ALPHA-1-ACID GLYCOPROTEIN AS A BIOMARKER FOR EARLY BREAST CANCER

Submitted by

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.

Signed:

Date:

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Abstract

In spite of major advances in detection and treatment, deaths from breast cancer are still high therefore there is a very definite requirement for the identification of a breast cancer specific biomarker to indicate the onset of the disease. We hypothesise that alpha-1-acid glycoprotein (AGP), which is a common constituent in all blood, could be a diagnostic marker for early breast cancer. AGP is present in all plasma and is extensively glycosylated with oligosaccharide chains covalently attached to the protein backbone. During several physiological and pathological conditions, not only does the total concentration of AGP increase two to five fold, but the relative proportions of the normal glycoforms or oligosaccharide "fingerprint" changes, as well as an increased expression of abnormal glycoforms. The discovery of a novel breast cancer biomarker would significantly advance the early detection and treatment of breast cancer and improve survival rates for the disease.

Plasma samples from normal, benign breast disease and breast cancer populations were obtained. AGP was isolated by low pressure chromatography and the differences in AGP glycosylation between and within the patient and control populations was determined by high pH anion-exchange chromatography. The monosaccharide composition of the AGP oligosaccharide chains was found to differ between normal, benign and cancer groups. Of all the samples containing fucose, the breast cancer group displayed the highest average level of this monosaccharide compared to the normal and benign breast disease group. Both the benign and breast cancer groups had N-acetylgalactosamine present; this monosaccharide is not usually found in AGP and was completely absent from the normal samples analysed in the study. The AGP oligosaccharide profiles differed between the normal, benign and breast cancer groups. There was a progressive shift towards greater expression of bi-sialylated chains and a reduction in the overall branching of the chains.

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Abbreviations

AA	amino acid
AAL	Aleuria aurantia Lectin
AAT	α_1-antitrypsin
AGP	α_1-acid glycoprotein
APP	acute phase proteins
APR	acute phase response
Asn	asparagine
BC	breast cancer
bFGF	basic fibroblast growth factor
BRCA-1	breast cancer-1
BRCA-2	breast cancer-2
CAIE	crossed affinity immunoelectrophoresis
CDG	congenital disorder of glycosylation
CEA	carcinoembryonic antigen
CM	carboxymethyl
Con A	Concanavalin A
CRP	C-reactive protein
DEAE	diethylaminoethyl
DCIS	ductal carcinoma in situ
Dol-P	dolichol pyrophosphate
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ER	endoplasmic reticulum
FDA	Food and Drug Administration

FFDM	full field digital mammography
FISH	fluorescent in situ hybridization
FNA	fine needle aspiration
Fuc	fucose
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HER-2/neu	human epidermal growth factor receptor 2
HPAEC	high pH anion-exchange chromatography
HPLC	high performance liquid chromatography
HSA	human serum albumin
IEF	isoelectric focusing
IFN-γ	interferon-gamma
IHC	immunohistochemistry
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IS	internal standard
KCl	potassium chloride
KSCN	potassium thiocyanate
LCIS	lobular carcinoma in situ
LN	lymph nodes
LPS	lipopolysaccharide

Man	mannose
M-CSF	macrophage-colony stimulating factor
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MUC-1	mucin 1
NaCl	sodium chloride
NAF	nipple aspirate fluid
NaOAc	sodium acetate
NaOH	sodium hydroxide
NeuAc	N-acetylneuraminic acid
NH_2	amino group
OH	hydroxyl
OR	oestrogen receptor
OST	oligosaccharyltransferase
PED	pulsed electrochemical detection
PEG	polyethylene glycol
PHA	mitogens phytohemagglutinin
pI	isoelectric point
PNGase F	Peptide-N-Glycosidase F
PR	progesterone receptor
PSA	prostate-specific antigen
PWM	pokeweed mitogen
R	retained
R^2	Pearson correlation coefficient
RA	rheumatoid arthritis

RCP	riboflavin carrier protein
rtPA	recombinant tPA
RT-PCR	reverse transcription polymerase chain reaction
SA	sialic acid
SAA	Serum amyloid A
S.D	standard deviation
SDS	sodium dodecyl sulphate
Ser	serine
SLe^x	Sialyl Lewis X
STn	Sialyl Tn
TAA	tumour-associated antigens
TFA	trifluoroaceteic acid
Thr	threonine
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumour necrosis factor-alpha
TNM	tumour-node-metastasis
tPA	tissue plasminogen activator
TPA	tissue polypeptide antigen
TPS	tissue polypeptide-specific antigen
UR	unretained
WR	weakly retained
VEGF	vascular endothelial growth factor

Publications

Refereed Journals

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Conference Posters

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CHAPTER 1

INTRODUCTION

1.1 Glycosylation

1.1.1 Introduction to Glycobiology

Today, there is a vast array of research targeted towards all aspects of glycosylation with an emphasis on its involvement in disease, but the discipline itself is relatively new when compared to the investigation of proteins and the genes which code for them. Sugar chains, also referred to as oligosaccharides or glycans were long thought to be an unimportant modification, merely a decoration of the underlying protein. Of the few who did recognise their importance, their apparent complexity when compared to that of DNA and protein chemistry made them secondary routes of research in many cases. The development of new technology capable of studying the intricacies of sugar chains enabled the scientific world to develop a greater understanding of these molecules and allowed investigation into their influence on protein function. The discipline flourished and sugar chains finally gained the respect and recognition they deserved. In 1988, the field of science concerned with determining the structure, biosynthesis and biological function of sugar chains was termed ‘glycobiology’ by Rademacher *et al*.

Oligosaccharides exist mainly in covalent association with proteins or lipids generating a variety of glycoconjugates in the form of glycoproteins and glycolipids respectively. These glycoconjugates have varying functions and some classes include enzymes, hormones, adhesion molecules, immunoglobulin’s and transport proteins. This modification of proteins and lipids is termed glycosylation and is a co/post translational process, the biosynthesis of which occurs in the endoplasmic reticulum (ER) and the Golgi apparatus (Kornfeld and Kornfeld, 1985).

Glycosylation occurs mainly on the surface of glycoconjugates and in some cases can significantly contribute to the mass of the molecule (Rademacher *et al*., 1988) with for example, the oligosaccharides of alpha-1-acid glycoprotein (AGP) accounting for 45% of its overall molecular weight. A modification with such an imposing presence could not simply be there as a consequence of chance. This observation lead to an array of work targeted at determining the functional significance of the oligosaccharide chains of glycoconjugates (Varki, 1993). Various techniques have been employed to study their function including the use of glycosylation inhibitors such as tunicamycin (Olden *et al*., 1979) and the development of mouse models with defective glycosylation machinery (Taniguchi *et al*., 2006).

In general a number of intra- and intermolecular properties such as solubility, conformation stability, circulatory lifespan and immune modulation have been attributed to the presence of the oligosaccharide chains of glycoconjugates (Lis and Sharon, 1993). Studies on congenital disorders of glycosylation (CDG) have provided strong evidence that the modification is essential for normal mammalian development (see Jaeken and Matthijs, 2007 for a recent review). CDG, previously termed carbohydrate-deficient glycoprotein syndromes, are inherited disorders involving defective processing of glycosylation. There are many different forms of CDG relating to varying processing defects but the predominant clinical manifestation of each are that of central nervous system abnormalities.

Glycosylation is a secondary gene product regulated by glycosyltranseferases, resident membrane proteins of the Golgi apparatus and ER (Paulson and Colley, 1989). Different tissue and cell types have varying complements of these enzymes resulting in the production of a number of glycoforms of a particular glycoconjugate. In the case of glycoproteins these glycoforms have the same amino acid sequence but vary with respect to the oligosaccharide chains attached to the polypeptide backbone (Rademacher *et al*., 1988, Lis and Sharon, 1993). This variation in glycosylation is

referred to as heterogeneity and the repertoire of glycoforms produced by glycoconjugates in tissues or organisms is known as the glycome.

Glycobiology is a rapidly growing field in biomedical science, one which may lead to new approaches in the diagnosis and prognosis of human diseases. The emergence of glycotechnology and the exploitation of glycosylation in the production of recombinant glycoproteins have allowed these glycoconjugates to become major targets of therapeutics. The first industrially produced recombinant glycoprotein targeted for clinical use was the glycoprotein hormone erythropoietin (EPO) used in the treatment of anaemia resulting from renal failure (Eschbach *et al.*, 1989, Adamson and Eschbach, 1989). The thrombolytic agent tissue plasminogen activator (tPA) was also discovered to be effective in the clinical setting with recombinant tPA (rtPA) used to prevent blood clots following myocardial infarction and in the treatment of strokes (Williams et al., 1986).

Due to the heterogeneous nature of glycoproteins and the expression of disease specific glycoforms, the oligosaccharide chains themselves have been extensively studied over the years as potential prognostic and diagnostic indicators of disease (Roberts *et al*., 1975, Moule *et al*., 1987, Turner and Goodarzi, 1998).

In the chapters to follow I hope to introduce the various aspects of glycoconjugate biochemistry and synthesis with an intricate discussion of the glycoprotein class with an emphasis on N-linked glycoproteins, discuss in detail the structure and function of the serum glycoprotein alpha-1-acid glycoprotein (AGP), give an overview of breast cancer detailing current diagnostic techniques and most importantly, present data supporting the clinical relevance of AGP with regards to the detection and management of breast cancer.

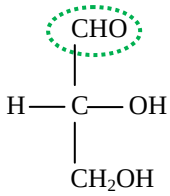
1.1.2 Oligosaccharides

Oligosaccharide chains are ordered structures composed of monosaccharides, the simplest monomer unit. Monosaccharides generally have the empirical formula $(CH_2O)_n$ where n can range anywhere from 3 to 9. Most simple monosaccharides can be grouped into two general classes, either the aldoses containing the (-CH=O) aldehyde group, or the ketoses with the (>C=O) functional ketone group (Manzi and van Halbeek, 1999). Figure 1.1a illustrates the structure of glyceraldehyde, the simplest aldose and of dihydroxyacetone, the simplest ketose. The number of carbon atoms in any given monosaccharide determines the name it is given. In the case of glycoconjugates, the most common constituents of the oligosaccharide chains contain 6 carbon atoms and are called hexoses (Taylor and Drickamer, 2003).

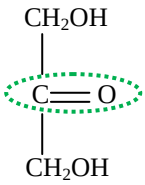
Mannose (Man), galactose (Gal), glucose (Glc) and fucose (Fuc) are common hexoses found in oligosaccharide chains, although fucose is technically termed a deoxyhexose since it does not have a hydroxyl group on carbon 6. Two possible configurations of the monosaccharide exist and are termed either D- or L- depending on the orientation of the CHOH group furthest from the functional aldehyde group with those in the D- conformation most commonly found in nature. The linear structures or Fischer projections of Man, Gal and Glc are presented in Figure 1.1b.

Glucosamine and galactosamine are hexosamines and differ in structure to their hexose counterpart by the addition of an amino group on carbon 2. In glycoconjugates these hexosamines are commonly N-acetylated to form N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) respectively. N-acetyl neuraminic acid (NeuAc) is a nine-carbon sugar acid from a family of sialic acids and is also a constituent of oligosaccharide chains (Varki, 1992).

a)

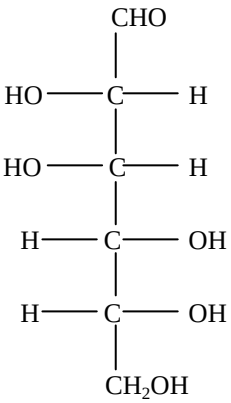


Glyceraldehyde

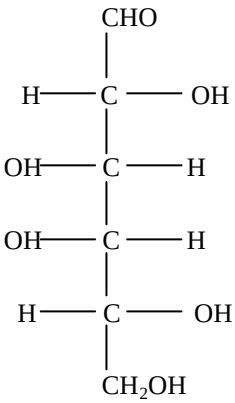


Dihydroxyacetone

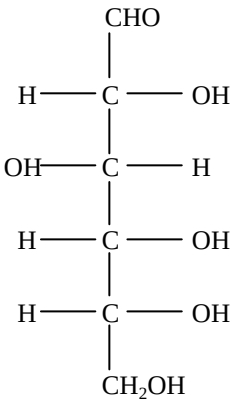
b)



D-mannose



D-galactose



D-glucose

Figure 1.1 a) Structure of simplest aldose, glyceraldehyde and simplest ketose, dihydroxyacetone with the corresponding aldehyde and ketone functional groups circled in green. b) Fischer projections or linear structures of common monosaccharides in the D- conformation.

The hexoses found in glycoconjugates do not exist in nature as linear structures with free aldehyde groups as detailed in Figure 1.1b. The more common structure is that of a 6 membered ring formed by the reaction of the hydroxyl group of carbon 5 with the aldehyde group of carbon 1, creating a stable cyclic hemiacetal. Figure 1.2 illustrates the ring structure of the common monosaccharides found in glycoconjugates. In this formation, carbon 1 becomes a chiral centre and is referred to as the anomeric carbon which is capable of two conformations termed alpha (α) when the hydroxyl (OH) group is below the plane of the ring and beta (β) when the OH group is above.

Monosaccharides join together to form oligosaccharides via glycosidic bonds. This reaction involves the reducing group of the anomeric carbon of one monosaccharide and any OH group from another monosaccharide with the elimination of a water molecule (Figure 1.3). The generation of oligosaccharides can provide huge structural diversity with not only differences in anomery (α, β) but also in the position of the linkages e.g. (β1, 4), (α1, 6). Since monosaccharides can form more than one glycosidic linkage, further structural diversity of oligosaccharides can be achieved through the formation of branched structures.

Glycoproteins are capable of containing oligosaccharides with 2, 3 and 4 branches termed bi-, tri- and tetra-antennary respectively. The huge variation available to oligosaccharides means that relatively few monosaccharides are required to generate a vast number of glycan structures (Lis and Sharon, 1993).

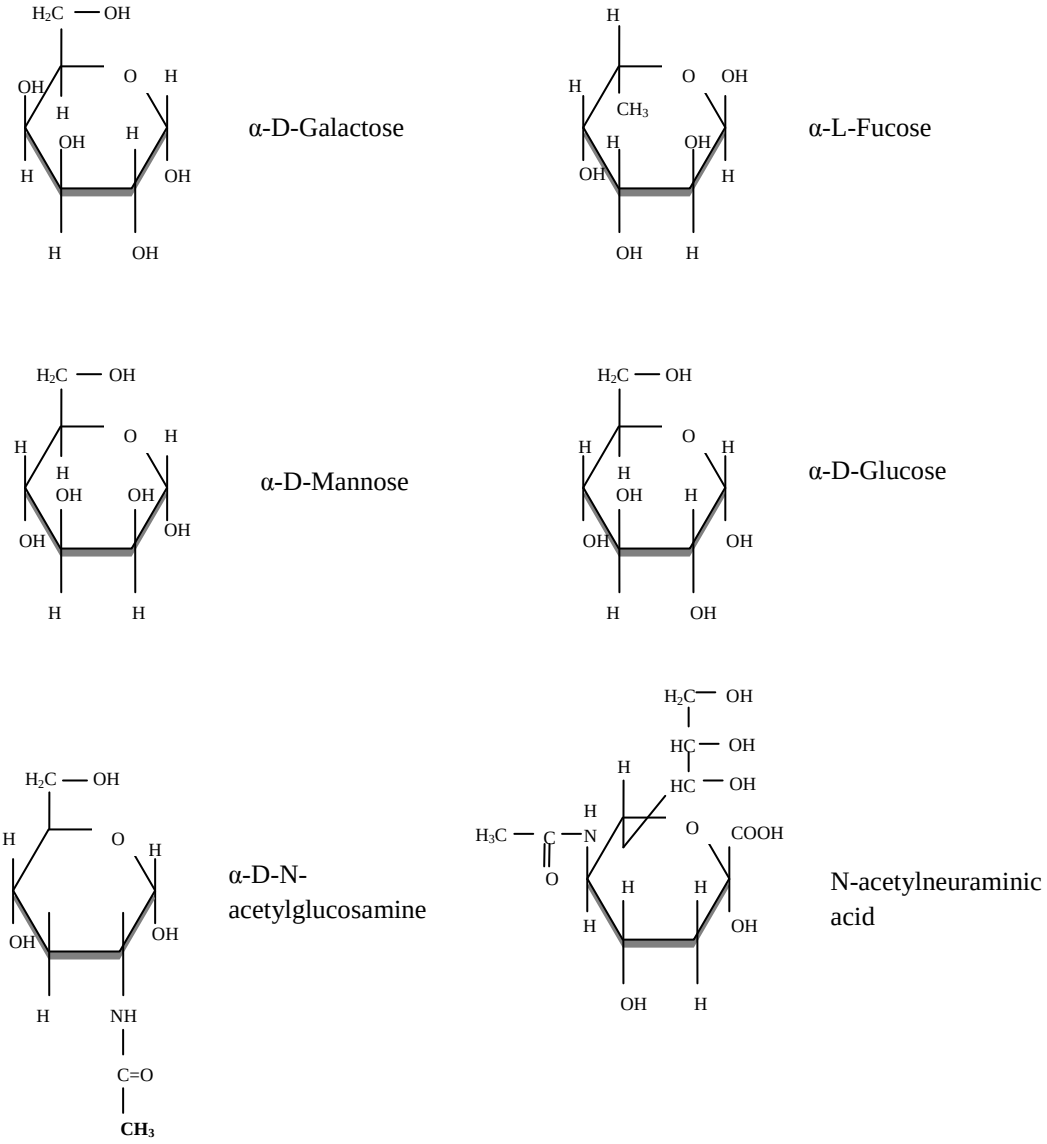


Figure 1.2 Ring structures of the common monosaccharide constituents of glycoconjugates.

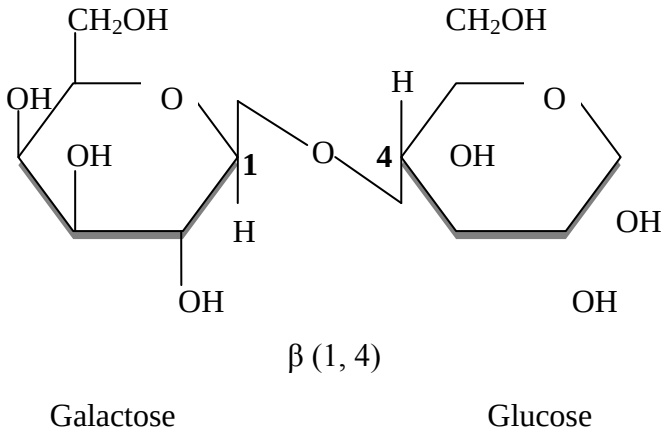


Figure 1.3 β (1, 4) glycosidic bond formation between galactose and glucose.

1.1.3 Glycoproteins

As mentioned previously, glycoproteins are formed by the covalent attachment of oligosaccharides or glycan chains to the polypeptide backbone of proteins. The addition of a large glycan to a protein backbone can dramatically change the structure and consequently the overall function of the respective protein (Imperiali and O'Connor, 1999). They also have the ability to play a major role in the protein-folding pathway allowing stabilization of the protein to which they are attached (Wormald and Dwek, 1999).

Glycoproteins can be divided into two groups depending on how the oligosaccharide chains are attached to the protein backbone. N-linked glycans are attached via the amide nitrogen of asparagine (Asn) residues and O-linked glycans to serine/threonine (Ser/Thr) residues. The generation of N-linked glycoproteins is a complex procedure requiring not only available Asn residues, but the presence of a specific tripeptide sequence**.** O-linked glycan synthesis is far less complex and involves the initial attachment of GalNAc to the Ser/Thr in question without the prerequisite of a consensus sequence. Glycoproteins generated via the N-linked pathway are predominantly serum glycoproteins and those of O-linked are mainly of epithelial origin.

The glycan chains of glycoproteins can be exclusively N-linked as in the case of AGP and alpha-1-antitrypsin (AAT) or all O-linked as with interleukin-2 and granulocyte colony stimulating factor but it is possible for a combination of both to exist on the same protein. The glycoprotein hormone EPO contains 3 N-linked and 1 O-linked chain attached at amino acid positions 24, 38 and 83 for the N-linked chains and position 126 for the O-linked chain. A study by Dubé *et al*. (1988) demonstrated that all chains both N- and O-linked were essential for optimal biological activity of EPO.

Serum glycoprotein levels can fluctuate depending on the body's patho-physiological state allowing them to play an important role in the modern clinical environment by aiding the detection and management of a number of diseases. This fluctuation in serum concentration is achieved by either an increase or decrease in transcription of the gene coding for the protein and likewise, an increase or decrease in transcription of the genes coding for the glycosyltransferase enzymes required for their subsequent glycosylation. Table 1.1a details a number of these proteins, their principal function and the diseases and disorders with which they are associated. Tumours have the ability to secrete a number of hormones and enzymes into the circulation which can subsequently be detected in the sera of cancer patients. A number of these tumour markers are glycoproteins and Table 1.1b details a few routinely measured by clinical laboratories.

Changes in serum concentration are not the only clinically useful attribute of glycoproteins. The structure of their oligosaccharide chains have also been shown to change during various physiological and pathological states (Varki, 1999c); of interest are the disease-specific changes which may be of diagnostic and/or prognostic significance. When compared to healthy individuals, patients suffering from acute inflammation have demonstrated decreased branching of the glycan chains of the serum glycoprotein alpha-1-acid glycoprotein and an increase in branching among those exhibiting chronic inflammation conditions (Nicollet *et al*., 1981; Fassbender *et al*., 1991). Similarly, studies on patients with liver disease have discovered highly branched chains and an increase in the overall content of the monosaccharide Fuc (Anderson *et al.*, 2002; Mooney *et al*., 2006).

In disease such as cancer, glycoproteins can display tumour associated antigens (TAA), the expression of which are often associated with poor prognosis (Itzkowitz *et al*., 1989) and which may give an indication of the metastatic potential of a tumour (Kim and Varki, 1997). Of these TAAs a number are expressed on the heavily O-glycosylated mucin-type glycoproteins which reside on the surface of a variety of

Table 1.1 a) Serum glycoproteins currently measured in clinical practice to evaluate various diseases and disorders and their associated functions. b) Tumour markers routinely measured in clinical laboratories for the management of disease. (Beckett *et al*., 2005; Gaw *et al*., 2004).

a)

Glycoprotein	Function	Disease Investigation
α_1-antitrypsin (AAT)	Protease Inhibitor	Reduced levels in AAT deficiency
Prothrombin	Blood Clotting	Liver Function Test and Coagulation Screening
Ceruloplasmin	Copper Transport	Reduced levels in Wilson's disease
Haptoglobin	Binds Haemoglobin	Reduced levels in haemolytic disorders
Transferrin	Iron Transport	Assessment of iron status-deficiency/excess

b)

Tumour Marker	Associated Malignancy	Marker Properties
Carcinoembryonic antigen (CEA)	Colorectal Cancer	Prognostic, monitoring and response to treatment
CA-125	Ovarian Cancer	Response to treatment and disease progression
Prostate-specific antigen (PSA)	Prostate Cancer	Aid diagnosis and monitoring
CA 15-3	Breast Cancer	Prognostic, detect recurrent disease and monitoring treatment
Human chorionic gonadotropin (hCG)	Choriocarcinoma	Prognostic and response to treatment

blood group antigens, expression of the Lewis antigens (Le^a, Le^b, Le^x, Le^y) and an increase in the expression of terminal structures such as Sialyl Lewis X (Muramatsu, 1993; Nakagoe *et al*., 2002).

epithelial cells. The tumour specific nature of these glycoproteins assumes the form of truncated versions of the normal O-linked chains generating a number of different TAAs termed T, sialyl T, Tn and sialyl Tn (Brockhausen, 1999). Other TAAs with valuable prognostic information include the altered expression or loss of the ABH

The vast array of information that the serum glycoproteins offer with regards to disease ensure that they are still at the forefront of biomarker research. The majority of these proteins are N-linked therefore an in-depth discussion of this glycosylation pathway will follow.

1.1.3.1 N-Linked Glycosylation

The best understood pathway of protein glycosylation is that of N-linked biosynthesis, a highly conserved process in eukaryotic cells (Burda and Aebi, 1999). Rademacher *et al*. (1988) details N-glycosylation as a post-translational modification that serves to create discrete subsets (glycoforms) of a glycoprotein that have different physical and biochemical properties that may lead to functional diversity. The process of N-linked biosynthesis is a co/post translational event and the addition of glycan chains to a partially folded polypeptide can impact on, or even facilitate the protein-folding process (Imperiali and O'Connor, 1999, Mitra *et al*., 2006, Varki, 1993).

N-linked glycoproteins are formed by the covalent attachment of the amino group (-NH_2) of the Asn residue and the OH group of the first GlcNAc of the oligosaccharide chain (Figure 1.4). The major requirement for this attachment is the presence of the initial Asn residue in the consensus sequence Asn-X-Ser/ Thr (Hart *et al*., 1979). As may be expected, there are many potential glycosylation sites on any given polypeptide, but not all are glycosylated. Kornfeld and Kornfeld (1985) stated that only one third of N-linked consensus sequences are inevitably glycosylated since they must also be present in the correct conformation. Work by Beeley (1977) and Bause *et al*. (1982) utilised peptide studies and space-filling models to determine that the correct conformation was in fact a β turn or loop since this secondary structure allowed for a hydrogen bond interaction between the hydroxy amino acid (Ser or Thr) and the side chain of Asn. X in the tripeptide sequence represents any amino acid (AA) except proline. Studies involving proline containing peptides (Bause, 1983) discovered that glycosylation did not occur when this AA was present in the X position. Investigations with space filling models by the same group demonstrated that ineffective glycosylation in the presence of this AA might be due to its inability to adopt the correct conformation of a β turn or loop.

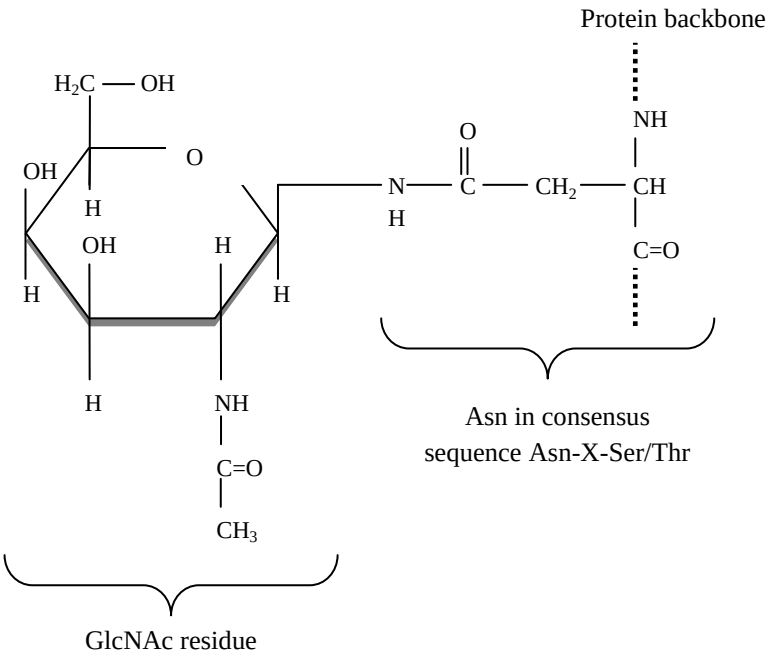


Figure 1.4 Diagram of a glycosidic bond formed between the amino group of an Asn residue and the hydroxyl group of the first GlcNAc residue of the oligosaccharide chain (adapted from Taylor and Drickamer, 2003; Hames and Hooper, 2000).

1.1.3.2 N-linked Biosynthesis

All N-linked glycoproteins have a common conserved core region of $Man_3GlcNAc_2$ but vary in respect to the outer chains generating three main glycoprotein structures, termed high mannose, hybrid and complex, as the pathway progresses. The biosynthetic pathway involved in the production of the pentasaccharide core and the cellular events which determine the final glycoprotein structure are outlined as follows.

The initiation of N-linked biosynthesis involves the production of a lipid-linked oligosaccharide precursor that is transferred *en bloc* to a growing polypeptide backbone in the ER (Tabas *et al*., 1978). The lipid in question is dolichol pyrophosphate (Dol-P) which acts as an anchor for the oligosaccharide precursor whilst it is being assembled and subsequently transferred to the Asn residues of the nascent polypeptide.

The initial stage in the formation of the lipid-linked precursor occurs on the cytosolic side of the ER and involves the addition of two GlcNAc residues by GlcNAc-1-phosphotransferase and GlcNAc-transferase from the monosaccharide donor UDP-GlcNAc. GDP-Man then acts as a donor for the sequential addition of five mannose residues. At this stage the $Man_5GlcNAc_2$-Dol-P flips across the membrane to the luminal side of the ER where a further four mannose residues are rapidly added utilising Dol-P-Man as a donor. Three Glc residues provided by Dol-P-Glc are added in the final stage of assembly producing the complete $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor (Li *et al*., 1978) after which transfer to the nascent protein can begin.

Transfer of the oligosaccharide from Dol-P to the Asn residues in the consensus sequence Asn-X-Ser/Thr is facilitated by a membrane protein complex termed the oligosaccharyltransferase (OST) which cleaves the initial GlcNAc-P bond and releases Dol-PP (Marth, 1999). After this transfer, the oligosaccharide is subject to a series of processing and trimming reactions.

Processing is initiated by the rapid removal of the outer two Glc followed minutes later by the removal of the third (Kornfeld *et al*., 1978). Glucosidase I removes the outermost Glc and glucosidase II removes the remaining two. This step is associated with protein folding with improperly folded proteins undergoing reglucosylation and refolding or degradation. Rapid folding of the protein at this stage may precede the access of processing enzymes and render a potential glycosylation site inactive (Taylor and Drickamer, 2003). The resulting oligosaccharide of $Man_9GlcNAc_2$-Asn is exposed to the action of the mannosidases which may remove some or all four α1, 2 linked Man residues. For some glycoproteins, processing events end here resulting in mannose rich glycans containing between five and nine Man. The production of the high mannose type glycoprotein is complete.

Removal of the first Man generating $Man_8GlcNAc_2$-Asn can initiate transfer of the growing glycoprotein to the *cis* Golgi via vesicles where it is destined for one of two biosynthetic routes. One such route targets the glycoprotein to the lysosomal compartment with the removal of the GlcNAc residues and subsequent production of Mannose-6-phosphate (Man-6-P). The alternative route involves removal of three Man residues ($Man_5GlcNAc_2$-Asn) and addition of a GlcNAc in the *medial* Golgi with the removal of a further two Man residues generating $GlcNAc_1Man_3GlcNAc_2$-Asn. Finally another GlcNAc can be added (and possibly a Fuc residue to the innermost GlcNAc; Marth, 1999) and the processing reactions leading to the development of complex-type glycoproteins can begin.

It is possible for further GlcNAc residues to be added in the medial Golgi generating a variety of branched structures. The enzymes necessary for elongation of the chains are present in the *trans* Golgi and transportation of the growing glycoprotein via vesicles to this region allows Gal and sialic acid (SA) residues to be added terminating the complex-type glycoprotein structure (Taylor and Drickamer, 2003) The glycoproteins then exit the Golgi and are transported to their site of action (Kornfeld and Kornfeld 1985). A diagrammatic representation of the N-linked biosynthesis of glycoproteins is presented in Figure 1.5.

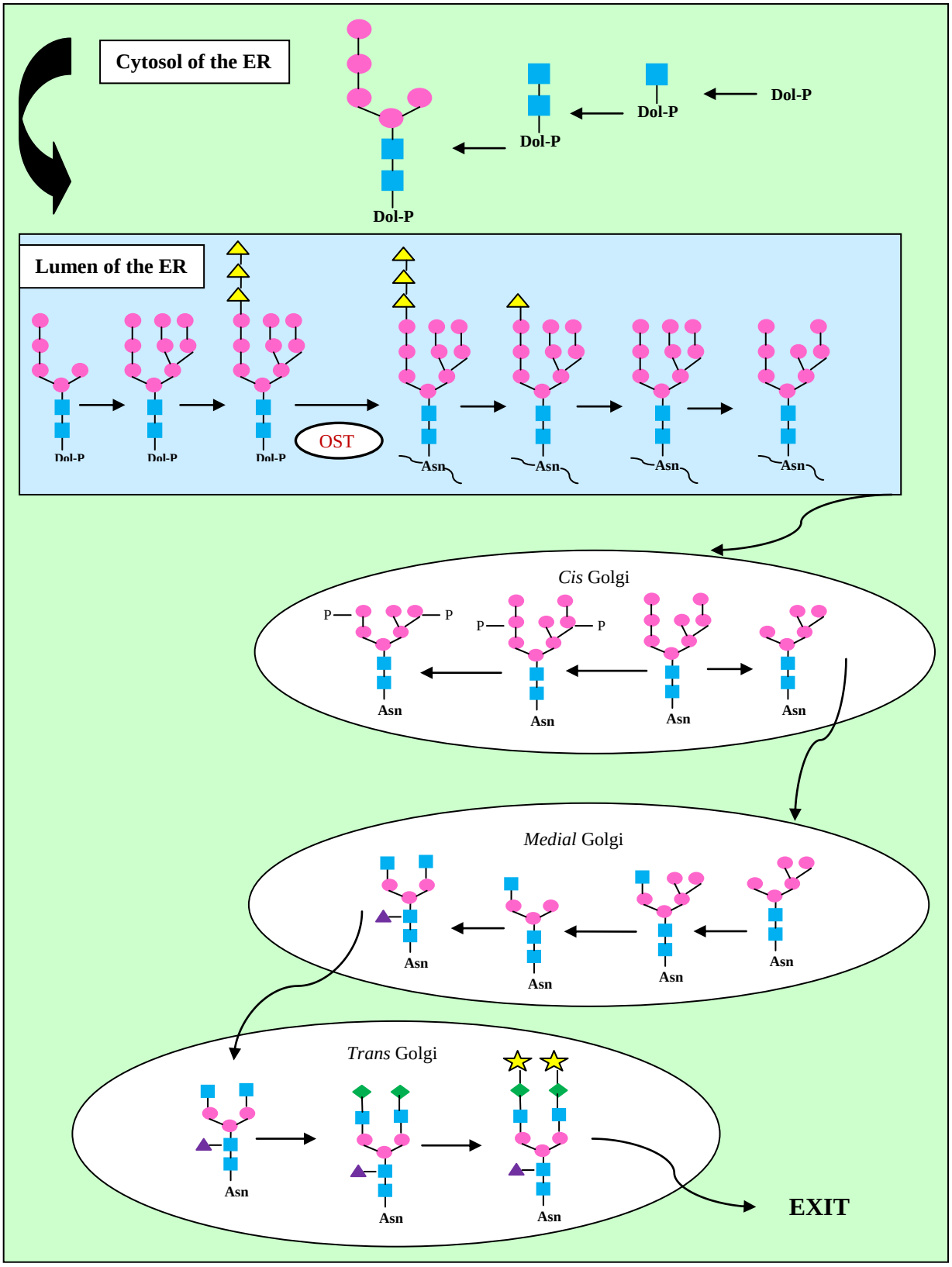


Figure 1.5 Pathway of N-linked glycoprotein glycosylation. GlcNAc Man Glc Gal N-acetylneuraminic acid (NeuAc) Fuc

1.1.3.3 Structure of N-linked Glycans

The N-linked biosynthetic pathway generates three distinct glycoprotein structures through a series of processing and trimming reactions in the ER and Golgi catalysed by a number of glycosyltransferase enzymes. These enzymes are typically grouped into families based on the type of sugar they transfer; galactosyltransferase catalyses the addition of Gal to GlcNAc residues of the oligosaccharide chain and sialyltransferases are responsible for terminating the chains with SA (Paulson and Colley, 1989). Figure 1.6 illustrates the structure of the high mannose, hybrid and complex-type glycoproteins highlighting the common pentasaccharide core of $Man_3GlcNAc_2$-Asn.

The outer chains of high mannose-type glycans consist of only Man residues in addition to the pentasaccharide core. No other monosaccharides are found in these chains therefore branch elongation is achieved through the attachment of an additional two to six α-linked Man residues. Complex-type glycans contain no Man residues other than the three present in the core. The addition of GlcNAc to the two outer Man residues of the developing glycan allows the production of branched structures; the greater the number of GlcNAc, the higher the number of branches. Bi-antennary glycans are the most abundant but tri- and tetra-antennary glycans are also very common features of complex glycans (Taylor and Drickamer, 2003). The extension of the branches can be achieved with the subsequent addition of Gal and the presence or absence of terminating sialic acid residues in the form of NeuAc. Other common structural features of complex glycans are represented by core fucosylation where a Fuc residue is attached to the first GlcNAc of the core (Marth, 1999) and of bisecting GlcNAc which can be found attached to the initial Man residue of the core.

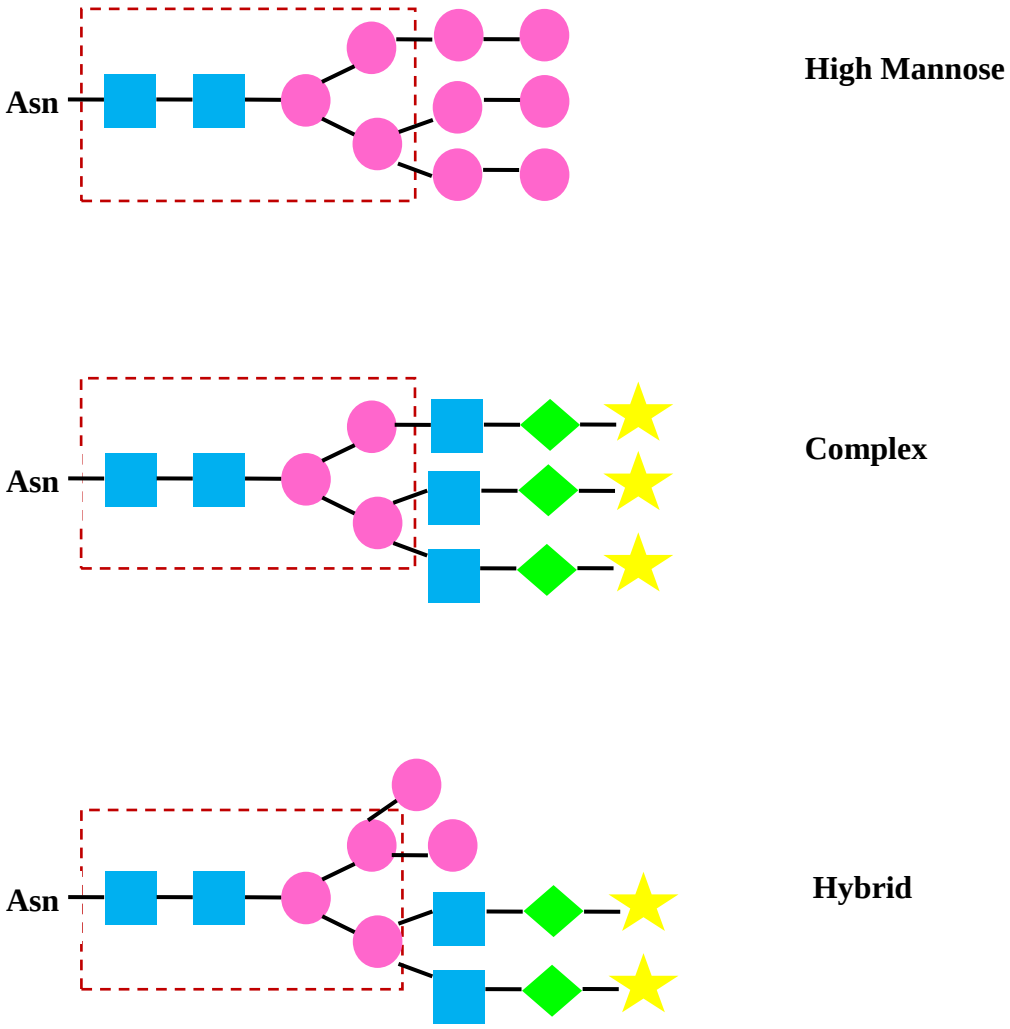


Figure 1.6 Structure of the three types of N-linked glycoprotein. The conserved pentasaccharide core is surrounding by the red box (Adapted from Kobata, 2000).

