ALPHA-1-ACID GLYCOPROTEIN AS A POTENTIAL BIOMARKER OF BREAST CANCER IN AT RISK INDIVIDUALS.

Submitted by

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Declaration

It is hereby declared that 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 led to the award of a degree.

Signed:

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"Happiness can be found, even in the darkest of times, if one only remembers to turn on the light"

Abstract

The identification of a blood-based diagnostic biomarker for breast cancer (BC) would be particularly beneficial to those at increased risk of developing BC and could result in earlier detection which may increase survival rates due to earlier treatment. Alterations in α-1-acid glycoprotein (AGP) glycosylation levels occur during disease and this study sought to determine the diagnostic potential of AGP glycan variation in triple negative breast cancer (TNBC) compared to BC of unknown type and healthy controls as well as women at increased risk of developing BC compared to age-matched healthy controls.

AGP was isolated from blood of two different sample populations using low pressure chromatography. AGP purity was confirmed using SDS-PAGE and concentration determined using spectrophotometry. Structural analysis of AGP glycan monosaccharide and oligosaccharide content was undertaken using high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Increased AGP concentrations were observed, in comparison to their controls, in BC of unknown type, TNBC and “at-risk” samples. Quantitative alterations in monosaccharide composition were also present. N-acetylgalactosamine (GalNAc) was present in over 88% of TNBC samples and was inversely correlated with age. For the TNBC groups, GalNAc was also present at higher levels in samples of individuals with family history of BC. There was an overall increase in GlcNAc levels compared to age-matched healthy controls and GalNAc presence in 81% of “at risk” samples.

Oligosaccharide analysis revealed increased branching in BC of unknown type and TNBC <35 years of age, whereas the “normal” healthy population and TNBC >35 possessed less branching. A similar trend was observed between the “at risk” samples and the age-matched controls. These branching patterns aligned well with the corresponding monosaccharide data.

Overall, this study indicated that alterations in AGP levels and glycosylation exist between TNBC compared to BC of unknown type and “normal” healthy controls as well as an “at risk” population and age-matched healthy controls. The data could underpin the development of a new diagnostic BC biomarker.

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Abbreviations

AAT	alpha-1-antitrypsin
AGP	alpha-1-acid glycoprotein
ANOVA	analysis of variance
APR	acute phase response
Arg	Arginine
Asn	Asparagine
BC	breast cancer
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
BMI	body mass index
CA15.3	cancer antigen 15.3
CA27.29	cancer antigen 27.29
CDH1	cadherin 1, E-cadherin
CEA	carcinoembryonic antigen
CGD	Clinical Genetics Department
CHEK2	checkpoint kinase 2
CI	Chief Investigator
CMF	cyclophosphamide, methotrexate and fluorouracil
Con A	conclavin A
DCIS	ductal carcinoma *in situ*
DNA	deoxyribonucleic acid
Dol-P	dolichylphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENU	Edinburgh Napier University
EPO	erythropoeitin
ER	oestrogen receptor
fMLP	*N*-formylmethionyl-leucyl-phenylalanine
Fuc	fucose
Gal	galactose

GalNAc	*N*-acetylgalactosamine
GCSF	granulocyte colony stimulating factor
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GlcNAcT-V	*N*-acetylglucosaminyltransferase V
GP	general practitioner
HCl	hydrochloric acid
HER2	human epidermal growth factor 2
HPAEC-PAD	high pH anion-exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
HR	hormone receptor
HRT	hormone replacement therapy
HSA	human serum albumin
IDC	invasive ductal carcinoma
IHC	immunohistochemistry
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
ILC	invasive lobular carcinoma
KCl	potassium chloride
kDa	kilo Dalton
KSCN	potassium thiocyanate
LCIS	lobular carcinoma *in situ*
LPS	lipopolysaccharide
Lys	Lysine
Man	mannose
MCA	mucin-like carcinoma associated antigen
MRI	magnetic resonance imaging
MUC-1	mucin 1
NaCl	sodium chloride

NaOAc	sodium acetate
NaOH	sodium hydroxide
NeuAc	neuraminic acid
nm	nanometres
NPI	Nottingham Prognostic Indicator
NTAC	neoadjuvant Taxotere®, Adriamycin® and cyclophosphamide
OH	hydroxyl group
ORM1	orosomucoid 1
ORM 2	orosomucoid 2
OST	oligosaccharyltransferase
PEG	polyethylene glycol
PHA	phytohaemagglutinin
Phe	phenylalanine
pI	isoelectric point
PIS	participant information sheet
pKa	dissociation constant
PNGase F	peptide-*N*-glycosidase F
PR	progesterone receptor
Pro	Proline
PTEN	phosphatase and tensin homologue
PWM	pokeweed mitogen
R&D	research and development
REC	research ethics committee
RER	rough endoplasmic reticulum
ROS	reactive oxygen species
RT	radiotherapy
SA	sialic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser	Serine
SLe^x	sialyl lewis X antigen
STK11	serine-threonine kinase 11
TAC	Taxotere®, Adriamycin® and cyclophosphamide
TFA	trifluoroacetic acid
Thr	Threonine
TMED	tetramethylethylenediamine
TNBC	triple negative breast cancer
TNF-α	tumour necrosis factor alpha
TNM	tumour node metastasis
TP53	tumour protein 53
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
UDP	uridine diphosphate
UICC	Union for International Cancer Control
UK	United Kingdom
UV	ultra-violet

Preface

This thesis is a written account of independent postgraduate research undertaken by the author, investigating Alpha-1-acid glycoprotein (AGP) glycosylation as a potential biomarker of breast cancer (BC) in women at risk of BC development.

Acute phase reaction (APR) as a result of pathological and physiological conditions causes AGP (a positive acute phase protein) plasma concentration to increase 2-5 fold, with increased expression of abnormal AGP glycoforms compared to normal AGP glycoforms. Raised AGP levels have been identified in BC patients and progression of the disease has been linked to increasing AGP plasma levels. Additionally, there is evidence that unique alterations of AGP glycosylation are present in other disease conditions thus there is a strong possibility that the same is true of BC.

Chapter 1 provides an overview of current knowledge regarding glycan biosynthesis and their attachment to proteins as well as more detailed information about AGP's structure and known functions. Furthermore, the recent links between triple negative breast cancer (TNBC) occurrence and BRCA1 and BRCA2 mutations are introduced with discussion of the patient pathway and current guidelines for increased surveillance of these individuals.

Chapter 2 summarises the materials and methodology used to conduct the research including low pressure chromatography for isolation of AGP, SDS-PAGE to prove successful AGP isolation, spectrophotometry for calculation of concentration of AGP present in samples, acid hydrolysis and enzyme digestion to cleave monosaccharides and oligosaccharide for analysis and finally high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for analysis of AGP glycan composition (monosaccharides) and structure (oligosaccharides).

Chapter 3 discusses the two datasets of samples recruited for the study. Dataset 1 was comprised of plasma samples from women suffering from TNBC at various ages compared to "normal" healthy samples and women with BC with an unknown molecular subtype. Dataset 2 was composed of women at increased risk developing BC due to family history risk. This cohort were recruited from a local NHS familial breast screening clinic (for women under age

50 with an increased risk due to genetic testing or family history risk assessment) who then were compared to age-matched healthy controls in a case-control style manner.

Chapter 4 illustrates the results of AGP isolation and level determination from both datasets. Discussion is given regarding the variations or similarities in AGP levels in different sample populations of both dataset 1 and dataset 2 as well as the isolation method chosen.

Chapter 5 provides a comprehensive overview of HPAEC-PAD and its use for analysis of monosaccharide content of glycans. Monosaccharide content results of AGP glycans using HPAEC-PAD are displayed and discussed, as variation in these levels can indicate alterations in structure.

Chapter 6 presents the versatility of HPAEC-PAD and its subsequent use for structural analysis of intact AGP glycans (oligosaccharide). An explanation of the technical aspects of the technique is given as well as presentation and discussion of sample results from datasets 1 and 2.

Lastly, chapter 7 details the conclusions drawn from this study in terms of AGP's potential use a BC biomarker in "at risk" individuals. Additionally this chapter give suggestions for future work that could supplement the current research.

The results of this study have been published in part in the following publication:

Dewar, E., Matthews-Smith, G., Cetnarskyj, R., and Smith, K.D., (2014). Alpha-1-Acid Glycoprotein as a Breast Cancer Biomarker in At Risk Individuals. 9th European Breast Cancer Conference (abstract number 240), Glasgow, March

2014.

Aims

To evaluate the diagnostic potential of alterations to AGP levels and glycosylation patterns in TNBC compared to BC of an unknown molecular type as well as “normal” healthy controls and women at increased risk of BC development compared to a ‘normal’ healthy age-matched control population.

Hypothesis

This study hypothesised that significant differences in AGP levels, monosaccharide content and oligosaccharide (glycan) structure may be present in TNBC samples compared to BC of an unknown molecular type and “normal” healthy controls. It was also hypothesised that modifications to AGP glycan content, structure and levels occur in women at increased risk of BC development, which may be seen at the early stages of BC development, compared to “normal” healthy age-matched controls.

Contents

Chapter 1 – Literature Review

1.1 Breast Cancer.

1.1.1 Breast Cancer Overview.

Breast cancer (BC) is the most common cancer in women in the UK with over 49,000 new cases in 2011 and accounts for almost a third of cancers diagnosed in women (Information Services Division: ISD Scotland., 2014; Northern Ireland Cancer Registry., 2013; Office for National Statistics., 2011; Welsh Cancer Intelligence and Surveillance Unit., 2014). This type of cancer occurs when the DNA of ductal epithelial cells of the breast is altered from proto-oncogenes to oncogenes, leading to formation of malignant masses, or tumours, in the breast tissue. These DNA modifications can be sporadic or inherited with family history being a significant risk factor in BC development. Inherited BCs are often diagnosed at a young age and are more commonly bilateral (National Cancer Institute., 2014). However, the majority of BCs are sporadic and tend to occur when somatic cells i.e. non-germline cells that are not inherited become mutated. This may be due to the individual having several risk factors linked to BC development (see Table 1.1), with scientists estimating that 42% of BC incidences could be prevented by being physically active, drinking less alcohol and reducing obesity (World Cancer Research Fund., 2011). Other risk factors for the general population include being female (Siegel*, et al.*, 2012), aging (Sasieni*, et al.*, 2011) and hormone replacement therapy (Beral, 2003; Ritte*, et al.*, 2012).

1.1.2 High Risk Factors for Breast Cancer Development.

Assessing increased risk using family history is formally defined using a specific criteria. In Scotland the criteria used are strong family history of BC as well as mutations of BRCA1 and BRCA2 genes which are considered high risk factors for BC development (The Scottish Government., 2009). Currently Clinical Genetics departments (CGD) in Scotland are the gatekeepers for early breast screening based on family history which has been assessed using the criteria. If a person has a family history of BC or ovarian cancer their GP can refer them to clinical genetics after consulting guidelines for risk assessment and CGD will formally take and confirm the cancers in a family history to then apply a risk of low, moderate, high or very high risk of the individual developing BC (see Table

1.1) devised by Scottish Intercollegiate Guidelines Network (SIGN) which is an adaptation of the guidelines used in England and Wales provided by the National Institute for Health and Care Excellence (NICE; National Institute for Health and Care Excellence. (2013)). Once risk level is determined, if individuals have at least a 20% chance of having a BRCA1 and BRCA2 mutation, the two genes most associated with BC development, and there is a living affected family member that person will be offered the option to have genetic testing (The Scottish Government., 2009).

Low Risk	Moderate Risk	High Risk	Very High Risk / Gene Carrier
• Anyone not fulfilling moderate, high or very high risk criteria	• One first degree relative with breast cancer diagnosed under the age of 40, or one first degree relative with male breast cancer diagnosed at any age; • Two first or one first and one second degree relative with breast cancer diagnosed under 60, or ovarian cancer at any age, on the same side of the family; • Three first or second degree relatives with breast or ovarian cancer on the same side of the family where one is a first degree relative of the proband or of the proband's father; A case of bilateral breast cancer should be treated as the equivalent of 2 affected relatives. In this context a first degree female relative is mother, sister or daughter. A second degree female relative is grandmother, granddaughter, aunt or niece.	• Families with four or more relatives affected with either breast cancer under age 60, or ovarian cancer at any age, in three generations; Families where one individual has had both breast and ovarian cancer; • Families where there is an estimated 20% likelihood of a BRCA1, BRCA2 or p53 mutation; • Individuals with a lifetime risk of developing breast cancer of 30% or more. The individual being assessed should be a first degree relative of an affected family member or a second degree relative through an unaffected male. Affected individuals should be first degree relatives of each other or related through unaffected males.	• Female carrier of a mutation in BRCA1, BRCA2 or p53; • At 50% risk of carrying a mutation in BRCA1 or p53; • Women in their thirties whose 10 year risk is greater than 8% as assessed at age 30, or in their forties and whose 10 year risk is greater than 20% as assessed at age 40, or 12% where there is a dense mammographic pattern.

Table 1.1 Scottish Government Breast Cancer Risk Stratification.

Criteria for assessment of risk of breast cancer development in Scotland (The Scottish Government., 2009).

A family history of BC has long been recognised as a predictor of BC development. A first degree relative i.e. mother or sister diagnosed with BC over age 50 effectively doubles an individual's risk of developing BC, or trebles if the relative was premenopausal at diagnosis or if there were two first-degree relatives diagnosed. There is a lesser risk (1.5 fold) associated with a second degree relative i.e. a grandparent or aunt diagnosed with BC (Pharoah*, et al.*, 1997). The risk also increases if multiple first degree relatives are diagnosed with almost a 4 fold risk if three or more first degree relatives are diagnosed (Collaborative Group on Hormonal Factors in Breast Cancer., 2001). The aforementioned risk is also inversely proportional to the age of the relative and is far higher if a brother rather than a sister is diagnosed with BC (Bevier*, et al.*, 2012).

If a BRCA1 or BRCA2 mutation is detected the affected individual will be offered extra surveillance and possibly prophylactic surgery in the form of a mastectomy to remove breast tissue to aid the prevention of BC development. BRCA1 of BRCA2 mutation carriers are also at increased risk of ovarian cancer (Gayther and Pharoah, 2010) and as such may also be offered a salpingo-oophorectomy (removal of fallopian tubes and ovaries) to reduce the risk of developing ovarian cancer. Women who are considered low risk can enrol in the National Breast Cancer Screening Programme from age 50, for screening every three years. Unaffected individuals with a family history assessed as moderate will have screening offered from the age of 40 occurring every two years, increasing to annually between ages 40-50. In contrast, the very high risk BC group can be offered screening from age 25, with mammography every two years increasing to annually then eighteen months when they reach age 50 as this is an interim screen alongside the national screening service (see Table 1.2).

Low Risk	Moderate Risk	High Risk	Gene carrier or Very High Risk
• Reassurance. • Provision of Information. • National Breast Cancer Screening Programme from age 50.	• Surveillance should be offered from the age of 40 or 5 years younger than the earliest age of onset of cancer in the family. • Mammography should be two yearly below age 40, and annually age 40 to age 50. • Mammography should not usually be commenced before age 35, and all women in this group should be offered mammography by the age of 40. • Breast examination should be offered where possible, and may be appropriate before the age of 35 where there is a family history of early onset cancer. The surveillance programme should be audited as part of the national cancer genetics audit and where possible patients should be recruited into national studies to investigate the efficacy of such surveillance.	• Surveillance should start at age 35 or 5 years younger than the youngest age of onset in the family. • Mammography should be 2 yearly below age 40, annually age 40 to 50, and subsequently 18 monthly from 50 to 70. • Mammography should usually start at age 35, and should not be offered under the age of 30. • Breast examination should be offered, particularly to women who are considered too young for mammography, who come from families where there has been onset of cancer before age 35. Genetic testing should be offered in these families, if a sample is available from an affected relative. Where mutation testing cannot be offered, the possibility that the woman may be at sufficiently high risk to be offered MRI (see next column and risk table) should be considered.	• Surveillance should start from age 25, or individualised according to the family. • Annual breast examination from 25 or 5 years younger than earliest age of onset, whichever is younger. • Mammography should be 2 yearly from age 30 to 39, annually from age 40 to 50, and subsequently 18 monthly from 50 to 70. • Breast MRI should be offered in addition to mammography, and should be annually from age 30-49. [a] Breast MRI should only be offered under 30 in rare very high risk situations eg p53 gene carrier. • Prophylactic mastectomy or, where appropriate, salpingo-oophorectomy should be discussed with the patient. Bilateral mastectomy should be considered if a woman who is a gene carrier, or is at very high risk, is found to have a breast cancer.

Table 1.2 Scottish Breast Cancer Surveillance Guidelines. (The Scottish Government., 2009).

Mutations of BRCA1 and BRCA2 are dominantly inherited, accounting for approximately 20-25% of hereditary BCs (Easton, 1999) and 5-10% of BCs overall (Campeau*, et al.*, 2008). BRCA1 and BRCA2 are genes (located on chromosomes 17q21 and 13q12-13 respectively) found in breast cells and other

tissue which produce tumour suppressor proteins that aid the repair of DNA when cells divide. Individuals have two copies of these genes, one inherited from their mother and one from their father. If a mutation occurs in either copy, the function may be lost and damaged DNA is not repaired. It is the accumulation of unrepaired DNA that causes BC when these genes are mutated, not the genes themselves (Hall*, et al.*, 1990; Wooster*, et al.*, 1994).

These genes have been extensively studied to assess the risk of BC development in mutation carriers. Initially it was concluded there was an 84% chance of developing BC for individuals carrying a BRCA1 or BRCA2 mutation (Ford*, et al.*, 1994). However, further studies examined the BC risk of each gene separately, and discovered that BRCA1 mutations conferred a 54-65% risk of developing BC by aged 70 or 45% if a BRCA2 mutation was present (Antoniou*, et al.*, 2003; Easton*, et al.*, 1995). Conversely, a recent, UK based study known as EMBRACE (Mavaddat*, et al.*, 2013) found that by age 70, BRCA1 mutation carriers had a 65% risk of BC development while BRCA2 mutation carriers had a 45% risk. Other genetic mutations are associated with BC development to a lesser extent – CHEK2, TP53, PTEN, STK11 and CDH1 (Campeau*, et al.*, 2008; Walsh*, et al.*, 2006) – however not all BCs occurring among relatives can be attributed to a genetic mutation.

Prophylactic mastectomy and salpingo-oophorectomy are offered to mutation carriers and high risk relatives. Studies have shown that preventative surgery significantly lowers risk of BC development by approximately 90%, but does not entirely eradicate it (Hartmann*, et al.*, 1999; Heemskerk-Gerritsen*, et al.*, 2013; Rebbeck*, et al.*, 2004). However, even after risk-reducing mastectomies a small number of women still go on to develop BC (Rebbeck*, et al.*, 2004; Skytte*, et al.*, 2011).

While mastectomy is considered the most effective risk-reducing strategy for BC, there is evidence that oophorectomy can also reduce risk of BC development. This procedure is primarily used for prevention of ovarian cancer but several studies have shown protective effects for breast cancer as well particularly if performed before age 50. For instance oophorectomy prior to age 40 reduced the risk of BC development by 64% and 31% in BRCA1 and BRCA2 mutation carriers respectively (Domchek*, et al.*, 2010; Rebbeck*, et al.*, 2009). This risk reduction does not appear to be affected by hormone replacement

therapy which may be given to prevent the effects of oophorectomy induced menopause (Eisen*, et al.*, 2008; Rebbeck*, et al.*, 2005). However, in spite of the aforementioned preventative measures some individuals still develop cancers of the breast or peritoneum (Finch*, et al.*, 2006; Olivier*, et al.*, 2004; Rutter*, et al.*, 2003).

1.1.2.1 Hodgkin's Lymphoma

A less well known high risk factor for BC development is radiotherapy treatment for Hodgkin's lymphoma. Hodgkin's lymphoma is the uncontrolled proliferation of B-lymphocytes and their congregation in sections of the lymphatic systems such as lymph nodes. These proliferated B-lymphocytes are unable to fight infection leaving an individual vulnerable to disease. Hodgkin's lymphoma usually presents at a young age and is commonly treated with radiotherapy to the chest area. Studies have shown that having radiotherapy for Hodgkin's lymphoma puts individuals at a comparable risk of BC development to those with mutations of BRCA1 and BRCA2 (Swerdlow*, et al.*, 2012). It has also been found that risk of BC is inversely correlated with age at treatment and reducing the proportion of breast tissue exposed during treatment may reduce the risk of BC in the future (De Bruin*, et al.*, 2009; Swerdlow*, et al.*, 2012). Maximum risk of BC development was found at ages 50-59 and while an individual would be eligible to participate in the national screening programme, there are suggestions that they would benefit from more frequent screening which should continue for more than 40 years after radiotherapy (De Bruin*, et al.*, 2009; Swerdlow*, et al.*, 2012). Currently. in Scotland, if a person's risk of BC development is more than 30%, (as for individuals who had been treated with radiotherapy for Hodgkin's lymphoma) they are eligible for increased surveillance as detailed in the guidelines for high and very high risk individuals above in Table 1.2 (The Scottish Government., 2009).

1.1.2.2 Histological and Molecular Breast Cancer Types.

BC can occur as either a non-invasive or invasive tumour. Non-invasive cancers are further subdivided into ductal carcinoma *in situ* (DCIS) and lobular

carcinoma *in situ* (LCIS). *In situ* refers to the state and location of the breast epithelial cells; they have gone through malignant transformation and are now proliferating but remain at the site of origin and do not penetrate the basement membrane into surrounding tissues. DCIS and LCIS are not at risk of metastatic spread as there are no blood vessels or lymphatics in the epithelial layer of the breast (Sakorafas and Farley, 2003).

DCIS is also referred to as intraductal cancer and originates from the terminal duct lobular epithelium and is a significant risk factor for development of invasive BC (Leonard and Swain, 2004). DCIS usually presents as micro-calcifications in a mammogram where calcium deposits have formed during areas of rapid epithelial proliferation. Difficulties can arise when determining whether DCIS will become invasive and therefore whether treatment for DCIS is necessary. Treatment usually involves a small excision of cancerous ducts but in some cases mastectomy is required. LCIS is rarer than DCIS and is a proliferation of identical small cells forming a mass in multiple breast lobules and sometimes in ducts. There is strong evidence to suggest that untreated LCIS can develop into BC (Coopey*, et al.*, 2012; Simpson*, et al.*, 2003). In contrast to DCIS, LCIS is almost impossible to detect via mammography as it does not cause micro-calcifications and is not a substantial lesion however areas of increased density in mammograms have been linked to LCIS (Beute*, et al.*, 1991; Georgian-Smith and Lawton, 2001; Sonnenfeld*, et al.*, 1991).

The two most common types of invasive BC are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). Approximately 75% of BCs are IDC with ILC accounting for a further 10%. The remaining 15% of diagnosed invasive BCs are made up of rare types such as Mucinous, Medullary, Tubular, and Papillary (Underwood, 2004).

IDC commonly presents as irregularly dense area of soft tissue with pointed surface projections or as a palpable lump. As the name suggests IDCs originate in the ductal epithelium but ultimately invade the surrounding breast tissue allowing cancerous cells to infiltrate vascular and lymphatics systems which can result in carriage to secondary locations forming metastases (King and Robins, 2006). A diagnosis of IDC often occurs when a tumour does not fit one of the “special types” such as ILC since IDCs do not possess distinguishing features. ILC is typically an asymmetrical thickening or bumpiness of the breast stroma

rather than a lump in the breast (IDC) that results in patients having much larger tumours by the time they are symptomatic compared to patients with IDC. ILCs do not usually present with microcalcification so are difficult to identify in mammograms but a characteristic feature of ILC cells is their lack of E-cadherin, a cell adhesion molecule (de Leeuw*, et al.*, 1997). Treatment for both IDC and ILC involves surgery and usually chemotherapy, radiotherapy or hormone therapy depending on the severity and hormone receptor status of the tumour.

In recent years research studies have provided a further classification of tumours (mainly IDCs) in the form of molecular subtypes (Curtis*, et al.*, 2012). Four molecular subtypes of BC have been identified: normal breast-like, luminal, basal-like and human epidermal growth factor 2 (HER2) with the luminal subtype later being subdivided into luminal A and luminal B (Hu*, et al.*, 2006; Perou*, et al.*, 2000; Sørlie*, et al.*, 2001; Sørlie*, et al.*, 2003). Of the four molecular subtypes, luminal (both A and B) is the only subtype possessing oestrogen receptors (ER) and are identified by having genes activated that are linked to an ER pathway, low molecular weight cytokeratins. When subdivided, Luminal A is usually a low histological grade, has high expression of ER-activated genes, low expression of proliferation genes, and is associated with a good prognosis. However, Luminal B subtypes have a worse prognosis and display higher proliferation rates and histological grades (Sotiriou*, et al.*, 2003).

The remaining three molecular subtypes (normal breast-like, HER2 and basal-like) do not possess ERs. Normal breast-like subtypes show high expression of genes usually found in adipose tissue and show similar gene expression to normal breast samples during micro-array analysis (Peppercorn*, et al.*, 2008). This subtype tends to have a good prognosis but in many cases this may be a false negative result due to the fact that there are not enough cancer cells present in the sample. Therefore, further investigation is required to assess the clinical significance of this subtype (Correa Geyer and Reis-Filho, 2009; Pusztai*, et al.*, 2006). HER2 and basal-like subtypes are linked with aggressive clinical features such as larger tumour size and higher tumour grade (Voduc*, et al.*, 2010). The HER2 subtype is determined by overexpression of HER2 and its associated genes as well as lack of ER and progesterone receptors (PR) (Weigelt*, et al.*, 2010). However some tumours with HER2 overexpressed are

not categorized as HER subtypes but are considered Luminal B if they have an ER positive status (Parker*, et al.*, 2009; Rouzier*, et al.*, 2005). Lastly, despite extensive research, the definition of basal-like tumours varies widely. They are typically defined as tumour cells expressing genes found in normal basal epithelial cells and tend to be triple negative (discussed in more depth in section 1.1.3.1) meaning they lack ER, PR or HER2 although this is not always the case (Rakha*, et al.*, 2008; Weigelt*, et al.*, 2010).

1.1.2.3 Histological Breast Cancer Staging.

Patients can be grouped by the severity of their disease which allows clinicians to construct a suitable treatment plan, give an indication of prognosis, evaluate the effect of treatment, exchange information with other treatment centres and contribute to cancer research. The TNM system of cancer classification was developed by the Union for International Cancer Control (UICC; previously the International Union Against Cancer) and is based on three main components: extent of the primary **T**umour; presence or absence and extent of surrounding lymph **N**ode metastasis and presence or absence of distant **M**etastasis. This is one of the most commonly accepted approaches for determining the stage of cancer and is normally presented as groups of stages that indicate chances of patient survival (see Table 1.3). Other classification systems include the Nottingham Prognostic Indicator (NPI), an index combining tumour size, lymph node status and grade to divide patients into three prognostic groups (good, moderate and poor) using the calculation 0.2 x tumour size (cm) + grade (1-3) + lymph node status (1= no nodes; 2= 1-3 nodes; 3**=** ≥3 lymph nodes are involved) (Harmer, 2011).

	T	N	M
Stage 0	0	0	0
Stage 1	1	0	0
Stage 2A	0	1	0
	1	1	0
	2	0	0
Stage 2B	2	1	0
	3	0	0
Stage 3A	0	2	0
	1	2	0
	2	2	0
	3	1/2	0
Stage 3B	4	0/1/2	0
Stage 3C	Any	3	0
Stage 4	Any	Any	1

Table 1.3 TNM System of Classification. Method of grouping cancer patients based on tumour dimension (no primary tumour present i.e. T0 and ≥5cm or progression into chest wall/oedema/ulceration being T4); lymph node infiltration with N0= no lymph node metastasis to N3= infiltration into internal mammary lymph nodes and distant metastasis where M0= no metastasis and M1= distant metastasis (Sobin*, et al.*, 2009).

1.1.3 Clinical Features of High Risk Breast Cancer.

Higher risk of BC development is associated with early age onset and aggressive clinical features. While there is some debate surrounding what age confers “young”, most studies agree that this refers to women below the age of 35-40. Approximately 7% of all BCs are diagnosed in women younger than 40 (National Cancer Institute., 2012) and there is evidence to suggest that this number is rising (Leclère*, et al.*, 2013). BC within this group tends to be aggressive, have a high histological grade, be triple negative (TN), and possess a basal-like phenotype.

Research has revealed that BC in younger women arises through distinct biological pathways compared to older women (Anders*, et al.*, 2008). Younger women (<40) with BC had an altered molecular phenotype distribution in comparison to a general population with BC (Collins*, et al.*, 2012). The age of diagnosis has been shown to inversely correlate with poor prognosis; the risk of mortality increasing by 5% for every year reduction in age of diagnosis (Gajdos*, et al.*, 2000; Han*, et al.*, 2010). Younger patients often have much larger, more

invasive tumours, have nodal involvement with more nodes affected and the majority were diagnosed at stage II or III. A further feature differing between young and older BC sufferers is that young patients are more likely to present with a palpable mass as opposed to a mammographic lesion but this is due to mammography screening not being routinely recommended for young women unless they are at increased risk of BC development (Gajdos*, et al.*, 2000). Additionally, risk of local and distant recurrence is increased if BC develops at a young age (Dubsky*, et al.*, 2002; Nixon*, et al.*, 1994). Age is not an independent risk factor for BC development but biological factors alone fail to explain the differences between BC in younger and older women.

1.1.3.1 Triple Negative BC.

TN tumour status is also a key clinical feature of BC presentation in high risk women. TN tumours are characterised by lack of ER and PR (known collectively as hormone receptors) as well as HER-2 through immunohistological tests. Triple negative breast cancer (TNBC) accounts for approximately 15% of all diagnosed BC (Anders*, et al.*, 2009), with up to 80% of BRCA1 related tumours being TN (Lakhani*, et al.*, 2002a) whereas BRCA2 linked tumours do not favour a particular BC subtype (Armes*, et al.*, 1999). TNBC is associated with younger age at diagnosis, higher tumour grade, rapid growth, early metastasis, poorer prognosis and increased risk of recurrence (Bauer*, et al.*, 2007; Blows*, et al.*, 2010; Chacon and Costanzo, 2010; Dent*, et al.*, 2007; Schneider*, et al.*, 2008). Dent*, et al.* (2007) key investigation highlights many of the aggressive features linked to TNBC. For instance, the mean age of TNBC diagnosis was significantly younger, with tumours more likely to be grade III and larger in size when diagnosed as two thirds of TNBC's were ≥3cm. Additionally the TNBC group were almost 10% more likely to have lymph node invasion with no correlation between node status and tumour size as even small tumours had at least one affected lymph node. This provides evidence that TNBC carries an increased risk of early metastasis. The study also showed that TNBC sufferers were almost twice as likely to die from the disease within 10 years compared to other BC types, which may be due limited treatment options. Distant and local recurrence was also analysed with TNBC patients having a higher rate of

distant recurrence primarily in the immediate three years after diagnosis while risk of local recurrence was similar to that of other BC types.

There is some overlap between triple negative histological subtype and basal-like molecular subtypes in the literature but the two are not synonymous. There is controversy surrounding the definition of basal-like BCs (for a recent review see Badve*, et al.* (2011) while there is definitive agreement TNBCs are ER, PR and HER2 negative. Furthermore, in a clinical setting immunohistological staining is more readily available than testing used to determine molecular subtypes, thus tumours are more likely to be classified as TN than basal-like (Nielsen*, et al.*, 2004). This study will refer to TNBCs throughout.

1.1.4 Current Breast Cancer Biomarkers.

A biomarker is defined as a measurable characteristic that can be evaluated to indicate normal biological processes, pathogenic processes or monitor therapeutic interventions (Atkinson*, et al.*, 2001). Biomarker research is thought to be imperative as clinical benefits could include earlier disease detection, improved monitoring of disease detection and treatment (Popescu*, et al.*, 2010; Whelan*, et al.*, 2008). A main priority of cancer detection is to develop biomarkers that can be detected via less invasive and complex methods and which also have the sensitivity and specificity to identify cancer earlier (Somasundaram*, et al.*, 2009), thus improving mortality rates, cost-effectiveness and potentially reducing the need for invasive treatment. However, there are very few biomarkers currently capable of this and those that are, often require improvement through the use of a biomarker panel to be successful (Anderson, 2005; Rifai*, et al.*, 2006).

With regards to BC, finding a suitable serum biomarker would be of great importance to those with an increased risk since the current methods, although greatly improving detection, such as palpation, mammography and MRI scanning (magnetic resonance imaging) are not capable of identifying all tumours. Additionally, repeated exposure to radiation from mammography screening increases risk of developing a cancer by 0.3-0.6 fold (Berrington de González, 2011). For instance, calcium deposits surround a lesion that could indicate BC onset cannot be visualised using MRI, smaller tumours in the early

stages may not be palpable and mammography has decreased sensitivity in women with dense breasts (Checka*, et al.*, 2012). A BC biomarker, particularly one that is a blood constituent, could enable identification of disease presence where imaging technologies fail and could allow more frequent screening starting at a younger age than can be provided using mammography.

Presently, there are only two clinically adopted biomarkers capable of distinguishing BC existence from "healthy" individuals but not for early diagnosis only for evidence of metastasis and evaluating treatment success. The first is carcinoembryonic antigen (CEA), a glycoprotein generated during foetal development by the large intestine although it has also been found in adult human blood at very low levels. Serum concentrations of CEA are increased in patients with ovarian, lung, pancreatic, liver, colorectal, prostate and BCs. In particular, women with metastatic BC show a 30-50% increase in CEA levels and a positive correlation between therapeutic response and CEA in women with metastasis (Cheung*, et al.*, 2000; Mughal*, et al.*, 1983; Williams*, et al.*, 1988). The second marker for BC is mucin 1 (MUC-1), a glycoprotein bound to the surface of epithelial cells of the stomach, pancreas, ovaries, bladder, respiratory tract and breast. Production of MUC-1 is upregulated and glycan chains of MUC-1 become truncated during BC compared to MUC-1 from normal breast epithelials. Cancer antigen 15.3 (CA15.3) is a secreted and soluble form of MUC-1, the concentration of which can be used to determine prognosis. CA15.3 has a similar specificity but much higher sensitivity when compared to CEA testing alone and is regarded as the "gold standard" test for BC management (Seregni*, et al.*, 2008).

Over 80% sensitivity is achieved for detection of metastasis when measuring CEA and CA15.3 together (Robertson*, et al.*, 1999). An investigation by Ebeling*, et al.* (2002) revealed that elevated levels of CA15.3 and serum CEA prior to surgical intervention were positively correlated with death from BC and early relapse. A decline in levels of CA15.3 and CEA were noted post-surgery. However, in patients where the concentrations of CEA decreased by over 33% were at significantly higher risk of death and relapse, which multivariate analysis showed to be an independent prognostic indicator. Research by Nicolini*, et al.* (2006) showed that a combination of classical BC biomarkers CEA, CA15.3 and tissue polypeptide (TPA) was more effective than the MCA (mucin-like carcinoma associated antigen)-CA15.3 biomarker panel at detecting early BC

relapse. Using all three markers greatly improved overall sensitivity with only a minor decrease in specificity. Another study revealed that combined measurement of CA15.3 and SLe^x might be better at detecting metastasis and monitoring BC than the CEA-CA15.3 combination (Kurebayashi*, et al.*, 2006). In 2001 Gion and colleagues published a paper confirming their earlier work in 1999 that cancer antigen 27.29 (CA27.29) was of comparable quality to CA15.3 for management of BC patients and was more sensitive when there was a limited antigen concentration (Gion*, et al.*, 1999; Gion*, et al.*, 2001).

Earlier research has already revealed several potential BC biomarkers. However, most of these are not sensitive or specific enough. Yet, advances in proteomic technologies that detect prospective biomarkers have enabled analysis of new biomarker possibilities. For instance, identification of tumour antigens through a proteomics approach allows for post-translational modifications to be assessed whereas previous methods detection methods such as analysis of recombinant proteins did not. This enables detection of autoantibodies that react to naturally occurring proteins, including lysates from tumours and their cell lines, allowing antigenicity towards changes in post translational modifications such as glycosylation to be uncovered (Misek and Kim, 2011). Profiling of serum and nipple aspirate fluid has been enhanced by using mass spectrometry to interrogate bodily fluids for possible biomarkers. Mass spectrometry permits direct protein content analysis of human biofluids enabling fast detection of prospective biomarkers (Gast*, et al.*, 2009; Pawlik*, et al.*, 2006). Recently, several new biomarker candidates have been investigated (see Table 1.4).

Biomarker	Type
CA15.3	Serum Protein
HER-2/neu	Serum Protein
RS/DJ-1	Serum Protein/Autoantibody
p53	Autoantibody
HSP60	Autoantibody
HSP90	Autoantibody
Mucin-related	Autoantibody
α-2-HS-glycoprotein	Ductal Protein
Lipophilin B	Ductal Protein
β-globin	Ductal Protein
Hemopexin	Ductal Protein
Vitamin D-binding protein	Ductal Protein

Table 1.4 Prospective Breast Cancer Biomarkers. [Adapted from Misek and Kim (2011)] Recently investigated potential biomarkers for breast cancer.

While these biomarkers are appropriate for monitoring BC progression and treatment efficacy they are all unsuitable for detecting the onset of BC, and there are currently no biomarkers that can.

AGP is a potential BC biomarker due to it displaying aberrant glycosylation during diseases such as cancer (Turner, *et al*., 1985; Hashimoto, *et al*., 2004; Saldova, *et al*., 2007) and its suitability as a BC biomarker is discussed further in section 1.3.4.

1.1.5 Need for a Diagnostic Breast Cancer Biomarker.

The overview provided earlier in this chapter highlights a need for increased surveillance for those at greater risk of BC. While mammography is a highly specific and sensitive method of detection for BC in national screening programmes, there are some BCs that go unnoticed and screening can only be offered every few years to avoid excess radiation exposure that could induce cancer (Berrington de González, 2011; NHS Breast Screening Programme, 2003). Magnetic resonance imaging (MRI) can also be used for detection of BC in addition to mammography and can be offered at a younger age and more frequently that mammography. However, due to cost, this is not currently funded in Scotland for women at increased risk of BC due to family history or mutation status. Both these detection methods run the risk of over diagnosing patients resulting in an individual receiving unnecessary treatment for a lesion

that would not have developed into cancer. However, at present there is no way of telling whether an abnormality detected during screening will develop into cancer and so the best option is to proceed with treatment (Marmot*, et al.*, 2012). Therefore, there is still a need for a more cost-effective, less harmful method of BC screening that could be used more often for women at increased risk of BC or the general population. A model biomarker would be located in the blood with the ability to be used for both detection and prognosis. A blood based biomarker would require minimally invasive procedures to retrieve and components could be easily isolated for analysis. To date, no suitable blood-based biomarkers have been identified for the detection of BC. While biomarkers such as CEA and CA15.3 glycoprotein are routinely used clinically, there are none suitable for early diagnosis of BC or screening programmes. This is because they may also be raised in benign conditions and have very low sensitivity and specificity [for a recent review see Mirabelli and Incoronato (2013)]. Absence of an effective blood-based biomarker leads to late diagnosis of many BC sufferers, limiting their treatment options and increasing their mortality. As both CEA and CA15.3 are glycoproteins capable of evaluating BC prognosis, investigation of other glycoproteins as potential biomarkers may be beneficial.

1.2 Glycosylation and Cancer

Investigation into the altered metabolism of cancer cells has enabled the definition of six hallmarks of cancer which have been widely researched and were originally defined as: sustaining proliferative signalling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis and resisting cell death (Hanahan and Weinberg, 2000). More recently, these six hallmarks have been expanded by a further four to include avoiding immune destruction; genome instability and mutation; tumour-promoting inflammation and deregulating cellular energetics such as the Warburg effect (Hanahan and Weinberg, 2011; Warburg, *et al*., 1928) giving a new total of ten cancer hallmarks. Aberrant glycosylation of a number of molecules is a known consequence of cancer (Dube and Bertozzi, 2005) and contributes to several of the above hallmarks of cancer.

Changes in glycosylation may be due to a number of factors including differing levels of glycosyltransferases and glycosidases as well as their location within the cell, availability and activity of nucleotide sugars, availability of protein substrates and expression of molecules that regulate glycoprotein folding (Varki, *et al*., 2009). The altered glycosylation may disrupt normal cellular functions and contribute to tumour growth, metastasis and immune evasion or may be a consequence of altered cell metabolism that could be used as biomarkers (Varki, *et al*., 2009).

Glycosylated molecules such as glycoproteins exist as secretory molecules or as glycoconjugates on cell surfaces; therefore it is important to consider both when discussing their involvement in cancer. The most common glycan alterations during cancer occur in *N*- and *O*- linked glycans and include sialylation, fucosylation, truncation and branching (Stowell, *et al*., 2015). Additionally, cancer cells often revert to expression of oncofetal antigens which are more commonly expressed in foetal tissue during development as opposed to normal adult tissue (Sapra, *et al*., 2012).

Sialylation is the addition of sialic acid residues usually as terminal structures, such as Neu5ac, to glycans which are involved in cell adhesion, cell to cell signalling and cellular recognition (Varki, *et al*., 2009). Increases in sialylation have been identified in active cancer and this correlates with a decreased cell adhesion which may aid the metastasis of tumour cells (Dennis, *et al*., 1982; Nadanaka, *et al*., 2001; Renkonen, *et al*., 1997 and Ugorski, *et al*., 2002). Furthermore, the viability of cancer cells may be increased by alterations of α2-6 sialylation expression that contribute to tumour growth via mechanisms independent of glycoproteins (Kroes, *et al*., 2010, Bobowski, *et al*., 2012). It has also been proposed that increased sialylation of cancer cells provides protection from the immune system and subsequent eradication (Bull, *et al*., 2014).

Similarly, fucosylation is the incorporation of the monosaccharide fucose into glycan chains and this process has been shown to occur at differing rates during the early and late stages of cancer. Early stage cancer appears to favour increased fucosylation as research has shown an upregulation of fucosyltransferase 8 (FUT8) which may aid cell proliferation (Chen, *et al*., 2013). However, once established, tumours display less fucosylation which may assist their survival via NK cell evasion through interaction with a secondary signalling

pathway known as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL; Moriwaki, *et al*., 2009). Furthermore, increased fucosylation is associated with an increase in Sialyl Lewis^X (SLe^X) terminal structures. SLeX is a ligand involved in cell adhesion, therefore increased expression may play a role in metastasis through increased binding of tumour cells in secondary organs (Julien, *et al*., 2011).

Another characteristic of cancer cells is truncated glycans, particularly *O*-linked glycans. These usually exist as more complex structures however during cancer, mucin-like *O*-linked glycans become truncated resulting in formation of Tn or Sialyl Tn (STn) stuctures. A solitary GalNAc confers Tn while and GalNAc joined to a sialic acid denotes STn. In both instances GalNAc remains attached to the protein via a linkage to the hydroxyl group of Serine or Threonine. Research has indicated that truncation of *O*-glycans may enhance malignant properties of cells including proliferation rate, invasive growth, loss of growth inhibition and tissue architecture as well as alterations in the adhesion of basement membranes (Radhakrishnan, *et al*., 2014).

Additionally, the Warburg effect describes the increased uptake and anaerobic metabolism of glucose by cancer cells for energy to facilitate proliferation (Warburg, *et al*., 1928). Recently, the Warburg effect has been linked to increased *O*-GlcNAc transferase which catalyses the addition of *N*-acetylglucosamine to proteins and lipids (Jozwiak, *et al*., 2014). It has been hypothesised that *O*-GlcNAcylation acts as a nutrient sensor and may link cell metabolism to transcription and signal transduction (Butkinaree, *et al*., 2010; Hanover, *et al*., 2010) resulting in enhanced proliferation, growth, angiogenesis and metastasis (Khan, *et al*., 2013; Porta, *et al*., 2014).

Increases in β1-6 branching of *N*-linked glycans arises from amplified expression GlcNAc tranferase V and can explain the increased size of glycopeptides derived from tumour cells. GlcNAc transferase V is encoded by MGAT5 and studies have shown their involvement in tumour growth and metastasis when increased. For example, increased β1-6 branching induced by elevated GlcNAc transferase V resulted in more cell migration as well as decreased cell-cell and cell-matrix adhesion (Demetriou, *et al*, 1995) while mice deficient in MGAT5 had reduced tumour proliferation and metastasis

(Granovsky, *et al*., 2000). Thus highlighting the role of increased β1-6 *N*-linked glycan branching in cancer progression.

Finally, tumour cells often display markers normally only seen during foetal development. These markers are known as oncofoetal antigens and the two most evident in the research are alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA), both of which are glycoproteins (Zhang, *et al*., 2015). AFP has a similar amino acid sequence to albumin, a minimal carbohydrate content of 3% and is present at low levels in normal adults. However, serum AFP concentrations of above 500ng/ml are linked with malignancy (Farinati, *et al*., 2006). Similarly, CEA is also expressed in malignancy as well as foetal development and is currently used clinically as an indicator and monitor of cancer. For example, persistently elevated CEA may indicate metastasis or ineffective therapy (Dallas, *et al*., 2012; Prager, *et al*., 2014) and pre-operative CEA can give an indication of patient prognosis (Amri, *et al*., 2013).

1.2.1 Glycosylation and Breast Cancer

Breast cancer remains the leading and most common cancer diagnosed in women (Information Services Division: ISD Scotland., 2014; Northern Ireland Cancer Registry., 2013; Office for National Statistics., 2011; Welsh Cancer Intelligence and Surveillance Unit., 2014) and as such, extensive research has been undertaken to identify potential biomarkers of diagnosis and prognosis as well as therapeutic targets. Much of this research has investigated altered glycosylation during BC using antibodies and lectins to recognise specific carbohydrate structures which unveiled variations in cell surface and serum protein glycosylation between BC and normal individuals. Elevated expression of truncated *O*-linked glycans, such as Tn and STn antigens, as well as increased *N*-linked β1-6 branching have been observed in breast cancer (Wu, *et al*., 2010, Saldova, *et al*., 2011). Increased sialic acid and fucose content was also noted due to the over expression of Lewis type structures such as SLe^x (Saldova, *et al*., 2011). Observations in serum proteins during BC have also indicated variations in mannose content and it is hypothesised that changes in processing of glycans which would normally trim Man9 structures leads to an increased expression of high mannose structures in place of complex and

hybrid *N*-linked glycans (de Loez, *et al*., 2011). All of the aforementioned glycan alterations correlated with changed expression of the corresponding glycosyltransferases that contribute to their formation – galactosyltransferases, fucosyltransferases, sialyltransferases and *N*-acetylglucosaminyltransferases (Potapenko, *et al*., 2010; Wu, *et al*., 2010; Burchell, *et al*., 1999).

The effects of these glycan alterations on patient prognosis and metastasis have also been investigated. Such research has shown that increased *N*-linked β1-6 branching and over expression of Lewis type epitopes is associated with poor outcomes in BC patients (Madjid, *et al*., 2005; Cui, *et al*., 2011) with expression of both SLe^x and SLe^a linked to higher risk of metastasis (Julien, *et al*., 2011; Nakagoe, *et al*., 2002). Additionally, the increased presence of high mannose structures have been shown to correlate with breast cancer progression (de Loez, *et al*., 2011).

Finally, aberrant glycosylation observed during BC has led to the use of glycoproteins such as CEA and CA 15-3 as serum BC biomarkers (discussed in more detail earlier in section 1.1.4). However, these glycoproteins lack the specificity and sensitivity required of diagnostic biomarkers so are not suitable for screening purposes (Anderson, 2005; Rifai, *et al*., 2006). Similarly, changes in glycosylation observed during BC are also displayed in other cancer types as mentioned in the previous section of this literature review (1.2). Therefore, a clear necessity for further investigation of altered glycosylation during BC has been highlighted.

1.2.2 *O*- and *N*- Glycosylation as Drivers for the Breast Cancer Subtypes Discussed in This Thesis.

The main BC subtype discussed throughout this thesis is TNBC, which may also be referred to as basal-like BC in the literature. As discussed in section 1.2, *O*-linked glycosylation is the post-translational addition of GalNAc to hydroxyl (-OH) groups of Ser/Thr followed by attachment of further monosaccharides by various glycosyltransferases (Varki, *et al*., 2009). *N*-linked glycosylation is more complex (see Figure 1.4) and occurs as the transfer of a donor molecule ($Glc_3Man_9GlcNAc_2$) to the amino groups of Asn residues at sites where the correct amino acid sequence exists (Asn-X-Ser/Thr) (Varki, *et al*., 2009).

Currently, little research has been performed with regards to glycosylation patterns within BC subtypes. Yet, *O*- and *N*-linked glycosylation of particular cell surface molecules have been found to aid the progression of certain BC subtypes (Srinivasan, *et al*., 2014).

A recent study carried out by Lee, *et al*., 2014 revealed structural glycan alterations of luminal A, HER2 and basal-like cell secretions compared to normal breast epithelial cells using LC-MS. Their research showed an increase in hybrid *N*-linked structures, higher levels of glycan branching, increased $SLe^{x/a}$ terminal structures as well as elevated fucosylation and overall degree of sialylation in basal-like BC cells. However, similar results were also seen in luminal and HER2 subtypes apart from basal-like cells having bisecting GlcNAc present and higher concentrations of α2-6 sialylation. Alpha 2-6 sialylation can occur in both *O*- and *N*-linked glycans with increases being linked to late stage BC (Alley and Novotny, 2010). As glycan sialylation is believed to aid tumour growth and contribute to metastasis through its functions in cell-cell recognition, cell adhesion and cellular recognition, increases in α2-6 sialylation are not unexpected in late stage cancer. Furthermore, TNBC is notoriously aggressive, fast growing and is linked with poor patient outcomes (Bauer, *et al*., 2007; Blows, *et al*., 2010; Chacon and Costanzo, 2010; Dent, *et al*., 2007; Schneider, *et al*., 2008). This also fits with the hypothesis that increased α2-6 sialylation of *O*- and *N*-linked glycans could be a contributing factor.

