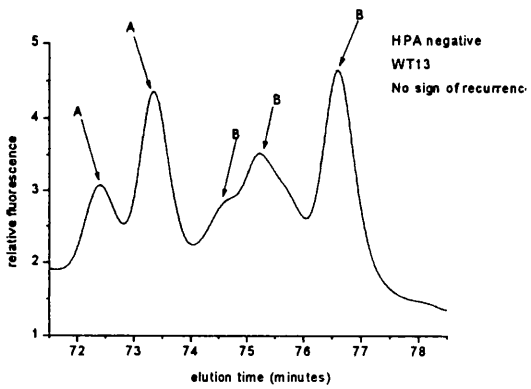

HPA negative
AS7017
No sign of recurrence
relative fluorescence
elution time (minutes)

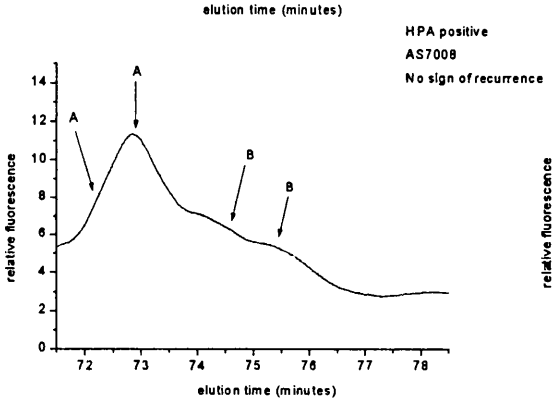

HPA negative
wt60
No sign of recurrence
relative fluorescence
elution time (minutes)

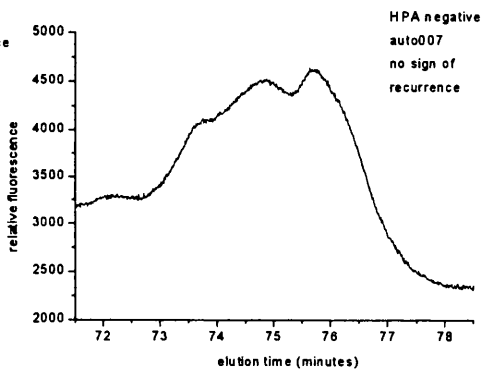

HPA negative
AS4016
No sign of
recurrence
relative fluorescence
elution time (minutes)

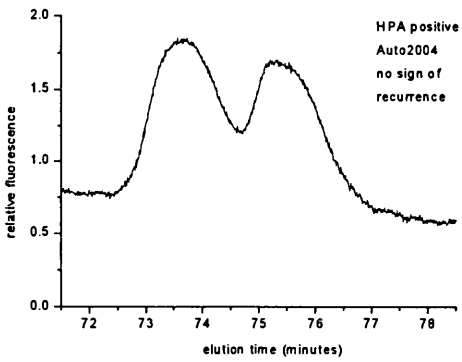

HPA negative
Auto006
No sign of
recurrence
relative fluorescence
elution time (minutes)

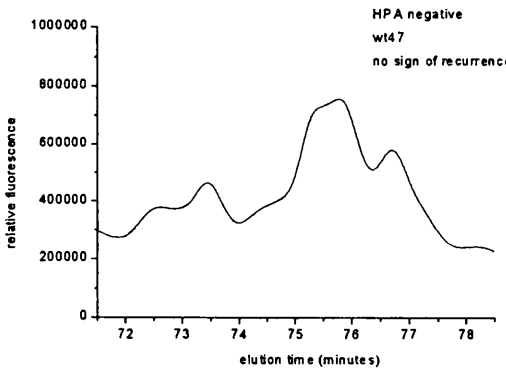

HPA negative
AS4011
No sign of recurrence
relative fluorescence
elution time (minutes)

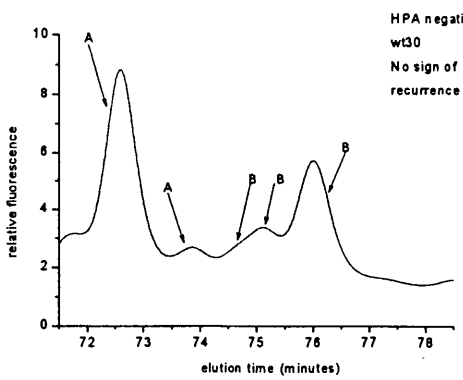

HPA negative
WT13
No sign of recurrenc
A
B
relative fluorescence
elution time (minutes)

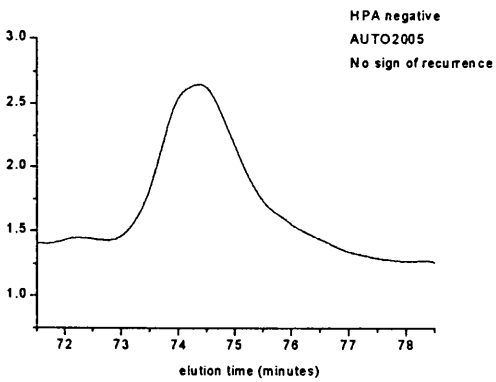

HPA positive
AS7008
No sign of recurrence
A
B
relative fluorescence
elution time (minutes)

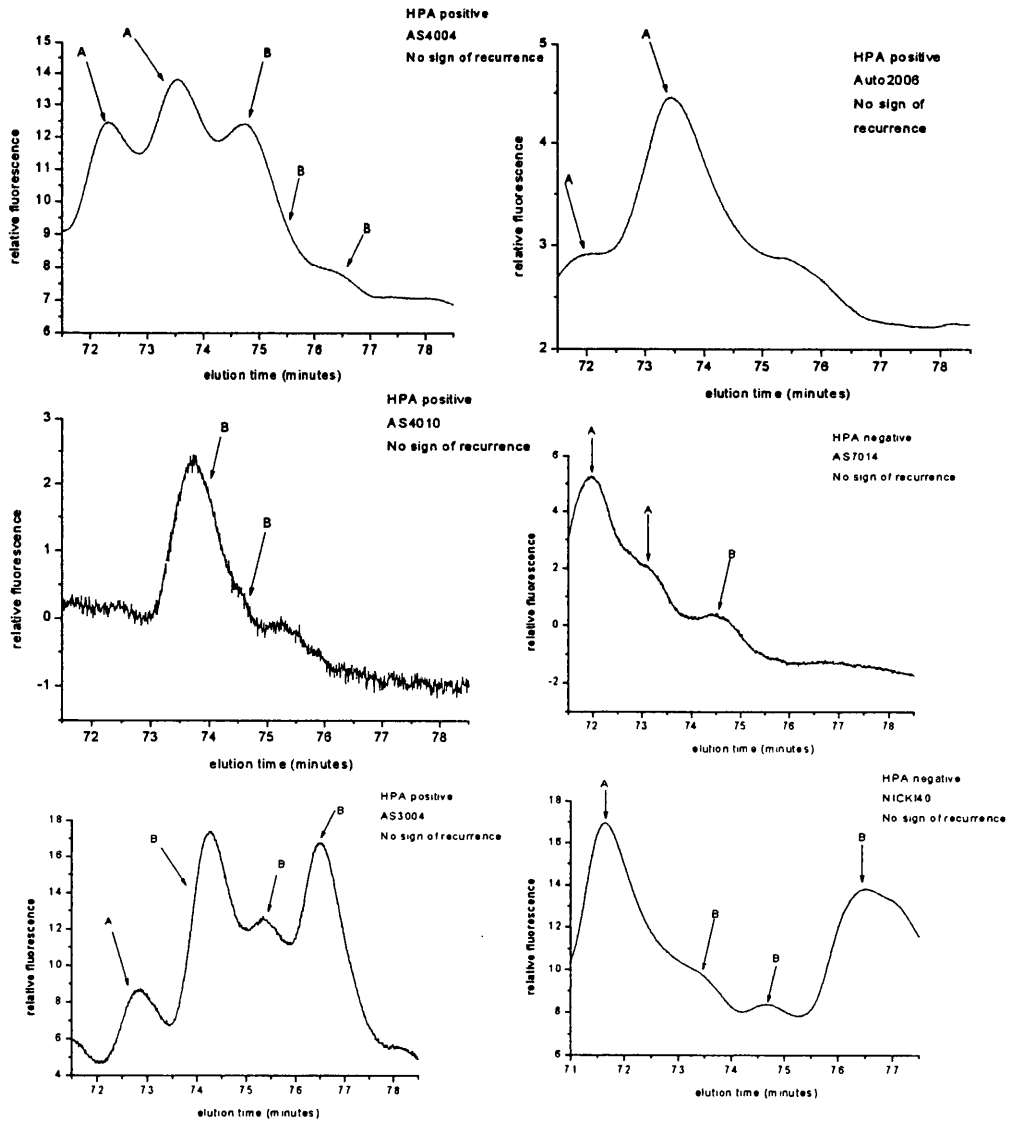

HPA negative
auto007
no sign of
recurrence
relative fluorescence
elution time (minutes)


HPA positive
Auto2004
no sign of
recurrence
relative fluorescence
elution time (minutes)


HPA negative
wt47
no sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
wt30
No sign of
recurrence
A
B
relative fluorescence
elution time (minutes)


HPA negative
AUTO2005
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA positive
AS4004
No sign of recurrence
relative fluorescence
elution time (minutes)
HPA positive
Auto2006
No sign of
recurrence
relative fluorescence
elution time (minutes)
HPA positive
AS4010
No sign of recurrence
relative fluorescence
elution time (minutes)
HPA negative
AS7014
No sign of recurrence
relative fluorescence
elution time (minutes)
HPA positive
AS3004
No sign of recurrence
relative fluorescence
elution time (minutes)
HPA negative
NICKI40
No sign of recurrence
relative fluorescence
elution time (minutes)