The hybrid –type glycans are so-called because they have characteristic features of both high-mannose and complex glycans, representing an intermediate between the two in the biosynthesis pathway. They contain one branch that has the complex structure and one or more branches with the high mannose structure. Among the three subgroups, complex glycans are the most abundant and account for the largest structural variation in N-linked glycoproteins (Kobata, 2000).

Lis and Sharon (1993) stated that tremendous structural diversity of N-linked glycans can be achieved by variations in number, composition and sequence of the substituents attached to the pentasaccharide core. The potential number of glycoforms of any given glycoprotein is vast and can dramatically increase as either the number of glycosylation sites or heterogeneity at each site increases (Rademacher *et al*., 1988). This heterogeneity can be separated into a number of different groups. Site heterogeneity refers to the many different glycan structures found at the same glycosylation site. Microheterogeneity is concerned with the oligosaccharide pattern of the glycans along any given glycoprotein backbone which can be further subdivided into major (extent of branching) and minor microheterogeneity (SA, Gal and Fuc content; van Dijk *et al*., 1994).

The glycoforms expressed on an individual polypeptide is cell and tissue specific and is dependent on the patho-physiological conditions. Alterations in the glycan structure of glycoproteins has been reported in certain disease states such as cancer and Kobata (2000) introduced the term "glycopathology" to describe these bio-synthetic abnormalities. Abnormal expression of glycoforms on a given glycoprotein is due to altered expression of the glycosyltransferase enzymes in the liver, a process controlled by a number of regulatory molecules termed cytokines. These cytokines can alter the transcription of the genes coding for individual glycosyltransferase enzymes which then determine the varying, and sometimes disease-specific, expression of glycoforms of a particular glycoprotein. The cytokines are themselves

up-regulated in response to injury and infection and play a major role in the body's acute phase response.

1.2 Alpha-1-Acid Glycoprotein

1.2.1 The Acute Phase Response

The acute phase response (APR) is a complex reaction initiated in response to injury or infection. It is thought to play an important adaptive and defensive role contributing to the isolation and destruction of pathogens and activation of tissue repair processes. The APR can be initiated by a number of stimuli including bacterial and viral infections, surgical trauma, bone fracture, burns injury, various inflammatory states, neoplasms, childbirth and strenuous exercise (Kushner and Rzewnicki, 1994). Initiation proceeds several minutes after the presentation of stressful stimuli and continues for 1-2 days to ensure normal homeostatic order is resumed (Kushner, 1982).

The local response of tissues to injury and infection manifests as acute inflammation resulting from increased vascular permeability and migration of leukocytes to the site of injury. The tissue macrophage is the cell commonly associated with the initiation of the APR although the role of other cells such as polymorphonuclear leukocytes, T-lymphocytes, endothelial cells, platelets and fibroblasts should also be considered (Koj, 1996). Characteristic features of the systemic APR include a large number of physiological and biochemical changes such as fever, anorexia, somnolence, altered serum concentration of copper, zinc and iron, altered synthesis of a number of endocrine hormones and plasma proteins, gluconeogenesis and leukocytosis (Kushner, 1982). Figure 1.7 summarises the initiation of the APR and its local and systemic effects.

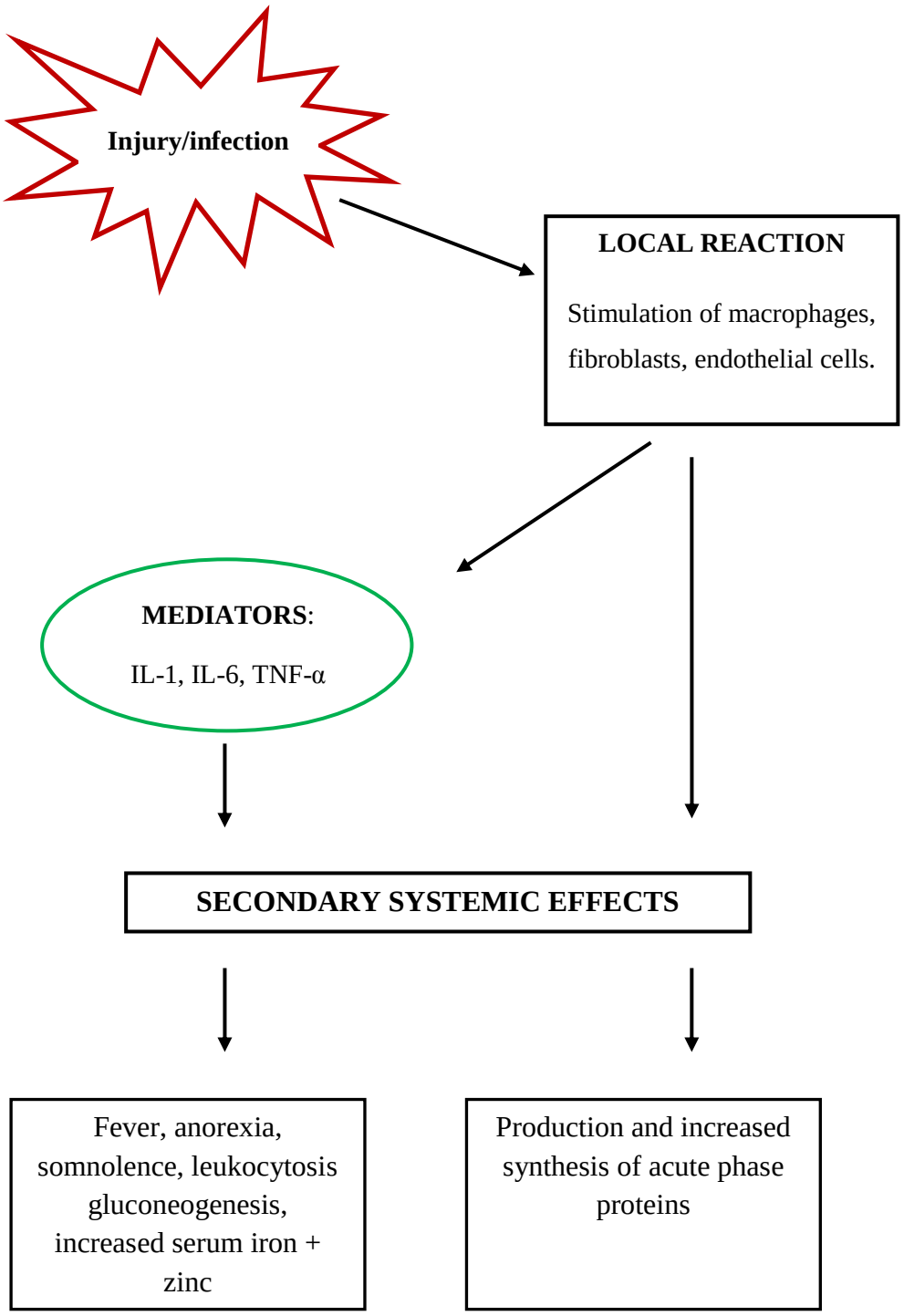


Figure 1.7 Summary of the acute phase response. (Adapted from Koj, 1996)

During the APR the inflammatory cells (mainly macrophages and neutrophils) together with endothelial cells secrete cytokines that induce the local and systemic responses detailed in Figure 1.7. These inflammatory agents can be divided into 3 groups. The first are the pro-inflammatory cytokines consisting of tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interferon-gamma (IFN-γ), and interleukin-8 (IL-8) which are mainly involved in the initiation of the APR. The second are interleukin-6-type cytokines (IL-6, leukaemia inhibitory factor, IL-11, oncostatin M, ciliary neutrophic factor and cardiotrophin-1) which are responsible for the majority of the systemic features of the APR. IL-10, IL-4, IL-13 and transforming growth factor-β make up the anti-inflammatory cytokines which serve to down regulate the APR (Koj, 1996). The cytokine network involved in the APR is complex with numerous overlapping expression pathways (Trey and Kushner, 1995) and it is therefore very difficult to determine which group plays a key role in its regulation. IL-1, IL-6 and TNF-α are initially produced following the presentation of a stressful stimulus and therefore may have an important role in the control of the early stages of the APR. Work by Castell *et al*. (1989) on the three monokines IL-1, TNF- α and IL-6 concluded that IL-6 plays the key role in the regulation of the APR in general but that IL-1 and TNF-α may exert their effect by acting on other cells types to increase their synthesis of IL-6.

One of the most interesting and important features of the APR is that the concentrations of a number of plasma proteins known as the acute phase proteins (APP) are altered during the process. APP are hepatic in origin and the overexpression of them by the liver is also known as the hepatic acute phase since their relative changes in concentrations are largely due to changes in their production by the hepatocytes. The synthesis and subsequent increased release of APP by the liver during the APR is regulated by inflammatory mediators such as the cytokines, glucocorticoids and growth factors which serve to increase gene transcription of the various APP as well as any increase in glycosyltransferase expression required for their production. Pathological states such as cancer induce an acute phase response

with subsequent release of cytokines which act at the transcription level to increase the expression of the APP.

APP have been defined as a particular set of proteins whose plasma concentration increases by at least 25% during the APR (Kushner, 1982). Up-regulated proteins are termed positive APP but there are also instances where the plasma concentrations of proteins decrease. These down-regulated proteins are termed negative APP. Within this classification there are also proteins termed major APP. An example of such a protein is serum amyloid A (SAA) the concentration of which can increase as much as 1000 fold during the APR. The majority of the APP produce only moderate increases in their concentration ranging from 50% to 2-5 fold. It is thought that the APP exert beneficial effects during the APR such as wound healing and may in fact have the potential to influence some stages of inflammation. Table 1.2 details the various APP, their classification as either positive or negative with respect to the APR and their associated function.

Disease-dependent alterations in the serum concentration of APP have targeted them as potential markers of disease prognosis, diagnosis and progression (van Dijk *et al*., 1994). A study by Ward and co-workers in 1977 proposed the use of APP profiling in the staging and monitoring of patients with prostate cancer and Cooper and Stone (1979) detailed the use of APP tests to assess prognosis and possible recurrence of variety of cancers. More recently, a study assessing the clinical significance of measuring SAA compared to the classic C-reactive protein (CRP) as a marker of the APR in elderly patients concluded that a combined measurement of the two in patients suffering from infection provided enhanced information with regards to monitoring the APR (Hogarth *et al*., 1997). In clinical practice today, measurement of serum APP concentrations can provide valuable information on the inflammatory status of a patient. Levels of CRP can change very rapidly and serum measurements of the protein can not only indicate the presence of inflammation but can assess the

Table 1.2 Classification of the acute phase proteins. (Adapted from Raynes, 1993; Suffredini *et al*., 1999 and Ebersole and Cappelli, 2000)

Acute Phase Protein Class	Protein	Function
Positive (major)	C-reactive protein	Opsonin, immunmodulation
	Serum amyloid A	Leukocyte activation, chemotaxis, phagocytosis
	α_2-macroglobulin	Antiprotease
	α_1-acid glycoprotein	Transport, immunomodulation
Positive (moderate)	Fibrinogen	Coagulation
	α_1-antichymotrypsin	Antiprotease
	α_1-protease inhibitor	Antiprotease
	Haptoglobin	Binds haemoglobin
	Complement 3	Opsonin
	Ceruloplasmin	Copper transport protein
Negative	Albumin	Transport
	Transferrin	Transport

patients' response to treatment and monitor their recovery from infection or body trauma induced by a surgical procedure (Gaw *et al*., 2004).

It is now accepted that during the APR not only are the levels of APP increased but the secondary structure attached to the protein backbone is altered. Since the majority of APP are glycoproteins, this secondary structural alteration is likely to be with respect to glycosylation and has been found to be dependent on patho-physiological conditions (van Dijk *et al*., 1994). As with the increase in APP expression during the APR, alterations in their glycosylated structure can also be attributed to the inflammatory cytokines. They alter the extent of transcription of the various glycosyltransferase genes and therefore alter the glycoforms expressed on an individual APP.

Changes in the major microheterogeneity (extent of chain branching) of APP have been demonstrated in a number of diseases. Decreased branching was detected in patients with chronic inflammatory conditions like rheumatoid arthritis (Pawlowski *et al*., 1989) and systemic lupus erythematosus (Mackiewicz *et al*., 1987) which was shown to be useful in the detection of intercurrent infection in these patients. Increased branching has been observed in the sera of pregnant women (Raynes, 1982) and in patients with liver diseases such as hepatitis (Biou *et al*., 1987). Changes in the minor microheterogeneity of APP during disease have been widely reported. Moule *et al*. (1987) reported a reduction in sialylation in patients with rheumatoid arthritis (RA) but an increase in the sialic acid content in patients with cancer. Increased fucosylation of APP and expression of sialyl Lewis X ($SLe^{X)}$ have been observed in acute inflammation (De Graaf *et al*., 1993) and in liver cirrhosis (Biou *et al*., 1987)

As noted in Table 1.2, α_1-acid glycoprotein (AGP) is a positive acute phase protein that undergoes a 2-5 fold increase in plasma concentration following the APR.

Despite this relatively moderate increase in concentration, AGP is viewed as one of the major APP (Ceciliani *et al*., 2002) and is considered one of the best proteins to study the modifications induced by the APR due to its 45% carbohydrate content**.**

1.2.2 Alpha-1-Acid Glycoprotein

Alpha-1-acid glycoprotein (AGP) also referred to as orosomucoid, is one of the most common serum proteins, second only to albumin and is one of the most extensively studied APP due to its high glycan content. The first isolation and characterisation of the protein was in 1950 by Weimer and co-workers who described the protein as a human plasma mucoprotein with a high carbohydrate content and a low isoelectric point. A vast array of research was undertaken in the years to follow allowing discovery of the proteins molecular weight (41-43kDa), 45% of which was made up of carbohydrate (Schmid *et al*., 1977). For several decades, AGP was thought to contain the greatest carbohydrate content until Karl Schmid and colleagues (Schmid *et al*., 1980) discovered galactoglycoprotein which has a staggering 76% share of its molecular weight in the form of a carbohydrate moiety.

AGP is a serum glycoprotein synthesised by the parenchymal cells of the liver and in healthy individuals, has a mean concentration of 0.77mg/ml and ranges from 0.36-1.46mg/ml (Blain *et al*., 1985). A study by Yost and DeVane (1985) on the diurnal variation of AGP concentration in a group of healthy volunteers reported that all mean concentrations were within the normal range but that deviation of the mean ranged from 6.4% to 48.9%. Work by Johnson *et al*. (1969) observed that the AGP levels of family members and those of identical twins were not correlated and concluded that fluctuation in the levels of the protein was not under genetic control. Blaine *et al*. (1985) concluded that variance in individual AGP concentrations could be attributed more to environmental, rather than genetic factors.

The serum concentration of AGP can increase 2-5 fold during a number of physiological and pathological conditions in response to the APR. Increases have been observed in acute inflammatory conditions (severe burns, trauma and infection) and chronic pathological conditions such as RA and cancer (van Dijk, 1995).

Increased levels have also been reported during periods of stress, pregnancy and in myocardial infarction patients (Duche *et al*., 2000) reinforcing its classification as a positive APP. Higher than normal AGP concentrations have been observed in obese individuals (Benedek *et al*., 1984) whereas lower than normal concentrations have been demonstrated after the intake of oestrogen containing oral contraceptives. This idea was used to explain the results of a study (Routledge *et al*., 1981) where slightly higher AGP concentrations were found in males compared with females.

AGP is a major APP in a number of other species such as rats and mice but the level of induction is variable with Dente *et al*. (1985) reporting a several hundred fold increase in the concentration in rats. The species-specific nature of AGP glycosylation has been demonstrated by Yoshima *et al*. (1981) in studies involving rat AGP where it was discovered that it contained six N-linked glycans compared with the five present in human AGP. This group also reported that the oligosaccharide pattern of AGP appeared to be consistent within the same species. Determination of the nucleotide sequence of rat AGP (Ricca and Taylor, 1981) in the same year detailed a 59% amino acid sequence homology between that of rat and human AGP.

The increase of AGP in serum concentration during the APR has been correlated with increased gene expression and subsequent hepatic synthesis in response to cytokines (IL-1, IL-6, TNF-α) and corticosteroids released during the initial stages of the APR (Kulkarni *et al*., 1985; Baumann and Gauldie, 1990)**.**

There is evidence to suggest that the synthesis of AGP is not purely hepatic. Early work by Twining and Brecher (1977) reported that AGP was a component of malignant tissue of the human breast, anus and colon and also a component of the surrounding normal tissue. It was also found to be present in malignant tissue of the stomach, ileum and lung and was thought to potentially play a regulatory role.

Studies by Gahmberg and Andersson (1978) discovered a 52kDa membrane form of AGP on normal human lymphocytes, granulocytes and monocytes, thought to be synthesised by the lymphocytes themselves. The authors suggested that this membrane form was cleaved into the normal 41kDa form and released into the serum to contribute to the host defence during the APR. Gendler *et al*. (1982) provided evidence of extra-hepatic production of AGP by demonstrating the synthesis of the protein by breast epithelial cells, thought to provide a protective role and Sörensson *et al*. (1999) detailed the production of AGP by human microvascular endothelial cells in vitro suggesting that the protein may play an important role in the endothelial glycocalyx.

1.2.2.1 Structure of AGP

Initial studies by Schmid *et al*. (1973) reported that AGP comprised of a single polypeptide of 181 amino acids. Further work by Dente *et al*. in 1985 concluded that it was in fact 183 amino acids in length and contained a possible 22 amino acid substitutions (Figure 1.8). Southern blot analysis of human DNA (Dente *et al*., 1985) identified two genes coding for the protein (AGP-1 and AGP-2), with the gene products present in normal plasma in the molar ratio 3:1 respectively. This group also studied the DNA sequences of two other APP's, α_1-antitrypsin and haptoglobin and found a conserved sequence in the 5' untranslated region of the three mRNA's which they concluded may play a significant role in the induction of the APR. Further studies concluded that two variants of AGP (ORM-1 and ORM-2) were coded for by a cluster of three adjacent genes which were termed AGP-A, AGP-B and AGP-B'. The AGP-A gene is expressed in the liver and codes for ORM-1, the major component of human serum. The ORM-2 variant differs by 22 base substitutions and is coded for by the identical genes AGP-B/B' and is expressed 100 fold less than AGP-A (Dente *et al*., 1987). A possible explanation for this could be that transcription is limited to the first gene in the cluster. A variety of amino acids can be found at positions 32 and 47 on the polypeptide chain and it is thought that these differences represent polymorphisms in the human population. Although differences in the amino acid sequence of the AGP genetic variants (ORM1/ORM2) exist, the glycosylation sites remain constant in both therefore under identical conditions, there is no difference in the glycoforms expressed.

Glu – Ile – Pro – Leu – Cys – Ala – Asn – Leu – Val – Pro – Val – Pro – Ile – Thr – Asn – Ala – Thr – Leu – Asp – Gln/Arg – Ile – Thr – Gly – Lys – Trp – Phe – Tyr – Ile – Ala – Aer – Ala – Phe/Ala – Arg – Asn – Glu – Glu – Try – Asn – Lys – Ser – Val – Gln – Glu – Ile – Gln – Ala – Thr/Ala – Phe – Phe – Try – Phe – Thr – Pro – Asn – Lys – Thr – Glu – Asp – Thr – Ile – Phe – Leu – Arg – Glu – Tyr – Gln – Thr – Arg – Gln- Asp/Asn – Gln – Cys – Ile/Phe – Try – Asn – Thr/Ser – Tyr – Leu – Asn- Val – Gln – Arg – Glu – Asn – Gly – Thr – Ile/Val – Ser – Arg – Tyr – Val/Glu – Gly – Gly – Gln/Arg – Glu – His – Phe/Val – Ala – His – Leu – Leu – Ile – Leu – Arg – Asp – Thr – Lys – Thr – Tyr/Leu – Met – Leu/Phe – Ala/Gly – Phe/Ser – Asp/Tyr – Val/Leu – Asn/Asp – Asp – Glu – Lys – Asn – Trp – Gly – Leu – Ser – Val/Phe – Tyr – Ala – Asp – Lys – Pro – Glu – Thr – Lys – Glu – Gln – Leu – Gly – Phe – Tyr – Glu – Ala – Leu – Asp – Cys – Leu – Arg/Cys – Ile – Pro – Lys/Arg – Ser – Asp – Val – Val/Met – Tyr – Thr – Asp – Trp – Lys – Lys – Asp – Lys – Cys – Glu – Pro – Leu – Glu – Lys – Gln – His – Glu – Lys – Glu – Arg – Lys – Gln – Glu – Glu – Gly – Ser – COOH

Figure 1.8 Amino acid sequence of AGP (Dente *et al*., 1985). The sites of amino acid substitution are highlighted in blue and the five Asn residues home to the 5 N-linked linked glycans are labelled in green.

AGP is a heavily glycosylated protein containing five highly sialylated complex N-linked oligosaccharide chains (Yoshima *et al*., 1981) accounting for 45% of its 41-43kDa weight. As previously mentioned a prerequisite of N-linked glycosylation is that the initial Asn residue occurs within the consensus sequence Asn-X-Ser/Thr. The five N-linked chains of AGP are therefore located on Asn 15, 38, 54, 75 and 85 (Figure 1.9).

The oligosaccharide chains of AGP display some heterogeneity and have undergone extensive study since this is one of the few serum glycoproteins capable of containing bi-, tri- and tetra-antennary glycans. Major microheterogeneity has been detected by determining the relative proportions of bi- tri- and tetra-antennary glycans present using Concanavalin A (Con A), a protein lectin isolated from jack bean which preferentially binds bi-antennary glycans. Bierhuizen *et al*. (1988) differentiated between three types of AGP by their reaction with Con A and reported that in healthy plasma the unretained (UR) form accounts for 46% of total AGP, the weakly retained (WR) 39% and the retained (R) 15%.

Considerable site heterogeneity has also been observed at the five glycosylation sites and individual site analysis studies have revealed that not all sites are capable of expressing all of the antennary structures available (Treuheit *et al*., 1992). Glycosylation site 1 is not capable of carrying tetra-antennary glycans, site 2 cannot display fucose-containing glycans, bi-antennary glycans are completely absent on site 4 and highly fucosylated glycans are only capable of being displayed on sites 4 and 5 (Fournier *et al*., 2000). As a result of this only 12-20 glycoforms of AGP are present in normal human serum and the relative occurrence of them is strongly dependent on patho-physiological conditions (van Dijk, 1995).

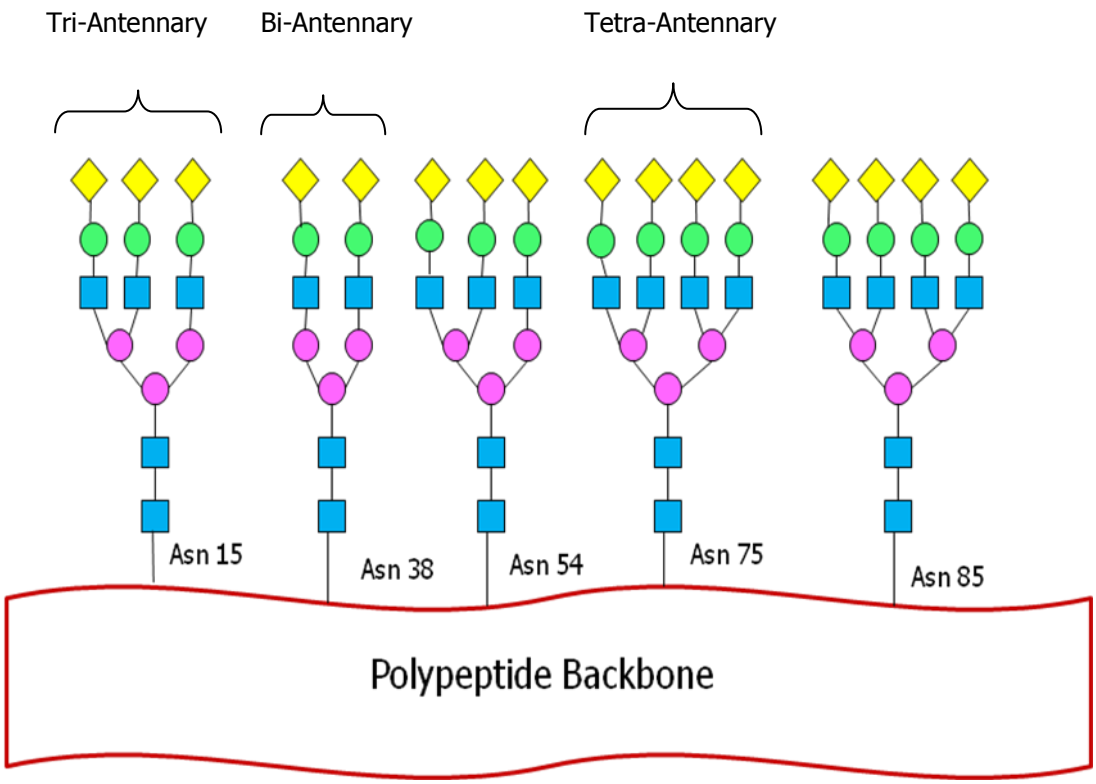


Figure 1.9 Structure of the N-linked oligosaccharide chains of AGP detailing the Asn residues to which they are attached.

GlcNAc Man Gal N-acetylneuraminic acid (NeuAc)

The microheterogeneity of AGP is also considerably variable with regards to the extent of branching and fucose and SA content (Treuheit *et al*., 1992, van Dijk, 1995 and van Dijk *et al*., 1995). The negative charge and low isoelectric point (pI, 2.8-3.8) of AGP is attributed to its relatively large SA content. SA in the form of neuraminic acid (NeuAc) accounts for 12% of the oligosaccharide moiety. This terminal monosaccharide can be found attached to outer Gal residues by either an α (2, 3) or α (2, 6) linkage. Fuc can be linked α (1, 3) and α (1, 2) to outer chain GlcNAc and Gal residues respectively and it has been reported that high Fuc levels are associated with a greater number of tri-and tetra-antennary glycans and low, or total absence of bi-antennary glycans (Fournier *et al*., 2000). The extent of AGP fucosylation in a normal healthy population varies dramatically with 30-40% possessing no Fuc at all (De Graaf *et al*., 1993; Fournier *et al*., 2000). This may be due to a lack of, or reduced expression of the enzyme required to fucosylated the AGP glycans. The species-specific nature of AGP microheterogeneity was demonstrated by Nakano *et al*. (2004) who analysed the oligosaccharide pattern of chains from human, bovine, sheep and rat AGP and found that they showed distinct variations.

AGP microheterogeneity can be easily determined through the use of techniques such as crossed affinity-immunoelectrophoresis (CAIE) (Van der Linden *et al*., 1994; Mackiewicz and Mackiewicz, 1995). This technique involves lectins, substances which recognize and interact with specific monosaccharides/sequences without altering their structure. Minor microheterogeneity, or the degree of fucosylation in a given AGP sample, can be measured by its binding to *Aleuria aurantia* (AAL) (Hashimoto *et al*., 2004). The development of such techniques has enabled the variable proportions of AGP glycoforms expressed in disease states such as cancer to be measured.

1.2.2.2 Function of AGP

The exact *in vivo* function of AGP is as yet unknown but there have been a number of activities associated with the protein such as various immunomodulatory effects (Eap and Baumann, 1993, Hochepied *et al*., 2003) all of which seem to be dependent on the oligosaccharide moiety of the glycoprotein (Bennett and Schmid, 1980).

Several pro- and anti-inflammatory properties have been related to AGP suggesting that it may play an important role in the regulation of the immune system with different immunomodulatory activities associated with different glycosylated variants of the protein (Eap and Baumann, 1993; Shiyan and Bovin, 1997). In the presence of lipopolysaccharide (LPS) AGP has been shown to significantly enhance (2-7 fold) the production of a number of cytokines (IL-1β, IL-6, TNF-α) in monocytes (Boutten *et al*., 1992). In the absence of LPS, little or no effect was observed. This enhancement of cytokine (LPS-induced) production by both peritoneal and alveolar macrophages suggested that AGP could have the ability to alter the function of monocytes and possibly play a role in the regulation of the immune and inflammatory response. It has been shown that AGP can induce the secretion of TNF-α by human monocytes (Su and Yeh, 1996) with the authors concluding that this may play a role in the initiation of inflammation. Further work by this group (Su *et al*., 1999) demonstrated that the ability of AGP to induce TNF-α production by human monocytes was dependent on the activation of protein tyrosine kinase in the signal transduction pathway.

Anti-apoptotic and anti-inflammatory functions of AGP have been described (van Dijk, 1995, Daemen *et al*., 2000). The protective role played by the protein in renal ischemia and reperfusion injury was demonstrated by Daemen *et al*. (2000) with the prevention of apoptosis and inflammation. It was shown that exogenous administration of AGP and α_1-antitrypsin in a mouse model inhibited both early and

late apoptosis. The anti-inflammatory effects were demonstrated by a reduction in TNF-α expression and neutrophil influx 24 hours after administration. Another instance of AGP behaving as an anti-inflammatory agent was discovered when a recombinant form of AGP terminated with SLe^X was injected into rats suffering from intestinal ischemia (Williams *et al*., 1997). The authors quoted a 62% reduction in lung injury and a 28% reduction in complement-dependent intestinal injury compared with controls and therefore concluded that this form of AGP could improve the response to injury induced by both complement and neutrophils.

Modulation of lymphocytes has been proposed as another immunomodulatory function of AGP (Hochepied *et al*., 2003). An early study by Chiu *et al*. (1977) presented evidence on the ability of AGP isolated from human ascitic fluid to significantly inhibit lymphocyte responsiveness in the presence of mitogens phytohemagglutinin (PHA). This effect was found to be reversible at high mitogen concentrations demonstrating that AGP, although inhibitory, was not cytotoxic to the lymphocytes. The same group demonstrated that AGP was also capable of inhibiting the blastogenic response of lymphocytes induced by the lectins Con A and pokeweed mitogen (PWM). Studies on the effect of the glycosylated variants of AGP on lymphocyte proliferation have detailed the greatest inhibition by the Con-A non-reactive, or UR form (Pos *et al*., 1990). Conversely, this variant also stimulated the proliferation of lymphocytes induced by low concentrations of AGP with desialylation of the protein abolishing both effects suggesting that in this instance, the terminal modifications of the glycan chains of AGP play an important role in its immunomodulatory effects.

Variation in the immunomodulatory roles of AGP variants is supported by Pukhal'skii et al. (1994) who demonstrate that glycan structure has an impact on the overall function of the glycoprotein. This group separated AGP isolated from the blood of normal donors and from ascitic fluid of patients with stomach cancer into the three Con A fractions (UR, WR and R) and assessed its effect on lymphocyte

responsiveness. They reported that cancer AGP had a greater inhibitory effect on lymphocyte proliferation compared to normal AGP and that the R variant produced the most potent effect. The UR variant was found to affect the action of interleukin-2 (IL-2) by inhibiting secretion of the cytokine and the WR variant was found to have a stimulatory effect on both these actions. Elg *et al*. (1997) reported that there was a positive correlation between AGP inhibition of lymphocyte proliferation and reduced synthesis of interleukin-2 (IL-2) by the lymphocytes.

The anti-inflammatory properties of AGP in some instances have the potential to be immunosuppressive. Bennett and Schmid (1980) reported on the inhibitory action of AGP towards lymphocytes, noting that the strongest inhibition occurred when exposed to a derivative of AGP lacking in Gal and SA. The authors stated that this could have implications in cancer management with the possibility of tumour cells containing neuraminidase and galactosidase allowing the creation of an extremely immunosuppressive environment which could potentially aid the tumours immune escape. Studies by Elg *et al*. (1997) have targeted AGP as an immunosuppressive factor in ascites from ovarian carcinoma. Converse to these detrimental effects AGP has been shown to be an important factor in resistance to Gram-negative infection (*Klebsiella pneumonia*). Prior to infection, exogenous administration of AGP resulted in significantly decreased mortality rates in mice and over-expression of rat-AGP by transgenic mice appeared to protect the animals from infection (Hochepied *et al*., 2000).

Structural studies on AGP have discovered a high affinity binding site (Kopecký Jr. *et al*., 2003) capable of binding in the plasma, a variety of basic and neutral drugs as well as steroid hormones (Israili and Dayton, 2001). Due to this drug binding ability it is thought to be a member of the lipocalin family; a large group of small extracellular transport proteins with immunomodulatory properties (Lögdberg and Wester, 2000). This too has great implications in the management of disease since increased levels of AGP during the APR may decrease the free plasma concentration

of the drug and significantly reduce the desired pharmacological effect. A study by Chatterjee and Harris (1990) demonstrated the ability of AGP to bind the breast cancer drug tamoxifen and on-going investigations in the lab have supported this work. These finding suggest that AGP may have an impact on the efficacy of the drug and may propose a possible mechanism of resistance to tamoxifen presented by a number of women with breast cancer.

To date, all of the proposed functions of AGP have been reported to be dependent on the composition of the glycan chains (Fournier, 2000) therefore determination of the oligosaccharide fingerprint of the chains may provide new evidence to the function of the protein with regard to specific disease states.

1.2.2.3 AGP in Disease

There have been a vast number of studies concerned with quantifying the levels and characterising the microheterogeneity of AGP in a number of diseases (Table 1.3). Variation in the branching of the chains, levels of fucose, sialic acid and the presence or absence of Sialyl Lewis X (SLe^X) are commonly reported and represent the potential exploitation of AGP heterogeneity as prognostic and diagnostic markers of disease (Ceciliani and Pocacqua, 2007).

Reports of increased levels of AGP in patients suffering from a number of malignancies are consistent with the idea that during the APR it acts as a positive acute phase protein. An early study by Rudman *et al*. (1974) found an increased level of AGP in patients suffering from a variety of cancers (lung, breast, ovary, pancreas and lymphoma) and stated that the increase was 2-5 times greater in malignant fluid compared with normal controls. Roberts *et al*. (1975) and Turner *et al*. (1985) also reported the finding of increased AGP concentrations in patients with breast cancer with the latter stating a 35.8% increase compared to the normal reference group and the former demonstrating that the level increased with the clinical stage of breast cancer (ranging from early to disseminated disease). A more recent study (Duché *et al*., 2000) demonstrated that the concentration of AGP in the serum of breast cancer patients was 2.5 times greater than that of healthy donors. Increased concentrations of AGP have also been observed in malignant and benign disease of the lung (Hansen *et al*., 1984; Bleasby *et al*., 1985; Van den Heuvel *et al*., 2000) and in the ascitic fluid of patients with liver cirrhosis and cancer (Fujii *et al*., 1988).

As may be expected, reports of increases in AGP concentration have not been limited to cancer. Benedek *et al*. (1984) demonstrated a 2 fold increase in the serum concentration of moderately obese males (body mass index, BMI, > 30 and < 45) compared with healthy controls which was shown to correlate with increased drug

Table 1.3 Heterogeneity of AGP in a variety of pathological and physiological conditions.

Pathological/ Physiological Condition	Observed changes in AGP heterogeneity	References
Acute Inflammation	↑ bi-antennary glycans ↑ fucosylation ↑ Sialyl Lewis X	Nicollet *et al*., 1981 Fassbender *et al*., 1991 De Graaf *et al*., 1993 Van der Linden *et al*., 1994 Higai *et al*., 2003 Higai *et al*., 2005
Chronic Inflammation	↑ tri- and tetra-antennary glycans ↑ fucosylation ↑ sialic acid in rheumatoid arthritis	Fassbender *et al*., 1991 Smith *et al* 1994 Elliot *et al*., 1997 Elliot *et al*., 1998 Jørgensen *et al*., 1998 Smith *et al*., 2002 Rydén *et al*., 2002 Haston *et al*., 2002 Haston *et al*., 2003 Higai *et al*., 2005
	↑ bi-antennary glycans in systemic lupus erythematosus	Mackiewicz *et al.*, 1987
	↑ tri- and tetra-antennary glycans in asthma	Van den Heuvel *et al*., 2000
Cancer	↑ bi-antennary glycans in lung and liver cancer ↑ fucosylation ↑ sialic acid	Hansen *et al*., 1984 Turner *et al*., 1985 Moule *et al*., 1987 Fujii *et al*., 1988 Hashimoto *et al*., 2004

Table 1.3 contd. Heterogeneity of AGP in a variety of pathological and physiological conditions.

Pathological/ Physiological Condition	Observed changes in AGP heterogeneity	References
Liver Disease	↑ tri- and tetra-antennary glycans ↑ fucosylation	Biou *et al*., 1987 Anderson *et al*., 2002 Rydén *et al*., 2002 Mooney *et al*., 2006
Pregnancy	↓ bi-antennary glycans	Raynes, 1982
	↑ tri- and tetra-antennary glycans late term	Biou *et al*., 1991
	↑ tri- and tetra-antennary glycans in amniotic fluid from 3rd trimester	Pawiliwicz *et al*., 2006
Burn Injury	↓ bi-antennary glycans	French *et al*., 2002
Oestrogen Treatment	↑ tri- and tetra-antennary glycans ↓ fucosylation ↓ Sialyl Lewis X	Brinkman-Van der Linden *et al*., 1996

binding which could potentially lead to reduced efficacy of drug therapy in these individuals. Cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS) was found to contain a higher AGP concentration than controls (Adam *et al*., 2003) and patients suffering from burns injury (French *et al*., 2003) have demonstrated higher serum levels of the protein also.