With regards to cell surface molecule glycosylation driving BC subtypes, research by Srinivasan *et al* (2014) revealed that *N*-linked glycosylation of ADAM8, a transmembrane metalloproteinase, occurred in ER negative tumours but not in ER positive tumours and that ADAM8 activity was reliant on correct *N*-linked glycosylation. ADAM8 has been shown to be upregulated in TNBC and is involved in cell adhesion as well as activation of growth pathways such as β1-integrin which promotes invasion and metastasis (Romagnoli, *et al*, 2014). Furthermore, MUC1 - a heavily *O*-glycosylated protein located in cell membranes – has been shown to express aberrant *O*-linked glycans on its surface after malignant transformation of cells (Gendler, *et al*., 1988). Recent research has shown a high expression of MUC-1 in TNBC tumour cells which correlated with tumour grade (Siroy, *et al*., 2013). However, other studies have noted an inverse correlation between MUC1 expression and tumour grade

(Rahn, *et al*., 2001). Throughout cancer MUC1 has been shown to interact with p53 to inhibit apoptosis which enables malignant cells to proliferate unimpeded (Wei, *et al*., 2005) and promote tumour invasion through beta-catenin. This association with beta-catenin results in the promotion of metastasis through epithelial-mesenchymal transition. This is the transformation of epithelial cells to mesenchymal stem cells through loss of cell-cell adhesion and cell polarity with enhancement of invasive and migratory mechanisms (Schroeder, *et al*., 2003 and Roy, *et al*., 2011). Therefore, increased expression of MUC1 in TNBC may contribute to the aggressive development and poor patient outcome linked with this BC subtype.

1.2.3 Glycobiology

Glycobiology is the study of proteins and lipids which have covalently linked, oligosaccharide chains added to their surface catalysed by enzymes (Rademacher*, et al.*, 1988). Oligosaccharide chains (also known as glycans) are composed of monosaccharides that share a $(CH_2O)_n$ chemical formula. The chains are synthesised as a co-translational and/or a post-translational modification. Glycosylation takes place in the endoplasmic reticulum and Golgi apparatus, generating a variety of particular glycoconjugates in the form of either glycolipids or glycoproteins. At a molecular level, glycosylation is controlled by enzyme clusters known as glycosyltransferases, within the endoplasmic reticulum and Golgi, which catalyse the joining of monosaccharides, arranging them in precise sequences to generate oligosaccharide chains. Therefore, glycosylation is not directly regulated genetically like the majority of protein or lipid alterations (Kobata, 1992; Sasisekharan and Myette, 2003). The variety of glycoconjugates (i.e. glycoproteins or glycolipids) produced can be affected by similar enzymes being present in other cell and tissue types causing trimming and/or additional monosaccharides being added to a glycan resulting in glycoforms. Glycoforms are proteins with the same amino acid sequences but possess different glycans (Lis and Sharon, 1993; Rademacher*, et al.*, 1988). Variation between glycoconjugates is known as heterogeneity and the collections of glycoforms that arise are referred to as the glycome.

Glycoconjugates are essential, having a vast array of functions such as acting as adhesion molecules and sometimes as enzymes. Generation of glycoconjugates uses large amounts of energy in an ordered and complex manner (Kornfeld and Kornfeld, 1985; Taylor and Drickamer, 2003; Varki, 2009) with glycosylation often being responsible for large amounts of a protein's (or lipid's) mass (Rademacher*, et al.*, 1988). For instance, oligosaccharides account for 45% of the molecular mass α-1-acid glycoprotein (AGP). It is also reported that approximately 70% of proteins undergo glycosylation (Wormald and Dwek, 1999).

These factors, imply that glycosylation of macromolecules is purposeful. For example, cancer progression has been shown to rely on glycans being present. For instance, increased sialyl Lewis X antigen (SLe^x; an inflammation derived terminal antigen structure containing fucose) present on glycans during cancer amplifies interactions with P-selectins which aids dissemination of tumours (Kim*, et al.*, 1998). This has enabled links to be drawn between glycan function and many pathophysiological or physiological conditions (Varki, 1993).

Overall, glycobiology is an important area of biomedical research that has yet to be fully explored. An accumulation of research in this area highlights that glycosylation is involved in many aspects of human life, from development to cancer progression. Therefore, it is possible that glycan composition could be used as a biomarker for BC onset.

1.2.4 Glycan Components

As discussed in section 1.2.1 glycosylation is the post-translation modification of a molecule through the addition of glycans (sugar chains). Glycans are comprised of monomeric units known as monosaccharides which can be discriminated between by their functional groups. There are two fundamentally different monosaccharides that differ in their functional groups at carbon 1 (*C*1; the anomeric carbon atom), namely, keto and aldehyde, which are composed of the *C*1 carbon atom linked covalently by a double bond to oxygen. When the structure of a monosaccharide is considered in its open-chain form, if a keto group is present, the double bond is located within a chain (>C=O) and the

monosaccharide is described as a ketose. Whereas aldoses have an aldehyde group with the double bond situated at the *C*1 end (-CH=O) (Bertozzi and Rabuka, 2009).

Oligosaccharides occur when eight or less monosaccharides undergo polymerisation (Bertozzi and Rabuka, 2009). The number of carbon atoms within the structure can be used to sub classify monosaccharides for instance; galactose (Gal), glucose (Glc) and mannose (Man) possess six and are therefore termed hexoses. Fucose (Fuc) also has six carbons but is referred to as a deoxyhexose as carbon 6 (*C*6) is missing a hydroxyl group (-OH), while sialic acids (SA; Gal with common terminating sugars α-2-3 or α-2-6 linked, also known as neuraminic acid NeuAc) are located on glycan termini and have nine carbons (Varki, 1992). Furthermore, a second group of monosaccharides known as hexosamines incorporate an amino group on the second of six carbons and tend to occur as *N*-acetylated structures such as *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc).These are the most common sugars found within glycoconjugates (see Figure 1.1 below for summary). Monosaccharides can exist as one of two stereoisomers, either D- or L- (with the former being the most common within nature), depending on whether the -OH group on the chiral centre found farthest away from the –CHO functional group has the D-glyceraldehyde configuration (OH to the right in the Standard Fischer Projection formula) or the L-glyceraldehyde configuration (OH to the left in the Standard Fischer Projection formula) (Bertozzi and Rabuka, 2009).

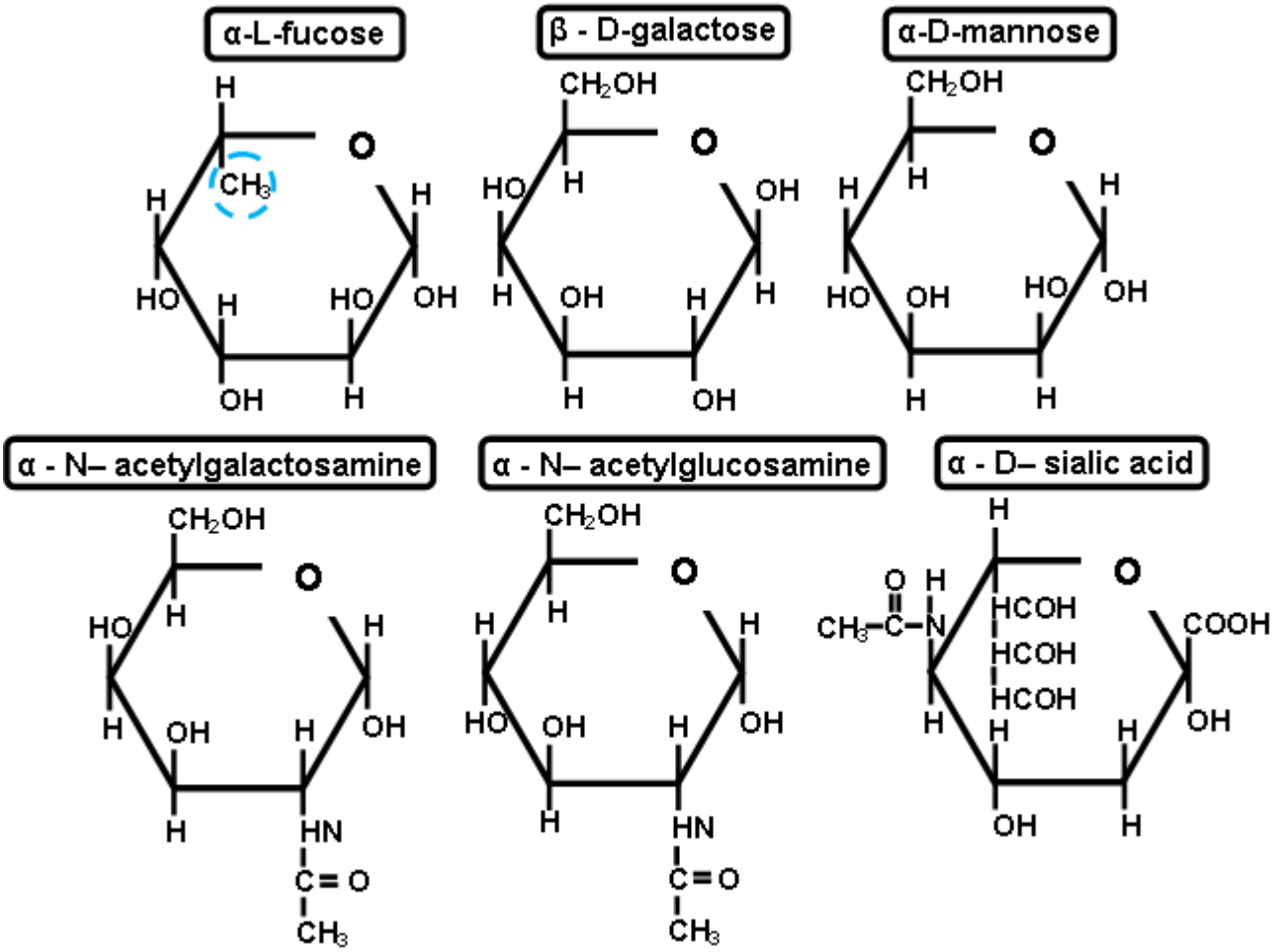


Figure 1.1 Hemiacetal Configuration of Common Monosaccharides within Glycans. Chemical structures of a selection of monosaccharides that normally compose glycoprotein oligosaccharide chains, as stable hemiacetal rings. As Fuc is a deoxyhexose the "missing" –OH group on C6 has been emphasised using .

The formation of glycan chains occurs when monosaccharides join together through the –OH group of *C*1 and another –OH group of a neighbouring residue forming a glycosidic bond through the loss of a H_2O molecule (condensation reaction) shown in Figure 1.2. Depending on the conformation of the –OH group of *C*1, the adjacent –OH must be in either the α or β anomeric position and the types of monosaccharides that are linked during the reaction determine whether the subsequent oligosaccharides are homo- or heterogenous. This creates a large assortment of glycans from few monosaccharides (termed heterogeneity), as the structure can vary marginally in length of chains, sequence, number of chains attached or type of bond. This structural diversity is illustrated by the fact that only six tripeptides can be generated from three

amino acids but 27, 648 trisaccharides can be produced from just three hexoses (Maeder, 2002). Furthermore, sugars are able to possess various linkage positions resulting in more complex structures being produced and by displaying α and β anomers even more glycan varieties can be formed.

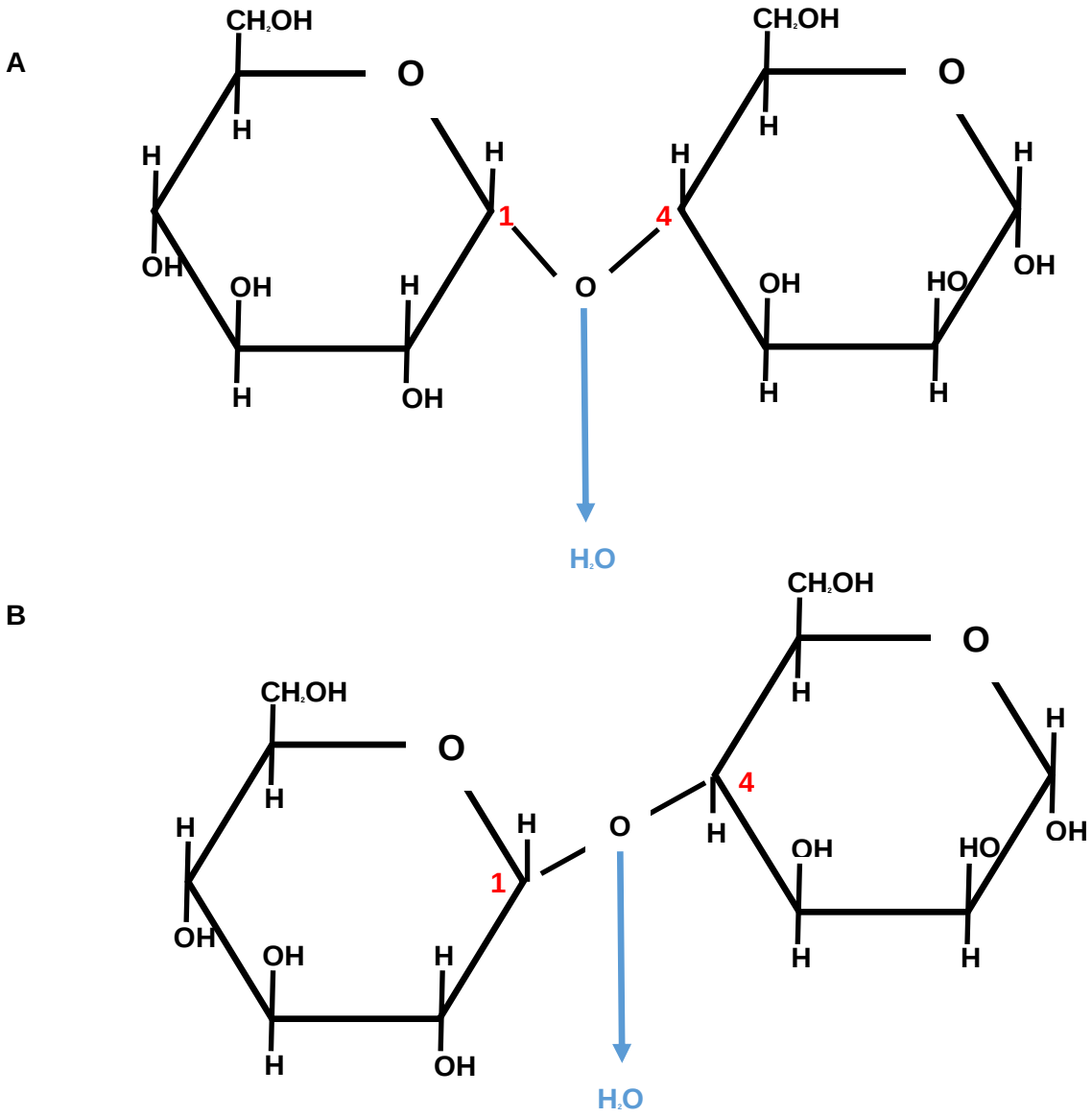


Figure 1.2 Formation of Glycosidic Linkages. Examples of α- (A) and β- (B) 1, 4-linkages between two sugar molecules. Linkages can also occur between carbon 1 and carbon 6 (1, 6-linkage).

Overall, it is remarkable that oligosaccharide structures are capable of such wide variation in composition. It is this variation in structure and glycan attachment to proteins to form glycoproteins that is of great interest for discovery of a BC biomarker.

1.2.5 Glycoproteins

Glycoproteins are polypeptides covalently linked to oligosaccharide moieties that usually have a SA residue at their terminus. The biosynthesis of glycans is a complex process but necessary as glycans contribute to stability and function of the recipient polypeptide. The addition of glycans to the polypeptide backbone can occur as a co- and/or post-translational modification.

Glycans commonly become linked to polypeptides via either *N*- or *O*-linkages at the reducing end of the oligosaccharide chain. At specific asparagine residues within the protein portion, *N*-linkages can occur through the amide nitrogen, while *O*-linkages arise via an –OH group of serine/threonine (Ser/Thr). It is known that approximately 97% of all blood plasma glycoproteins possess *N*-linked glycans (Finne and Krusius, 1979). Their synthesis is complex and attachment to the polypeptide requires the specific tripeptide sequence Asn-X-Ser(Thr) in order to occur. This sequence can be used to predict which Asn residues in the protein backbone will become glycosylated via an *N*-link (Gavel and von Heijne, 1990). Due to specific requirements such as an appropriately positioned consensus sequence, sufficient oligosaccharyltransferase activity and variety of fully assembled, glycosylated lipid-linked oligosaccharide donors, glycosylation only occurs on roughly one third of Asn residues (Kornfeld and Kornfeld, 1985). The intricate synthesis of *N*-linked glycans from a common precursor results in the possession of a characteristic core structure of $GlcNAc_2Man_3$.

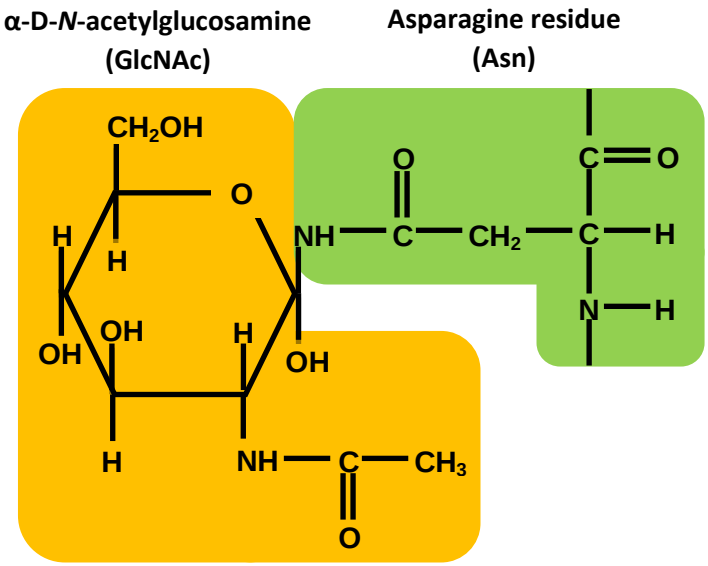


Figure 1.3 Representation of Bond Between Terminal GlcNAc and Asn Residue. [Adapted from Garrett and Grisham (2005)] Depiction of linkage between terminal monosaccharide GlcNAc and the Asn residue in the consensus sequence of the recipient protein to form a basic glycoprotein prior to processing.

In comparison, *O*-linked glycan synthesis is much simpler, without the requirement for a consensus sequence in the inner core; it is GalNAc normally attached to Ser/Thr. However, thereafter glycan diversity is increased due to numerous core structures being generated by such a simple synthesis. *O*-linked glycans may contain Glc within the oligosaccharide chain and tend to exist on glycoproteins originating from epithelial cells (Dell and Morris, 2001).

Many glycoproteins are exclusively either *N*- or *O*- linked. For example AGP, α-1-antitrypsin (AAT), tissue plasminogen activator, interferon β and interferon γ only possess *N*-linkages while granulocyte colony stimulating factor (GCSF) and interleukin-2 (IL-2) are solely *O*-linked. It is possible for glycoproteins to carry an assortment of *N*- and *O*-linkages as is the case of coagulation factor VIII and the hormone erythropoietin (EPO) (Lis and Sharon, 1993).

Integration of glycoproteins in different cell types strongly influences their expression. The polypeptide backbone is synthesised through the transcription and translation of particular genes and co-ordinates glycosylation sites (Freeze, 2001). Glycoform expression from different cell types is capable of producing a wide variety of glycans as each type has a diverse assortment of enzymes which catalyse glycan formation, resulting in a large amount of heterogeneity.

Therefore, variance between glycan compositions can reveal the biochemical and physiological conditions present during production as well as tissue or cell origin (van Dijk*, et al.*, 1994). Glycosylation of proteins with a diverse assortment of glycans is essential for normal mammalian function as glycans are involved in biomolecule and cellular recognition, movement of enzymes within cells and ceasing leukocyte rolling during inflammation. However, to gain a wide variety of glycans, a complex mode of synthesis is required which is fully explored in the following section. Glycan heterogeneity affects the conformation of a glycoprotein which changes its physical properties such as solubility or stability. This influences functions such as cell-cell, protein-protein or protein-saccharide recognition; intracellular enzyme trafficking and inhibition of leukocyte rolling during inflammation (Durand and Seta, 2000; Sears and Wong, 1998).

The majority of serum proteins possess surface glycans. The glycosylation of proteins is the final stage in the maturation of the protein structure. If glycoproteins fail to become glycosylated, their function may be altered or lost entirely. EPO is an example of a protein whose activity is reduced when it is has incomplete glycosylation. In order to function correctly EPO must be glycosylated at three *N*-linked sites (positions 24, 38 and 83) and one *O*-linked site (126). EPO regulates red blood cell synthesis upon release from the kidneys, but is rapidly eliminated by the liver if reduced SA content of the glycans occurs. Removal of SA residues can occur during or after biosynthesis during recycling of cell surface molecules (Varki, 2009). This highlights the importance of glycosylation as anaemia can occur in an individual if EPO is removed before it can participate in the synthesis of red blood cells (Macdougall and Eckardt, 2006).

1.2.5.1 Biosynthesis of *N*-linked Glycans.

N-linked glycosylation occurs in the membrane of the rough endoplasmic reticulum (RER) and Golgi. It is the *en bloc* transferal of a $Glc_3Man_9GlcNAc_2$ (a tetradecasaccharide) from a polyisoprenyl donor onto the amide nitrogen located at the terminus of an Asn side chain acting as an acceptor protein.

In order for *N*-linked glycosylation to occur on the amide group of Asn residues, β-turns within the secondary structure of polypeptides must be formed. β-Turns are formed when hydrogen bonds arise between the hydroxyl (-OH) group of Ser/Thr and an Asn residue amide ($CO\text{-}NH_2$) group (Smith*, et al.*, 1997). Therefore, the consensus sequence (Asn-X-Ser/Thr) resides as a β-turn, where X can be any amino acid except proline (Pro) as it is missing an α-amide proton preventing β-turn configuration (Bause, 1983). A bond can now form between the –NH group of the Asn amide residue and terminal monosaccharide (GlcNAc) of a basic glycan chain resulting in the beginning of a glycoprotein (see Figure 1.3). It may also be possible for *N*-linked oligosaccharides to react with neutral, aromatic amino acid side chains in the three amino acids adjacent to glycosylated Asn residues (Yet and Wold, 1990). Newly added glycans are then processed and trimmed in the RER and Golgi by competitive enzymes known as glycosidases and glycosyltranserases e.g. mannosidase, glucosidase and galactotransferase. These enzymes can catalyse several varied reactions due to their competitive nature, increasing microheterogeneity of the glycans produced (Couldrey and Green, 2000; Guile*, et al.*, 1996).

N-linked glycosylation occurs in four locations (summarised in Figure 1.4). In the cytosol of the RER, monosaccharides become bound to nucleotides to create high energy molecules such as GDP-Man and UDP-GlcNAc that attach to the lipid dolichylphosphate (Dol-P) situated in the RER membrane, forming $Man_5GlcNAc_2$-Dol-P. The transfer of nucleotide-bound monosaccharides to Dol-P is catalysed by GlcNAc-transferase, GlcNAc-1-phosphotransferase and mannosyltransferase. Transporter proteins termed flippases then cause the structure to move from the RER cytosol to the RER lumen (Sanyal and Menon, 2009) where another seven monosaccharides are joined to the structure via glycosidic bonds creating the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$. Oligosaccharyltransferase (OST) is a multimeric complex that transfers the oligosaccharide precursor from Dol-P to Asn in the consensus sequence (Asn-X-Ser/Thr) of nascent glycoproteins (Dennis*, et al.*, 1999; Imperiali and O'Connor, 1999). The structure is then trimmed by glycosidases whilst being translocated through the RER and Golgi. In the medial Golgi GlcNAc-transferases perform substitutions and lastly, in the trans Golgi network the structure is elongated before leaving the Golgi.

Regulatory stages take place during synthesis to ensure that defective glycans are not created. For instance, glycoproteins enter the calnexin/calreticulin cycle, once they have two Glc removed in the RER by α-1,2-glucosidase I and α-1,3-glucosidase II, which prevents incorrectly folded proteins from remaining in the pathway. If an improperly folded protein is detected, a Glc residue is added to the protein in the RER lumen by a glycosyltransferase targeting it to re-enter the calnexin/calreticulin cycle. The faulty protein remains in the calnexin/calreticulin cycle until folded correctly, at which point the added Glc residue is removed by glucosidase II (Deprez*, et al.*, 2005; Sousa*, et al.*, 1992).

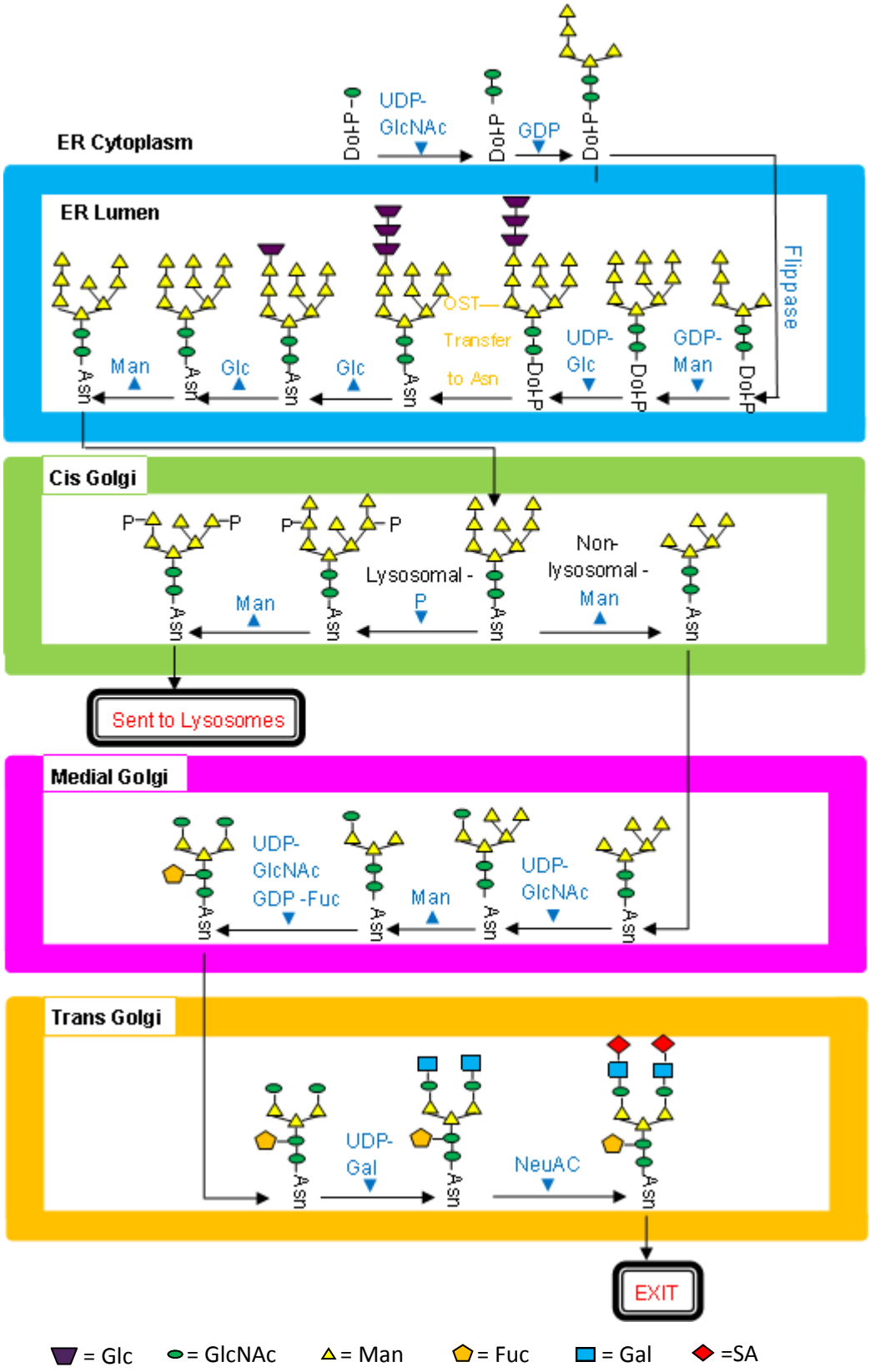


Figure 1.4 *N*-linked Glycosylation. [Adapted from (Larkin and Imperiali, 2011; Rich and Withers, 2009; Varki, 2009)] A summary of the synthesis and processing of *N*-linked glycans in the RER and Golgi which differ depending on the type of *N*-linked glycan attached.

1.2.5.2 Types of *N*-linked Glycans: High-Mannose, Complex and Hybrid.

N-linked glycans share a common pentasaccharide core ($Man_3GlcNAc_2$) but can differ in overall structure due to the biosynthetic process of trimming and adding monosaccharides (Albani and Plancke, 1998) as shown in Figure 1.4. Three types of *N*-linked glycans termed high-mannose, complex or hybrid (shown in Figure 1.5) are produced via trimming and processing of the glycan precursor by enzymes in the RER and Golgi.

High-mannose structures are distinguished by the possession of a heptasaccharide core which is formed when the pentasaccharide core gains between two and six Man residues (see Figure 1.5). A maximum of four Man residues can join to the Man located at the non-reducing terminus of the core and as suggested by the name high-mannose, the only monosaccharides found in these chains are Man (Imperiali and O'Connor, 1999; Kobata, 1992). If high-mannose structures become phosphorylated, producing mannose-6-phosphate, they are targeted towards the lysosomes through binding to the Man-6-phosphate receptor and exit from the cis golgi without being involved in the rest of the processing pathway (Smith*, et al.*, 1997).

Synthesis of complex *N*-linked glycans requires more processing as they tend be capable of a wider assortment of linkages and monosaccharides. They consist of a trimannosyl core (three Man) with two GlcNAc residues linked to a glycoprotein and during processing have sugars added to them such as GlcNAc, Gal, Fuc and SA as shown in Figure 1.5. Structural variation of the glycan may be increased by a Fuc residue bonding to the innermost GlcNAc (Kobata, 1992). In comparison to high-mannose structures which tend to express only 2 GlcNAc residues and between 5-9 Man, complex glycans usually have 4-6 GlcNAc residues and 5-7 neutral sugars (Fan*, et al.*, 1994). Complex glycans can exist as bi-, tri-, tetra- and penta-antennary chains and can be subdivided as such, with a SA residue at the terminus allowing the glycans to avoid being destroyed by the liver and circulate in the blood (Morell*, et al.*, 1971).

Hybrid structures arise when mannosidase (hydrolyses Man) does not act upon addition of a GlcNAc residue to the core structure in the Golgi. Similar to high-mannose, Man residues can join core outer Man residues as well as GlcNAc,

while Fuc can bind to the innermost GlcNAc residue in a similar manner to complex glycans (see Figure 1.5). This results in a combination of high-mannose and complex characteristics as hybrids possess a Man α-1,6 branch (high-mannose) and a Man α-1,3 branch incorporating Gal and more GlcNAc residues comparable to complex glycans (Durand and Seta, 2000; Kobata, 2000).

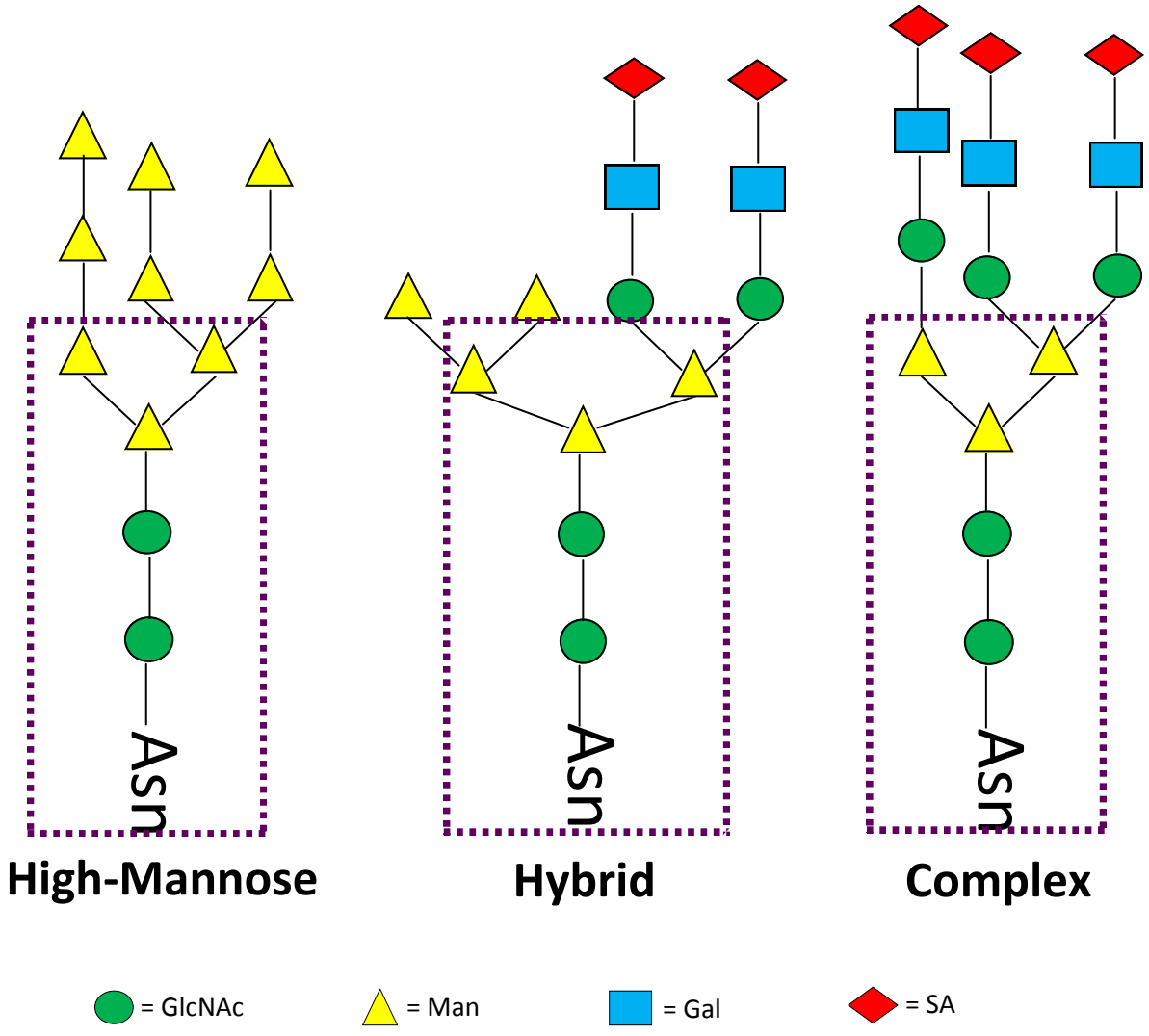


Figure 1.5 Types of *N*-linked Glycan Structure. [Adapted from Varki *et al*., 2009] Illustration of high-mannose, complex and hybrid *N*-linked glycan structures emphasising the common pentasaccharide core.

1.3 Alpha-1-acid Glycoprotein

Alpha-1-acid glycoprotein (AGP) is an *N*-linked glycoprotein, exclusively expressing complex glycans that can be bi-, tri- or tetra-antennary, which occurs

naturally in plasma through secretion by hepatocytes (primarily), breast tissue (Gendler, *et al*., 1982), cardiac cells (Siegal, *et al*., 1985), testes, gastrointestinal tract (Twining, *et al*., 1977) and number of immune cells such as monocytes and lymphocytes (Gahmberg, *et al*., 1978). Therefore, AGP glycosylation could prove to be a suitable biomarker for BC detection due to secretions of AGP carrying aberrant glycosylation from malignant BC tumour cells.

AGP is predominantly secreted by hepatic parenchymal cells and while its primary function has yet to be discovered, it is known that AGP is involved in plasma protein binding of drugs for transportation, regulation of homeostasis, and immunomodulation (Fournier, 2000). For instance, AGP has been shown to increase in concentration during inflammation suggesting its implication as an anti-inflammatory and immunomodulatory molecule. Increased AGP concentrations as well as changes in glycosylation have been shown to occur during several pathophysiological and physiological states such as cancer.

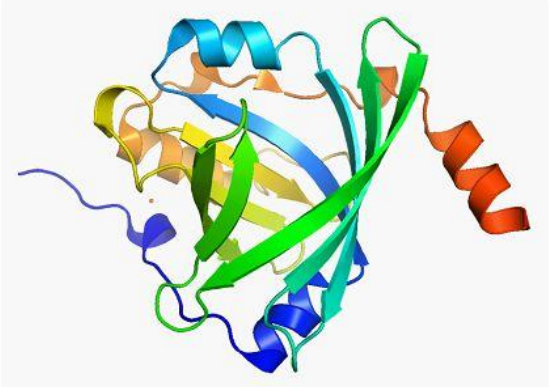
1.3.1 AGP Structure

AGP is named due to its migration with the α-1 protein group during plasma protein electrophoresis as it has a low pI of 2.8-3.8 (Nakano*, et al.*, 2004). AGP is formed when a 201 residue single polypeptide precursor is cleaved after the first 18 residues (Ceciliani and Pocacqua, 2007). The remaining protein portion contains roughly 59% of AGP's structure and 23kDa of its total mass (Albani*, et al.*, 2000; Liao*, et al.*, 1985) with the rest being composed of carbohydrate. Human AGP was initially thought to be composed of 181 amino acids creating a single polypeptide (Schmid*, et al.*, 1973). However, research by Dente*, et al.* (1985) proved that AGP was 183 amino acids in length due to an extra Lys and an Arg residue at positions 173 and 174 respectively. There is also the potential for 22 amino acid substitutions within the molecule. These 22 sites of substitution are linked to the differences between the genes that code for AGP (AGP 1 and 2) and the gene products are present in a 3:1 ratio in human plasma under normal physiological conditions.

There are three main genetic variants of AGP with expression of these variants controlled by three adjacent genes located on chromosome 9. AGP-A (also

referred to as ORM-1) encodes variants F1, F2 and S while AGP-B and AGP-B' (ORM-2) code for A (Colombo*, et al.*, 2006; Katori*, et al.*, 2011). The three genes are very similar in structure with AGP-A only differing from identical genes AGP-B/B' by 22 base substitutions. As F1, F2 and S are all encoded by two alleles of AGP-A, they are collectively known as F1*S and differ by less than five amino acids each. However, variant A has approximately 20 amino acids substituted but with all variants containing 183 amino acids in total this is only a minor difference (nearly 11%) (Schmid, 1989). It is not yet known whether these substitutions affect gene product glycosylation but, it is thought that they may be located within AGP's glycosylation sites at positions 15, 75 and 85 as shown in Figure 1.8 (van Dijk*, et al.*, 1991). F1*S is the main constituent of serum with a 3:1 ratio compared to A but this may be due to AGP-B/B' being expressed 100 times less than AGP-A (possibly owing to transcription occurring only at the first gene within the cluster) (Dente*, et al.*, 1987). However, the level of each variant has been shown to change during diseases such as lymphoma, ovarian cancer and melanoma with ratios of up to 8:1 reported (Budai*, et al.*, 2009). Figure 1.6 shows a structural representation of the F1*S variant (A) and the A variant (B) acquired by x-ray crystallography which reveals the folding pattern of F1*S and A are the same but the binding pockets differ as F1*S has three lobes and is wide but A only has two lobes thus is narrower and may affect binding function (Taguchi*, et al.*, 2013). Figure 1.6 A/B shows unglycosylated AGP variants as the extensive glycosylation makes x-ray crystallography difficult therefore glycans are removed.

A – F1*S variant



B – A variant

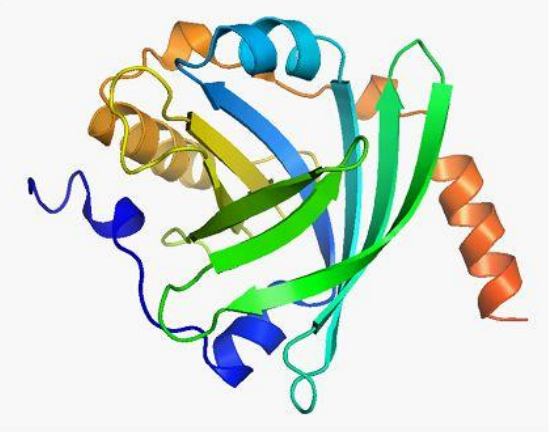


Figure 1.6 Unglycosylated Structure of AGP Variants. X-ray Crystallographic structure of AGP variants F1*S (AGP-A) and A (AGP-B/B') from Taguchi *et al*., (2013).

The molecular structure of AGP is comprised of α-helix (15%), β-sheet (41%), reversed β-turns (12%), bands (8%) and unordered structures (24%) (Kopecký Jr*, et al.*, 2003). This structure shows similarities with lipocalins (of which AGP is a subfamily via the immunocalin group) which also contain a large number of β-sheets and have been shown to have significant immunomodulatory effects. Similar to lipocalins, AGP folds as a highly symmetrical β-sheet structure containing a single antiparallel β-sheet with eight strands (Flower, 1996; Kopecký Jr*, et al.*, 2003).

AGP has a carbohydrate content of over 40% of the molecular weight as it is extensively glycosylated with five highly sialylated complex *N*-linked oligosaccharide chains (Yoshima*, et al.*, 1981) with four of these being linked to Asn residues in areas with a high number of polar residues or inside reverse β-

turns (Ceciliani and Pocacqua, 2007; Kremer, *et al.*, 1988). The consensus sequence Asn-X-Ser(Thr) where X cannot contain a proline (Pro) residue due to it preventing a β-turn, necessary for glycan attachment.

A number of amino acids such as all three tryptophan (Trp) residues, certain tyrosine (Tyr) residues and the majority of phenylalanine (Phe) residues have preferences of location within AGP's folded structure (see Figure 1.7) due to molecular interactions within the polypeptide backbone (Albani, 2003). While some residues lie close to or above the surface of AGP such as Trp 122 which is located between the surface and the hydrophobic binding site and Trp 160 (exposed), Trp 25 is buried in the hydrophobic binding site. Trp residues are more capable of rotation the closer they reside to the surface (Friedman, *et al.*, 1985). Furthermore, 5-7 Tyr and the majority of the Phe residues lie buried – partially or completely – in the native state.

```
AGP-A           MQIPLCANLVPVPITNATLDQITGKWFYIASAFRNEEYNKSVQEIQATFFYFTPNKTEDTI 60
AGP-B/B'        MQIPLCANLVPVPITNATLDRITGKWFYIASAFRNEEYNKSVQEIQATFFYFTPNKTEDTI 60
                 ******************:****************************************

AGP-A            FLREYQTRQDQCIYNTTYLNVQREN-TISRYVGGQEHFAHLLILRDTKTYMLAFDVNDEK 119
AGP-B/B'         FLREYQTRQDQCFYNSSYLNVQRENGTVSRYEGGREHVAHLLFLRDTKTLMFG---DDEK 117
                 ************:**::******** *:*** **:**.****:****** *:.   :***

AGP-A            NWGLSVYADKPETTKEQLGEFSYLEALDCLRIPKSDVVYTDWKKDKCEPLEKQHEKERKQ 179
AGP-B/B'         NWGLSFYADKPETTKEQLGEF--YEALDCLCIPRSDVMYTDWKKDKCEPLEKQHEKERKQ 175
                 *****.***************   ****** **:***:**********************

AGP-A            EEGES 184
AGP-B/B'         EEGES 180
                 *****
```

Figure 1.7 Protein Alignment of AGP Variants. [adapted from Taguchi, *et al.*, 2013 and created using Clustal 2.1 software] The start methionine is highlight in **green** and sites of transcript variation are highlighted in **red**. ":" (colon) indicates conservation between groups of strongly similar properties. "." (period) indicates conservation between groups of weakly similar properties.

The remaining 41-45% of the AGP molecule is composed of glycan chains and galactoglycoprotein (Schmid, *et al.*, 1980) is the only molecule to surpass AGP in its unusually high glycan content with 76%. At residues 15, 38, 54, 75 and 85 there are five variable, highly sialylated heteropolysaccharide complex-type *N*-linked glycans attached to Asn residues usually in the first half (from the *N*-terminus) of the polypeptide (Eap and Baumann, 1993; Israili and Dayton, 2001). The negative charge and low pI (2.8-3.8) is due to C1 of SA possessing a –COOH group as well as acidic amino acids being present (Elg, *et al.*, 1997;

Hochepied*, et al.*, 2003). It is estimated that 11-12% of AGP is comprised of up to 16 SAs, which results in the low pI of AGP, with each having an approximate weight of 314Da and if these are lost through desialylation, AGP becomes inactive. Mature unglycosylated AGP has a higher pI of 4.97 (Devereux *et al*., 1984). The glycan structure of AGP is also composed of Gal, GlcNAc and Man (14%) as well as Fuc (1%). (Dente*, et al.*, 1987; Kremer*, et al.*, 1988). The innermost pentasaccharide core is common to all *N*-linked glycans.

Fournier*, et al.* (2000) revealed that Fuc can be linked via the external branch to GlcNAc using an α-1-3 bond as well as being linked to a core GlcNAc or Gal via a α-1-6 and α-1-2 bond respectively. This may account for the degree of variation in fucosylation between individuals which tends to increase when greater numbers of tri- and tetra antennary chains are present in the structure. Approximately 30% of human serum proteins do not contain Fuc but AGP is in the minority of glycoproteins that can possess tetra antennary *N*-linked glycans along with bi- and tri- antennary, therefore allowing for the presence of Fuc. Fuc accounts for 0.8% of AGP with Gal, GlcNAc, Man and SA completing the molecule with 6.3%, 14.9%, 5.2% and 11.3% respectively.

The presence of glycans allows for the vast structural variability of AGP creating the potential for 10^5 different glycoforms. This is known as microheterogeneity. Only 12-20 of these are expressed during normal (non-pathological) circumstances with varying levels of fucosylation, branching and sialylation demonstrated by each (Albani, 1997). AGP expresses two types of microheterogeneity: type I and type II which can be sub-classified and are not associated with the peptide backbone but rather the glycan chains. Type I is the major form, responsible for the reduced tri- and tetra-antennary chain expression in favour of bi-antennary branching. However, type II (the minor form) is associated with the degree of sialylation and fucosylation of AGPs five oligosaccharide chains (Albani, 1997). As shown in Figure 1.8, Asn residues express glycans selectively depending on their degree of branching which reduces the possible number of glycoforms. Asn 15 and 38 are the first and second sites of glycan expression and usually have bi-antennary glycans linked to them. Additionally, Asn 15 never carries tetra-antennary chains just as Asn 38 will not bind fucosylated glycans. However, branched glycans are favoured by Asn 75, which only binds tri- and tetra-antennary chains, and Asn 85 which

displays the highest levels of α-1,3- fucosylation (Higai*, et al.*, 2005). Fournier *et al*., (2000) proposed that these are the only two sites with the ability to bind tetra-antennary glycans with multiple Fuc residues due to increased branching. AGP glycans are 10-15% bi-antennary with the majority (85-90%) being either tri- or tetra-antennary (Perkins*, et al.*, 1985).

Smith*, et al.* (2002) demonstrated that AGP is more metabolically stable than other glycoconjugates as tri- and tetra-antennary chains are bound more tightly by their termini than bi-antennary chains to the polypeptide core. As well as this, it was shown that glycan microheterogeneity variation is established by sugar nucleotide availability, tissue-specific glycosyltransferase gene regulation and enzyme competition during the processing of the glycan.

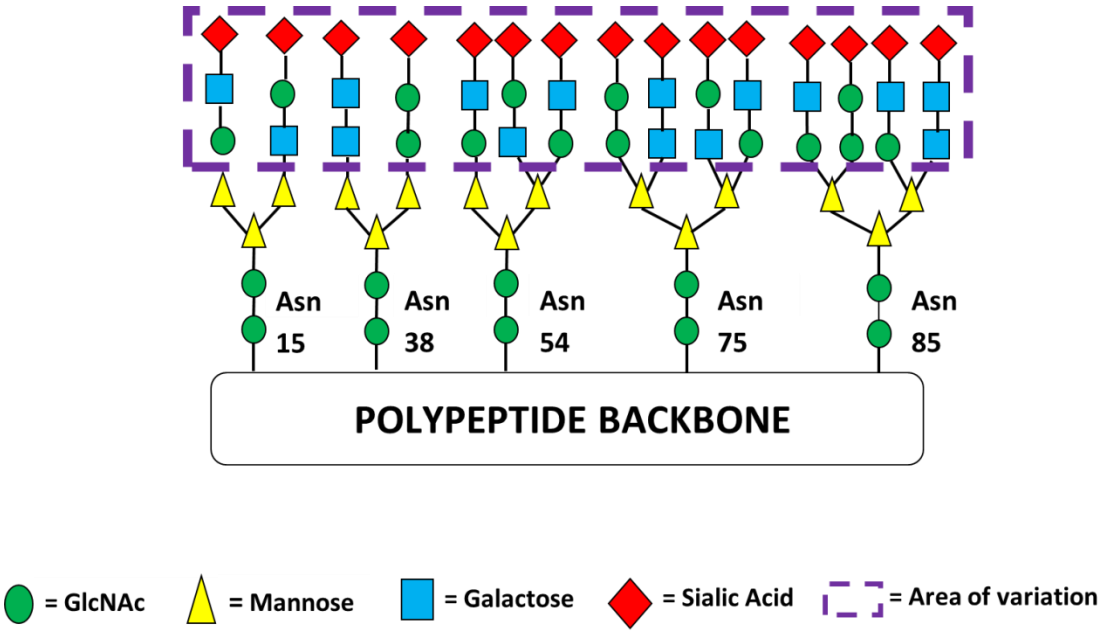


Figure 1.8 Illustration of AGP *N*-linked Glycosylation Possibilities.
Glycan branching is shown as described previously in the text, however chain length can differ. The particular Asn sites that glycans link to are also labelled with certain residues only expressing a specific type of glycan branching (bi-, tri- and tetra-antennary).