Appendix 6

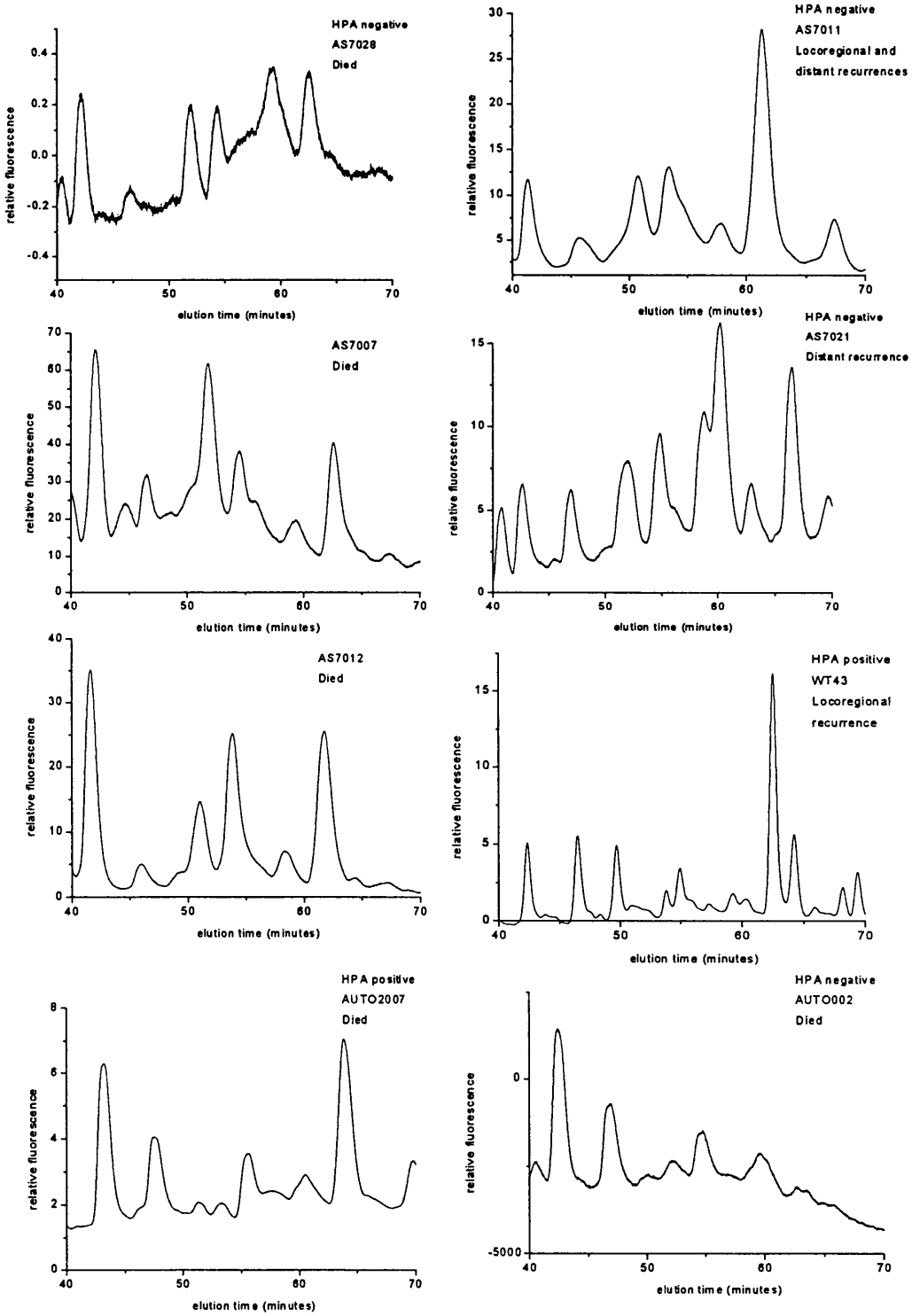
The relative quantities of oligosaccharides A and B extracted from the different breast cancer specimens.

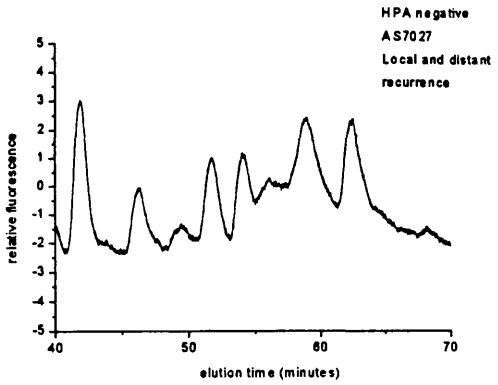
Patient / Run Code	HPA stainer y/n	Status at follow-up	% 3.5 GU	%3.67 GU	Sum A	% 3.77 GU	%3.86 GU	%3.96 GU	Sum B
	N	patient deceased			0.00				0.00
AS7 028		patient deceased			0.00				0.00
AS7 011	N	loco regional and distant			0.00	5.65		0.45	6.10
AS7 007	Y	patient deceased		0.12	0.12	1.52			1.52
AS7 021	N	distant recurrence	2.46	0.85	3.31		2.59		2.59
AS7 012	disregard	patient deceased				4.93			
WT 43	Y	loco regional recurrence	2.52		2.52	0.11	3.74		3.85
AUTO2 007	Y	patient deceased		3.26	3.26				0.00
AUTO 002	N	patient deceased			0.00				0.00
AS7 027	N	loco regional and distant			0.00				0.00
AS7 023	N	loco regional recurrence			0.00				0.00
AS7 025	Y	patient deceased	0.33	1.63	1.96	0.20	2.06	1.06	3.32
AS4 018	N	loco regional and distant		1.06	1.06		1.41	1.60	3.01
AS4 008	Y	loco regional recurrence		3.35	3.35	0.55			0.55
WT 33	Y	patient deceased	0.64	3.86	4.50	1.75	0.50	0.96	3.21
WT 46	Y	distant recurrence	0.39	2.05	2.44	0.45	0.24	1.70	2.39
AS7 022	disregard	loco regional recurrence							
WT 41	Y	patient deceased	0.34	2.55	2.89		1.44	0.20	1.64
AUTO 004	N	loco regional recurrence			0.00				0.00
WT 50	N	patient deceased	0.05	0.17	0.22	0.03	0.02	0.61	0.66
AUTO 005	disregard	patient deceased							
WT 29	Y	patient deceased	1.10	3.40	4.50	1.00	0.21	3.04	4.25
WT 37	N	patient deceased	3.02	5.42	8.44	4.54	0.24		4.78
AS4 015	Y	patient deceased			0.00				0.00
AS4 005	Y	loco regional recurrence	0.74		0.74	0.23	0.02	0.07	0.32
WT 003	disregard	patient deceased							
WT 44	Y	patient deceased	1.42	1.96	3.38	0.93	0.25	0.07	1.25
AS7 004	N	patient deceased			0.00	0.40	1.37	0.06	1.83
WT 27	Y	patient deceased	0.30	1.07	1.37	0.60	1.07	0.06	1.73
WT 36	Y	patient deceased	0.65	3.51	4.16	0.38	0.76		1.14
AS4 007	Y	loco regional recurrence	0.37	0.94	1.31				0.00
WT 55	Y	loco regional recurrence	3.10	0.11	3.21		1.61		1.61
AS4 014	N	patient deceased		3.41	3.41		0.28		0.28
WT 23	N	patient deceased			0.00				0.00
AS7 024	Y	distant recurrence			0.00				0.00
AS4 002	Y	loco regional recurrence		0.14	0.14		0.95	0.03	0.98
WT 31	N	patient deceased	0.28	6.84	7.12	0.39		0.83	1.22
AUTO 008	Y	patient deceased			0.00				0.00
AS3 005	N	patient deceased			0.00	1.66		8.13	9.79
AS7 030	N	patient deceased			0.00				0.00
		mean	1.11	2.29	1.76	1.41	1.04	1.26	1.61
		sd	1.06	1.85	2.16	1.76	1.00	2.08	2.13
		2sd	2.12	3.70	4.33	3.52	2.01	4.15	4.25
		mean-2sd	-1.01	-1.42	-2.57	-2.11	-0.96	-2.90	-2.64
		mean+2sd	3.22	5.99	6.09	4.93	3.05	5.41	5.86