The observed increase of AGP levels during pathological states has been correlated to an increase in the expression of its genetic variants. Duche *et al*. (2000) reported a 2.5 fold increase in the ORM-1/ORM-2 variants (see section 1.2.2.1) in breast and lung cancer and a 1.6 fold increase in ovarian cancer but stated that the overall proportions of ORM-1/ORM-2 was not significantly different to that observed in healthy individuals. Hervé *et al*. (1998) and van Dijk *et al*. (1991) have also demonstrated this increase in expression of the genetic variants of AGP in plasma from malignant mesothelioma and burns patients respectively.

AGP Heterogeneity in Acute and Chronic Inflammation

A variety of changes in major and minor micro-heterogeneity have been observed during inflammatory conditions. In 1981 Nicollet and colleagues reported an increase of Con-A reactive forms of AGP in acute inflammation which initially hinted at increased expression of bi-antennary glycans which was later supported by Fassbender *et al*. (1991), Higai *et al*. (2003) and Higai *et al*. (2005). Increased fucosylation of the glycan chains of AGP have also been demonstrated in acute inflammation (Higai *et al*., 2003; Higai *et al*., 2005) which in some studies has been positively correlated to increased expression of SLe^X (De Graaf *et al*., 1993; Van der Linden *et al*., 1994). SLe^X is a blood group antigen expressed on a number of white blood cells and plays an important role in inflammation. When SLe^X binds its ligand, E-Selectin, it can aid the extravasation of leukocytes into tissues to mount their inflammatory response.

Chronic inflammatory states such as those found in patients with RA are characterised by an increase in branching (Elliot *et al*., 1997; Smith *et al*., 2002), increased fucosylation of the chains (Jørgensen *et al*., 1998; Rydén *et al*, 2002) and increased sialic acid content (Elliot *et al*., 1998; Haston *et al*., 2003) which is thought to possibly be due to an increase in the expression of SLe^X. De Graaf and colleagues (1993) proposed that the increased expression of this antigen on AGP represents a negative feedback response during inflammatory conditions. Further work by Jørgensen *et al*. (1998) using a microtitre cell-protein binding assay discovered that SLe^X-containing AGP expressed in patients with RA was able to inhibit binding of SLe^X presenting cells to E-Selectin. Thus, during inflammatory states such as those in RA, the abnormally glycosylated form of AGP has the ability to competitively bind to E-Selectin and inhibit leukocyte extravasation exerting an overall anti-inflammatory effect.

AGP Heterogeneity in Cancer

Investigations into the heterogeneity of cancer AGP have found an increase in bi-antennary glycan content with increased fucosylation and sialylation. An early study by Hansen and co-workers (1984) reported that the major microheterogeneity of AGP from malignant, benign and normal groups was significantly different from each other. When compared with normal controls, sera from patients with inflammatory lung disease (benign group) exhibited an increase in Con A unreactive forms signifying an increase in tri-and tetra-antennary glycans and in sera from lung cancer patients (malignant group) an increase in Con A reactive forms (increased bi-antennary expression). This work was contradicted the following year in a study by Bleasby *et al*. (1985) who repeated the study including an evaluation of the microheterogeneity of AGP in colorectal cancer and reported that there was no significant difference in the microheterogeneity of AGP from malignant and non-malignant inflammatory disease. Evidence to support the cancer-induced expression of bi-antennary glycans on AGP detailed by Hansen and colleagues was provided by Fujii *et al.* (1988) who reported a similar finding on AGP from the ascitic fluid of liver cancer patients.

Reports of changes in the minor microheterogeneity of AGP from studies of the oligosaccharide content of the chains in cancer have been detailed. Moule *et al*. (1987) and Turner *et al*. (1985) observed an increase in the sialic acid content of cancer AGP glycans compared to normal controls with Turner and co-workers (1985) and Hashimoto *et al*. (2004) also noting an increase in the fucosylation of the chains.

Although the limited research to date has discovered structural differences in the glycosylation in AGP isolated from different cancers and early and advanced stages of the same cancer, very few studies have looked at breast cancer samples.

There are very definite disease-specific changes in AGP which has allowed the protein to be targeted in a number of studies as a potentially useful indicator of disease but it is the disease-specific changes in the microheterogeneity of AGP glycosylation which may have significance in the development of useful diagnostic and prognostic markers of disease and in the monitoring of its progression.

1.2.2.4 AGP as a Disease Marker

An early study by Rudman and colleagues (1974) reported that the measurement of AGP levels in effusions from patients suffering from a number of pathological states provided valuable information when compared with measurement of total protein content or measurement of other plasma proteins. The authors concluded that the AGP content of effusions allowed for discrimination between neoplastic (lung, breast, ovarian and pancreatic cancer) and non-neoplastic non-inflammatory disease (cirrhosis and congestive heart failure). This group also reported that the carbohydrate composition of a high percentage of the neoplastic fluids was abnormal exhibiting a lower than normal sialic acid, hexose and hexosamine content and a higher than normal hexosamine content in some samples. They suggested that these abnormalities could possibly be correlated to the invasive properties of cancer cells in patients with disseminated disease.

An early serum assay study by Roberts *et al*. (1975) indicated that the serum concentration of AGP in breast cancer patients may provide a useful marker of disseminated disease and may have a valuable role in assessing prognosis and in monitoring the progress of patients with early, recurrent and disseminated breast cancer. They demonstrated that patients with early breast cancer with tumours < 5cm (Group A) had a significantly higher serum AGP concentration than the normal control group and that early breast cancer patients with tumours > 5cm (Group B) had a significantly higher AGP concentration than those in Group A. Furthermore, the serum AGP concentration of patients with recurrent disease (Group C) was significantly higher than group A and disseminated disease (Group D), was associated with serum concentrations significantly higher than those found in Group C. The authors concluded that the abnormal level of AGP demonstrated by a number of the breast cancer patients may have been induced by a number of factors and suggest that a normal AGP level is of more clinical value than an elevated level with

a normal serum AGP concentration correlating to a more favourable prognosis in patients with both early and disseminated disease.

As detailed above, AGP levels have long been considered useful markers in pathological conditions but changes in the serum concentration of the protein may not necessarily relate to disease progression. A number of studies have introduced the idea of using AGP glycosylation patterns as markers in diagnosis, prognosis, disease progression and recovery (Turner and Goodarzi, 1998).

Discrimination between benign lung disease, lung carcinoma and normal health, on the basis of differences in the branching of their oligosaccharide chains was proposed by Hansen *et al*. (1984). This study separated AGP by crossed immuno-affinoelectrophoresis using the lectin Con-A into four distinct forms and suggested that, along with measurement of the total AGP concentration, analysis of the individual AGP profiles could discriminate between normal, benign and cancer groups. Compared with normal, benign sera displayed an increase in non-Con A binding AGP and a decrease in Con A binding AGP. Cancer sera produced a reduction in the weakly Con A bound AGP variant and an increase in the strongly bound variant. Since a relatively small number of subjects were recruited in this study, the authors concluded that it was impossible to draw any definite conclusions from the results.

The following year Bleasby and co-workers (1985) repeated the study by Hansen *et al*. (1984) increasing the number of subjects and extending the analysis to include colorectal cancer patients and pleural effusions from patients with benign lung disease and lung cancer. As with the previous study, Bleasby *et al*. (1985) reported an increased AGP concentration in the benign and malignant groups compared with normal controls but the AGP microheterogeneity data contradicted that of Hansen *et al*. (1984) by demonstrating that there were no significant differences in the Con A

binding profiles of AGP from benign and malignant disease such as to allow discrimination between them.

In 1985, Turner *et al*. measured the general serum concentration of Fuc and SA and eight acute phase proteins to determine if an increase in the sugars correlated to increased concentrations of the APP. They reported that in cancer, serum fucose levels were increased more in patients with advanced stages of the disease. This group also noted an increase in serum SA among cancer patients and in some cases this increase in SA complemented the observed increase in Fuc. The authors concluded that the combined measurement of the two markers in serum (improved only slightly by including measurement of the APP) may prove valuable in monitoring individuals with the disease suggesting that SA content could be indicative of the presence of the APR and that measurement of fucose levels could provide information on the extent of tumour spread. Mackiewicz and Mackiewicz (1995) reported that valuable information could be obtained from AGP glycoforms by differentiating between primary and secondary liver cancer. This could also be used in the assessment of the stage of ovarian cancer and subsequent effectiveness of therapy in stage IV of the disease.

Further work by Hashimoto *et al*. (2004) on AGP branching and fucosylation in cancer concluded that changes in microheterogeneity could be used as a marker of carcinoma progression and prognosis. Patients with advanced stages of the disease who displayed highly branched glycans with a high fucose content for a significant duration post-surgery were associated with poor prognosis whereas, patients who did not display this increase in branching and fucosylation were likely to have a good, more reassuring prognosis. Attempts have been made to use the detection of AGP glycoforms for the diagnosis and management of cancer and there has been some evidence that CAIE of AGP can differentiate between colorectal cancer and chronic colorectal inflammatory conditions such as ulcerative colitis and Chrohns disease (Hansen *et al*., 1986).

The clinical significance of changes in the microheterogeneity of AGP has not been limited to cancer studies. van Dijk *et al*. (1995) suggested that measurements of AGP branching and fucosylation could be useful clinical parameters in following the recovery of patients from severe trauma and/or surgery.

In 1987 Mackiewicz and colleagues investigated the relative amount of Con A variants present in different grades of RA concluding that AGP microheterogeneity could be more useful than that of CRP levels as an indicator of RA activity. In the same year this group published similar data relating to Systemic Lupus Erythematosus (SLE) activity (Mackiewicz *et al.,* 1987) where they reported that serum AGP microheterogeneity and more specifically an increase in Con-A bound serum AGP, had valuable marker status compared with elevated CRP levels in assessing intercurrent infection in these patients.

More recently, a study assessing the fucosylation of AGP in male and female patients with early RA reported that the level of fucose in the RA patients was significantly higher than healthy controls and that there was a weak correlation between fucosylation and disease activity among the male participants (Rydén *et al*., 2001). The authors concluded that in men suffering from RA, AGP fucosylation could provide useful prognostic information with regards to disease progression when coupled with the assessment of traditional clinical markers.

AGP microheterogeneity has been found to be useful in discriminating between patients with liver cirrhosis and those with liver disease (Anderson *et al*., 2002; Rydén *et al*., 2002). By calculating the AGP fucosylation index the authors demonstrated the presence of a higher fucose content in patients with liver cirrhosis compared with normal and liver disease groups and reported that this difference had a high diagnostic accuracy and specificity such that it could be useful in clinical investigations. Further liver studies by Mooney *et al*. (2006) demonstrated that the

Fuc and SA content of the AGP glycans were influenced by the degree of fibrosis of the liver and that these changes may provide a valuable prognostic indicator of the development of cirrhosis.

Studies dedicated to investigating and exploiting the microheterogeneity of AGP in disease are ongoing and although the results from previous studies are encouraging, there is still a long way to go in discovering suitable disease-specific biomarkers which could be used in clinical practice to aid the diagnosis and prognosis of disease with the overall aim of improving survival rates.

1.3 Breast Cancer

1.3.1 Introduction to Breast Cancer

The female breast is predominantly composed of stroma made up of fatty and connective tissue which surrounds a network of structures termed lobes. These lobes contain milk producing glands known as lobules, connected to tiny tubes or ducts which aid the transport of milk from the lobes to the nipple. The breast also contains a complementary network of blood and lymph vessels which serve to nourish the cells and remove waste products respectively. The lymph vessels are connected to lymph nodes located in a number of regions around the body, the most important of which with regards to breast cancer are located under the arms, above the collar bone and in the neck area. Breast cancer (BC) develops from excessive proliferation of the resident cells of the ducts or lobes resulting in the production of a malignant tumour and can be characterised by the part of the breast from which it originated e.g. ductal or lobular carcinomas.

BC is the most prevalent cancer worldwide (Parkin *et al*., 2005) and although it predominantly affects women it has also been found to occur in men with the UK Office of National Statistics attributing 314 cases (approximately 1%) of newly diagnosed breast cancer in 2006 to male breast cancer. 45,822 new cases of breast cancer were diagnosed in 2006 and a study conducted in February 2009 by the Cancer Research UK Statistical Information Team have estimated that the lifetime risk of developing the disease is 1 in 9 for women and 1 in 1,014 for men.

In 1988 the UK Department of Health started the world's first national breast screening programme with the overall aim of reducing the high mortality rates of women with BC. The scheme was initially set up in response to a report commissioned by the government termed the Forrest Report (Breast Cancer Screening, Department of Health and Social Security, November 1986) which concluded that the introduction of screening mammography had the potential to prolong the life of women aged 50 and over. In the United Kingdom currently all women between the age of 50 and 70 are invited to attend a screening mammography appointment every three years with the age range due to be extended to 47-73 years of age gradually by 2012 (www.cancerscreening.nhs.uk).

1.3.2 Benign and Malignant Disease

There are a number of benign breast conditions the majority of which are not associated with an increased risk of subsequently developing BC however; there are a few which do pose an increased risk. Epithelial ductal hyperplasia involves a mild to moderate proliferation of the cells lining the ducts and often represents a 1.6 times increased risk of developing BC which can increase to a 2 times risk if there is a family history of the disease. Individuals demonstrating the presence of atypical hyperplasia have around a 4 times increased risk which can rise considerably when coupled with a positive family history of BC. This benign breast condition is representative of proliferative lesions of the breast which develop an unusual pattern and shape. It can occur in both the ducts and lobules and has the ability to spread to the surrounding tissue. It is possible for ductal atypical hyperplasia and lobular atypical hyperplasia to share features and in some cases be mistaken for ductal carcinoma in situ and lobular carcinoma in situ respectively. The increase in mammographic screening has seen a rise in the number of individuals diagnosed with this benign disease. Generally, the condition can be managed through increased monitoring to check on its progress but in some cases excision of the area may be advised to limit the chance of it developing further.

Ductal carcinoma in situ (DCIS) is caused by the malignant transformation and excessive proliferation of cells in the ducts of the breast. The growing mass is confined to the ducts (‘*in situ*’) and is termed non-invasive since it is not capable of producing metastatic spread and may represent an intermediate stage of carcinoma progression from atypical hyperplasia to invasive disease. There are various types of DCIS termed comedo, cribiform, micropapillary, papillary and solid which are most often detected as tiny specks or microcalcifications on screening mammograms.

Lobular carcinoma in situ (LCIS) is represented by the malignant transformation of the cells present in the breast lobes. Again, this is termed non-invasive cancer but does have the ability to develop into an invasive carcinoma if left untreated. Unlike DCIS, LCIS cannot be detected by mammogram and is usually found when a breast biopsy is performed for another condition.

A diagnosis of invasive or infiltrating carcinoma indicates that the cancer is no longer confined to the ducts and/or lobes and has spread to the surrounding tissue with increased ability to invade the blood and lymphatic system and ultimately form metastasise in distant organs and tissues. There are various forms of invasive carcinoma which represent approximately 80% of diagnosed cases.

Paget's disease of the breast is associated with eczema-like changes of the nipple which manifest as a rash. This disease is almost always linked with an underlying ductal or invasive carcinoma. Inflammatory breast cancer is a rare but aggressive form of the disease in which the cancer cells grow along and block the lymph vessels which may result in the breast becoming red and inflamed. This type of breast cancer does not usually manifest as a lump and often goes undetected by mammography. The skin of the breast may take on an orange peel effect due to an underlying accumulation of fluid and is often represented by unusual warmth and/or pain in the breast.

The analysis of a cancerous biopsy specimen will yield information on how far the cancer has progressed (stage) and the speed at which the cancer is growing (histologic grade) (Brown *et al.*, 2007). The grading of a tumour ranges from 1 to 3 and provides an indication of the arrangement of the cells and details some of their characteristics. Grade 1 tumours are termed '*well differentiated'* where the cells of the tumour are relatively slow growing and resemble normal breast cells in many ways. Grade 2 tumours are termed '*moderately differentiated'* and Grade 3 are

termed '*poorly differentiated'*. At this point the cells are abnormal and have an erratic arrangement with the ability to spread aggressively. The staging of a tumour takes into account the tumour size and type, LN involvement and metastatic spread and is based on the tumour-node-metastasis (TNM) classification system.

Stage 0- represents DCIS and LCIS with no LN involvement or tissue spread.

Stage 1- early stage, tumour is < 2cm in diameter with no LN involvement.

Stage 2- early stage, tumour is between 2-5cm in diameter with possible LN involvement but no evidence of spread to surrounding tissues.

Stage 3- advanced stage (also termed locally advanced disease), tumour is > 5cm in diameter with extensive LN involvement with possible spread to surrounding tissues. Inflammatory BC is categorised by this stage of locally advanced BC.

Stage 4- metastatic disease, size and LN involvement are no longer as important since the cancer has spread from the breast to involve other organs of the body.

The combined evaluation of the grade and stage of a cancer can determine the treatment regime adopted since an early stage, grade 3 cancer has a more reassuring prognosis than a late stage, grade 1 cancer since metastasis has already occurred.

1.3.3 Breast Cancer Biomarkers

In addition to the prognostic information provided from the determination of hormone receptor status a number of serum biomarkers or blood tumour markers, are currently measured in clinical practice for the management of BC. Unfortunately, none of these markers have proven sensitive or specific enough to allow them to be applied to the detection of the disease.

Carcinoembryonic antigen (CEA) and mucin 1 (MUC-1) are the most widely measured of the markers and can diagnose metastatic disease and monitor the response of an individual to systemic therapy. CEA is a glycoprotein produced by the large intestine during foetal development but can be present in very low concentrations in the blood of human adults. Increased serum levels have been found in patients with colorectal, lung, ovarian, prostatic, pancreatic, liver and breast cancer and can be elevated 30-50% in women with metastatic disease of the breast (Mughal *et al*., 1983; Williams *et al*., 1988). A positive correlation between serum CEA and therapeutic response has been reported in a number of studies investigating women with metastatic breast disease (Cheung *et al*., 2000).

MUC-1 is a protein produced by the epithelial cells of the breast, bladder, stomach, pancreas, ovary and respiratory tract. In BC the glycosylation chains of MUC1 are much shorter (truncated) compared with the normal breast epithelial variant and this aberrant glycosylation coupled with up-regulation of the protein are characteristic features of malignancy. The level of MUC-1 in BC is determined by the CA15-3 enzyme-linked immunosorbent assay (ELISA) which contains a monoclonal antibody capable of reacting with a specific epitope of the MUC-1 protein. With regards to the management of BC this ‘gold standard’ test (Seregni *et al*., 2004) is more sensitive than measurement of CEA but has a similar specificity. Combined measurement of CA15-3 and CEA is routine in clinical practice for detecting

metastatic disease, increasing sensitivity to over 80% (Robertson *et al*., 1999). A recent study by Kurebayashi and colleagues (2006) demonstrated that measurement of SLe^X coupled with CA15-3 may be more useful in monitoring BC patients and in detecting metastasis than CEA. The CA27-29 test can also assay MUC-1 and in 1998 the United States of America Food and Drug Administration (FDA) approved it for use in patients with stage II and III BC to detect recurrent disease. A preliminary investigation by Gion *et al*. (1999) reported that this 'new' test was better than CA15-3 in discriminating between healthy individuals and those with primary BC. Further work by the same group confirmed that measurement of CA27-29 was suitable for routine use in the management of individuals with BC producing comparable results to CA15-3 but demonstrating that it may be slightly more sensitive in the presence of low antigen concentrations (Gion *et al*., 2001). A group of intermediate filament proteins termed the cytokeratins have also been approved for use by the FDA for use in monitoring BC therapy and prognosis (Cheung *et al*., 2000; Ludwig and Weinstein, 2005) and includes tissue polypeptide antigen (TPA) and tissue polypeptide-specific antigen (TPS). Unfortunately, the routinely measured tumour markers lack sensitivity and specificity in early stages of the disease, which means that currently there is no serum breast cancer biomarker capable of screening high risk populations and/or diagnosing early BC. In recent years a huge amount of investment and research has been targeted at finding this elusive 'breast cancer biomarker' to aid detection of the disease (Chatterjee and Zetter, 2005).

An ideal biomarker is one that can be easily detected in the blood providing diagnostic, as well as prognostic information and whose presence could possibly precede the presentation of a tumour mass or cells allowing its use in the screening of high risk individuals. Such a marker would allow the development of a non-invasive serologic test for the early diagnosis of BC. To date, the only biomarker routinely used for the detection of early disease is prostate-specific antigen (PSA) which is used widely in screening men for the presence of prostate cancer. Recent advances in breast cancer biomarker discovery have been facilitated by the development of new technologies such as protein/antibody microarrays (Anderson *et al*., 2008), gene

expression profiling (Martin *et al*., 2000) and proteomic techniques such as mass spectrometry (Srinivas *et al*., 2001) which has been used to generate glycan profiles to aid the discovery of specific glycan biomarkers for BC (Kirmiz *et al*., 2007).

A vast number of preliminary studies have presented a number of potential prognostic and diagnostic markers for BC but to date, none have been approved for use. The discovery that PSA was not prostate-specific but was produced in the tissue and secretions of the female breast as well as breast tumours led to investigations targeting PSA as a potential prognostic indicator of BC. Romppanen and colleagues (1999) demonstrated that measurement of total serum PSA was unable to distinguish healthy women and/ or women with benign breast disease or BC but data presented by Black and Diamandis (2000) suggested that measurement of the level of PSA in nipple aspirate fluid (NAF) could be used as a prognostic tool since PSA concentration in NAF was inversely related to BC risk.

Tumour progression and metastatic disease are problematic in BC and represent a poorer prognosis. The matrix metalloproteinases (MMPs) are a large family of zinc and calcium-dependent extracellular enzymes which have roles in wound healing and angiogenesis (growth of new blood vessels) and along with their inhibitors (TIMPs), have been implicated in breast tumour progression and metastasis. Measurement of the serum concentration of MMP-9 and TIMP-1 has demonstrated that their levels are significantly higher in BC patients compared to healthy controls and women with benign breast disease. High serum levels of both MMP-9 and TIMP-1 were associated with LN metastasis and tumours of higher stage as was high expression of the MMP-9 protein (Wu *et al*., 2008). The authors suggested that these two markers may be useful in predicting progression and prognosis of BC and that future studies may further evaluate their findings. Endothelial growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have also been linked to tumour progression and spread due to their potent angiogenic properties. A preliminary study by Granato *et al*. (2003) proposed bFGF as a non-invasive diagnostic tool for BC since its presence in the serum appeared to be consistent with BC and although it was also detected in the serum of the control

group, the level was significantly different to that of healthy subjects. The authors concluded that further investigation was required in order to determine the usefulness of bFGF in the detection of early disease.

A number of studies have proposed serum riboflavin carrier protein (RCP), a protein required for cell growth and development, as a potential diagnostic and prognostic factor in women with BC (Rao *et al*., 1999; Karande *et al*., 2001). The authors reported significantly elevated RCP serum levels (3-4 fold) among BC patients compared to controls and indicated a good correlation between disease progression and increasing levels of the protein. The data suggested that exploitation of serum RCP levels may provide a useful diagnostic/prognostic marker of BC and in addition to current methods could be useful in screening.

The diagnostic value of Macrophage-colony stimulating factor (M-CSF) in BC has been investigated (Ławicki *et al*., 2006; Ławicki *et al*., 2008). M-CSF is a cytokine involved in the regulation of the growth and differentiation of haematopoietic cells and is referred to as a haematopoietic growth factor. Significantly different levels of serum M-CSF measured by ELISA were found in BC patients compared to that of benign breast disease patients and healthy controls with the level among the BC patients appearing to correlate to the stage of disease. Both groups also reported a positive correlation between serum M-CSF and CA15-3 levels. The combined data suggested that M-CSF may be useful in the diagnosis of BC and in distinguishing between malignant and benign disease.

The tumour suppressor gene p53 has long been implicated in breast cancer since its mutation is one of the most widely accepted genetic alterations in human cancer. P53 is a transcription factor involved in DNA repair mechanisms and the mutated protein has been associated with more aggressive tumours and worse prognosis. Many studies have indicated p53 mutant protein expression as a prognostic factor in BC but a recent article by Balogh and co-workers (2006) proposed its diagnostic potential by reporting that serum mutant p53 protein was increased in patients with invasive

breast cancer and that there was a strong correlation between this level and the accumulation of p53 in breast tumour tissue detected by IHC. The authors concluded that further investigations would be required to fully evaluate its potential use in BC.

Based on its relatively breast-specific pattern of expression (O'Brien *et al*., 2002), the glycoprotein mammaglobin has been investigated as a potentially useful breast tumour marker. The expression of the protein in primary and metastatic breast tumours was studied (Watson *et al*., 1999) with 81/100 primary tumours testing positive for the protein and 10/11 LN from patients with metastatic disease producing detectable amounts of mammaglobin mRNA which was absent from uninvolved LN's. The prognostic potential of the protein was demonstrated with the discovery of a significant correlation between high mammaglobin expression and characteristic features of less aggressive tumour phenotypes such as OR/PR expression, nuclear grade and absence of axillary node involvement (Núñez-Villar *et al*., 2003). The development of a reverse transcription polymerase chain reaction (RT-PCR) assay and a sandwich ELISA for mammaglobin were suggested to be valuable tools in the diagnosis and prognosis of BC with the RT-PCR assay indicating a positive result for mammaglobin in 77% of BC blood samples and the ELISA, positive for the protein in 68% of BC sera studied. When the tests were combined, the sensitivity increased to 84% and the specificity to 97%. In 2005, mammaglobin was investigated for its potential as a serum biomarker for the detection of BC (Bernstein *et al*., 2005). The group demonstrated that their ELISA was highly sensitive and specific for detecting the protein in the sera of BC patients and in differentiating between healthy and BC patients. The authors proposed that mammaglobin has a significant future in the management of the disease and could potentially be used in BC screening. Currently, the serum and tissue levels of an extracellular matrix protein termed tenascin-W are being investigated as diagnostic and prognostic biomarkers in colorectal and breast cancer (Degen *et al*., 2008).

Despite the vast investment into breast cancer biomarker research, the results have yet to yield a specific and sensitive enough factor or combination of factors capable of detecting early disease. The importance of glycosylation with regards to BC has been presented and the emergence of glycomics (Abd Hamid *et al*., 2008; An and Lebrilla, 2010) has ensured that this area of science is ripe for exploitation. The following study sought to address the biomarker problem by targeting AGP microheterogeneity as a potential marker of early breast cancer.

1.4 Research Strategy

During severe physiological and pathological conditions, not only does the total concentration of AGP increase several fold (due to the APR), but the relative proportions of the normal AGP glycoforms have been found to change with abnormal glycoforms being expressed. Increased levels of AGP have been detected in patients with breast cancer and plasma levels have been shown to increase with disease progression. Given the observations that AGP glycosylation is uniquely altered in other disease conditions, it is more than likely that this will also occur in breast cancer.

The main objective of the project is to determine whether disease specific alterations in the glycosylation pattern of AGP could be diagnostic for the detection of breast cancer at an earlier stage than existing methods, and whether it could become the basis for a diagnostic assay based on microarray technology. Verification of this novel hypothesis will significantly advance early detection and treatment of breast cancer and improve survival rates for the disease.

The initial stage in testing this hypothesis will be to isolate AGP from plasma obtained from patients with varying breast conditions such as benign breast disease and breast cancer. AGP will also be isolated from the plasma of healthy donors to act as normal controls and to allow any disease-specific changes in AGP glycosylation to be determined. The method of isolation adopted has previously been shown to maintain the structural integrity of the protein by avoiding desialylation and denaturation reflecting the *in vivo* state.

The structural analysis of AGP glycosylation will be achieved by determining the monosaccharide and oligosaccharide composition of the glycan chains.

Monosaccharide analysis will provide quantitative information on any changes in the monosaccharide composition of the chains in relation to disease and oligosaccharide analysis will determine any disease-specific shift in major microheterogeneity by providing an insight into the extent of branching of the chains. Figure 1.10 summarizes the research strategy of the project.

In summary, the ultimate aim of the following project is to investigate the presence of any structural changes in breast cancer AGP glycosylation, uniquely altered from normal such that an AGP-directed biomarker for early detection of the disease could be developed.

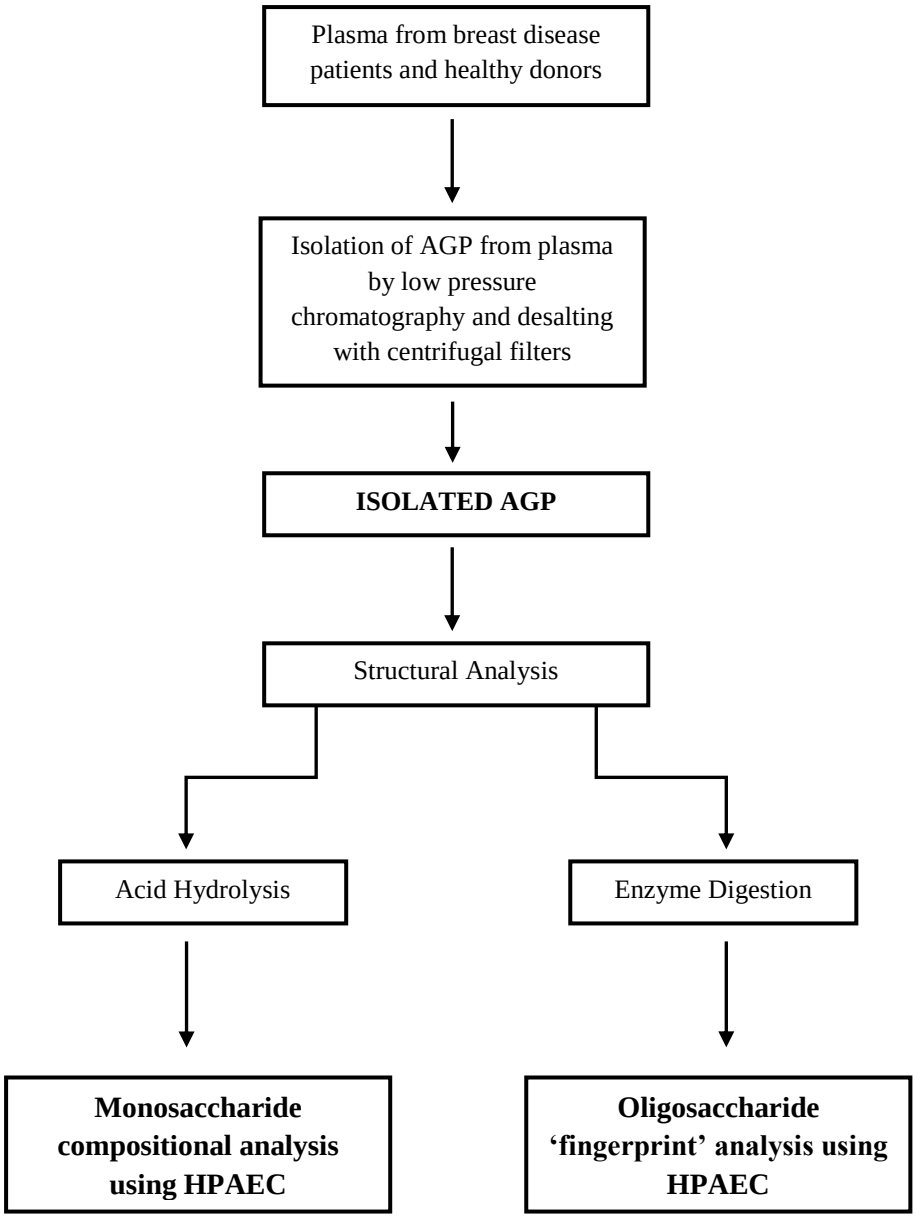


Figure 1.10 Summary of Research Strategy

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Patient Samples

Plasma samples (~500µl of each) representing benign and breast cancer populations were supplied by Dr. Tony Magliocco of the Tom Baker Cancer Institute, Calgary, Canada. Samples were taken from patients attending Dr. Magliocco's clinic in Canada with required consent prior to commencing breast cancer treatment. Table 2.1 details the patient samples obtained with their corresponding diagnosis and classification as either benign breast disease (benign) or invasive breast cancer (invasive). Plasma samples (~1ml of each) representing a 'normal' or breast cancer free population were obtained from Professor David George of the Glasgow Western Infirmary. These samples were taken from an elderly female population living in the Glasgow area. All samples were stored at -20^{o}C prior to use.

Table 2.1 Patient demographics.

Sample Number	Age	Diagnosis	Study Group
S1	62	Benign proliferative breast disease	Benign
S2	48	Benign proliferative breast disease	Benign
S3	77	Invasive ductal carcinoma	Invasive
S4	51	Invasive ductal carcinoma	Invasive
S5	35	Invasive ductal carcinoma	Invasive
S6	39	Benign proliferative breast disease	Benign
S7	35	Malignant phyllodes tumour	Invasive
S8	53	DCIS + invasive ductal carcinoma	Invasive
S9	49	Invasive ductal carcinoma	Invasive
S10	76	Invasive ductal carcinoma	Invasive
S11	65	Invasive ductal carcinoma	Invasive
S12	32	Invasive ductal carcinoma	Invasive
S13	43	Invasive ductal carcinoma	Invasive
S14	40	Biphasic fibroepithelial lesions	Benign
S15	48	Benign proliferative breast disease	Benign
S16	50	Invasive ductal carcinoma	Invasive
S17	69	Invasive ductal carcinoma	Invasive
S18	45	Invasive ductal carcinoma	Invasive
SUB 1	NA	Benign proliferative breast disease	Benign
SUB 2	NA	Malignant phyllodes tumour	Invasive
SUB 3	NA	Invasive ductal carcinoma	Invasive
SUB 4	NA	Infiltrating ductal carcinoma	Invasive
SUB 5	NA	Invasive ductal carcinoma	Invasive
SUB 6	NA	Invasive lobular carcinoma	Invasive
SUB 7	NA	Infiltrating ductal carcinoma	Invasive
SUB 8	NA	Infiltrating ductal carcinoma	Invasive
SUB 9	NA	Fibrocystic	Benign
SUB 10	NA	Fibrocystic	Benign

Data not available (NA).

2.1.2 AGP Isolation

Polyethylene glycol (PEG) 3350, Reactive Blue Sepharose (Cibacron Blue 3GA), potassium chloride (KCl), potassium thiocyanate (KSCN), sodium azide, Trizma base, sodium acetate (NaOAc), sodium chloride (NaCl) and glacial acetic acid were purchased from Sigma-Aldrich, Pool, UK. Disposable 10ml poly prep columns were obtained from Bio-Rad Laboratories Ltd., Hemel Hempstead, UK and Red Sepharose CL-6B was supplied by Amersham Biosciences, UK. Samples and buffer were loaded onto the column using a Pharmacia LKB Pump P-1. The peaks were measured by a single path optical UV-1 monitor and control unit and were recorded by a Servogor 120 chart recorder all of which were obtained from Pharmacia, UK. Rathburn Chemicals Ltd, Walkerburn, UK supplied the high performance liquid chromatography (HPLC) grade water and Millipore Ltd, Hertfordshire, UK supplied the Centricon YM-10 centrifugal filter devices (10,000Da cut-off filters).

Column eluents were concentrated using a Christ RVC 2-18 Concentrator purchased from SciQuip Ltd., Shrewsbury, UK. The samples were spun in an Eppendorf Centrifuge 5415C and a MSE Centaur 2 (large scale).

2.1.3 High pH Anion-Exchange Chromatography

2.1.3.1 Monosaccharide Analysis

Trifluoroacetic acid (TFA) was purchased from Fisher Scientific, Loughborough, UK. Constant boiling hydrochloric acid (HCl), 2ml hydrolysis vials, aluminium lined screw caps and Dowex®-50WX8 cation exchange resin (hydrogen form, 12% cross linked, dry mesh 50-100) were supplied by Sigma-Aldrich, Poole, UK. 50% w/v sodium hydroxide was obtained from VWR International Ltd. Lutterworth, UK. Monosaccharide standards, 2-deoxy-D-galactose, fucose, glucosamine,

galactosamine, galactose and mannose were purchased from Sigma-Aldrich, Poole, UK as was the commercial human AGP standard. HPLC grade water was supplied by Rathburn Chemicals Ltd. Walkerburn, UK. High pH anion exchange chromatography (HPAEC) was carried out on a Dionex 600™ system with pulsed electrochemical detection (PED) purchased from Dionex, Camberley, UK. The system contained a GP50 gradient pump and ED40 electrochemical detector with PeakNet™ software controlled via a Dell OptiPlex GX110 computer. The separation of monosaccharides was carried out using a CarboPac™ PA-100 analytical column (4x250mm) and guard column (4x50mm) obtained from Dionex, Camberley, UK.

2.1.3.2 Oligosaccharide Analysis

Peptide-N-Glycosidase F (PNGase F) purified from *Flavobacterium meningosepticum*, 10% NP-40 and NE Buffer G7 were supplied by New England Biolabs Inc., Hitchin, Hertfordshire, UK. Ethanol for the ethanol precipitation step was purchased from Bamford Laboratories Ltd. Rochdale, UK. The AGP N-linked glycan library was obtained from Prozyme® and distributed by Europa Bioproducts Ltd. Ely, Cambridgeshire, UK. Rathburn Chemicals Ltd. Walkerburn, UK supplied the HPLC grade water. Sodium hydroxide 50% w/v was obtained from VWR International Ltd. Lutterworth, UK. Sodium acetate was purchased from Sigma-Aldrich, Poole, UK. High pH anion exchange chromatography (HPAEC) was carried out on a Dionex 500™ system with pulsed electrochemical detection (PED) purchased from Dionex, Camberley, UK. The system contained a GP50 gradient pump and ED40 electrochemical detector with PeakNet™ software controlled via a Vtech 486X25 computer (Viglen, UK). The separation of oligosaccharides was carried out using a CarboPac™ PA-100 analytical column (4x250mm) and guard column (4x50mm) obtained from Dionex, Camberley, UK.

2.1.4 Immunodiffusion

Multiple pattern immunodiffusion plates were purchased from Fisher Scientific, Loughborough, UK. Anti-human AGP, anti-human albumin and anti-human alpha-1-antitrypsin were obtained from Sigma-Aldrich, Poole, UK as were the commercial standards of human serum AGP, human serum albumin (HSA) and human serum alpha-1-antitrypsin (AAT). Blood group antigen A antibody and Sialyl Tn antibody were purchased from Abcam®, Cambridge, UK.