1.3.2 AGP Function

The primary *in vivo* function of AGP is still debated despite rigorous investigation, however, it is reported that AGP production contributes to regulation of homeostasis, plasma protein binding to drugs and immunomodulation. With immunomodulatory functions being influenced by

glycosylation pattern (Ceciliani and Pocacqua, 2007) as summarised in Table 1.5. The structure of the glycans AGP carries can affect its function (Chiu*, et al.*, 1977) and as a member of the lipocalin family, AGP has a highly conserved structure with a largely varied protein sequence allowing it to perform the transportation of small hydrophobic molecules (Flower*, et al.*, 2000; Treuheit*, et al.*, 1992).

Activity	AGP Involvement Examples	Study
Anti-inflammatory	• Injury healing carried out by neutrophils and complement is reliant on SLe^x groups. • AGP with little or no bi-antennary glycans enables inhibition of CD3-induced lymphocyte proliferation. • Reduces neutrophil influx and TNF-α expression in mice with renal ischemia and reperfusion injury. • Inhibits complement cascade if carrying the correct glycan composition.	Pos, *et al.* (1990b) Kalmovarin, *et al.* (1991) Williams, *et al.* (1997) Daemen, *et al.* (2000)
Pro-inflammatory	• AGP co-induces monocyte production of IL-1β, IL-6 and TNF-α in the presence of LPS. • Can stimulate fMLP preactivated neutrophils to produce a secondary ROS response. • Local AGP production by monocytes in response to cytokines.	Boutten, *et al.* (1992) Drenth, *et al.* (1996) Su and Yeh (1996) Hochepied, *et al.* (2003) Gunnarsson, *et al.* (2010)
Immunomodulation	• Modulation of lymphocytes by decreasing their responsiveness to PHA, Con A and PWM. • Lymphocyte function is maximally suppressed by agalactosylated and asialylated AGP. • Inhibits activation and migration of neutrophils. • Inhibits apoptosis and the resulting inflammation. • Inhibits phagocytosis and platelet aggregation.	Snyder and Coodley (1976) Chiu, *et al.* (1977) Bennett and Schmid (1980) Athamna, *et al.* (1996) Hochepied, *et al.* (2003) de Vries, *et al.* (2004) Mestriner, *et al.* (2007) Matsumoto, *et al.* (2007)
Drug Binding	• High affinity binding sites can bind neutral and basic drugs as well as steroids and plasma. • Can bind tamoxifen, imipramine, warfarin, quinine, imatinib, disopyramide and methadone. • Elevated AGP can reduce adverse effects of toxic drug doses by binding to the toxic component and subsequently inactivating it. • Binds toxic lectins and LPS produced by microorganisms.	Otagiri, *et al.* (1987) Chatterjee and Harris (1990) Pos, *et al.* (1990a) Silamut, *et al.* (1991) Hervé, *et al.* (1998) Libert, *et al.* (1994) Moore, *et al.* (1997) Frantz, *et al.* (2000) Israili and Dayton (2001) Matsumoto, *et al.* (2002) Kopecký Jr, *et al.* (2003) Gambacorti-Passerini, *et al.* (2003) Widmer, *et al.* (2006)

Table 1.5 Summary of AGP Functions.

AGP glycoforms have differing immunomodulatory roles due to varying glycan structure, particularly in cancer patients. For example, Pukhal'skiĭ, *et al.* (1994) isolated AGP from ascitic fluid in stomach cancer patients as well as from the blood of a healthy population and investigated the effects of lymphocyte

responsiveness. AGP isolated from patients with stomach cancer contained more bi-antennary glycans and proved a better inhibitor of lymphocyte proliferation than AGP from healthy individuals. However, AGP possessing two bi-antennary glycans stimulated both IL-2 and lymphocyte proliferation. Therefore, AGP can have an immunomodulatory effect through the inhibition of IL-2 secretion; however this effect seems to be influenced by structural differences of AGP. Additionally, positive correlation between inhibition of lymphocyte proliferation by AGP and decreased IL-2 secretions has also been noted (Elg*, et al.*, 1997).

The immunosuppressive actions of AGP may have implications in cancer management as the immune system is not activated against cancer cells allowing them to proliferate unnoticed. AGP lacking Gal and SA gave the most potent inhibition of lymphocyte proliferation (Bennett and Schmid, 1980) which suggests that tumours may create a highly immunosuppressive environment if their cells contain galactosidase and neuraminidase. AGP has already been shown to act as an immunosuppressant in ovarian cancer by decreasing lymphocyte proliferation and IL-2 production (Elg*, et al.*, 1997). Saldova*, et al.* (2008) revealed patients with ovarian cancer possessed AGP with increased SLe^x when plasma concentration was amplified. Increased SLe^x contributes to high AGP plasma levels as it reduces the accessibility of free Gal to Kupffer cells in the liver decreasing the rate of AGP clearance from plasma (Coombs*, et al.*, 2006). Furthermore, increased endogenous sialylation has been revealed as a possible anti-apoptotic mechanism involved in transforming tumours to a more malignant phenotype in type B lymphoma cells (Keppler*, et al.*, 1999). This links with research by Kato*, et al.* (2001) showing that sialidase expression is inversely correlated with tumour growth and metastatic potential of murine cancer cells, perhaps via a regulatory mechanism promoting apoptosis and suppressing cell growth.

Overall, while the exact function of AGP remains unknown its minor functions contribute to cancer development and progression. AGP can suppress aspects of the immune system resulting in cancer cells remaining undetected and can potentially create more malignant tumours.

1.3.3 AGP in Cancer.

A significant number of research studies have been undertaken to assess whether the subtle structure changes in AGP could act as a disease specific biomarker. Normally this equates to measuring SA and Fuc levels in comparison to healthy individuals; the variation in the degree of branching (bi,-tri-, and tetra-antennary) of oligosaccharide chains; or the occurrence of antigen structures such as SLe^x. Research to date has shown that AGP glycosylation is altered during acute and chronic inflammation, diabetes and liver disease (for example increased Fuc content) as well as pregnancy (increased tri- and tetra-antennary branching) and burns victims (increased bi-antennary branching) (Higai*, et al.*, 2003; Mooney*, et al.*, 2006; Pawiłowicz*, et al.*, 2006; Poland*, et al.*, 2001; Pos*, et al.*, 1990b; Saroha*, et al.*, 2011).

The majority of research into AGP as a potential biomarker in cancer initially focussed on changes in AGP plasma concentration during disease. AGP plasma levels have been shown to increase 2-5 fold in the presence of cancer (Rudman*, et al.*, 1974); be significantly augmented (compared to healthy individuals) in cancer of the ovary, breast or lung (Duché*, et al.*, 2000; Routledge, 1989) as well as increased AGP levels in malignant lung and breast tissue (Twining and Brecher, 1977). Furthermore, it was noted by Turner *et al*., (1985) that BC patients have a 35.8% increase in AGP concentrations compared to a healthy population. These results are consistent with the notion that AGP operates as a positive acute phase protein during an APR.

Moreover, rising AGP levels have been shown to correlate with increased clinical stages of primary BC (early to disseminated disease) which had metastasised to the liver (Chandrasekaran*, et al.*, 1984; Roberts*, et al.*, 1975). Comparison of these findings with AGP from healthy individuals, resulted in the discovery of two AGP forms with different molecular masses. A 45kDa form, possibly carrying 3-4 oligosaccharide chains and a 37kDa form potentially having 5-6 oligosaccharide chains attached. Similarly, increased expression of AGPs genetic variants has been shown when levels of AGP increase during disease. F1*S and A variants of AGP have been shown to increase 2.5 fold in lung and BC with a 1.6 fold increase being recorded in ovarian cancer.

However, the overall ratio of F1*S to A was not significantly altered to that of a healthy population (Duché*, et al.*, 2000).

In addition to increased AGP concentration, disease specific modifications of AGP glycosylation and expression have been observed between cancer, and healthy populations. AGP in cancer patients has been shown to carry higher levels of bi-antennary glycans, fucosylation and sialylation (Turner*, et al.*, 1985). Increased SA was related to the occurrence of the APR, while increased Fuc seemed to be linked to the spread of malignancy, thus indicating the potential of AGP as a cancer biomarker. Croce*, et al.* (2005) revealed that AGP purified from colorectal cancer patients carries SLe^x groups which is a terminal structure containing Fuc. Shiyan and Bovin (1997) showed that unusual glycoforms of AGP, such as glycans with GlcNAc as terminal residues (mono-agalacto glycans), also occur; and that AGP carrying the maximum number of bi-antennary glycans present in cancer patients showed more immunomodulatory activity than AGP from healthy individuals.

Levels of AGP in fluid from patients with various diseases enabled Rudman*, et al.* (1974) to distinguish congestive heart failure and liver cirrhosis (which do not result in excessive cell proliferation) from pancreatic, breast, lung and ovarian cancer. They also found that the majority of the fluids isolated from cancer patients had an abnormal monosaccharide content displaying decreased hexosamines (GlcNAc and GalNAc), hexoses and SA while a small number of samples showed increased hexosamines. Rudman, *et al.* (1974) concluded that invasive mechanisms used by cancer cells when the disease spreads could be correlated with their results.

In parallel, an investigation by Roberts, *et al*. (1975) showed that BC progression and prognosis could potentially be monitored by analysing serum AGP concentration. Patients with early tumours <5cm had greatly increased concentrations of serum AGP compared to a healthy control group, and patients with early tumours >5cm had even higher levels of serum AGP than those with smaller tumours. Patients with recurrent BC had higher serum AGP levels when compared to the previous two groups and the last group (disseminated BC) had the biggest increase in serum AGP concentration out of all the cohorts tested. Therefore, showing increased AGP levels are good indicators of BC progression and prognosis.

The studies reviewed to date conclude that AGP serum concentration can be used as an indicator of disease however the changes in concentration are not capable of being disease specific or definitively monitoring prognosis. Therefore, AGP surface glycosylation patterns have been analysed in a number of conditions in order to determine validity of AGP as a biomarker.

Investigations by Hansen *et al*., (1984) showed that variations in oligosaccharide branching allowed researchers to differentiate between a healthy population and those with lung carcinoma or benign lung disease. Benign lung disease and healthy populations had less bi-antennary branching but AGP from cancer cohorts contained more bi-antennary branches. This investigation was repeated using the same method by Bleasby *et al*., (1985) however; their findings for AGP microheterogeneity disputed those of the previous study with AGP showing no significant differences to enable discrimination between benign diseases and cancer.

Turner, *et al*., (1985) reported that patients with advanced stage cancer had higher serum concentrations of Fuc. Elevated SA levels were also noted in cancer patients and occasionally it was noted that rises is SA accompanied increases in Fuc. Turner, *et al*., (1985) concluded that measuring these two together (along with APP concentrations) could prove useful for monitoring individuals with cancer. They proposed that Fuc concentrations may predict the degree of tumour infiltration and that incidence of APR could be revealed by SA content. Furthermore, research revealed that primary and secondary liver cancer could be distinguished between by analysis of AGP glycoforms and these findings could be used to determine the stage of ovarian cancer and monitor the success of therapy in advanced ovarian cancer (Mackiewicz and Mackiewicz, 1995).

Additional investigation into AGP fucosylation and branching in cancer by Hashimoto*, et al.* (2004) demonstrated that cancer prognosis and progression could be assessed by changes to AGP microheterogeneity. Good prognosis was associated with advanced stage cancer for patients who possessed normal AGP branching and fucosylation for the majority of time after surgery, while poor prognosis was linked to those with highly branched AGP glycans and who also had elevated fucosylation. Furthermore, Saldova, *et al*. (2007) showed that increased expression of SLe^x structures by AGP could be used in combination

with elevated FA2 (fucosylated, agalactosylbiantennaryglycan) levels on IgG antibodies to distinguish between ovarian cancer and benign gynaecological conditions.

Therefore, increased AGP concentration on its own is merely a non-specific indication that something has changed within an individual and cannot be diagnostic for a certain pathophysiological condition. However, modifications of AGP glycosylation in various diseases, such as cancer, have the potential to be manipulated as biomarkers for diagnosis and progression of disease and changes in glycosylation structure can alter the function of glycoproteins which may also link to particular diseases. There is still a need for further research to discover more disease-specific markers to enhance survival rates by improving diagnosis, prognosis and monitoring of disease. While the primary function of AGP remains elusive there is evidence linking altered AGP glycosylation to various diseases including cancer. Therefore, AGP glycosylation is an ideal candidate for investigation as a diagnostic BC biomarker.

1.3.4 AGP is an Ideal Candidate for a BC Biomarker.

Throughout the previous sections several features of AGP have been discussed that make it an excellent candidate for investigation as a BC biomarker in this research study. Firstly, AGP is not only produced by hepatocytes but extra hepatic production has been reported in breast tissue as well (Gendler, *et al*., 1982), inferring that alterations in AGP glycosylation may result during BC from breast tumour secretions as well as hepatocellular AGP secretions. Concentrations of AGP have also been shown to rise in line with BC progression (Roberts, *et al*., 1975) which may further indicate the role of BC tumour secretions. Additionally, AGP is one of the most heavily glycosylated proteins in existence (approximately 45%) expressing various bi-, tri- and tetra-antennary complex *N*-linked glycans (Yoshima, *et al*., 1981; Schmid, *et al*., 1980) making AGP a highly suitable model for glycosylation studies. Research has shown that AGP displays aberrant glycosylation during a variety of pathophysiological conditions including cancer (Turner, *et al*., 1985 and Hashimoto, *et al*., 2004). Therefore, it is possible that AGP may display uniquely altered glycosylation patterns during TNBC or BRCA1/2 related BC. Lastly,

acquisition of AGP from BC patients would be minimally invasive as AGP can be isolated from whole blood. Thus individuals could be tested through the provision of a small blood sample which is a key aim of biomarker development (Misek and Kim, 2011). Overall, the reasons listed above provide evidence of AGP's suitability for the current research study investigating the potential of AGP glycosylation as a biomarker in TNBC and women at increased risk of BC development due to family history and/or genetic predisposition via BRCA1 and BRCA2 mutations.

1.4 Research Strategy.

Glycoproteins are present in all human blood and are the most heterogeneous group of posttranslational modifications known. Glycans reflect fluctuations in a person's health via high structural diversity which has been proven to correlate with the development or progression of cancer as well as other disease states (Block*, et al.*, 2005). Plus, there is increasing evidence that glycoprotein glycans are modified during BC (Abbott*, et al.*, 2008; Burchell*, et al.*, 2001; Dennis and Laferté, 1989; Fernandes*, et al.*, 1991; Goodarzi and Turner, 1995; Hayes*, et al.*, 1989; Hull*, et al.*, 1989; Perey*, et al.*, 1992a; Perey*, et al.*, 1992b; Sewell*, et al.*, 2006). As mentioned previously, BC affects thousands of women every year, particularly those carrying BRCA1/2 mutations. Individuals afflicted with these mutations are far more likely to develop aggressive forms of BC. By detecting cancer development earlier, more treatments would be available to BRCA1/2 mutations carriers increasing their chance of survival. AGP is a circulatory glycoprotein whose *N*-linked glycosylation undergoes modifications between normal and diseased states such as cancer. By assessing whether a unique change in AGP glycosylation occurs prior to or after development of BC in BRCA1/2 mutation carriers a new biomarker could be discovered. This is why investigation of AGP as a biomarker for proactive detection of BC in BRCA1/2 mutation carriers is vital.

The purpose of the study was to determine any changes to AGP glycosylation in women with family history of BC. To facilitate this, a case control style study was performed after collection of initial data from validation of laboratory protocols to isolate and analyse AGP glycosylation. This type of approach has

been adopted in many other studies and was consistent with the range of samples that were available for this study.

Preliminary findings were accumulated using plasma samples from women with family history of BC and/or TNBC, due to the previously described link between BRCA1/2 mutations and TNBC. Further to this, blood samples were collected from women with family history of BC attending the High Risk Familial Breast Screening Clinic at Edinburgh's Western General Hospital and age-matched healthy controls from Edinburgh Napier University to form a case-control style approach. A summary flow diagram of the isolation and analytical process is shown in Figure 1.9 below,

To summarise, this study sought to identify alterations to AGP glycosylation that may be indicative of BC onset, particularly in women at increased risk of BC development or those with an aggressive from of BC such as TNBC. It was hoped that the results of this study could lead to further investigations using larger cohort sizes with annual or bi-annual follow-up to monitor any BC development.

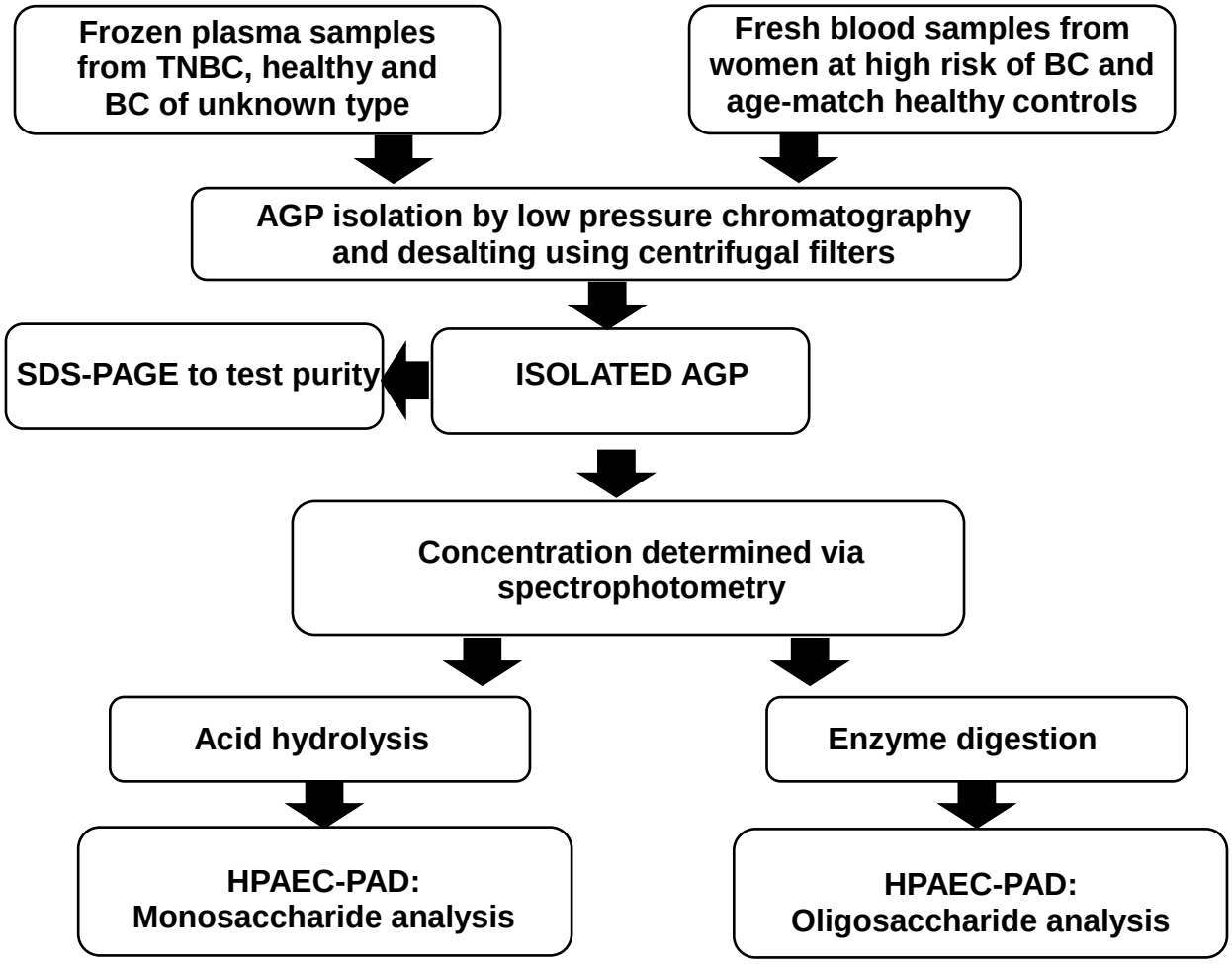


Figure 1.9 Summary Flow Diagram of AGP Isolation and Analysis conducted in this research. SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis and HPAEC-PAD = high pH anion-exchange chromatography with pulsed amperometric detection.

Chapter 2 – Materials and Methodology.

2.1 Introduction.

This chapter details all methods used for this study along with justifications of any deviations from the original research strategy. The objective of this chapter is to provide a detailed and transparent description of the methods used to enable the collection and interpretation of all data presented in the chapters that follow.

2.1.1 Dataset 1 Sample Acquisition and Demographics.

As detailed previously, a strong link between TNBC and BRCA1 mutations has been unveiled by recent research (Anders*, et al.*, 2009; Lakhani*, et al.*, 2002b). Therefore, as this study could not obtain confirmed BRCA1/2 mutation carrier samples, samples of plasma were purchased from the Canadian Breast Cancer Foundation Tumour Bank (Alberta) from patients with TNBC with and without family history of BC (n=18). Patient's family history was not determined genetically but through patient's selecting a known family history on a data collection sheet. TNBC status was confirmed by pathology laboratories in Canada as part of a patient's clinical care using immunohistochemistry (IHC; Edmonton) or Allred assay system (Calgary) (Gazinska*, et al.*, 2013; Oakman*, et al.*, 2010) prior to blood draw. Samples from across the age spectrum were requested as there is an even stronger link between BRCA1 mutations and TBNC in young women than those with TNBC at a later age.

Positive control samples in the form of generic BC were purchased from Capital Biosciences (Maryland, USA). Samples were largely from the age range expected of BC sufferers (age 50-70). Samples were of a low-medium TNM stage and were all invasive. Similarly the ethnicity of all patient samples was Caucasian.

Additionally, negative control samples used in Dataset 1 were obtained from the Scottish Blood Transfusion Service. No information was provided other than blood type but it was thought that any blood drawn with the intent of transfusion would be from individuals free from disease and thus inflammation. To aid the collection of reliable results, n=5 samples were selected for use, one of each blood type (A, B, O and AB) and a second type B, selected at random. All

samples were of positive Rhesus type as no negative Rhesus type samples were available.

All samples were subjected to laboratory methods (described in detail later in this chapter) to isolate AGP and analyse glycan content.

2.1.2 Dataset 2 NHS Sample Acquisition.

2.1.2.1 Population Identification.

Edinburgh's Western General Hospital is home to the High Risk Familial Breast Screening Clinic run by consultant breast surgeon Miss Elaine Anderson. Women attending this clinic had been referred due to having family history of BC and required more frequent and earlier screening for BC development, thus this was identified as a recruitment site for potential participants. Any participants recruited from this site would represent a population who were at increased risk of BC development but were yet to develop BC. While potential participants were identified at this site, they were invited to attend Edinburgh Napier University (ENU) to donate a blood sample for the study.

2.1.2.2 Sample Size.

Sufficient case sample size of n=12 was decided upon based on an 80% statistical power of detecting differences in AGP glycosylation using a 5% statistical significance level and by estimating the degree of agreement between the test system through determination of the Kappa co-efficient greater than 0.81.

2.1.2.2 Ethical Approval.

To access a clinical cohort ethical approval is needed from a local NHS Research Ethics Committee (REC) and Research and Development (R&D) department is necessary. Ethical approval was granted by NHS Lothian's REC in April 2013 and shortly thereafter NHS Lothian R&D approval was received also (see Appendix 1). One requirement of favourable ethical opinion was for

the Chief Investigator (CI; myself) to obtain a Research Passport – previously known as an Honorary Contract – prior to undertaking the study. A Research Passport for the CI was obtained in October 2013. Further ethical approval was granted by NHS Lothian's REC and R&D in December 2013 to increase the maximum number of study participants to n=20 (see Appendix 2).

2.1.2.3 Recruitment and Informed Consent.

The consultant surgeon identified potential research participants with the aid of clerical staff from Ardmillan House Breast Screening Centre, Edinburgh. An invitation letter (see Appendix 3) from the consultant was included in an information pack containing a patient information sheet (PIS, Appendix 4), draft consent form (Appendix 5), reply slip (Appendix 11) and a pre-paid reply envelope. Information packs were then posted out to 50 potential participants with the aim of recruiting a minimum of n=12. Women who were willing to take part were asked to fill in the reply slip and provide contact details allowing them to be contacted by the research team to arrange a suitable appointment time to donate a blood sample and fill in a data collection sheet (Appendix 6). By deciding to take part, women were agreeing that they met the inclusion criteria and did not knowingly have a condition that would exclude them from the study.

At the participant's clinic appointment an informed consent form (Appendix 5) was completed by a trained member of the research team including ensuring each potential participant had read the PIS, understood the implications of participating and that they had the right to withdraw without consequence at any time. Participants were also asked to consent to whether their GP should be informed of their involvement in the study. If participants consented, they were asked to provide contact details for their GP to enable the research team to post a letter and PIS to inform GP's of the individual's participation in the study. Consent was taken prior to blood draw and filling in of the data collection sheet. Blood samples were then subjected to the same laboratory methods as samples from Phase 1 (described later in this chapter) and participants offered a Lothian Bus Day ticket for attendance.

2.1.2.4 Inclusion Criteria.

Women, over the age of 18 who attended the High Risk Familial Breast Screening Clinic at Edinburgh's Western General Hospital due to increased risk of BC development.

2.1.2.5 Exclusion Criteria.

Women could not participate in the study if they were diabetic, pregnant or had a chronic inflammatory condition such as rheumatoid arthritis, Crohn's disease or liver disease. This was due to previous research detailing changes to AGP glycosylation in these conditions which could have provided misleading results for this study.

2.1.2.6 Blood Draw and Data Collection Sheet.

Once informed consent had been obtained, participants were asked provide answers to the questions within the data collection sheet (Appendix 6), transcribed by a member of the research team, to provide information that was relevant to the study e.g. height, weight and questions relating to medical history and a 3-4 ml blood sample was drawn by a qualified member of the research team.

2.1.3 Phase 2 ENU Age-Matched Negative Control Sample Acquisition.

2.1.3.1 Population Identification.

To complete this case control study an age-matched negative control population i.e. healthy participants were recruited from ENU. These participants would represent a population without family history of BC and without underlying health conditions that could affect AGP glycosylation. As before, participants would be required to provide a blood sample for analysis and fill in a data collection sheet with information relevant to the study.

2.1.3.2 Ethical Approval.

Ethical approval was granted by ENU Faculty of Health, Life and Social Sciences Ethics Committee on 15th January 2014.

2.1.3.3 Recruitment and Informed Consent.

Posters (Appendix 7) were placed around ENU describing the study and what would be involved for potential participants. Posters detailed the ages that were required for age-matched participants and asked interested individuals to contact the research team. The research team then gave a PIS (Appendix 8) to potential participants to read, if they were still interested an appointment would be arranged for the individual to give informed consent (Appendix 9), have their family history taken (by a trained member of the research team), have a blood sample drawn and fill in a data collection sheet (Appendix 10) with information relevant to the study. Informed consent was taken by a trained member of the research team as was the blood sample. By taking family history the research team could be sure that participants had no family history of BC. Blood samples were again subjected to laboratory procedures described later in this chapter.

2.1.3.4 Inclusion Criteria.

An individual who is an age-match to one of the increased risk participants, thus acting as a negative control group for this study.

2.1.3.5 Exclusion Criteria.

Individuals were not eligible for the study if they had increased risk of BC, were diabetic or pregnant, had had recent surgery or had an inflammatory condition such as arthritis or Crohn's disease.

2.1.3.6 Family History, Blood Draw and Data Collection Sheet.

During a participants appointment and once informed written consent had been given, a trained member of the research team took a detailed family history to check that participants did not have an unknown family history of BC. If a participant was still eligible after family history was taken, a 3-4 ml blood sample was drawn and a data collection sheet filled in by the participant, providing relevant information for the study e.g. height, weight or any medication being taken.

2.2 Laboratory Materials and Methods.

2.2.1 Samples

Dataset 1 comprised of eighteen TNBC plasma samples purchased from the Alberta Tumour Bank – six from patients below 35 years of age, six from patients aged 35-60 and six from patients over 60. Five BC samples were acquired from Capital Biosciences Inc., Rockville, Maryland, USA and used as positive controls while five “normal” plasma samples from donor without cancer were pooled and sourced from the Scottish National Blood Transfusion Service to act as negative controls.

Dataset 2 included sixteen whole blood samples obtained from female volunteers at increased risk of BC due to family history and/or a genetic mutation who were recruited at a local High Risk Familial Breast Screening clinic. Ten age-matched healthy controls were recruited from members of staff at ENU.

All samples were stored at -20^{0} C until further analysis was able to take place.

2.2.2 AGP Isolation

2.2.2.1 Polyethylene Glycol Precipitation.

One millilitre whole blood, serum or plasma samples were transferred into fresh eppendorf tubes from their collection vials. PEG 3350 (Sigma-Aldrich, Poole, UK) was added to samples at 40% w/v (0.4g to 1ml of sample or 0.04g to 100µl

sample) then samples were vortexed to ensure even distribution and refrigerated at 4°C for a minimum of 24 hours. The samples were centrifuged at 14,000 RPM for 30 minutes in an Eppendorf Centrifuge 5415C, the supernatant moved to fresh eppendorfs and the pellets discarded. Samples were frozen at -20°C until protein purification could take place.

2.2.2.2 Buffers.

Buffer	Components
Cibracron Blue Elution	One hundred millimolar potassium chloride, 50mM Trizma and 3.07mM sodium azide (pH7)
Cibacron Blue Desorption	Five hundred millimolar of potassium thiocyanate to 250ml of Cibacron Blue Elution buffer
Red Sepharose Elution	Twenty three millimolar of sodium acetate to 250ml of distilled water and 200mM of glacial acetic acid to a separate beaker containing 100ml distilled water.136ml of sodium acetate dilution was added to 13.5ml of glacial acetic dilution in a fresh beaker. 1000ml of distilled water was added to the solution (pH 5.7)
Red Sepharose Desorption	One molar sodium chloride to 100ml Red Sepharose Elution buffer

Table 2.1 Composition of Low Pressure Chromatography Elution and Desorption Buffers. Composition of all buffers utilised for isolation of AGP by low pressure affinity chromatography are detailed in Table 2.1. All buffers were stored in Duran jars until use.

2.2.2.3 Low Pressure Chromatography.

A 10ml disposable Poly-Prep column sourced from Bio-Rad Laboratories Ltd., Hemel Hempstead, UK, was filled to approximately 5ml with Reactive Blue Sepharose (Cibacron Blue 3GA) resin purchased from Sigma-Aldrich, Poole, UK, and this was carried out for Red Sepharose CL-6B resin (provided by Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) columns also. The previously prepared Cibacron Blue 3GA column was equilibrated using 5ml of the appropriate elution buffer (compositions presented in Table 2.1), added at a flow rate of 0.5 ml per minute by a Pharmacia LKB Pump P-1 for 10 minutes, before adding approximately 1ml supernatant, resulting from PEG precipitation, simultaneously with 12.5ml elution buffer for 25 minutes at a flow rate of 0.5ml per minute. Changes in absorbance were monitored with a single path Pharmacia optical UV-1 monitor and were noted as a peak on a Kipp and Zonen BD41 chart recorder (see Figure 4.1 in Chapter 4) set at 2mm/min indicating when supernatant had passed through the column and the eluted fraction (approximately 9ml) was collected in a 15ml centrifuge tube. Human serum albumin (HSA) is bound by Cibacron Blue 3GA resin within the column resulting in AGP being eluted. The column was then regenerated using 7.5ml desorption buffer, in place of elution buffer, for 15 minutes at a flow rate of 0.5ml per minute, to remove previously bound HSA from the column. This was shown on the Kipp and Zonen BD41 chart recorder as a baseline. Before addition of further samples, the column was equilibrated using elution buffer.

The eluted fraction (approximately 9ml) was concentrated to approximately 2ml using a Christ RVC 2-18 Concentrator at -60^0 C (SciQuip Ltd., Shrewsbury, UK) and the time taken for this to occur varied between samples. The concentrated 2ml fraction added to a previously prepared Red Sepharose CL-6B resin column which was equilibrated with 5ml of the appropriate elution buffer (see Table 2.1) for 10 minutes at a flow rate of 0.5ml per minute. 2ml of sample concentrated from the Cibacron Blue 3GA column was added simultaneously with 12.5ml elution buffer for 25 minutes at a flow rate of 0.5ml per minute .The movement of the sample through the column was monitored with a single path Pharmacia optical UV-1 monitor and were noted as peaks on a Kipp and Zonen BD41 chart recorder (see Figure 4.1 in Chapter 4). Approximately a 9ml eluted fraction was again collected in a 15ml centrifuge tube. Red Sepharose CL-6B

resin binds AAT and transferrin thus the eluted fraction contained isolated AGP.Transferrin and AAT were removed from the column by washing with 7.5ml of the appropriate desorption buffer (see Table 2.1) for 15 minutes at a flow rate of 0.5ml per minute. All of the above purification methods were performed at room temperature. Both columns were stored in 10% ethanol at 4°C until further use to prevent bacterial growth and drying out of the resin.

Purified AGP in the eluted fraction (approximately 9ml) was concentrated in the Christ RVC 2-18 Concentrator at -60^{0} C to approximately 2ml and the time taken for this to occur varied between samples. Once concentrated to 2ml the purified AGP was ready for desalting.

2.2.2.4 Desalting.

Excess salt was removed from samples of isolated AGP using a Centricon® YM-10 centrifugal filter device with a 10,000Da cut-off filter purchased from Millipore Ltd., Hertfordshire, UK. Initially, the filter membrane was dampened with one or two drops of HPLC grade water (sourced from Rathburn Chemicals Ltd., Walkerburn, UK), the 2ml concentrated eluted fraction containing isolated AGP is then added and topped up with 2ml of HPLC grade water. To prevent cross contamination only one filter device was used per sample. Filter devices containing samples were spun in a Zentrifugen Universal 320 centrifuge by Hettich for 30 minute cycles at 4000 rpm, being topped up with 1ml of HPLC grade water after every cycle, until the full 1ml of HPLC grade water added prior to a cycle had filtered through. This took approximately six cycles and indicated that de-salting was complete. Excess salt water collected at the bottom of the tube was disposed of and the desalted sample was transferred into a fresh eppendorf then dried to completion in a Christ RVC-2-18 Concentrator.

2.2.2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed in line with the protocol devised by Laemmli (1970) and all reagents were purchased from Sigma-Aldrich, Poole, UK, unless stated otherwise. The resolving gel was composed of 10% (w/v) acrylamide (30%

protogel; purchased from ThermoFisher Scientific, Loughborough, UK), 0.1% (w/v) SDS, 0.08% (w/v) ammonium peroxodisulfate and 0.375 Tris-HCl, pH 8.85. The stacking gels were formed using 5% (w/v) acrylamide (30% protogel), 0.2% (w/v) SDS, 0.12% (w/v) ammonium peroxodisulfate and 0.17M Tris-HCl pH 6.8. Polymerisation of resolving and stacking gels was achieved by adding N,N,N',N' – tetramethylethylenediamine (TEMED) to give a final concentration of 0.05% (v/v) and 0.02% (v/v) respectively. Samples were denatured for 5 minutes at 95°C after the addition of 2 x loading sample buffer (LSB) containing 20% glycerol, 4% SDS, 200mM Tris-HCl pH 6.8 and 10mM ethylenediaminetetraacetic acid (EDTA). Samples were then loaded onto the gel and electrophoresed for 50 minutes at 185V in running buffer (0.1% SDS, 25mM Tris, 192 mM glycine, pH8.3). Gels were stained with Coomassie brilliant blue R.

2.2.2.6 Coomassie Staining.

Gels were submerged in Coomassie brilliant blue staining solution sourced from Sigma-Aldrich, Poole, UK (45% methanol, 10% acetic acid, 0.2% Coomassie R250) after electrophoresis. Gels were then incubated at room temperature and gently agitated for 30 minutes. Following incubation, dH_2O was used to wash gels which were then immersed in destaining solution (Sigma-Aldrich, Poole, UK; 20% methanol, 10% acetic acid in dH_2O) to enable visualisation of protein and through reduction of back ground staining.

2.2.3 Determination of Sample AGP Concentration.

A standard calibration curve of absorbance against AGP concentration (mg/ml) was produced by dissolving commercial human AGP sourced from Sigma-Aldrich, Poole, UK, at concentrations from 0-1.5 mg/ml, in 1ml HPLC water and reading the absorbance of each at 280nm. De-salted and concentrated AGP samples were reconstituted in 1ml HPLC grade water then the absorbance measured at 280nm on a Thermo Scientific™ Genesys™ 10S UV-Vis Spectrophotometer using a quartz cuvette with a path length of 1cm. The

equation of the standard curve was used to calculate the concentration of AGP in samples from their absorbance value in accordance with the linear relationship between absorbance and concentration outlined by the Beer-Lambert law.

2.2.4 High pH Anion-Exchange Chromatography.

2.2.4.1 Acid Hydrolysis.

Once the concentration of AGP in each sample was determined an aliquot of each sample was diluted to give 50µg/ml using HPLC water, dried down to completion and reconstituted in 100µl HPLC water. The 50µg reconstituted sample was transferred to a glass vial with an aluminium lid (to prevent evaporation; purchased from Sigma-Aldrich, Poole, UK) and to this 100µl of 2M TFA acid (Fisher Scientific, Loughborough, UK) and 50µl 4M HCl (Sigma-Aldrich, Poole, UK) were added. Vials were then placed in a heat block set at 100°C for 4 hours to allow glycans to separate from the polypeptide back bone of AGP and the bonds between glycan monosaccharides to be hydrolysed.

2.2.4.2 Dowex Ion Exchange.

Dowex-50 WX8 H^+cation exchange resin (1 ml; purchased from Sigma-Aldrich, Poole, UK) was added to a Pasteur pipette packed with approximately 1 cm of glass wool to produce a Dowex column. The column was washed with 6ml of HPLC water and then the sample added. The polypeptide scaffold was bound by the resin while the separated glycans passed through the column and were collected in a fresh eppendorf. A further 1 ml of HPLC water was added to the column to flush out any remaining glycans which were collected in another fresh Eppendorf. If using multiple samples, a separate Dowex column was used for each sample to prevent cross contamination. Collected monosaccharides from each sample were dried down to completion using a Christ RVC-2-18 Concentrator at -60^0 C in preparation for monosaccharide analysis. Time taken to dry down samples to completion varied between samples.

2.2.4.3 Monosaccharide Analysis.

Analysis of sample AGP monosaccharide content was achieved using HPAEC-PAD on a Dionex 3000™ system with a GP50 gradient pump, an ED40 electrochemical detector, a CarboPac™ PA-100 analytical column (4x250mm) and a guard column (4x50mm) (Dionex, Camberley, UK) and used in conjunction with a HP Compaq computer with Chromeleon™ software to control the automated system. This instrument separated monosaccharides based on the formation of oxyanions from contact with the alkaline eluents and detection occurred from the oxidation of these oxyanions on the gold electrode surface which generated a measurable electric current. During PAD the pulse potentials used were 0 seconds (sec), 0.05 V; 0.29 sec, 0.05 V; 0.49 sec, 0.05 V; 0.5 sec, 0.05 V; 0.51 sec, 0.6 V; 0.61 sec, -0.6 V; 0.65 sec, -0.6 V; 0.66 sec, 0.05 V.

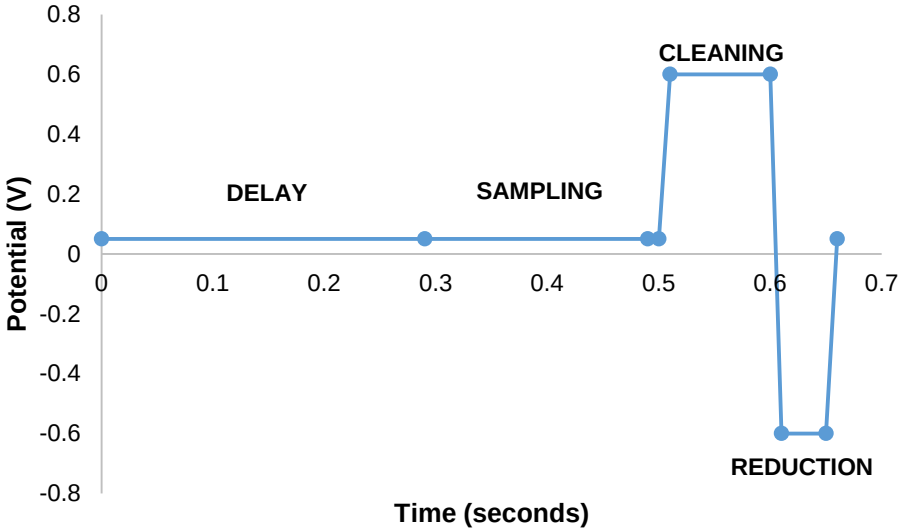


Figure 2.1 Pulsed Potentials of the PAD System During HPAEC.

A 15 minute regeneration of a CarboPac PA-100 pellicular anion exchange column was performed using 50% 0.5M NaOH (Sigma-Aldrich, Poole, UK) and 50% HPLC water. Each dried AGP glycan sample had 45 µl HPLC water and 5µl internal standard (2-deoxy-D-galactose, 1 mg/ml; Sigma-Aldrich, Poole, UK) added before 50 µl was transferred into fresh plastic Dionex vials with septum ready for monosaccharide analysis. The vials were then placed into the auto-sampler of the Dionex analyser and 10 µl volumes applied to the column.

Monosaccharides were separated at pH13 with an isocratic elution with 30 mM NaOH at a flow rate of 0.5 ml/min for 35 minutes. The HPAEC column was regenerated for 15 minutes using 50% 0.5M NaOH/ 50% HPLC prior to further analyses.

Monosaccharide peaks were identified by dividing elution time of peaks by the internal standard, then comparing the ratios to that of known monosaccharide standards purchased from Sigma-Aldrich, Poole, UK (Fuc, Man, Glucosamine, Galactosamine, Gal). Standard curves of each monosaccharide present on AGP were produced by plotting monosaccharide amount (µg) versus peak area in order to quantify levels of each monosaccharide within AGP samples.

2.2.4.4 Enzymatic Digestion.

One hundred microlitres of HPLC grade water was used to reconstitute approximately 100µg of AGP and the solution was transferred from an eppendorf into a glass vial with an aluminium lined cap which were purchased from Sigma-Aldrich, Poole, UK. The vial was then placed in a heating block at 100°C for 3 hours to denature AGP. After denaturation the AGP solution was dried down to completion using a Christ RVC-2-18 Concentrator at -60^0 C and the time required to accomplish this varied between samples.

To perform enzyme digestion, the denatured AGP was reconstituted in 79% (v/v) HPLC grade water and the following reagents (New England Biolabs Inc., Hitchin, Hertfordshire, UK) - 10% (v/v) NP-40, 10% (v/v) NE Buffer G7 and 5 units (U) of PNGase F enzyme giving a total volume of 100µl. One unit of PNGase F is defined by the manufacturer as the amount of enzyme required to remove >95% of the carbohydrate from 10µg of denatured RNase B in 1 hour at 37^0 C in a total reaction volume of 10µl. This mixture was incubated in water bath for 24 hours at 37°C, following which, a further 5U of PNGase was added and incubation resumed for another 24 hours at 37°C.

Once enzyme digestion had occurred, protein fragments were separated from the oligosaccharides by ethanol precipitation. Three hundred microlitres of ice-cold 80% ethanol was added to the 100 µl reaction mixture (3:1), the solution mixed thoroughly and then stored at -20°C overnight. The processed AGP was

then centrifuged for 30 minutes at 14000 rpm. The supernatant containing oligosaccharides was transferred to a fresh Eppendorf and dried to completion under a vacuum while the protein pellet was discarded.

2.2.4.5 Oligosaccharide Analysis.

HPAEC-PAD was performed on a Dionex ICS 3000™ system with a GP50 gradient pump, an ED40 electrochemical detector, a CarboPac™ PA-100 analytical column (4x250mm) and a guard column (4x50mm) which was purchased from Dionex, Camberley, UK. This was used in conjunction with a HP Compaq computer with Chromeleon™ software to control the automated system. This instrument separated oligosaccharides based size and charge due to the formation of oxyanions from contact with the alkaline eluents and detection occurred from the oxidation of these oxyanions on the gold electrode surface which generated a measurable electric current. The same pulsed potentials as monosaccharide analysis were used (see Figure 2.1) to analyse oligosaccharide content of samples. Initial regeneration of the CarboPac PA-100 pellicular anion exchange column was achieved by running 50% 1M NaOH and 50% HPLC grade water through the column for 20 minutes prior to use. Samples were reconstituted in 25 µl of HPLC grade water before being loaded into the CarboPac PA-100 column. Oligosaccharides were separated using the following eluent settings – equilibration for 10 minutes with 10% NaOH/ 5% 1M NaOAc/ 85% HPLC grade water; separation for 45 minutes with a linear gradient to a concluding eluent composition of 10% NaOH/20% NaOAc/ 70% HPLC grade water; regeneration for 10 minutes using 50% NaOH/ 50% HPLC grade water and lastly equilibration for 5 minutes using 10% NaOH/ 5% NaOAc/ 85% HPLC grade water. All of these steps occurred at a flow rate of 1 ml/min. NaOAc and NaOH were sourced from Sigma-Aldrich, Poole, UK.

A reference profile comprised of approximately 5µg of a human *N*-linked oligosaccharide library purchased from ProZyme Inc., California, USA was analysed for comparison with commercial AGP and patient-derived sample profiles.

2.3 Statistical Analysis.

Microsoft® Excel 2010 was used to calculate the Pearson correlation coefficient (r^2). Pearson correlation coefficient values of 0.7-1 represent strong linear relationships with 1 denoting a perfectly linear fit. Mean and standard deviation were also established using Microsoft® Excel 2010. Statistical significance ($p<0.05$) was determined using SPSS statistical analysis software package version 20.

Chapter 3 – Participant Demographics

3.1 Dataset 1 Sample Demographics.

As detailed previously, a strong link between TNBC and BRCA1 mutations has been unveiled by recent research. Fully anonymised samples of plasma were purchased from the Alberta Tumour Bank from patients with TNBC with and without family history of BC. TNBC status had been determined during the patients' clinical care process using IHC or Allred assay system prior to blood draw (Gazinska*, et al.*, 2013; Oakman*, et al.*, 2010). Family history was recorded by patients' answering "Yes" or "No" during completion of a questionnaire upon their initial clinic visit, however no further details were requested. Table 3.1 below gives further details regarding TNBC samples. Samples from a variety of ages were requested as there is an even stronger link between BRCA1 mutations and TBNC in young women than those with TNBC at a later age. Samples were separated by age into categories ≤ 35 years old (n=6); >35 to 60 years old (n=6) and >60 years old (n=6) giving a total TNBC sample size of n=18. Age of samples ranged from 22 to 81 years of age with a mean ± standard deviation of 53 ± 19. Eight patients (44%) had received treatments prior to donation of a blood sample.

Sample	Age*	TNBC	Family History	Pre/Post Menopausal	TNM Stage	BMI	Treatment Prior to Blood Draw
MT3625	22	✓	Yes	Pre	2A	22.3	TAC
CT79	26	✓	Yes	Pre	1	24.5	-
MT2621	27	✓	Unknown	Pre	2A	25.5	TAC
F5412	32	✓	Yes	Pre	1	23.0	-
MT3965	34	✓	Yes	Pre	2A	26.4	TAC and RT
MT2305	35	✓	Unknown	Pre	2B	29.0	NTAC
MT1177	51	✓	Yes	Post	2A	30.6	-
MT1178	52	✓	Unknown	Post	1	30.9	-
MT3601	54	✓	-	Post	1	33.9	TC and RT
MT3795	57	✓	-	Post	2B	37.2	TC
MT1937	59	✓	-	Post	1	36.6	-
MT1106	60	✓	-	Post	1	Unknown	-
MT3352	68	✓	-	Post	3B	20.5	CMF
MT3959	69	✓	Yes	Post	2B	26.0	-
MT3442	71	✓	-	Post	1	29.9	TC
MT3362	72	✓	-	Post	2B	27.6	-
MT2569	75	✓	-	Post	1	34.4	-
MT3497	81	✓	Yes	Post	2A	41.5	-

Table 3.1 TNBC Sample Information. Details provided from sample provider considered relevant for this study n=18. TAC (Taxotere®, Adriamycin®, cyclophosphamide), RT (radiotherapy), CMF (cyclophosphamide, methotrexate, fluorouracil), NTAC (neoadjuvant TAC).

Positive control samples in the form of BC of an unknown histological type were obtained to aid the identification of any glycan similarities when compared to women without BC, because cancer may be in present in early stages. As detailed in Table 3.2 samples were largely from the age range expected of BC sufferers with a mean age ± standard deviation of 57 ± 8.5, bar I8 from a 46 year old patient. Samples were of a low-medium TNM stage and were all invasive. Similarly the ethnicity of all patient samples was Caucasian. No treatment was received prior to sample collection meaning positive control samples were truly reflective of AGP glycosylation during BC.

Sample	Age*	BC Type	TNM Stage	Treatment Prior to Blood Draw	Ethnicity
H8	61	IDC	2A	-	Caucasian
I2	54	IDC	2A	-	Caucasian
I3	56	IDC	2	-	Caucasian
I5	69	IDLC	2A	-	Caucasian
I8	46	IDC	1A	-	Caucasian

Table 3.2 Positive Control Sample Information. Relevant details provided by sample supplier (Capital Bioscience) n=5. All samples were taken from women suffering from BC of low to moderate stage.

Negative control samples were obtained from the Blood Transfusion Service and used to represent a healthy population. Thus their AGP glycosylation patterns are likely to mirror those of a healthy individual.

Sample	Blood Type
N1	A+
N2	B+
N3	AB+
N4	O+
N5	B+

Table 3.3 Negative Control Sample Information. Blood type of each sample provided for information n=5.

3.2 Dataset 2 Sample Demographics.

In addition to samples collected for dataset 1, a population of 50 women at increased risk of BC development were identified at a local High Risk Familial Breast Screening Clinic. 16 women agreed to participate in the study giving a recruitment rate of 32%. Table 3.4 shows the information collected from each participant providing various factors for analysis. Ages of participants ranged from 25-68 years old with a mean age ± standard deviation of 48.5 ± 11.8 with the majority of participants (93.75%, n=15) being classed as high risk due to family history and/or a genetic mutation. Within the sample set (n=16) half of the participants had confirmed BRCA1 or BRCA2 mutations (n=8), others (n=7) had no confirmed mutation but one participant had a BRCA1 mutation in the family

but had chosen not to be tested yet. The remaining participant (n=1) was considered high risk due to previous treatment for Hodgkin's lymphoma. A number of participants had opted for preventative surgical measures (n=9) which is reflected in the menopause column of Table 3.4. Further information including smoker status and BMI (calculated from height and weight data collected) are also shown in Table 3.4.