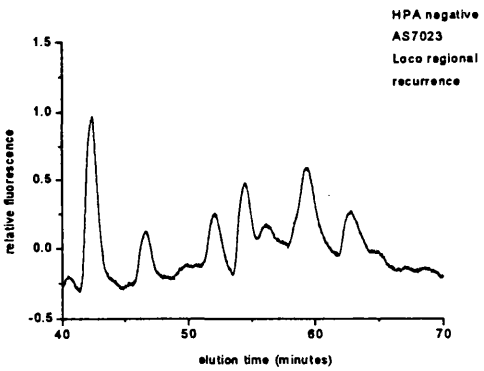
Patient / Run Code	HPA stainer y/n	Status at follow-up	% 3.5 GU	%3.67 GU	Sum A	% 3.77 GU	%3.86 GU	%3.96 GU	Sum B
AS4 019	N	no sign of recurrence		0.13	0.13	1.58	0.44		2.02
AS7 006	N	no sign of recurrence		0.07	0.07		1.48		1.48
AS4 003	N	no sign of recurrence		1.13	1.13	3.26		0.21	3.47
AS7 013	N	no sign of recurrence		0.37	0.37	0.61	0.65	0.14	1.40
AS4 013	N	no sign of recurrence	0.15	1.50	1.65		0.25	0.35	0.60
AS 005	N	no sign of recurrence		1.14	1.14	5.38	1.49		6.87
AS4 012	N	no sign of recurrence			0.00	2.29	0.13	0.22	2.64
AS3 003	N	no sign of recurrence		0.28	0.28	2.37	0.05	2.73	5.15
AS7 019	disregard	no sign of recurrence							
WT 059	Y	no sign of recurrence	0.39	3.51	3.90	0.22	0.52	1.04	1.78
AS4 006	Y	no sign of recurrence	2.18	1.45	3.63	1.61			1.61
AS7 003	Y	no sign of recurrence			0.00				0.00
WT 4	disregard	no sign of recurrence			0.00				
AUTO 003	N	no sign of recurrence			0.00				0.00
AS7 016	Y	no sign of recurrence		0.09	0.09	0.64	0.47		1.11
AS7 005	N	no sign of recurrence			0.00	0.58	0.65	0.70	1.93
AS7 009	N	no sign of recurrence			0.00		1.07	0.36	1.43
AS7 017	N	no sign of recurrence	0.08	1.77	1.85	0.09			0.09
WT 060	N	no sign of recurrence			0.00				0.00
AS4 016	N	no sign of recurrence	0.14	1.59	1.73	0.35			0.35
AUTO 006	N	no sign of recurrence			0.00				0.00
AUTO2 002	Y	no sign of recurrence	0.23	13.65	13.88				0.00
AS4 011	N	no sign of recurrence	0.24		0.24		0.17	0.28	0.45
WT 13	N	no sign of recurrence	0.39	0.99	1.38		0.42	1.33	1.75
AS7 008	Y	no sign of recurrence	0.04		0.04	2.61	0.05	0.16	2.82
AUTO 007	N	no sign of recurrence			0.00				0.00
AUTO2 004	Y	no sign of recurrence		1.79	1.79	1.23			1.23
WT 47	N	no sign of recurrence	0.10	0.27	0.37		1.37	0.37	1.74
WT 30	N	no sign of recurrence	0.18	5.18	5.36	0.18	0.38	2.69	3.25
AUTO2 005	N	no sign of recurrence			0.00				0.00
AS4 004	Y	no sign of recurrence	0.21	0.44	0.65		0.47	0.42	0.89
AUTO2 006	Y	no sign of recurrence	0.20	5.47	5.67	0.22			0.22
AS4 010	Y	no sign of recurrence			0.00	0.92		2.92	3.84
AS7 014	N	no sign of recurrence		0.96	0.96	4.08			4.08
AS3 004	Y	no sign of recurrence		1.07	1.07	3.29	0.15	3.20	6.64
NICKI 40	N	no sign of recurrence		0.06	0.06		1.52	0.90	2.42
		mean	0.35	1.95	1.36	1.66	0.62	1.06	1.80
		sd	0.56	3.02	2.64	1.51	0.51	1.10	1.85
		2sd	1.12	6.03	5.28	3.03	1.02	2.20	3.69
		mean-2sd	-0.77	-4.08	-3.93	-1.37	-0.41	-1.14	-1.89
		mean+2sd	1.47	7.98	6.64	4.69	1.64	3.26	5.49

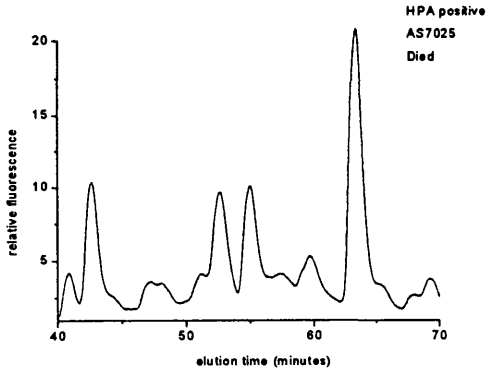
Appendix 7

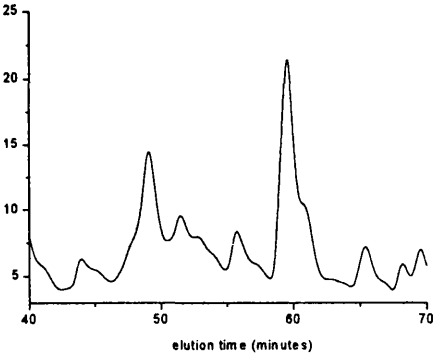
Normal-phase HPLC separation of oligosaccharides C and D.

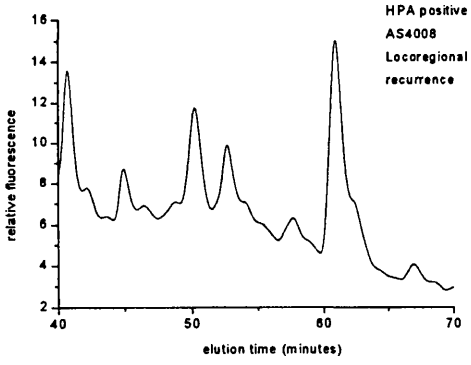


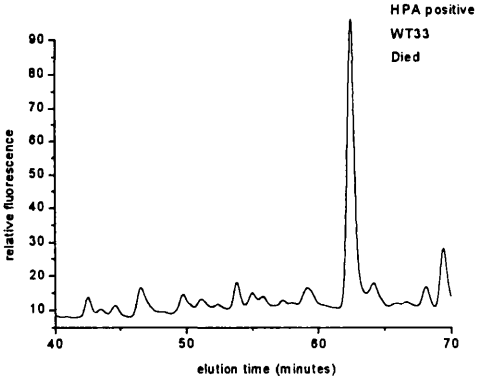

HPA negative
AS7027
Local and distant
recurrence
relative fluorescence
elution time (minutes)

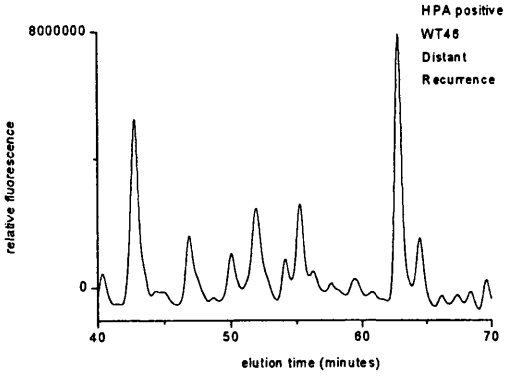

HPA negative
AS7023
Loco regional
recurrence
relative fluorescence
elution time (minutes)

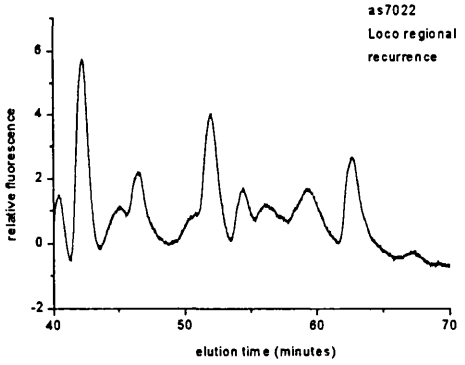

HPA positive
AS7025
Died
relative fluorescence
elution time (minutes)

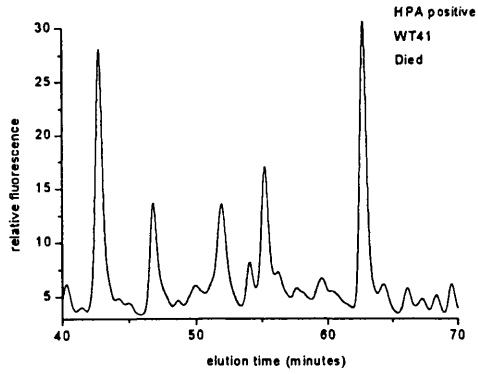

elution time (minutes)

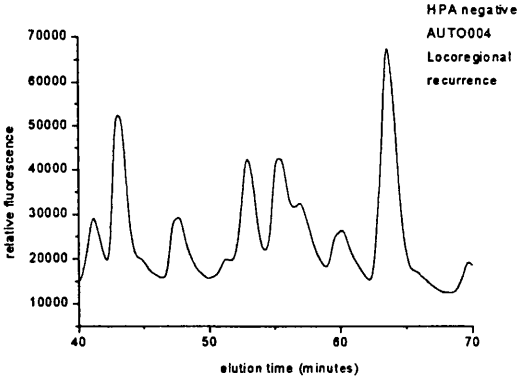

HPA positive
AS4008
Locoregional
recurrence
relative fluorescence
elution time (minutes)