2.2 Methods

2.2.1 AGP Isolation

2.2.1.1 Polyethylene Glycol Precipitation

Plasma samples were transferred from their collection vials into clean eppendorf tubes and PEG 3350 added to a final concentration of 40% w/v (0.2g PEG added to 500µl patient serum samples and 0.4g PEG added to 1000µl normal serum samples). The contents were mixed thoroughly and refrigerated at 4^{o}C overnight. Following the incubation period, the samples were centrifuged for 30 minutes at 14,000 rpm, the supernatants removed to clean eppendorf tubes and the pellets discarded. The samples were then stored at -20^{o}C until required for protein purification.

2.2.1.2 Low Pressure Chromatography

A 10ml disposable Poly-prep column was packed to approximately 5ml with Cibacron Blue resin. A similar column was also prepared for the Red Sepharose resin. The appropriate elution buffer (Table 2.1) was added to equilibrate the column with the aid of a Pharmacia LKB pump at a flow rate of 0.5ml/min. A Pharmacia optical unit was used to monitor the change in absorbance at 280nm and a Servogor chart recorder set at 12cm/min used to record the separation of the sample.

Table 2.2 details the reagents used in the preparation of all buffers utilised in the following low pressure chromatography procedures.

Table 2.2 Summary of low pressure chromatography isolation buffers.

Chromatography Resin	Buffer	Reagents
Cibacron Blue 3GA Sepharose	Elution	50mM Trizma 0.1mM Potassium Chloride 0.02% Sodium Azide pH 7.0
	Albumin Desorption	0.5M Potassium Thiocyanate 50mM Trizma 0.1mM Potassium Chloride 0.02% Sodium Azide pH 7.0
Red Sepharose CL-6B	Elution	30mM Sodium Acetate 1.15% Glacial Acetic Acid pH 5.7
	Cleaning Buffer	30mM Sodium Acetate 1M Sodium Chloride pH 5.7

The supernatant from the PEG precipitation step was loaded directly onto the prepared Cibacron Blue column and allowed to run through the column with elution buffer. Cibacron Blue binds to the bilirubin binding sites of HSA allowing the AGP containing fraction to elute unchallenged which manifested as a peak on the chart recorder. The eluted peak fractions were collected in 15ml centrifuge tubes and concentrated in a centrifugal concentrator to approximately 2ml. Regeneration was achieved by passing desorption buffer (Table 2.1) through the column to remove the bound HSA. The column was then equilibrated again with elution buffer ready for the next sample.

The concentrated Cibacron Blue column eluents were then loaded onto the Red Sepharose column which had previously been equilibrated with elution buffer (Table 2.1). Red Sepharose was used to remove any remaining AAT allowing a pure AGP fraction to be collected. As with the Cibacron Blue column, AGP eluted in the first peak which was subsequently collected in 15ml centrifuge tubes. Cleaning buffer (Table 2.1) was added to regenerate the column for the next sample by removing any bound AAT. The Red Sepharose column eluents (purified AGP) were concentrated to 2ml in a centrifugal concentrator ready for desalting.

The chromatography columns were stored at 4^{o}C to preserve them for future use and stored in 10% ethanol to limit bacterial growth and to prevent the resin from drying out.

2.2.1.3 Immunodiffusion

The purity of the newly isolated AGP was determined by an immunodiffusion technique on Ouchterlony plates. Aliquots of samples were taken from various stages of the isolation procedure- original plasma, PEG supernatant, Cibacron Blue Sepharose AGP fraction and Red Sepharose AGP fraction, along with appropriate controls- 1mg/ml solutions of human AGP standard, human AAT standard and a HSA standard. Each sample was placed individually into a central antigen well cut into the immunodiffusion plate and was surrounded by AGP, AAT or HSA antisera (undiluted stock supplied by the manufacturer) in the outer wells. Each sample was tested separately with each of the antisera to avoid any cross-reaction. The plates were placed in a moist chamber at 4^{o}C for 48 hours to allow sufficient time for antibody-antigen reactions to occur. A positive reaction was indicated by a white line of precipitation between the central antigen containing wells and the outer antibody/antisera wells.

2.2.1.4 Desalting

Centricon® centrifugal filters were used to remove excess salt from the samples as a consequence of the isolating procedure. Initially, two or three drops of HPLC grade water were added to the cartridge to moisten the membrane. The 2ml concentrated fraction from the Red Sepharose isolation step was then added to the cartridge and spun at 4,000rpm in a centrifuge until all of the sample had passed through the membrane. A separate cartridge was used for each sample to avoid cross-contamination. The sample was washed several times with HPLC grade water before the cartridge was reversed and the protein washed off the membrane. The desalted sample was transferred to a clean eppendorf tube and the contents dried to completion in a centrifugal concentrator. At this stage 1ml of HPLC grade water was

added to reconstitute the sample. The absorbance was measured at 280nm and the AGP concentration determined using a standard curve.

2.2.2 AGP Concentration Determination

A set of commercial human AGP standards ranging from 0-3mg/ml were prepared in HPLC grade water. The absorbance was measured at 280nm and a standard curve of AGP concentration (mg/ml) versus absorbance was produced. The absorbance of the isolated AGP (reconstituted in 1ml HPLC grade water) from each sample was measured and the AGP concentration determined from the standard curve.

2.2.3 High pH Anion-Exchange Chromatography

2.2.3.1 Acid Hydrolysis

100μl 2M trifluoroacetic acid and 50μl 4M hydrochloric acid were added to 50μg of AGP in a glass reaction vial. An aluminium lined cap was added and the vials placed on a heat block for 4 hours at 100^{o}C to undergo the hydrolysis reaction.

Glass wool was packed into glass Pasteur pipettes to a depth of approximately 1cm. Dowex-50 WX8 H^{+} cation exchange resin was added to a bed volume of 1ml. A Dowex column was prepared for each separate sample to avoid any cross-sample contamination. The column was washed with 6ml of HPLC grade water before application of the sample. Once the AGP sample was loaded, the column was washed with a further 2ml of HPLC grade water and the released monosaccharide's collected

in eppendorf tubes. The samples were dried to completion under a vacuum ready for monosaccharide analysis.

2.2.3.2 Monosaccharide Analysis

Analysis of the monosaccharide content of an AGP sample was performed by HPAEC on a Dionex 600^{TM} system with PED. The pulse potentials utilised during PED were as follows- 0 seconds (sec), 0.05V; 0.29sec, 0.05V; 0.49sec, 0.05V; 0.5sec, 0.05V; 0.51sec, 0.6V; 0.6sec, 0.6V; 0.61sec, -0.6V; 0.65sec, -0.6V; 0.66sec, 0.05V (see Figure 2.1).

The CarboPac PA-100 pellicular anion exchange column was initially regenerated with 50% 1M NaOH and 50% HPLC grade water for 15 minutes. 20μl of HPLC grade water and 5μl of internal standard (2-deoxy-D-galactose, 1mg/ml) was added to each of the dried hydrolysed AGP samples. This 25μl solution was then applied to the column and the monosaccharide's separated at pH 13 with an isocratic elution of 3% 1M NaOH/ 97% HPLC grade water for 35 minutes at a flow rate of 0.5ml/min. The column was then regenerated with 50% NaOH/ 50% HPLC grade water for 15 min ready for the next separation.

Identification of the monosaccharide peaks was achieved by dividing the elution time of the peaks with that of the internal standard and comparing the ratios to that of known monosaccharide standards. For each of the monosaccharide's present on AGP (fucose, galactose, glucosamine and mannose) standard curves of monosaccharide amount (μg) versus peak area were produced in order to quantify the amount of each monosaccharide present in the AGP samples.

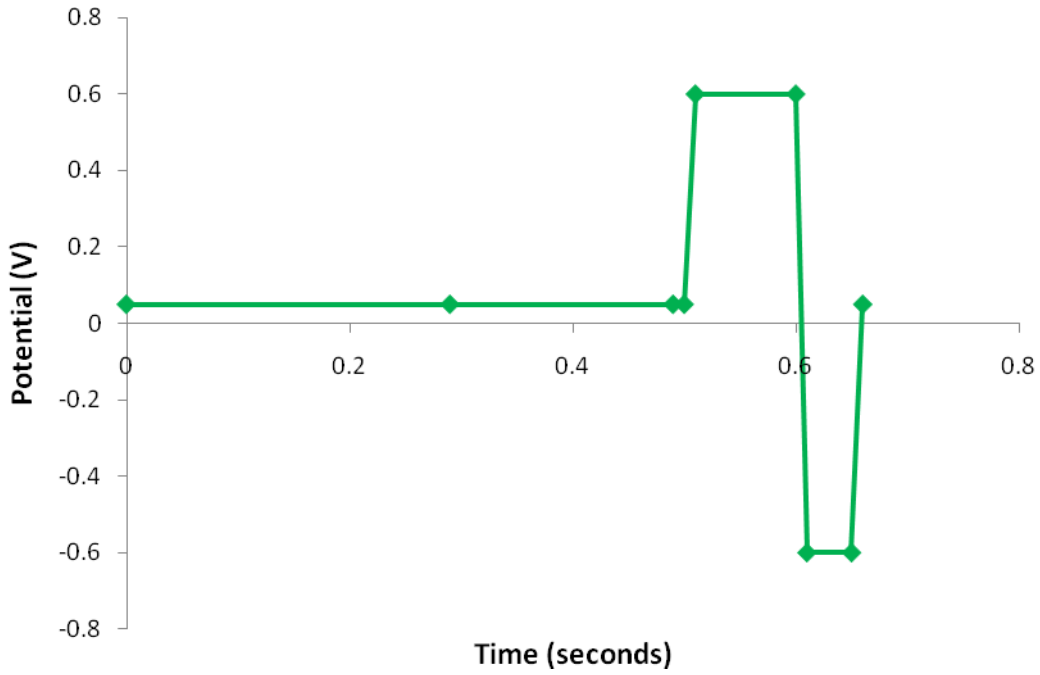


Figure 2.1 Pulsed electrochemical detection. Pulsed potentials utilised during high pH anion exchange chromatography.

2.2.3.3 Immunodiffusion

In order to characterise the GalNAc containing component observed during monosaccharide analysis of the patient samples an immunodiffusion technique was employed. This allowed investigation into potential antibody-antigen reactions between the patient samples and antibodies against known GalNAc containing structures such as the tumour-associated antigen Sialyl Tn (STn) and Blood Group A antigen. Due to the large number of samples to be tested, the antibodies (undiluted stock supplied by the manufacturer) were placed in the individual central wells of the immunodiffusion plates and the samples (isolated AGP samples) of interest in the surrounding outer wells. Each sample was tested with both antibodies. The plates were placed in a moist chamber at 4^{o}C for 48 hours to allow sufficient time for antibody-antigen reactions to occur. A positive reaction was indicated by a white line of precipitation between the central antibody containing wells and the outer sample/antigen wells.

2.2.3.4 Enzymatic Digestion

Approximately 100µg of AGP was reconstituted in 100µl of HPLC grade water. The solution was transferred to a glass vial, topped with an aluminium lined cap, placed on a heating block and denatured at 100^{o}C for 3 hours. The denatured AGP solution was then dried to completion under a vacuum.

The denatured AGP was prepared for enzyme digestion by reconstituting in 79µl HPLC grade water, 10µl NE Buffer G7 and 10µl 10% NP-40 (supplied by New England Biolabs with the PNGase F enzyme). Approximately 5 units (U) of PNGase F enzyme was added to make a total volume of 100µl. The reaction mixture was

incubated at 37^{o}C for 24 hours after which another 5U of PNGase F was added and the reaction mixture incubated for a further 24 hours at 37^{o}C.

After incubation with the enzyme, an ethanol precipitation step was required to separate the protein fragments from the oligosaccharides of interest. Ice-cold 80% ethanol was added to the reaction mixture in the ratio of 3 parts ethanol to 1 part reaction mixture (the total volume of the reaction mixture was approximately 100µl therefore 300µl of ethanol was added). The samples were mixed thoroughly and placed at -20^{o}C overnight. The samples were then centrifuged at 14000rpm for 30 minutes and the oligosaccharide containing supernatant removed to clean eppendorf tubes. The pellet was discarded and the supernatant dried to completion under a vacuum ready for oligosaccharide analysis.

2.2.3.5 Oligosaccharide Analysis

Analysis of the AGP oligosaccharide profiles was performed by HPAEC on a Dionex 500^{TM} system with PED. The pulsed potentials were the same as for monosaccharide analysis (see section 2.2.3.2). The CarboPac PA-100 pellicular anion exchange column was initially regenerated with 50% 1M NaOH and 50% HPLC grade water for 20 minutes before use. The digested AGP samples were reconstituted in 25µl HPLC grade water and applied to the CarboPac PA-100 column. The oligosaccharides were separated with eluent conditions as follows- equilibration with 10% NaOH/ 5% 1M NaOAc/ 85% HPLC grade water for 10 minutes; separation for 45 minutes with a linear gradient to a final eluent composition of 10% NaOH/ 20% NaOAc/ 70% HPLC grade water; regeneration for 10 minutes with 50% NaOH/ 50% HPLC grade water and finally equilibration with 10% NaOH/ 5% NaOAc/ 85% HPLC grade water for 5 minutes, all at a flow rate of 1ml/min.

A human N-linked oligosaccharide library (approximately 5µg) was run as a reference profile to which the sample and commercial AGP profiles could be compared.

2.2.4 Statistical Analysis

Microsoft® Excel 2000 was used to calculate the Pearson correlation coefficient (R^2). A Pearson correlation coefficient of 1 represents a perfect linear fit, with correlations ranging from 0.7 to 1 representative of a strong linear relationship. The mean and standard deviations were calculated using Microsoft® Excel 2007. The statistical significance of the data was determined by Mann-Whitney U test on the statistical software package, Analyse-it for Microsoft® Excel.

CHAPTER 3

PATIENT DEMOGRAPHICS

3.1 Patient Details

Twenty-eight patient plasma samples were obtained from females prior to the initiation of therapy or treatment. Table 3.1 details the clinical information obtained for each subject recruited in the study including age, individual diagnosis and receptor status. The age of patients at enrolment ranged from 32 to 77 years with a mean and standard deviation (S.D) of 52.4 ± 14.7 years. Data on the age of ten of the subjects in the study group was unavailable. The sample group consisted of patients with a number of benign breast conditions such as fibrocystic and benign proliferative breast disease and invasive carcinomas such as invasive ductal/ lobular carcinoma and phyllodes tumour. The patient samples were grouped into two populations termed benign and invasive depending on their clinical diagnosis. Table 3.1 details the group to which each patient/sample was assigned.

The benign group consisted of 8 subjects (n=8) with a mean age of 47.4 years (S.D ± 9.2 years) accounting for 28.6% of the total patient population. Twenty patient samples (n=20) with an age averaging 52.3 years (S.D ± 15.2 years) were assigned to the invasive group. This was the largest group in the study (71.4% of total patient population) and consisted predominantly of patients with invasive ductal carcinoma (>80% of invasive group). Table 3.2 summarises the data detailed above on the individual sample populations used in the study.

Table 3.1 Patient clinical data.

Sample Number	Age	Diagnosis	Study Group	OR/PR Status	HER-2 Status
S1	62	Benign proliferative breast disease	Benign	-	-
S2	48	Benign proliferative breast disease	Benign	-	-
S3	77	Invasive ductal carcinoma	Invasive	OR+ / PR-	Negative
S4	51	Invasive ductal carcinoma	Invasive	OR+ / PR+	Negative
S5	35	Invasive ductal carcinoma	Invasive	OR+ / PR+	Negative
S6	39	Benign proliferative breast disease	Benign	-	-
S7	35	Malignant phyllodes tumour	Invasive	NA	Negative
S8	53	DCIS + invasive ductal carcinoma	Invasive	OR+ / PR+	Negative
S9	49	Invasive ductal carcinoma	Invasive	OR+ / PR+	Positive
S10	76	Invasive ductal carcinoma	Invasive	OR+ / PR+	Negative
S11	65	Invasive ductal carcinoma	Invasive	OR+ / PR+	Negative
S12	32	Invasive ductal carcinoma	Invasive	OR+ /PR+	Positive
S13	43	Invasive ductal carcinoma	Invasive	OR+ / PR-	Negative
S14	40	Biphasic fibroepithelial lesions	Benign	-	-
S15	48	Benign proliferative breast disease	Benign	-	-
S16	50	Invasive ductal carcinoma	Invasive	OR+ /PR+	Positive
S17	69	Invasive ductal carcinoma	Invasive	OR+ /PR+	Negative
S18	45	Invasive ductal carcinoma	Invasive	OR- / PR-	Positive
SUB 1	NA	Benign proliferative breast disease	Benign	-	-
SUB 2	NA	Malignant phyllodes tumour	Invasive	NA	Negative
SUB 3	NA	Invasive ductal carcinoma	Invasive	OR+ / PR+	Positive
SUB 4	NA	Infiltrating ductal carcinoma	Invasive	OR+ / PR-	Negative
SUB 5	NA	Invasive ductal carcinoma	Invasive	NA	Negative
SUB 6	NA	Invasive lobular carcinoma	Invasive	OR+ / PR+	Negative
SUB 7	NA	Infiltrating ductal carcinoma	Invasive	OR- / PR-	Positive
SUB 8	NA	Infiltrating ductal carcinoma	Invasive	OR+ / PR+	Negative
SUB 9	NA	Fibrocystic	Benign	-	-
SUB 10	NA	Fibrocystic	Benign	-	-

Oestrogen receptor / progesterone receptor status (OR/PR), tumour tested positive (+) or negative (-). HER-2/neu protein status (HER-2). Data not available (NA).

Table 3.2 Summary of sample populations.

Sample Population	Number of Samples	% of Cohort	Average Age (years)
Benign	8	28.6	47.4
Invasive	20	71.4	52.3

The receptor status of the patients within the breast cancer population is presented in Table 3.1. Patients with oestrogen receptor positive (OR+) tumours had an average age of 54.5 ± 15.4 years and represented 88.2% of the total cancer population. Tumours testing positive for the HER-2/neu protein (HER-2) were presented by slightly younger women (average age 44.0 ± 7.2 years) and represented 28.6% of the cancer population. Four patients tested positive for the presence of both the OR and HER-2 receptor.

The patient samples utilised in the study were allocated a new identification number to reflect the patient population (benign and invasive) to which they were assigned. Table 3.3 details the samples present in each of the two groups, their previous identification number and the new study identification number used to identify the sample throughout the duration of the investigation.

Table 3.3 Classification of breast disease samples. B- Benign, I- Invasive.

Sample Group	Identification Number	Study Identification
Benign	S1	B1
	S2	B2
	S6	B3
	S14	B4
	S15	B5
	SUB 1	B6
	SUB 9	B7
	SUB 10	B8
Invasive	S3	I1
	S4	I2
	S5	I3
	S7	I4
	S8	I5
	S9	I6
	S10	I7
	S11	I8
	S12	I9
	S13	I10
	S16	I11
	S17	I12
	S18	I13
	SUB 2	I14
	SUB 3	I15
	SUB 4	I16
	SUB 5	I17
	SUB 6	I18
	SUB 7	I19
	SUB 8	I20

Discussion

There are a number of signs and symptoms of BC the most common being the presence of an unexplained lump. Malignant masses in most cases are hard and painless with irregular edges and are difficult to move. Benign masses are usually rubbery with distinct margins and can in the majority of cases, be moved easily under the skin. Generalised swelling of the breast may be noticed in malignant disease, and swelling under the arm may indicate the spread of the cancer to the lymph nodes even before the initial lump is found in the breast tissue. Irritation or dimpling of the skin and redness/scaliness of the nipple are also common signs as well as pain, discharge or retractions of the nipple.

A complete medical history and physical exam are the initial steps when evaluating a woman with suspected breast cancer. The breasts are thoroughly examined to locate any lumps and to determine their size and texture. Changes to the nipples or skin as well as any swelling of the breast and lymph areas are also noted. A variety of imaging tests are then carried out and possibly a breast biopsy performed in order to reach a definitive diagnosis (Houssami *et al*., 2006).

Mammography is the most widely recognised technique in BC diagnosis and is used generally in the national screening programme of women who are at high risk of developing the disease. Currently it is the only technique proven to be effective in population screening of asymptomatic individuals. The process utilises low dose X-rays and compresses the breast between two plates allowing the best possible image to be taken. It is now common for mammograms to be generated digitally (Full-field digital mammography, FFDM) which speeds up the process by removing the need for film development and also ensures that the images are sent straight to the specialist for analysis. The introduction of screening mammography has been attributed to a reduction in BC mortality rates since the disease is now diagnosed at

an earlier stage where prognosis is improved (Silva and Zurrida, 2005, Chapter 5). A diagnostic mammogram will be performed on symptomatic individuals or if a screening mammogram requires further investigation but it is important to note that this technique cannot reliably distinguish benign from malignant lesions but is useful for defining the size of the lesion, determining its location prior to biopsy and in evaluating the presence of new masses or calcifications after surgery.

Ultrasound utilises high frequency sound waves to generate an image of the breast and can be used to target areas of concern from mammograms or in the screening of younger women with high breast density since a clear image cannot be produced with mammography. It is an extremely useful technique with the ability to distinguish between harmless fluid-filled cysts and solid masses which warrant further investigation.

Magnetic resonance imaging (MRI) has been approved as a supplementary tool to mammography and ultrasound and uses magnetic fields to generate a detailed image of the breast and has proven useful in the staging of cancer (Goethem *et al*., 2006) and in testing women with dense breasts. The use of MRI in screening is currently being investigated due to its high sensitivity but the technique does have reduced specificity compared with others and has resulted in a high number of false positives and subsequent biopsies. In addition to this, MRI does not detect calcifications which may be indicative of non-invasive carcinomas. Despite its limitations recent studies have identified breast MRI as an important tool in the pre-operative assessment and management of patients with BC (Goethem *et al*., 2006; Mukherjee *et al*., 2008; Vallow *et al*., 2008; Crowe *et al*., 2009).

If cancer is suspected from the results of the physical exam, mammogram or ultrasound then a biopsy will be performed. This procedure involves removing a sample of cells or tissue from the suspect mass to examine under the microscope and

is the only technique able to give a definitive diagnosis of cancer. There are three types of biopsies termed fine needle aspiration (FNA) biopsy, core biopsy and surgical biopsy. FNA biopsy involves the insertion of a very fine needle into the mass to remove fluid or cells for examination. Ultrasound may be utilised during this technique to guide the needle to the area of concern. If the mass contains fluid, then this is indicative of a cyst but if the mass is solid then laboratory tests are required to determine if it is benign or malignant. Advantages of FNA biopsy include its simplicity, minimal patient discomfort and relatively low cost but this procedure cannot reliably distinguish non-invasive from invasive carcinoma.

If results from FNA biopsy are inconclusive a core needle biopsy may be performed to remove a small cylinder of tissue from the lump to aid further analysis. Surgical biopsy removes a large amount of tissue and can be either incisional, where only part of the lump is removed or excisional where the entire lump is removed. The biopsy of a suspect mass can yield a number of results. If cancer is not present then continuation of routine mammograms will be advised, if the lump is not cancerous but the cells were abnormal then increased surveillance in the form of regular breast exams will be advised. If laboratory analysis of the mass tests positive for cancer and if an appropriate amount of surrounding healthy tissue has been removed during an excisional biopsy then this can serve as a lumpectomy in many cases and follow-up treatment can begin.

A variety of laboratory tests can be performed on malignant tissue removed during a biopsy procedure which can provide valuable prognostic information. Prognostic factors provide information on the behaviour of a disease and its likely outcome in the presence and absence of therapy (Silva and Zurrida, 2005, Chapter 10). The primary prognostic factors with regards to BC include hormone receptor status, lymph node (LN) status, tumour size and tumour grade.

The axillary lymph nodes are located under the arm and are the primary site of breast lymph drainage. The nodes are large and vary in number between twenty and thirty. Determination of LN involvement in BC can provide valuable prognostic information on an individual with a low number of positive lobes (1-3) correlated to a reassuring prognosis (Valagussa *et al*., 1978). Determination of tumour size can give some indication of potential nodal involvement with increasing size correlated to increased probability of positive axillary lymph nodes. Any invasive carcinoma, ductal or lobular, less than 1cm in diameter correlates with a good prognosis.

The hormone receptor status of a tumour can be assessed by subjecting a biopsy tissue specimen to a technique termed immunohistochemistry (IHC) which using antibodies to locate receptors and tissues of interest. A positive test result for the oestrogen receptor (OR) and the progesterone receptor (PR) indicates that the tumour is fuelled by the presence of the hormones oestrogen and progesterone and may respond to hormonal therapy the ultimate aim of which is to block their action. OR positive tumours tend to occur in post-menopausal women and have a more reassuring prognosis than OR negative tumours. Assessment of the receptor status of the protein human epidermal growth factor receptor 2 (HER-2/neu) can also provide a good indication of prognosis and response to therapy. This protein is involved in epithelial cell growth and differentiation and overexpression of the HER-2/neu gene occurs in 20-30% of BC cases. Two techniques are available to determine the receptor status of the HER-2 protein. IHC is used to measure tumour production of the HER-2 protein and Fluorescent In Situ Hybridization (FISH) is used to measure the gene amplification of the protein through the use of fluorescent probes. HER-2 positive tumours are associated with more aggressive forms of BC and are commonly found in inflammatory BC and in Paget's disease of the nipple. A positive test result for the HER-2 protein can also indicate a positive response to therapy with Herceptin® (trastuzumab).

In the current study it was vital that the samples obtained were from newly diagnosed breast cancer patients (representing an early breast cancer population) who had not yet entered into their schedule of treatment. AGP has been reported to bind a number of different drugs (Israili and Dayton, 2001, Johnson and Smith, 2006) including the breast cancer drug Tamoxifen, used to treat hormone receptor positive disease (Chatterjee and Harris, 1990). This binding has the ability to not only reduce the pharmalogical effectiveness of the drug in question but also lower the free plasma concentration of AGP, masking the true level present. The wide array of drugs used in the treatment of breast cancer may have an impact on any disease-specific glycoforms demonstrated by AGP, so in order to ensure that the plasma level, glycosylation patterns and expressed glycoforms were representative of the disease, the analysis was carried out on treatment-free patient samples.

After gender, age is the single biggest risk factor for BC with 80% of cases occurring in post-menopausal women (generally > 50 years of age). The sample population in this study covered a wide range of ages from 32-77 years with 47.4% representative of women 50 or older. The relatively small sample population and the random nature in which the samples were chosen may explain why the study population was not representative of the breast cancer population as a whole.

Invasive (or infiltrating) ductal carcinoma is the most common form of BC and represents approximately 75-80% of all diagnosed cases (Silva and Zurrida, 2005; www.cancerscreening.nhs.uk). The cancer population in the study cohort contained 80.9% patients with a diagnosis of invasive ductal carcinoma which is therefore in agreement with the national statistic.

Patients exhibiting OR+ tumours tend to be older and present with a good prognosis (Silva and Zurrida, 2005) indicating that hormonal therapy with Tamoxifen may be a successful route of treatment. Of the patients in this study 88.2% were OR+ with an

average age of 54.5 years, consistent with the idea that this receptor status is most common amongst post-menopausal women. Overexpression of the HER-2/neu protein occurs in 20-30% of BC cases (Silva and Zurrida, 2005) and is associated with a more aggressive form of the disease. 28.6% of patients in the sample population tested positive for the HER-2/neu protein and were associated with younger women at time of diagnosis (average age 44.0 years).

CHAPTER 4

AGP ISOLATION

RESULTS AND DISCUSSION

4.1 Isolation and Purification of AGP

RESULTS

In order to accurately investigate the glycosylation of AGP in relation to breast cancer, the protein must initially be separated from constituent plasma proteins, the majority of which are also glycosylated. An essential requirement of the isolation process is that it does not degrade or structurally alter the AGP protein or its glycan chains to aid investigation of the native, *in vivo* state. Since its discovery, there have been a wide array of methods described for the isolation and purification of the protein utilising various techniques, some of which have been more successful than others in retaining the desired *in vivo* structure.

The first isolation and subsequent characterisation of AGP was carried out in the 1950's (Schmid, 1950; Weimer *et al*., 1950) and utilised ammonium sulphate precipitation to isolate the protein. The various steps employed during this procedure exposed the protein to harsh acidic conditions which would have inevitably resulted in desialylation. The first large scale fractionation of AGP was presented in 1973 by Hao and Wickerhauser who described a two-stage purification procedure of Cohn Fraction V supernatant, of which AGP was a component, using ion-exchange chromatography. The first step involved absorption of AGP onto diethylaminoethyl (DEAE)-Sephadex with the subsequent DEAE eluate subject to ion-exchange chromatography on a carboxymethyl (CM)-cellulose column. The formation of Cohn Fraction V required an acidic pH (pH4.7) as did the first step in the purification process since the successful removal of albumin from the preparation required that the conditions were kept close to the proteins isoelectric point (~4.7). Structural alteration of the glycan chains of AGP would occur as a result of these acidic conditions.

A decade later, a three-stage purification procedure was documented (Laurent *et al*., 1984) using pseudo-ligand affinity chromatography on Cibacron Blue F3GA and Procion Red HE3B (Red Sepharose) followed by isoelectric focusing (IEF) to isolate and purify AGP from the plasma. The eluent from the Cibacron Blue chromatography was found to contain the desired AGP as well as traces of AAT, prealbumin, α2-HS glycoprotein, transferrin, ceruloplasmin, IgA and IgG. Application of the Cibacron Blue fraction to the Procion Red column previously equilibrated with NaOAc buffer yielded an unbound fraction containing AGP together with AAT and trace quantities of prealbumin. Preparative IEF was used to remove the residual contaminants of the chromatography procedures. The purity of the protein was determined by gel and immunoelectrophoresis and a final yield of 88% was quoted by the authors. The method described by Laurent and colleagues managed to avoid the use of acidic conditions (pH 7.0 and 5.8) therefore allowing the integrity of the AGP structure to be maintained throughout the process, which was confirmed by CAIE analysis of the glycan chains using the Con-A lectin.

A two-step chromatographic purification of AGP was detailed by Hervé *et al*. (1996) using Cibacron Blue F3GA cross-linked to agarose followed by chromatography on hydroxyapatite. This isolation technique was viewed by the authors as advantageous when compared with previously published methods due to the small number of steps involved and the commercial availability of the chromatography gels. The group reported an AGP yield of 80% and managed to avoid structural degradation of the protein (confirmed by analytical IEF) by committing to the use of buffers outwith the acidic range. More recently, a technique developed to isolate and separate the genetic variants of AGP has been described (Azzimonti *et al*., 2003). This single-step procedure involves Cibacron Blue F3GA chromatography and requires a change in the pH of the buffer from 4.0 to 4.95 to elute the ORM2 and ORM1 variants respectively. Acidic buffers were an obvious feature of this method inevitably resulting in structural degradation to the protein.

The AGP isolation technique adopted in this research study was based on the method developed by Smith *et al.* (1994). This method utilised a three column chromatography procedure, similar to the Laurent *et al.* (1984) method, avoiding the use of acidic buffers allowing the production of a final AGP preparation free from structural alteration. Smith and colleagues could not reproduce the results obtained by the Laurent group which resulted in the removal of the IEF step and addition of a Q-sepharose column. By basing the isolation procedure on this technique we were confident that subsequent results obtained from the glycosylation analysis of patient samples were representative of the disease state and not due to any alteration induced by the purification procedure.

The initial volume of patient plasma (~ 500µl) obtained made it necessary to adapt and develop a micro-scale version of the Smith method in order to recover the greatest yield of AGP for subsequent investigation. Initially the plasma samples were precipitated overnight with PEG 3350 and the protein purified from the remaining plasma components by a two-step low pressure chromatography procedure (Figure 4.1). The supernatant was applied to a column containing Cibacron Blue 3GA Sepharose which bound human serum albumin (HSA) allowing the AGP containing fraction to elute unhindered. The chart recorder trace presented in Figure 4.1a demonstrates that elution of the AGP fraction occurred approximately 10 minutes after sample application upon which, collection was initiated and continued until a baseline measurement was reached. At this stage the application of desorption buffer (Table 2.1) was necessary to regenerate the column with the removal of bound albumin a process which took approximately 40 minutes to complete (Figure 4.1a). The retained fraction was then applied to a column containing Red Sepharose CL-6B to remove remaining impurities such as α_1-antitrypsin (AAT). The AGP fraction eluted approximately 10 minutes after sample application (Figure 4.1b) and was collected for further analysis. Column regeneration was achieved in approximately 35 minutes with the addition of a cleaning buffer (Table 2.1).

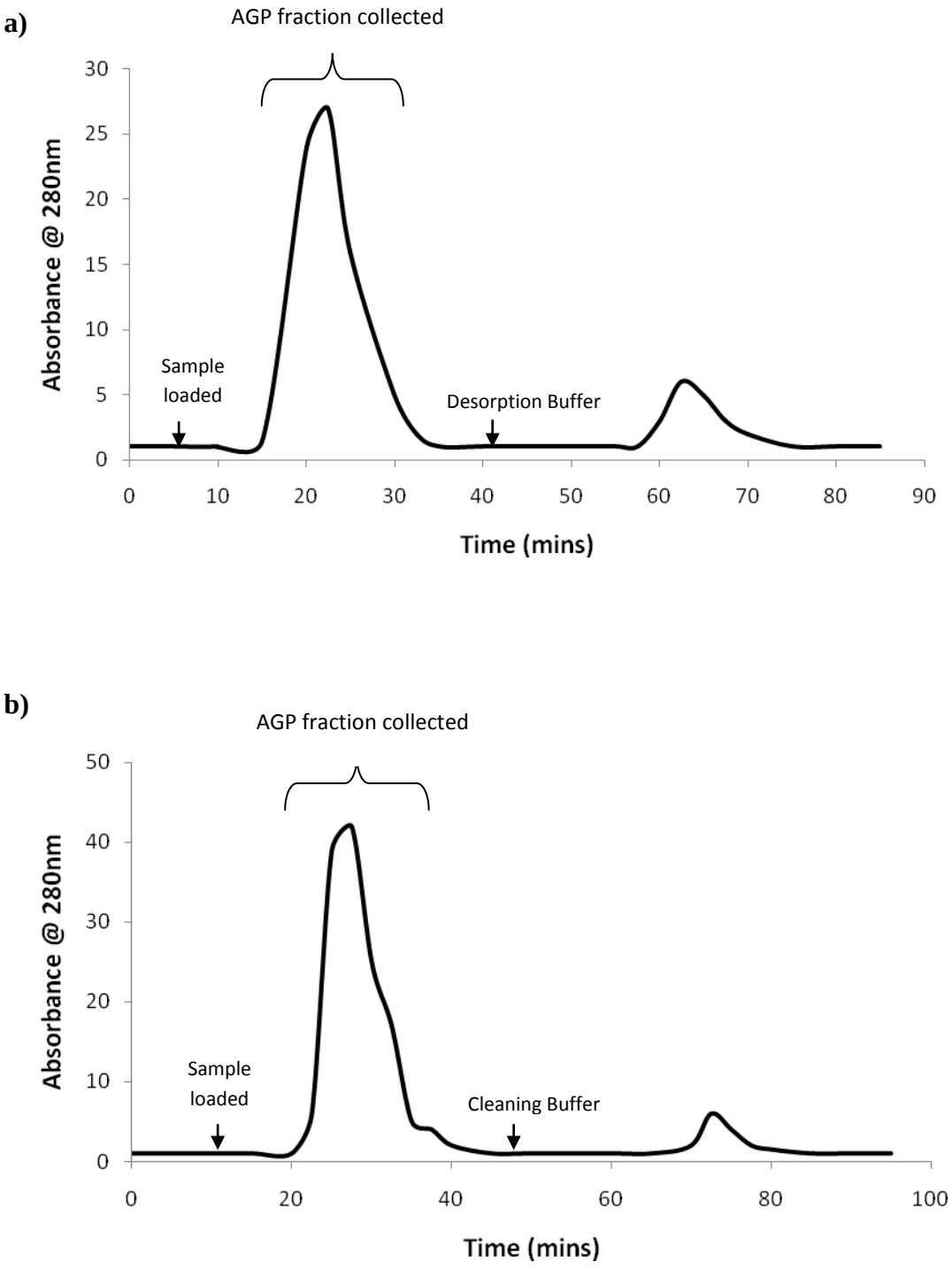


Figure 4.1 Isolation of AGP by low pressure chromatography. Chart recorder traces from a) Cibacron Blue Sepharose column and b) Red Sepharose column.

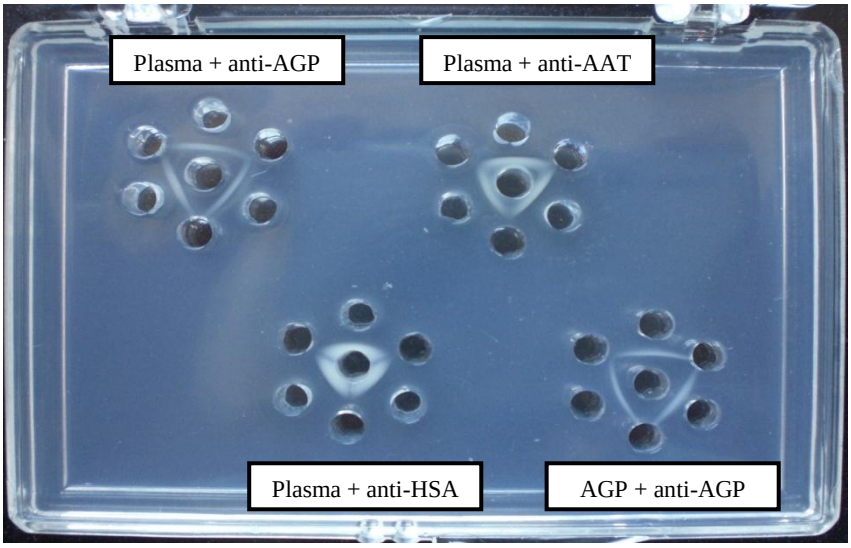
As a consequence of the low pressure chromatography procedure, the newly isolated AGP must be subject to further purification to remove excess salt introduced by the sodium acetate (NaOAc) elution buffer utilised during the Red Sepharose chromatography step. It is essential that this contaminant is removed as it may interfere with the subsequent absorbance calculation of the sample resulting in an inaccurate representation of the total amount of AGP isolated. Due to the small volume of the isolated AGP preparations, Centricon centrifugal filter cartridges were used to desalt and concentrate the samples.