Sample	Age	Risk	Gene Mutation	BMI	Smoker	Prophylactic surgery	Pre/Post Menopausal
W1	47	High	BRCA1	28.2	N	Full Hysterectomy	Surgery
W2	68	High	BRCA 2	20.2	N	Full Hysterectomy	Post
W3	68	High	-	23.7	N	-	Post
W4	43	High	BRCA2	32.4	N	Oopherectomy	No HRT
W5	25	High	Not tested but BRCA1 in family	27.4	N	-	Pre
W6	42	High	BRCA2	20.9	N	Oopherectomy	No HRT
W7	47	High	Hodgkins lymphoma	26.1	N		Pre
W8	37	High	BRCA1	24	N	Mastectomy	Pre
W9	52	Mod	-	23	N	-	Peri
W10	45	High	-	24.8	N	-	Pre
W11	51	High	BRCA2	20.4	Y	Full Hysterectomy	Surgery
W12	43	High	-	25.2	N	-	Pre
W13	63	High	-	24.1	N	-	Post
W14	34	High	BRCA1	34.2	N	Full Hysterectomy	Surgery
W15	53	High	BRCA1	21.9	N	Oopherectomy	Surgery
W16	58	High	-	28.2	N	Full Hysterectomy	Surgery

Table 3.4 At-Risk Sample Demographics Details from data collection sheet completed by participants (n=16) at clinic appointment. Collected information includes age, BC risk status, genetic mutation (if any), height and weight to calculate BMI, smoker status, preventative surgery and menopausal status.

Sample	Age	BMI	Smoker	Pre/Post Menopausal
C1	47	22.4	**Y - not for 21 years**	Peri
C2	68	25.6	**Y – not for 33 years**	Post
C4	43	27.7	**Y - not for 8 years**	Peri
C5	25	25.6	**Y - not for 4 years**	Pre
C6	42	23.5	**No**	Pre
C7	47	28.5	**No**	Pre
C8	37	20.6	**Y - not for 8 years**	Pre
C11	51	32.5	**No**	Peri
C12	43	26.4	**N**	Pre
C13	63	29.3	**Y - not for 20 years**	Post

Table 3.5 Age-Matched Healthy Control Sample Demographics. Table provides demographic information about recruited age-matched control samples (n=10) for dataset 2.

3.3 Patient Demographic Discussion.

Current BC detection methods struggle to detect BC in its very early stages. Detecting BC as early as possible is particularly important for women at high risk of BC development as these women often develop more aggressive tumours at a younger age and would greatly benefit from earlier diagnosis that could be provided by a blood-based biomarker such as AGP. Earlier detection would increase treatment options and likelihood of survival.

Dataset 1 of this study investigated AGP glycosylation in a patient population with BC to examine AGP glycosylation during disease. The ideal sample population for phase 1 would have been high risk women with family history or BRCA1/BRCA2 mutations who had developed BC however, despite extensive searching this population was not accessible. Thus, with recent research indicating up to 80% of BRCA1 mutation carriers develop TNBC, samples from women of varying ages with TNBC with and without family history were obtained. Triple negative status was determined using IHC and All Red Assays at pathology laboratories in Canada as part of a clinical process. IHC is the staining of histological tissue samples using antibodies for the detection of various cellular components such as ER and PR. While IHC diagnostic thresholds differ between laboratories, there are suggestions that any result >1% should be considered positive for hormone receptors. The Allred system is a form of IHC results are given as a score from 0-8 calculated from percentage

of cells stained and intensity of staining. A higher score indicates greater presence of hormone receptors and high staining intensity. A TN result is given if no staining occurs (<1%) and an Allred score of 0 or 1 (depending on laboratory diagnostic protocols) (Gazinska*, et al.*, 2013; Oakman*, et al.*, 2010). All TNBC samples in Phase 1 were reported as testing negative for ER, PR and HER2 however the diagnostic thresholds used were not obtainable.

Within the dataset 1 sample population, approximately half (55.5%) had not undergone any treatment prior to blood draw. Of the remaining samples, various treatments had been employed. TAC, CMF and NTAC are different combinations of chemotherapy drugs Taxotere®, Adriamycin® (known generically as Docetaxel and Doxorubicin respectively), Cyclophosphamide, Methotrexate, and Fluorouracil. NTAC means chemotherapy was given prior to surgery in an attempt to reduce the size of the tumour. The treatments given to this sample group reflect that chemotherapy and RT are the treatments of choice for TNBC as hormone therapies such as Tamoxifen are not feasible due to lack of ER, PR and HER2. It has been shown previously by this research team that altered AGP glycosylation during BC returns to a normal glycosylation pattern upon treatment with Tamoxifen (Doak, 2008). Therefore, by choosing untreated and treated BC samples a comparison can be drawn to investigate differences in AGP glycosylation.

Dataset 1 samples were mainly of low TNM staging with n=8 stage 1, n=9 stage 2 and n=1 stage 3 and were all invasive ductal cancers. This was highly beneficial for assessment of AGP glycosylation as a detection biomarker because by choosing early stage TNBC, the AGP glycosylation patterns and composition were reflective of that during the beginnings of TNBC development.

Furthermore, Phase 1 samples were from TNBC sufferers with ages ranging from 22-81 giving a mean age of 53. Samples were equally separated into three age categories of ≤35, >35-60 and >60 years of age. As discussed in section 1.1.3, approximately 7% of BCs are diagnosed in women below the age of 40 and this figure may be rising. BC diagnosed in young women is more likely to be aggressive, of a high grade, TN and is associated with poor prognosis. Recent research has revealed distinct differences between BC in younger women compared to older women and each is thought to develop via different biological pathways (Anders*, et al.*, 2008; Anders*, et al.*, 2009). Recently, it has

been suggested, in the United States that women who develop BC below the age of 35 to be referred for genetic testing for BRCA1 of BRCA2 mutations due to the links discovered between early presentation of BC and mutations of these genes.

The positive control for dataset 1 was samples from women who had developed BC; no information was available on the molecular or histological subtype of the tumour (n=5). This was to determine whether changes in AGP glycosylation occurred differently between generic BC and samples known to be TN. Additionally the samples chosen to represent a positive control were all of low stage BC, again to provide a better representation of AGP glycosylation during early stage BC as well as enabling a better comparison with TNBC samples.

Blood samples donated by the Blood Transfusion Service were used to represent a healthy population as a negative control (n=5). No information other than blood type was provided for these samples however, individuals are screened prior to blood donation to assess their health status and suitability for donation. Therefore, it is highly likely that these samples were provided by healthy individuals and suitable for use as a negative control in this study.

Dataset 2 samples were collected in a case-control manner, where each case (a woman at increased risk of BC) was age-matched to a healthy control for comparison. This was to examine if altered AGP glycosylation in the at-risk population could be an indicator of BC development and opposing that whether similarities in AGP glycosylation to healthy control samples could indicate no BC development. As discussed in Chapter 1, women can be considered at increased risk of BC development for several reasons. In this study women were recruited from a High Risk Familial Breast Screening Clinic and those that chose to participate were at increased risk because of a known BRCA1 or BRCA2 mutation, strong family history of BC but no mutation detected or tested for and one participant was at increased risk due to previous treatment for Hodgkin's lymphoma which involves radiation to the chest area. A variety of ages were recruited ranging from 25 to 68 years of age with a mean age of 47 which is approaching the age that women at normal risk of BC would be sent for screening in Scotland. These women were all under increased surveillance as stated in the screening guidelines with some having had prophylactic surgery to aid BC prevention. None of the at-risk population were taking any type of

preventative drugs such as Tamoxifen. Unfortunately, no follow up of the “at risk” participants was possible because if BC is detected during screening they are referred to another clinic for treatment and no further information is provided to the screening clinic regarding their treatment or progression.

Use of an age-matched healthy control population allowed for a direct comparison to be drawn between AGP glycosylation in at-risk and not at-risk individuals increasing the likelihood that any changes in AGP glycosylation seen in the at-risk population was due to disease occurrence. Unfortunately due to a poor recruitment rate, not all age-matched controls could be recruited for this study. However, of the participants recruited, there was variation in ages and pre/peri/post-menopausal status. The age of participants ranged from 25-68 years and the mean age was 46 which was very similar to that of the at-risk population recruited. Therefore, despite not all age-matched samples being successfully recruited, a sufficient variation of control samples were collected. Smoking status was recorded as inflammation of the lungs due to smoke inhalation could alter AGP glycosylation producing a false result.

Dataset 2 of the study required individuals to self-select to participate which removed any bias from the investigator but samples could still be biased in terms of other factors such as age. Participants were also responsible for reporting whether they met any of the exclusion criteria listed. Participants may have had undiagnosed conditions that could result in altered AGP glycosylation and thus affect results of the study.

Overall, it was considered that the samples recruited for both datasets would be able to effectively test the hypothesis and aims of the study.

Chapter 4 – AGP Isolation and Level Determination

4.1 AGP Isolation.

This study used an adapted version of Smith*, et al.* (1994) for the isolation of AGP from blood samples. This utilised a low pressure chromatography procedure to isolate AGP which preserved the structural integrity of both the protein and glycans.

Samples were precipitated with PEG 3350 and the supernatant collected prior to further purification using low pressure chromatography. Sample supernatants were applied to a Cibacron Blue 3GA Sepharose low pressure chromatography column which binds HSA and the fraction containing AGP was collected when a peak was illustrated by the chart recorder approximately 5 minutes (according to defined operating conditions) after addition of a sample to the column (Figure 4.1 A). Eluted fractions were collected until baseline absorbance was reached. The column was then regenerated for approximately 20 minutes by the addition of desorption buffer to remove HSA bound to the Cibacron Blue 3GA Sepharose resin. The eluted fraction was concentrated before application to a Red Sepharose CL6B to further remove impurities including AAT. Approximately 10 minutes after sample application the AGP fraction was eluted and collected when a peak was illustrated by the chart recorder (Figure 4.1 B). Again, the column was regenerated using a desorption buffer.

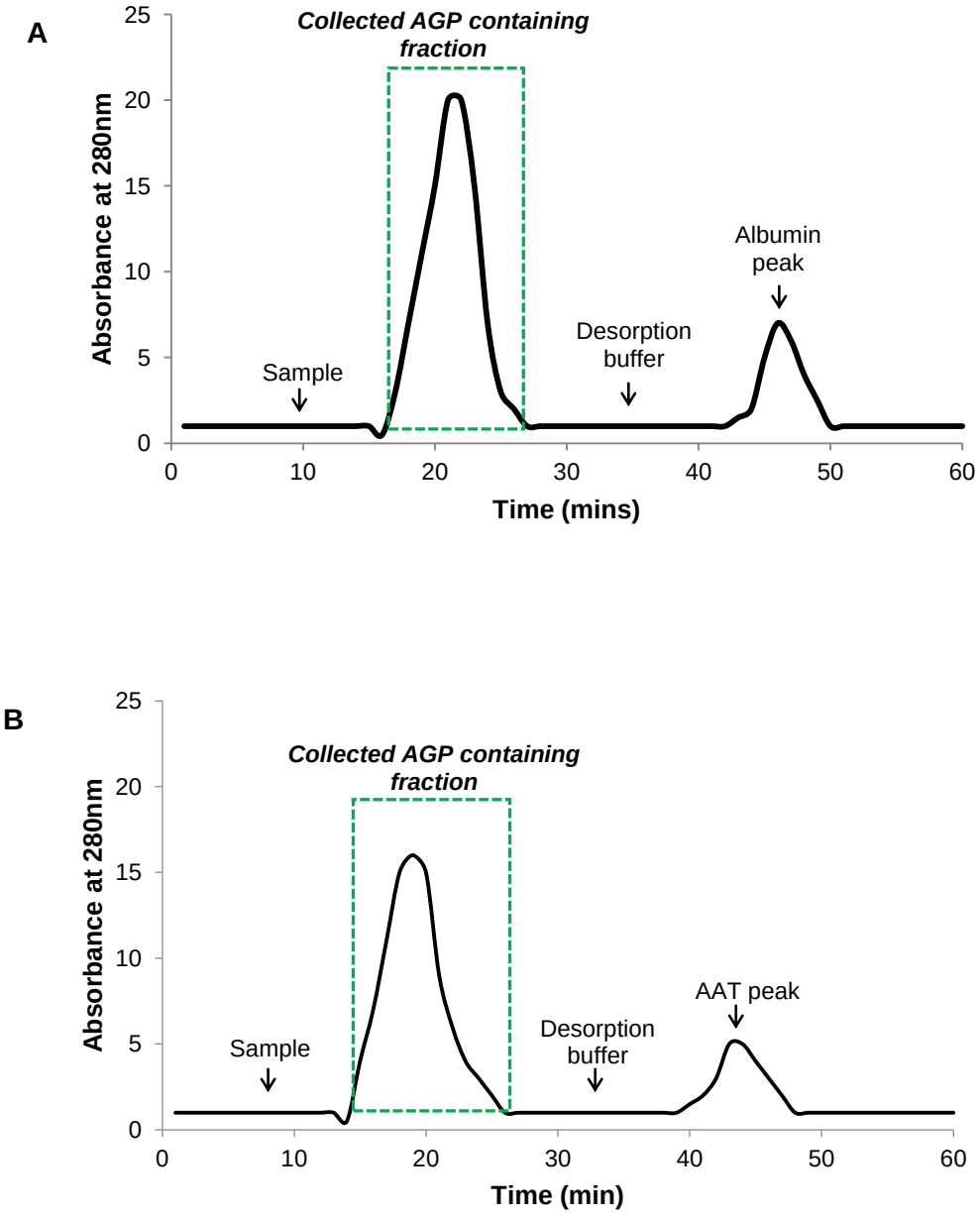


Figure 4.1 Low Pressure Chromatography Chromatograms Produced During AGP Isolation A) Cibacron Blue 3GA Sepharose B) Red Sepharose CL6B

Following isolation of AGP all samples were subjected to desalting using Centricon filter devices to prevent sample contamination and remove excess salts that could interfere with absorbance readings at 280nm, thus altering AGP concentration calculated from those results.

AGP purity and effectiveness of the isolation technique stated above were proved using SDS-PAGE as shown in Figure 4.2.

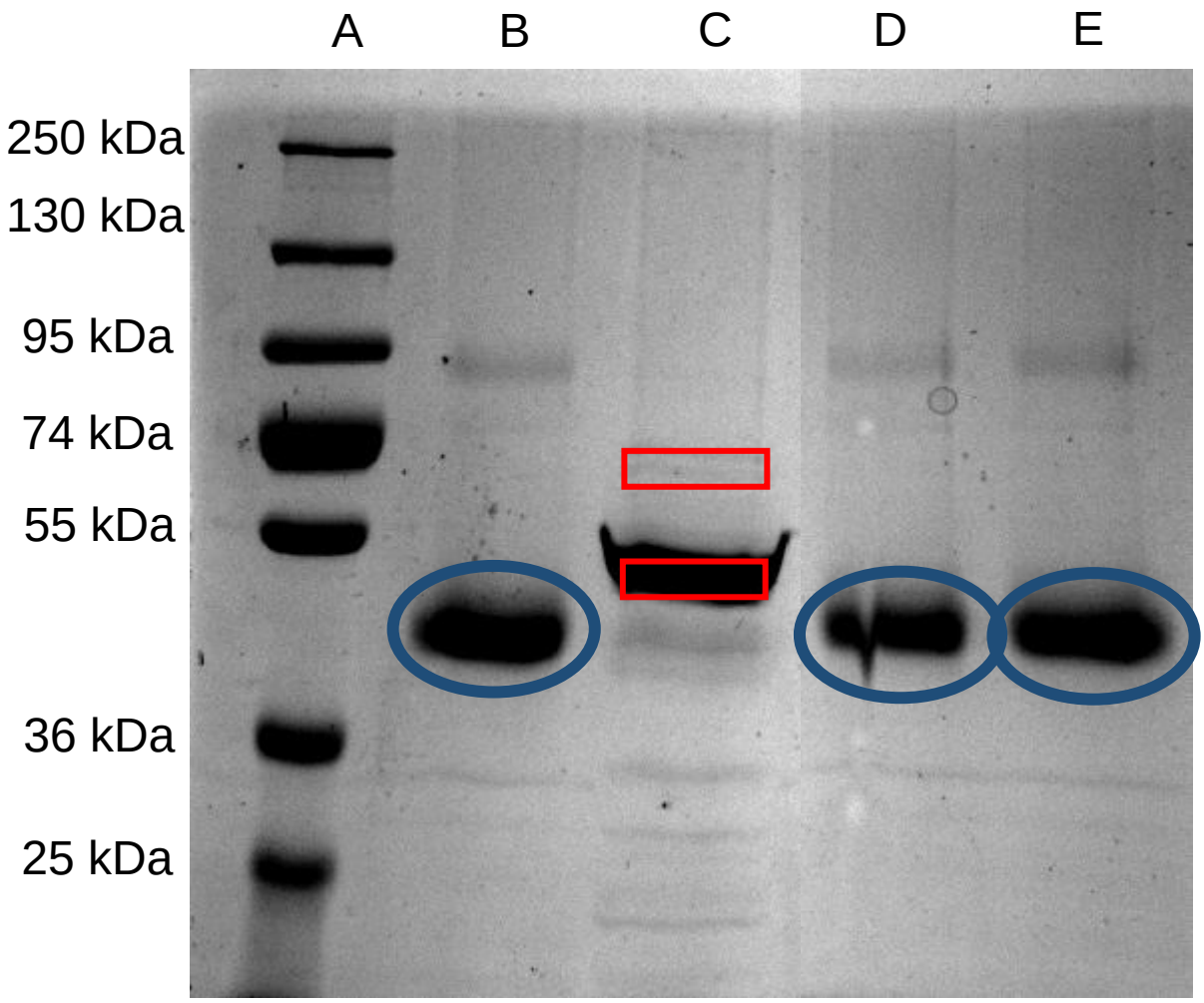


Figure 4.2 SDS-PAGE Gel Proving AGP Purification. Gel shows protein molecular mass marker ladder (lane A), commercially available AGP (lane B), PEG-precipitated plasma (lane C) and two samples that have undergone the isolation technique (lanes D and E). Purified AGP is highlighted using a ⬭ while bands likely to be HSA and AAT are indicated by a ▭ .

The SDS-PAGE gel showed AGP had been isolated to the same standard as commercially available AGP and that unpurified plasma (lane C) contains several impurities that have been removed from the isolated AGP samples. The main impurities removed by the isolation technique are HSA (67kDa) and AAT (52kDa).

4.2 Calculation of Sample AGP Concentration.

A calibration curve of absorbance values of commercial AGP ranging from 0.0625-1.5 mg/ml at 280nm was used to produce a line of best fit (Figure 4.3) with equation of $y = 0.5663 x$ ($R^2 = 0.99$). Isolated concentrations of AGP from normal blood samples obtained from the blood transfusion service (negative

control; n=4), samples from individuals with varying degrees of BC (positive control; n=5), and samples from individuals with TNBC (n=18) with or without family history as well and the means of each group are shown in Table 4.1.

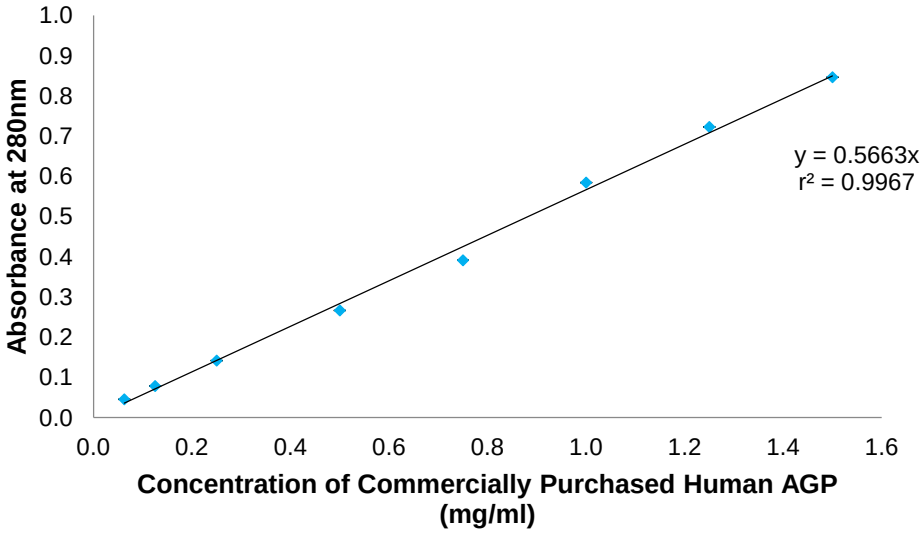


Figure 4.3 Plot of Variation of Absorbance with Concentration of Commercially Purchased Human AGP (n=3). R^2>0.9. Relative amount of AGP in a sample (x): x = Absorbance at 280nm/ 0.5663.

4.3 Dataset 1 AGP Concentration.

Using the equation shown in Figure 4.3 AGP concentrations from dataset 1 were determined as shown below in Table 4.1.

Sample	Concentration (µg/ml)	Level Compared to Mean Healthy AGP
N1	178	-
N2	331	-
N3	16	-
N4	68	-
N5	234	-
Negative Control Mean ± SD	**165.5 ± 126.5**	
Breast Cancer H8	4156	+ 4007.7
Breast Cancer I2	4417	+ 4268.7
Breast Cancer I3	536	+ 387.7
Breast Cancer I5	161	+ 12.7
Breast Cancer I8	137	- 11.2
Positive Control Mean ± SD	**1881.4 ± 2203.1**	
MT 3965	474.1	+ 325.8
MT 3625	280.2	+ 131.9
MT 2305	544.6	+ 396.3
CT 79	1073.5	+ 925.2
F 5412	579.9	+ 431.6
MT 2621	333.1	+183.0
TNBC Under 35 Mean ± SD	**547.5 ± 316.4**	
MT 1177	562.3	+ 414.0
MT 1178	474.1	+ 325.8
MT 3601	685.7	+ 537.4
MT 3795	421.2	+ 272.9
MT 1937	650.4	+ 502.1
MT 1106	403.6	+ 255.3
TNBC 35-60 Mean ± SD	**532.9 ± 128.9**	
MT 3352	262.6	+ 114.3
MT 3497	791.4	+ 643.1
MT 3442	685.7	+ 537.4
MT 3362	579.9	+ 431.6
MT 3959	456.5	+ 308.2
MT 2569	315.5	+ 167.2
TNBC 60 and Over Mean ± SD	**515.3 ± 230.5**	

Table 4.1 Summary of Isolated AGP Concentrations. AGP concentrations of BC (n=5), healthy (n=5) and TNBC (n=18) samples are given as µg/ml with the difference between each sample and the mean ± SD AGP concentration of healthy samples provided in a separate column.

All TNBC samples showed AGP concentrations elevated above mean healthy controls, with over 80% having over double the mean healthy AGP concentration. AGP concentration of TNBC samples was comparable across all age categories with only a small decline in mean concentration with increasing

age. All except one of the BC positive control samples had AGP concentrations above that of the mean healthy controls. Samples H8, I2 and I3 were greatly increased above mean healthy control concentration whereas sample I5 was comparable to the mean heathy controls and sample I8 revealed a slightly lower AGP concentration. As only one repeat of TNBC samples was possible due to small sample volume, mean AGP concentration values were calculated for age category for comparison with each control (BC and healthy) and are shown below in Figure 4.4.

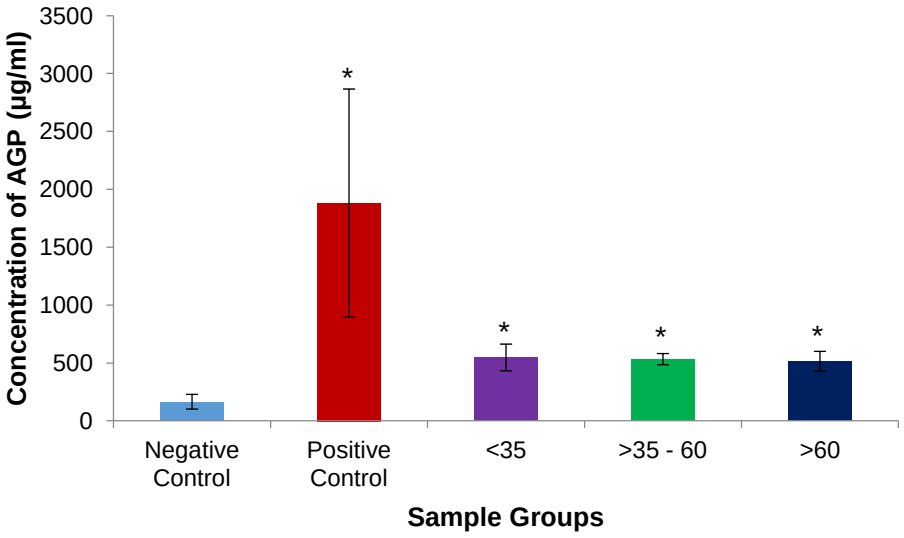


Figure 4.4 **Plot of Variation of Mean Isolated AGP Concentrations** from all sample groups (n=3), significance at $p<0.05$ indicated by *.

Mean concentrations of isolated AGP from normal blood samples was significantly lower ($p<0.05$) than isolated AGP from both BC samples (positive controls) and samples from individuals with TNBC with/without family history. Similarly, there was a significant difference between all TNBC groups and positive control. There was no significant variation between mean AGP concentrations in TNBC groups ($p<0.05$) when a one-way analysis of variance (ANOVA) was used although a slight decrease in concentration with increasing age was noted.

4.4 Dataset 2 AGP Concentration.

Sample	At Risk (µg/ml)	Age Matched Healthy Control (µg/ml)	Difference Between "At Risk" Sample and Healthy Control (µg/ml)
1	455.5	243.6	+ 211.9
2	441.5	305.5	+ 136.0
3	384.9	-	+ 111.2 *
4	443.2	183.6	+ 259.6
5	471.4	356.7	+ 114.7
6	517.3	303.7	+ 213.6
7	342.6	247.2	+ 95.4
8	226.0	151.8	+ 74.2
9	459.1	-	+ 185.4 *
10	799.9	-	+ 526.2 *
11	420.2	499.7	- 79.5
12	706.3	220.7	+ 485.6
13	148.3	224.2	- 75.9
14	148.3	-	- 125.4 *
15	287.8	-	+ 14.1 *
16	277.2	-	+ 3.5 *
Mean ± SD	**408.1 ± 196.5**	**273.7 ± 99.8**	

Table 4.2 Summary of Isolated AGP Concentrations of At-Risk Samples Compared to Age-Matched Healthy Control Samples. AGP concentrations of "at risk" (n=16) and healthy controls (n=10) are given as µg/ml. Where comparison to an age-matched control was not possible, a comparison was drawn between the mean healthy AGP concentration (273.7µg/ml) and indicated by an *.

The majority of "at risk" samples (87.5%) revealed increased AGP concentrations compared to age-matched healthy controls or mean AGP concentration of healthy controls. However, samples W11 and W13 showed AGP concentrations below that of healthy controls while samples W15 and W16 were only marginally elevated above healthy control concentrations. Sample W10 had the highest AGP concentration at 799.9µg/ml closely followed by W12 at 706.3µg/ml, and samples W13 and W14 both had the lowest AGP concentration of 148.3µg/ml. Of the age-matched healthy controls C11 possessed highest AGP concentration of 499.7µg.ml with C8 having the lowest at 151.8µg/ml.

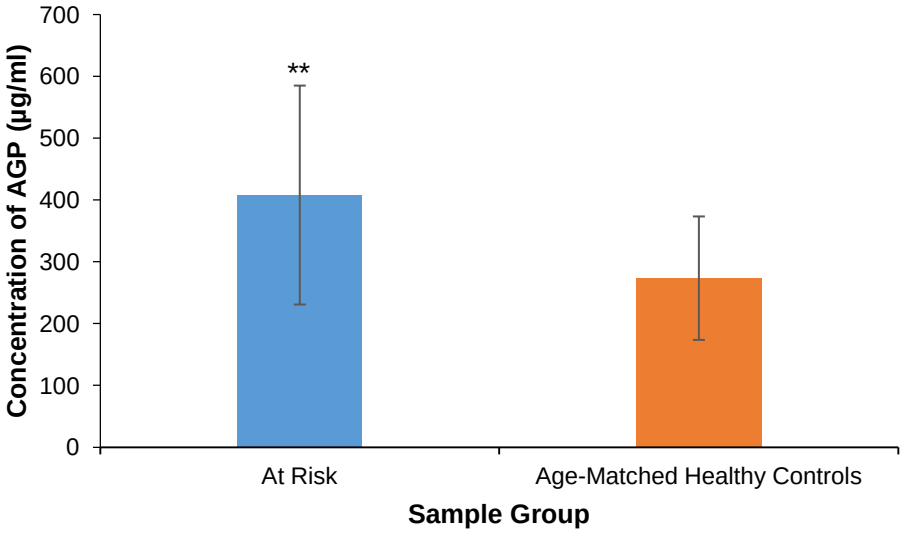


Figure 4.5 Mean AGP Concentration of "At Risk" and Age-Matched Healthy Control Samples. Mean AGP concentration of "at risk" (n=16) and healthy control (n=10) samples is given in µg/ml. Data was gathered in triplicate and calculated using the equation in Figure 4.3 as above. Statistical significance of $p<0.01$ indicated by **.

Use of a one-way ANOVA determined a significant difference between mean AGP concentrations of "at risk" samples and healthy controls ($p<0.01$) with the "at risk" population having significantly elevated AGP levels.

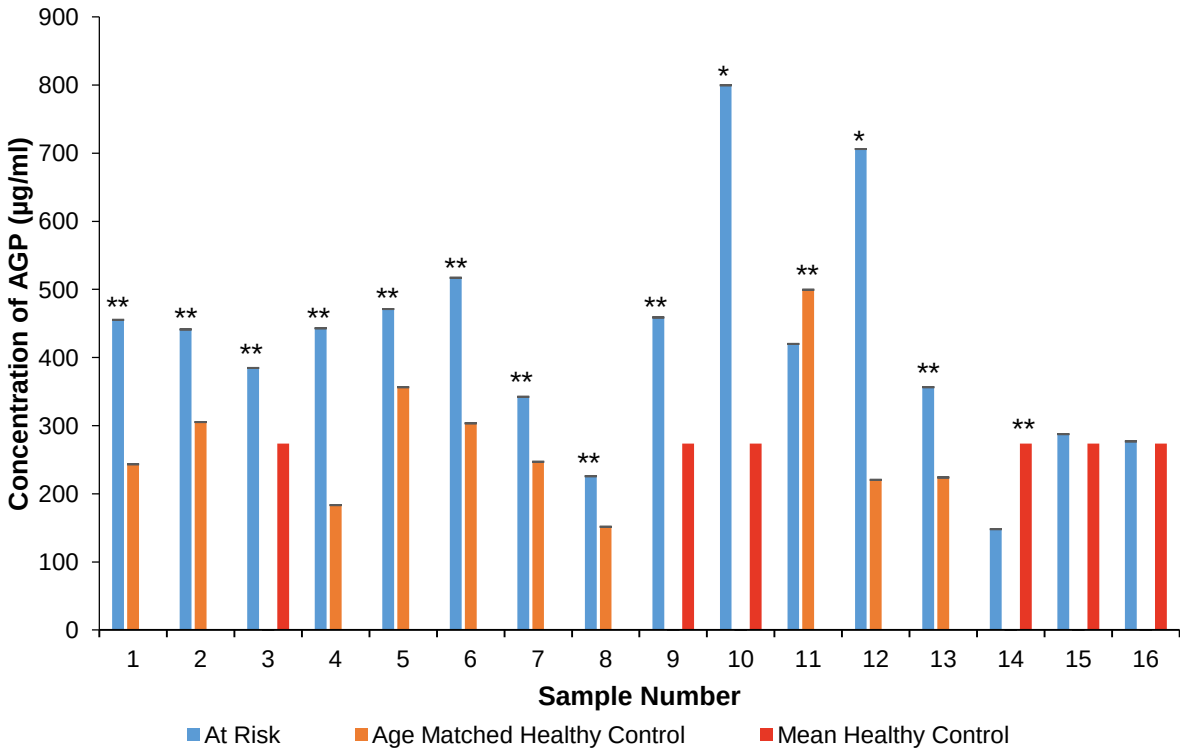


Figure 4.6 Isolated AGP Levels of At-Risk Samples Compared to Age-Matched Healthy Controls or Mean Healthy Control. Graph shows a direct comparison between AGP concentrations from "at risk" sample and an age-matched healthy control. Where an age-matched healthy control was not possible, "at risk" samples are compared to the mean AGP concentration of the healthy control population. Significant differences are indicated as * for $p<0.05$ and ** for $p<0.01$

.

The majority of "at risk" samples (12/16) showed significantly higher levels of AGP ($p<0.01$; W10 and W12 $p<0.05$) compared to controls, However, W11 and W14 had significantly lower AGP levels and W15 and W16 showed no significant difference. As mentioned previously, absorbance measurements were taken in triplicate and used to calculate AGP concentration with little variation displayed between readings as shown by small error bars in Figure 4.6. Overall AGP concentrations of the "at risk" population show a 1.4 fold increase compared to the healthy control population.

4.5 Discussion.

4.5.1 Purification of AGP.

The aim of the research discussed in this chapter was to confirm purification of AGP and determine concentrations of isolated AGP to assess the variation in levels from two sample populations - Dataset 1 included of samples from

healthy individuals, women with BC of unknown type and women with TNBC as well as Dataset 2 containing samples from women at increased risk of BC development and age-matched healthy controls. This was performed using low pressure chromatographic isolation of AGP, desalting, SDS-PAGE and spectrophotometry.

The isolation of plasma proteins from whole blood was once a problematic and laborious task, due to the presence of large amounts of HSA in plasma (approximately 50%) (Travis *et al*., 1976). HSA was difficult to remove completely as it has a similar molecular mass and charge to other plasma proteins, therefore effective removal techniques are essential (Travis *et al*., 1976).

There are several protocols of AGP isolation available, most of which exploit AGP's low pI or extremely high solubility (Chan and Yu., Charlwood *et al*., 1976; Hellerstein *et al*., 1985; 1991; Sugiyama *et al*., 1985; Routledge *et al*., 1980 and Weimer *et al*., 1950). However, the majority use harsh acidic conditions that will affect the structural integrity of glycans bound to AGP which would affect investigation of glycosylation. For instance, an initial AGP isolation method used by Weimer*, et al.* (1950) used ammonium sulphate to precipitate AGP at low pH. Low pH could result in glycan structure alterations such as desialylation (Varki, *et al*., 2009). Other methods of AGP isolation include hot phenol extraction (Chan and Yu, 1991) but this was shown to be ineffective as AGP purified in this way was more readily cleared upon injection into mice which indicated a change in glycan structure such as desialylation (McCurdy*, et al.*, 2011).

Another method was proposed by Herve*, et al.* (1996), who created a two-step purification of AGP from plasma comparable with the protocol of Smith, *et al.* (1994).

The method utilised in this study was an adapted form of Smith and colleagues (1994) protocol which in turn adapted Laurent*, et al.* (1984) method using PEG 3350 and low pressure affinity chromatography to isolate AGP without denaturation or desialylation. Thus allowing AGP's *in vivo* glycan structure to be analysed for unique characteristics that could indicate presence of BC. Previous studies by Doak (2008) and Gallacher (2009) have shown this AGP isolation

method is suitable for both fresh and frozen samples as AGP remains stable. Subsequently methods such as Herve, *et al*. (1996) were comparable with Smith, *et al*. (1994).

Initial addition of PEG 3350 to samples at 40% w/v enables precipitation of proteins less soluble than AGP and blood cells, leaving AGP and other major plasma constituents such as HSA and AAT (Smith, *et al*. 1994). PEG 3350 functions by drawing water molecules away from proteins in the suspension, increasing the number of protein to protein interactions in the plasma. Proteins can no longer dissolve when these interactions become abundant leading them to precipitate (Ingham, 1990). The main advantages of PEG 3350 are two-fold: firstly, PEG 3350 does not denature the proteins it interacts with, preserving the *in vivo* nature of AGP when used. This is imperative when performing a structural analysis such as this study. Secondly, PEG 3350 removes a number of potentially interfering plasma components in one simple step (Smith, *et al*., 1994) as shown in Figure 4.2. However, after centrifugation, HSA and AAT remain in the removed supernatant, both of which can interfere with AGP analysis and thus require removal.

This study employed low pressure affinity chromatography in the form of a Cibracron Blue Sepharose 3GA column which facilitated the removal of HSA from samples, further isolating AGP. As shown in Figure 4.1a, after addition of a sample to the column, the first fraction containing AGP eluted at approximately 15 minutes, represented by a peak deviating from the baseline. The addition of a desorption buffer (see Table 2.1) resulted in column-bound HSA eluting from the column shown as a second peak on the chromatogram. Complete regeneration of the column occurred within 20 minutes.

Use of Cibacron Blue for removal of HSA has been recognised for several years with Travis*, et al.* (1976) reporting removal of up to 98% HSA when using a Cibacron blue column. HSA possesses bilirubin binding sites and as Cibacron Blue dye is similar to bilirubin as it contains negatively charged planar aromatic ring systems, HSA binds to the dye (Leatherbarrow and Dean, 1980). Cibacron Blue dye works well for this study as it removes HSA allowing the remaining plasma constituents to pass through the column unhindered.

The original method used by Smith, *et al.* (1994) contained a Q-sepharose step to further isolate AGP after removal of HSA describe previously. However, the method was designed for larger sample volumes (50ml) which could afford the loss of sample at this stage but due to small sample quantity the step was removed to preserve sample volume. Removal of this step has been shown to have little effect on AGP purity by members of the research team through immunodiffusion and SDS-PAGE (Behan, 2010; Gallacher, 2009). Therefore, the next stage of AGP isolation was elimination of AAT from samples.

For removal of AAT, concentrated samples from the previous step were applied to a Red Sepharose CL-6B column; a peak indicating sample elution from the column was seen between 13-25 minutes and collected as before. Application of a desorption buffer to the column resulted in a second peak illustrating the elution of AAT previously bound to the column. Whilst in the column, AAT and proteins such as kinases, dehydrogenases and transferases bind to the resin. However, AGP is so heavily glycosylated there is not enough of the protein portion available to facilitate binding, allowing AGP to pass through the column unimpeded. The eluted sample fraction now contained only purified AGP as shown in Smith, *et al.* (1994) study by the appearance of a single band on SDS-PAGE at the 45kDa mark and detection of only AGP through immunodiffusion. The current study confirmed the successful isolation of AGP using SDS-PAGE, which in figure 4.2 shows a single band at approximately 45kDa in line with previous investigations Smith, *et al.* (1994).

4.5.1.1 Potential Problems with Purification Method

The purification method described in section 2.2.2 successfully purified AGP from whole blood and plasma samples to facilitate the current study. However, issues did arise that could be improved in the future. Firstly, due to small sample volumes there was limited unused sample to perform techniques such as SDS-PAGE or lectin ELISA's to confirm AGP purification. Therefore, use of this purification method in subsequent studies would benefit from larger initial sample volumes of approximately 3-4ml. Another potential problem with this purification method was that the same Cibracron Blue Sepharose 3GA and Red Sepharose CL-6B columns were used for purification of all samples used in this

study. Despite rigorous cleaning of the columns with desorption buffers after application of each sample, sample remnants may have remained in the column which could have resulted in cross-contamination. However, use of a freshly prepared column for each sample to avoid cross-contamination entirely would have proved costly and so was not feasible for this study. Additionally, due to the introduction of salts, from use of a NaOAc buffer during the isolation process, it was necessary to filter isolated samples using Centricon filter devices with a 10kDa molecular weight cut off. Presence of salt in isolated AGP samples would interfere with absorbance readings at 280nm, as both NaOAc and AGP absorb at this wavelength, which would subsequently affect calculation of AGP total mass in samples. Although desalting of isolated AGP samples was performed there was no way to ensure all salt had been removed which could have falsely elevated AGP concentrations measure spectrophotometrically.

Despite the potential problems with the purification method it is unlikely that there was any negative implications for AGP glycosylation as several studies have successfully utilised this purification methods for analysis of AGP glycan content without detriment to glycosylation (Behan., 2010; Behan and Smith., 2011; Behan, *et al*., 2013; Doak., 2008; Gallacher., 2009; Smith *et al*., 1997; Smith *et al*., 2002).

4.5.2 Determination of Isolated AGP Levels.

AGP levels were determined after purification through a standard curve created using varying concentrations of commercially available AGP. Phe, Tyr and Trp residues present in AGP's protein backbone absorb at 280nm, the protein backbone is not distorted by isolation techniques used making the use of spectrophotometry more reliable (Smith *et al*.,1994; Behan., 2010).

A standard curve was constructed using concentrations of commercial AGP at 0.0625-1.5 mg/ml as these are similar to *in vivo* AGP levels and gave absorbance readings <1. The linearity of the Beer Lambert equation breaks down with absorbance values above 1.0 indicating that the sample is too saturated to give a dependable result (Williams and Fleming, 2007). There is

good correlation between commercial AGP concentration and absorbance at 280nm with an r^2 value of 0.99.

4.5.3 Alternative Supportive Methodologies.

Sample volume and funding permitting, additional methodologies exist that may have supported the purification method used in this study to aid the confirmation of results and enhance the outcomes. Efficiency of the low pressure chromatography columns used could have been verified through the use of plasma samples spiked with known concentrations of AGP to measure the percentage of AGP recovered from the column. SDS-PAGE of all fractions after application to each low pressure chromatography column would have evidenced the removal of different unwanted sample components by each column as opposed to the before and after purification samples shown in figure 2.4. Western Blotting of SDS-PAGE gels using AGP specific antibodies could have confirmed purification of AGP (Behan, 2010). Use of a Bradford assay (Bradford, 1976) to determine total protein concentration or a sandwich ELISA incorporating antihuman AGP and antihuman AGP conjugated with horseradish peroxidase could have been utilised (Hashimoto, 2004) to measure AGP concentration (McCurdy *et al*., 2011) and could have been used to corroborate calculated AGP concentrations from absorbance measurements and improve the reliability of results. However, this would have reduced the already limited sample volume available for this study.

4.5.4 Variation of Isolated AGP Content in Healthy Individuals.

Isolated AGP from healthy control samples in dataset 1 ranged from 16-331µg/ml with a mean ± SD of 165.5 ± 126.5µg/ml indicating large variation of isolated AGP levels in samples from healthy individuals within this dataset. However, levels of isolated AGP from age-matched healthy controls in dataset 2 were more consistent, ranging from 151.8-499.7µg/ml with a mean ± SD of 273.7 ± 99.8 µg/ml. Differences in isolate AGP levels between healthy controls of both datasets could be due to the way samples were stored and the length of storage prior to use. Healthy control samples for dataset 1 were stored at in a freezer at -20^0 C for several months prior to purification whereas samples from

age-matched healthy controls used in dataset 2 were collected fresh and the purification process started immediately without prior freezing. Therefore it is possible that degradation of AGP occurred while healthy samples from dataset 1 were stored at -20^0 C as proteins should be stored at -80^0 C to prevent degradation (Saraswathy and Ramalingam, 2011). Degradation could also account for the large disparities between healthy control samples in dataset 1 depending on length of storage. Additionally, as AGP concentration is known to increase during inflammation and several diseases (Cecilani and Pocacqua, 2007) asymptomatic volunteers may have considered themselves healthy but been unaware of having pathological conditions that could have falsely elevated levels of AGP isolated from samples used as healthy controls and increased variation between samples.

Interestingly, studies by Kishino *et al*., (2002) and Orzyck-Powiiowicz (2006) revealed that gender may influence AGP levels in healthy individuals in that men may have higher concentrations of AGP than women and that AGP content can vary throughout a woman's menstrual cycle. As no gender information was supplied with healthy samples for dataset 1, variation in isolated AGP levels could be due to a mixture of male and female samples being supplied by the Blood Transfusion Service for this study.

Previous studies have shown the mean concentration of AGP in healthy participants to range between 360-1460µg/ml (Blain*, et al.*, 1985; Jørgensen*, et al.*, 2002). However, earlier research using an identical purification method has shown mean levels of AGP in healthy populations at 53µg/ml, 78µg/ml and 830µg/ml (Behan, 2010; Doak, 2008; Smith *et al*., 2002). In this study, mean isolated AGP levels of healthy participants were marginally lower than the majority of previously published results. This may be because samples were obtained from the Blood Transfusion which implements a strict health criterion prior to blood donation, leading to very low AGP concentrations. However, it is more likely that some sample loss has occurred during the purification process or ineffective sample storage.

4.5.5 Isolated AGP Levels from Dataset 1.

Isolated levels of AGP from all sample groups in dataset 1 are shown in table 4.1 and were calculated using the standard curve in figure 4.3 which revealed that mean AGP levels in a healthy population (165.5 ± 126.5µg/ml) were significantly lower than those of both BC samples (positive control; 1881.4 ± 2203.1µg/ml) and TNBC samples regardless of age (547.5 ± 316.4; 532.8 ± 128.9 and 515.2 ± 230.5µg/ml respectively).

4.5.5.1 Isolated AGP Levels of BC Positive Control Samples

In comparison to healthy controls used in this study, BC samples acting as positive controls had a significant ($p<0.05$) 11 fold greater mean concentration. However, research by Blain*, et al.*, (1985), Jørgensen*, et al.*, (2002) and Smith *et al*., (2002) showed AGP levels of 360-1460µg/ml isolated from healthy individuals. Therefore, if applying this healthy AGP reference range to BC samples in this study, only two out of five samples (H8 and I2) had AGP concentrations above this range, both showing an approximate 3 fold increase. The elevation of isolated AGP in H8 and I2 correlates with results from a study by Rudman*, et al.* (1974) which investigated AGP in various disease states and showed a 2-5 fold increase in AGP concentration in the course of cancer. Furthermore, the results for H8 and I2 connect well to research that illustrates plasma AGP concentration in BC being 2.5 times higher than healthy controls (Duché*, et al.*, 2000), while Twining and Brecher (1977) illustrated a 35.8% increase in AGP concentration in BC sufferers compared to healthy individuals.

However, the findings of this study do not show association an with research conducted by Roberts *et al*., (1975) which highlighted a correlation between increasing plasma AGP concentration and increasing severity of BC, as the remaining BC samples from the current study (I3, I5 and I8) have isolated AGP levels within or below the range indicated by Blain*, et al.*, (1985), Jørgensen*, et al.*, (2002) and Smith *et al*., (2002). All positive control BC samples were from women with low grade tumours of 1A to 2A using TNM staging. Therefore, given the results from the study by Roberts *et al*., (1975), it was expected that all positive control BC samples would contain similar levels of isolated AGP

after purification. As discussed in section 4.3.5, reasons for this unexpected variation could be due to storage of samples prior to purification. BC samples used as positive controls for this study were purchased in advance of the current research study beginning and were kept frozen at -20^0 C which may have caused degradation of AGP, subsequently affecting the detected concentration after purification (Saraswathy and Ramalingam, 2011).

4.5.5.2 Isolated AGP Levels of TNBC Samples.

AGP levels isolated from TNBC samples ranged from 262.6-791µg/ml with mean concentrations of 547.5 ± 316.4; 532.8 ± 128.9 and 515.2 ± 230.5µg/ml when grouped by age as <35, 35- 60 and >60 years old respectively. All TNBC samples contained significantly ($p<0.05$) elevated levels of isolated AGP of approximately a 3 fold difference compared to healthy control samples. To date no other studies have investigated AGP concentration in TNBC. Yet, these increases in AGP concentration are similar to those published by Rudman *et al*., (1974) and Duche *et al*., (2000) who found AGP levels rose by 2-5 fold during cancer and 2.5 fold in BC specifically. However, if relating these results to isolated AGP levels from healthy individuals in other studies (Blain*, et al.*, 1985, Jørgensen*, et al.*, 2002 and Smith *et al*., 2002), AGP concentrations from TNBC samples lie below or within healthy AGP concentration ranges. Yet, unlike the healthy and BC control samples, lower levels of isolated AGP are unlikely to be due to degradation of the protein during storage. This is because samples were stored at -80^0 C upon collection at the facility in Canada and transported on ice within seven days to Edinburgh Napier University where the purification process began immediately upon reciept of samples. While the possibility of sample loss during purification could be attributed to lower levels of AGP being isolated from these samples, recovery experiments were not performed prior to sample purification it so remains unclear whether sample loss was a factor.

Interestingly, despite elevation of AGP concentration in the TNBC samples being equivalent to previous studies compared to healthy controls, isolated AGP levels from these samples were significantly ($p<0.05$) lower than BC positive controls. Postive controls contained 3.5 fold greater AGP concentration than TNBC samples. All TNBC samples were from patients with low TNM stage

tumours (1-2B) except MT3352 which was stage 3B. This indicated an initial difference between TNBC and BC of an unknown type and links to research by Perou *et al*., (2000) suggesting that perhaps BC subtypes should be considered separate diseases due to the different molecular mechanisms involved in their development. If TNBC tumours are secreting different levels of AGP than other BC subtypes this could be used to further differentiate between BC subtypes and aid early diagnosis.

While mean levels throughout the TNBC age groups were consistent showing no significant differences, standard deviations highlighted variance within the groups. Eight patients with TNBC who donated blood samples underwent chemotherapy prior to blood draw. This may have accounted for variation in AGP concentrations in samples as AGP concentration has been shown to return to healthy, pre-cancer levels upon treatment (Kailajarva *et al*., 2000). However, this hypothesis could only be attributed to TNBC samples from women <35 years old as low AGP levels corresponded with patients who had received treatment prior to blood draw while those who had not received treatment had higher levels of AGP. This may account for the large variation between samples in this group (indicated by a SD of 316.4). In the other age groups there appeared to be no correlation between treatment and AGP levels. If examining AGP concentrations in TNBC samples by stage there also appeared to be no links between TNM stage and AGP concentration. This contradicts findings by Roberts *et al*., (1975). For example MT3352 was TNM stage 3B but contained 262.6µg/ml isolated AGP and CT79 was TNM stage 1 yet had an isolated AGP concentration of 1073.5µg/ml.