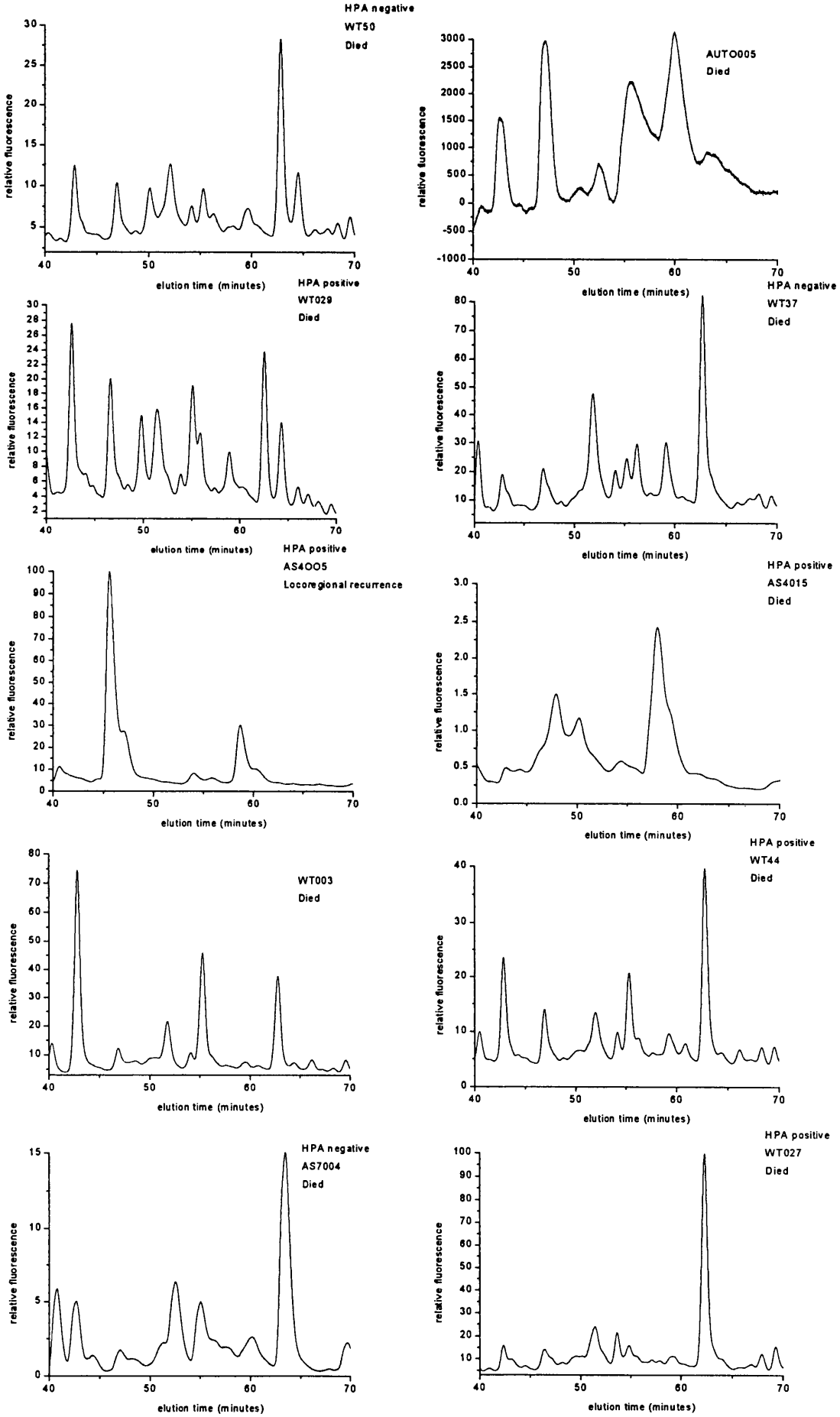

HPA positive
WT33
Died
relative fluorescence
elution time (minutes)

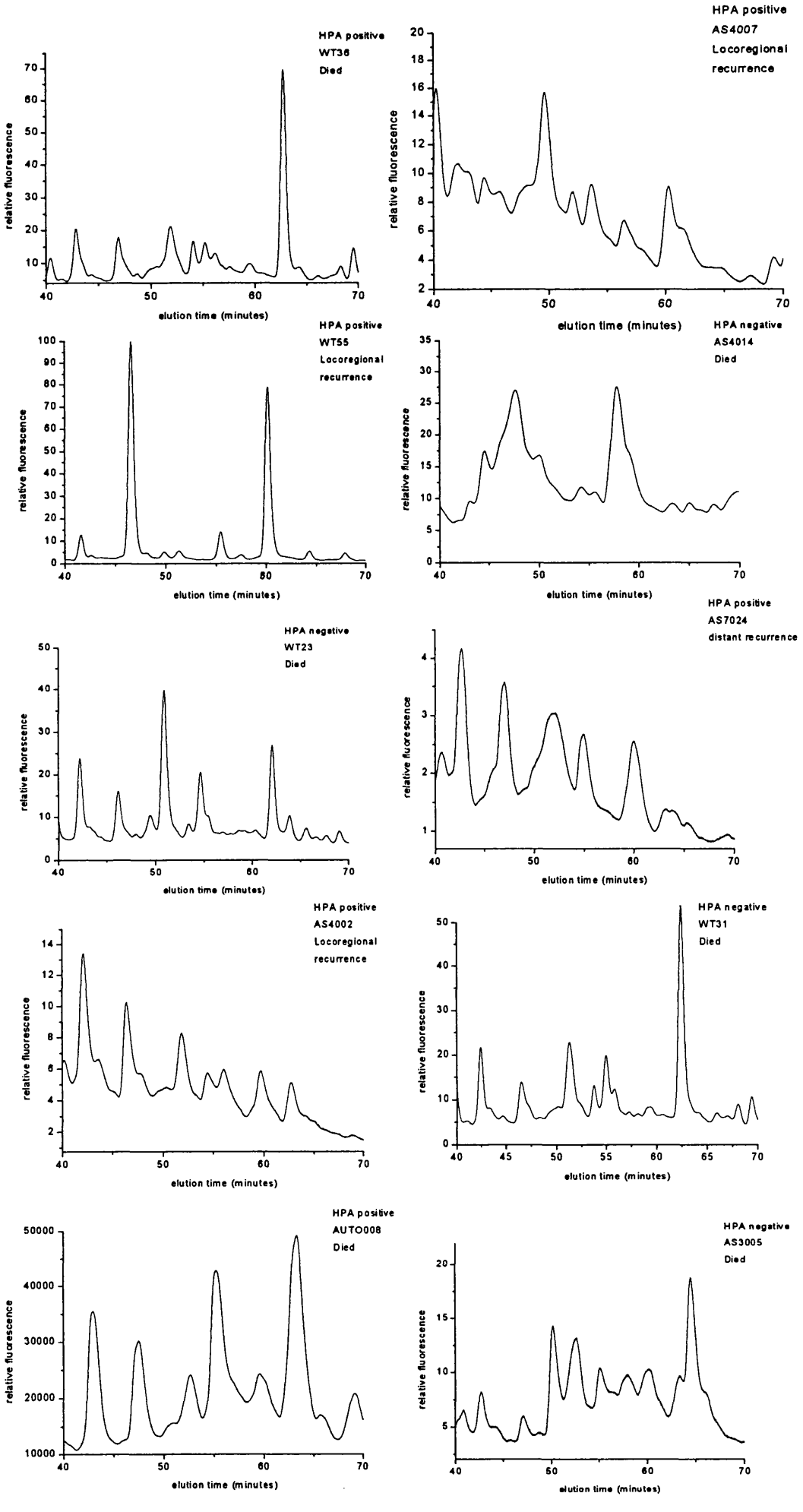

HPA positive
WT46
Distant
Recurrence
relative fluorescence
elution time (minutes)

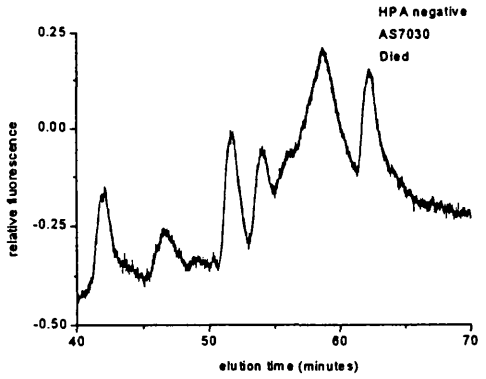

as7022
Loco regional
recurrence
relative fluorescence
elution time (minutes)

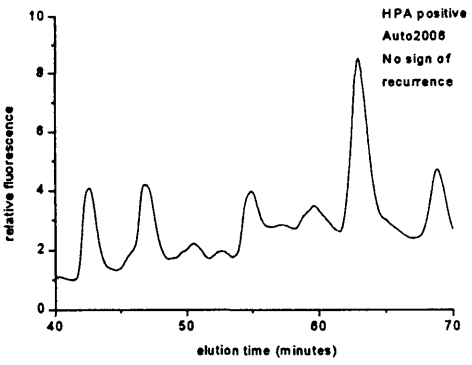

HPA positive
WT41
Died
relative fluorescence
elution time (minutes)

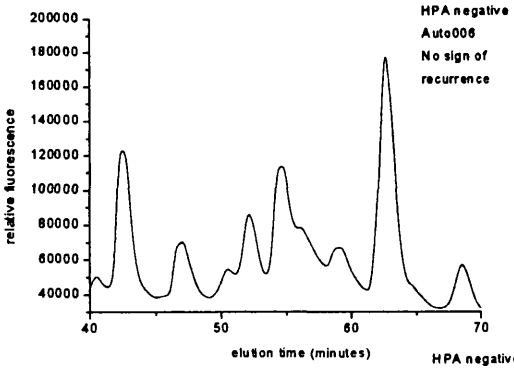

HPA negative
AUTO004
Locoregional
recurrence
relative fluorescence
elution time (minutes)

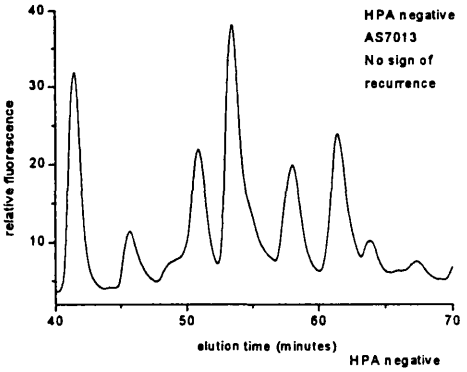

HPA negative
WT50
Died
relative fluorescence
elution time (minutes)
AUTO005
Died
HPA positive
WT029
Died
HPA negative
WT37
Died
HPA positive
AS4005
Locoregional recurrence
HPA positive
AS4015
Died
WT003
Died
HPA positive
WT44
Died
HPA negative
AS7004
Died
HPA positive
WT027
Died