4.1.1 Determination of AGP Purity

RESULTS

For the purpose of this research study, it was essential to preserve as much AGP from patient samples as possible to aid thorough analysis of the proteins glycosylation so that any apparent disease-specific alterations could be identified and evaluated. In order to address this issue it was deemed necessary to remove the Q-Sepharose chromatographic step from the isolation procedure since in this instance, a slight reduction in protein purity was a more favourable consequence than a loss of total protein. Immunodiffusion experiments were carried out in order to ascertain the extent of purity sacrificed by the removal of this step. Figure 4.2 details the results of the immunodiffusion experiment.

a) Plate 1



b) Plate 2

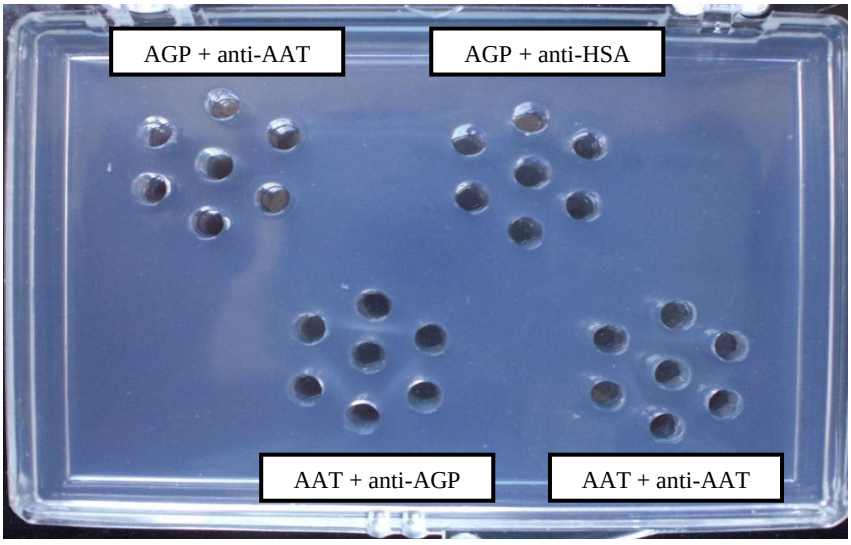
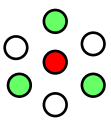
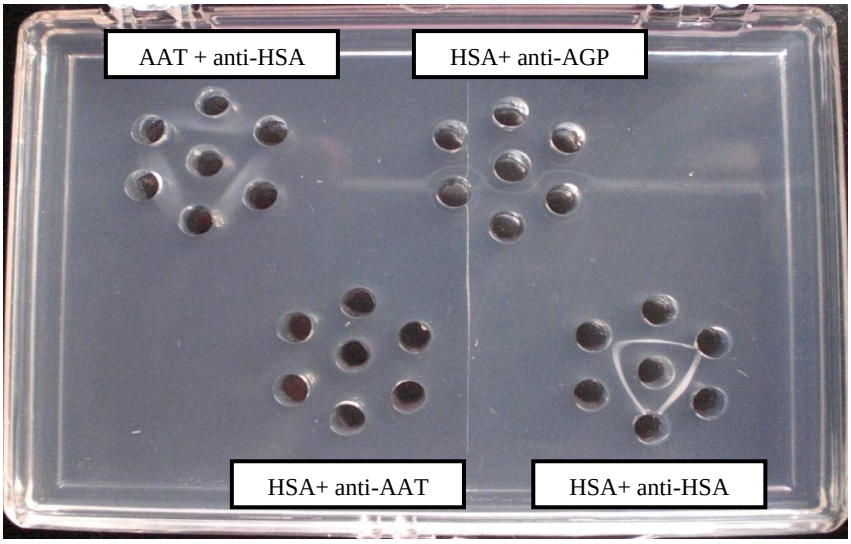


Figure 4.2 Immunodiffusion plate results for a) original plasma and AGP standard b) AGP and AAT standards. Antigens (protein standard or aliquot from isolation procedure) placed in central red wells and antibodies (AGP, AAT or HSA) placed in outer green wells as shown. A positive antibody-antigen reaction was indicated by a white line of precipitation.



c) Plate 3



d) Plate 4

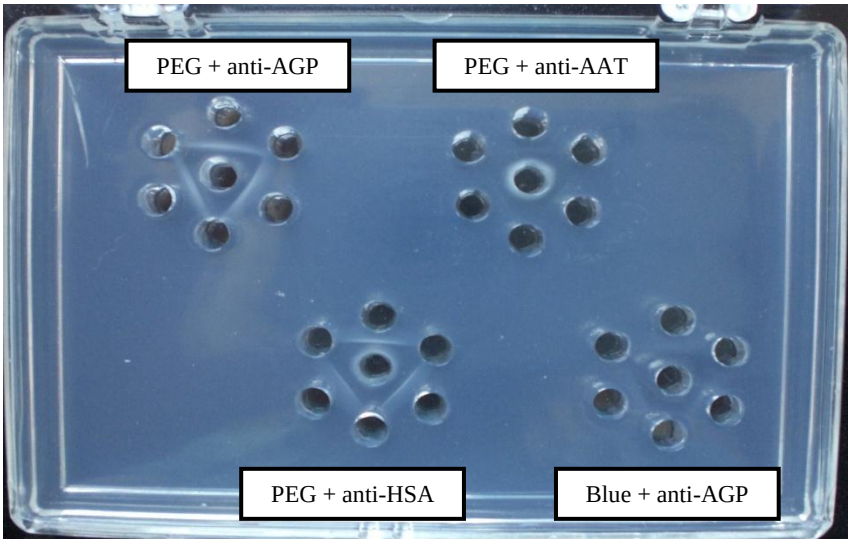
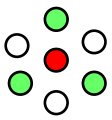
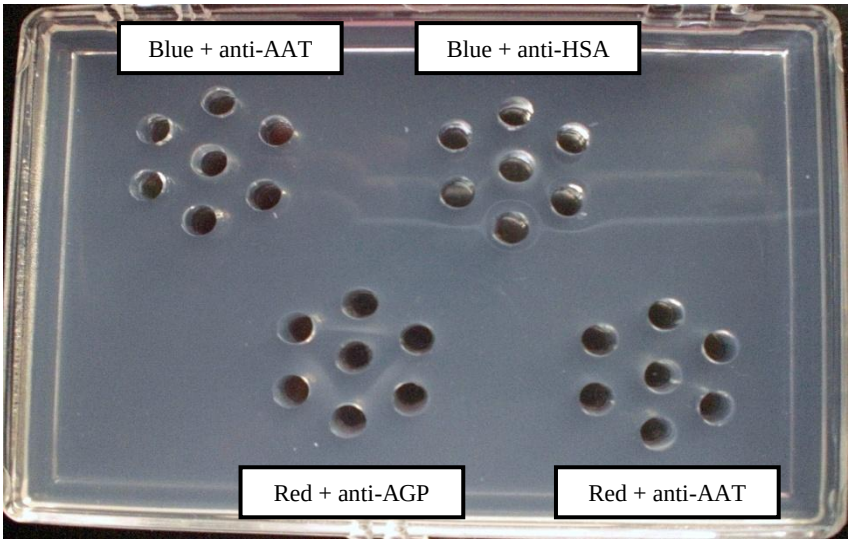


Figure 4.2 cont. Immunodiffusion plate results for c) AAT and HSA standards d) PEG supernatant (PEG) and Cibacron Blue AGP fraction (Blue). Antigens placed in central red wells and antibodies placed in outer green wells as shown. A positive antibody-antigen reaction was indicated by a white line of precipitation.



e) Plate 5



f) Plate 6

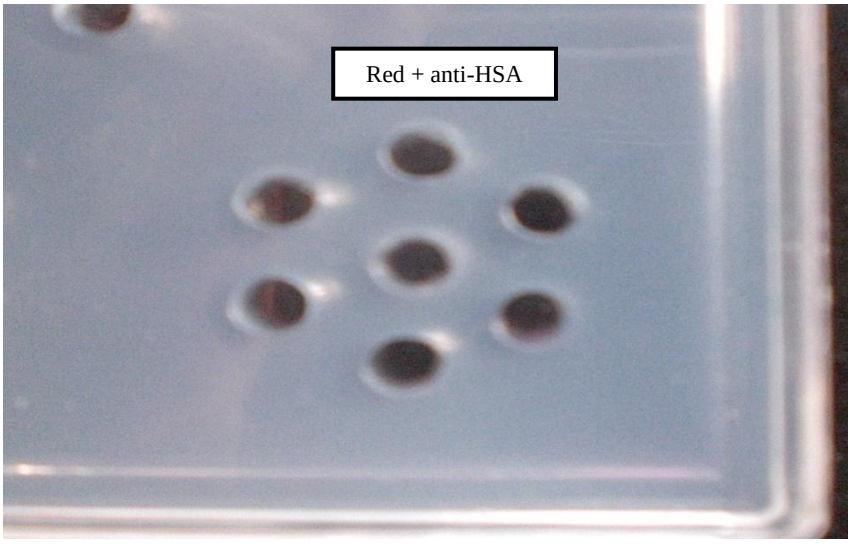
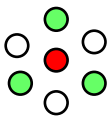


Figure 4.2 cont. Immunodiffusion plate results for e) Cibacron Blue AGP fraction (Blue) and Red Sepharose AGP fraction (Red) f) Red Sepharose AGP fraction (Red). Antigens placed in central red wells and antibodies placed in outer green wells as shown. A positive antibody-antigen reaction was indicated by a white line of precipitation.



The immunodiffusion results for the original plasma sample presented in Figure 4.2a show strong lines of precipitation between the central antigen well and the outer antibody wells containing AGP AAT and HSA antisera. This demonstrates the presence of the AGP, AAT and HSA proteins in the initial plasma sample since a positive reaction to all three antisera was produced. A positive reaction between the commercial AGP standard and the anti-AGP antibody was produced demonstrating the ability of the antibody to identify and confirm the presence of the protein (Figure 4.2a). No reaction (no precipitation) was observed with the AGP standard and the AAT and HSA antisera indicating that the commercial preparation of AGP was free from both of these proteins (Figure 4.2b). Commercial HSA reacted only with its own antiserum (anti-HSA) demonstrating the ability of the antibody to recognise its antigen (Figure 4.2c). The lack of reaction between the HSA standard and the AGP and AAT antisera indicates that the preparation was free from these protein contaminants. The commercial AAT standard produced a positive reaction with all three antisera (Figure 4.2b and c). The AAT antiserum did not react with the AGP or the HSA standards (Figure 4.2 b and c) which therefore rules out any specificity issues of the AAT antibody. In this case the results appear to indicate that both AGP and HSA are present in the AAT preparation probably as contaminants of the large-scale isolation of the protein.

PEG precipitation removes the majority of unwanted plasma proteins from a sample but leaves behind highly soluble proteins such as AGP in the supernatant. The immunodiffusion results from the PEG supernatant tests appear to be consistent with this idea since a positive reaction was produced between an aliquot of the supernatant and the AGP antiserum (Figure 4.2d). AAT and HSA were also identified as components of the supernatant (Figure 4.2d) reaffirming the need for subsequent isolation procedures to remove both these proteins from the desired AGP preparation. The reaction of the Cibacron Blue collected fraction with the AGP antiserum (Figure 4.2d) demonstrates the continued presence of AGP at this stage of the isolation procedure. Slight reactions of the AAT and HSA antisera with the Cibacron Blue fraction was observed (Figure 4.2e) indicating that HSA was not

completely removed by the Cibacron Blue Sepharose chromatographic step and that as expected, AAT was still present in the preparation. After the final isolation and chromatographic step with Red Sepharose a relatively pure AGP preparation was sought for subsequent analysis. The results from the immunodiffusion tests on an aliquot of the Red Sepharose fraction demonstrate that AGP was in fact present in the final sample as indicated by a positive result with the AGP antiserum (Figure 4.2 e). Barely visible precipitation bands were observed in the reaction of the Red Sepharose fraction and the AAT antiserum (Figure 4.2e) but no reaction was produced with the HSA antiserum (Figure 4.2f). These results indicate that the final AGP preparation was free from detectable albumin but contained trace amounts of contaminating AAT.

Discussion

In order to accurately analyse the glycosylation patterns of AGP from breast cancer plasma, the method of isolation must not denature or structurally altered the protein or its glycan chains. The isolation method developed by Smith and colleagues (1994) meets these essential requirements and was therefore adopted as the method of choice in this research study.

The initial step in isolating the desired AGP involved precipitating the plasma with PEG 3350 allowing the plasma proteins to be separated based on their relative solubility (Ingham, 1990). PEG is a non-ionic, water soluble polymer which allows precipitation of the proteins based on an excluded volume effect. The polymer forms micelle-like structures forcing the proteins into the extra-polymer space where they are concentrated until their solubility is exceeded and they precipitate. A 40% w/v PEG solution was found to be sufficient to precipitate unwanted proteins and leave AGP in solution (Smith *et al*., 1994). The precipitated proteins formed a pellet upon centrifugation which was subsequently discarded. The AGP-containing supernatant was retained for further purification.

Firstly Cibacron Blue Sepharose column chromatography was used to remove HSA from the preparation. Cibacron Blue F3GA has been found to bind to the bilirubin binding sites present on HSA (Leatherbarrow and Dean, 1980) allowing the AGP-containing fraction to elute unchallenged. Travis *et al*. (1976) demonstrated the removal of approximately 98% of HSA when whole human plasma was applied to a column of Cibacron Blue Sepharose. Immunodiffusion experiments conducted by Smith *et al*. (1994) confirmed the successful removal of albumin from the AGP preparation.

Originally the unbound Cibacron Blue fraction was then subject to anion-exchange chromatography on a Q-Sepharose fast flow column which further separated the proteins on the basis of charge. The Q-Sepharose resin is made up of beads which contain charged ammonium groups to which AGP can bind. The application of an increasing salt gradient (NaCl) increases the affinity of the salt for the charges on the beads resulting in the removal of the protein from the column. Three fractions are eluted during this process with AGP found to be a constituent of fraction two. AAT was still present in the preparation at this stage therefore a final dye-ligand chromatography step utilising the resin Red Sepharose, was included to aid the complete removal of this contaminating protein. Immunodiffusion experiments demonstrated that the unbound fraction contained AGP and a single band produced by the SDS-PAGE of the fraction confirmed the purity of the protein (Smith *et al*., 1994).

For the purposes of this project, it was decided to remove the Q-Sepharose chromatographic step in order to preserve the maximum yield of AGP from patient samples due to the small volumes obtained for analysis. The Smith method had previously been developed for the large scale isolation (50mls) of AGP from blood and its components and although translation of the Cibacron Blue and Red Sepharose chromatography columns into the micro-scale versions proved successful, the utility of the Q-Sepharose column with regards to the subsequent yield of AGP was questioned. Further separation of the Cibacron Blue AGP fraction with Q-Sepharose required the induction of an ion gradient (NaCl) resulting in the production of three eluted peaks. Immunodiffusion experiments identified AGP as a component of the second peak (Smith *et al*., 1994) which was collected and applied to the final Red Sepharose column. It was in the production of the ion gradient and the subsequent identification of the initiation of peak two (i.e. when to start collecting the sample) which generated problems with small sample volumes since there were concerns that AGP-containing eluent was being lost during the intermediary peak/elution stages.

Immunodiffusion experiments were employed to determine the effect this omission had on the purity of the final protein. The immunodiffusion technique employed was based on the theory of double diffusion (Bailey, 1996) in which antigens and antibodies have the ability to diffuse towards each other if placed into separate wells cut into agarose gel and generate a line of precipitation if a positive reaction occurs (i.e. the antibody recognises the antigen). The final AGP preparation was tested for the presence of both HSA and AAT since these proteins were the most likely residual contaminants. Antiserum was used to positively identify the presence of each of the proteins in the sample at various stages of the isolation procedure.

As expected, all three proteins (AGP, AAT and HSA) were found to be constituents of the original plasma sample (Figure 4.2a) and the PEG supernatant (Figure 4.2d) since all three have a high solubility and are not removed by the precipitation of the sample with PEG. The unbound fraction of the Cibacron Blue Sepharose column was found to contain AGP along with small traces of HSA (Figure 4.2e). This indicates that total removal of albumin from the preparation was not achieved which is in agreement with work by Travis *et al*. (1976) who demonstrated that only 98% of albumin was removed from a preparation of plasma applied to a Cibacron Blue Sepharose column. A positive result for the presence of AAT was also observed (Figure 4.2e) reaffirming the need for the Red Sepharose chromatography step. The final preparation from the unbound fraction of the Red Sepharose column tested positive for the desired protein AGP. Complete removal of albumin from the preparation was indicated by the lack of reaction of the fraction with the HSA antiserum. Very faint precipitation bands were observed in the reaction with the AAT antisera indicating that this protein was present in almost undetectable amounts in the final protein preparation. The removal of the Q-Sepharose chromatography step appears to have only slightly impacted the purity of the final AGP preparation. The contaminating protein AAT is also a glycoprotein but the interference, if any, in the glycosylation analysis of the patient samples should be negligible. Although an immunodiffusion technique was employed to determine protein purity in the current

study, sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) could also have been used to assess protein purity.

Due to the use of a NaOAc buffer in the final chromatographic step of the AGP isolation procedure, the AGP preparation contained an excessive amount of salt the removal of which was required to complete the purification process. The presence of salt in the AGP sample had the potential to interfere with subsequent analytical techniques such as in the determination of the total mass of AGP since both NaOAc and AGP absorb at 280nm. Centricon centrifugal filter devices with 10,000Da molecular weight cut-off filters were used to desalt and concentrate the samples. The filters were loaded with sample and centrifuged to force the small salt molecules through the membrane. The filter was washed thoroughly with water to ensure complete removal of the NaOAc from the AGP sample which was subsequently recovered from the filter and stored for further analysis.

CHAPTER 5

MONOSACCHARIDE ANALYSIS

RESULTS AND DISCUSSION

5.1 High pH Anion-Exchange Chromatography

RESULTS

The accurate analysis of the glycosylation pattern of glycoproteins is essential when investigating potential disease-specific changes and their subsequent evaluation as biomarkers of disease diagnosis, prognosis and progression. A variety of techniques have been employed to analyse the glycosylation profiles of glycoproteins including mass spectrometry (MS) and high performance liquid chromatography (HPLC) (Dage *et al*., 1998; Charlwood *et al*., 1999; Imre *et al*., 2008), nuclear magnetic resonance (NMR) (van Halbeek *et al*., 1981), and lectin ELISA (Rydén *et al*., 1999).

HPAEC is another well documented technique utilised in the structural analysis of glycoproteins, capable of providing information on both the monosaccharide and oligosaccharide composition of N-linked glycans (Hardy *et al*., 1988; Townsend *et al*., 1989; Smith *et al*., 1997). With respect to monosaccharide analysis this technique exploits the weakly acidic nature of individual monosaccharides. The alkaline environment created during HPAEC, can ionise the hydroxyl group (OH) attached to the anomeric carbon of a monosaccharide resulting in the formation of an oxyanion. The varying location of the OH groups results in slight differences in the pKa value (ranging from 12-14) of individual monosaccharides under these conditions. The interaction of the charged oxyanions with the strong anion-exchange CarboPac ™ PA-100 column and the slight differences in relative pKa values aids the chromatographic separation of individual monosaccharides, the lower the pKa; the longer it will be retained by the column (Lee, 1990).

HPAEC with PED (Figure 2.1) is an extremely sensitive technique capable of the rapid detection of picomolar quantities of monosaccharides without derivatisation

thus, allowing the accurate analysis of small quantities of sample. Due to the level of sensitivity and the relatively short analysis time, HPAEC was considered a superior method to aid thorough investigation of the AGP glycosylation patterns of samples in this research study. Normal, benign and breast cancer populations were analysed to determine if quantitative and/or qualitative differences exist between disease and normality.

Prior to HPAEC analysis, AGP samples were subject to an acid hydrolysis step to separate the glycan component from the protein backbone by cleaving them into their constituent monosaccharides and amino acids respectively. Figure 5.1 presents a HPAEC trace produced by the separation of a mixture of known monosaccharide standards (monomix). These standards were chosen since they are common constituents of N-linked glycan chains. Under the utilised alkaline conditions; the order of elution is consistent throughout.

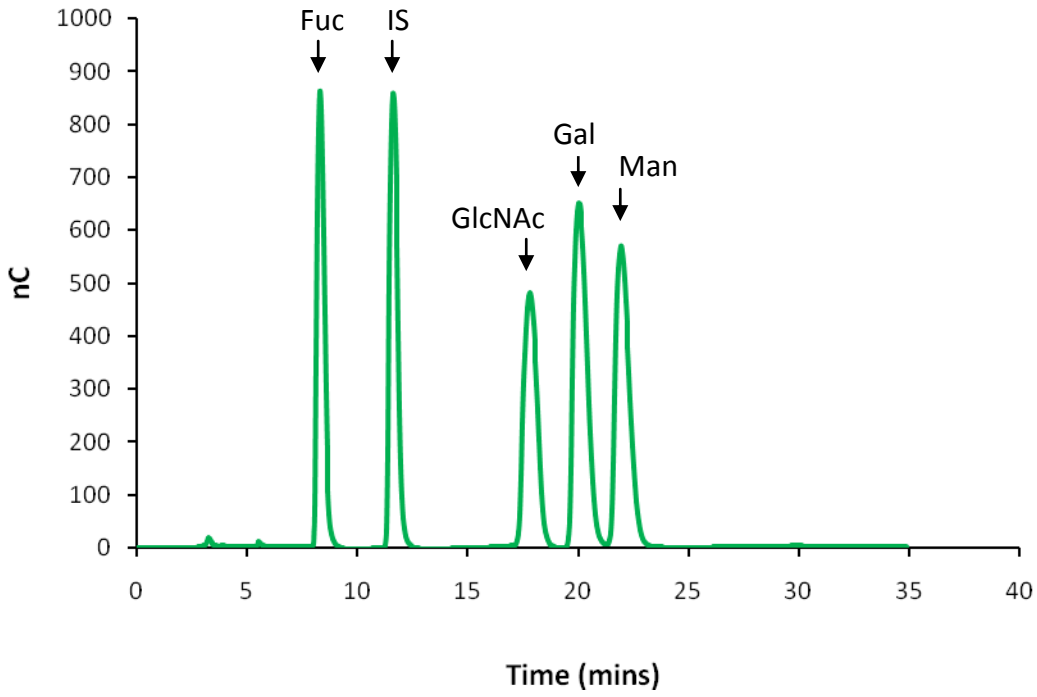


Figure 5.1 High pH anion-exchange chromatography trace of known monosaccharide standards (monomix). Fuc- Fucose; IS- Internal Standard, 2-deoxygalactose; GlcNAc- N-acetylglucosamine; Gal- Galactose; Man-Mannose.

Figure 5.2 demonstrates the resultant calibration curves for Fuc, IS, GlcNAc (glucosamine), Gal and Man along with their corresponding correlation coefficients (R^2). The data represents the mean of four HPAEC runs at each mass in the range 0-5µg. Standard deviations are represented by the error bars. In each case, increasing mass of monosaccharide yielded an increase in peak area.

The HPAEC profile from a patient sample is presented in Figure 5.3. Identification of the unknown peaks was achieved as described in methods section 2.2.3.2 by comparing the IS retention time ratio of each unknown to that of the standard monomix run. The peak area was noted and the relative mass of Fuc, GlcNAc, Gal and Man in each of the samples was determined by reference to the standard monosaccharide curves (Figure 5.2). During analysis of patient samples the recurrence of a reproducible unknown peak eluting between IS and GlcNAc prompted investigation into its identification. It was discovered to be N-acetylgalactosamine (GalNAc), a monosaccharide not usually considered a constituent of the N-linked glycans of AGP. Due to its persistent appearance, GalNAc was included in subsequent monomix runs and a calibration curve generated so that the mass of the monosaccharide present could be quantified. Figure 5.4 displays the HPAEC profile of a GalNAc-containing monomix and the calibration curve produced. As with the other standard curves, the data presented in Figure 5.4a represents the mean of four HPAEC runs at each mass in the range 0-2µg. It was not thought necessary to extend the range up to 5µg for GalNAc since the observed mass of the monosaccharide present in the samples was well within the 0-2µg range.

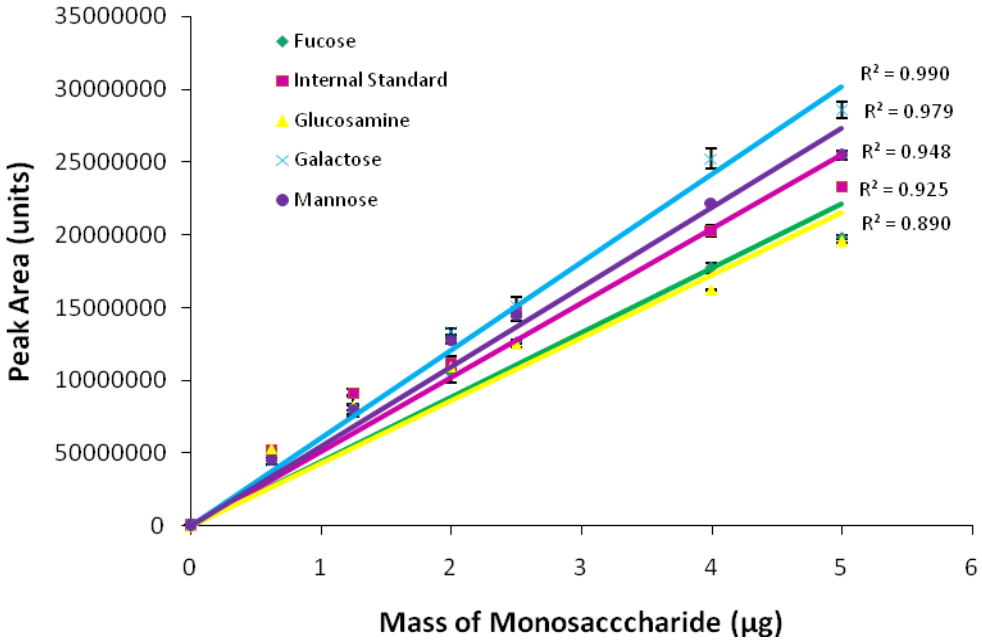


Figure 5.2 Monosaccharide calibration curves of mass of monosaccharide against peak area from the HPAEC chromatogram. Mean values (n=4) plotted for each monosaccharide amount with standard deviations represented by the error bars. R^2-correlation coefficient.

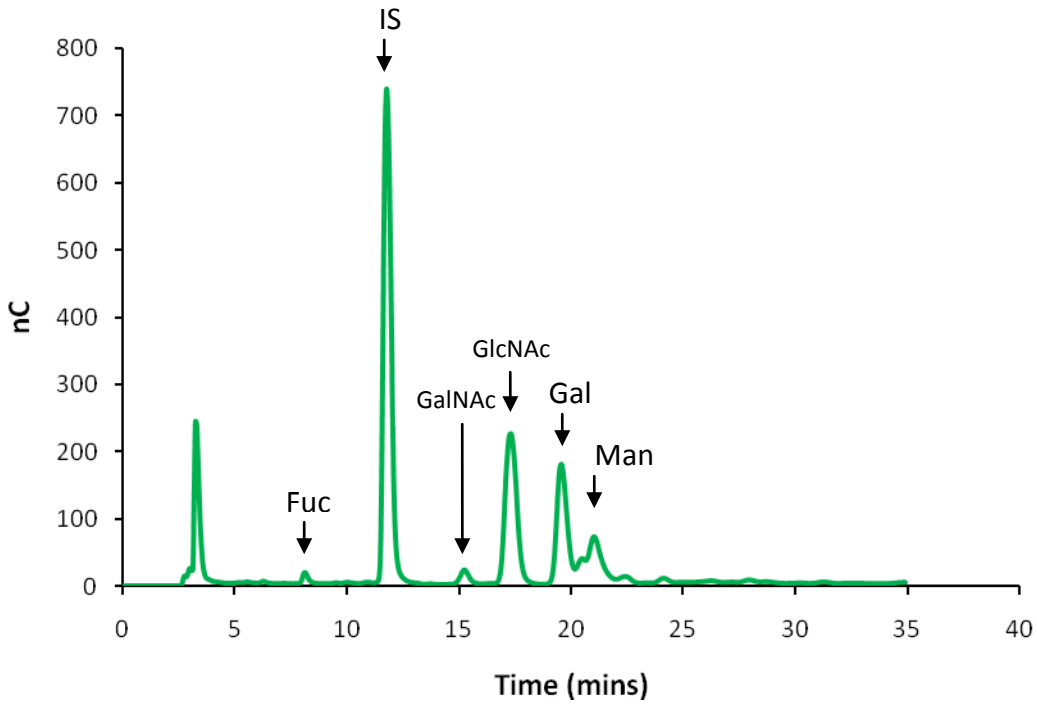
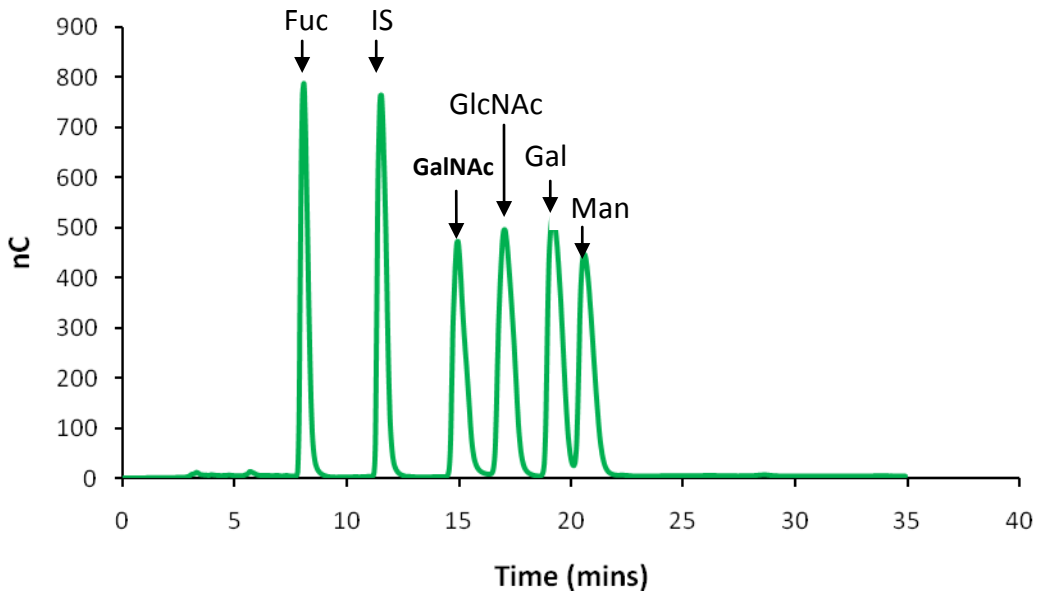


Figure 5.3 High pH anion-exchange chromatography trace of AGP from a patient sample. Fuc- Fucose; IS- Internal Standard, 2-deoxygalactose; GalNAc- N-acetylgalactosamine; GlcNAc- N-acetylglucosamine; Gal- Galactose; Man-Mannose.

a)



b)

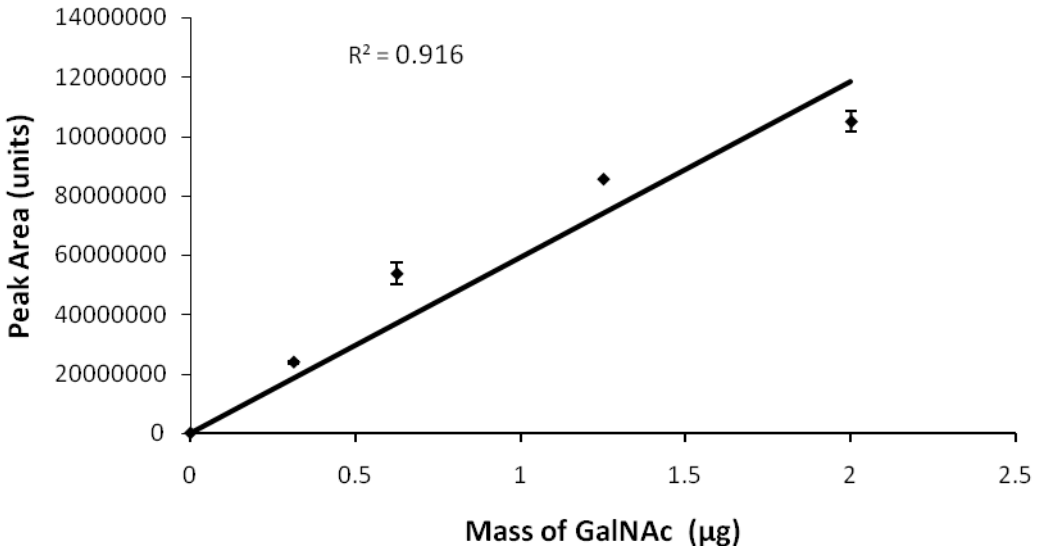


Figure 5.4 a) HPAEC trace of monomix containing GalNac. b) GalNAc calibration curve of mass of GalNAc against peak area. Mean values (n=4) plotted for each monosaccharide amount with standard deviations represented by the error bars. R^2- correlation coefficient.

The relative mass of Fuc, GalNAc, GlcNAc, Gal and Man in each sample was calculated. The composition results of patient samples in the benign breast disease (benign), breast cancer (invasive) and normal populations as well as results from a commercial AGP preparation are presented in Table 5.1 and 5.2. The data is expressed as moles of monosaccharide per mole of AGP and represents the mean and standard deviation of duplicate runs of each sample, and in the case of B7, I5 and I19, from a single analysis due to limited sample size. The designation of a monosaccharide as 'not detectable' indicates that it may be absent altogether from the sample or may be outwith the detectable range of analysis. In a number of cases quantification of the Fuc and GalNAc content of a sample yielded only one value from duplicate runs which may have been due to the presence of trace quantities near to or within the undetectable range.

The data in Table 5.1 and 5.2 demonstrates that GlcNAc, Gal and Man were present in all four groups analysed. The relative compositions of samples from the normal (N1-N4), benign and invasive groups are similar to that found in the commercial AGP sample, indicating that the protein was not structurally altered during the isolation procedure. Fuc was detected in samples from the commercial, normal, benign and invasive groups. The benign and invasive breast cancer population contained GalNAc hinting at the presence of disease-specific glycosylation of AGP since this monosaccharide was absent from both the commercial and normal groups. The presence of Fuc and GalNAc in the benign and invasive groups was not consistent throughout the populations with three (37.5%) of the benign and seven (35%) of the invasive group containing Fuc and five (62.5%) of the benign and eighteen (90%) of the invasive group containing GalNAc. The amount of Fuc and GalNAc present in the samples varied within and between the benign and invasive populations. Fuc ranged from 0.58- 0.93 mol/mol AGP in the benign and 0.63- 1.06 mol/mol AGP in the invasive group and GalNAc ranged from 0.39-1.89 mol/mol AGP and 0.26-1.27 mol/mol AGP in benign and invasive groups respectively. Figure 5.5 details the distribution of data, in the form of box plots, for each monosaccharide in the normal, benign and invasive groups.

Table 5.1 Monosaccharide composition of normal and benign AGP samples.

	Monosaccharide Composition (mol/mol AGP)				
Sample	**Fucose**	**GalNAc**	**GlcNAc**	**Galactose**	**Mannose**
Commercial	0.75 ± 0.06	-	9.96 ± 0.65	6.43 ± 0.20	3.91 ± 0.04
N1	1.29 ± 0.06	-	5.60 ± 0.82	1.29 ± 0.12	0.60 ± 0.32
N2	0.57 ± 0.20	-	5.70 ± 0.59	3.00 ± 0.47	0.75 ± 0.02
N3	0.70 ± 0.36	-	6.27 ± 1.71	2.41 ± 0.35	1.22 ± 0.90
N4	0.55 ± 0.26	-	4.62 ± 1.06	2.23 ± 0.03	1.08 ± 0.22
B1	-	-	6.55 ± 1.28	3.31 ± 0.47	2.71 ± 0.09
B2	-	-	7.05 ± 0.23	3.33 ± 0.45	1.97 ± 1.03
B3	0.73*	0.84*	5.69 ± 0.45	2.74 ± 0.15	1.21 ± 0.77
B4	-	1.89*	5.86 ± 1.32	2.95 ± 0.60	1.76 ± 0.07
B5	-	0.39 ± 0.09	5.75 ± 2.52	2.41 ± 1.90	1.01 ± 0.20
B6	0.93*	0.78*	6.74 ± 0.93	2.72 ± 0.33	1.54 ± 0.35
B7†	0.58	0.59	4.09	1.56	1.39
B8	-	-	4.87 ± 0.13	1.70 ± 0.31	0.66 ± 0.06

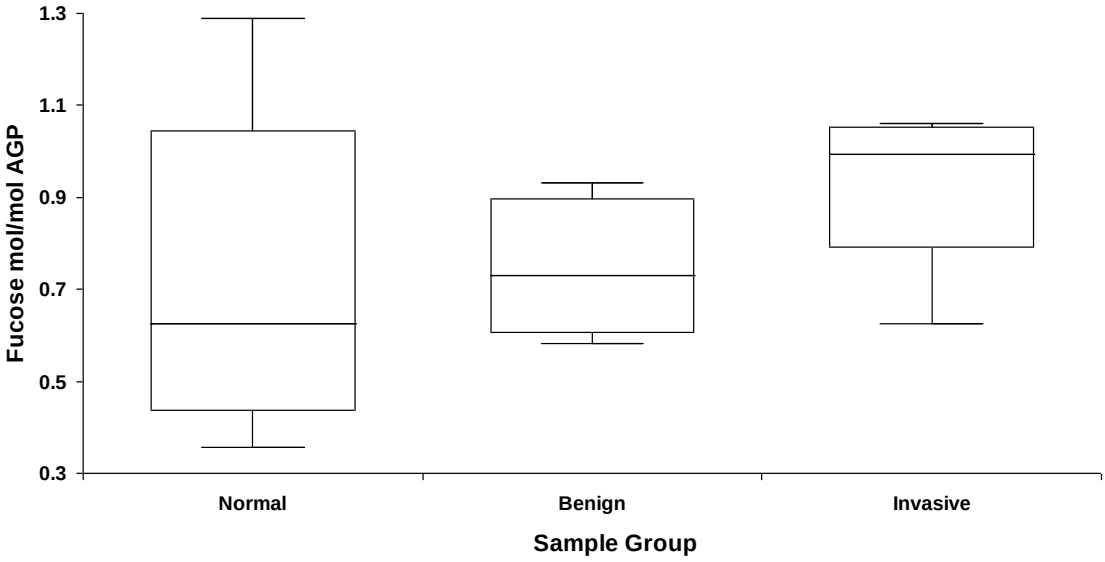
Data expressed as moles of monosaccharide per mole of AGP and unless stated, represents the mean and standard deviation (±) of each sample analysed in duplicate. N1-4 represents data for normal samples. † Single data set due to limited sample. * Detectable in one data set. - Not detectable.

Table 5.2 Monosaccharide composition of normal and invasive AGP samples.