4.5.6 Isolated AGP Levels from Dataset 2.

Dataset 2 was comprised of samples from a population at increased risk of BC development in comparison to samples from age-matched healthy controls. Where age-matched controls could not be recruited, comparisons were drawn using the mean AGP concentration of the age-matched healthy controls which in this dataset was 273.7 ± 99.8µg/ml. This is lower than the range of 360-1460µg/ml found in previous studies by Blain, *et al.* (1985) and Jorgensen, *et al.*

(2002) as well as being similar to levels isolated previously by this research group (Behan, 2010; Doak, 2008; Smith *et al*., 2002).

As this study was comparing two healthy populations, the only difference being that one population was it increased risk of BC, it was expected that no differences would be found in terms of AGP concentration. However, mean AGP levels isolated from “at risk” patients were significantly ($p<0.01$) increased compared to age-matched healthy controls .Similarly if examining “at risk” samples compared to their age-matched healthy control individually, 87.5% contained significantly (majority $p<0.01$, 2 samples $p<0.05$) elevated levels of isolated AGP. As with TNBC samples in dataset 1 isolated AGP levels from the ”at risk” population were within the normal range reported by previous studies (Blain, *et al.* 1985; Jorgensen, *et al.* 2002; Behan, 2010; Doak, 2008; Smith *et al*., 2002). Yet the majority of “at risk” sample showed at least a 1.3 fold rise in AGP concentration compared to age-matched controls which is not as high as the 2-5 fold or 2.5 fold increases reported by Rudman *et al*., (1974) and Duche *et al*., (2000) during cancer or seen in TNBC samples from dataset 1, but an increase in AGP concentration could still indicate the early stages of BC development. Relating to this, AGP is known to possess immunomodulatory functions (see Table 1.5) and increases in AGP concentration are associated with decreased proliferation of lymphocytes (Chiu *et al*., 1977), decreased lymphocytic secretion of IL-2 (Elg *et al*., 1997) which aids differentiation of self and non-self by the immune system (Liao *et al*., 2011) and preliminary data gathered by Zsila *et al*., (2009) demonstrates that increased AGP concentration may activate cell proliferation. Therefore, the small increases in AGP concentration in “at-risk” samples compared to age-matched healthy controls may be a result of early metastatic breast cells creating an immune suppressed micro-environment to enhance tumour development and survival. Furthermore, several studies have already shown AGP’s non-specific immunosuppressive actions during cancer may promote immune evasion and lower the effectiveness of immunotherapy based treatments (Tamura *et al*., 1981; Samal *et al*., 1982 and Elg *et al*., 1997). Yet in contrast, a study conducted in 1986 by Watanabe *et al*., implicated AGP expression with inhibition of tumour growth. However, as AGP protein synthesis and glycosylation are independently regulated (van Dijk *et al*., 1994 and van Dijk *et al*., 1995) it is possible that

expression of altered AGP glycosylation was responsible for tumour inhibition rather than increased AGP concentration.

Furthermore, previous research within this group by Doak (2008) examined untreated BC, tamoxifen treated BC and “at risk” individuals receiving tamoxifen as a preventative measure. Doak’s study revealed that an “at risk” population receiving tamoxifen had similar AGP levels to a “normal” healthy population (660µg/ml compared to 530µg/ml respectively). None of the current study’s “at risk” participants were receiving tamoxifen and the majority had AGP concentrations similar to or lower than “at risk” tamoxifen recipients in Doak's (2008) study. Therefore, it would appear that tamoxifen treatment has little effect on AGP levels in an “at risk” population. However, no information on why Doak’s “at risk” population were considered to be at risk of BC development was provided while the current study contained women who were “at risk” due to confirmed family history, BRCA1 or BRCA2 mutation or treatment for Hodgkin’s lymphoma.

Examination of sample AGP concentration in confirmed mutation carriers within dataset 2 showed no correlation between BRCA1 mutation and AGP concentration. Nevertheless, sample W1 had an isolated AGP level 1.5 fold higher than other samples from BRCA1 patients which may point to initial development of BC (Rudman *et al*., 1974 and Duche *et al*., 2000). BRCA2 mutation carriers had consistently higher concentrations of AGP compared to BRCA1 mutation carriers except for sample W1. As BRCA1 mutation carriers are at higher risk of BC development compared to BRCA2 carriers (Mavaddat *et al*., 2013) maybe the concentrations of AGP within these samples should be viewed differently. The majority of BRCA1 samples contained isolated AGP levels approximately 1.5 fold lower than BRCA2 samples, as BRCA1 carriers are more likely to develop BC, perhaps an initial decrease in AGP concentration is associated with BC occurrence. Similarly low levels of AGP were seen in samples W13 and W16 which had no known mutations. Yet, to date no other studies have investigated AGP in BRCA1 and BRCA2 mutation carriers so no comparisons can be drawn to test this hypothesis.

4.5.7 Variants of AGP.

When assessing AGP concentration in samples it is important to note that total AGP concentration is the result of variants produced by three genes known as AGP-A (ORM1) and AGP-B/B' (ORM2) (Katori *et al*., 2011). AGP-A encodes for F1, F2 and S variants which are more commonly referred to as F1*S and AGP-B/B' both encode for the A variant (Colombo *et al*., 2006; Taguchi *et al*., 2013). The F1*S and A variants differ by 20 amino acid substitutions (see Figure 1.7) but do not vary greatly in structure (see Figure 1.6). In healthy individuals the ratio of F1*S to A variants is approximately 3:1 (Dente *et al*., 1987) however these ratios may alter during cancer. As increases in isolated AGP levels were seen in BC of unknown type and TNBC samples (dataset 1) as well as "at-risk" samples (dataset 2) compared to healthy controls, it is possible that differences in AGP variant ratios was also occurring. Previous research has revealed that increased expression of F1*S and A variants occurs in breast, ovarian and lung cancer, however the overall ratio tends to remain the same (Duche *et al*., 1998 and Duche *et al*., 2000). In contrast to this, a more recent study by Budai *et al*., (2009) using ultra high performance liquid chromatography and mass spectrometry instead of the isoelectric focussing and immunoblotting method used by Duche *et al*., (1998;2000), unveiled F1*S to A variant ratios of up to 8:1 in lymphoma, melanoma and ovarian cancer. However, unfortunately it was not feasible to investigate the proportion of AGP variants within samples during this study to account for increases in overall levels of isolated AGP.

4.6 Summary Statement.

In summary this chapter accomplished its aim to confirm the isolation of AGP from blood samples using low pressure chromatography and assess variation in isolated AGP levels from different sample populations used in this study.

It was noted that other analytical techniques may have been employed to support the findings if sample volume was not so limited; that variation of isolated AGP levels in healthy controls and BC of unknown type in dataset 1 may have been due to ineffective storage and alterations in the ratios of AGP variants which could have accounted for increased isolated AGP levels.

Results from dataset 1 highlight differences in isolated AGP levels between healthy individuals, BC and TNBC samples. Samples from healthy individuals contained low mean levels of isolated AGP while BC samples had significantly increased AGP levels in comparison. Both sets of controls showed variation within the positive and negative control groups that may be a results of substandard storage or sample loss during purification. Interestingly, AGP concentration of TNBC samples were significantly raised above the healthy control but significantly lower than the BC control which could further differentiate a diagnosis using AGP in future. Age of individuals and TNM stage of TNBC tumour appeared to have no effect on AGP concentration while treatment prior to blood draw may have caused lower levels of AGP in the <35 years of age group.

In dataset 2 the majority of “at-risk” samples had increased isolated AGP levels compared to age-match healthy controls despite both sample populations being considered disease free. This unexpected increase in AGP concentration could be contributing to the development of BC by suppressing the immune system in the tumour vicinity to avoid destruction by the immune system. Additionally, a number of samples, particularly samples from BRCA1 mutation carriers, contained isolated AGP levels at least 1.5 fold lower than other “at-risk” samples which could infer that a decrease in AGP concentration occurs during the initial stages of BC development.

However, isolated AGP levels alone are not indicative of BC thus this study focuses on alterations in AGP glycosylation.

Chapter 5 – Monosaccharide Analysis Results and Discussion

5.1 Introduction.

Glycans attached as post-translational modifications to proteins can vary in structure and composition during certain physiological or pathological conditions. These changes can affect the conformation of the protein affecting function and circulation lifetime. Furthermore, a glycan pattern unique to a specific disease could be used as a biomarker to distinguish between healthy and diseased individuals. Therefore, analysis of glycan chain composition and structure (known as microheterogeneity) is vitally important. Analysis of individual sugars (monosaccharides) that are the components of glycan chains can indicate structural alterations of the chain as a whole (oligosaccharide). For example, Gal is exclusively present in the branches of AGP glycans, thus distinctly increased concentrations of Gal could indicate greater branching of AGP glycans.

HPAEC-PAD is a well utilised, reliable and documented technique for monosaccharide analysis of glycans (Cataldi*, et al.*, 2000; Corradini*, et al.*, 2012). While other methods of glycan composition analysis are available such as mass spectrometry, nuclear magnetic resonance, HPLC, gas chromatography and lectin ELISAs, these techniques often require derivatisation – the transformation of an analyte into a similar structure (derivative) that is more amenable to the analytical technique being used - or large sample quantities to obtain better separation and detection, with HPAEC-PAD continuing to be one of the most reliable and effective methods of glycan analysis. HPAEC-PAD was implemented for this study as no prior derivatisation of samples was necessary, which would have altered the structure of monosaccharides and may have affected results. Secondly, HPAEC-PAD is highly sensitive, able to detect 10-100 picomolar concentrations (Hardy*, et al.*, 1988) which was very beneficial for Phase 1 of this study as only small sample volumes could be obtained.

In order for monosaccharide analysis by HPAEC-PAD to occur, glycans must first be cleaved from the protein and the covalent bonds between monosaccharides disrupted. TFA and HCl are commonly used to cleave neutral and basic monosaccharides in preparation for analysis. This study incubated 50µg of isolated AGP for 4 hours at 100°C with 100µl 2M TFA and 50µl 4M HCl

in line with the protocol devised by Smith*, et al.* (1997), which was proven to effectively cleave individual monosaccharides for analysis.

To utilise HPAEC for monosaccharide analysis there are several requirements. Firstly, a highly mechanical and chemically stable stationary phase is necessary; commonly in the form of a column containing a polymer based pellicular resin. The active component of such resins is a quaternary ammonium ion. Secondly, an alkaline mobile phase such as 0.03M NaOH is required to be used in an isocratic gradient. Lastly, a detection system must be in place to identify separated monosaccharides. This study used NaOH in an isocratic gradient to induce electrocatalytic oxidation of weakly acidic sugars this occurred through the ionisation of hydroxyl groups (-OH) within monosaccharide structures, forming oxyanions at high pH (12 or above) (see Figure 5.1). Monosaccharides possess several –OH groups which vary in position between monosaccharides resulting in different dissociation constants (pKa) ranging from 12-14. HPAEC analysis exploits the pKa values of monosaccharides and their subsequent binding to the pellicular anion-exchange resin, to separate them for detection. Those with higher pKa values, such as Fuc, are less negatively charged and form weaker interactions with the cationic resin within the CarboPac™ PA-100 and are therefore eluted and detected earlier than a more negatively charged monosaccharide (Lee, 1990; McGuire*, et al.*, 1999).

Detection of eluted monosaccharides occurs on a gold electrode and prior to the invention of PAD the gold electrode would be fouled by a build-up of oxides that formed a coating on the surface, making it redundant. Since the 1980's PAD has overcome the issue of electrode fouling by allowing oxides to form when the potential is increased thus the monosaccharides are detected but cleaning the electrode surface using a low potential, greatly improving the reproducibility of the technique (Johnson and LaCourse, 1992; Rocklin and Pohl, 1983). Detection occurs when an oxyanion (an ionised –OH group on a monosaccharide) comes into contact with the gold electrode at a high potential, the electrical current generated by the oxidation of the monosaccharide on the electrode surface is then measured and recorded. The electrode surface is then cleaned by lowering the potential, readying it for the next eluted monosaccharide (see Figure 2.1).

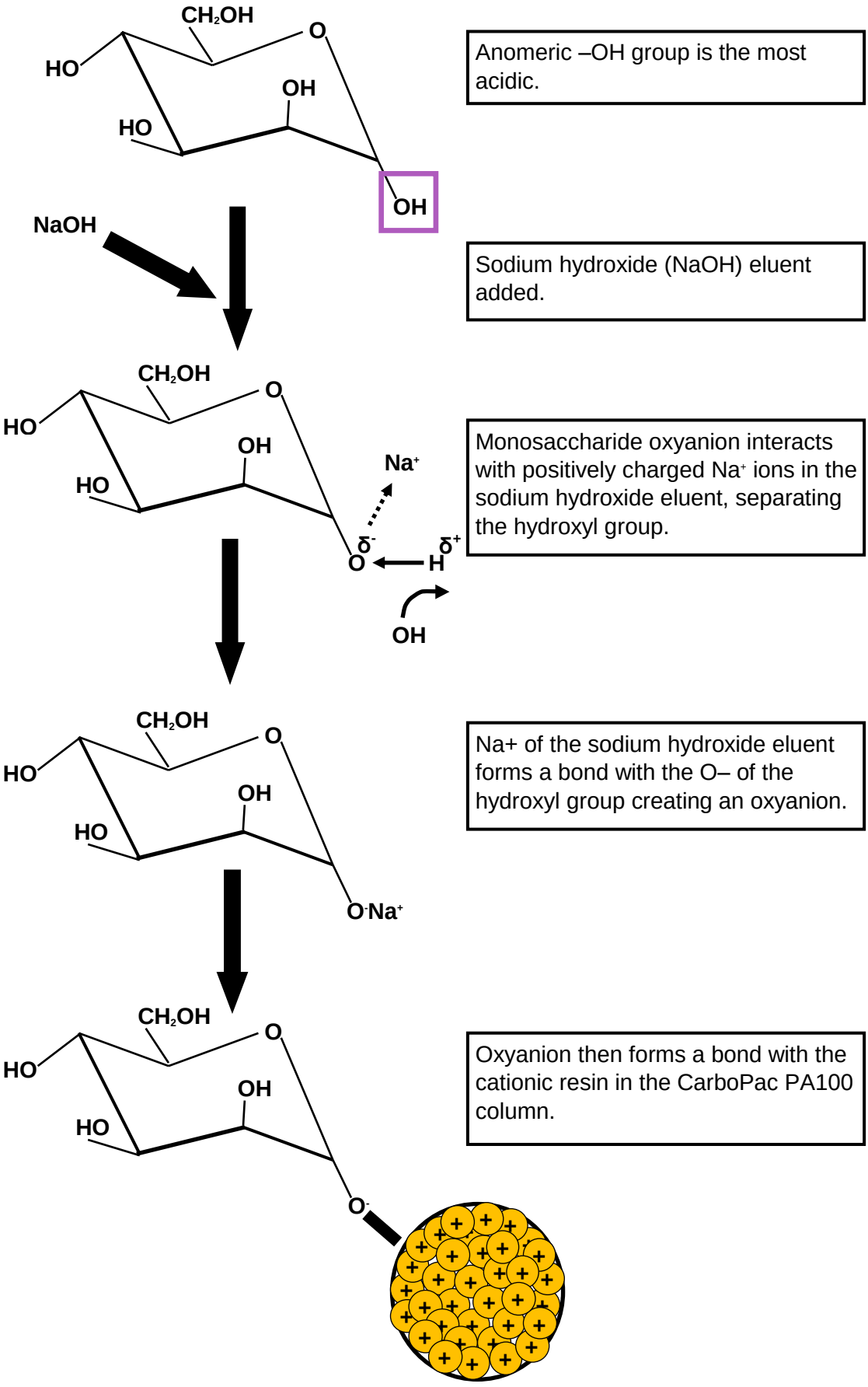


Figure 5.1 Oxyanion Formation. [Adapted from Behan, (2010)]

5.2 Dataset 1 Results.

The monosaccharide composition of AGP glycan chains was evaluated via HPAEC-PAD. Samples from dataset 1 and dataset 2 were analysed to establish any quantitative or qualitative differences in composition that may form the basis of a novel BC biomarker.

Performing such analysis is reliant on complete separation of glycan chains from the polypeptide backbone of AGP and degradation of the bonds between individual monosaccharides. Uncleaved or degraded monosaccharides will not be detected by HPAEC-PAD which could alter results. Figure 5.2 shows a representative chromatogram of monosaccharides common to complex *N*-linked glycans – Fuc, glucosamine, Gal and Man. To represent the deacetylation of *N*-acetylated hexosamines by HCl and NaOH during acid hydrolysis and HPAEC respectively, glucosamine was used as a standard for GlcNAc. The chromatogram below illustrates that HPAEC is capable of separating stereoisomers such as Gal and Man which are isomers of one another differing by the orientation of their atoms in space (see Figure 1.1 in Chapter 1). 2-Deoxy-galactose was used as an internal standard to aid the identification of sample monosaccharides through retention time ratios. The alkaline conditions selected, provided consistent elution order of monosaccharides throughout this study.

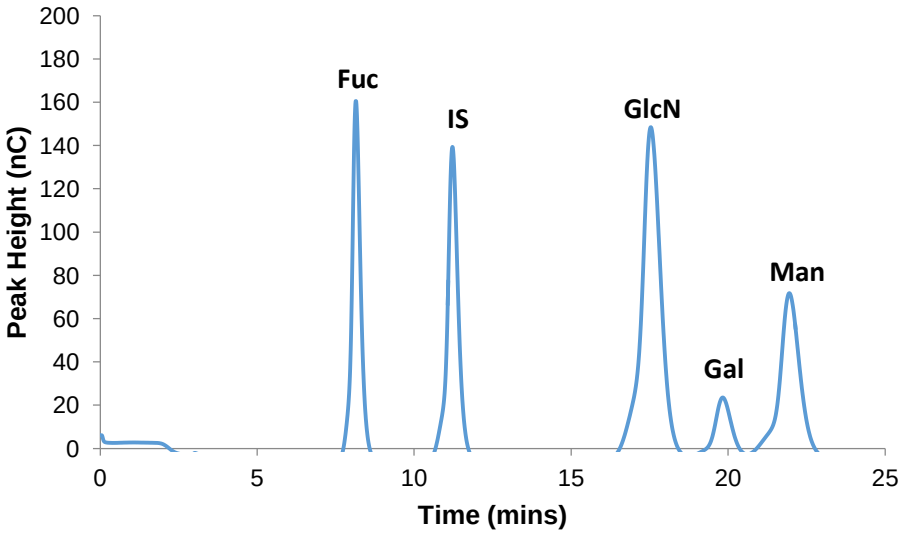


Figure 5.2 HPAEC Separation Chromatogram of Common Complex *N*-linked Glycan Monosaccharides Fuc – Fucose; IS – Internal standard (2-Deoxy-D-galactose); GlcNAc – *N*-acetylglucosamine; Gal – Galactose; Man- Mannose.

However, upon monosaccharide analysis of TNBC samples a peak was consistently detected at approximately 15 minutes between IS and GlcNAc. Further investigation revealed this to be *N*-acetylgalactosamine (GalNAc) which, as shown below in Figure 5.3, has a similar retention time to that of the unexpected peak. As with GlcNAc, galactosamine (GalN) was used as a standard to represent GalNAc. GalNAc is not routinely present in *N*-linked complex glycans such as AGP but is commonly found in *O*-linked glycans.

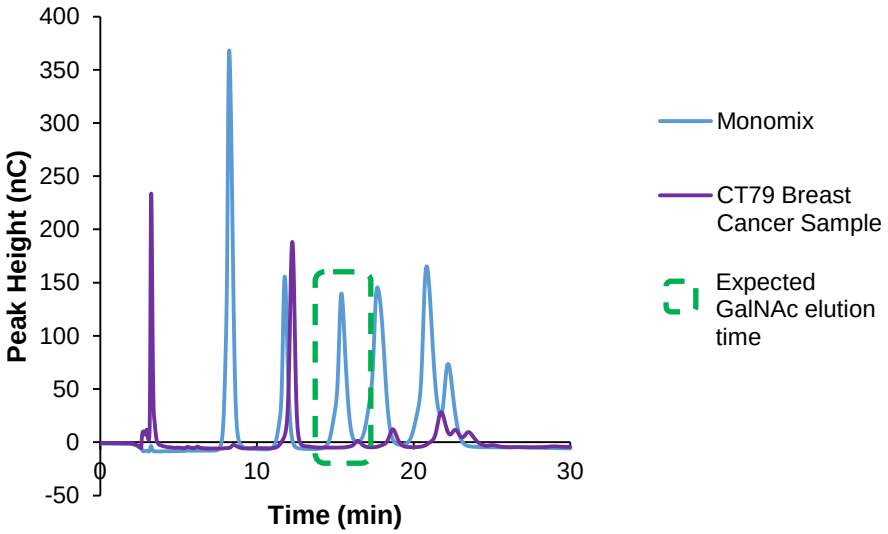


Figure 5.3 Identification of Unknown Chromatogram Peak. Continual appearance of unknown peak in TNBC samples was identified as Galactosamine as both had similar retention times and ratios.

Reliability of the acid hydrolysis technique and HPAEC instrument was critical for investigation of monosaccharide content during this study. Therefore, prior to Phase 1 sample analysis, commercially purchased AGP test samples were acid hydrolysed using methods previously devised by the research group (Behan*, et al.*, 2013) and the monosaccharide content analysed to ensure reliability of the methods.

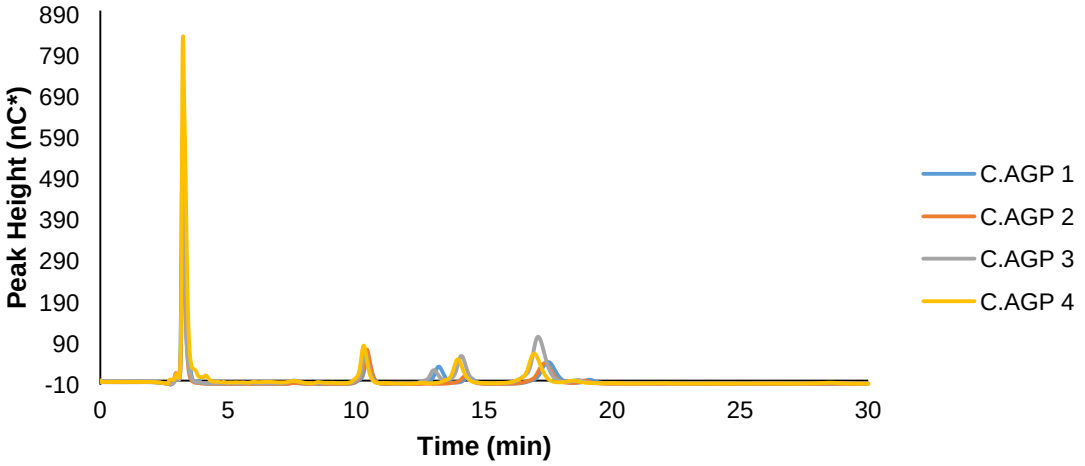


Figure 5.4 Chromatogram of Commercially Purchased AGP Samples to Highlight Dionex Reproducibility. Commercially purchased AGP samples (n=4; 50µg) were acid hydrolysed to release individual monosaccharides that were analysed by HPAEC-PAD to show consistent AGP chomatogram confirming reproducibility of technique.

After reproducibility of the technique was confirmed, standard curves were produced using known quantities of Fuc, GlcN, Gal and Man (shown below Figure 5.5) and equations devised for the calculation of unknown monosaccharide content from Phase 1 and Phase 2 samples. Galactosamine standard curve was calculated and added to Figure 5.5 after detection in TNBC samples. Analysis of monosaccharide concentrations of 0-2ug were repeated five times to calculated mean values and standard deviations to plot standard curves with error bars. As shown below increase in monosaccharide concentration caused an increase in peak area detected during HPAEC analysis. The majority of r^2 values were >0.9 indicating a strong linear association between monosaccharide concentration and detected peak area.

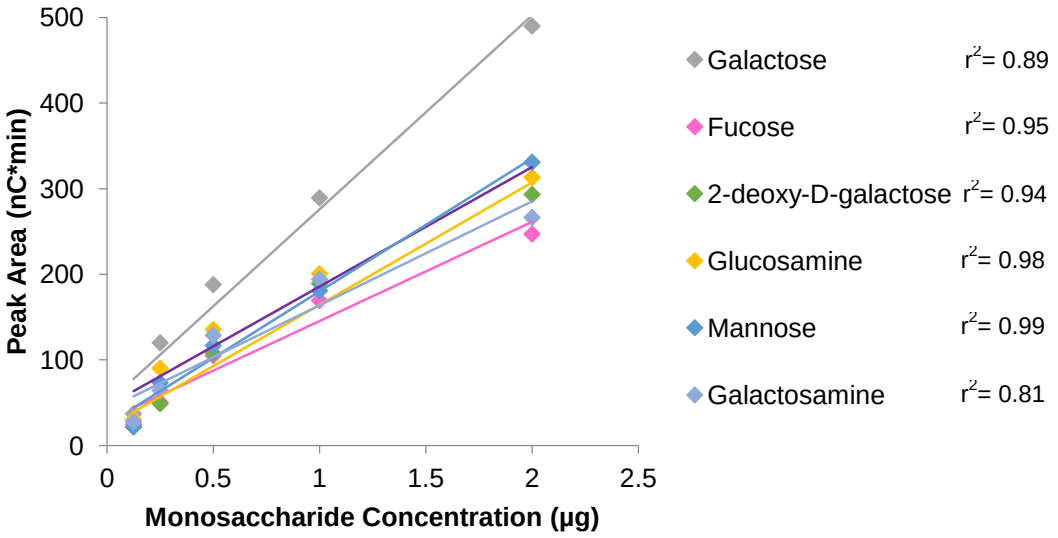


Figure 5.5 Plot of Peak Area Variation with Known Concentrations of Common *N*-linked Glycan Monosaccharides and Galactosamine. (n=5).

The equation of each monosaccharide curve was rearranged to give an equation for generating concentration of each monosaccharide in µg. Concentration in micrograms was then subsequently adapted to nanomoles for conversion to mol of sugar per mol AGP. This allowed for direct comparison of monosaccharide content between sample groups in dataset 1 and dataset 2 of the study.

Monosaccharide	Equation of Mono Curve	Equation for Mono Mass (µg)	Equation for µg to nmol	Equation for mol sugar/mol AGP
Fucose	y = 137.46x	x= Peak Area/137.46	x= (mono mass µg/MW of mono) X 1000	x= no. nmol AGP injected/ nmol mono
2-deoxy-galactosamine	y = 158.58x	x= Peak Area/158.58	"	"
Galactosamine	y = 152.32x	x= Peak Area/152.32	"	"
Glucosamine	y = 149.21x	x= Peak Area/149.21	"	"
Galactose	y = 215.78x	x= Peak Area/215.78	"	"
Mannose	y = 147.66x	x= Peak Area/147.66	"	"

Table 5.1 Equations Derived From Standard Curve to Determine mol monosaccharide per mol AGP Content of Each Sample.

5.3 Dataset 1 Monosaccharide Content Results.

Dataset 1 of the study compared the monosaccharide content of samples from women with TNBC (n=18), healthy individuals (n=5; negative control) and women with BC of an unknown hormone receptor type (n=5; positive control). For both positive and negative controls, analysis was carried out in triplicate and thus figures shown in Table 5.2 are means of three replicates and standard

deviation is shown. However, low TNBC sample volumes made repeat analysis impossible, therefore figures shown in Table 5.2 below are representative of only one analysis of each sample.

	Monosaccharide Content (mol monosaccharide/mol AGP)				
Sample	**Fucose**	**GalNAc**	**GlcNAc**	**Galactose**	**Mannose**
H8	Trace	Trace	0.01 ± 0.02	0.07 ± 0.13	0.11 ± 0.13
I2	0.02 ± 0.01	Trace	0.06 ± 0.05	0.14 ± 0.09	0.16 ± 0.24
I3	0.04 ± 0.00	Trace	0.01 ± 0.00	1.13 ± 0.09	0.15 ± 0.02
I5	Trace	Trace	0.01 ± 0.00	0.17 ± 0.14	0.15 ± 0.20
I8	0.01 ± 0.00	Trace	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.06
Mean Positive Control	**0.01 ± 0.01**	**-**	**0.02 ± 0.01**	**0.31 ± 0.20**	**0.13 ± 0.02**
N1	0.02 ± 0.01	Trace	Trace	0.05 ± 0.00	0.94 ± 0.07
N2	0.01 ± 0.00	Trace	0.11 ± 0.10	0.11 ± 0.01	0.07 ± 0.10
N3	0.01 ± 0.00	Trace	0.11 ± 0.04	0.12 ± 0.08	0.18 ± 0.17
N4	0.01 ± 0.02	Trace	0.03 ± 0.02	0.17 ± 0.00	0.42 ± 0.18
N5	Trace	Trace	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Mean Negative Control	**0.01 ± 0.00**	**-**	**0.05 ± 0.02**	**0.09 ± 0.03**	**0.33 ± 0.17**
F5412	0.21	0.27	0.56	1.54	0.87
MT3625	Trace	Trace	Trace	0.02	0.41
MT2305	0.04	0.04	0.16	0.60	1.13
CT79	0.04	0.08	0.27	0.62	0.29
MT2621	0.08	0.02	0.09	0.45	0.76
MT3965	0.02	0.45	0.06	0.83	0.25
Mean <35	**0.07 ± 0.08**	**0.17 ± 0.19**	**0.19 ± 0.20**	**0.67 ± 0.50**	**0.62 ± 0.35**
MT1106	0.04	0.02	0.35	0.71	1.76
MT1177	0.05	0.04	0.85	1.26	3.51
MT1178	0.05	0.38	0.24	0.57	1.45
MT3601	0.15	0.16	0.32	0.78	1.18
MT1937	0.05	0.12	0.20	0.38	0.80
MT3795	0.12	0.02	0.43	0.59	1.90
Mean Over 35-60	**0.07 ± 0.05**	**0.12 ± 0.14**	**0.39 ± 0.23**	**0.72 ± 0.30**	**1.77 ± 0.94**
MT3362	0.02	0.01	0.03	0.34	0.69
MT3497	0.12	0.08	0.46	0.66	1.80
MT3442	0.01	0.01	0.25	0.45	0.74
MT3959	0.04	0.02	0.26	0.13	3.03
MT3352	Trace	Trace	Trace	Trace	1.35
MT2569	Trace	0.03	0.03	0.51	0.44
Mean >60	**0.03 ± 0.05**	**0.03 ± 0.02**	**0.17 ± 0.18**	**0.35 ± 0.19**	**1.34 ± 0.96**

Table 5.2 Monosaccharide Content of Dataset 1 Samples. Table shows mean of three replicates of each positive and negative control sample ± SD. While TNBC figures are the result of one analysis.

Man is only present in the pentasaccharide core of complex *N*-linked glycans, thus consistent levels are an indication that similar amounts of AGP are being analysed each time. While Man levels differed slightly between the three groups, variation was not significant and alterations in level may be due to the sheer accuracy of the HPAEC technique. As no significant difference was found this suggested that the *N*-linked pentasaccharide core structures were consistent in all samples implying that the levels of AGP being analysed were as intended and reliable.

Due to only one repeat of each TNBC sample being possible, statistical analysis of differences between individual TNBC samples was not possible. However, TNBC samples were sub-divided into varying categories such as age, family history status, cancer grade and treatment status to statistically examine any alterations in monosaccharide content that could lead to a biomarker for BC in "at risk" individuals. Healthy and BC of unknown type samples were then compared to these categories using one-way ANOVA with a Tukey's post hoc test to examine any statistical differences between the groups. Statistical significance is indicated on the graphs that follow (Figures 5.6, 5.7, 5.8, and 5.9) by an * symbol above the relevant column for significance at $p<0.05$ and ** symbol if significance is at $p<0.001$.

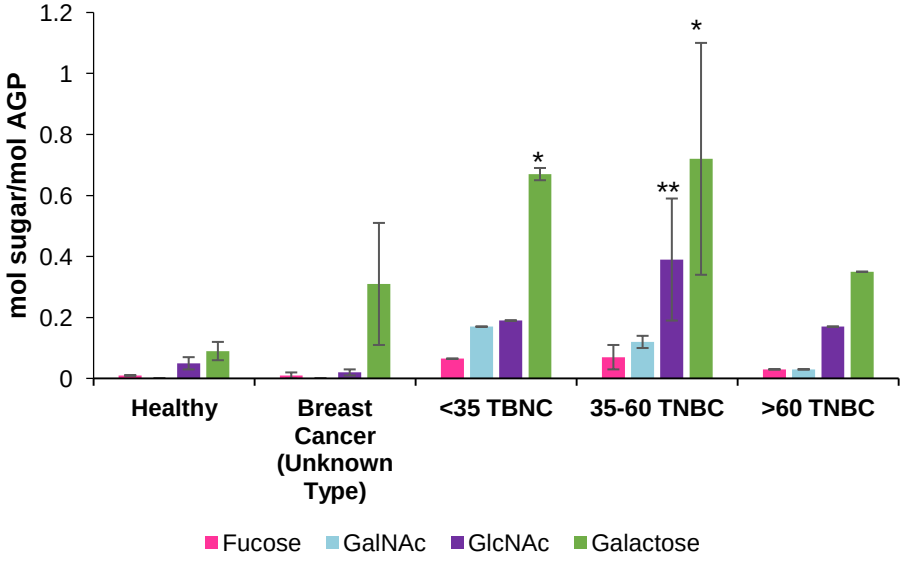


Figure 5.6 Mean Monosaccharide Content of TNBC Samples by Age Compared to Controls. TNBC samples were separated into age categories <35, >35-60 and >60 (n=6 per category) for comparison with healthy and BC of unknown type. Statistical significance indicated by * for $p<0.05$ and ** for $p<0.01$.

Differences in monosaccharide content were observed between both negative and positive controls as well as across all age categories of TNBC. Healthy samples showed low levels of all monosaccharides whereas BC of an unknown type showed increased levels of Gal although these differences were not significant. Increased levels of Gal may indicate increased branching of glycan chains within a sample as Gal is only present in the branches of complex *N*-linked glycans such as those on AGP. Interestingly, TNBC is shown to have elevated levels of all monosaccharides compared to not only the healthy population, but that of the BC unknown type as well. TNBC samples aged <35 and 35-60 show similar monosaccharide content trends with levels of monosaccharides increasing slightly with age. However, TNBC samples from women >60 years of age contained lower levels of all monosaccharides than both the other TNBC age categories but levels were still elevated above those of the healthy population. Furthermore, the unusual presence of GalNAc appears to decrease with age, but again this was not significant. GlcNAc levels of the TNBC 35-60 category were found to be significantly higher ($p<0.05$) to both the healthy and BC of unknown type. Similarly levels of Gal were significantly higher in TNBC <35 and 35-60 age groups. This suggested that TNBC has a different monosaccharide content to that of BC that may be of a different type, significantly so at younger ages (<60) but not in the age range that BC commonly occurs (usually 50-70). When comparing TNBC from patients below and over 50 years old, only Gal was significantly higher in <50 group compared to both controls. Analysis of samples MT3625 and MT3352 showed unquantifiable trace amounts of the majority of monosaccharides that were expected to have been detected. The explanation for this is unknown but it is unlikely to be a true reflection of monosaccharide content.

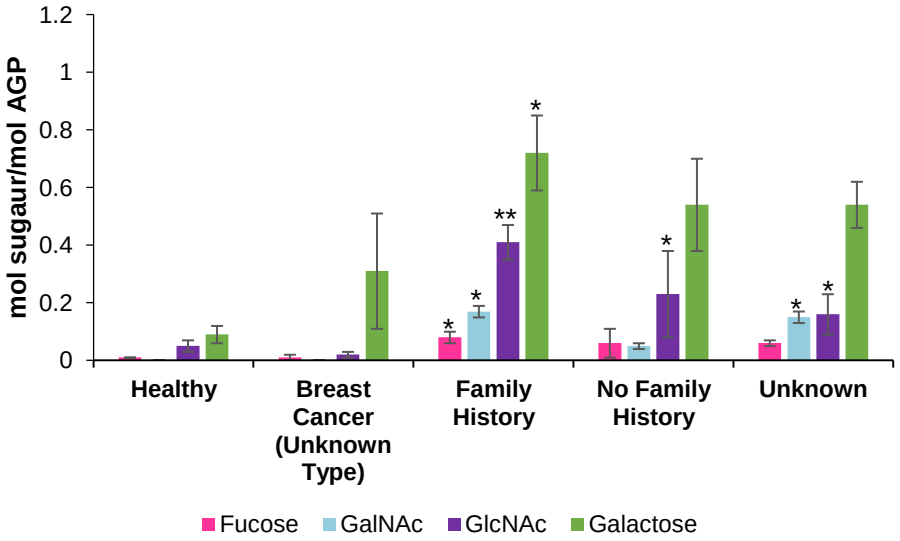


Figure 5.7 Mean Monosaccharide Content of Samples With and Without Family History. TNBC samples were separated into groups with and without family history (n=7 and n=8) as well as unknown family history status (n=3) for comparison with healthy and BC of unknown type. Statistical significance indicated by * for $p<0.05$ and ** for $p<0.01$.

Examining the monosaccharide content of TNBC samples categorised by family history status, highlighted several significant differences between those with family history and both controls. As with TNBC separated by age, levels of all monosaccharides were elevated compared to healthy and BC of unknown type controls. Furthermore, samples from patients with family history of BC have higher levels of all monosaccharides compared to those with no family history (not significant). However, when compared to controls, samples from patients with family history have significantly higher Fuc compared to BC of unknown type. GalNAc is also significantly increased in those with family history and unknown family history status compared to both controls. Both controls contain significantly lower levels of GlcNAc compared to family history TNBC samples but the BC of unknown type also has signficantly lower GlcNAc levels than those without family history. A significantly higher level of Gal was also observed in family history compared to healthy samples. Samples with unknown family history exhibit monosaccharide trends similar to both family history and non-family history samples such as elevated GalNAc levels similar to family history samples and Gal level comparable to those of non-family history TNBC. Therefore there appears to be a distinct difference in the monosaccahride content of TNBC samples with family history compared to BC of unknown type.

Additionally there are few differences between non family-history TNBC samples and BC of unknown type other than significantly higher GlcNAc levels.

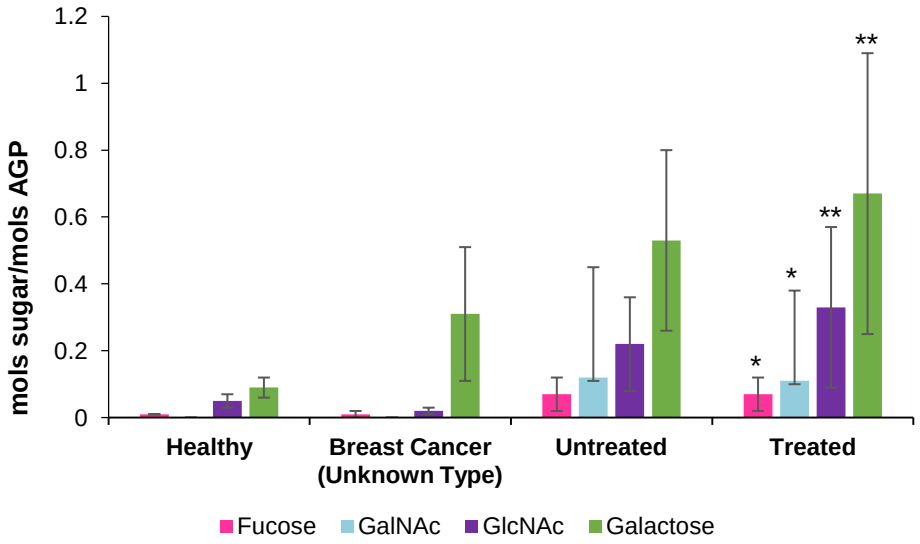


Figure 5.8 Mean Monosaccharide Content of Treated and Untreated TNBC Compared to Controls. Statistical significance indicated by * for $p<0.05$ and ** for $p<0.01$.

To assess whether treatment prior to blood draw had an effect on AGP monosaccharide content in TNBC, samples were split into treated and untreated samples and compared to both controls. When sub-categorised in this manner, treated and untreated TNBC samples appeared to follow similar trends in monosaccharide content that are distinctly different to either control. There were no significant differences between monosaccharide content in treated and untreated samples implying that at this stage treatment was having no effect on AGP monosaccharide content in TNBC samples. However, significantly lower levels of all monosaccharides were found in both controls compared to treated TNBC samples other than Gal which was only significantly lower in healthy samples compared to treated TNBC. There were also no significant differences in monosaccharide content found in either control compared to untreated samples.

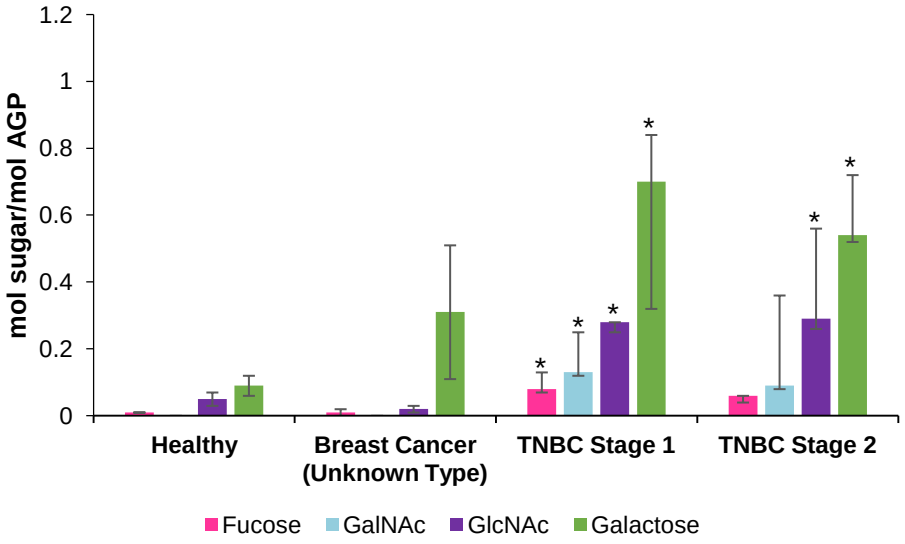


Figure 5.9 Mean Monosaccharide Content of TNBC Samples by Stage Compared to Controls.

As in Figure 5.9, when TNBC samples are separated into cancer stage there are similar trends in monosaccharide content between Stage 1 and Stage 2 TNBC. As there was only one Stage 3 TNBC in the cohort (MT3352) this sample was not used for comparison as the sample would not be a reliable representation of Stage 3 TNBC. Stage 1 TNBC possessed significantly higher levels of Fuc, GalNAc and GlcNAc ($p<0.05$) in comparison to both controls with Gal being significantly higher than the healthy population as well. Stage 2 TNBC also had significantly higher levels of GlcNAc ($p<0.05$) compared to both controls and Gal in comparison to the healthy population. No significant difference was found between Stage 1 and Stage 2 TNBC but GalNAc and Gal showed a 0.4 and 1.29 fold decrease in Stage 2 TNBC which may indicate a decrease in glycan branching.

5.4 Dataset 2 Monosaccharide Results.

Dataset 2 of this study examined the monosaccharide content of a population of “at risk” women (n=16) compared to (where possible) age-matched healthy controls (n=10) to determine any differences between the groups as well as any patterns that may indicate BC onset. Analysis using HPAEC-PAD was conducted five times for each sample to improve the reliability of results.

Therefore, figures in Table 5.3 are the means of five replicates and the standard deviation is given.

	Monosaccharide Content (mol monosaccharide/mol AGP)				
	Fucose	**GalNAc**	**GlcNAc**	**Galactose**	**Mannose**
W1	0.127 ± 0.06	0	0.351 ± 0.37	1.424 ± 0.64	0.930 ± 0.73
C1	0.174 ± 0.08	0	1.180 ± 0.15	1.367 ± 0.42	0.631 ± 0.45
W2	0.078 ± 0.04	0.133 ± 0.11	1.315 ± 0.85	1.518 ± 0.84	1.052 ± 0.52
C2	0.155 ± 0.11	0	0.508 ± 0.28	0.814 ± 0.56	1.003 ± 0.52
W3	0.146 ± 0.09	0.740 ± 0.70	1.217 ± 0.08	1.571 ± 0.26	1.264 ± 0.44
C3	-	-	-	-	-
W4	0.159 ± 0.13	0.213 ± 0.20	1.052 ± 0.67	1.546 ± 1.13	1.251 ± 0.74
C4	0.174 ± 0.12	0	1.014 ± 0.15	1.448 ± 0.29	0.857 ± 0.19
W5	0.163 ± 0.10	0.157 ± 0.09	1.120 ± 0.58	1.661 ± 0.79	1.204 ± 0.59
C5	0.208 ± 0.19	0	1.251 ± 0.36	0.981 ± 0.90	1.497 ± 1.29
W6	0.136 ± 0.06	0.933 ± 0.53	1.106 ± 0.58	1.086 ± 0.26	1.055 ± 0.67
C6	0.114 ± 0.09	0	0.849 ± 0.27	0.984 ± 0.48	1.822 ± 0.63
W7	0.116 ± 0.06	0	1.246 ± 0.66	1.670 ± 0.93	1.347 ± 0.71
C7	0.137 ± 0.09	0	0.817 ± 0.41	1.323 ± 0.65	1.010 ± 0.49
W8	0.086 ± 0.05	0	0.641 ± 0.37	0.430 ± 0.28	0.629 ± 0.34
C8	0.061 ± 0.03	0	0.438 ± 0.20	0.798 ± 0.19	1.006 ± 0.59
W9	0.050 ± 0.00	0.162 ± 0.27	0.954 ± 0.48	1.205 ± 0.63	0.936 ± 0.49
C9	-	-	-	-	-
W10	0.088 ± 0.02	0.298 ± 0.251	0.894 ± 0.24	1.057 ± 0.20	0.895 ± 0.19
C10	-	-	-	-	-
W11	0.041 ± 0.02	0.638 ± 0.37	1.257 ± 0.27	0.646 ± 0.59	0.853 ± 0.31
C11	0.097 ± 0.11	0	0.333 ± 0.54	1.140 ± 0.49	0.742 ± 0.48
W12	0.053 ± 0.01	0.068 ± 0.03	1.230 ± 0.17	0.098 ± 0.09	0.863 ± 0.19
C12	0.229 ± 0.06	0	0.780 ± 0.20	1.288 ± 0.20	1.128 ± 0.59
W13	0.081 ± 0.02	0.058 ± 0.04	1.357 ± 0.04	1.354 ± 0.14	1.224 ± 0.02
C13	0.069 ± 0.01	0	0.924 ± 0.27	1.266 ± 0.55	0.734 ± 0.55
W14	0.091 ± 0.01	0.777 ± 0.67	0.990 ± 0.07	1.350 ± 0.38	1.047 ± 0.13
C14	-	-	-	-	-
W15	0.192 ± 0.19	0.407 ± 0.434	1.309 ± 0.44	1.469 ± 1.15	1.202 ± 1.12
C15	-	-	-	-	-
W16	0.157 ± 0.08	0.561 ± 0.46	1.001 ± 0.13	1.614 ± 0.14	1.033 ± 0.17
C16	-	-	-	-	-

Table 5.3 Monosaccharide Content of Dataset 2 Samples. Table shows the mean of five replicates of “at risk” samples and their age-matched controls ± SD. “-“ indicates no age-matched controls were recruited.

As discussed for dataset 1, Man is only present in the pentasaccharide core of AGP glycans and therefore the consistent levels indicate similar concentrations of AGP being analysed. There was no significant variation between sample Man levels in dataset 2 implying that consistent concentrations of AGP were analysed and the results of monosaccharide analysis were reliable.

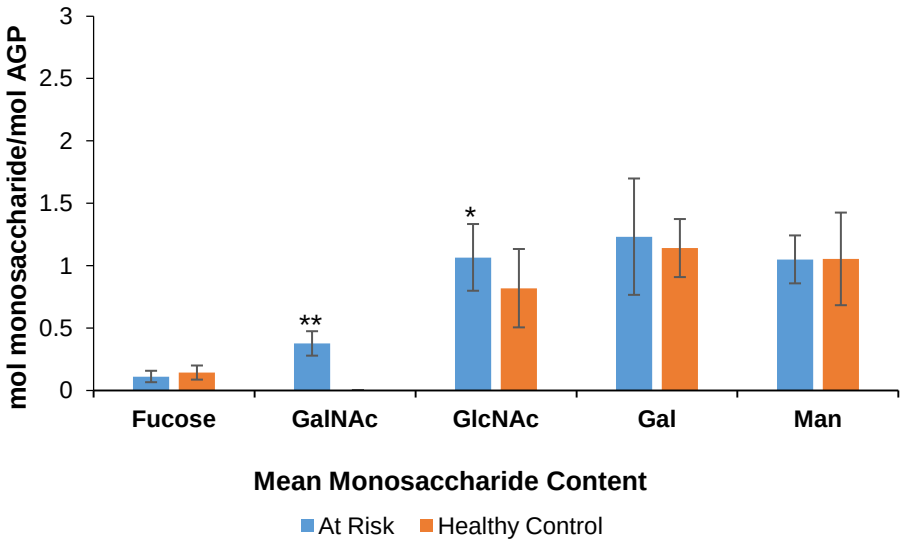
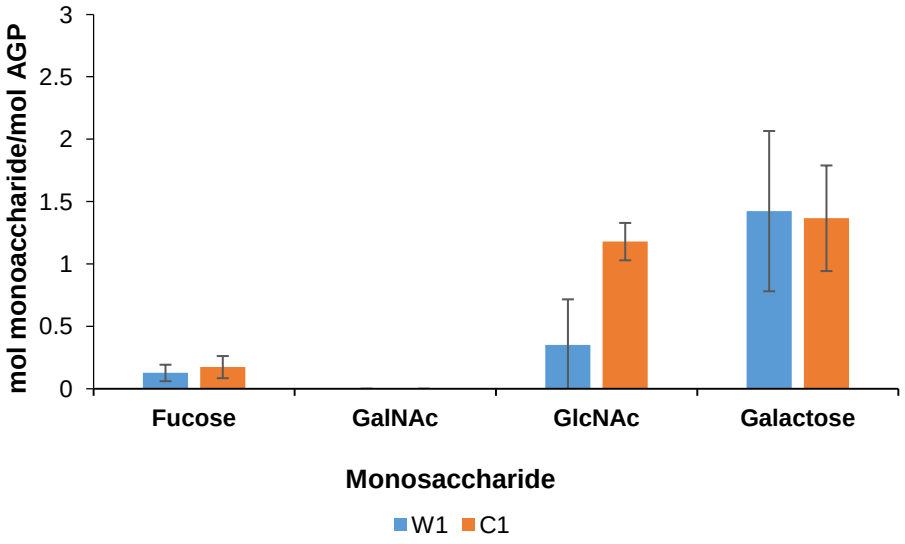


Figure 5.10 Mean Monosaccharide Content of At-Risk and Healthy Control Populations. Mean monosaccharide content in mol monosaccharide/mol AGP of a population at risk of BC (n=16) compared to a healthy control population (n=10) ± SD.