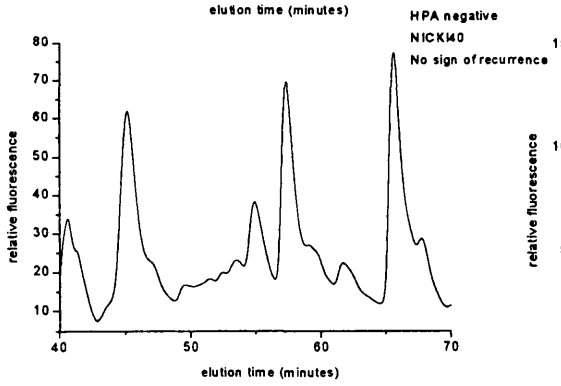

HPA positive
WT36
Died
relative fluorescence
elution time (minutes)
HPA positive
AS4007
Locoregional
recurrence
relative fluorescence
elution time (minutes)
HPA positive
WT55
Locoregional
recurrence
relative fluorescence
elution time (minutes)
HPA negative
AS4014
Died
relative fluorescence
elution time (minutes)
HPA negative
WT23
Died
relative fluorescence
elution time (minutes)
HPA positive
AS7024
distant recurrence
relative fluorescence
elution time (minutes)
HPA positive
AS4002
Locoregional
recurrence
relative fluorescence
elution time (minutes)
HPA negative
WT31
Died
relative fluorescence
elution time (minutes)
HPA positive
AUTO008
Died
relative fluorescence
elution time (minutes)
HPA negative
AS3005
Died
relative fluorescence
elution time (minutes)

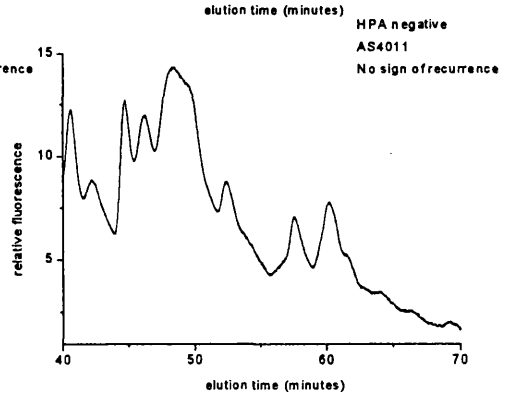

HPA negative
AS7030
Died
relative fluorescence
elution time (minutes)

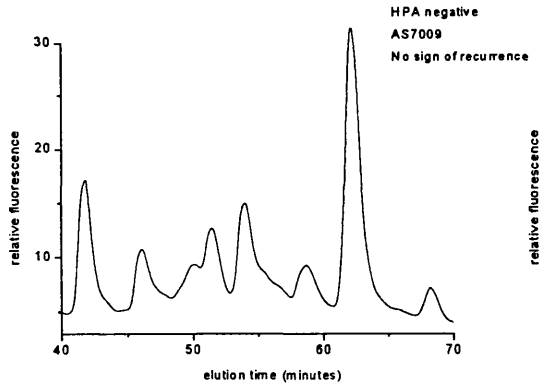

HPA positive
Auto2006
No sign of
recurrence
relative fluorescence
elution time (minutes)

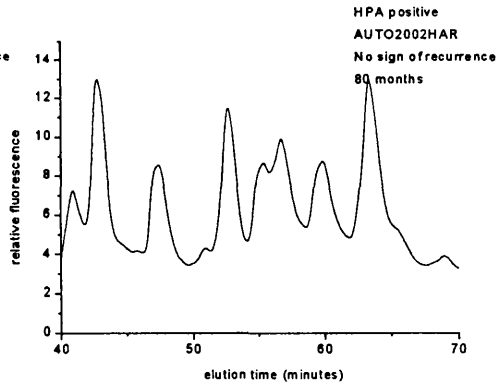

HPA negative
Auto006
No sign of
recurrence
relative fluorescence
elution time (minutes)

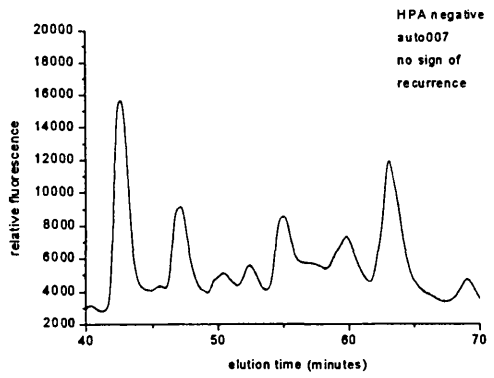

HPA negative
AS7013
No sign of
recurrence
relative fluorescence
elution time (minutes)

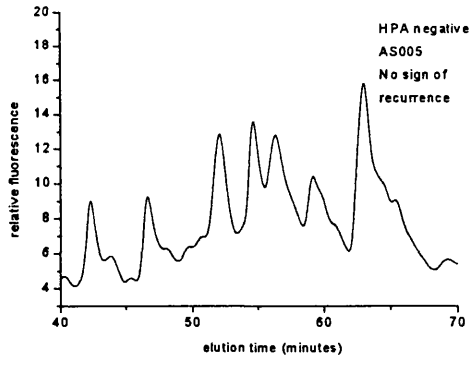

HPA negative
NICKI40
No sign of recurrence
relative fluorescence
elution time (minutes)

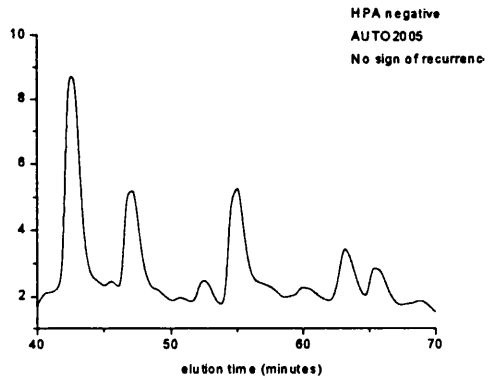

HPA negative
AS4011
No sign of recurrence
relative fluorescence
elution time (minutes)

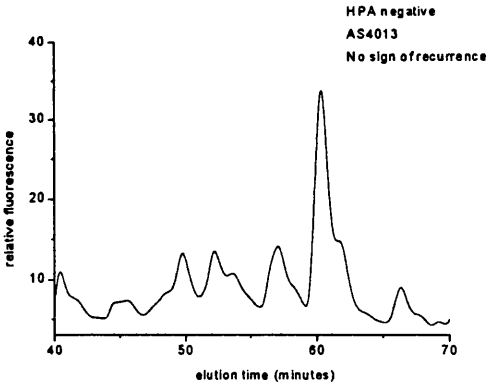

HPA negative
AS7009
No sign of recurrence
relative fluorescence
elution time (minutes)

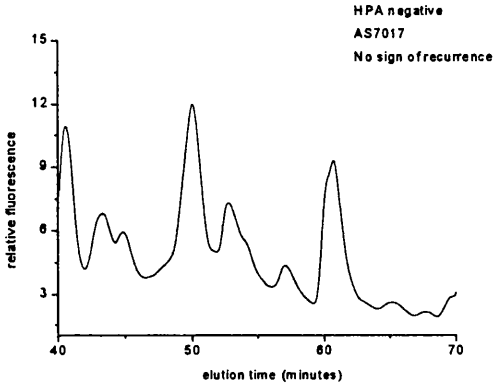

HPA positive
AUTO2002HAR
No sign of recurrence
80 months
relative fluorescence
elution time (minutes)

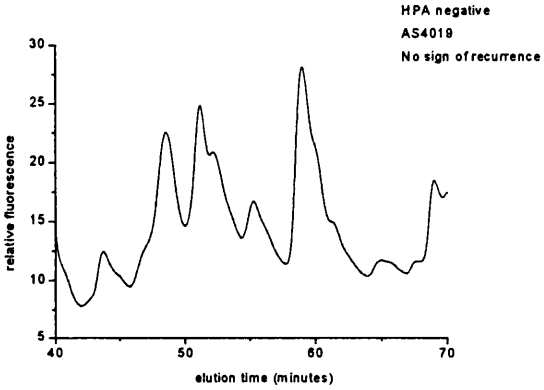

HPA negative
auto007
no sign of
recurrence
relative fluorescence
elution time (minutes)

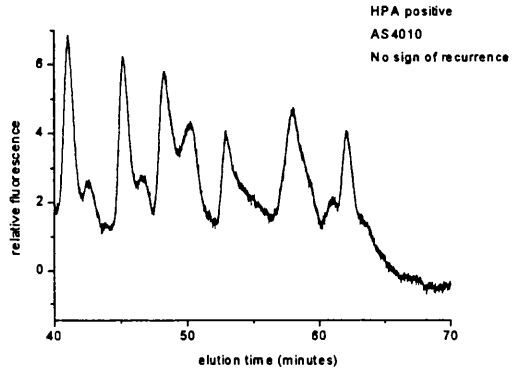

HPA negative
AS005
No sign of
recurrence
relative fluorescence
elution time (minutes)