	Monosaccharide Composition (mol/mol AGP)				
Sample	**Fucose**	**GalNAc**	**GlcNAc**	**Galactose**	**Mannose**
Commercial	0.75 ± 0.06	-	9.96 ± 0.65	6.43 ± 0.20	3.91 ± 0.04
N1	1.29 ± 0.06	-	5.60 ± 0.82	1.29 ± 0.12	0.60 ± 0.32
N2	0.57 ± 0.20	-	5.70 ± 0.59	3.00 ± 0.47	0.75 ± 0.02
N3	0.70 ± 0.36	-	6.27 ± 1.71	2.41 ± 0.35	1.22 ± 0.90
N4	0.55 ± 0.26	-	4.62 ± 1.06	2.23 ± 0.03	1.08 ± 0.22
I1	-	0.29*	5.82 ± 2.01	2.77 ± 0.73	1.64 ± 1.40
I2	-	-	7.35 ± 1.34	3.55 ± 1.20	2.89 ± 1.49
I3	0.63*	0.54*	6.50 ± 2.00	2.51 ± 1.01	1.17 ± 1.05
I4	0.89*	1.02*	5.53 ± 0.34	2.57 ± 0.57	1.78 ± 0.82
I5†	-	0.49	6.67	3.15	1.53
I6	0.77*	0.90*	6.77 ± 0.26	3.04 ± 0.06	1.44 ± 0.04
I7	-	0.26*	6.80 ± 0.81	2.90 ± 0.70	1.47 ± 0.21
I8	0.99*	1.27*	6.82 ± 0.27	3.46 ± 0.30	1.79 ± 0.54
I9	-	0.34*	7.12 ± 1.80	2.97 ± 1.74	1.66 ± 0.75
I10	1.06*	0.94*	7.25 ± 0.98	3.52 ± 0.95	2.11 ± 0.82
I11	-	0.31*	6.55 ± 1.06	2.81 ± 0.87	1.71 ± 0.63
I12	-	0.36*	7.60 ± 2.18	3.38 ± 1.57	1.56 ± 1.07
I13	-	0.31*	5.43 ± 0.78	2.34 ± 1.39	1.31 ± 0.99
I14	-	0.28*	7.46 ± 0.71	3.40 ± 0.50	2.69 ± 0.36
I15	-	0.65*	6.73 ± 1.62	2.81 ± 0.37	2.08 ± 0.64
I16	-	0.38*	5.50 ± 0.72	2.05 ± 0.68	1.05 ± 0.40
I17	1.06*	1.13*	6.85 ± 1.54	2.95 ± 0.23	2.26 ± 0.94
I18	-	-	6.50 ± 1.59	2.91 ± 0.53	1.71 ± 0.99
I19†	1.03	1.04	6.84	2.82	2.05
I20	-	0.71*	6.48 ± 1.09	3.06 ± 0.63	2.18 ± 0.78

Data expressed as moles of monosaccharide per mole of AGP and unless stated, represents the mean and standard deviation (±) of each sample analysed in duplicate. N1-4 represents data for normal samples. † Single data set due to limited sample. * Detectable in one data set. – Not detectable.

a) Fucose



b) N-acetylglucosamine

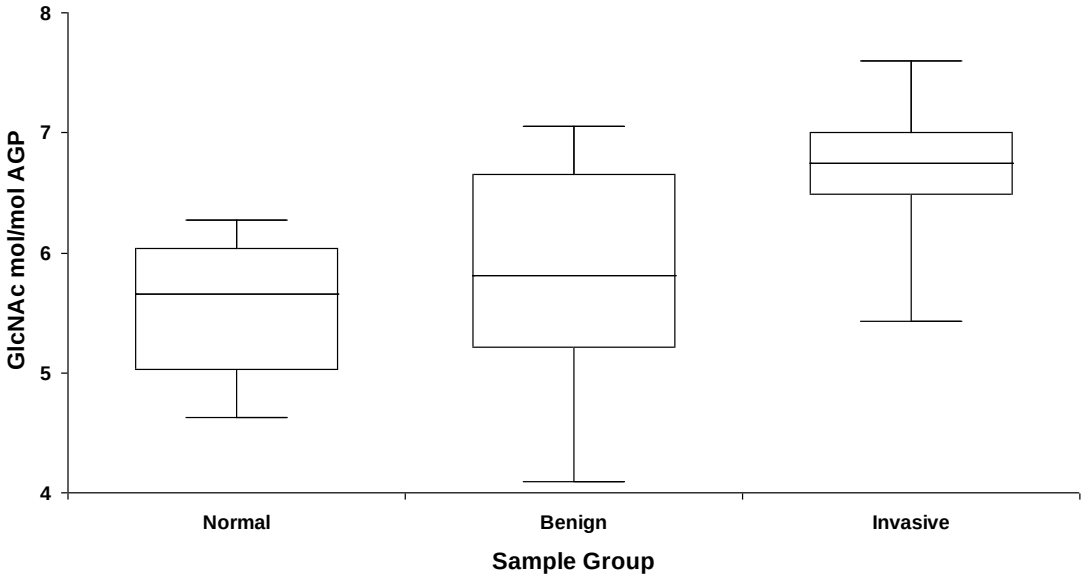
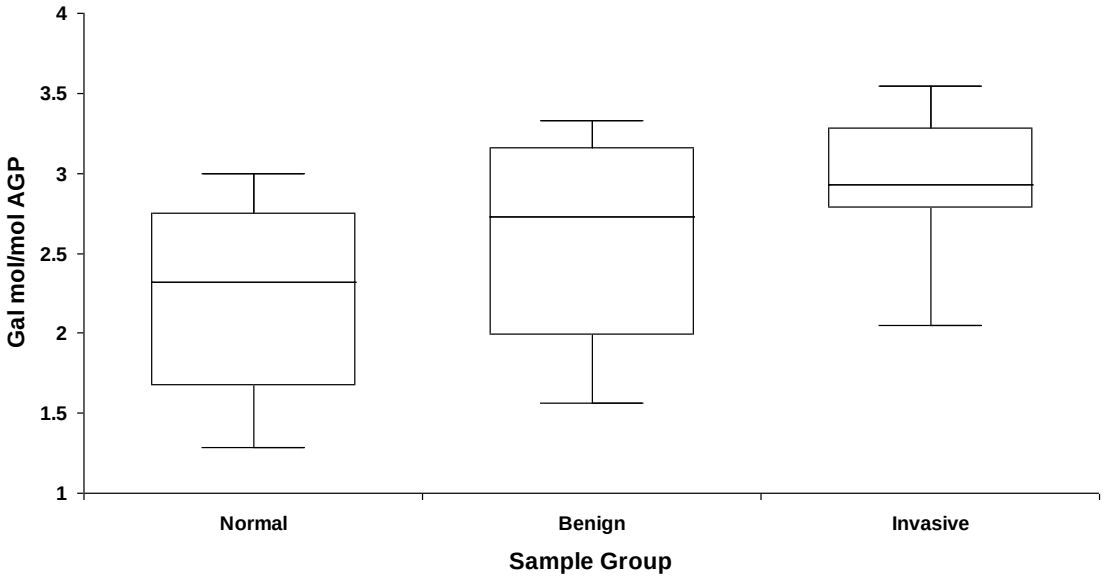


Figure 5.5 Box plots of variation in **a)** fucose (Fuc) and **b)** N-acetylglucosamine (GlcNAc) composition within and between normal, benign and invasive groups.

c) Galactose



d)

Mannose

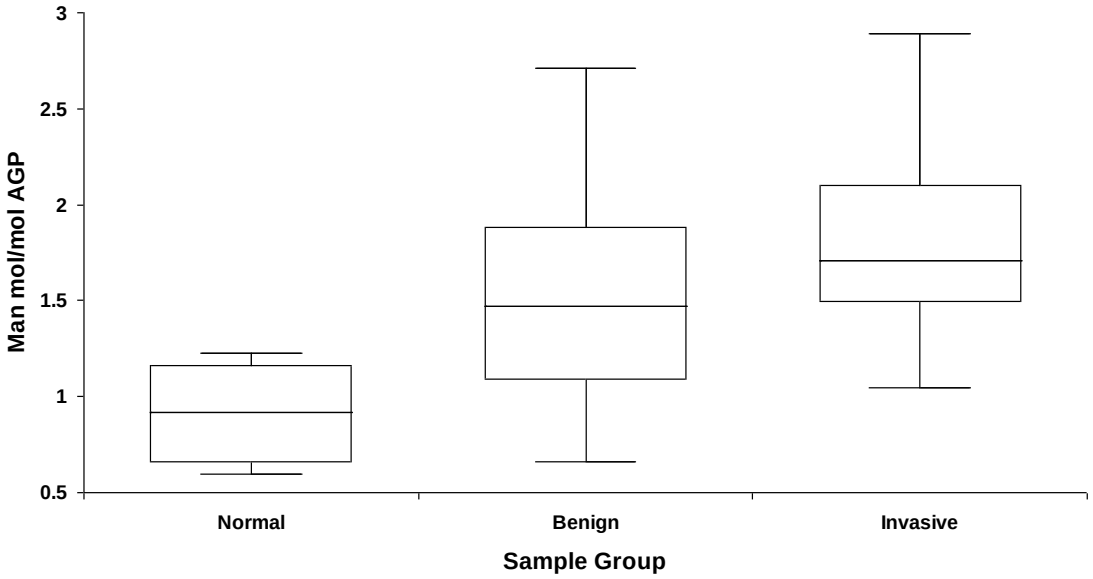


Figure 5.5 cont. Box plots of variation in **c)** galactose (Gal) and **d)** mannose (Man) composition within and between normal, benign and invasive groups.

e) N-acetygalactosamine

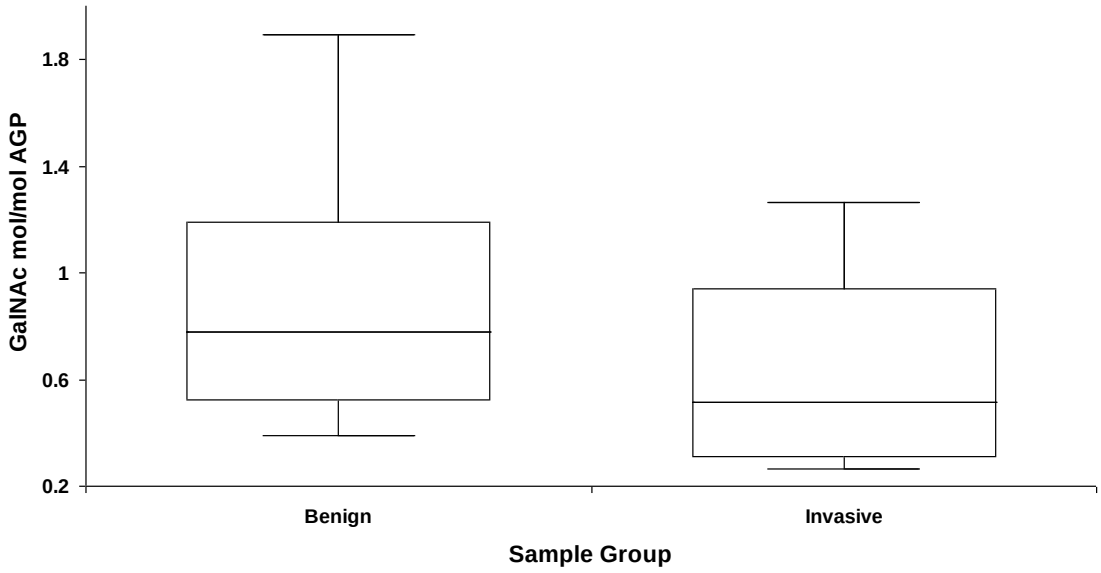


Figure 5.5 cont. Box plot of variation in **e)** N-acetylgalactosamine (GalNAc) composition within and between benign and invasive groups.

Table 5.3 details the mean composition of Fuc, GalNAc, GlcNAc and Gal in the various sample groups (expressed as moles of monosaccharide per mole of AGP). The mean values of Fuc and GalNAc quoted for the benign and invasive group were calculated solely from samples positive for the presence of each monosaccharide and not from the population as a whole in order to give a true representation of the average level displayed when the monosaccharide is present.

The data presented in Table 5.3 demonstrates that AGP from the commercial group had the highest average composition of GlcNAc and Gal compared to the other three groups. These levels were found to be significantly ($p < 0.05$) higher compared to the benign and invasive groups. AGP from the normal and benign groups had a similar GlcNAc and Gal content but the invasive group demonstrated a higher average composition compared to these groups and a statistically ($p < 0.05$) higher composition of GlcNAc compared to the normal group.

The commercial, normal and benign groups all had approximately the same Fuc content but the invasive group, although not significantly different to that of the others, demonstrated an increased level of this monosaccharide. Of the two groups containing GalNAc, the benign group had a greater average composition than the invasive group but again, this difference was not found to be significant.

Table 5.3 Average monosaccharide composition of sample groups.

	Monosaccharide Composition (mol/mol AGP)			
Sample Group	**Fucose**	**GalNAc**	**GlcNAc**	**Galactose**
Commercial	0.75 ± 0.06	-	9.96 ± 0.65	6.43 ± 0.20
Normal	0.78 ± 0.35	-	5.55 ± 0.68■	2.23 ± 0.71
Benign	0.75 ± 0.18^	0.90 ± 0.58♦	5.83 ± 0.99†	2.59 ± 0.67†
Invasive	0.92 ± 0.17*	0.62 ± 0.34●	6.63 ± 0.63†	2.95 ± 0.40†

Data expressed as moles of monosaccharide per mole of AGP and unless stated represents the mean (commercial, n=2; normal, n=4; benign, n=8; invasive, n=20) and standard deviation (±) of each group. Mean calculated from samples positive for fucose ^ n=3, * n=7. Mean calculated for samples positive for GalNAc ♦ n=5, ● n=18. † Significantly ($p < 0.05$) different from commercial AGP sample. ■ Significantly ($p < 0.05$) different from invasive AGP sample. - Not detectable.

5.2 Characterisation of N-acetylgalactosamine Structure

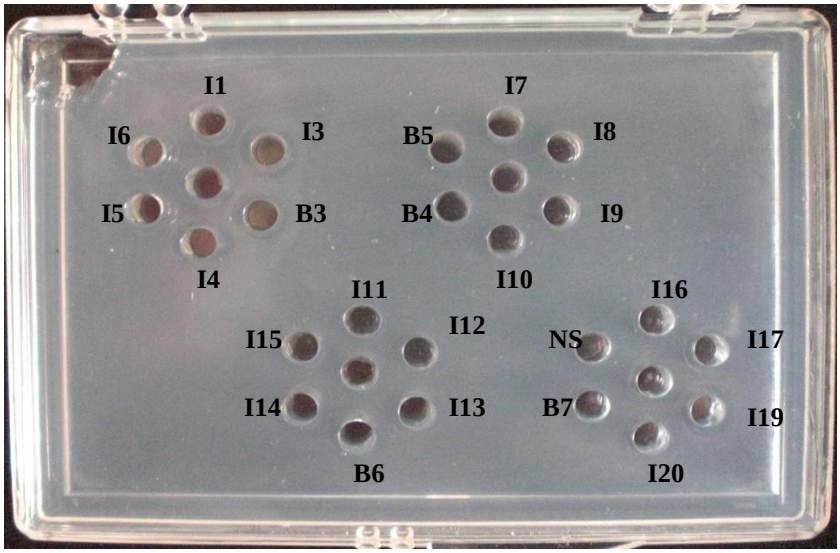
RESULTS

The five glycans chains of AGP are of the complex-type (see section 1.1.3.3) N-linked via asparagines residues to the protein backbone consisting of a pentasaccharide core (composed of two GlcNAc and three Man residues) and outer branches extended by GlcNAc, Gal and possibly Fuc and N-acetylneuraminic acid (NeuAc). To date, no other glycosylation linkages (i.e. O-linked) or constituent monosaccharides have been associated with normal AGP glycans. The discovery and positive identification of N-acetylgalactosamine (GalNAc) in a number of the benign and invasive breast cancer AGP samples prompted an investigation into determining the possible source of this likely terminal antigenic structure.

Immunodiffusion experiments were conducted to determine if a terminal antigen was responsible for the GalNAc detected in the majority of the benign and invasive patient samples. Blood group A antigen and the tumour associated antigen Sialyl Tn (STn) are two of the most common GalNAc-containing structures and were therefore evaluated as potential sources of the monosaccharide observed in this research study. Figure 5.6 demonstrates the results of the immunodiffusion experiment.

No lines of precipitation were observed between any of the sample and antibody wells indicating a lack of antibody-antigen reaction and therefore a lack of either blood group A and/or STn antigen in the samples.

a)



b)

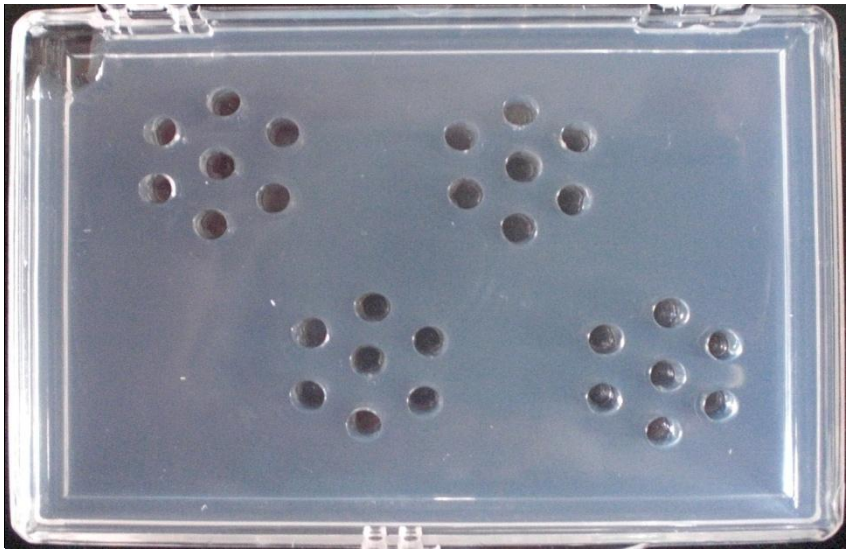
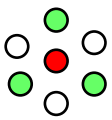


Figure 5.6 Immunodiffusion plate results of patient samples with a) blood group A antibody b) Sialyl Tn antibody. Antibodies placed in central red wells and samples placed in outer green wells as shown. Samples loaded in the orientation demonstrated in a). NS- no sample.



Discussion

Prior to HPAEC analysis the isolated AGP was subject to acid hydrolysis to separate the glycan chains from the protein backbone. Trifluoroaceteic acid (TFA) was used to release the neutral monosaccharides such as Fuc, Gal and Man and HCl was used to release the amino monosaccharides such as GlcNAc. Previous work in this research group has demonstrated the relative stability of the monosaccharides under these conditions with the exception of NeuAc which is destroyed in this strongly acidic environment. The resulting hydrolysate was applied to a column of Dowex® cation-exchange resin which aided the release of the sample monosaccharides by retaining the charged amino acids. The sample was then ready to be applied to the Dionex-600 system for analysis (see Table 5.1 and 5.2 for results).

The monosaccharides GlcNAc and Gal are components of the outer branches of AGP, therefore any increase or decrease in their levels could be indicative of changes in the branching of the chains. Table 5.2 demonstrates that the commercial AGP population had the highest average composition of GlcNAc and Gal compared to the benign and invasive populations. HPAEC analysis of a commercial AGP sample was conducted to provide additional normal population data. The lower than normal levels of GlcNAc and Gal present in the benign and invasive groups appears to indicate a decrease in the branching of the AGP glycan chains in patients from these groups. This finding is in agreement with work by Hansen *et al*. (1984) and Fujii *et al.* (1988) who demonstrated a decrease in the branching (increase in bi-antennary content) of AGP glycans in the sera of patients with lung cancer and in the ascitic fluid of patients with liver cancer respectively.

Although there were no significant differences in the GlcNAc and Gal content of the benign and invasive groups, having the same monosaccharide composition does not

necessarily equate to identical oligosaccharide chains with regards to monosaccharide sequence, linkage and extent of branching.

Interestingly, the normal sample group had a similar GlcNAc and Gal composition to the benign and non-invasive cancer groups and a lower level of both compared to the commercial AGP group. Although it was hoped that the sample group represented a normal population, in conjunction with the commercial AGP sample, the results from Table 5.2 do not support this idea. The samples allocated to this normal group were obtained from an apparently healthy, cancer free population of elderly females but it is possible that at the time of collection an acute phase response was present which may have induced the observed differences between the commercial and normal sample. As with the breast disease populations, a reduction in the average GlcNAc and Gal content of the normal group indicates a decrease in the branching of the AGP chains. AGP glycosylation studies have demonstrated a decrease in branching (increase in bi-antennary content) due to the APR in patients suffering from acute inflammatory conditions such as rheumatoid arthritis and in those with acute bacterial infections (Fassbender *et al*., 1991; Higai *et al*., 2003; Higai *et al*., 2005). Oligosaccharide analysis may provide more information on the branching of the AGP chains and demonstrate any significant differences between the normal and the commercial group analysed in the present study.

van Dijk *et al*. (1994) stated that Fuc levels show the greatest change in disease conditions. In cancer, Fuc levels have been shown to increase (Turner *et al*., 1985; Hashimoto *et al*., 2004). In the current study, the invasive breast cancer group was found to have the highest level (not significant) of Fuc compared to the others although not all samples in the benign and invasive groups contained detectable amounts of this monosaccharide. Turner and colleagues (1985) investigated the Fuc content of a number of cancers, 21 of which were from patients with breast cancer of varying severity. The group reported a 45.6% increase in Fuc of the cancer group compared with normal controls and demonstrated a 70% increase in patients with

advanced disease. The authors concluded that measurement of the Fuc content in cancer patients may give an indication of the extent of cancer spread. The results in the present study agree in part with that of Turner *et al*. (1985). When present, the invasive breast cancer group does indeed have the highest average level of the monosaccharide indicating a correlation between Fuc levels and disease stage (fucose increased 22.7% compared to commercial group), but the benign group has a level very similar to that of the normal and commercial populations demonstrating a lack of correlation between the level and general progression of the disease.

A study by Hashimoto and colleagues (2004) investigated the AGP fucosylation of a variety of different cancer patients (one stage II breast cancer) to assess its potential as a marker of disease progression and prognosis. They reported that patients with advanced disease displaying highly branched and fucosylated glycans for longer durations after surgery were associated with a poorer prognosis. The authors concluded that combined measurement of the branching and fucosylation of AGP chains could provide a very significant prognostic marker useful in monitoring patients after surgery.

The unexpected presence of GalNAc in the majority of the benign and invasive patient samples and lack thereof in the normal and commercial groups indicated that AGP was displaying a glycosylation alteration which may form the basis of a serum biomarker for breast disease; currently there is no breast disease/cancer-specific biomarker in clinical use. Previous studies utilising HPAEC to investigate AGP glycosylation patterns in various liver diseases identified GalNAc as a recurring component of AGP samples from hepatitis C (Anderson *et al*., 2002) and fibrosis patients (Mooney *et al*., 2006). Relatively small sample cohorts were analysed in both studies but Anderson *et al*. (2002) reported the presence of GalNAc in 64% of hepatitis C samples and Mooney *et al*. (2006) detected the monosaccharide in 33% of samples from patients with varying degrees of fibrosis. The lack of GalNAc in cirrhotic samples from both studies lead to the suggestion that the presence and

subsequent disappearance of the monosaccharide could perhaps indicate the progression of liver disease to cirrhosis. Another possible explanation for the lack of GalNAc in some of the hepatitis C and fibrosis samples could be that these patients are simply lacking the glycosyltransferase gene or lack the enzyme activity required to produce this modification.

GalNAc was present in almost all (90%) of the invasive breast cancer samples but the modification was not specific to malignant disease since it was also found in samples from the benign group. Due to the high percentage of invasive samples containing the monosaccharide, its presence in patients with benign disease of the breast may possibly indicate an increased risk of subsequent malignant transformation. Unfortunately, information on patient follow-up was unavailable therefore any correlation between GalNAc level and subsequent clinical status could not be determined in order to investigate this hypothesis.

A higher average GalNAc content (not statistically significant) was observed among the benign group compared to the invasive group. This difference may simply be due to the small number of benign samples analysed compared to that of the invasive population (8 samples versus 20 samples) or could possibly represent a disease-specific change of clinical relevance. As with investigations into liver disease (Anderson *et al*., 2002; Mooney *et al*., 2006) the appearance of the monosaccharide in the benign group and subsequent disappearance, or reduction in the average level in the invasive group may have prognostic potential in indicating extent of disease. The diagnostic/prognostic data available on the two invasive samples lacking GalNAc demonstrates that although invasive, the tumours are OR and PR positive which usually reflects a good prognosis and positive response to hormone therapy. Further information on the stage, grade and metastatic status of both patients was unavailable therefore correlations between a lack of GalNAc and prognosis could not be determined. Detailed analysis on patients with non-invasive breast cancer as well as those presenting with benign breast disease and various stages of invasive

carcinoma will have to be undertaken to determine if there is a progressive reduction and positive association between GalNAc levels and progression of breast cancer.

To date, GalNAc has not been described as a component of the N-linked chains of AGP and as previously discussed (section 1.2.2.1) structural studies have attributed the glycosylated component of AGP solely to N-linked glycans. GalNAc is typically associated with O-glycosylated proteins (see section 1.1.3) but since the method of isolation adopted in this study results in the successful removal of unwanted proteins from the final AGP preparation it was concluded that the GalNAc peak observed during HPAEC analysis of patient samples was not induced by a contaminating O-linked glycoprotein. O-linked glycan chains are produced by a different biosynthetic pathway to that of N-linked chains, requiring the sequential addition of monosaccharides to an initial serine or threonine residue on the polypeptide backbone. As with N-linked glycosylation this process is regulated by glycosyltransferase enzymes. Any suggestion that the observed GalNac component was added to the protein in the form a novel O-linked glycan chain would be extremely unlikely as this would require complete alteration of the co/post-translational biosynthetic machinery utilised in the production of the glycoprotein.

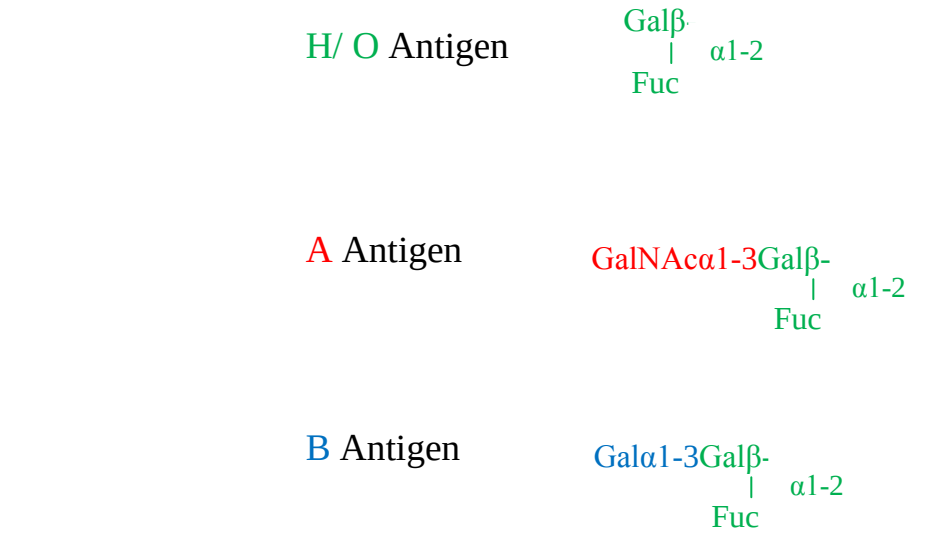
The studies conducted by Anderson *et al.* (2002) and Mooney *et al.* (2006) were unable to investigate the possible source of the observed GalNAc due to limited sample obtained for analysis but both groups suggested that its presence may be explained by the attachment of a terminal structure to the glycan chains of AGP during disease, possible in the form of an antigen such as blood group A.

The discovery of the three blood group antigens A, B and O was first reported by Karl Landsteiner in the early 1900's with subsequent description of the ABO blood group system (Watkins, 2001). These antigens are carbohydrate in origin associated with glycoproteins and glycolipids and further investigations concluded that the O

antigen (later termed the H antigen) was in fact a precursor for the production of the A and B antigens differing only in their terminal monosaccharide. The A antigen requires the addition of GalNAc to the precursor H molecule with the B antigen requiring the addition of Gal (Figure 5.7). The blood group antigens are normally found on the surface of red blood cells and on many human epithelial cells. Aberrant expression of the antigens, in particular the A antigen, has been reported in diseases such as cancer where it has been discovered in patients not of this blood type (Greenwell, 1997). Loss of expression of the ABO/H antigens has been associated with poor prognosis.

There are also a number of tumour associated antigens which may provide a possible source of GalNAc. These antigens are associated with truncated forms of O-linked glycan chains induced by incomplete glycosylation of the mucin protein to which they are attached. GalNAc linked to serine or threonine on the polypeptide backbone generates the Tn antigen which can be sialylated to produce Sialyl Tn (Figure 5.7). Expression of the Sialyl Tn (STn) antigen has been observed in breast cancer (Brocke and Kunz, 2002) and has been found to correlate with poor prognosis (Kim and Varki, 1997).

a)



b)

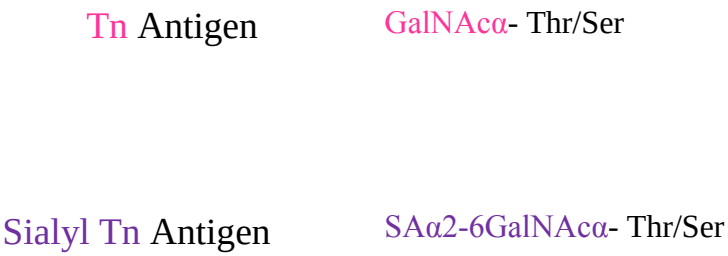


Figure 5.7 a) Monosaccharide composition of the ABH/O blood group antigens. B) Structure of the tumour-associated Tn and Sialyl Tn antigens. Gal- galactose; Fuc- fucose; GalNAc- N-acetylgalactosamine SA, sialic acid; Thr- threonine; Ser- serine.

To date the Fuc occupying the outer branches of AGP has only been found to be linked α (1, 3)and not α (1, 2) to Gal as would be required for the attachment of the blood group A antigen to the terminal end of the chains. Attachment of the tumour-associated STn antigen would require the novel expression of a GalNAc-transferase to catalyse the reaction. The APR-induced change in hepatic production of AGP is regulated by a network of cytokines which subsequently control the expression of the glycosyltransferase enzymes and therefore the overall expression of potential disease-specific glycoforms. It may therefore be possible for the liver to produce varying complements of these synthesising enzymes in response to the breast cancer-induced APR and aid the expression of these highly unusual antigenic structures.

In this study, the terminal antigens blood group A and STn were not present (Figure 5.6). It is possible that the isolation procedure detailed previously changed the integrity of the antigenic structure, altering it in such a way that it could no longer be recognised by its antibody. The development and optimization of a blood group A and STn antigen ELISA may allow further investigation into the presence or absence of these antigenic structures on benign and malignant patient AGP and aid the evaluation of GalNAc as a potential disease marker.

Monosaccharide compositional analysis using HPAEC-PED has highlighted the differences that exist between normal and breast disease populations. The results suggest a reduction in chain branching and demonstrate an increase in fucosylation of malignant AGP glycans compared to control populations. The presence of GalNAc in the benign and invasive samples and its absence from normal samples suggests that the modification could form the basis of a serum biomarker for breast disease/cancer with potential prognostic utility.

CHAPTER 6

OLIGOSACCHARIDE ANALYSIS

RESULTS AND DISCUSSION

6.1 High pH Anion-Exchange Chromatography

RESULTS

During physiological and pathological conditions, not only are the levels of AGP increased (2-5 fold) but the relative proportions of the normal glycoforms are altered coupled with expression of abnormal glycoforms (Ceciliani and Pocacqua 2007). Determination of the glycoforms expressed in normal and disease populations may aid the discovery of disease-specific alterations with diagnostic or prognostic potential.

As with monosaccharide analysis, the AGP oligosaccharide profiles of AGP were determined by HPAEC-PED demonstrating the further utility and flexibility of the technique. The addition of PED is of great advantage allowing picomolar quantities of underivatized oligosaccharides to be detected. In this instance, the alkaline environment created during HPAEC allows the efficient separation of oligosaccharide chains on the basis of their size, monosaccharide composition and intra-chain linkages (Hardy and Townsend, 1988). Unlike monosaccharide analysis, the information obtained is qualitative in nature allowing an AGP oligosaccharide 'fingerprint' to be determined.

AGP has a high content of negatively charged, terminating sialic acid (SA) which allows the oligosaccharide chains to be separated on the basis of their charge. In the case of AGP this SA is present in the form of N-acetylneuraminic acid (NeuAc). Separation of the oligosaccharides is also achieved through exploitation of the hydroxyl groups of the individual monosaccharides in the chains and their oxyanion formation at alkaline pH (~13) It is possible for the oligosaccharides to be separated at a lower pH (4.6) purely on the basis of their NeuAc content (Townsend *et al*., 1988), but the additional oxyanion charge at alkaline pH enhances resolution of the

oligosaccharide mixture by allowing separation of the chains based on isomeric differences (Townsend *et al*., 1989).

The application of oligosaccharide chains to an anion-exchange column results in the negatively charged glycans binding strongly to the positively charged beads of the column resin. Removal from the column requires the addition of an eluent that can compete with the glycans for the positively charged beads and displace them from the resin. The strong interaction of the negatively charged NeuAc with the quaternary ammonium-bonded pellicular resin used in HPAEC ensures that stronger elution conditions are required to displace the charged molecules from the column (Smith *et al*., 1997). This is achieved through the addition of a sodium acetate gradient; the greater the negative charge (greater the number of NeuAc) the higher the sodium acetate concentration required to displace the oligosaccharides from the column.

The sialylated portion of the oligosaccharide allows separation of the chains into distinct charge bands; the greater the number of NeuAc, the greater the overall negative charge resulting in a longer retention time. Within these charge bands, the oligosaccharides can be separated according to size with larger structures demonstrating greater retention times. On this basis, bi-antennary, bi-sialylated structures will elute earlier than tri-antennary, bi-sialylated structures. It is also possible for this technique to detect and successfully separate structures within charge bands which differ in one linkage. NeuAc can be linked ether α (2, 6) or α (2, 3) to an outer galactose residue. The presence, or increased proportion of α (2, 6) NeuAc-linked chains results in a reduction in retention time (Townsend *et al*., 1988). It is thought that when NeuAc is added α (2, 6) the oxyanion effect of the 6-OH of Gal is blocked resulting in an overall reduction in charge and a decrease in the retention time when compared to α (2, 3)-linked NeuAc (Townsend *et al.*, 1989).

Further separation based on the neutral portion of the oligosaccharide can also be achieved. This is demonstrated by the greater retention time of sialylated structures containing Galβ (1, 3) linked to GlcNAc compared to Galβ (1, 4)-linked (Townsend *et al*., 1988; Townsend *et al*., 1989). It was suggested that this difference in retention time was due to the conformation of the OH groups in the Galβ (1, 3)-GlcNAc allowing for greater interaction with the column. The presence of Fuc on an oligosaccharide chain has also been shown to affect retention time by reducing it quite considerably. It is thought that this is due to a 'masking' effect of the charges present on surrounding monosaccharides and therefore a reduction in the overall charge of the oligosaccharide (Hardy and Townsend, 1988; Lee, 1990).

The oligosaccharide chains of AGP were separated from the protein backbone and analysed using HPAEC coupled to PED. This technique provides an AGP oligosaccharide 'fingerprint' by initially separating the released oligosaccharides on the basis of their charge (determined by the number of terminal sialic acid residues) and thereafter on the basis of size and isomeric linkages within each distinct charge band. Mono-sialylated glycans are retained to a lesser degree than the others and elute between 10-20 minutes; bi-sialylated elute between 20-30 minutes; tri-sialylated elute between 30-40 minutes and tetra-sialylated between 40-50 minutes.

Figure 6.1 shows a chromatogram of an AGP oligosaccharide library containing bi-tri- and tetra-sialylated structures. AGP samples from all other groups were compared to this library. Figure 6.2 demonstrates the HPAEC chromatogram of a commercial and a normal AGP sample compared to the oligosaccharide library. The samples are comparable with the library in that they both display peaks in the bi-, tri- and tetra-sialylated regions. The normal AGP sample appears to display a slight shift towards bi-sialylated structures compared to the commercial sample but both have a decreased proportion of tetra-sialylated structures compared to the library.

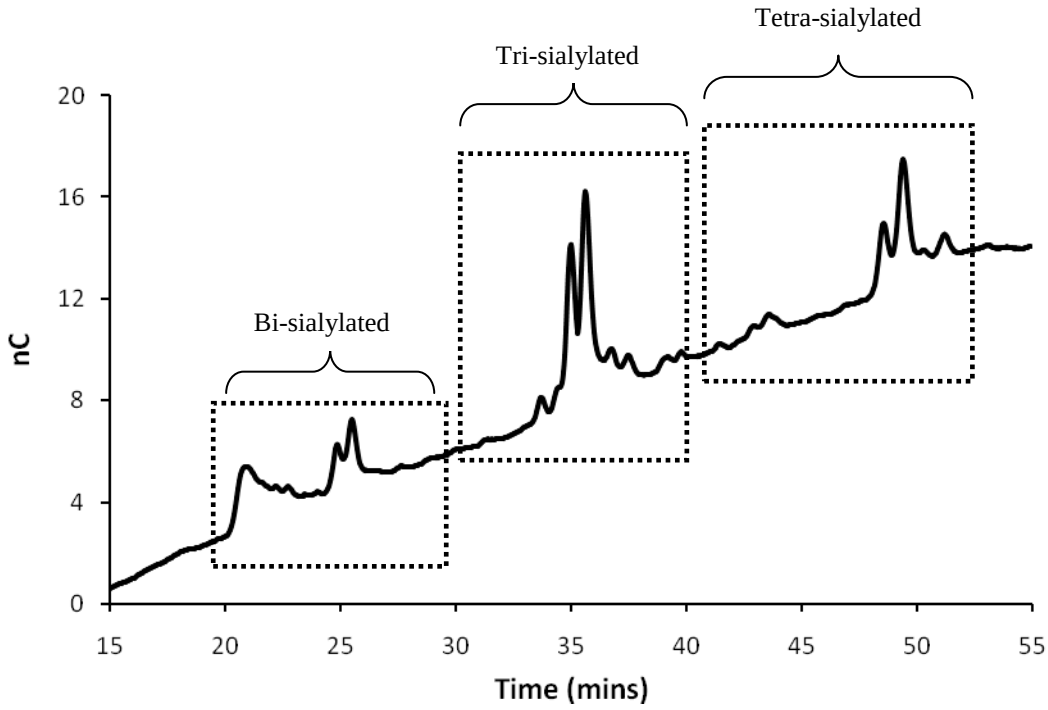


Figure 6.1 HPEAC trace of an oligosaccharide library.