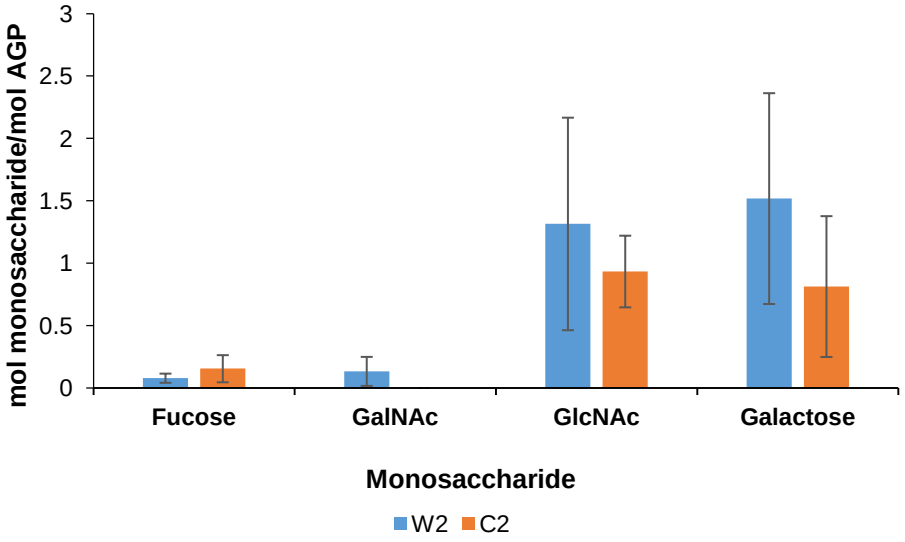
Comparison of mean monosaccharide content of the “at risk” population and healthy controls as well as a one-way ANOVA revealed alterations between both groups. The “at risk” population had slightly decreased Fuc levels compared to the healthy controls (not significant), but had significantly elevated concentrations of GalNAc ($p<0.01$) and GlcNAc ($p<0.05$). GalNAc was present in 81% of “at risk” samples but not detected in any of the healthy control samples and mean GlcNAc levels were 1.3 times higher in the ”at risk” population. No significant difference was found between either group for Gal or Man, highlighting the same levels of AGP were analysed.

The following graphs (Figures 5.11 A-P) give direct comparisons of AGP monosaccharide content between “at risk” samples and their age-matched healthy control or the mean monosaccharide content if an age-matched healthy control was unavailable.

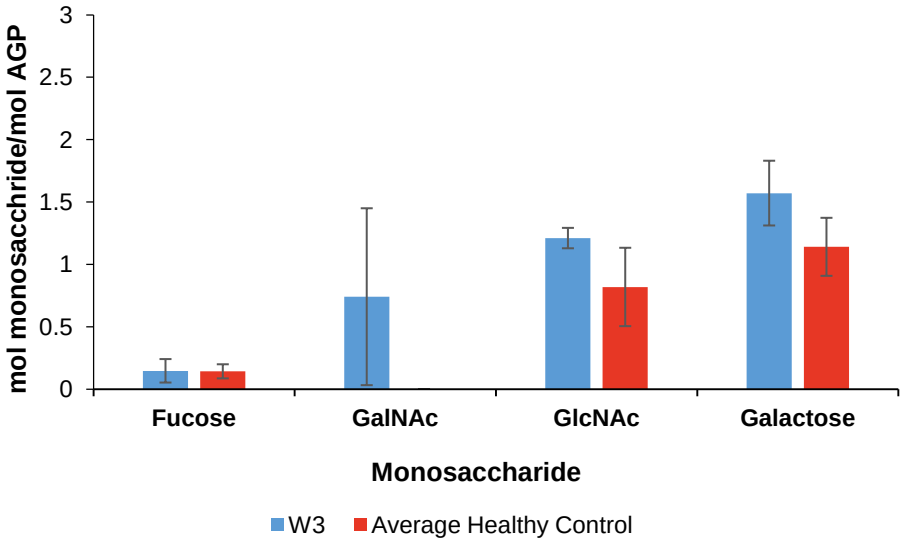
A


mol monoaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W1
C1

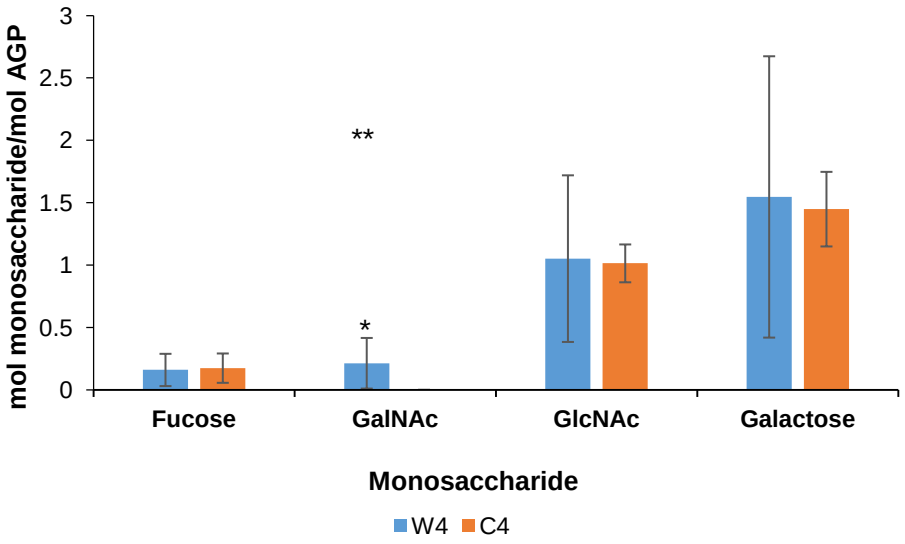
B


mol monosaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W2
C2

C


mol monosacchride/mol AGP
3
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W3
Average Healthy Control

D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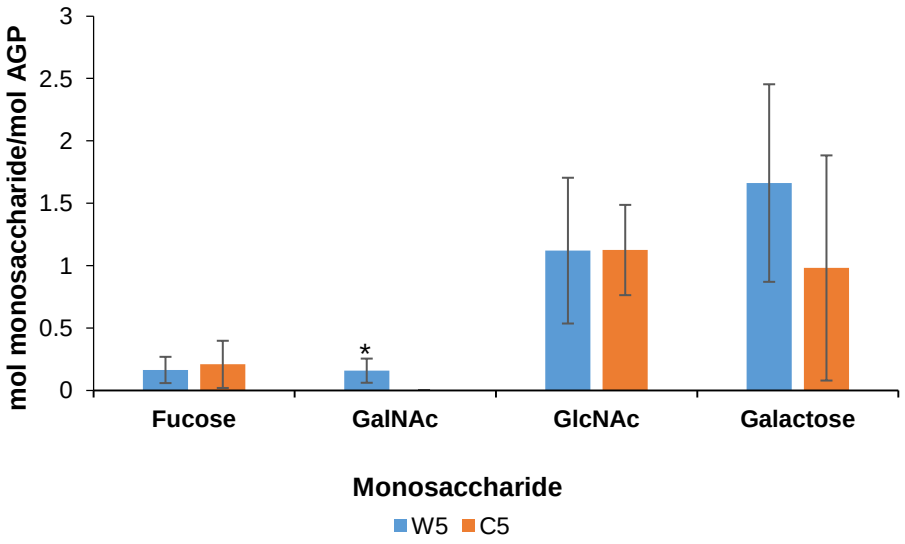
3
2.5
2
1.5
1
0.5
0
**
*
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W4
C4

E


mol monosaccharide/mol AGP
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2
1.5
1
0.5
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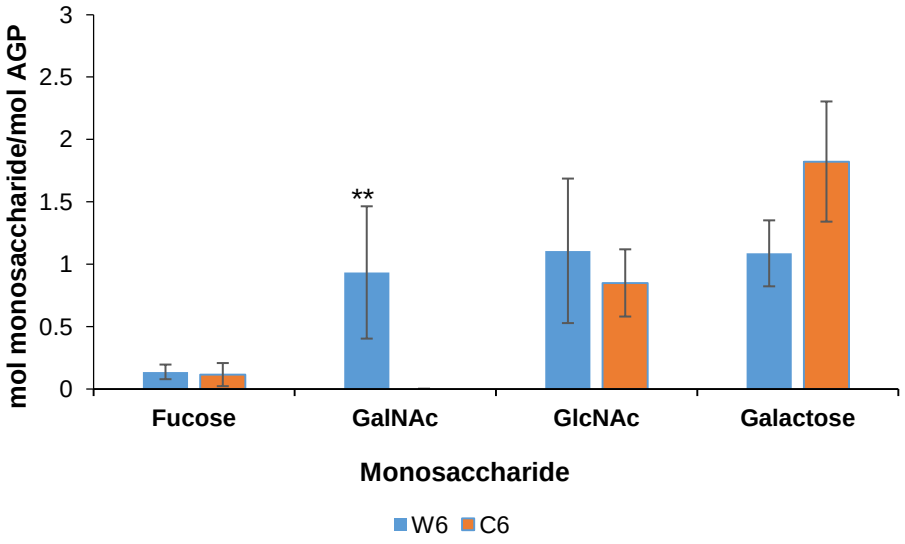
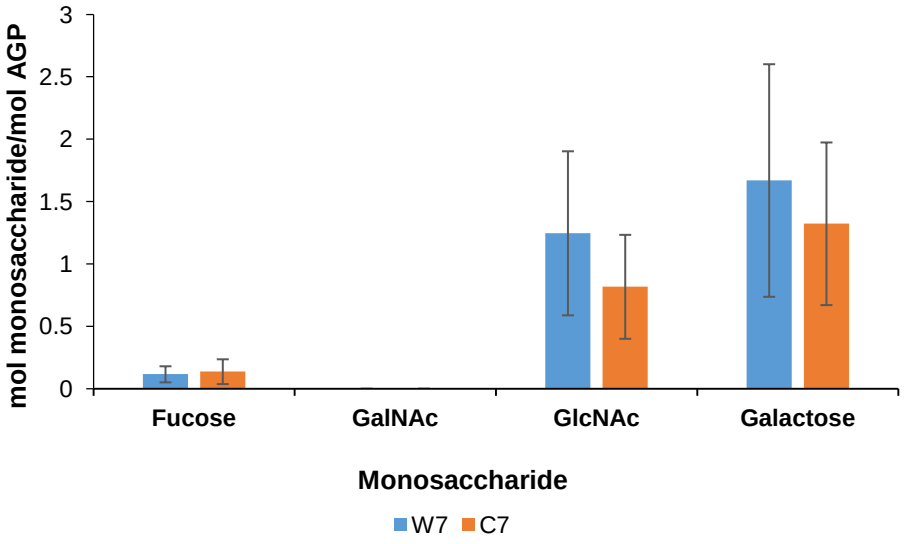
*
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W5
C5

F


mol monosaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
**
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W6
C6

G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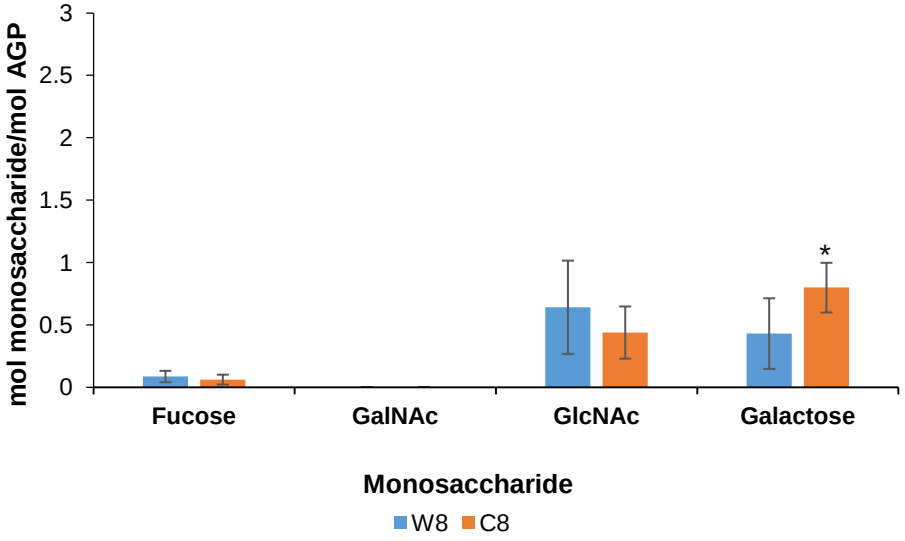
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W7
C7

H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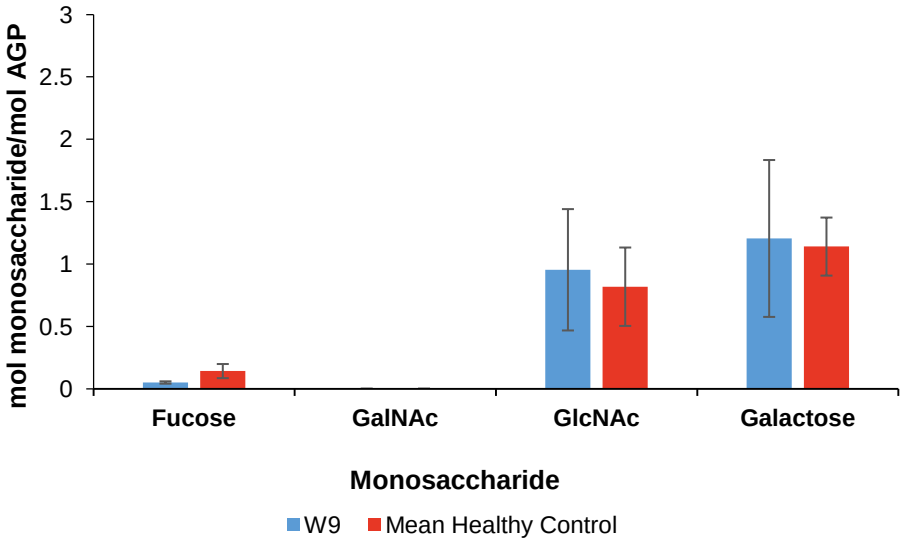
2.5
2
1.5
1
0.5
0
*
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W8
C8

I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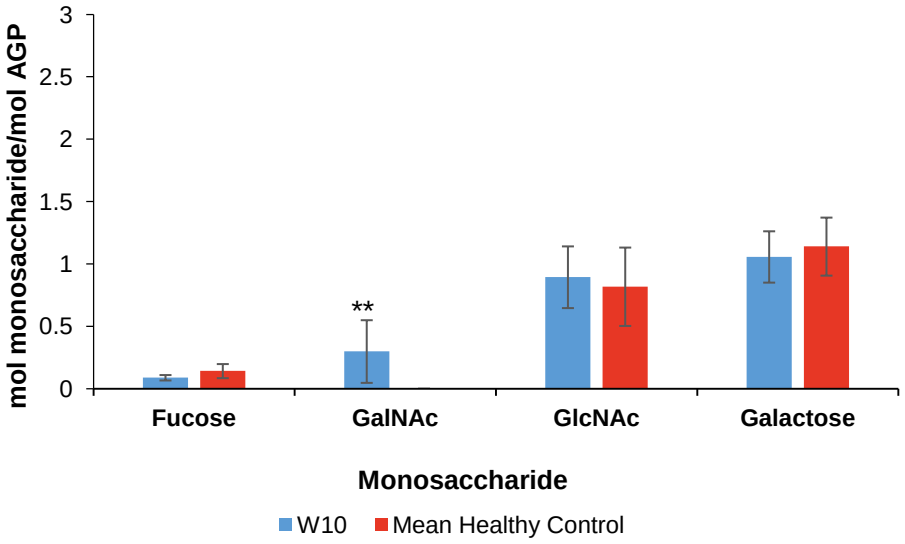
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W9
Mean Healthy Control

J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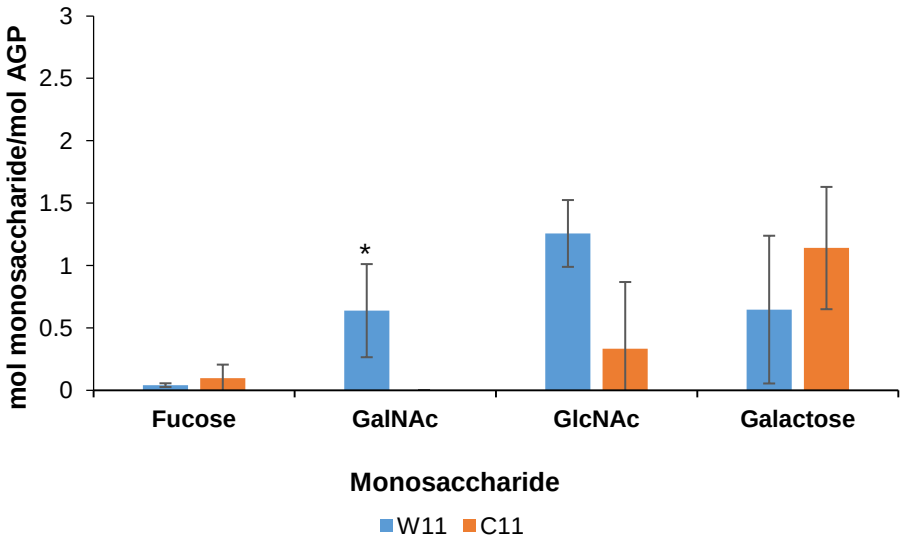
3
2.5
2
1.5
1
0.5
0
**
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W10
Mean Healthy Control

K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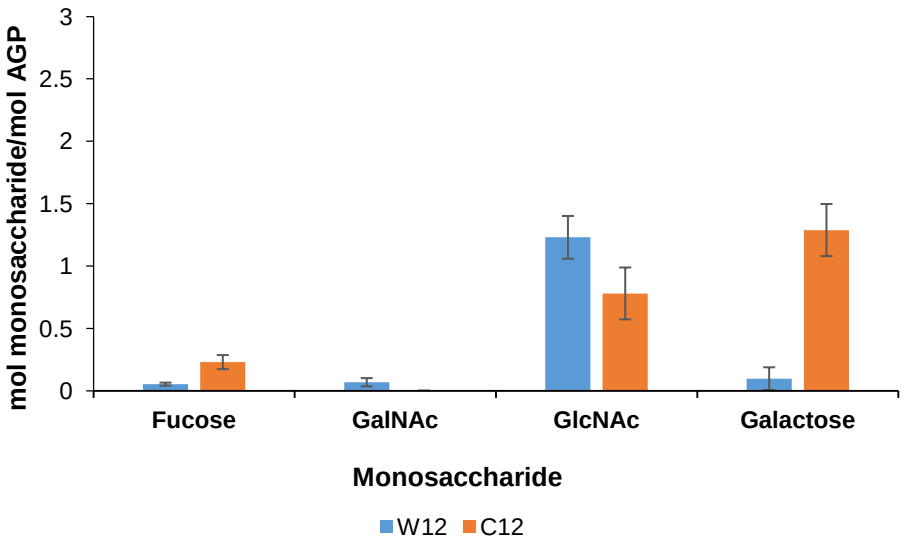
3
2.5
2
1.5
1
0.5
0
*
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W11
C11

L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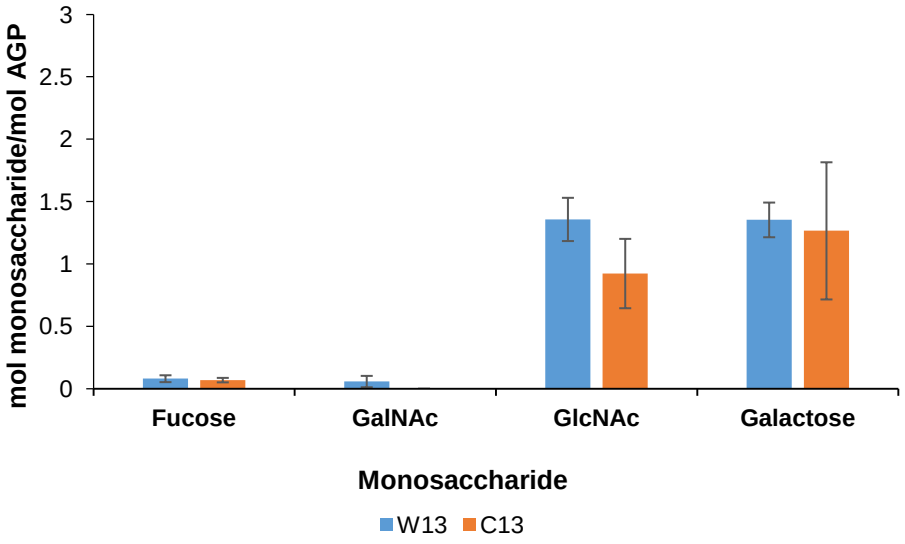
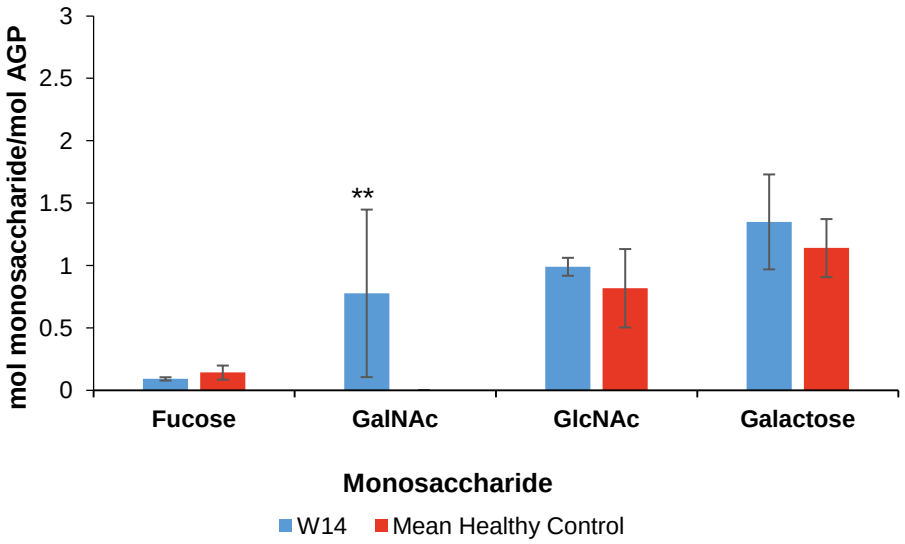
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W12
C12

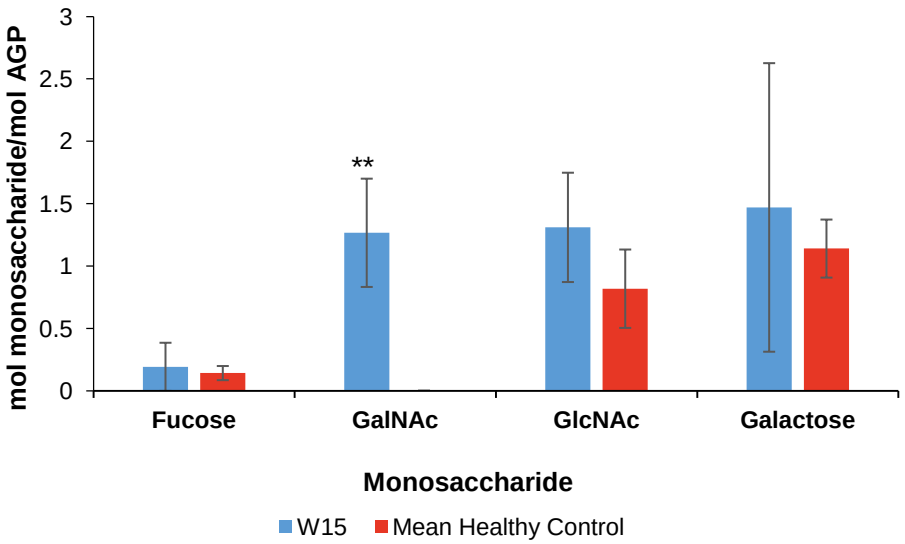
M


mol monosaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W13
C13

N


mol monosaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
**
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W14
Mean Healthy Control

O


mol monosaccharide/mol AGP
3
2.5
2
1.5
1
0.5
0
**
Fucose
GalNAc
GlcNAc
Galactose
Monosaccharide
W15
Mean Healthy Control

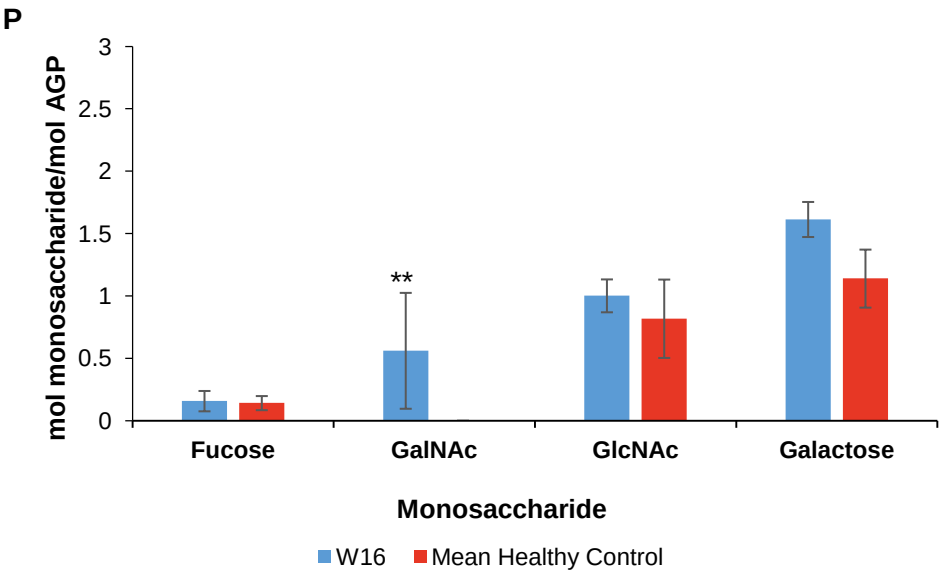


Figure 5.11 Comparison of Monosaccharide Content of At-Risk Samples with Age-Matched Healthy Controls. Graphs A-P show direct comparisons of sample AGP monosaccharide content between "at risk" samples (n=16) and their age-matched healthy controls (n=10). Where age-matched healthy controls could not be recruited (n=6), "at risk" samples were compared to the mean monosaccharide content of all healthy controls. Figures shown represent mean of the five replicates ± SD. Statistical significance indicated by * if $p<0.05$ or ** if $p<0.01$.

In line with previous statistical analysis in this chapter (Figure 5.10), comparisons between the monosaccharide content of "at risk" samples and age-matched controls (or mean monosaccharide content where no age-matched control was available) were analysed using a one-way ANOVA. Results were considered statistically significant at $p<0.05$.

Sample W1 showed no significant differences in monosaccharide levels compared to age-matched healthy control C1. However, GlcNAc levels were 70% decreased in W1 compared to C1. Fuc and Gal levels were similar in both samples and no GalNAc was detected in either.

Comparison of sample W2 to age-matched control sample C2 revealed no significant differences in monosaccharide content. While Fuc levels were comparable in both samples, GlcNAc and Gal were elevated in W2 compared to C2. Additionally, GalNAc was present in W2 but not C2 though not at significant levels.

No age-matched healthy control was available for comparison to sample W3. Therefore, as stated, W3 was compared to the mean monosaccharide levels of all healthy controls. GalNAc was present at significant levels ($p<0.01$) in W3

and both GlcNAc and Gal were also elevated by 47% and 38% respectively (not significant).

Sample W4 had GalNAc present (significant at $p<0.05$) however GalNAc was not present in C4. No other significant differences in monosaccharide content were found between W4 and C4 as levels of Fuc, GlcNAc and Gal were almost identical.

Assessment of W5 and C5 revealed significant GalNAc presence ($p<0.05$) in W5 but not C5. Fuc levels were marginally lower in W5 – 0.16 compared to 0.2 mol monosaccharide/mol AGP – and GlcNAc levels were similar in both samples. In contrast, Gal levels of W5 were almost double those in C5.

Significant GalNAc levels ($p<0.01$) were detected in W6 whereas no GalNAc was found in C6. W6 also showed an increase in GlcNAc compared to C6 however, Gal was 40% decreased in W6. Fuc levels were comparable in both samples.

W7 and C7 had no significant differences in monosaccharide content, with both lacking GalNAc and possessing similar Fuc levels. However, GlcNAc and Gal levels in W7 were increased compared to C7.

Interestingly, samples W8 and C8 had lower levels of all monosaccharides compared to other sample pairs. Neither sample contained GalNAc however W8 contained higher levels on GlcNAc (not significant). In contrast, C8 showed significantly higher levels on Gal ($p<0.05$). Again, Fuc levels were similar in W8 and C8

W9 was the second “at risk” sample not to recruit an age-matched healthy control, so as with W3, was compared to the mean monosaccharide content of all healthy controls. However, no significant differences were found in monosaccharide content between the groups. W9 showed lower levels of Fuc but increased GlcNAc, similar Gal levels to the control (not significant) and GalNAc was present.

W10 was also compared to the mean monosaccharide content of all healthy controls which revealed significant ($p<0.01$) GalNAc concentrations in W10. No other significant differences in monosaccharide content were discovered as levels on Fuc, GlcNAc and Gal were similar in both groups.

Comparison of W11 to C11 revealed that W11 contained 57.5% less Fuc than the age-matched healthy control. W11 also had significant levels of GalNAc present ($p<0.01$) whereas C11 contained none. When examining GlcNAc content, W11 had almost four times as much GlcNAc as C11 but 43% less Gal (not significant).

W12 was examined against age-matched healthy control C12 and showed GalNAc presence (not significant) and GlcNAc levels elevated above C12. However, W12 Fuc levels were four times lower than C12 and unusually, Gal levels were significantly lower than the healthy control. GalNAc presence was not detected in C12.

Similarly, GalNAc was not detected in C13 upon analysis but was present in W13 (not significant). No significant differences were found between the control and “at risk” sample for any monosaccharide despite a 32% elevation of GlcNAc in W13 compared to C13.

An age-matched healthy control could not be recruited for “at risk” sample W14, thus comparisons were drawn between the mean monosaccharide content of all healthy controls and W14. Fuc, GlcNAc and Gal levels were comparable between W14 and healthy controls with minimal increases in W14’s GlcNAc and Gal content. However, a significant ($p<0.01$) level of GalNAc was detected in W14, with no GalNAc detected in the healthy control samples.

Similarly, comparison of W15 with the mean monosaccharide content of all healthy controls showed significant presence of GalNAc ($p<0.01$) in W15 but none detected in the healthy control samples. W15 also showed elevation of Fuc, GlcNAc and Gal by 26%, 37% and 18% respectively, though no differences were significant.

Lastly, as with W14 and W15, significant ($p<0.01$) levels of GalNAc were detected in W16 when compared to the mean monosaccharide content of all healthy controls. A similar trend followed, where Fuc, GlcNAc and Gal were elevated in comparison to the controls. As with previous comparison, no GalNAc was present in any of the control samples.

5.5 Discussion.

5.5.1 Introduction.

The aim of the research discussed in this chapter was to determine the concentrations of monosaccharides and investigate changes in monosaccharide concentrations from isolated AGP samples from two sample populations – Dataset 1 comprised samples from healthy individuals, women with BC of unknown type and women with TNBC as well as Dataset 2 containing samples from women at increased risk of BC development and age-matched healthy controls. This was performed using acid hydrolysis and analysis by HPAEC-PAD.

To date several studies have explored changes to AGP glycan composition in BC (Croce*, et al.*, 2005; Hashimoto*, et al.*, 2004; Turner*, et al.*, 1985) but none have investigated monosaccharide levels in TNBC. As well as increases in *in vivo* AGP concentration, unique changes in monosaccharide content of AGP's five glycans occurring in a disease-dependent manner could potentially be used as a biomarker for disease diagnosis.

As discussed in section 5.1, monosaccharide levels of AGP glycans from TNBC samples, healthy individuals and women with BC of an unknown type were analysed by HPAEC-PAD after subjection to acid hydrolysis. While the exact monosaccharide sequence cannot be determined from the cleavage of glycan chains into individual monosaccharides, variations in monosaccharide levels can be assessed for suggestions of structural changes. This can be used in conjunction with data collected from analysis of whole oligosaccharide chains (explained in Chapter 6, section 6.4) to better assess structural changes.

While used earlier to confirm reproducibility of the HPAEC-PAD technique, commercial AGP was not a suitable representation of a normal, healthy population to act as a negative control in this study. This is because the method used to isolate commercial AGP was an adaptation of a large-scale methodology by Hao and Wickerhauser (1973) which differed from that used in this study. Therefore, AGP was isolated from samples sourced from the Blood Transfusion Service to represent a healthy population and ensure consistent isolation methods across all sample groups, reducing the risk of unreliable results.

5.5.2 Trends in AGP Monosaccharide Content.

5.5.2.1 Dataset 1

Analysis of sample monosaccharide content using HPAEC-PAD revealed low levels of monosaccharides (Fuc, GlcNAc, Galactose and Mannose) in the healthy controls. In comparison, BC of unknown type (acting as a positive control) showed increased Galactose and Mannose content. GalNAc was not observed in any control samples.

Overall, TNBC samples showed increased Fuc, GlcNAc, Gal and Man content compared to both controls and interestingly, 88% of TNBC samples revealed the unusual presence of GalNAc which is associated with *O*-linked glycosylation (Varki, *et al*., 2009) and discussed later in section 5.5.2.4.

5.5.2.1.1 Monosaccharide Content of TNBC Categorised by Age.

When comparing monosaccharide content of TNBC samples by age, individuals <60 years of age showed increased concentrations of all monosaccharides monitored, including significantly ($p<0.05$) increased Galactose and GlcNAc ($p<0.01$ in the 35-60 age group) compared to both controls whereas TNBC samples from women >60 years old possessed increased GlcNAc and Mannose content compared to controls but had similar Galactose and Fuc content.

TNBC is a notoriously aggressive form of BC particularly in young women. Mean Fuc levels of TNBC samples from women aged <35 and 35-60 were double that of those >60. Although this difference was not significant, these results fit with the idea that increased Fuc levels correlate with poorer prognosis associated with TNBC at a younger age (Anders *et al*., 2008 and Hashimoto *et al*., 2004). Furthermore, increased fucosylation may be due to increased presence of SLe^X terminal structures which are linked to increased risk of metastasis (Julien *et al*., 2011 and Nakagoe *et al*., 2002).

The current study also demonstrated increased GlcNAc and Gal in samples from women <60 which also confers to current evidence that BC at a younger age is more aggressive and associated with a poorer prognosis. For example,

data from Smith *et al*., 2012 showed that increased Gal sample content was correlated with increased AGP glycan branching in malignant BC samples which could define malignant samples from non-malignant samples. Additionally, increased *N*-linked β1-6 branching has been linked to poor outcomes in BC patients, further associating poor prognosis with younger TNBC patients as increases in glycan branching were observed in this patient population during this study (Madjid *et al*., 2005 and Cui *et al*., 2011).

5.5.2.1.2 Monosaccharide Content of TNBC Categorised by Family History.

Examination of TNBC sample monosaccharide concentrations categorised by family history of BC revealed TNBC samples from individuals with family history had significantly elevated monosaccharide ($p<0.05$; GlcNAc $p<0.01$) content compared to both controls. Increased levels of GalNAc, GlcNAc and Galactose in were observed in TNBC samples from individuals with family history of BC in comparison to samples without family history of BC. Yet no significant difference in Fuc content was observed between samples with or without family history. Monosaccharide content of TNBC samples from women with unknown family history of BC (n=3) did not fall distinctly into either trend seen in family history or no family history. Galactose and Fuc content were similar to that of samples without family history, yet concentrations of GalNAc were comparable to sample with family history of BC. Therefore, this group may have contained samples that could have been assigned to either the family history or no family history group based on monosaccharide content. Of the 3 TNBC samples with unknown family history MT1178 contained a monosaccharide content that reflected the family history category while MT2621 and MT2305 monosaccharide content resembled that of the no family history group. However, the current study cannot definitively associate monosaccharide content with family history because no follow up of this sample population was available.

Increased Fuc content of these samples could indicate worse prognosis and risk of metastasis associated with TNBC's poor outcomes (Hashimoto *et al*., 2004; Julien *et al*., 2011 and Nakagoe *et al*., 2002). However, comparable

levels of Fuc between TNBC samples from women with and without family history or unknown family history may indicate similar levels of metastasis risk between these groups and reflect overall poor prognosis of TNBC rather than conferring more risk to those with family history.

However, elevated GalNAc and Gal content in TNBC samples from women with family history compared to those without family history could imply increased branching of AGP in these samples (Smith, *et al*., 2012). This could correlate with previous literature indicating poor prognosis associated with increased glycan branching (Smith *et al*., 2012; Madjid *et al*., 2005 and Cui *et al*., 2011) however a recent, large study by Malone *et al*., (2011) revealed patients with family history of BC had a better prognosis than women without family history. Therefore, it is unclear whether the increased GalNAc and Gal in TNBC samples from patients with family history act as prognostic indicators of survival.

5.5.2.1.3 Monosaccharide Content of Treated and Untreated TNBC Samples.

Furthermore, comparison of treated and untreated TNBC samples delivered similar trends in monosaccharide content and the concentrations of all monosaccharides were increased above both controls. Previous research by Doak (2008) using the same methods as this study showed that altered glycosylation during BC returned to a normal glycosylation pattern similar to the healthy control population upon treatment. This may indicate that treatment administered prior to blood draw had no effect on glycosylation composition or that these treatments have not had an effect on the TNBC and therefore the glycosylation composition remains unchanged.

5.5.2.1.4 Monosaccharide Content of TNBC Samples Grouped by TNM Stage.

In this study, the data on monosaccharide content of TNBC samples were also separated by cancer stage to allow comparison of TNBC at stage 1 and stage 2. Comparison of mean Fuc content in controls and TNBC by cancer stage revealed that stage 1 TNBC had increased Fuc concentration compared to

stage 2. These results did correspond with previous research (Hashimoto*, et al.*, 2004; Turner*, et al.*, 1985), which showed that levels of Fuc increased with increasing cancer severity. However, given that no samples of advanced stage TNBC were available to this study for comparison, it remains unknown if elevated Fuc levels would have been seen compared to early stage TNBC.

Furthermore, with only one TNBC sample representing stage 3 a reliable comparison with stage 1 and 2 mean GlcNAc and Gal content could not be made. Stage 1 and stage 2 TNBC showed similar levels of GlcNAc while stage 2 had slightly lower levels of Gal. Although no significant differences were found between TNBC stages there were significant differences ($p<0.05$) between GlcNAc and Gal in controls and TNBC stages. Healthy and BC of unknown type samples had significantly ($p<0.05$) lower levels of GlcNAc compared to stage 1 and stage 2 TNBC. Mean Gal levels were significantly ($p<0.05$) higher in stage 1 and stage 2 TNBC compared to healthy controls but not BC of unknown type. Thus implying that not only do both stages of TNBC exhibit greater AGP glycan branching but that this may be similar to that of BC of unknown type.

Increased GlcNAc and Gal content of TNBC samples compared to the positive and negative controls could indicate increased branching of AGP glycans in these samples. The higher the TNM stage attributed to a tumour the more developed the cancer is, the worse the prognosis and there may be increased metastasis compared to lower stage tumours (Sobin, *et al*., 2009). Hashimoto, *et al*., (2004) highlighted that patients with various advanced malignancies showed more tri- and tetra-antennary branching and these results were confirmed via mass spectrometry in a later study (Asao, *et al*., 2013). A separate study by Hamid, *et al*., (2008) revealed increased tri-antennary branching of AGP was linked to BC progression. Therefore, the results of the current study contradict previous research as higher GlcNAc and Gal concentrations in stage 1 TNBC infer increased branching at lower stage TNBC compared to stage 2 TNBC.

5.5.2.2 Dataset 2.

Analysis of the monosaccharide content of samples from women at increased risk of BC development and age-matched healthy controls revealed two trends (see Table 5.4). Eleven of the sixteen “at risk” samples had similar Fuc concentrations but increased GlcNAc and Galactose content compared to their age-matched healthy controls (Trend 1). Meanwhile, four of the sixteen “at risk” samples followed another trend when compared to their age-matched healthy controls, again showing similar Fuc levels and elevated GlcNAc; however these samples had decreased Galactose concentrations (Trend 2). On the other hand, sample W1 did not fit either trend because although Fuc concentrations were comparable to those found in the age-matched healthy control (C1), the GlcNAc content was decreased and the Galactose level was similar to the control.

Samples		
Trend 1	**Trend 2**	**No Trend**
↔ Fuc, ↑GlcNAc and ↑ Gal	**↔ Fuc, ↑GlcNAc and ↓ Gal**	**↔ Fuc, ↓GlcNAc and ↔ Gal**
W2	W6	W1
W3	W8	
W4	W11	
W5	W12	
W7		
W9		
W10		
W13		
W14		
W15		
W16		

Table 5.4 Monosaccharide Content Trends Observed in At-Risk Samples. Entries in the table highlight sample monosaccharide variation (↔ = comparable; ↑ = increased; ↓ = decreased levels compared to age-matched healthy controls) per trend 1 (↔ Fuc, ↑GlcNAc and ↑ Gal), trend 2 (↔ Fuc, ↑GlcNAc and ↓ Gal) or no trend (↔ Fuc, ↓GlcNAc and ↔ Gal).

Both trends revealed little variation in Fuc concentration between “at risk” samples and age-matched healthy controls. This is consistent with previous research, given both populations are theoretically disease-free and fucosylation

has been shown to increase during malignancy (Hashimoto *et al*., 2004). Fuc is usually incorporated into a glycan chain as a component of a terminal structure, such as SLe^x or bound to the pentasaccharide core via the innermost GlcNAc of *N*-linked glycans. Fuc may also be integrated directly into *O*-linked glycans by *O*-fucosyltransferases such as POFUT1 or *O*-FUT2 (Wang *et al*., 2001; Roos *et al*., 2002). Studies have revealed that increased fucosylation in cancer patients increases with the stage of malignancy and is associated with a poor post-operative outcome (Asao *et al*., 2013; Ferens-Sieczkowska*, et al.*, 2013; Hashimoto*, et al.*, 2004; Turner*, et al.*, 1985). Furthermore, Hamid *et al*., (2008) and Royle *et al*., (2008) established that increased fucosylation of a number of serum proteins, including AGP, was the most significant change detected in serum samples from breast cancer patients. This can be attributed to the altered functions of aberrant fucosylation during cancer. For example, increases in the expression of fucosyltransferase FUT 8 may enhance malignant cell proliferation (Chen *et al*., 2013) and increases in SLe^X terminal structures may denote enhanced metastasis (Julien *et al*, 2011). However, a study conducted by Moriwaki *et al*., (2009) found decreased fucosylation on established tumours aided evasion from NK cells. Therefore, the Fuc content from samples in dataset 2 may imply that immune evasion by cancer cells is occurring in these participants. However, as similarly low levels of Fuc were observed in both the "at risk" and age-matched healthy controls it is more likely that decreased Fuc content in these samples implies lack of malignancy.

Variation in Mannose concentration was also observed between certain "at-risk" samples and age-matched healthy controls, for example W1, having approximately 32% more Mannose than the corresponding control. Increased expression of high mannose glycans during BC has been observed (de Loez *et al*., 2011) and despite AGP being known to solely complex type glycans (see Figure 5.12), expression of high mannose type glycans during BC onset cannot be ruled out. For instance a study by de Loez *et al*., (2011) used a mouse model and human serum to demonstrate the presence high mannose *N*-linked glycans during BC which may infer incomplete trimming of glycans during processing which would normally result in expression of complex and hybrid *N*-linked glycans (see Figure 1.4).

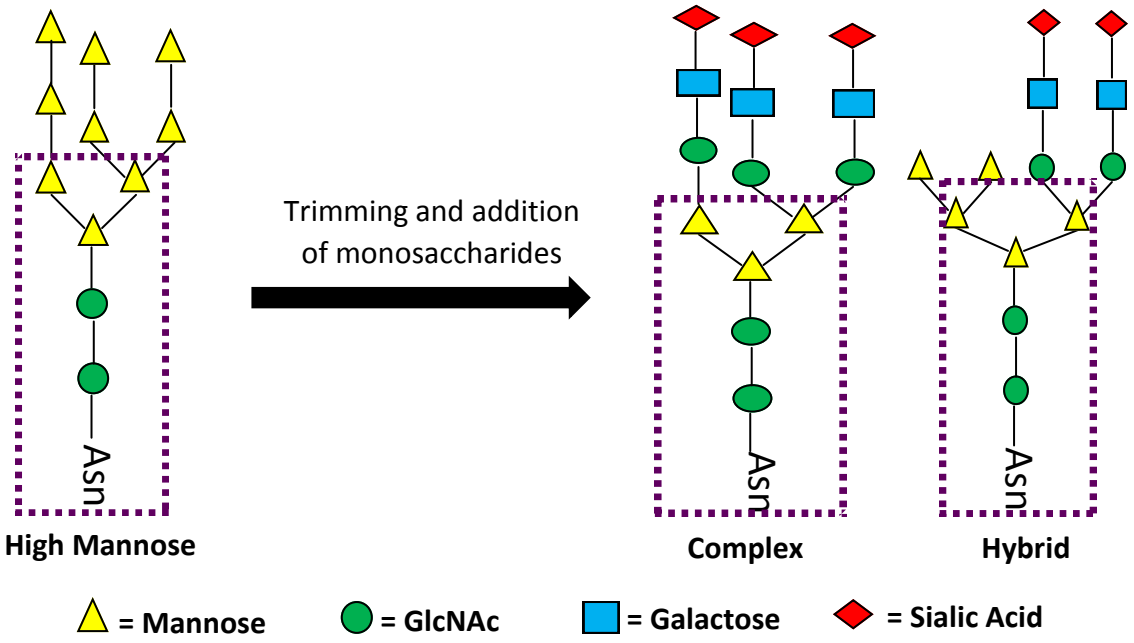


Figure 5.12 Illustration of Structural Differences Between High Mannose, Complex and Hybrid *N*-linked Glycan Types.

Moreover, inter-individual variation of GlcNAc and Galactose concentration was identified between age-matched healthy control samples with levels ranging from 0.333-1.251 mol monosaccharide/mol AGP and 0.798-1.448 mol monosaccharide/mol AGP respectively. Variation in GlcNAc and Galactose levels are associated with the degree of glycan branching. Galactose is present solely in the branches of *N*-linked glycans but GlcNAc is a component of the common pentasaccharide core as well as in glycan branches (Varki, *et al*., 2009. Therefore, changes in concentration in each of these monosaccharides are a more reliable indicator of alternative branching. Alterations in AGP glycan branching have been evaluated in a number of conditions such as cancer and inflammation (Higai *et al*., 2005, Smith *et al*., 2002, and Hashimoto *et al*., 2004). Studies have shown a decrease in branching during acute inflammation through the presence of increased bi-antennary glycans, yet glycans switch to displaying more tri- and tetra-antennary glycans during chronic inflammation (Higai *et al*., 2005; Fassbender *et al*., 1991 and Smith *et al*., 2002). In the present study, given that healthy volunteers self-selected to participate they may have been unaware of or offered incomplete information regarding possessing inflammatory conditions that may have implications for the quantification of AGP monosaccharide content. Therefore this possibly could account for decreased or increased levels of GlcNAc and Gal in certain age-

matched control samples. Since the age-matched healthy control samples for dataset 2 were collected fresh and purification began immediately, it is unlikely that ineffective storage may have caused alterations in AGP monosaccharide content of these samples (Hofbauerova, *et al*., 2003).

5.5.2.3 Protein Glycosylation in Cancer/Breast Cancer: Summary of the findings of the present study.

Previous research has highlighted various changes in glycosylation of proteins during cancer development (Dube and Bertozzi, 2005). The current study examined two sample populations – one with TNBC and the other at high risk of BC development due to family history and/or genetic mutations predisposing BC risk.

Monosaccharide analysis of TNBC samples revealed increased Fuc, GlcNAc, and Gal compared to healthy and BC of unknown type controls (see Table 5.2). Increased Mannose was also detected and possible reasons for this have been discussed in the previous section (5.5.2.2). The majority of TNBC samples (88%) had the unusual presence of GalNAc detected and the significance of this is discussed in section 5.5.2.4.

In contrast to this, investigation of monosaccharide content in at risk samples revealed two trends. Eleven samples conveyed trend 1 possessing similar levels of Fuc but increased GlcNAc and Gal compared to their age-matched healthy controls. Whereas 4 samples displayed trend 2 and showed similar Fuc content, increased GlcNAc but decreased Gal compared to age-matched healthy controls. Sample W1 monosaccharide content did not follow either of these trends as analysis revealed similar levels of Fuc and Gal with decreased GlcNAc compared to controls.