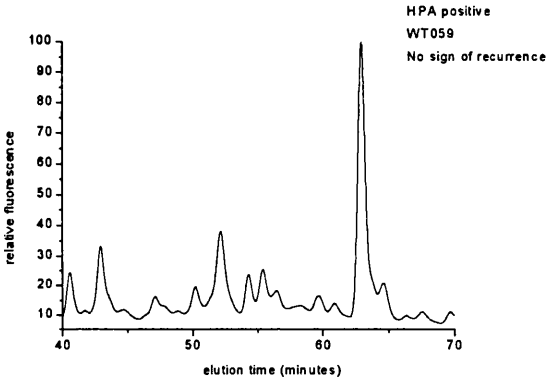

HPA negative
AUTO2005
No sign of recurrenc
relative fluorescence
elution time (minutes)

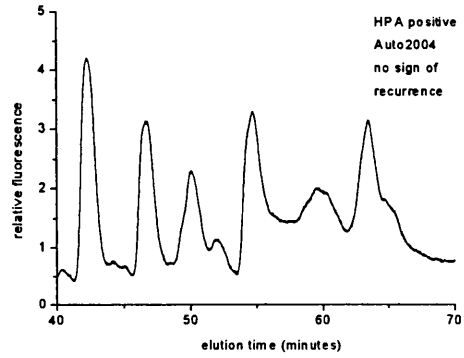

HPA negative
AS4013
No sign of recurrence
relative fluorescence
elution time (minutes)

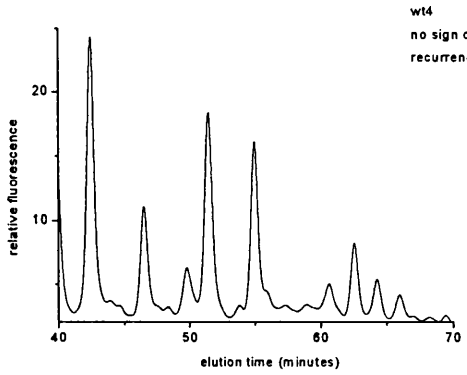

HPA negative
AS7017
No sign of recurrence
relative fluorescence
elution time (minutes)

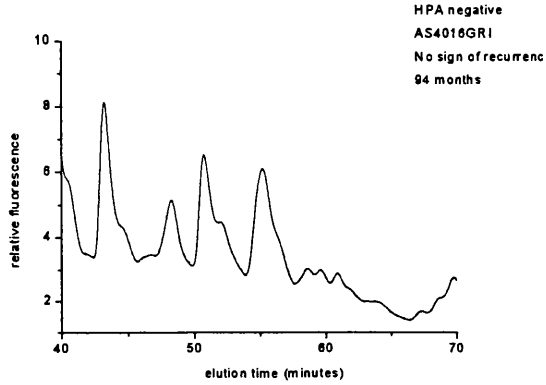

HPA negative
AS4019
No sign of recurrence
relative fluorescence
elution time (minutes)

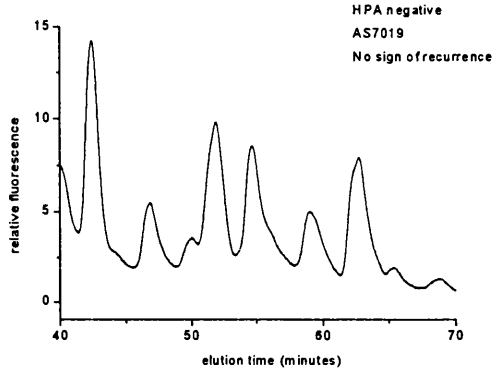

HPA positive
AS4010
No sign of recurrence
relative fluorescence
elution time (minutes)

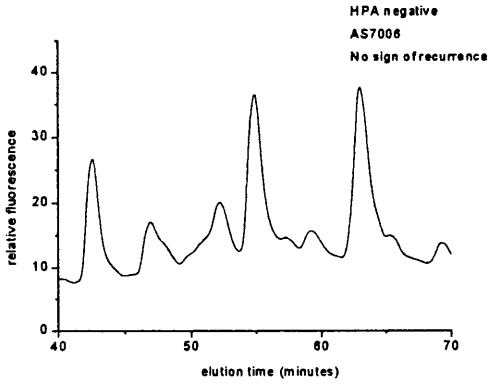

HPA positive
WT059
No sign of recurrence
relative fluorescence
elution time (minutes)

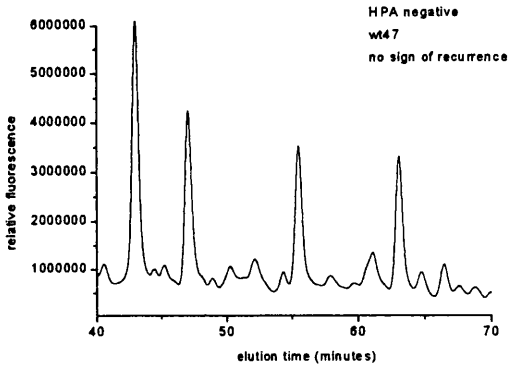

HPA positive
Auto2004
no sign of
recurrence
relative fluorescence
elution time (minutes)

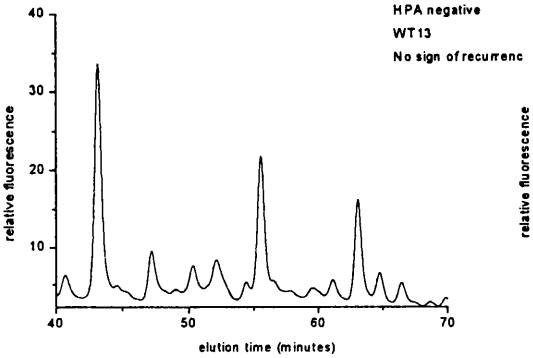

wt4
no sign of
recurrence
relative fluorescence
elution time (minutes)

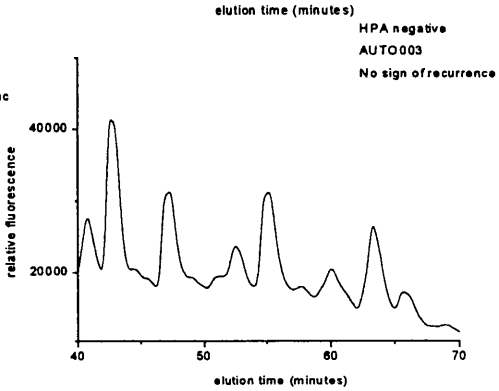

HPA negative
AS4016GRI
No sign of recurrenc
94 months
relative fluorescence
elution time (minutes)

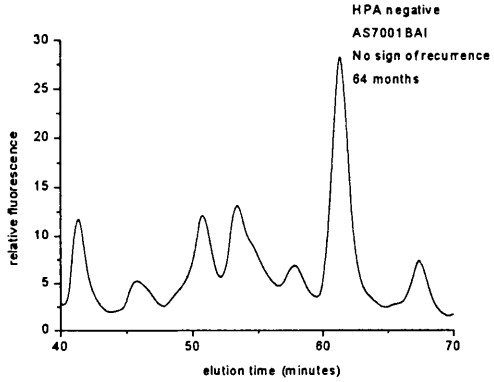

HPA negative
AS7019
No sign of recurrence
relative fluorescence
elution time (minutes)

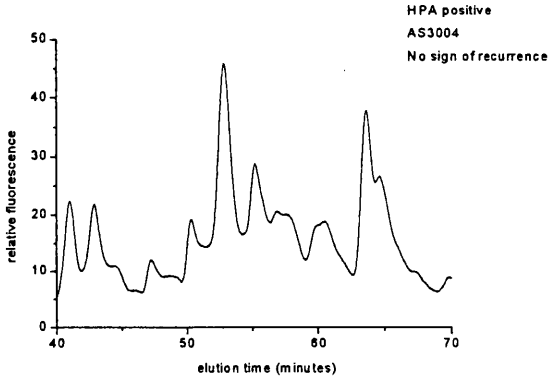

HPA negative
AS7006
No sign of recurrence
relative fluorescence
elution time (minutes)

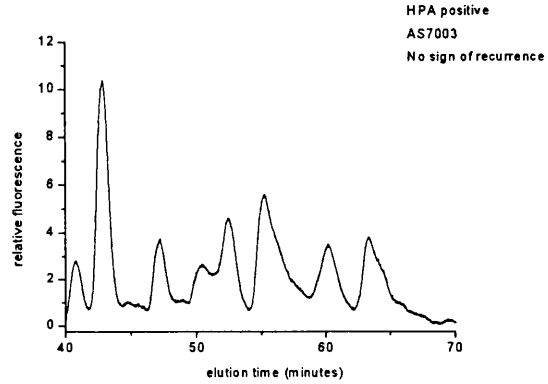

HPA negative
wt47
no sign of recurrence
relative fluorescence
elution time (minutes)

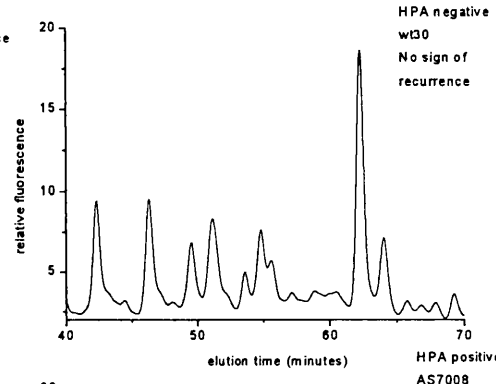

HPA negative
WT13
No sign of recurrenc
relative fluorescence
elution time (minutes)

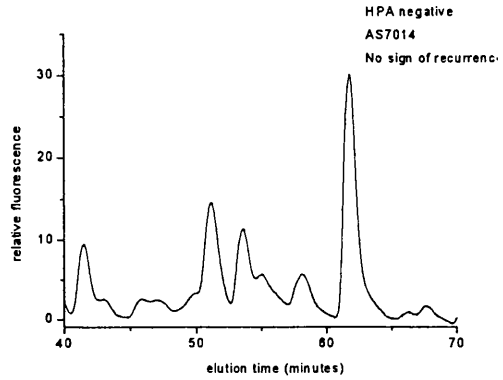

HPA negative
AUTO003
No sign of recurrence
relative fluorescence
elution time (minutes)