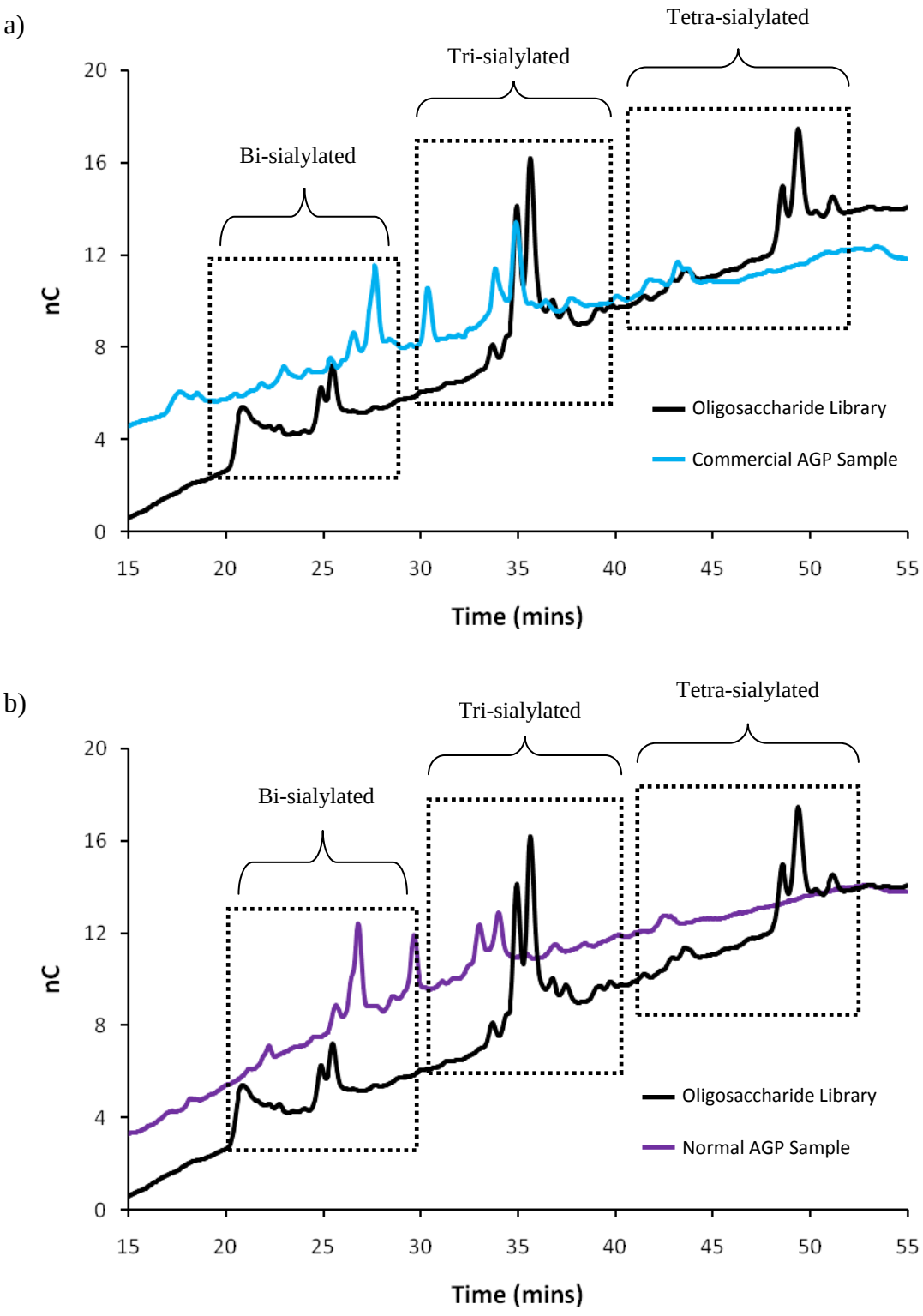


Figure 6.2 HPAEC trace of the oligosaccharide library compared to a) a commercial AGP sample and b) a normal AGP sample.

Figure 6.3 represents the results from oligosaccharide analysis of six of the benign patient AGP samples. Unfortunately data could not be obtained for B7 and B8. All benign data were plotted in green. Sample B1 (a) has oligosaccharides eluting to a higher degree in the bi- and tri-sialylated region with two peaks occurring late on in the tetra-sialylated region. The pattern in B2 (b) is similar with an increased activity in the bi- and tri- regions and two in the tetra-sialylated, although one has eluted considerable earlier compared to that of B1. Sample B3 has a high proportion of peaks eluting early in both the bi- and tri-sialylated charge bands and very little activity in the tetra-sialylated region. The shape and proportion of the peaks in B4 are very similar to that of B3, with the majority occurring in the bi- and tri-sialylated regions and no distinct peaks in the tetra-sialylated region. The chromatogram of B5 is again, very similar to that of B3 and B4 with increased activity in the bi- and tri-sialylated charge bands and very little in the tetra-sialylated region. B6 (f) displays a mono-sialylated peak eluting at 16 minutes and appears to have equal proportions of oligosaccharides in the bi-, tri- and tetra-sialylated region.

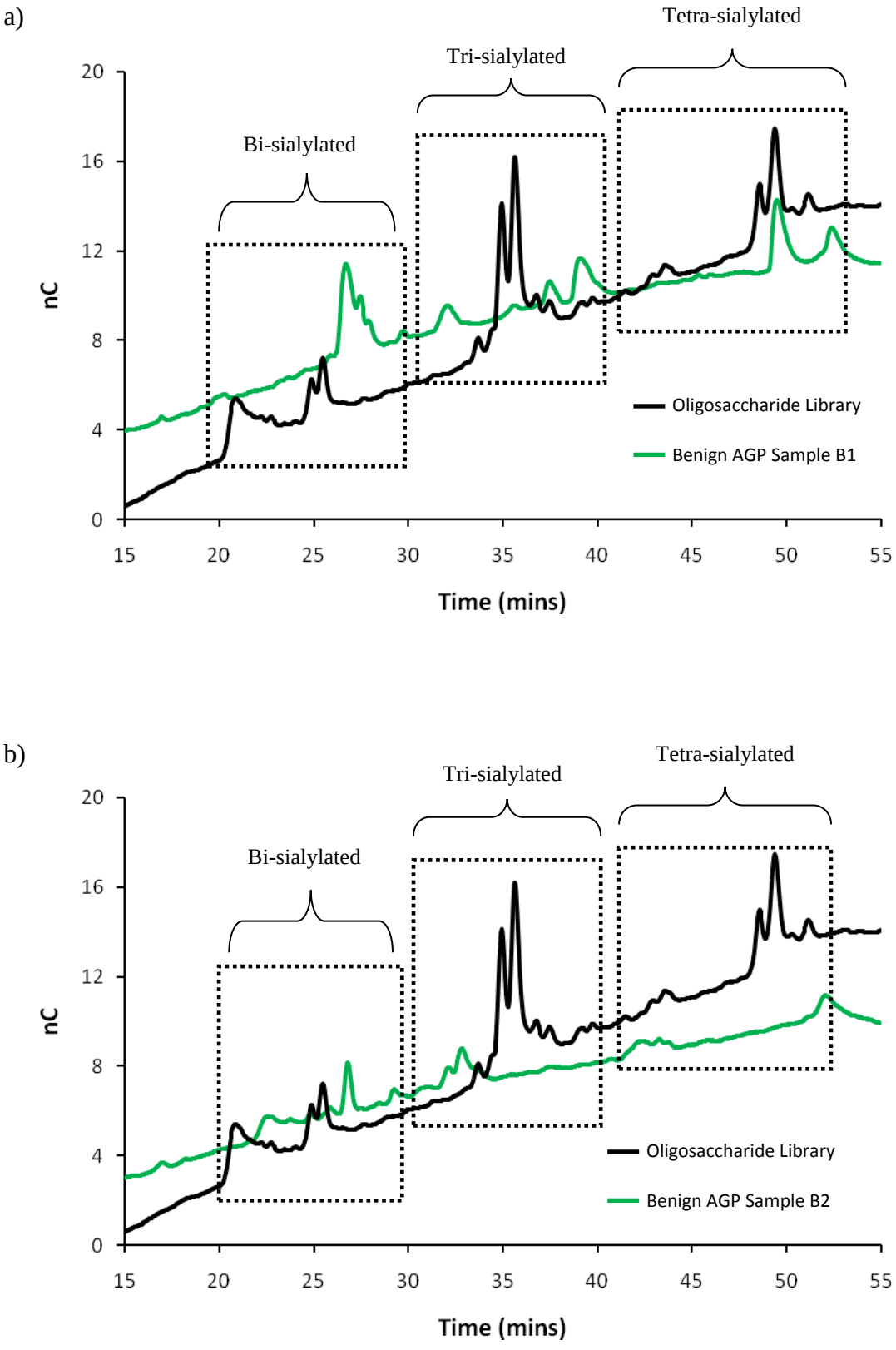


Figure 6.3 HPAEC trace of the oligosaccharide library compared to benign AGP samples a) B1 and b) B2.

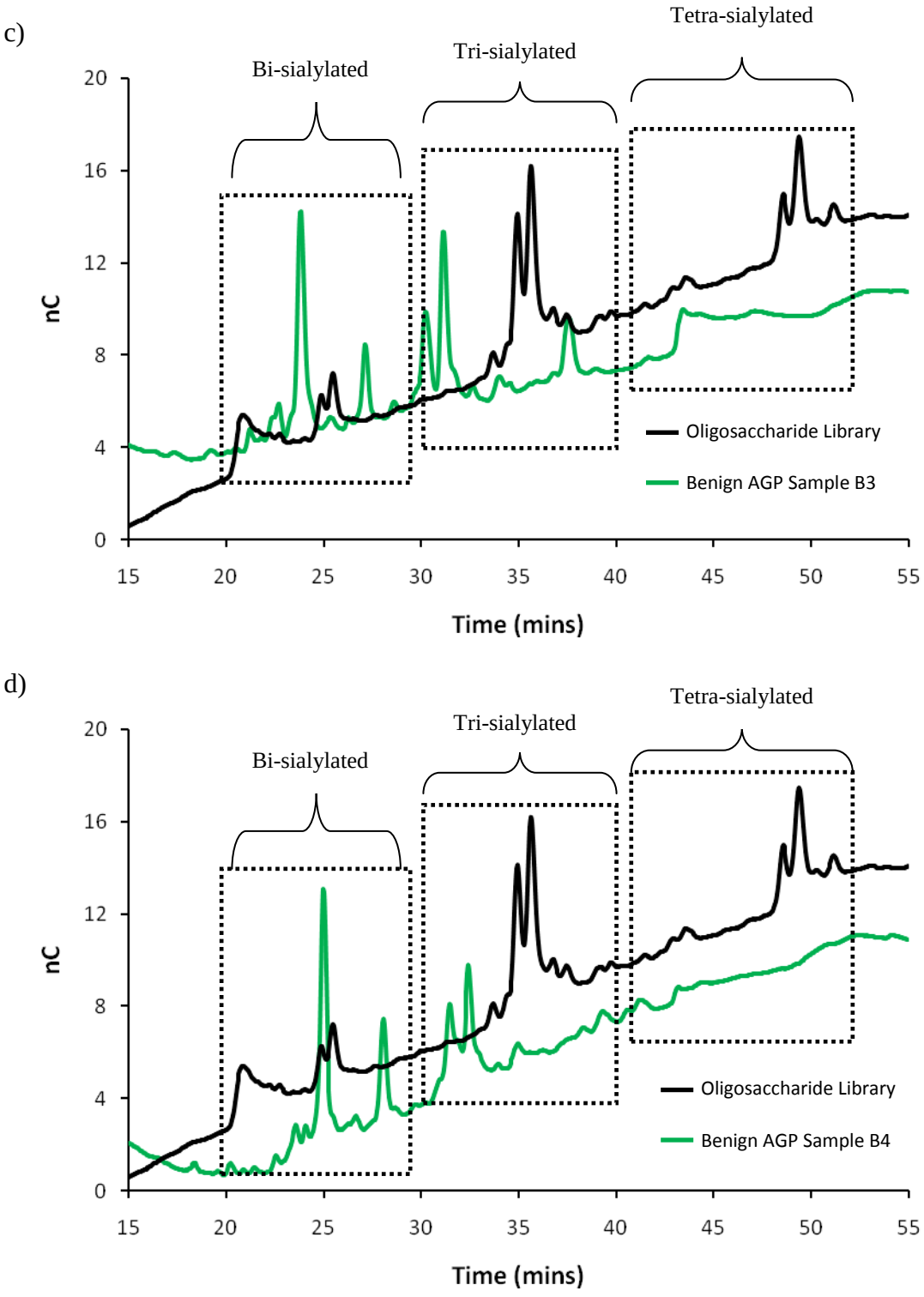


Figure 6.3 contd. HPAEC trace of the oligosaccharide library compared to benign AGP samples c) B3 and d) B4.

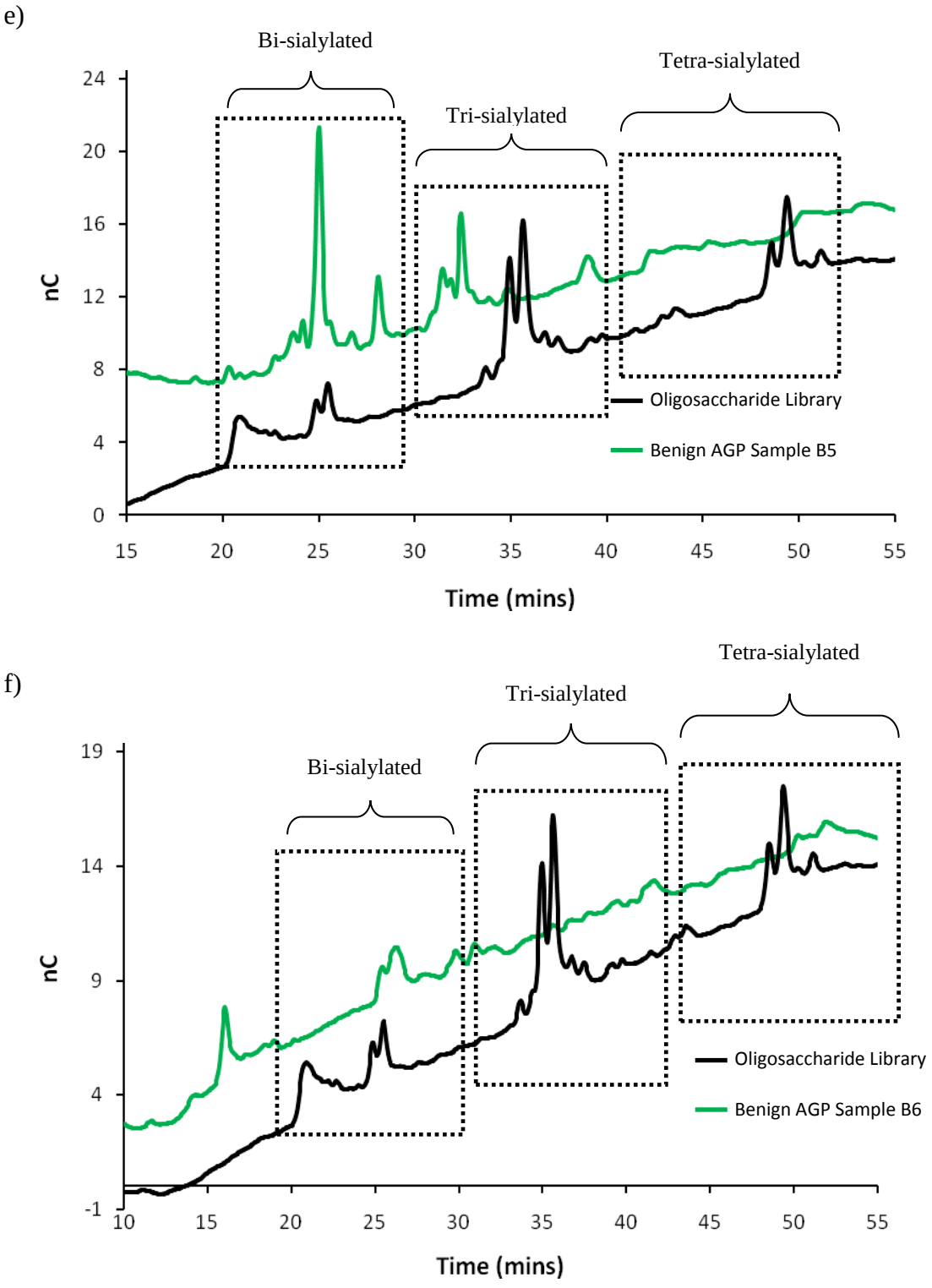
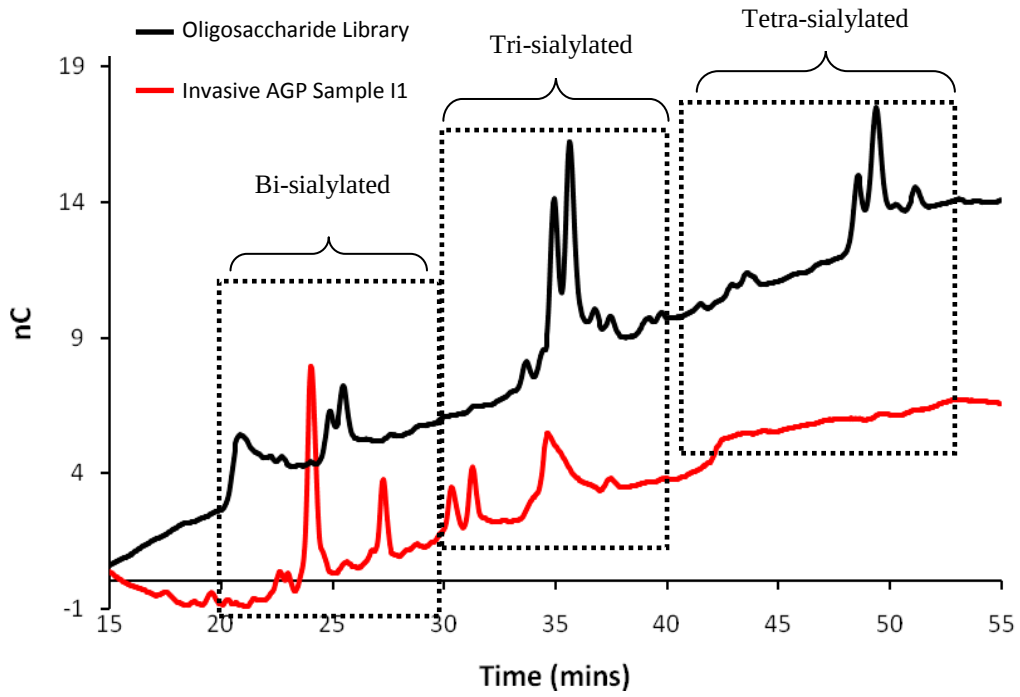


Figure 6.3 contd. HPAEC trace of the oligosaccharide library compared to benign AGP samples e) B5 and f) B6.

Figure 6.4 demonstrates the chromatograms produced from oligosaccharide analysis of the invasive breast cancer AGP samples. All invasive data were plotted in red. Unfortunately no data could be obtained for sample I5. In general there appears to be a greater proportion of bi-sialylated oligosaccharides with the proportion of tri-sialylated chains close behind. In the majority of samples there is little or no activity in the tetra-sialylated region with the exception of I11, 12, 13, 18 and 20 which all have a relatively distinct peak or number of peaks in this region. Mono-sialylated peaks were observed in I14, 15 and 17. Of the peaks in the bi- and tri-sialylated region, the majority eluted between 25 and 35 minutes.

a)



b)

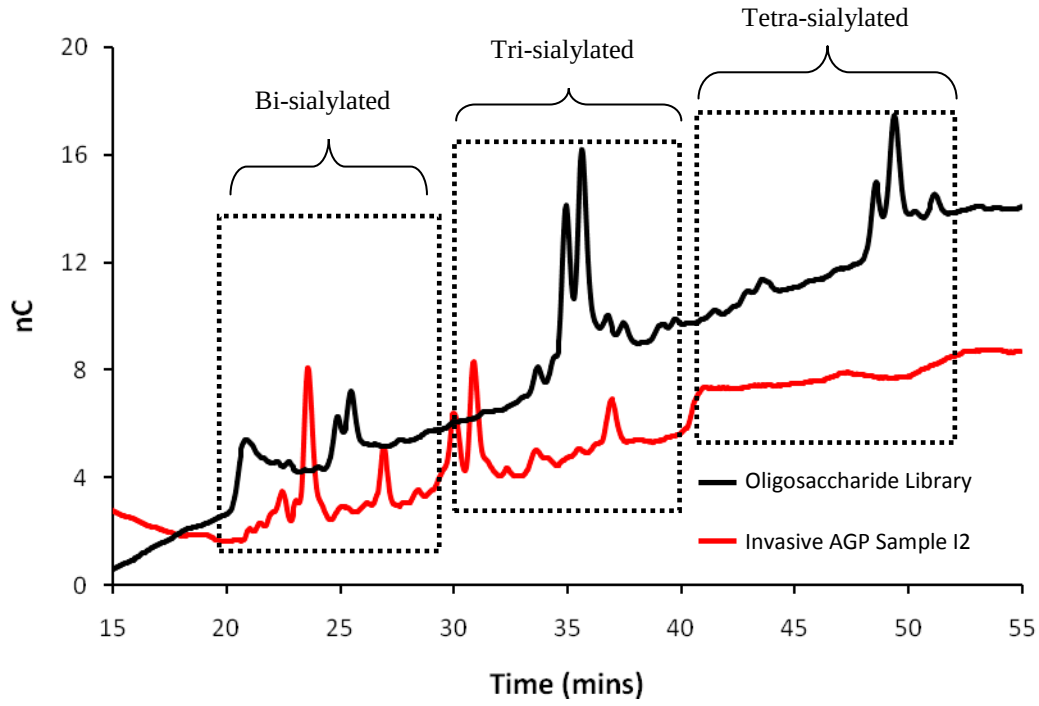
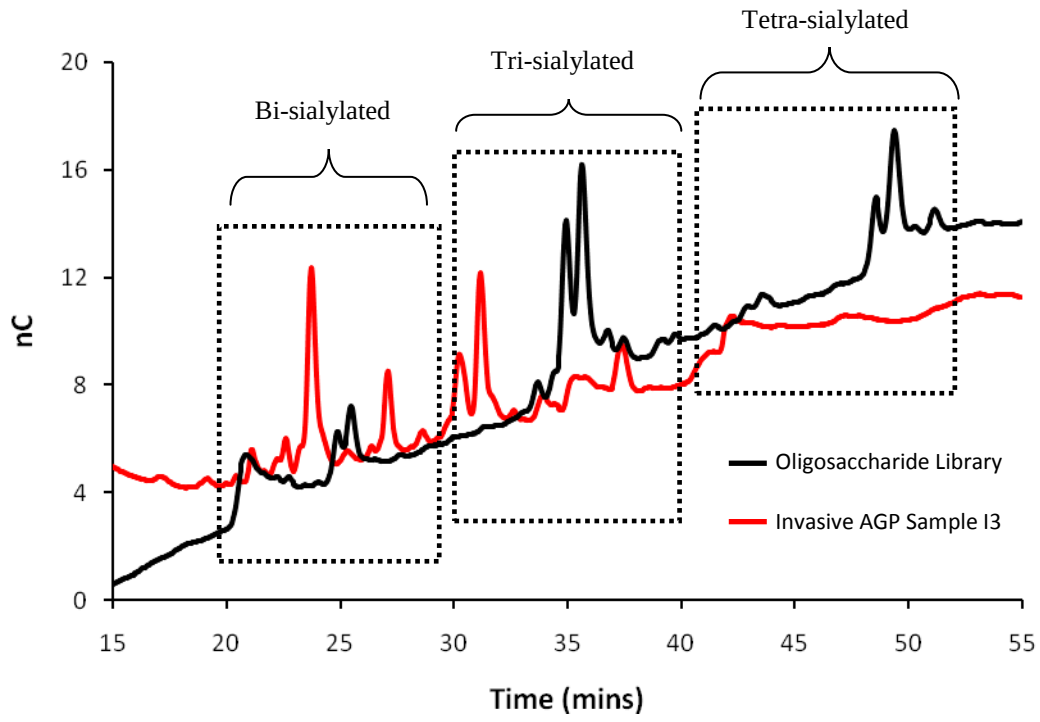


Figure 6.4 HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples a) I1 and b) I2.

c)



d)

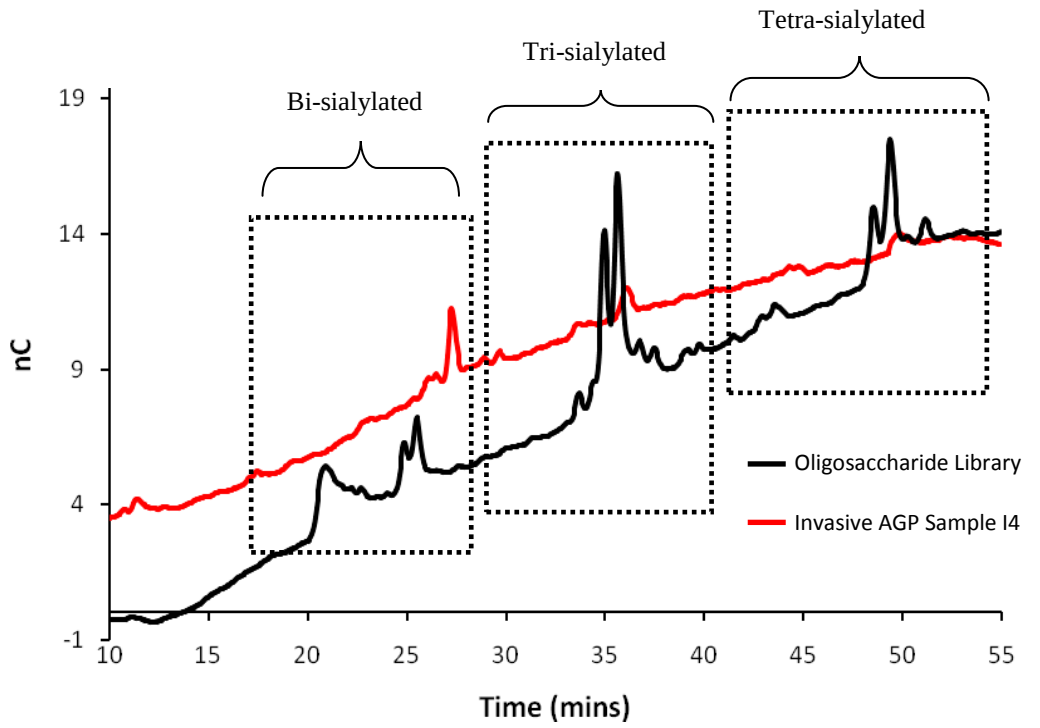
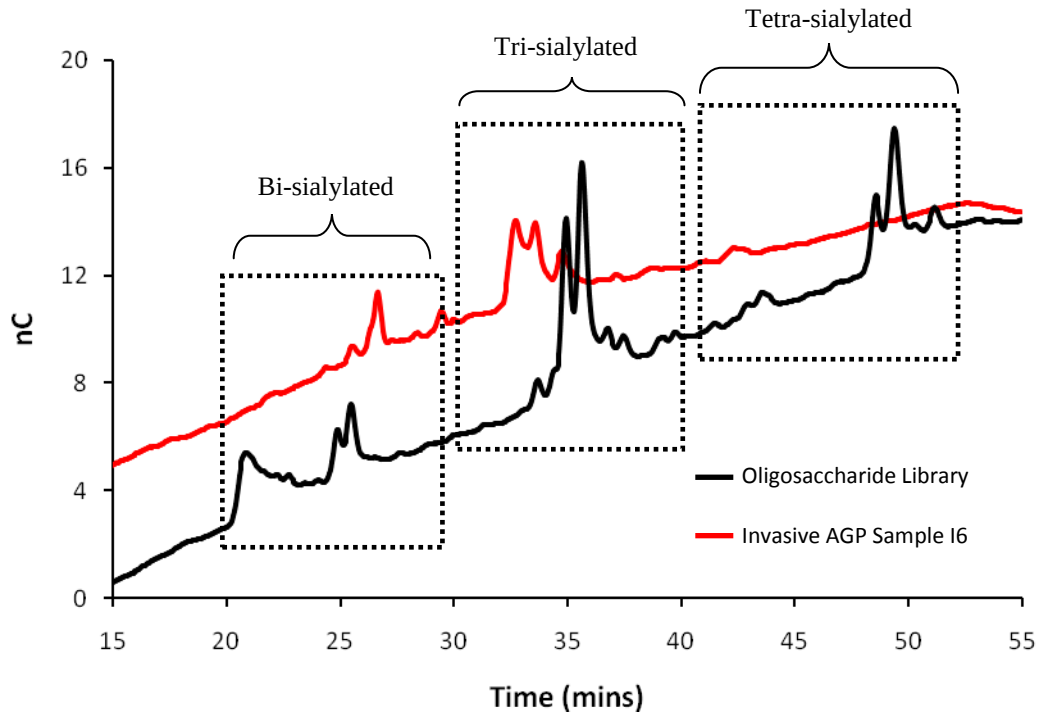


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples c) I3 and d) I4.

e)



f)

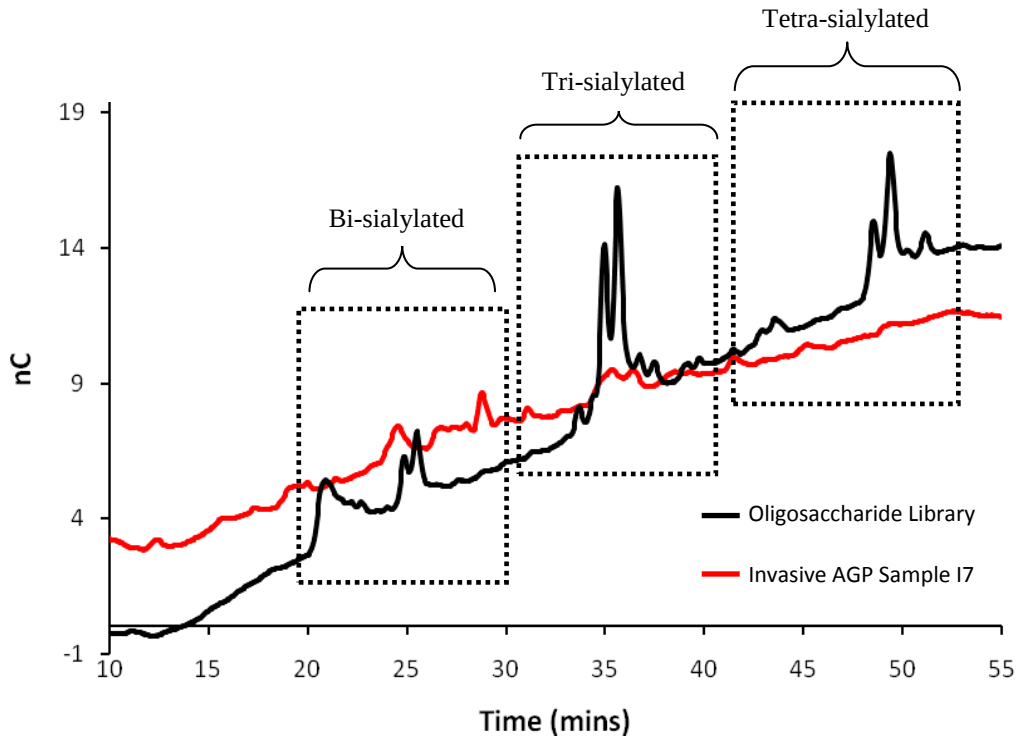
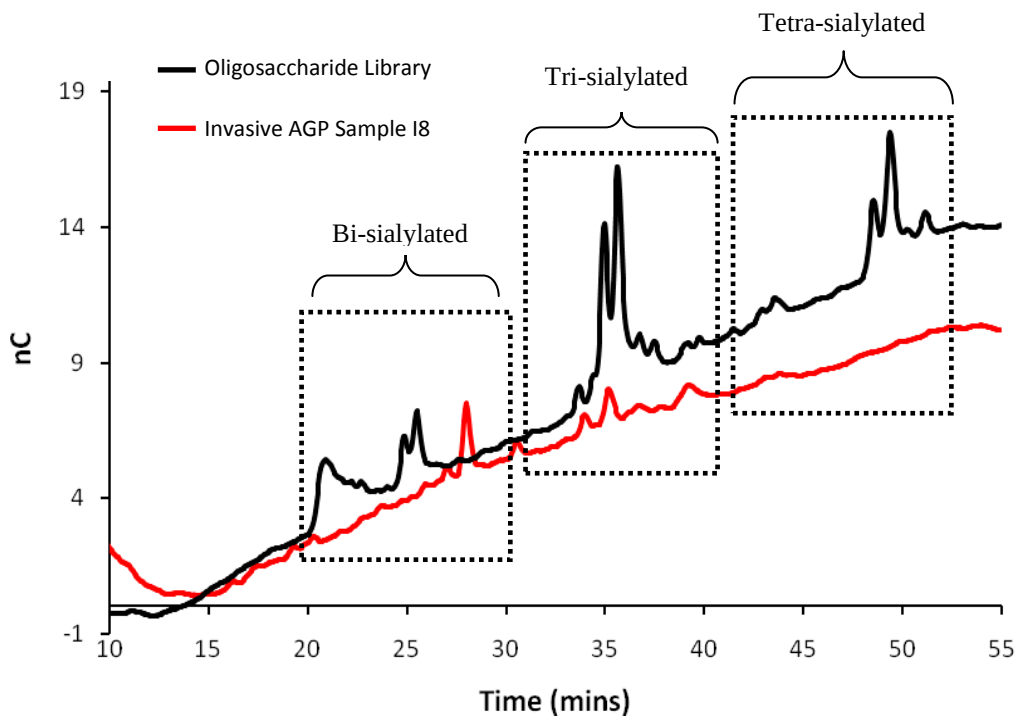


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples e) I6 and f) I7.

g)



h)

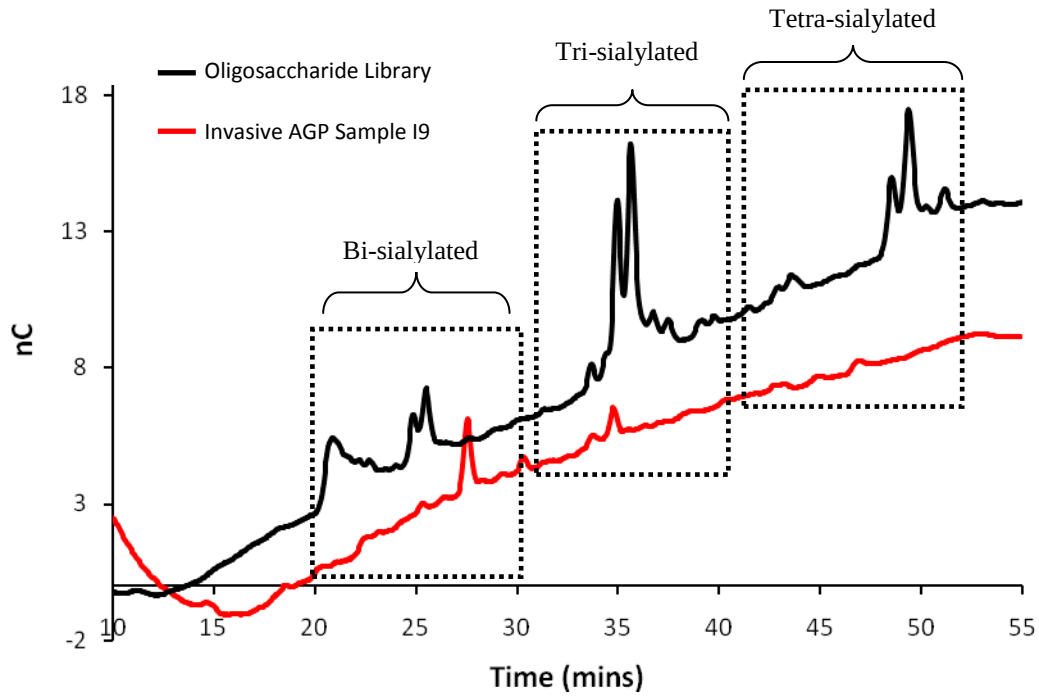
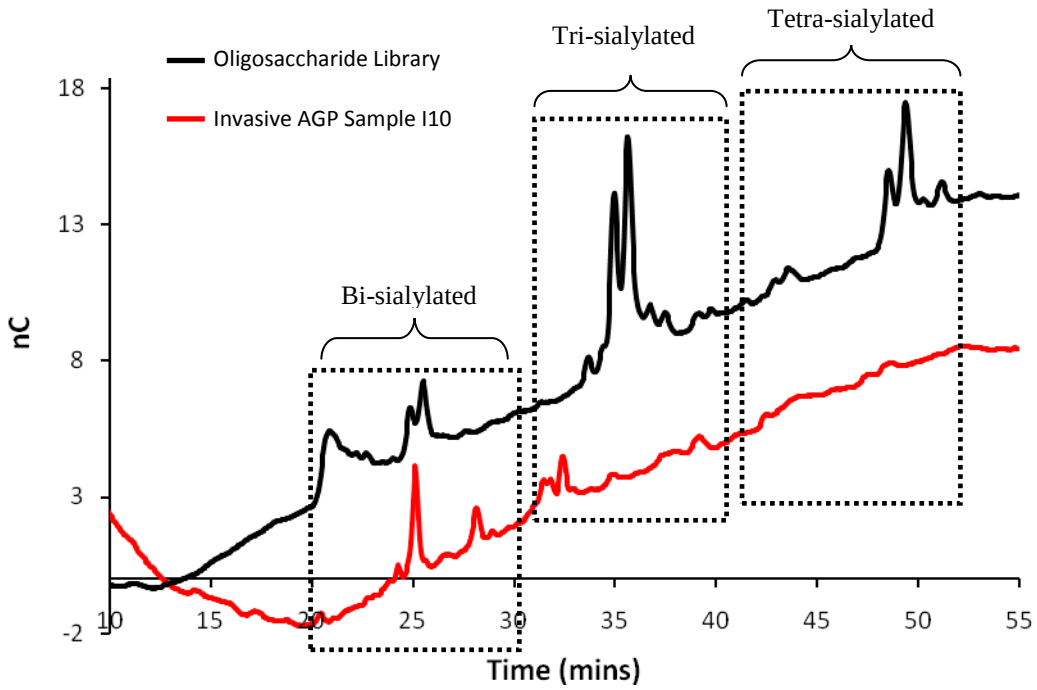