As discussed in previous sections (literature review sections as well as 5.5.2.1 and 5.5.2.2) changes in Fuc content have been noted in various cancers. Increased expression of FUT8 (a fucosyltransferase) was found by Chen, *et al*., (2013) during early stage cancer which may promote malignant cell proliferation through increased fucosylation. This correlates with data from previous studies that revealed increased fucosylation correlated with cancer severity and poor

prognosis (Asao, *et al*., 2013; Ferens-Sieczkowska*, et al.*, 2013; Hashimoto, *et al*., 2004; Turner, *et al*., 1985). However, a study by Moriwaki *et al*., (2009) highlighted a decrease in fucosylation once tumours are established which may protect tumours from destruction by NK cells. Thus the increased Fuc content seen in TNBC samples from dataset 1 may reflect that these early stage (TNM stage 1 and 2) samples are expressing increased Fuc to enhance the growth of tumours which may lead to a poor prognosis for the patient. This would align well with the current knowledge regarding TNBC's aggressive nature (Blows, *et al*., 2010; Dent, *et al*., 2007; Schnieder, *et al*., 2008). Meanwhile, the lack of change in Fuc content observed in at risk samples from the current study could indicate the creation of an immunosuppressive tumour environment through the avoidance of NK cell detection. If this is the case then use of Fuc as a marker of BC development in individuals at increased risk of BC may not be ideal.

Furthermore, alterations in GlcNAc and Gal content have been demonstrated during cancer (Asao, *et al*., 2013; Cui, *et al*., 2011; Hamid *et al*., 2008; Hashimoto, *et al*., 2004; Smith, *et al*., 2012) and changes in their levels are often correspond with changes in degree of branching (Behan, *et al*., 2013). Notably, increases in both monosaccharides taken together are a more reliable indicator of changes in branching as GlcNAc is also present in the common pentasaccharide core of *N*-linked glycans, whereas Gal is only present in glycan branches (Varki, *et al*., 2009; see Figures 1.5 and 5.12). A study by Hashimoto, *et al*., (2004) and a separate study by Asao, *et al*., (2013) showed correlation between increases in glycan branching and cancer stage. Similarly, research has shown that tri-antennary glycans are predominantly displayed as BC progresses (Hamid, *et al*., 2008). Furthermore, increases in *N*-linked β1-6 branching were demonstrated to correlate with increased mortality during cancer in studies by Majid, *et al*., (2005) and Cui, *et al*., (2011). In light of these previous findings, the increases in GlcNAc and Gal content observed in TNBC samples and eleven at risk samples (Trend 1) could be indicative of BC progression and poorer prognosis. However, at risk samples displaying trend 2 (increased GlcNAc and decreased Gal compared to controls) could suggest less glycan branching within these samples and potentially a better prognosis if the participant develops BC or even less risk of BC development to those displaying trend 1.

5.5.2.4 *O*-glycosylation of AGP: considerations for the present study.

For decades mature adult AGP has been described as solely expressing 5 complex *N*-linked glycans (Yoshima, *et al*., 1981). Despite this, GalNAc (a known component of *O*-linked glycans) was detected in 81% of TNBC sample and 88% of “at-risk” samples while no GalNAc was detected in any of the negative or positive control samples.

GalNAc is a known component of *O*-linked glycans through its addition to primary alcohol hydroxyl groups in the side-chains of Ser/Thr residues as the starting point for the addition of further monosaccharide moieties to create *O*-linked glycan chains (Varki, *et al*., 2009). As AGP contains Ser/Thr amino acids within its protein backbone sequence the possibility exists for AGP to possess *O*-linked glycans (see Figure 1.7). In fact during foetal development, AGP expresses 3 *N*-linked glycans and 3 *O*-linked glycans with the *O*-linked glycans subsequently being lost during development (Shiyan and Bovin, 1997).

Studies have revealed that certain proteins revert to foetal glycan expression during cancer due to rapid proliferation during tumour development (Sapra, *et al*., 2012; Farinati, *et al*., 2006). This is known as oncofoetal antigen expression and has been shown to occur in glycoproteins such as AFP and CEA (Farinati, *et al*., 2006 and Amri, *et al*., 2013). Thus, it is possible that the detection of GalNAc is the majority of TNBC and “at risk” samples could be due to AGP expressing oncofoetal antigens.

Additionally, as GalNAc is not a constituent of the pentasaccharide core perhaps GalNAc was integrating into AGP glycan branches. Previous studies investigating AGP glycosylation have found the presence of GalNAc in a variety of patient populations. Anderson*, et al.* (2002) reported 64% of AGP samples from individuals with hepatitis C contained GalNAc, however GalNAc was not present in cirrhosis samples leading them to speculate that disappearance of GalNAc from samples could indicate progression of liver disease to a cirrhotic state. When investigating AGP glycosylation in fibrosis patients, Mooney*, et al.* (2006) detected GalNAc in 34% of samples. Both studies had limited sample numbers meaning no investigation into GalNAc presence could be undertaken. However, both groups hypothesised that GalNAc presence could be due to the attachment of a terminal structure on AGP glycans. This theory was

investigated by Gallacher (2009) after GalNAc was detected when examining AGP glycosylation in benign, invasive and non-invasive BC samples. Benign and invasive BC samples had GalNAc present therefore immunodiffusion was used to investigate whether this was due to addition of terminal glycan structures. Blood group antigen A and sialyl Tn were tested for as they are the most common GalNAc containing terminal structures. However, neither were detected indicating neither of these terminal structures were responsible for GalNAc presence in Gallacher's samples. Unfortunately, it was not feasible as part of this study to investigate the source of GalNAc within TNBC samples. Nevertheless, in contrast to this study, Gallacher (2009) found an association between GalNAc presence and ER/PR positivity in tumours whereas this study only found GalNAc in TNBC samples and not BC of unknown type samples.

A number of TNBC samples showed elevated levels of GalNAc in conjunction with decreased levels of Fuc and vice versa (see Table 5.2). Therefore, it may be possible that GalNAc is binding in place of Fuc in AGP glycans. However, no significant differences were found among GalNAc content when comparing TNBC to controls by age. Contrary to this, significant differences ($p<0.05$) were seen in GalNAc levels between TNBC with family history of BC and TNBC with unknown family history status compared to healthy and BC of unknown type controls samples. GalNAc values were also significantly ($p<0.05$) higher in treated TNBC samples compared to both controls.

Additionally, when investigating monosaccharide content in TNBC cancer stages, stage 1 TNBC showed significantly ($p<0.05$) higher levels of GalNAc than healthy and BC of unknown type controls. Thus GalNAc was not only present in TNBC samples but was significantly different when examining the data in different categories.

AGP expression of *O*-glycans and hence detection of GalNAc links to recent literature regarding *O*-glycosylation of other proteins being involved in TNBC. For example, Milde-Langosch, *et al*., (2014) revealed that upregulation of *O*-glycosylation related enzymes were associated with TNBC subtype and metastasis. The group hypothesised that since a number of ligands are composed of *O*-linked glycans, increases in their expression could increase the movement and binding of malignant cells away from the primary tumour site. Similarly, elevated expression of MUC1 (an *O*-glycosylated protein) has been

shown in TNBC and increases were associated with tumour grade (Siroy, *et al*., 2013). Studies have revealed that MUC1 may promote tumour growth by inhibiting malignant cell apoptosis (Wei, *et al*., 2005) and enhance metastasis via epithelial-mesenchymal transition (Schroeder, *et al*., 2003 and Roy, *et al*., 2011). Consequently, detection of GalNAc and its association with *O*-glycosylation from TNBC samples in this study - such as F5412, MT3965, MT1178 and MT3601 which contained high levels of GalNAc - could indicate that these patients have a higher risk of metastasis and aggressive tumour growth which is already linked to TNBC (Blows, *et al*., 2010; Dent, *et al*., 2007; Schnieder, *et al*., 2008).

5.5.3 Benefits and Shortcomings in the Methodology Used.

The current study used the well-documented and highly sensitive technique, HPAEC-PAD, (Behan and Smith, 2011; Hardy and Townsend, 1988; Harazono, *et al*., 2011; Smith, *et al*., 2012; Townsend, *et al*., 1995) for the quantification of monosaccharides within purified AGP samples after acid hydrolysis. A benefit of both techniques is that they are non-discriminant - acid hydrolysis will release all monosaccharides from the protein backbone (Behan, 2010) just as HPAEC-PAD will detect all monosaccharides in a sample (Smith, *et al*., 2012; Harazono, *et al*., 2011; Behan and Smith, 2011; Townsend, *et al*., 1995). A further benefit of these techniques is that samples do not require derivitization to enhance detection as this would alter the structures of the monosaccharides (Lee, 1990). Use of commercial monosaccharide standards of Fuc, GalNAc, GlcNAc, Gal and Man with HPAEC-PAD enabled the identity and consistent elution time (retention times) of each monosaccharide to be established prior to each batch of samples being analysed (see Figure 5.2). Additionally, 5 commercial AGP samples were subjected to acid hydrolysis and HPAEC-PAD to confirm the reliability and reproducibility of the method (see Figure 5.4).

However, as with AGP concentration analysis, inadequate sample storage may have affected the monosaccharide content of AGP in dataset 1. The samples were purchased prior to the study commencing and it was acknowledged that post-translational modifications such as glycosylation may degrade if stored at -20°C instead of -80°C (Davies, 1968). Dataset 2 samples were processed

immediately and not stored for long periods of time, therefore, storage is unlikely to have affected the monosaccharide content in these samples.

Additionally, while monosaccharide standards were analysed prior to each batch of sample investigation, other controls such as commercial AGP could have been subjected to acid hydrolysis and HPAEC-PAD to ensure contamination was not occurring. However, commercial AGP is expensive and with a limited budget for this study it was not feasible to use commercial AGP as a control.

5.5.4 Additional Methodologies to Study Glycan Content.

Additional methodologies could, in principle, have been used throughout this study to support and confirm the results obtained. However, due to the small budget and small sample volumes this was not feasible.

Initially, given greater sample quantities, basic techniques could have been utilised to confirm removal of glycans from AGP by acid hydrolysis or to demonstrate increased branching within AGP samples. SDS-PAGE separates proteins by size on a gel through application of an electrical current and the protein bands can be visualised using stains (Laemmmli, 1970). Samples could have been analysed on an SDS-PAGE gel pre and post acid hydrolysis to detect the reduction of molecular mass that would have indicated successful removal of glycans from the protein. Futhermore, a periodic acid-Schiff stain, such as that proposed by (Hart, *et al*., 2003), could have been used in conjunction with SDS-PAGE and a nitrocellulose blot to measure glycosylation of AGP pre and post acid hydrolysis. Yet recovery of the AGP protein bound to Dowex-50 WX8 H^+cation exchange resin to enable separation of proteins and monosaccharides after acid hydrolysis proved difficult. Therefore, this along with the cost implication was a factor in choosing not to use such techniques. Likewise, analysis of each sample using SDS-PAGE could have indicated increased monosaccharide content and glycan branching as these samples would have possessed a higher molecular mass than their counterparts with lower monosaccharide content. Thus, samples with a higher molecular mass due to increased monosaccharide content would not have migrated as far through a SDS-PAGE gel, giving rise to different banding for each sample.

However, due to limited sample volume this was not practical. That said, the acid mediated hydrolysis of carbohydrates and glycoproteins is a well-accepted technique for oligosaccharide degradation and monosaccharide analysis (Manzi, 2001).

More specialised techniques could also have been used. For example, use of an ELISA specific for Fuc would have enabled confirmation of Fuc content of AGP isolated from samples (Olewicz-Gawlik, *et al*, 2007; Ryden, *et al*., 2002) while mass analysis of sample monosaccharides could have been carried out using various mass spectrometry methods after separation by HPAEC to quantify monosaccharide content (Hammad, *et al*., 2010; Yuan, *et al*., 2005; Zhu and Sato, 2007).

5.6 Summary Statement.

To conclude, this chapter accomplished its aim to determine the monosaccharide concentrations of AGP from blood samples using acid hydrolysis and assess variation in monosaccharide concentrations from different sample populations used in this study by HPAEC-PAD.

Dataset 1 showed low levels of all monosaccharides in the healthy negative control population as expected while BC of unknown type showed increased levels of Gal. Interestingly over 88% of TNBC samples showed the unusual presence of GalNAc which inversely correlated with age whereas other monosaccharide contents showed no relationship with age. GalNAc was also decreased in TNBC samples with no family history or unknown family history compared to TNBC samples with family history of BC. Furthermore, dataset 2 revealed that AGP glycans in women “at risk” of BC are composed of more monosaccharides than their healthy control counterparts thus indicating an increase in branching. The reason for this alteration remains unclear as theoretically both groups are healthy; the only difference being that one group is at increased risk of BC development. One explanation for this is that women at risk of BC persistently have altered AGP glycosylation compared to women not “at risk”. Another is that, samples such as W2, W3, W5, and W7 that possess GlcNAc and Gal contents greatly higher than those of their healthy controls may

be a sign of BC development. Furthermore, GalNAc presence in *N*-linked glycans, such as those is AGP, is unusual as it is more commonly present in *O*-linked glycans. Therefore, its presence in 81.2% of “at risk” samples may indicate BC development.

Chapter 6 – Oligosaccharide Analysis Results and Discussion.

6.1 Introduction.

Alterations to AGP oligosaccharide chain structure may occur during a disease state (Hashimoto*, et al.*, 2004; Turner*, et al.*, 1985). These structural changes can be used in conjunction with monosaccharide data to evaluate glycosylation changes in AGP that could be unique to BC.

HPAEC-PAD was implemented to separate and detect oligosaccharide chains, after cleavage from the polypeptide backbone of AGP using PNGaseF, due its highly reliable and sensitive capabilities (Hardy*, et al.*, 1988). Similar to monosaccharide analysis, HPAEC detection sensitivity was greatly improved by the addition of PAD to purge the gold electrode of contaminants between sample analyses. Oligosaccharide separation is reliant on interactions between the negatively charged carbohydrate molecules (due to pH13 mobile phase) and the positively charged stationary phase. The strength of interaction oligosaccharide interaction with the stationary phase is determined by negatively charged terminal 9-carbon carboxylated SA residues combined with the negative charge of component monosaccharides under alkaline conditions. Therefore (as shown in section 6.2 and 6.4) oligosaccharide chains with varying terminal SA residues and negative charge contribution from monosaccharide content, results in different elution times for each oligosaccharide chain. For instance a bi-antennary oligosaccharide chain with only one terminal SA residue will have a lower overall negative charge and will elute earlier than a tri-antennary oligosaccharide chain with three terminal SA residues. Therefore, separation is enhanced due to the formation of oxyanions under strong alkaline conditions, resulting in varying pKa values of monosaccharides within the chain to allow differentiation between glycan structures. Thus a “fingerprint” of the extent of branching can be seen using HPAEC-PAD without prior derivatisation of samples (Lee, 1990). As such, HPAEC-PAD has remained a valuable and sensitive technique for structural analysis of glycans after their removal from the protein portion using enzymes or chemicals.

Separation of oligosaccharide chains during HPAEC-PAD necessitates the removal of glycans bound to the anion exchange resin within the column. This removal is facilitated by an increasing linear gradient of NaOAc which acts as a pushing agent while 100mM NaOH provides an alkaline pH. The NaOAc

gradient is increased from 50-250 mM over a period of 30 minutes causing displacement of glycans from the column surface as the NaOAc competes for the anion-exchange resin. Increasing the NaOAc gradient to 250mM ensures the elution of all oligosaccharide chains including the highly branched and sialylated glycans which form the strongest bond with the column resin and hence are most difficult to remove. SA exists in 2 main forms that differ by a single OH group being present or absent, known as *N*-acetylneuraminic acid (NeuAc) and *N*-glycolneuraminic acid. Behan (2010) revealed that HPAEC-PAD is capable of separating these two SA forms by over 10 minutes in spite of the minute structural difference, further highlighting the sensitivity of the technique.

During analysis of glycan structure using HPAEC-PAD, similar oligosaccharide chains which vary only by the extent of sialylation will separate out into distinct charged bands due to considerably different overall negative charges of each structure. Additionally the size of glycans will affect elution time within a charged band. Therefore, glycan chains with increased monosaccharide content will have a later elution time than glycan chains with a lower monosaccharide content but same degree of sialylation. For instance, if mono-sialylated bi-, tri- and tetra-antennary chains were analysed simultaneous, this would be the order in which they elute. This is because glycans with greater monosaccharide content will contain higher numbers of carbon atoms and thus have more –OH groups and negative charge causing an increase in retention time through a stronger interaction with the stationary phase resin (Paskach*, et al.*, 1991). Similarly the reverse is also true whereby a highly branched chain with incomplete sialylation may elute prior to a less branched structure with full sialylation, as the overall charge of the former will be less negative due to missing SA residues. However, this would be a rare occurrence as lack of SA terminal structures usually results in degradation of the molecule, due to Gal being exposed at the terminus. If another terminal structure was present, thus hiding Gal, degradation would not occur.

Furthermore, separation of glycan chains can be affected by different linkages within chain structures. Townsend*, et al.* (1988) discovered that variation between linkages connecting an SA terminal residue to a glycan chain can increased retention time. An SA residue can bind to the outer most Gal of a glycan branch via an α-(2,3) or α-(2,6) linkage, with greater expression of α-

(2,3) giving a greater elution time due to increased interaction with the column. Column interaction is increased because if an α-(2,6) linkage binds SA as a terminal structure, the oxyanion effect at the *C*6 $-O^-$ group of Gal is removed as that carbon is facilitating the attachment of SA. Absence of this additional negative charge allows the glycan chain to elute faster than if an α(2,3) linkage occurred because an α(2,3) linkage will not utilise the oxyanion group of *C*6 (Townsend*, et al.*, 1989). Additionally the group also found when Gal is bound to the pentasaccharide core via a β-(1,4) bond to GlcNAc, the elution time is reduced compared to a β-(1,3) linkage, due to less association of the -OH groups with the column resin at pH13 during analysis. This allows separation of glycans when these linkages differ (Townsend*, et al.*, 1989; Townsend*, et al.*, 1988).

6.2 Dataset 1 Oligosaccharide Results.

Oligosaccharide analysis was carried out using HPAEC-PAD, as described in chapter 2, to provide a fingerprint of the extent of AGP glycan branching within samples and assess any alterations between sample groups. HPAEC-PAD initially separates glycans into specific charged bands. Eluting first are glycans expressing one SA residue (10-20 minutes), secondly are bi-sialylated glycans (20-30 minutes), followed by tri-sialylated (30-40 minutes) and tetra-sialylated glycans (40-50 minutes). Figure 6.1 illustrates an *N*-linked oligosaccharide (5µg) library with groups of bi-, tri- and tetra-sialylated chains labelled at the expected time ranges as described by the manufacturer. This *N*-linked library chromatogram was used as a comparison for all samples to enable identification of sialylation of AGP branches. Unlike monosaccharides, which can be quantified using calibration curves, oligosaccharides cannot be quantified as no pure forms for use as a reference were available. Commercially sourced AGP was not used for comparison due to the different isolation method used.

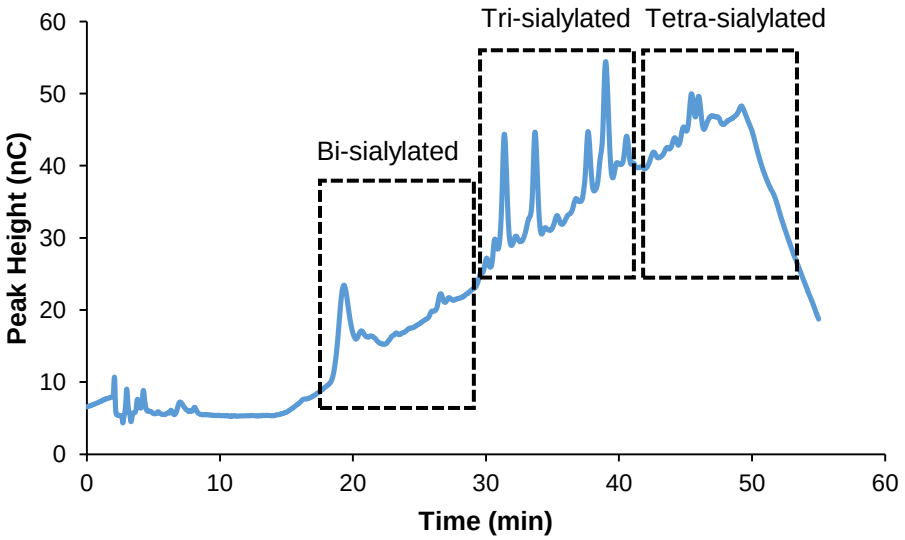


Figure 6.1 *N*-linked Oligosaccharide (5µg) Library. Illustrates the separation of varying degrees of branching as distinct charged bands, each labelled with their SA content: bi-sialylated, tri-sialylated and tetra-sialylated.

In the case of dataset 1, oligosaccharide chromatograms were collect from TNBC samples (n=18), BC of unknown type (n=5; positive control) and "normal" healthy individuals (n=5; negative control). As this method was not quantitative there was no requirement for sample repeats unless an erroneous chromatogram was produced, additionally there was only enough isolated AGP from TNBC samples for a single oligosaccharide analysis to be performed.

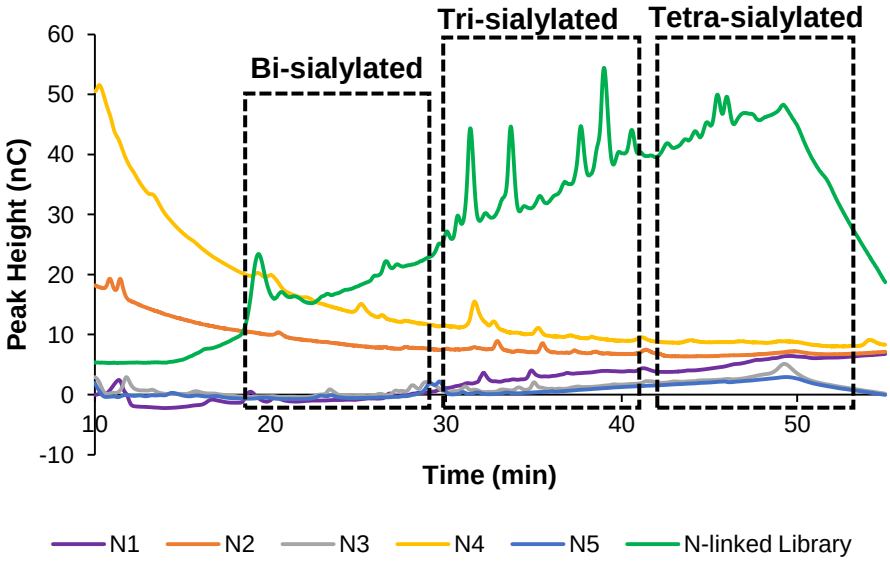


Figure 6.2 Oligosaccharide Chromatogram of "Normal" Healthy Controls Compared to an *N*-linked Oligosaccharide (5µg) Library.

Oligosaccharide analysis of “normal” healthy controls and comparison to the *N*-linked library revealed a tendency towards bi-sialylated and tri-sialylated glycan branches. The majority of peaks lay in the tri-sialylated time range, followed by bi-sialylated and lastly tetra-sialylated. Most of the healthy controls showed only one or two peaks in the tetra-sialylated time-frame compared to several in the tri-sialylated time-range.

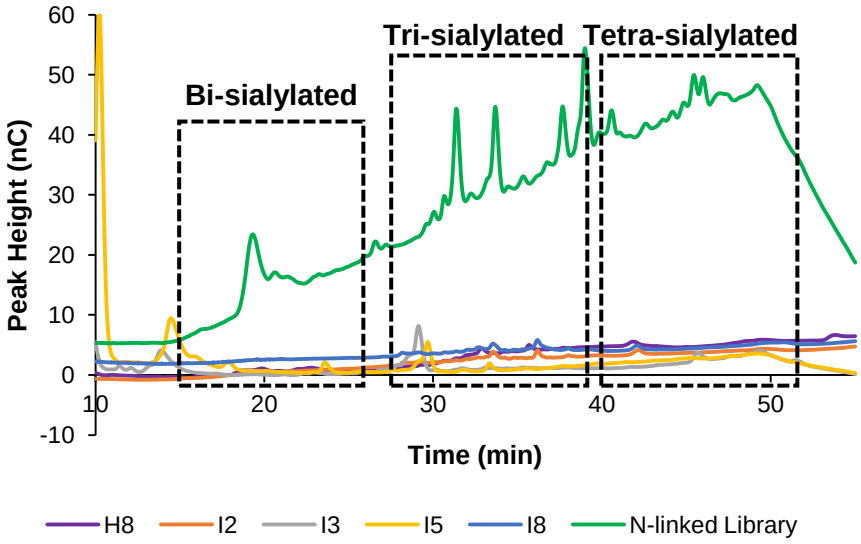


Figure 6.3 Oligosaccharide Chromatogram of BC of Unknown Type Controls Compared to an *N*-linked Oligosaccharide (5µg) Library.

Use of BC of an unknown type as a positive control highlighted variations in glycan branching compared to “normal” healthy samples (negative control). Comparison to the *N*-linked library standard revealed BC of an unknown type had an increase in tri-sialylated and tetra-sialylated branches with very few peaks detected in the bi-sialylated time range.

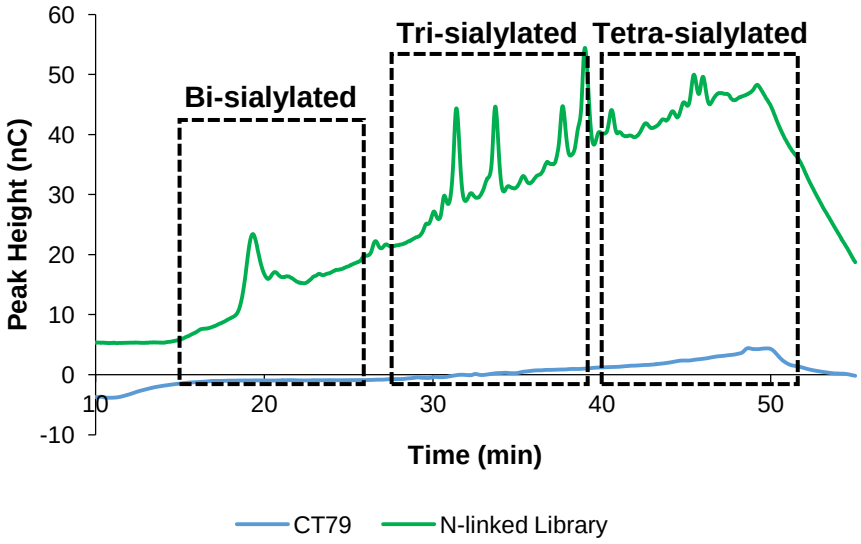


Figure 6.4 Oligosaccharide Chromatogram of TNBC Sample CT79 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Very few peaks were detected in TNBC sample CT79 but magnification of the chromatogram revealed peaks within the tri- and tetra-sialylated time ranges. No peaks were detected in the bi-sialylated time range. This right shift indicates increased branching within sample CT79.

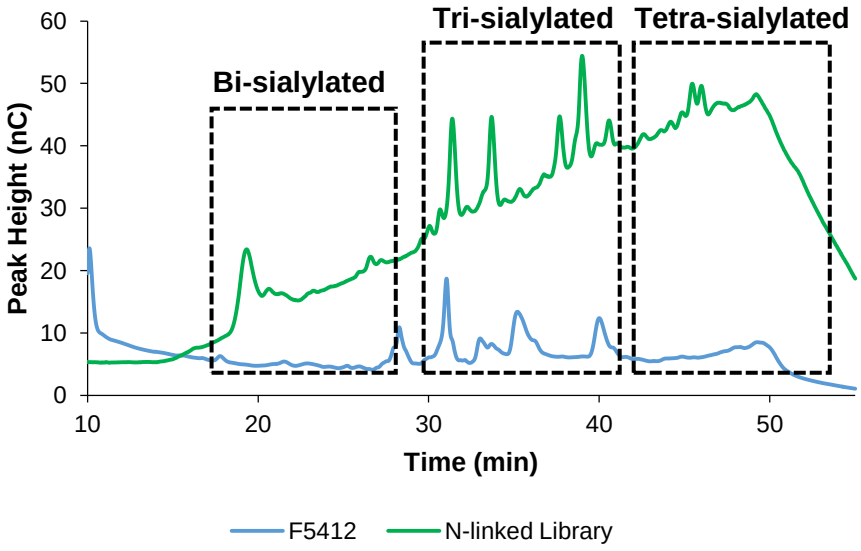


Figure 6.5 Oligosaccharide Chromatogram of TNBC Sample F5412 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample F5412 showed a significant number of tri-sialylated glycans with a smaller number of peaks in the bi- and tetra-sialylated area. Again this

showed an increase in branching similar to that of BC of unknown type as opposed to low levels of branching seen in healthy samples.

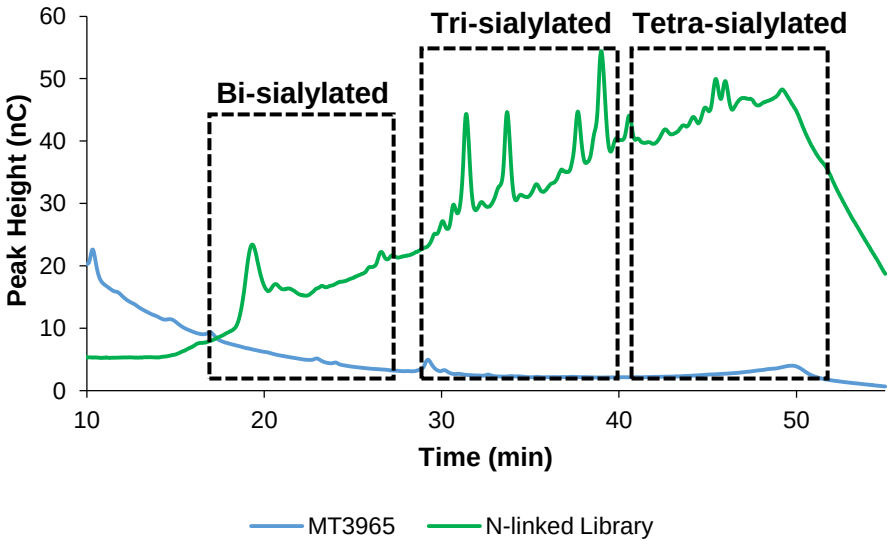


Figure 6.6 Oligosaccharide Chromatogram of TNBC Sample MT3965 Compared to an *N*-linked Oligosaccharide (5µg) Library.

A lower level of branching was seen in TNBC sample MT3965 resembling the extent of branching found in normal samples. No peaks were detected in the tetra-sialylated time range. However, bi-sialylated and tri-sialylated peaks were detected in the corresponding time frames, thus suggesting a lower degree of branching.

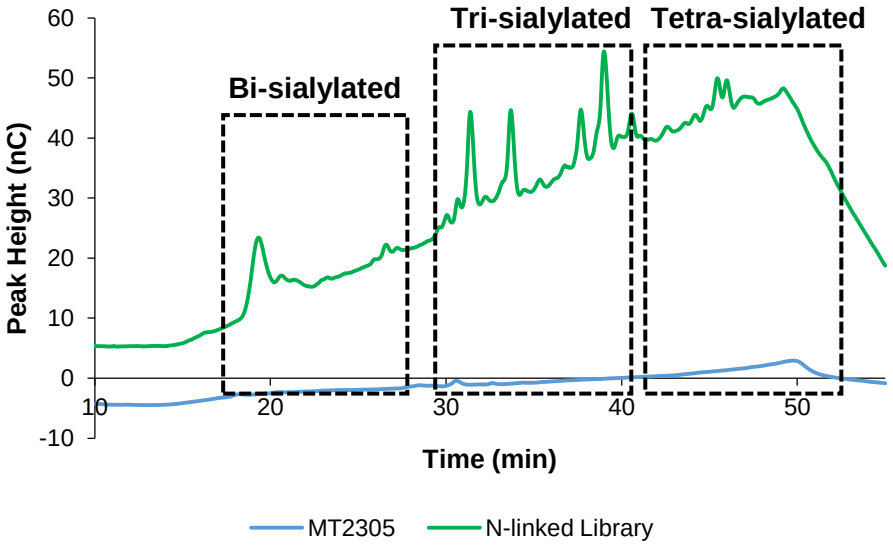


Figure 6.7 Oligosaccharide Chromatogram of TNBC Sample MT2305 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Oligosaccharide analysis of TNBC sample MT2305 highlighted similar oligosaccharide branching to that of TNBC sample MT3965 in that tri-sialylated branching was most prominent with few bi-sialylated branches and no tetra-sialylated branches detected. This degree of branching most closely reflects that of the normal population in this dataset.

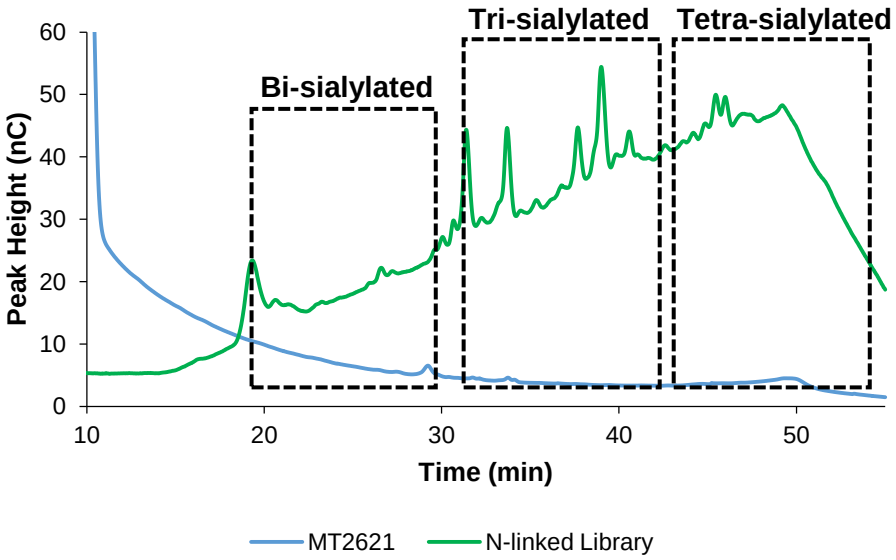


Figure 6.8 Oligosaccharide Chromatogram of TNBC sample MT2621 Compared to an *N*-linked Oligosaccharide (5µg) Library.

In TNBC sample MT2621 mostly tri-sialylated glycans were detected with three peaks detected late in the bi-sialylated time range. Again this corresponded with healthy samples' AGP glycan branching and is similar to that of samples MT3965 and MT2305 which both have less branching of glycans.

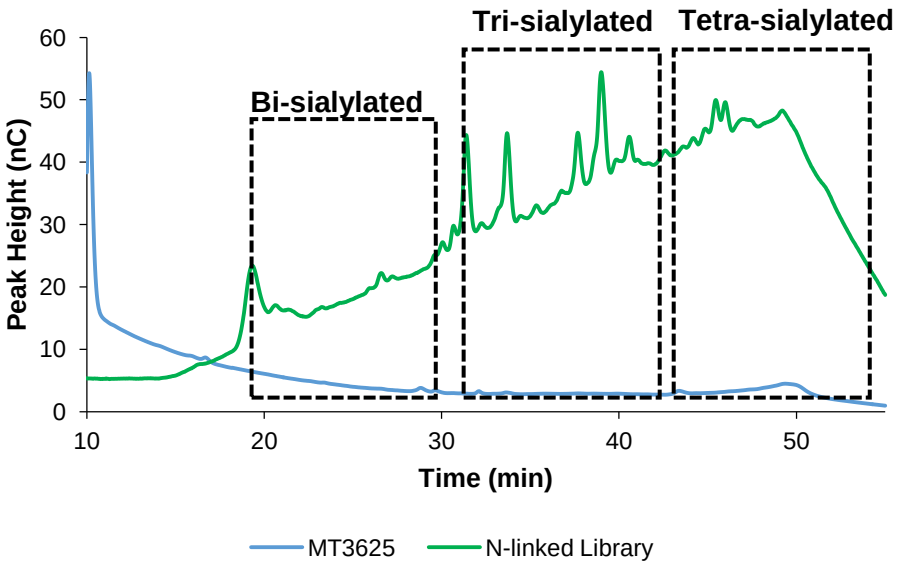


Figure 6.9 Oligosaccharide Chromatogram of TNBC Sample MT3625 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample MT3625 showed a similar oligosaccharide profile to MT3695, MT2305 and MT2621 whereby late bi-sialylated branching is detected as well as tri-sialylated branching. However, a peak was detected in the tetra-sialylated time range indicating the presence of a glycan with a higher degree of branching that the previous samples and healthy control samples.

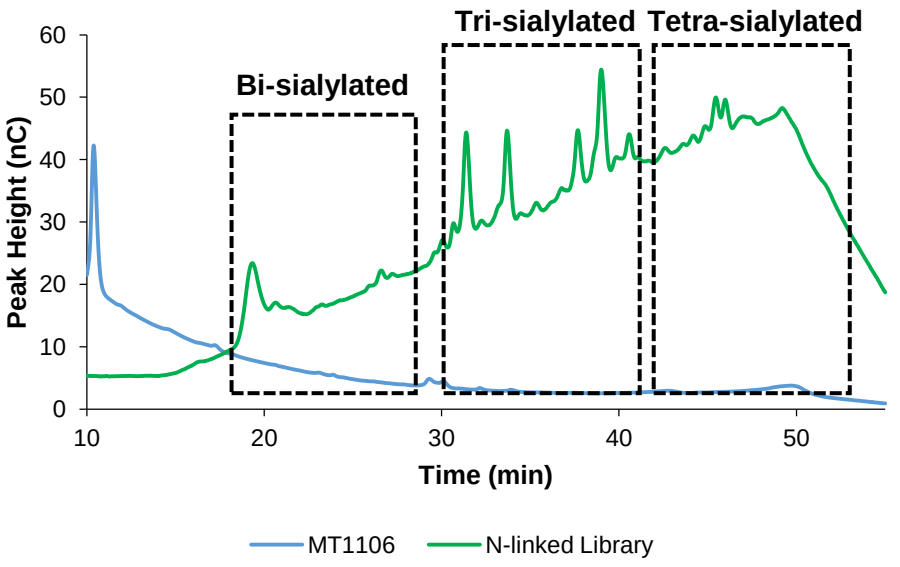


Figure 6.10 Oligosaccharide Chromatogram of TNBC Sample MT1106 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample MT1106 had a mostly tri-sialylated glycans with a small number of bi-sialylated peaks detected which became apparent upon magnification of the chromatogram. There was also a minor peak detected in the tetra-sialylated time area.

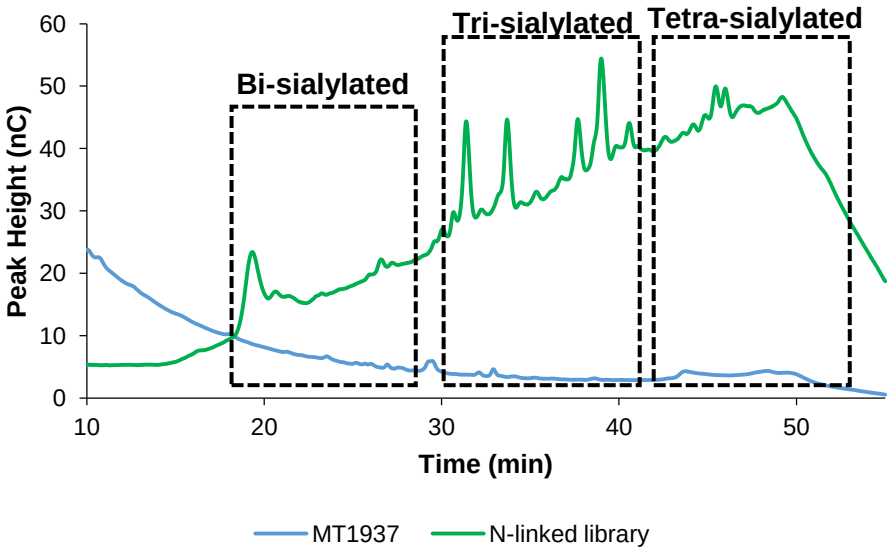


Figure 6.11 Oligosaccharide Chromatogram of TNBC sample MT1937 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Tetra-sialylated branching was identified in TNBC sample MT1937 but the majority of glycans still appeared in the tri-sialylated and bi-sialylated region of the chromatogram. However the chromatogram still indicated that that an increase in glycan branching was present in this sample, similar to that of the positive control.

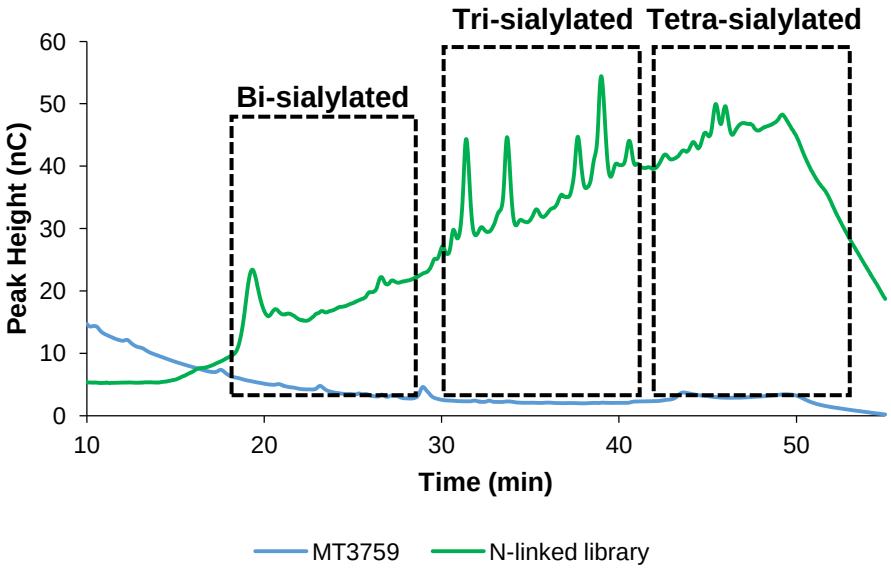


Figure 6.12 Oligosaccharide Chromatogram of TNBC Sample MT3759 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample MT3759 displayed an oligosaccharide profile with mainly bi- and tetra-sialylated glycans present. No peaks were detected in the tri-sialylated time period. Therefore, MT3759 appeared to have an increase in the degree of AGP glycan branching.

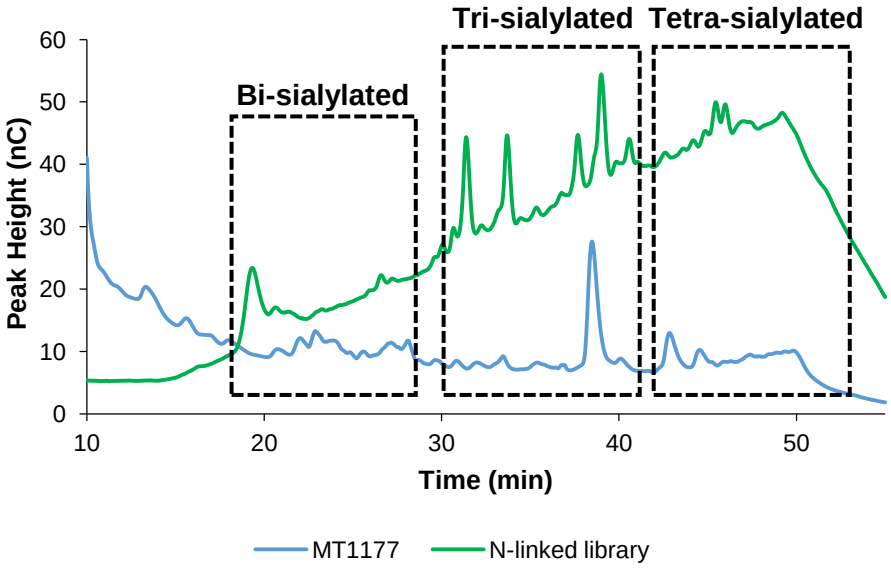


Figure 6.13 Oligosaccharide Chromatogram of TNBC Sample MT1177 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Interestingly, TNBC sample MT1177 showed a very clear oligosaccharide profile featuring a variety of types of glycan branching. Bi-sialylated and tri-sialylated branching featured more prominently than tetra-sialylated. However,

the presence of tetra-sialylated glycans indicated an increase in the degree of branching not commonly seen within the other TNBC samples.

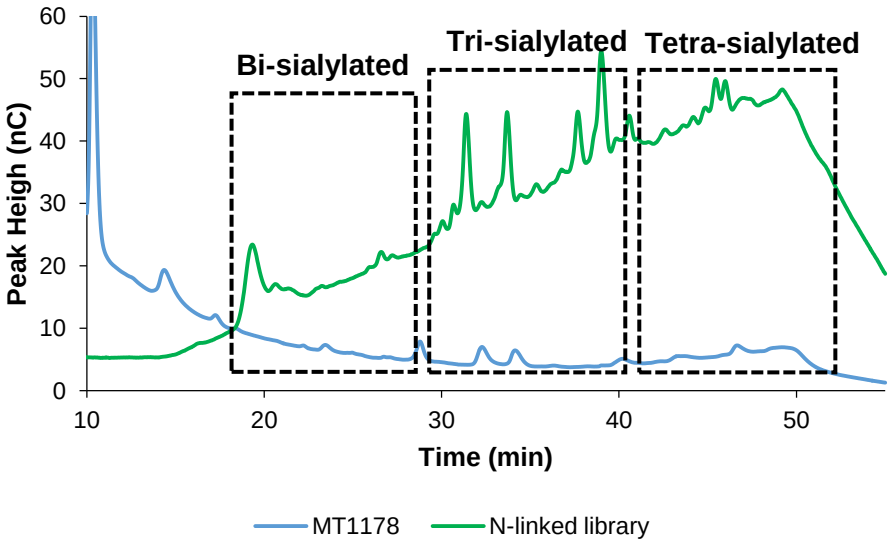


Figure 6.14 Oligosaccharide Chromatogram of TNBC Sample MT1178 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample MT1178 clearly exhibits all identified types of glycan branching with peaks visible in the bi-, tri and tetra-sialylated regions of the chromatogram. Peaks prior to the bi-sialylated area also indicated presence of mono-sialylated bi-antennary glycans. Therefore, MT1178 possessed a wide variety of glycan branching with larger peaks in the tri-sialylated time frame suggesting a preference for this level of glycan branching.

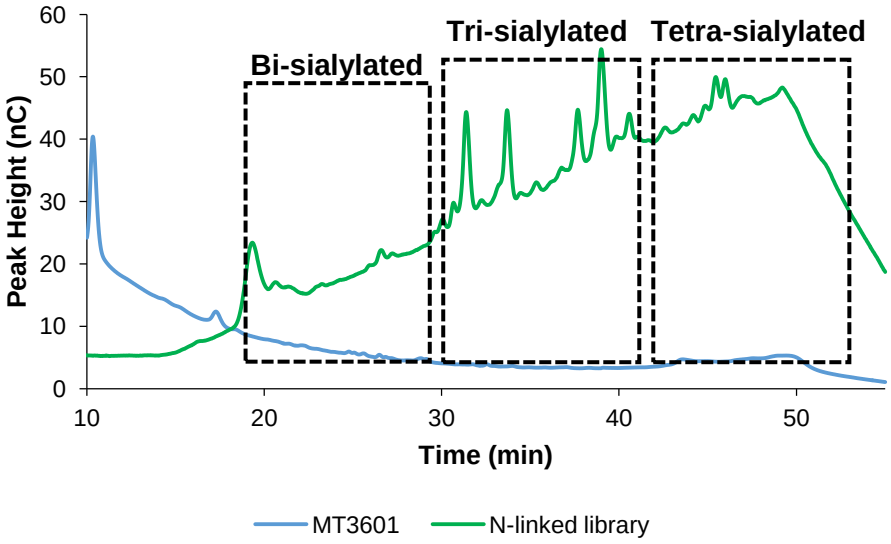


Figure 6.15 Oligosaccharide Chromatogram of TNBC Sample MT3601 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Oligosaccharide analysis of TNBC sample MT3601 highlighted larger peaks arising in the mono-sialylated, bi-sialylated and tetra-sialylated regions of the chromatogram. No peaks were detected in the tri-antennary region suggested that AGP isolated from this participant favoured a lesser degree of branching.

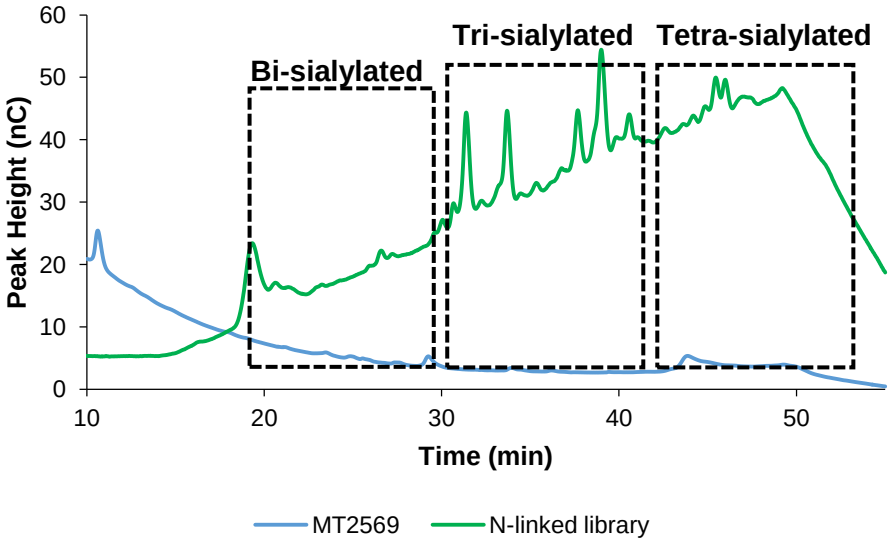


Figure 6.16 Oligosaccharide Chromatogram of TNBC Sample MT2569 Compared to an *N*-linked Oligosaccharide (5µg) Library.

A preference for bi-and tri-sialylated glycans was shown by TNBC sample MT2569 with only one peak being detected in the tetra-sialylated region of the

chromatogram. Again, indicating a lesser degree of branching displayed by TNBC samples.