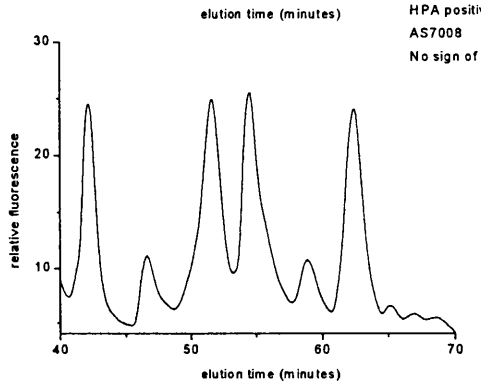

HPA negative
AS7001BAI
No sign of recurrence
64 months
relative fluorescence
elution time (minutes)

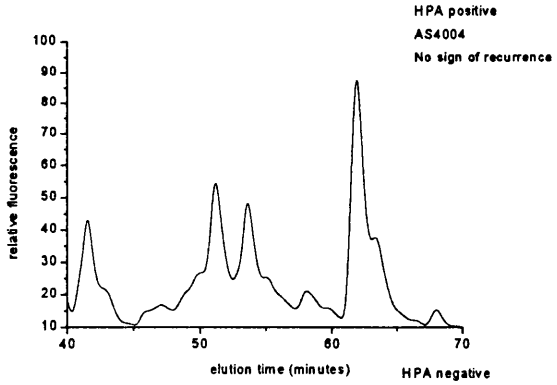

HPA positive
AS3004
No sign of recurrence
relative fluorescence
elution time (minutes)

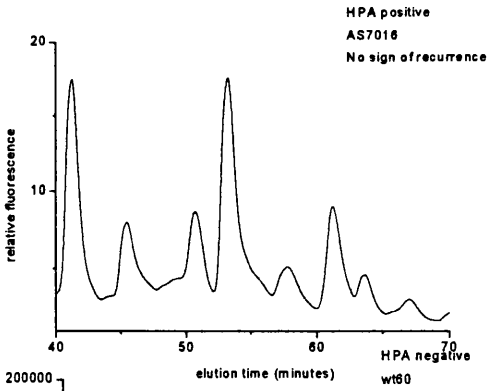

HPA positive
AS7003
No sign of recurrence
relative fluorescence
elution time (minutes)

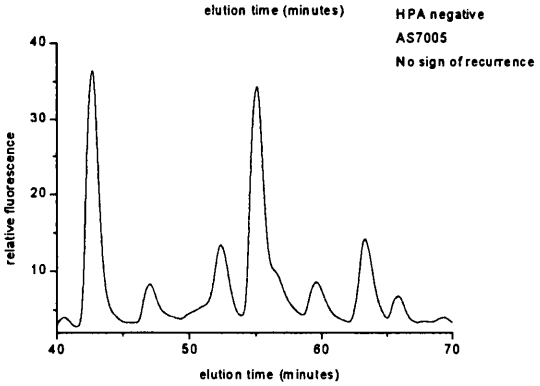

HPA negative
wt30
No sign of
recurrence
relative fluorescence
elution time (minutes)

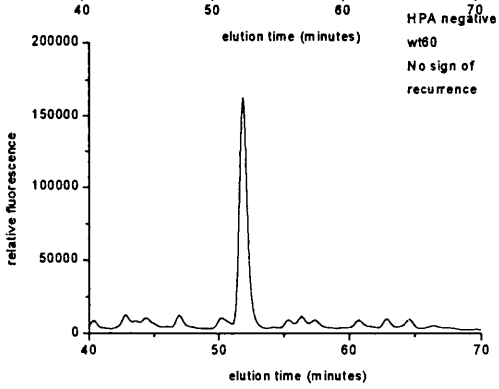

HPA negative
AS7014
No sign of recurrenc
relative fluorescence
elution time (minutes)

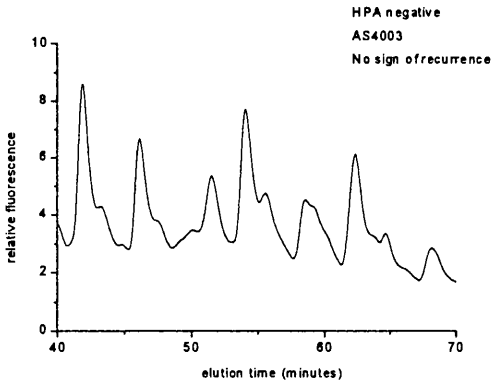

HPA positive
AS7008
No sign of recurrence
relative fluorescence
elution time (minutes)

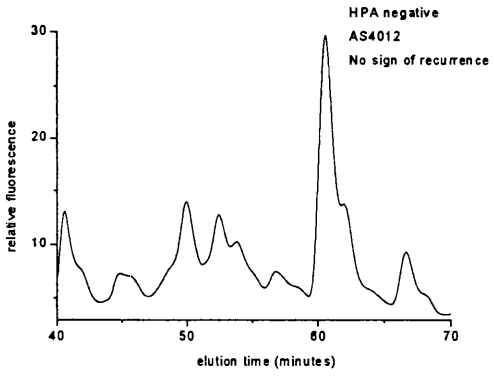

HPA positive
AS4004
No sign of recurrence
relative fluorescence
elution time (minutes)

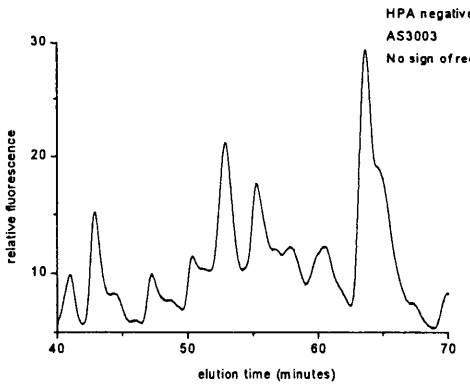

HPA positive
AS7016
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
AS7005
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
wt60
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
AS4003
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
AS4012
No sign of recurrence
relative fluorescence
elution time (minutes)


HPA negative
AS3003
No sign of recurrence
relative fluorescence
elution time (minutes)

Appendix 8

The relative quantities of oligosaccharides C and D extracted from the different breast cancer specimens.

Patient / Run code	Status at follow-up	%1.37GU	%1.55GU	%1.83GU	%2.17GU	SUM C
	patient deceased	n	0.03	n	0.26	0.29
AS7 028	patient deceased	n	12.07	1.96	9.57	23.60
AS7 011	loco regional and distant	1.06	8.19	3.61	8.01	20.87
AS7 007	patient deceased	2.42	9.40	1.64	7.09	20.55
AS7 021	distant recurrence	n	n	n	n	0.00
AS7 012	patient deceased	2.53	17.60	2.16	6.08	28.37
WT 43	loco regional recurrence	0.15	0.91	n	2.90	3.96
AUTO2 007	patient deceased	11.58	n	n	3.60	15.18
AUTO 002	patient deceased	8.62	n	0.30	n	8.92
AS7 027	loco regional and distant	1.49	9.52	3.87	5.51	20.39
AS7 023	loco regional recurrence	1.25	2.39	19.15	4.88	27.67
AS7 025	patient deceased	6.06	n	n	0.44	6.50
AS4 018	loco regional and distant	n	2.96	3.38	n	6.34
AS4 008	loco regional recurrence	7.94	0.44	0.46	n	8.84
WT 33	patient deceased	0.06	1.49	3.74	0.20	5.49
WT 46	distant recurrence	11.29	n	2.13	1.03	14.45
AS7 022	loco regional recurrence	0.78	7.08	1.44	4.47	13.77
WT 41	patient deceased	n	0.10	n	1.55	1.65
AUTO 004	loco regional recurrence	8.19	n	n	n	8.19
WT 50	patient deceased	5.99	n	3.00	1.03	10.02
AUTO 005	patient deceased	6.00	n	n	n	6.00
WT 29	patient deceased	0.06	n	0.21	0.61	0.88
WT 37	patient deceased	n	n	n	1.36	1.36
AS4 015	patient deceased	n	n	n	3.97	3.97
AS4 005	loco regional recurrence	0.06	n	n	n	0.06
WT 003	patient deceased	20.42	n	0.33	0.60	21.35
WT 44	patient deceased	10.24	0.04	0.54	1.56	12.38
AS7 004	patient deceased	5.00	5.80	1.40	0.35	12.55
WT 27	patient deceased	0.58	0.25	0.17	8.65	9.65
WT 36	patient deceased	n	n	n	2.70	2.70
AS4 007	loco regional recurrence	n	0.89	n	1.08	1.97
WT 55	loco regional recurrence	n	31.89	1.02	4.22	37.13
AS4 014	patient deceased	n	n	n	0.76	0.76
WT 23	patient deceased	n	n	0.09	n	0.09
AS7 024	distant recurrence	0.51	n	6.29	n	6.80
AS4 002	loco regional recurrence	1.26	4.87	3.99	3.24	13.36
WT 31	patient deceased	n	0.32	0.07	2.17	2.56
AUTO 008	patient deceased	12.32	n	n	n	12.32
AS3 005	patient deceased	n	2.15	1.56	6.25	9.96
AS7 030	patient deceased	n	6.96	4.46	n	11.42
	mean	5.03	5.70	2.68	3.25	10.31
	sd	5.24	7.51	3.81	2.77	8.99
	2sd	10.48	15.01	7.63	5.54	17.98
	mean-2sd	-5.45	-9.32	-4.95	-2.29	-7.68
	mean+2sd	15.52	20.71	10.31	8.78	28.29