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples g) I8 and h) I9.

i)



j)

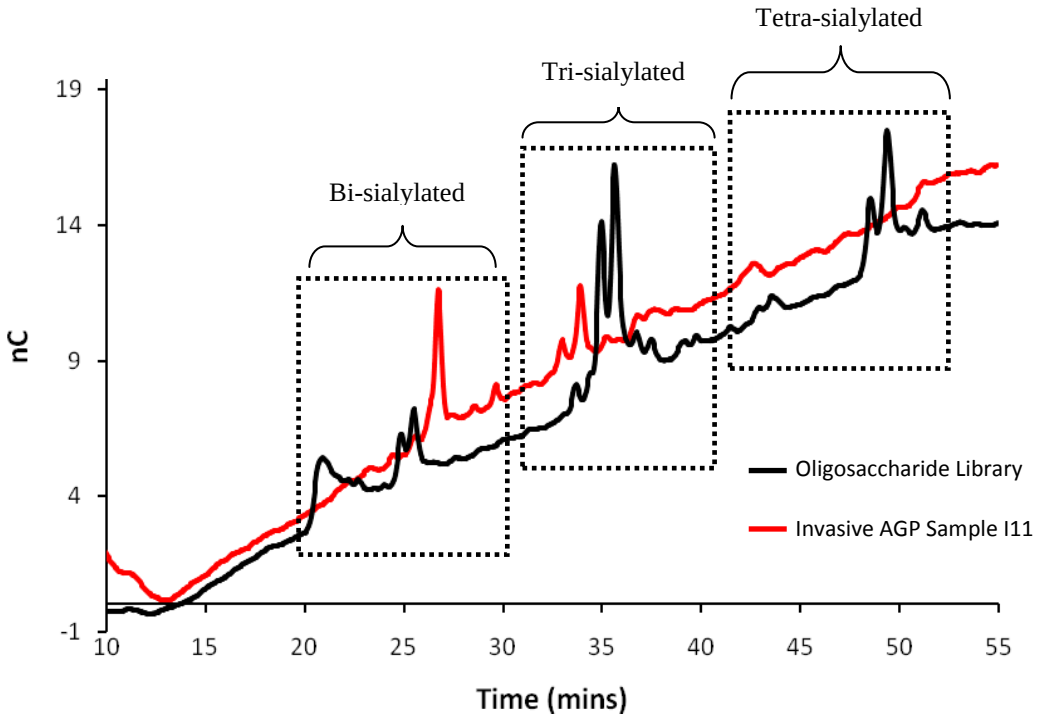
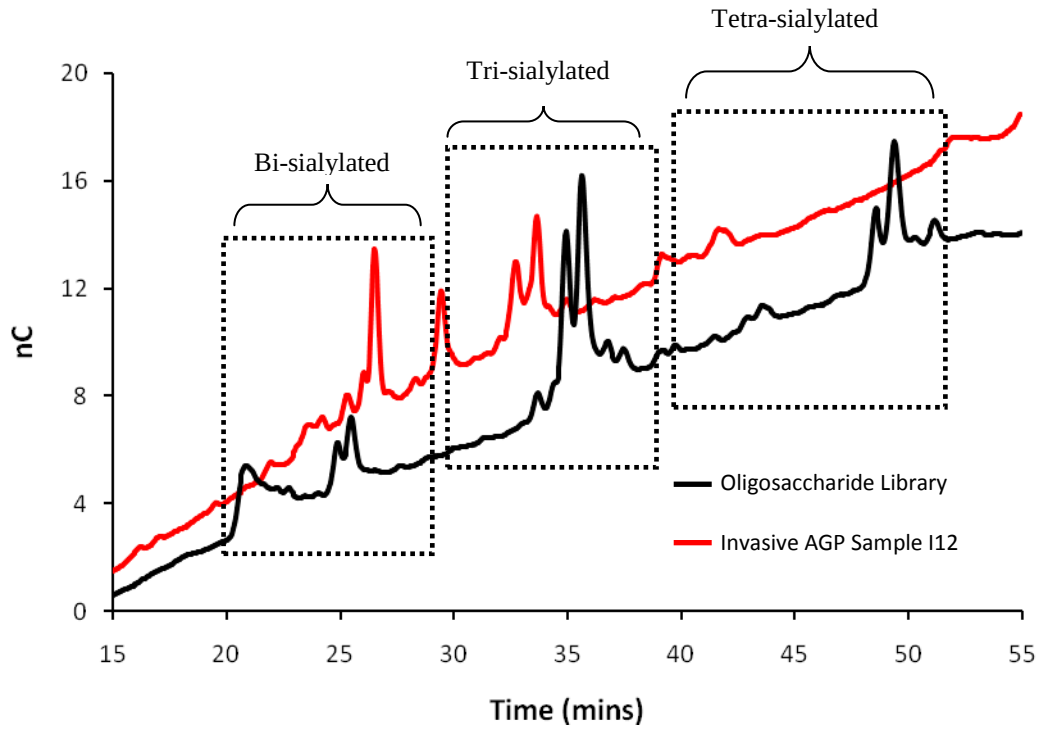


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples i) I10 and j) I11.

k)



l)

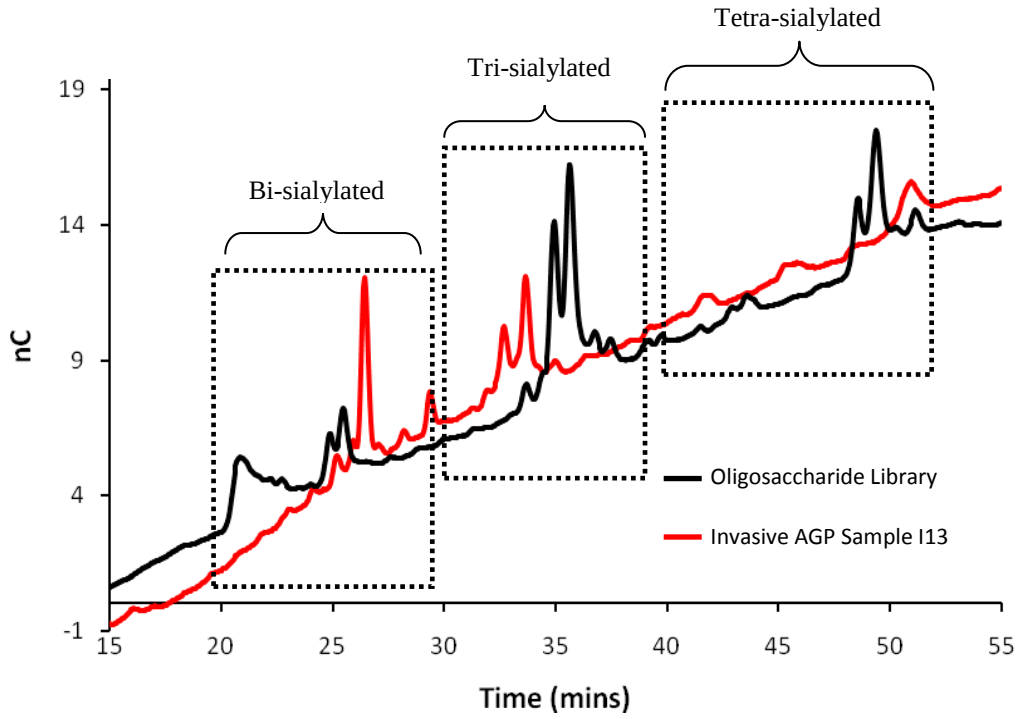
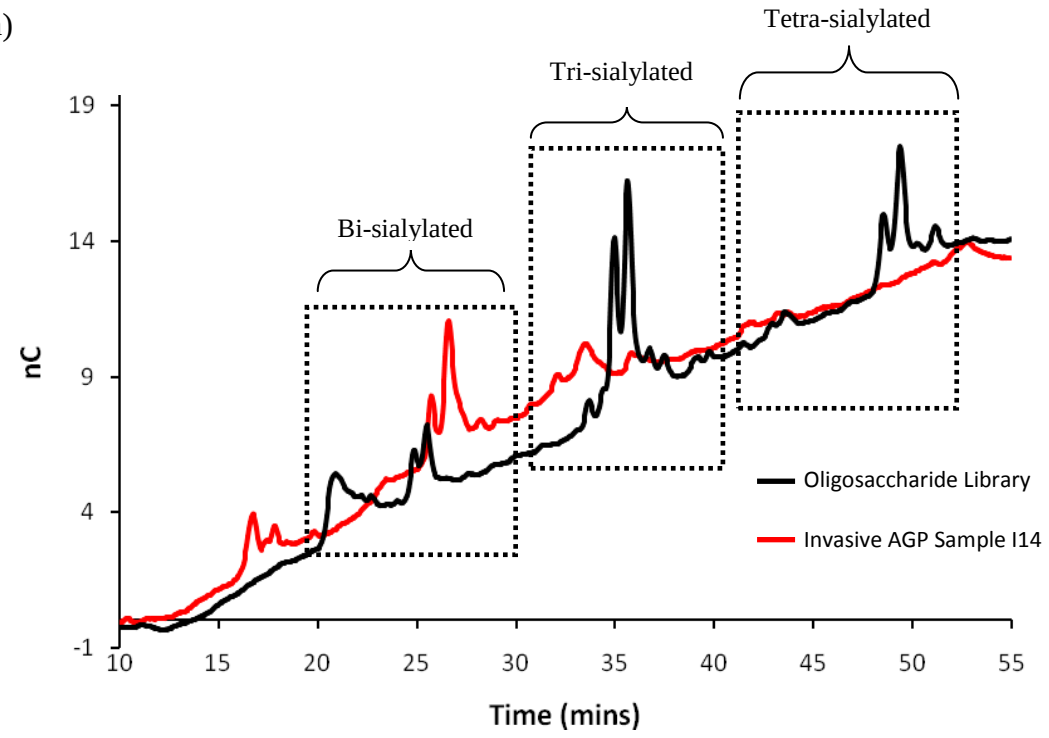


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples k) I12 and l) I13.

m)



n)

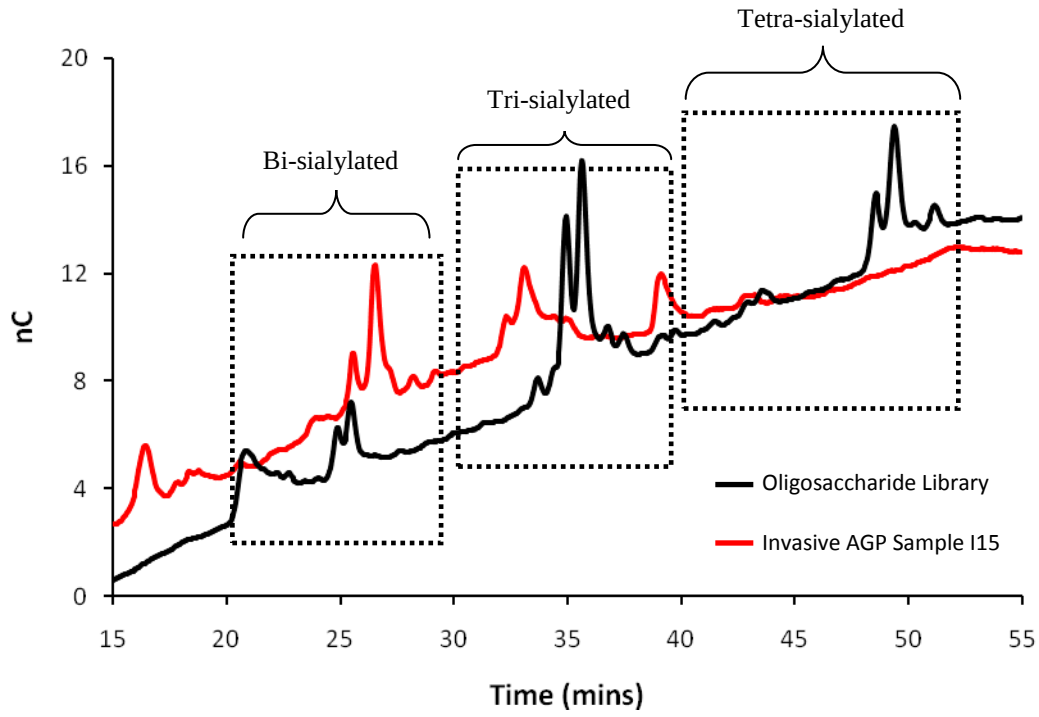
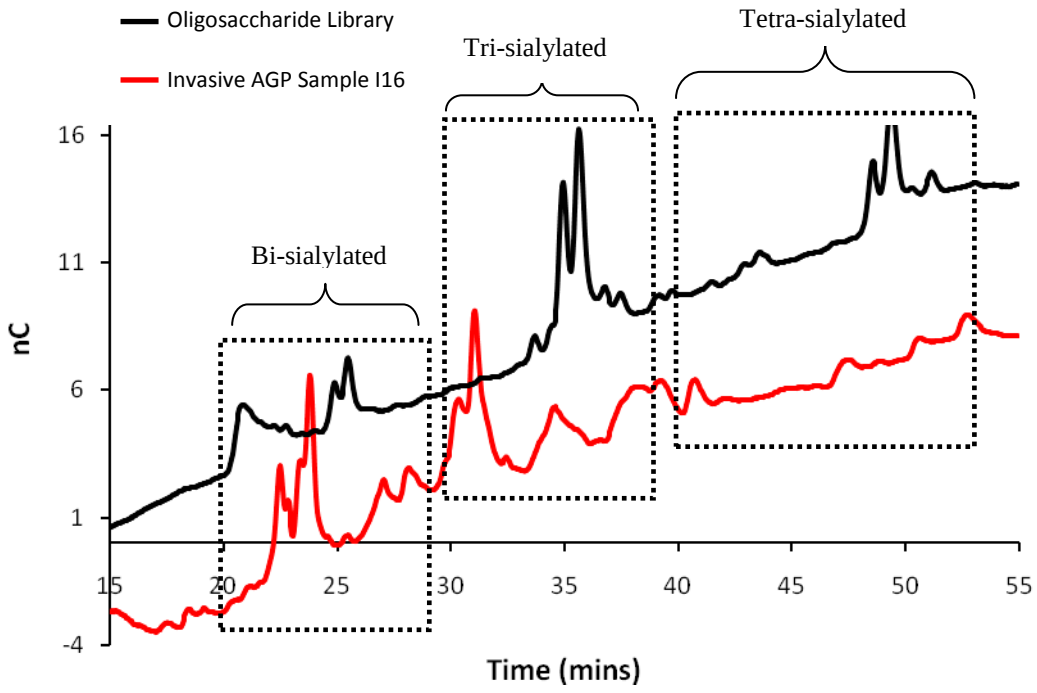


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples m) I14 and n) I15.

o)



p)

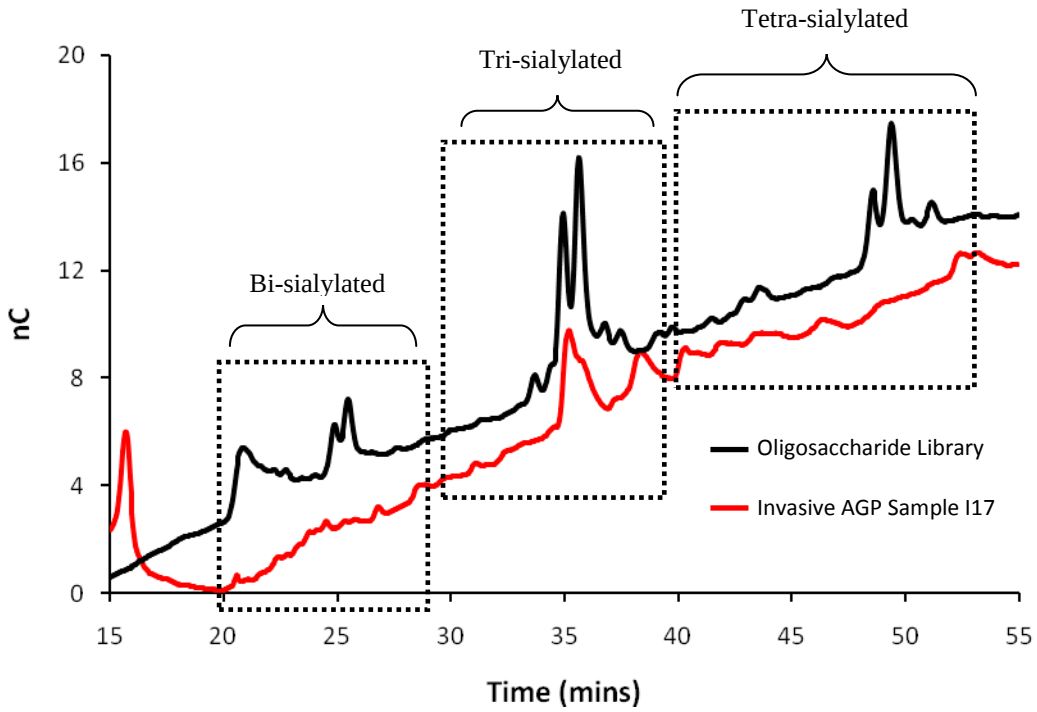
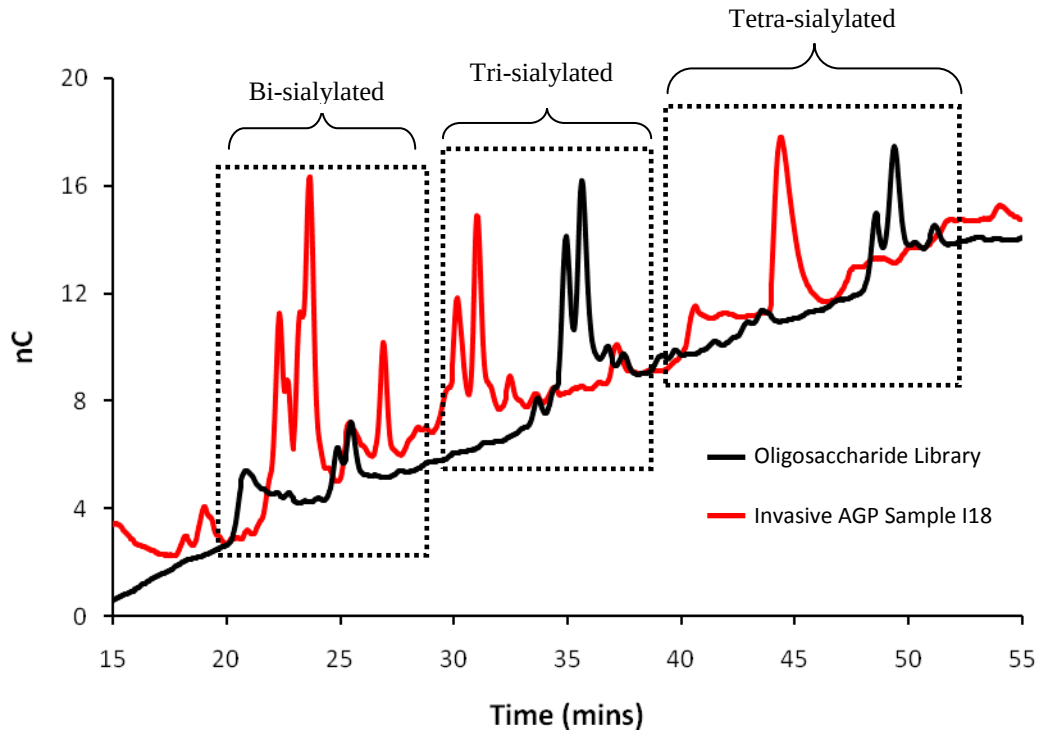


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples o) I16 and p) I17.

q)



r)

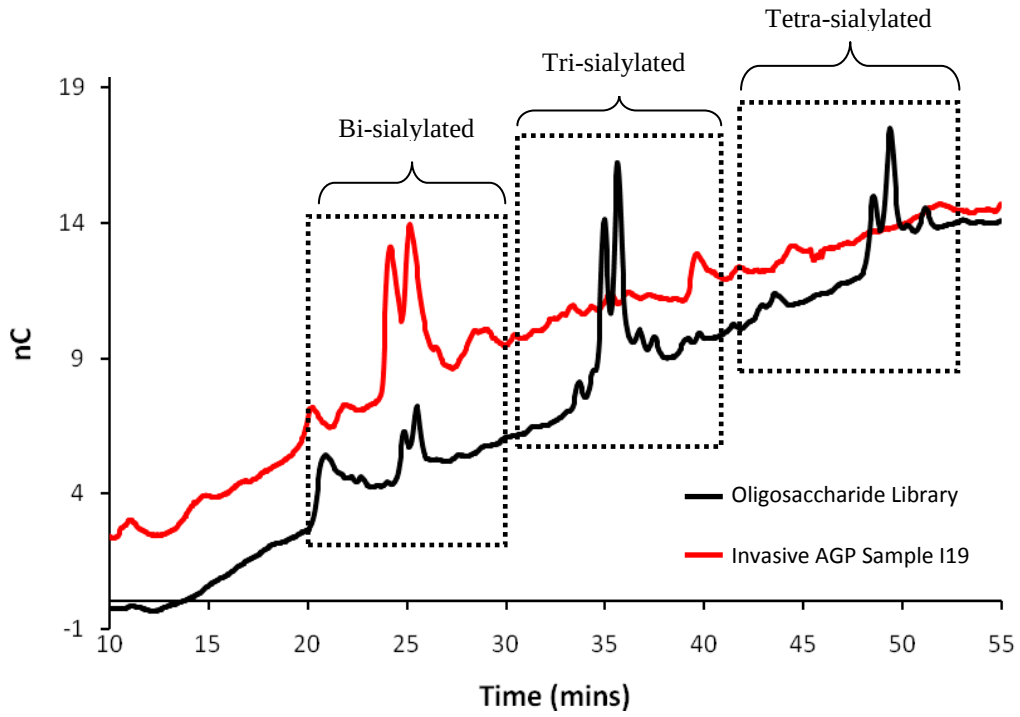


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to invasive breast cancer AGP samples q) I18 and r) I19.

s)

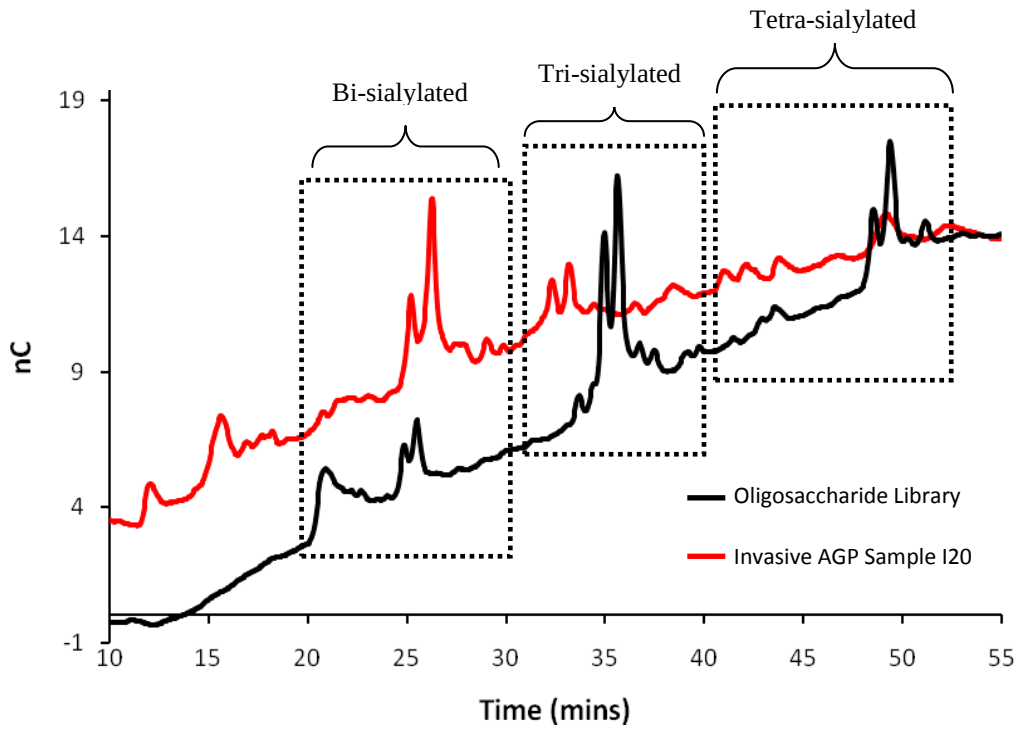


Figure 6.4 contd. HPAEC trace of the oligosaccharide library compared to s) invasive breast cancer AGP sample I20.

Discussion

Prior to HPAEC analysis, the oligosaccharides were denatured and subsequently enzymatically removed from the protein backbone. Denaturing conditions using sodium dodecyl sulphate (SDS) have been shown to partially desialylate N-glycans (Hermentin e*t al*., 1992) therefore a heat denaturation step was opted for instead. PNGase F was used to cleave the oligosaccharides from the protein. This enzyme is an amidase capable of hydrolysing the amide linkage between the Asn residue of the polypeptide backbone and the initial GlcNAc residue of the oligosaccharide chain resulting in the release of all N-linked glycans (Davis *et al.*, 1993).

The chromatogram produced from analysis of a commercial AGP sample (Figure 6.2a) demonstrates that the PNGase F enzyme successfully separated the glycans from the protein backbone to produce peaks in the same charge regions as the oligosaccharide library. The exact method of isolation and purification of the commercial sample was unknown. The normal AGP sample on the other hand, was subject to an identical isolation procedure to that of the patient samples (one which avoided desialylation of the glycans) therefore this chromatogram (Figure 6.2b), coupled with that of the oligosaccharide library was used to compare the sialylated structures present in normal and disease populations.

The slight difference in oligosaccharide profile of the commercial and normal AGP samples (Figure 6.2) is in agreement with the data obtained from monosaccharide compositional analysis. The normal sample displayed a reduction in the average GlcNAc and Gal content (Table 5.2) which indicated the possibility of decreased branching of the chains. The normal oligosaccharide profile displays a slight shift towards more bi-sialylated structures and a slight reduction in the retention time of the peaks within this region compared to the commercial sample. This may

demonstrate the presence of structures branched to a lesser degree than that of the commercial sample.

A number of the patient samples contain activity in the mono-sialylated region. This finding was highly unusual since the exposure of an outer Gal residue through the loss of a terminal NeuAc usually targets the molecule for degradation. Its presence in the sample may be explained by a branched chain possibly terminated with another structure e.g. a terminal antigen.

Figure 6.5 presents a comparison of HPAEC traces produced by the reference group (oligosaccharide library and normal AGP sample) to that of a representative trace from the benign and invasive breast cancer populations. Overall, the benign AGP samples appear to have an increased proportion of oligosaccharides displaying bi- and tri-sialylated structures and a lesser degree of tetra-sialylated structures. From this, benign sample B5 was chosen as a representative trace from this group. The invasive breast cancer group had a greater proportion of bi-sialylated oligosaccharides with the presence of tri-sialylated to a slightly lesser degree and little or no activity in the tetra-sialylated region. On this basis, invasive sample I3 was chosen as a representative trace for this group..

It is evident from Figure 6.5 that there is a degree of variability between the reference, benign and invasive populations. The benign trace is very similar to that of the normal trace with regards to the number and shape of the peaks produced although, within each charge band, the peaks eluted earlier in the benign sample.

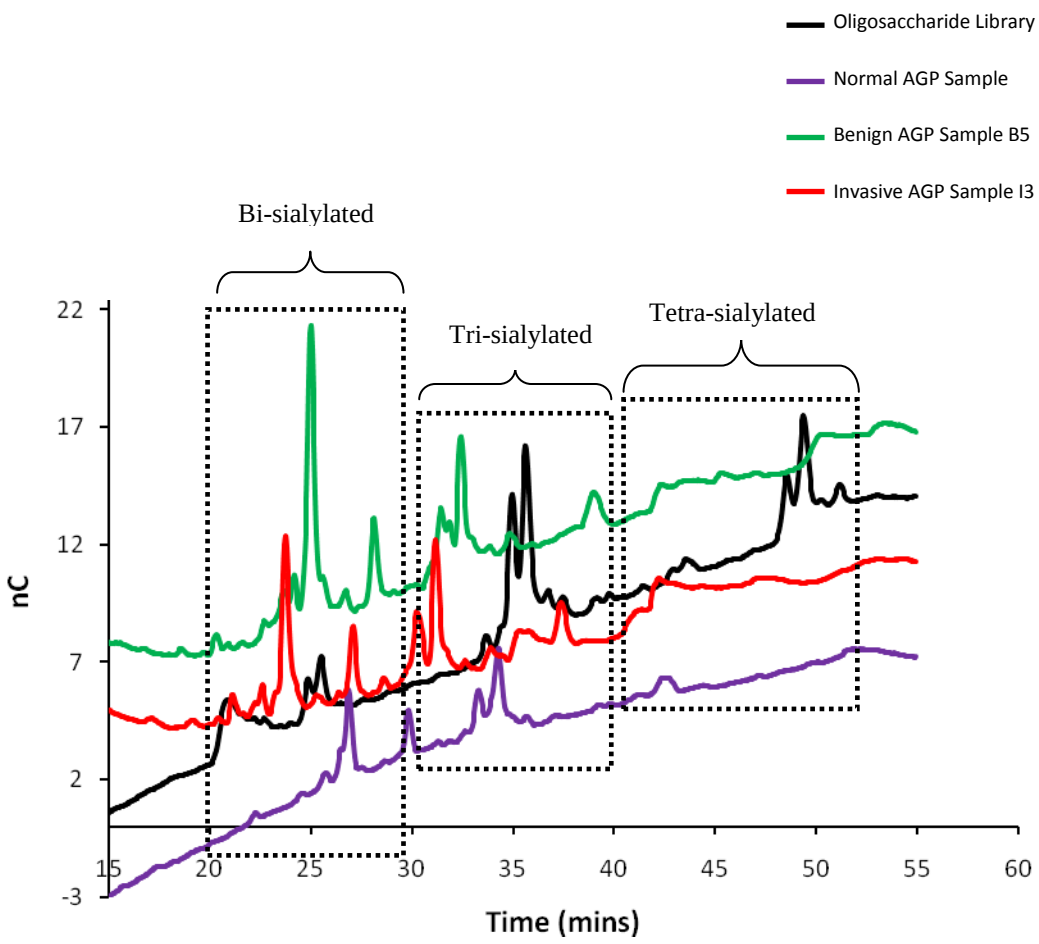


Figure 6.5 Comparison of HPAEC oligosaccharide analysis chromatograms of AGP from a normal, benign and invasive breast cancer sample as well as an oligosaccharide library.

Since the oligosaccharides are separated in order of increasing size, this could indicate a decrease in the branching of the structures in the benign sample. Referring to the average monosaccharide compositional data (Table 5.2) both the normal and benign groups have approximately the same GlcNAc and Gal content and differ only slightly in their Fuc content. The shift in the benign profile (decrease in peak retention time) may also be explained by this slightly higher fucose content observed in the benign population since as previously discussed, fucose has the ability to reduce the retention time of an oligosaccharide.

There is a very definite shift in the invasive breast cancer profile compared with the reference and benign groups. There appears to be greater activity in the bi-sialylated region compared with the tri- and tetra-sialylated regions. Within the bi- and tri-sialylated charge bands, there is a reduction in the retention time of the peaks compared to all other groups which may indicate a decrease in the branching of the oligosaccharide chains. Looking back at Table 5.2, the monosaccharide compositional data does not agree with this idea. The invasive group has a slightly higher level of GlcNAc and Gal compared to the normal and benign groups. Based solely on this data, the degree of branching of the invasive chains would be relatively similar compared to that of the normal and benign chains.

Of all the groups studied during monosaccharide analysis, the invasive breast cancer AGP population had the greatest average level of Fuc. The increased presence of this monosaccharide in the oligosaccharide chains may provide an explanation for the significant shift in the invasive AGP profile compared to that of normal and benign AGP.

The ability of HPAEC-PED to generate an AGP oligosaccharide ‘fingerprint’ allows alterations in the profile of normal and disease populations to be effectively monitored. The results from oligosaccharide analysis in the present study have

demonstrated that there are differences in the AGP profile between normal, benign and invasive breast cancer populations. These differences correlate to increasing severity of disease and are represented by a progressive increase in oligosaccharides displaying bi-sialylated chains and an overall decrease in the extent of branching.

CHAPTER 7

CONCLUSIONS

Conclusions

During the acute phase response, not only does the total serum concentration of AGP increase two to five fold, but the relative proportions of expressed glycoforms has been found to change with an increased expression of abnormal glycoforms. Higher than normal serum concentrations of AGP have been found in patients with breast cancer and the levels have been shown to increase with disease progression. Serum concentrations of AGP in patients with breast cancer are of no diagnostic value since the increased level may be due to a number of non-specific APR-inducing factors. AGP glycosylation has been found to be uniquely altered in disease conditions such as liver disease, inflammation and cancer however no extensive glycosylation studies have been performed on AGP from breast cancer patients.

Breast cancer is the most common cancer in women and currently diagnosis is reliant upon mammography and invasive techniques such as biopsy. There is no serum biomarker currently used in clinical practice for the detection and diagnosis of breast cancer. The discovery of a breast cancer-specific biomarker whose presence and/or altered expression in the serum precedes the appearance of a malignant mass would allow the development of a non-invasive serologic test and provide an invaluable screening tool in the assessment of high risk individuals.

The main aim of this research project was to determine whether quantitative and/or qualitative disease-specific alterations in the glycosylation pattern of AGP could be diagnostic for the detection of breast cancer at an earlier stage than existing methods, and whether it could form the basis for a diagnostic test.

As well as a normal patient population, samples were obtained from untreated patients with benign breast disease and invasive breast cancer. AGP was isolated

from plasma by a specifically chosen method that did not cause alteration to the native structure of the protein allowing analysis of the true *in vivo* state. The translation of the technique into a micro-scale version and subsequent omission of the Q-Sepharose chromatography column had only a minor impact on the purity of the final AGP preparation. Immunodiffusion experiments detected trace quantities of α_1-antitrypsin in the final sample and although this contaminant was also a glycoprotein, it is unlikely that the negligible amount present would have interfered with the glycosylation analysis of patient AGP.

Monosaccharide compositional analysis of the AGP oligosaccharide chains demonstrated that quantitative differences existed between patient populations. When present, the invasive breast cancer population displayed the highest level of the monosaccharide fucose compared to the commercial, normal and benign groups. Increased fucose levels have been observed in cancer and have been shown to correlate with the extent of cancer spread (Turner *et al*., 1985). In this study, fucose levels appear to correlate with the stage of disease (invasive disease has the highest level of the monosaccharide) but due to a lack of non-invasive breast cancer patient samples, a definite relationship between fucose levels and progression of the disease could not be determined. The monosaccharide GalNAc was detected in 90% of the invasive cancer samples but was absent from normal populations. Anderson *et al.* (2002) and Mooney *et al*. (2006) reported the presence of GalNAc upon analysis of AGP isolated from the plasma of hepatitis C and fibrosis patients respectively but there have been no such reports relating to breast cancer AGP. The novel modification identified in the current study was also found on AGP isolated from the benign group therefore, although not breast cancer-specific; it appears to be useful in identifying patients with breast disease.

GalNAc is not usually observed as a constituent of AGP oligosaccharide chains. We explained its presence by suggesting the possible attachment of a terminal antigen to the oligosaccharide chains. We performed immunodiffusion experiments, incubating

the AGP samples with antibodies against two known GalNAc-containing antigens, Blood Group A (Greenwell, 1997) and Sialyl Tn (Brocke and Kunz, 2002). The results demonstrated that neither antigen was present in any of the GalNAc positive patient samples.

Oligosaccharide analysis demonstrated the qualitative differences in the AGP profiles from patients in the normal, benign and invasive groups. There was a progressive disease-specific shift in the oligosaccharide 'fingerprint' from normal which correlated to the severity of disease. The changes were represented by a progressive increase in AGP oligosaccharides displaying bi-sialylated structures and an overall decrease in the branching of the chains. These results were in agreement with previous work by Hansen *et al*. (1984) and Fujii *et al*. (1988) who reported increased expression of bi-antennary glycans on AGP from lung and liver cancer patients respectively.

The current study has identified that the quantitative and qualitative changes in AGP glycosylation observed among patients with breast disease and breast cancer may in fact, provide the basis of a serum biomarker with potential prognostic utility.

Future Work

This study has demonstrated the potential use of AGP glycosylation as a biomarker for breast disease and possibly breast cancer. This preliminary project has used a relatively small patient cohort therefore further work in this area would allow validation of the conclusions drawn from the current study.

- The patient sample numbers could be increased ensuring that the results obtained are truly representative of the population as a whole.
- An age matched control population of healthy donors would provide a direct comparison between the glycosylation of AGP in health and disease.
- Analysis of samples from patients with non-invasive cancers such as ductal carcinoma in situ and lobular carcinoma in situ would allow the direct comparison of the AGP glycosylation of non-invasive and invasive carcinoma. This would also allow the evaluation of any disease-specific changes with regards to severity of disease and disease progression.
- Analysis of invasive breast cancer patient AGP with varying prognostic evaluations (various stages of disease) to determine if AGP glycosylation can provide prognostic information on the extent of tumour spread and possible lymph node involvement and/or metastatic spread.
- Tumours positive for the HER-2/neu receptor represent a more aggressive form of disease and a worse prognosis. AGP from patients with HER-2 positive and HER-2 negative tumours, within a given cancer population (non-invasive, invasive) could be analysed to determine if AGP glycosylation correlates to the presence of this aggressive phenotype.
- The follow-up of patients with benign breast disease and analysis of further samples over time may allow any correlation between the presence of GalNAc and subsequent breast cancer risk to be determined.

- Further immunodiffusion experiments with a variety of antibodies against known GalNAc structures may allow identification of the source of the modification.
- The development and optimization of an ELISA technique with suitable standards would allow for rapid analysis and quantification of the GalNAc present.
- The isolation of AGP from plasma detailed in this research study is a lengthy, time-consuming process. In order for AGP analysis to be considered a feasible test for use in a national breast screening programme a rapid, high through-put technique such as a protein microarray would have to be developed to cope with demand of such a service.

CHAPTER 8

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