Patient / Run code	Status at follow-up	%1.37GU	%1.55GU	%1.83GU	%2.17GU	SUM C
AS3 004	no sign of recurrence	n	5.31	1.79	16.88	23.98
AS7 014	no sign of recurrence	n	5.89	0.75	10.89	17.53
AS4 010	no sign of recurrence	n	5.41	4.61	2.40	12.42
AUTO2 006	no sign of recurrence	10.83	n	0.92	6.50	18.25
AUTO2 005	no sign of recurrence	26.66	0.10	n	14.42	41.18
AS4 004	no sign of recurrence	1.63	n	1.90	n	3.53
NICKI 40	no sign of recurrence	n	23.11	0.45	4.98	28.54
WT 47	no sign of recurrence	16.37	0.50	0.83	0.77	18.47
WT 30	no sign of recurrence	n	0.27	n	1.29	1.56
WT 13	no sign of recurrence	17.17	2.52	n	0.60	20.29
AS7 008	no sign of recurrence	1.06	11.20	3.91	14.38	30.55
AUTO 007	no sign of recurrence	22.78	0.13	1.69	n	24.60
AUTO2 004	no sign of recurrence	7.32	5.48	3.15	5.43	21.38
AS4 011	no sign of recurrence	1.77	3.79	1.37	n	6.93
AS4 016	no sign of recurrence	n	n	n	6.33	6.33
AUTO 006	no sign of recurrence	0.01	n	n	6.21	6.22
AUTO2 002	no sign of recurrence	8.80	n	0.20	1.70	10.70
AS7 016	no sign of recurrence	3.95	17.41	6.36	5.68	33.40
AS7 005	no sign of recurrence	n	28.12	3.47	7.24	38.83
AS7 009	no sign of recurrence	n	11.70	5.18	3.82	20.70
AS7 017	no sign of recurrence	2.67	n	n	n	2.67
WT 060	no sign of recurrence	n	0.80	n	0.61	1.41
AS7 019	no sign of recurrence	5.57	10.73	4.14	0.54	20.98
WT 059	no sign of recurrence	6.58	0.30	0.15	2.05	9.08
AS4 006	no sign of recurrence	11.43	3.36	7.10	0.82	22.71
AS7 003	no sign of recurrence	2.54	8.94	2.25	0.82	14.55
WT 4	no sign of recurrence	11.64	0.09	1.80	6.19	19.72
AUTO 003	no sign of recurrence	11.21	0.04	0.05	n	11.30
AS4 003	no sign of recurrence	1.73	9.87	n	0.64	12.24
AS7 013	no sign of recurrence	0.33	14.57	4.16	10.61	29.67
AS4 013	no sign of recurrence	n	8.41	3.56	4.99	16.96
AS 005	no sign of recurrence	0.53	3.45	3.60	6.43	14.01
AS4 012	no sign of recurrence	n	10.10	3.69	5.18	18.97
AS3 003	no sign of recurrence	5.27	7.10	5.39	11.43	29.19
AS4 019	no sign of recurrence	0.14	8.04	3.15	9.87	21.20
AS7 006	no sign of recurrence	0.14	12.30	6.60	7.94	26.98
	mean	7.13	7.30	2.94	5.73	18.25
	sd	7.37	6.95	2.05	4.57	10.18
	2sd	14.75	13.91	4.10	9.15	20.35
	mean-2sd	-7.62	-6.60	-1.16	-3.42	-2.10
	mean+2sd	21.87	21.21	7.03	14.88	38.60

Patient / Run code	Status at follow-up	%2.75GU	%2.88GU	%3.05GU	SUM D
	patient deceased	n	1.22	0.22	1.44
AS7 028	patient deceased	10.95	n	9.13	20.08
AS7 011	loco regional and distant	n	28.49	n	28.49
AS7 007	patient deceased	1.38	n	5.38	6.76
AS7 021	distant recurrence	19.96	n	2.71	22.67
AS7 012	patient deceased	2.78	n	15.94	18.72
WT 43	loco regional recurrence	22.36	0.49	0.31	23.16
AUTO2 007	patient deceased	n	12.32	n	12.32
AUTO 002	patient deceased	n	0.80	n	0.80
AS7 027	loco regional and distant	7.54	n	6.58	14.12
AS7 023	loco regional recurrence	13.36	n	6.26	19.62
AS7 025	patient deceased	3.26	n	18.49	21.75
AS4 018	loco regional and distant	2.96	n	32.00	34.96
AS4 008	loco regional recurrence	24.43	n	n	24.43
WT 33	patient deceased	2.30	30.95	1.49	34.74
WT 46	distant recurrence	13.94	2.79	0.36	17.09
AS7 022	loco regional recurrence	2.20	n	3.29	5.49
WT 41	patient deceased	17.66	0.81	1.20	19.67
AUTO 004	loco regional recurrence	n	17.29	n	17.29
WT 50	patient deceased	14.99	4.23	0.30	19.52
AUTO 005	patient deceased	1.78	n	n	1.78
WT 29	patient deceased	8.78		4.79	13.57
WT 37	patient deceased	0.14	20.98	0.24	21.36
AS4 015	patient deceased	42.51	n	0.29	42.80
AS4 005	loco regional recurrence	0.15	0.03	0.19	0.37
WT 003	patient deceased	8.82	0.34	0.79	9.95
WT 44	patient deceased	19.26	0.20	1.12	20.58
AS7 004	patient deceased	3.86	n	27.77	31.63
WT 27	patient deceased	3.63	40.82	n	44.45
WT 36	patient deceased	26.38	0.40	0.30	27.08
AS4 007	loco regional recurrence	2.99	0.22	0.45	3.66
WT 55	loco regional recurrence	1.19	n	1.05	2.24
AS4 014	patient deceased	21.78	n	n	21.78
WT 23	patient deceased	0.65	6.34	0.06	7.05
AS7 024	distant recurrence	7.43	n	1.72	9.15
AS4 002	loco regional recurrence	3.63	n	1.93	5.56
WT 31	patient deceased	0.13	24.42	0.16	24.71
AUTO 008	patient deceased	n	20.00	n	20.00
AS3 005	patient deceased	4.11	0.86	7.77	12.74
AS7 030	patient deceased	20.84	n	11.04	31.88
	mean	9.95	10.19	5.27	17.89
	sd	10.03	12.67	8.09	11.37
	2sd	20.07	25.35	16.18	22.75
	mean-2sd	-10.12	-15.16	-10.91	-4.86
	mean+2sd	30.01	35.54	21.45	40.63

Patient / Run code	Status at follow-up	%2.75GU	%2.88GU	%3.05GU	SUM D
AS3 004	no sign of recurrence	7.91	7.76	0.97	16.64
AS7 014	no sign of recurrence	n	29.12	n	29.12
AS4 010	no sign of recurrence	6.62	n	2.30	8.92
AUTO2 006	no sign of recurrence	27.21	n	n	27.21
AUTO2 005	no sign of recurrence	6.34	n	4.19	10.53
AS4 004	no sign of recurrence	0.22	40.89	n	41.11
NICKI 40	no sign of recurrence	n	n	n	0.00
WT 47	no sign of recurrence	8.48	1.13	1.84	11.45
WT 30	no sign of recurrence	13.64	3.61	0.56	17.81
WT 13	no sign of recurrence	8.01	n	1.95	9.96
AS7 008	no sign of recurrence	2.93	14.77	n	17.70
AUTO 007	no sign of recurrence	n	18.58	n	18.58
AUTO2 004	no sign of recurrence	6.50	n	n	6.50
AS4 011	no sign of recurrence	2.73	2.83	n	5.56
AS4 016	no sign of recurrence	0.56	0.41	0.60	1.57
AUTO 006	no sign of recurrence	n	21.98	n	21.98
AUTO2 002	no sign of recurrence	10.46	n	n	10.46
AS7 016	no sign of recurrence	8.01	1.93	1.68	11.62
AS7 005	no sign of recurrence	3.98	n	9.87	13.85
AS7 009	no sign of recurrence	3.82	28.76	n	32.58
AS7 017	no sign of recurrence	n	9.83	n	9.83
WT 060	no sign of recurrence	0.05	1.06	0.37	1.48
AS7 019	no sign of recurrence	4.42	n	9.18	13.60
WT 059	no sign of recurrence	23.31	1.50	0.25	25.06
AS4 006	no sign of recurrence	0.62	2.51	n	3.13
AS7 003	no sign of recurrence	3.26	n	3.68	6.94
WT 4	no sign of recurrence	3.01	1.55	0.97	5.53
AUTO 003	no sign of recurrence	3.30	7.02	1.76	12.08
AS4 003	no sign of recurrence	9.52	7.44	0.65	17.61
AS7 013	no sign of recurrence	8.42	10.22	1.07	19.71
AS4 013	no sign of recurrence	n	38.04	n	38.04
AS 005	no sign of recurrence	5.29	11.95	0.30	17.54
AS4 012	no sign of recurrence	1.17	18.32	0.89	20.38
AS3 003	no sign of recurrence	6.38	13.46	n	19.84
AS4 019	no sign of recurrence	3.14	n	14.72	17.86
AS7 006	no sign of recurrence	1.92	n	18.14	20.06
	mean	6.37	12.28	3.62	15.61
	sd	6.14	11.95	5.04	9.81
	2sd	12.27	23.91	10.07	19.62
	mean-2sd	-5.90	-11.63	-6.46	-4.02
	mean+2sd	18.65	36.19	13.69	35.23

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