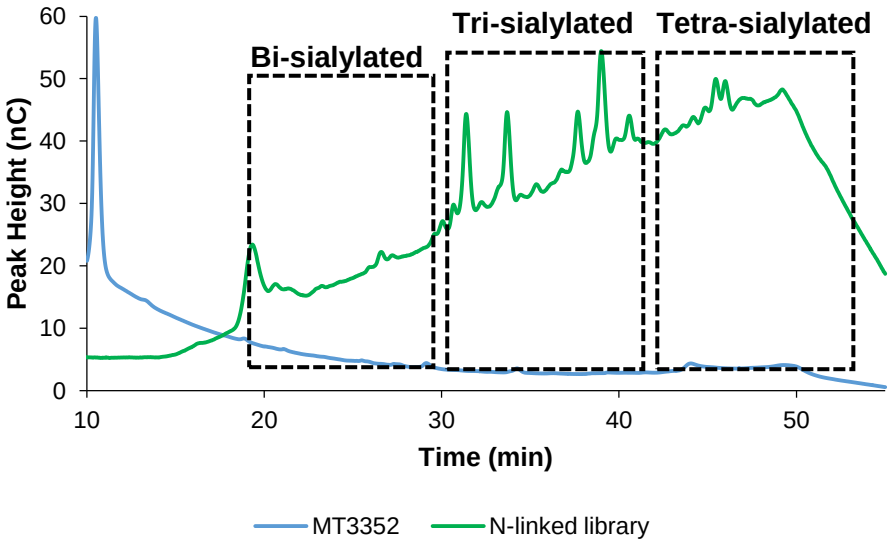


Figure 6.17 Oligosaccharide Chromatogram of TNBC Sample MT3352 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Analysis of TNBC sample MT3352's branching highlighted a shift towards more highly branched AGP glycan chains with an increase in peaks detected in the tri- and tetra-sialylated time frames of the chromatogram.

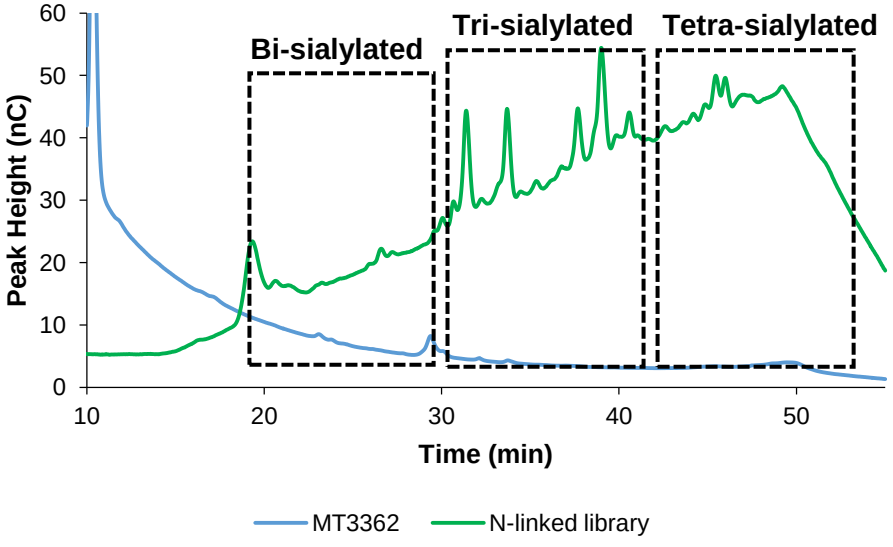


Figure 6.18 Oligosaccharide Chromatogram of TNBC Sample MT3362 Compared to an *N*-linked Oligosaccharide (5µg) Library.

TNBC sample MT3362 displayed a preference for bi-sialylated and tri-sialylated glycans with no peaks present in the tetra-sialylated region of the

chromatogram. Therefore, there was a predominance of less highly branched AGP glycan chains in this sample.

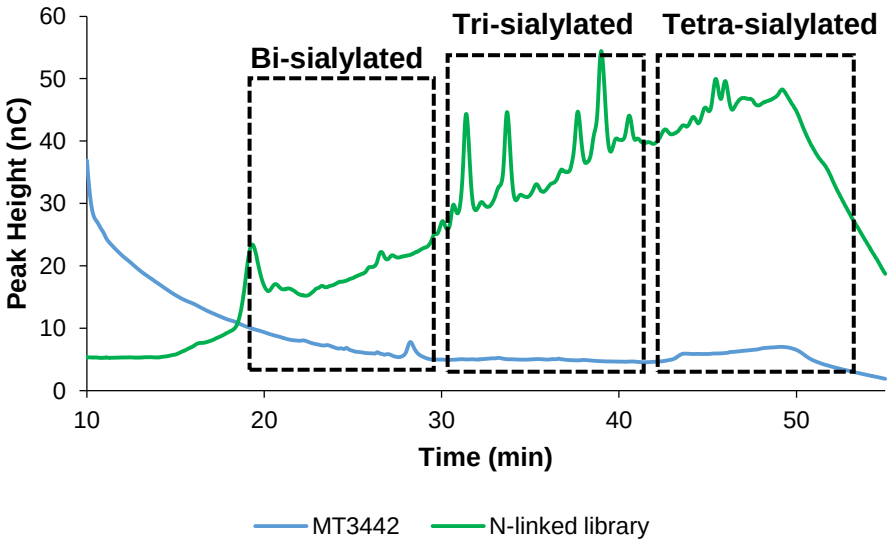


Figure 6.19 Oligosaccharide Chromatogram of TNBC samples MT3442 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Very few peaks were detected in TNBC sample MT3442 however peaks were visible during the bi-sialylated time frame and a minor peak was present in the tetra-sialylated area. No peaks were found in the tri-sialylated region of the chromatogram. Thus, a lesser degree of branching was present in this sample.

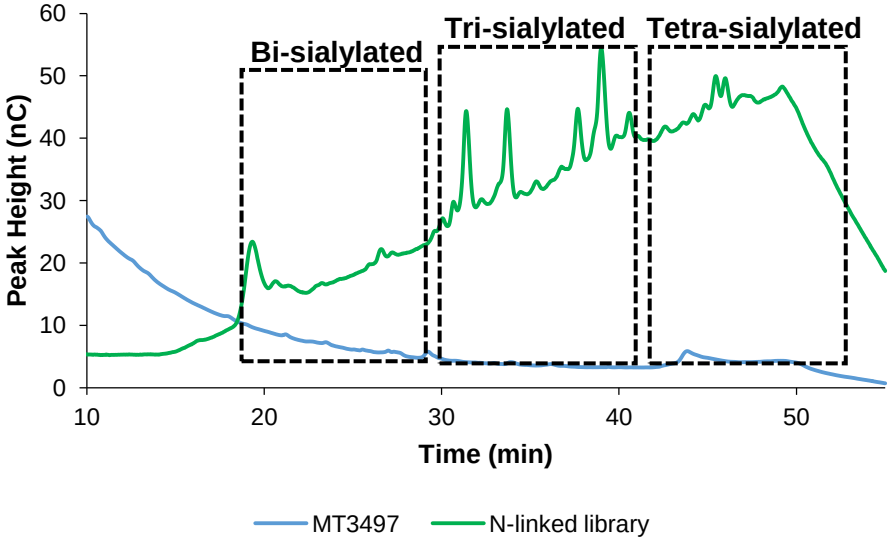


Figure 6.20 Oligosaccharide Chromatogram of TNBC Sample MT3497 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Several peaks were present in the bi-sialylated and tri-sialylated areas of the chromatogram with one peak also detected within the tetra-sialylated time point. The predominance of bi- and tri-antennary glycans detected indicated a lesser degree of branching present in MT3497.

6.3 Dataset 2 Oligosaccharide Results.

Oligosaccharide analysis of dataset 2 was performed using identical methods to dataset 1. HPAEC-PAD was utilised to separate AGP glycans (initially by charge) to deliver an indication of the extent of branching and any structural alterations to oligosaccharide chains between samples from women at-risk of BC development and age-matched healthy controls. The same *N*-linked (5µg) library chromatogram given in section 6.2 (Figure 6.1) was used for comparison as in dataset 1 to provide elution times of the charge bands of interest – bi-sialylated (20-30 min), tri-sialylated (30-40 min) and tetra-sialylated (40-50 min).

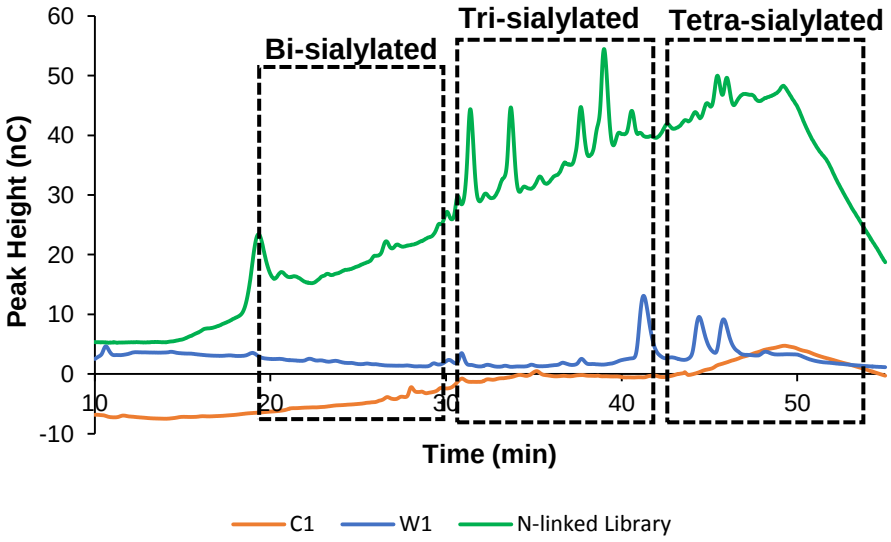


Figure 6.21 Oligosaccharide Chromatogram of W1 Compared to C1 and an *N*-linked Oligosaccharide (5µg) Library.

A clear difference in glycan structure could be seen between W1 and age-matched healthy control C1. W1 had a greater number of tri-sialylated and tetra-sialylated peaks, with the tetra-sialylated peaks also being far larger than any

peaks in C1. Whereas C1 showed a preference for less highly branched glycans with most peaks eluting in the bi- and tri-sialylated charge bands.

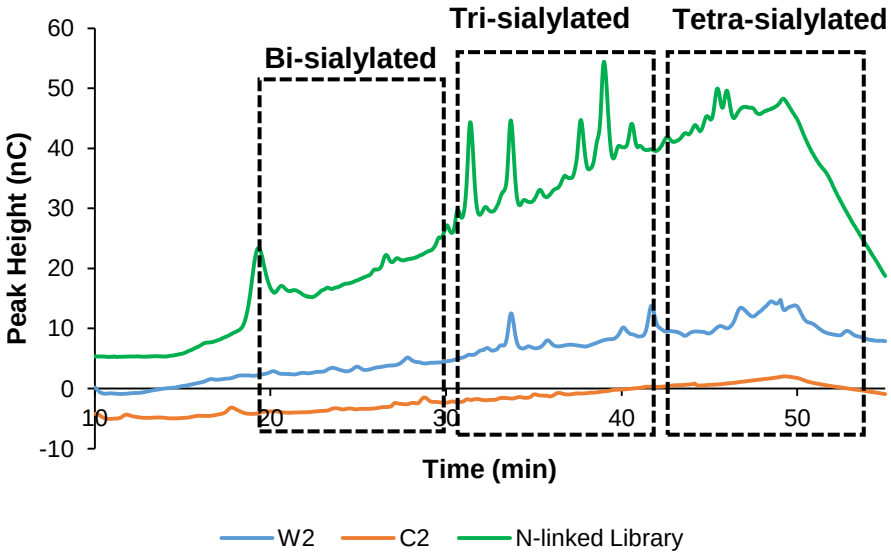


Figure 6.22 Oligosaccharide Chromatogram of W2 Compared to C2 and an *N*-linked Oligosaccharide (5µg) Library.

W2 also had a preference for highly branched glycans with more peaks detected in the tri- and tetra-sialylated charge bands. Bi-sialylated peaks were present in W2 however they were smaller and lower in numbers than peaks found in the tri- and tetra-sialylated charge bands indicating less of these types of glycans being present in the sample. C2 had no peaks in the tetra-sialylated charge band but had high numbers of peaks in the bi- and tri-sialylated elution region.

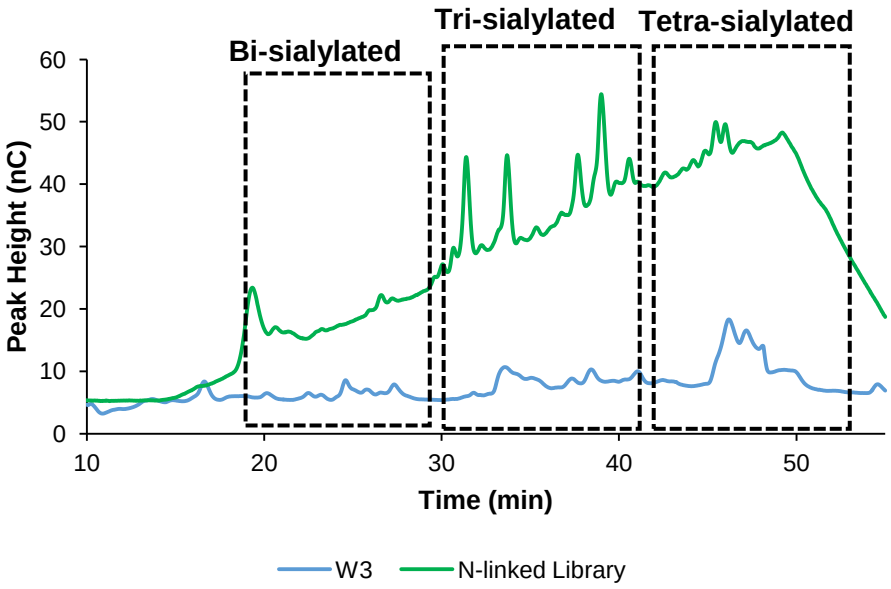


Figure 6.23 Oligosaccharide Chromatogram of W3 Compared to an *N*-linked Oligosaccharide (5µg) Library.

An age-matched healthy control was not available for comparison of oligosaccharide analysis of W3 and a mean of the healthy controls could not be calculated as the results are not quantitative. However, structural observation can still be gathered from analysis. W3 had peaks in all charged bands. More peaks were present in the bi-sialylated charge band however, these peaks were small in size. A lower number of larger peaks were detected in the tri- and tetra-sialylated elution regions. Therefore, it would appear that W3 has a preference for more highly branched glycans.

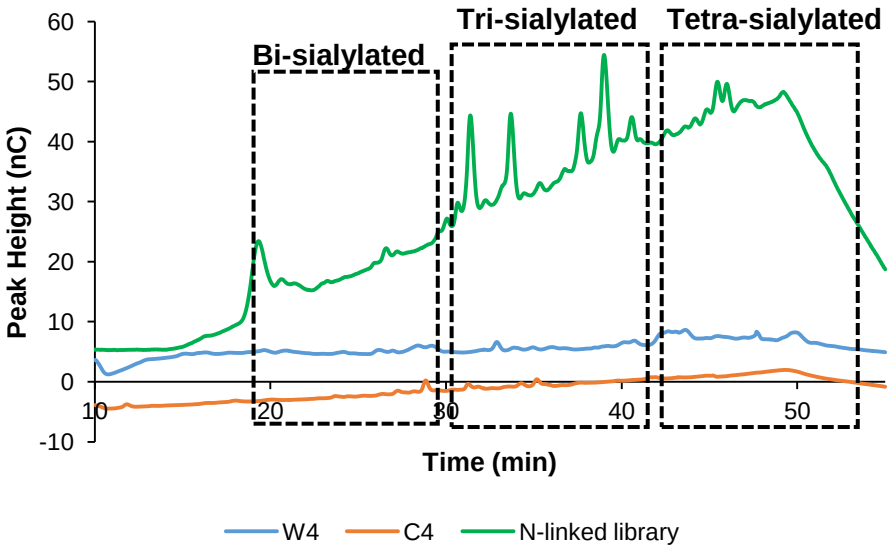


Figure 6.24 Oligosaccharide Chromatogram of W4 Compared to C4 and an *N*-linked Oligosaccharide (5µg) Library.

Alterations in glycan structure were apparent between W4 and C4 upon analysis. W4 had small peaks in every charge band with the majority appearing in the tri- and tetra-sialylated elution regions. Small peaks were present in the bi-sialylated charge band however these were smaller peaks than those in the other charge band and there were less of them. Thus indicating AGP glycans from W4 displayed a higher degree of branching. C4 revealed a different pattern whereby no peaks were detected in the tetra-sialylated region of the chromatogram however several peaks were present in the tri-sialylated charge band with a smaller number eluting late in the bi-sialylated band. Therefore, W4 has more highly branched AGP glycans than C4.

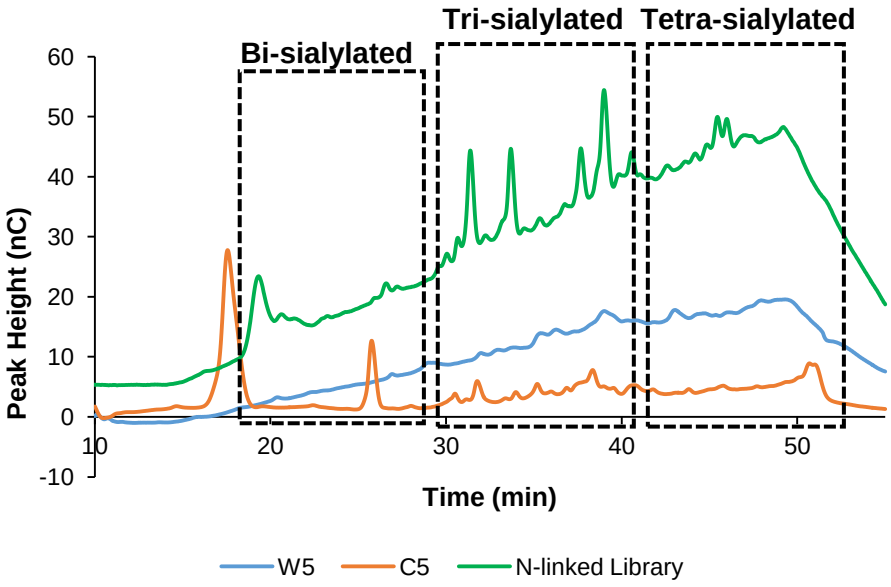


Figure 6.25 Oligosaccharide Chromatogram of W5 Compared to C5 and an *N*-linked Oligosaccharide (5µg) Library.

W5 had a larger number of peaks present in the tri- and tetra-sialylated charge bands upon oligosaccharide analysis showing a greater degree of AGP glycan branching in this sample. In comparison, C5 had a large peak in the bi-sialylated region and several smaller peaks in the tri-sialylated charge band suggesting less highly branched AGP glycans compared to W5. Interestingly a very large peak was detected prior to the bi-sialylated charge band which may have indicated a large presence of mono-sialylated bi-antennary glycans.

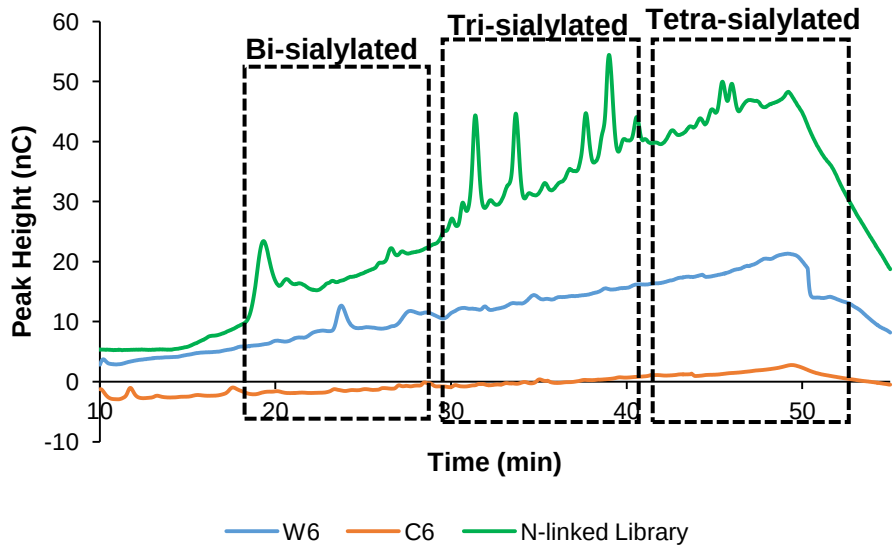


Figure 6.26 Oligosaccharide Chromatogram of W6 Compared to C6 and an *N*-linked Oligosaccharide (5µg) Library.

Oligosaccharide analysis of W6 revealed the majority of peaks lay within the bi- and tri-sialylated charge bands suggesting a lower level of branching compared to other “at risk” samples. Only two peaks were present in the tetra-sialylated region of W6’s chromatogram suggesting very little of this type of glycan branching existed in this sample. This profile was similar to that of C6 which also had very few peaks in the tetra-sialylated charge band but more peaks detected in the bi- and tri-sialylated region. Results showed that both W6 and C6 possessed a lower extent of AGP glycan branching.

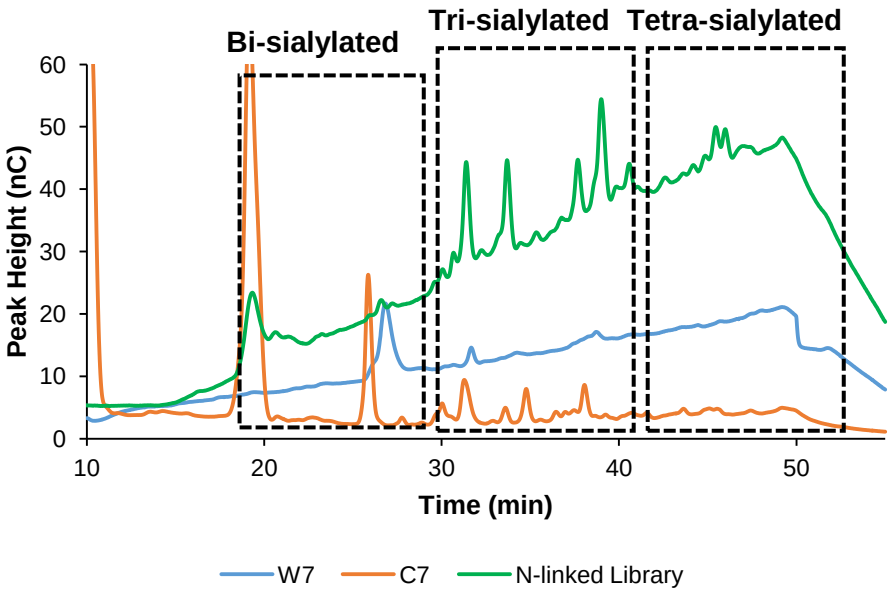


Figure 6.27 Oligosaccharide Chromatogram of W7 compared to C7 and an *N*-linked Oligosaccharide (5µg) Library.

Sample W7 possessed a large peak in the bi-sialylated charge band and several smaller peaks in the tri-sialylated charge band suggesting a lower level of glycan branching despite detection of peaks in the tetra-sialylated charge band as these were small and present in low numbers. C7 showed a wide variety of peaks present across all three charged bands with the majority occurring in the tri-sialylated charge band. However, two large peaks were detected in the bi-sialylated charge band which may highlight a preference for this type of AGP glycan branching. Several small peaks were detected in the tetra-sialylated charge band revealing low levels of higher branched structures.

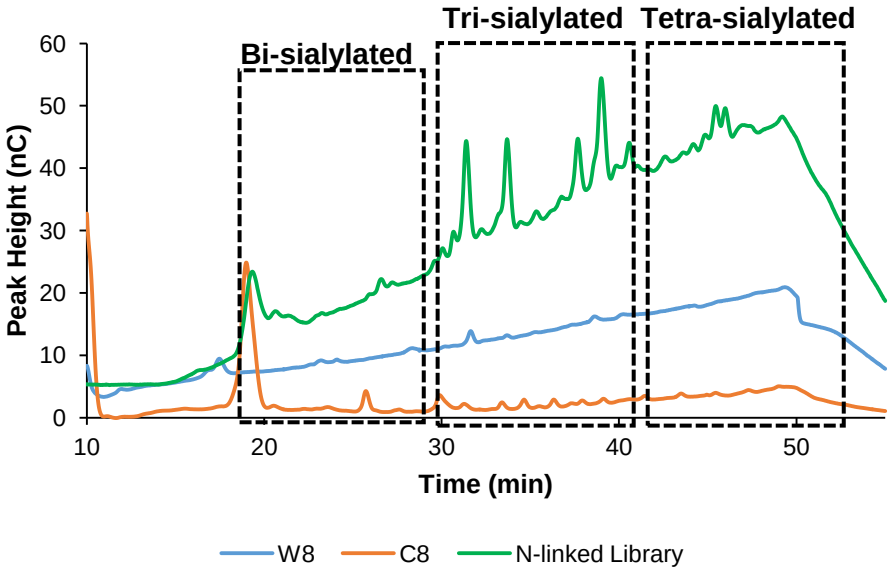


Figure 6.28 Oligosaccharide Chromatogram of W8 Compared to C8 and an *N*-linked Oligosaccharide (5µg) Library.

W8 had low numbers of peaks present within the sample, most of which lay in the tri-sialylated charge band and a less in the bi-sialylated charge band. One very small peak was detected in the tetra-sialylated charge band indicating an overall preference for a lower degree of AGP glycan branching in W8. C8 also showed this partiality to less highly branched structures as the greatest number of glycans were present in the bi- and tri-sialylated charge bands including one particularly large peak eluting early in the bi-sialylated region.

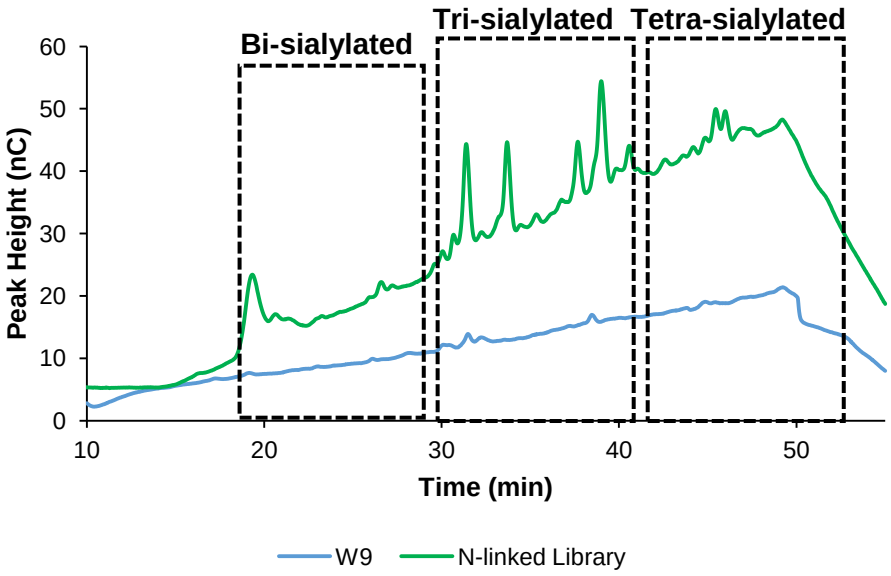


Figure 6.29 Oligosaccharide Chromatogram of W9 Compared to an *N*-linked Oligosaccharide (5µg) Library.

As with sample W3 no age-matched health control could be recruited for comparison to the at-risk sample W9 and no mean could be calculated so a general features were observed. Few peaks were detected in the bi-sialylated charge band however and those that were present eluted at longer retention times. However a great number of larger peaks were present in the tri- and tetra-sialylated charge bands showing W9 to have a preference for a greater degree of branching in AGP glycans.

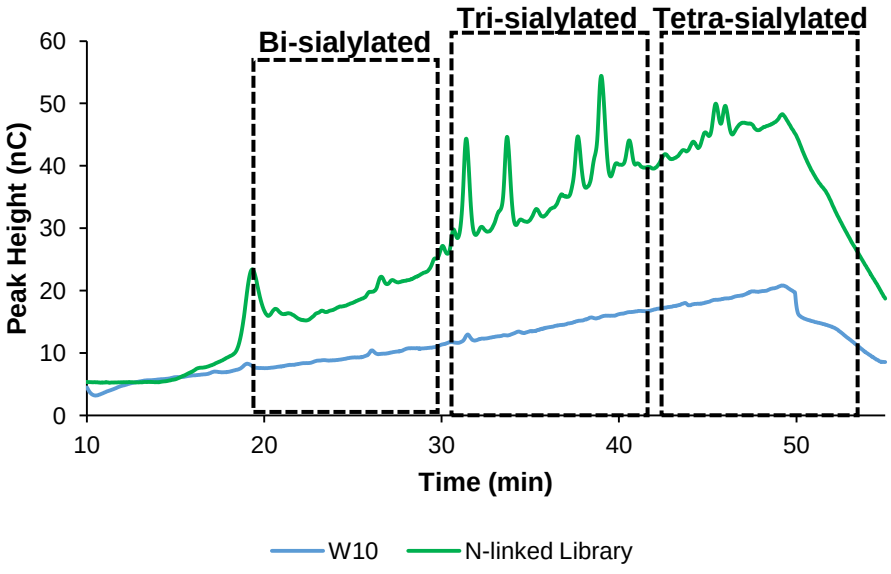


Figure 6.30 Oligosaccharide Chromatogram of W10 compared to an *N*-linked Oligosaccharide (5µg) Library.

It was not possible to recruit an age-matched healthy control for comparison to “at risk” sample W10. Few peaks were detected in the sample however those that were mostly occurred in the bi- and tri-sialylated charge bands suggesting low levels of branching in this sample. Two minute peaks were present in the tetra-sialylated charge band showing presence of more highly branched structures however the overall preference of W10 was for less branched AGP glycans.

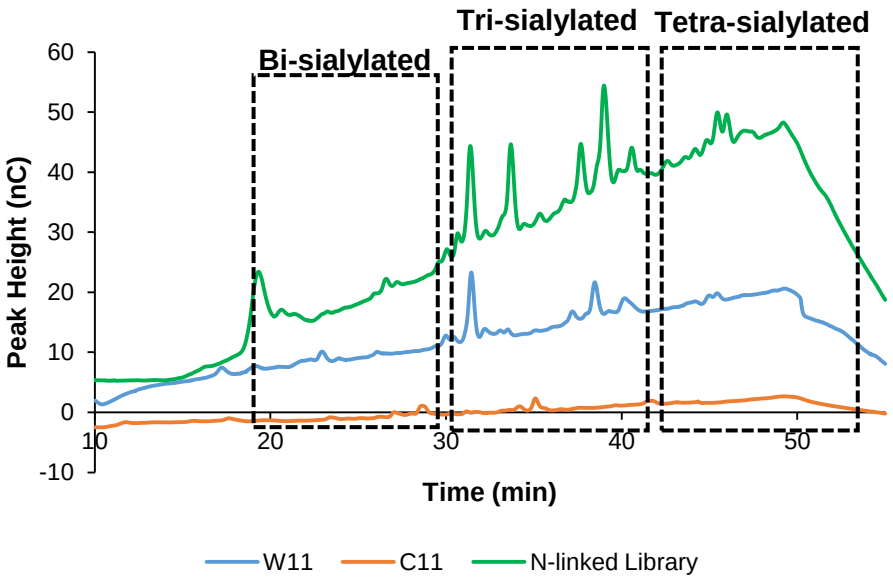


Figure 6.31 Oligosaccharide Chromatogram of W11 Compared to C11 and an *N*-linked Oligosaccharide (5µg) Library.

“At risk” sample W11 showed a greater tendency toward tri- and tetra-sialylated glycans due to the presence of higher numbers of larger peaks in these charge bands compared to fewer small peaks in the bi-sialylated charge band. Whereas no peaks were present in C11’s tetra-sialylated charge band but peaks were detected in the bi- and tri-sialylated regions. Therefore, W11 appeared to have more highly branched AGP glycans than C11.

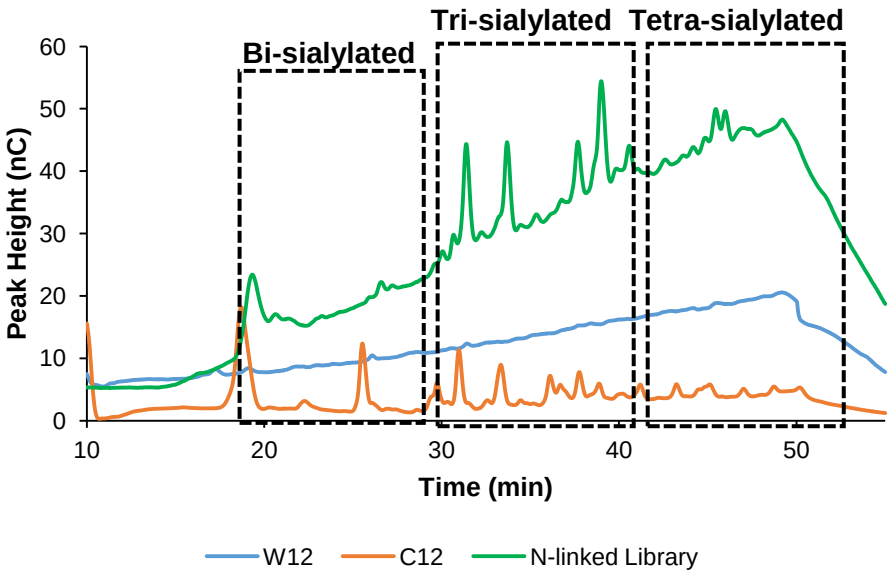


Figure 6.32 Oligosaccharide Chromatogram of W12 Compared to C12 and an *N*-linked Oligosaccharide (5µg) Library.

Minute peaks were detected in at-risk sample W12 and upon magnification, the most significant peaks were present in the bi- and tri-sialylated charge bands indicating low levels of branching. However, C12 had large peaks present throughout the charge bands with numerous large peaks in the lower glycan branching bi- and tri-sialylated charge bands. While peaks were detected in the tetra-sialylated charge band of C12, they were present in lesser numbers than those in other regions. Therefore, while both samples had peaks in the same charge bands, W12 appeared to have a lower level of branching than C12 due to smaller peak sizes in these elution regions.

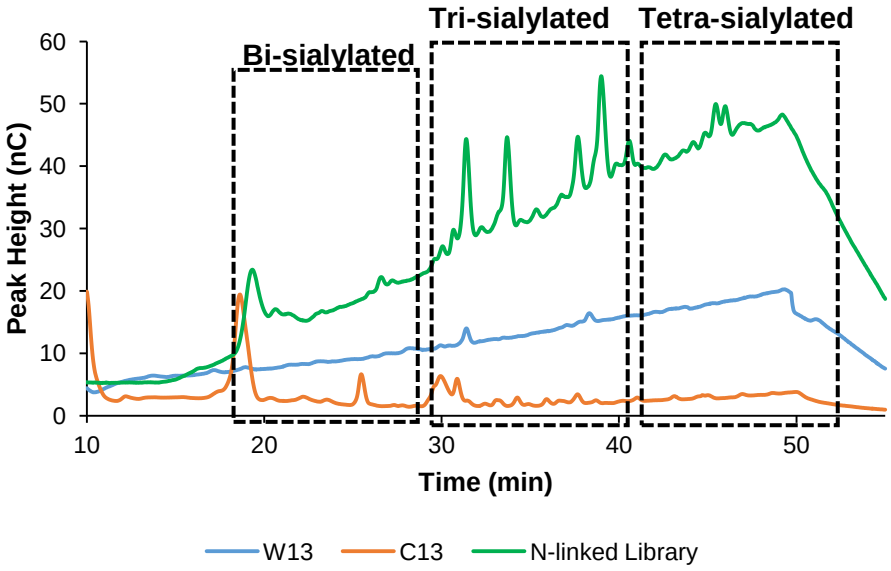


Figure 6.33 Oligosaccharide Chromatogram of W13 Compared to C13 and an *N*-linked Oligosaccharide (5µg) Library.

W13 possessed peaks in the tri-sialylated charge band with none detected in the tetra-sialylated band. Several small peaks were present in the bi-sialylated charge band however the tendency towards tri-sialylated glycans was still prevalent. However, several large peaks occurred in C13's bi- and tri-sialylated charge bands with lower numbers of smaller peaks detected in the tetra-sialylated region. Thus sample W13 indicated a preference for slightly higher branching of AGP glycans compared to C13 which had a greater presence of bi- and tri-sialylated glycans.

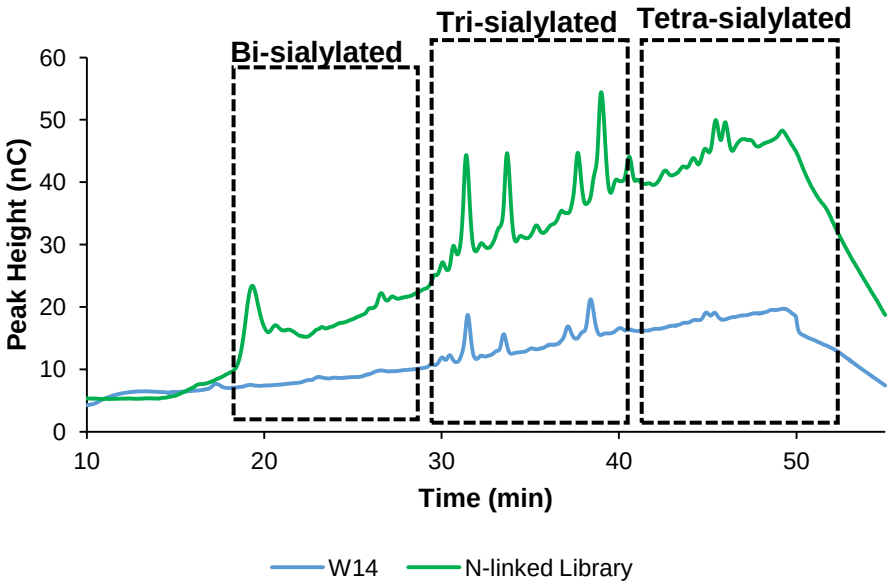


Figure 6.34 Oligosaccharide Chromatogram of W14 Compared to and *N*-linked Oligosaccharide (5µg) Library.

Age-matched healthy controls could not be recruited for W14, W15 or W16, therefore observations were made without comparison to a healthy control. Oligosaccharide analysis of W14 showed a penchant for tri-sialylated glycans with only one or two peaks detected in the bi- or tetra-sialylated charge bands.

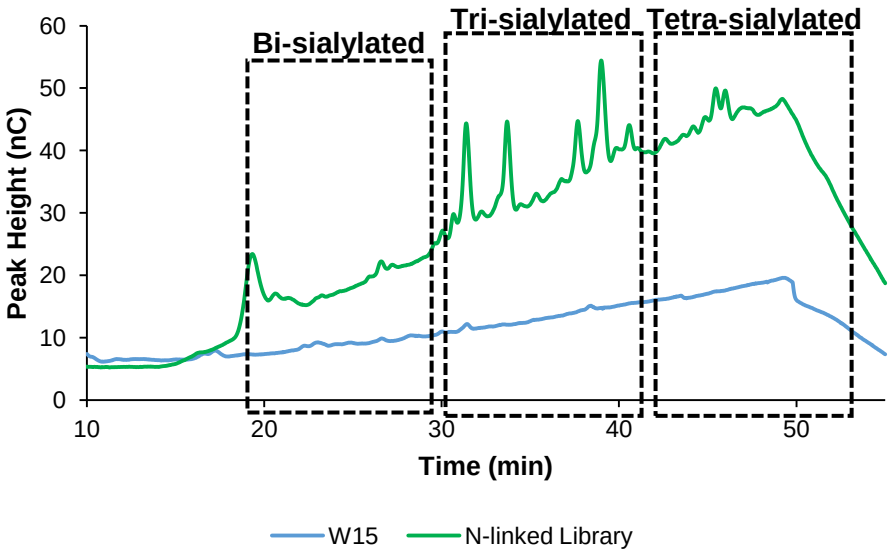


Figure 6.35 Oligosaccharide Chromatogram of W15 Compared to an *N*-linked Oligosaccharide (5µg) Library.

W15 possessed AGP glycans mainly with low levels of branching as indicated by prevalence of peaks in the bi-sialylated charge band. Few peaks occurred in the tri- of tetra-sialylated regions of the chromatogram.

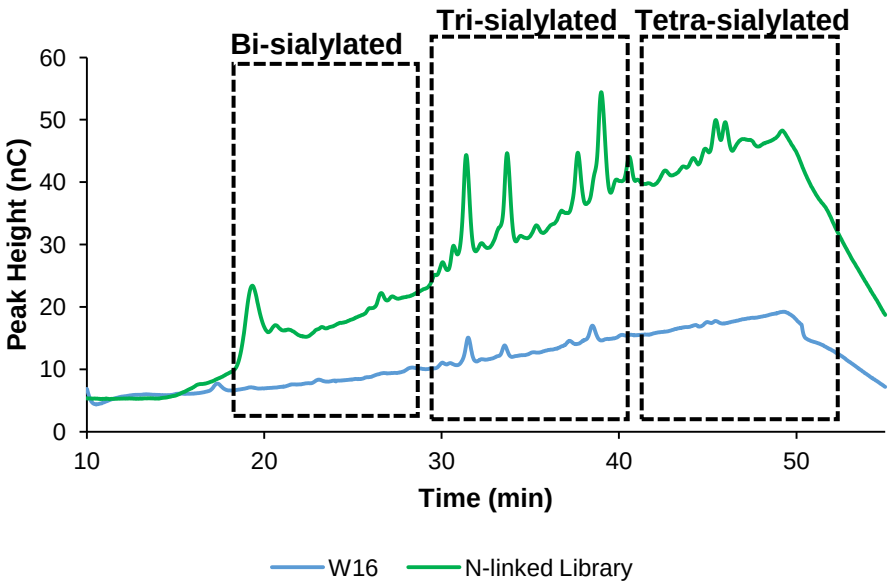


Figure 6.36 Oligosaccharide Chromatogram of W16 Compared to an *N*-linked Oligosaccharide (5µg) Library.

Tri-sialylated glycans were most predominant in “at risk” sample W16 with the majority of peaks occurring in this charged band. Few peak were detected in the bi-sialylated and tetra-sialylated region however the overall preference remained tri-sialylated glycans.

6.4 Discussion.

6.4.1 Introduction.

The aim of the research discussed in this chapter was to investigate variation in the extent of branching of oligosaccharide glycan chains of isolated AGP from two sample populations - Dataset 1 comprised samples from healthy individuals, women with BC of unknown type and women with TNBC as well as Dataset 2 containing samples from women at increased risk of BC development and age-matched healthy controls. This was performed through enzymatic release of *N*-linked glycans and analysis by HPAEC-PAD.

Release of intact AGP glycans from the polypeptide backbone is an essential prerequisite for investigation of their structure. This was achieved through denaturing isolated AGP then endoglycosidase PNGase F was used to cleave intact glycans from the polypeptide backbone. This study used heat to denature AGP as it promotes cleavage of glycans by PNGase F, whereas other methods

of denaturing, such as SDS, would cause destruction of glycan structures (Hermentin*, et al.*, 1992). Therefore, other denaturing methods would be unsuitable for the purposes of this study as they may produce unreliable results (Hermentin*, et al.*, 1992). PNGase F was used because the enzyme cleaves only the bond between the first GlcNAc of the pentasaccharide core and the Asn residue within the denatured polypeptide backbone (Plummer*, et al.*, 1984). A further condition of enzymatic release of glycans using PNGase F, is that the *N*-linked glycan consensus sequence Asn-X-Ser/Thr is present (Plummer*, et al.*, 1984). Therefore this ensured release of glycans in their entirety for analysis as well as guaranteeing every glycan was released. Separation of glycans by HPAEC-PAD was reliant on the negative charge of the ionised carboxylic acid group of terminal SA residues. Use of acidic conditions, such as those used to cleave AGP glycan monosaccharides, would have resulted in the loss of SA and unreliable results (Szabo, *et al*., 2012). However, PNGase F preserves SA residues allowing true *ex vivo* results to be collected.

HPAEC-PAD chromatograms of oligosaccharides in samples versus the N-linked library (Figures 6.1 to 6.20) show successful separation of glycans after PNGase F treatment into bands primarily based on the influence of the negative charges of SA residues. As discussed in the introduction to this chapter (section 6.1), glycan chains are not only separated by HPAEC due negative charge of SA residues and size, but by component monosaccharides and the linkages between them as well (Hardy and Townsend, 1988). To improve peak resolution during separation, strong alkaline conditions of pH13 were employed causing formation of oxyanions which enabled isomeric structures to be recognised. Another possible method of improving peak resolution would have been to reduce glycan chains prior to HPAEC analysis. However, this may have caused degradation and epimerisation of any glycans structures with sensitivity to alkaline conditions used during analysis as well as being more time consuming (Lee, 1996).

An *N*-linked library was analysed by HPAEC-PAD as a standard to indicate the elution time of bi-, tri- and tetra-sialylated glycans (see Figures 6.1 and 6.37). Sample chromatograms were subsequently compared to the chromatogram produced by this *N*-linked library to identify the presence of these glycan types

at the corresponding elution time points. As mentioned previously, commercially sourced AGP was not analysed due to the difference in isolation method and an inaccurate representation of glycan branching for instance, a lack of tetra-sialylated glycans (Behan, 2010).

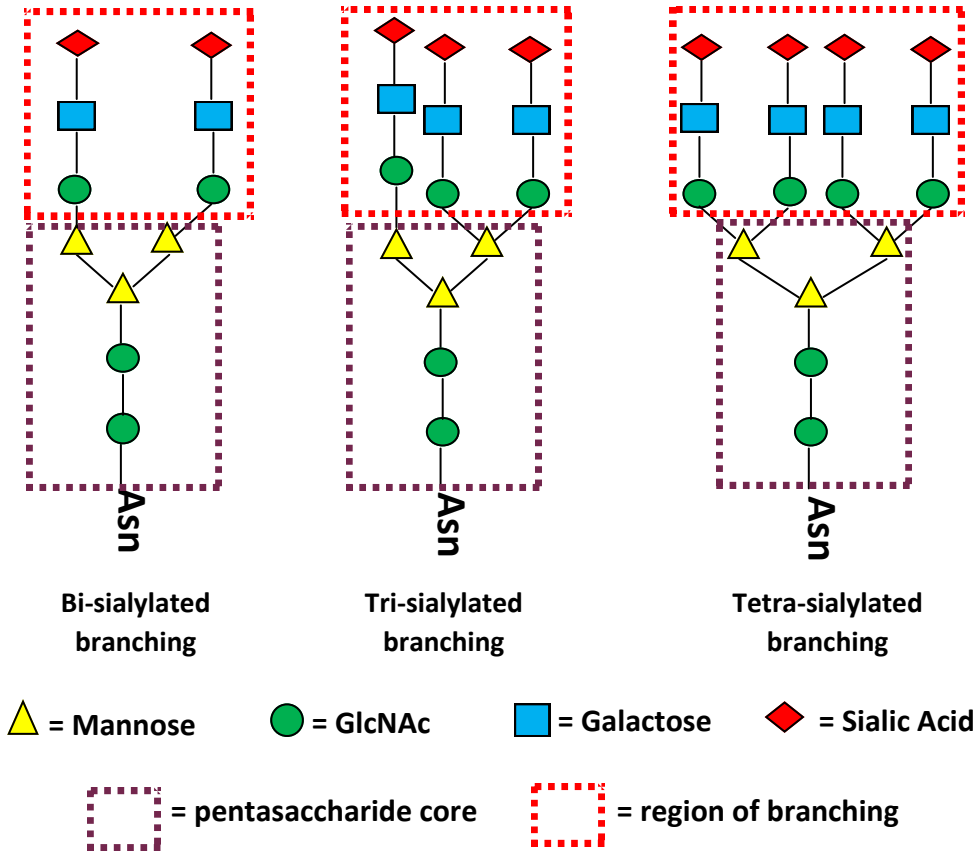


Figure 6.37 Illustration of Possible Glycan Branching in AGP. Diagram depicts bi-sialylated (2 branches), tri-sialylated (3 branches) and tetra-sialylated (4 branches) branching of complex oligosaccharides.

6.4.2 Trends in Oligosaccharide Branching Observed in Dataset 1.

Comparisons were drawn from oligosaccharide chromatograms of samples in all three groups from dataset 1 – BC of unknown type (positive control, n=5), “normal” healthy individuals (negative control n=5) and individuals suffering from TNBC (n=18).

Oligosaccharide analysis of samples from “normal” healthy individuals (see Figure 6.2) revealed a preference for bi- and tri-sialylated glycans (see Figure 6.37) as most peaks were present within the corresponding charge bands, with

few peaks detected in the tetra-sialylated time frame. This conformed to previous published research (Asao, *et al*., 2013; Behan, 2010; Kimura*, et al.*, 2006; Maresca, *et al*., 2012; Smith, *et al*., 2012) in which it was shown that AGP generally had less branched glycan structures in healthy volunteers. Interestingly, a small number of peaks were detected in the region preceding the bi-sialylated charge band which could indicate the presence of mono-sialylated bi-antennary glycans. However, previous research suggests that this is unlikely in AGP from healthy individuals as Gal would become exposed if an SA residue is missing causing the glycan to be targeted for destruction by lysosomes or repair by the calnexin/calreticulin cycles (Ware, *et al*., 1995). The oligosaccharide profiles generated from "normal" healthy samples correspond with the data collected through monosaccharide analysis in chapter 5 (see table 5.2) in that GlcNAc and Gal levels were generally low compared to BC of unknown type and TNBC samples, indicating less branched glycan structures.

BC of unknown type samples (see Figure 6.3) indicated a tendency for more highly branched structures upon oligosaccharide analysis (see Figure 6.37). Peaks were mainly present in the tri-sialylated and tetra-sialylated charge band with only samples H8 and I5 showing any presence of bi-sialylated glycans. H8 and I5 oligosaccharide chromatograms were in agreement with previous research (Fujii*, et al.*, 1988; Hansen*, et al.*, 1984) which showed an increase in bi-sialylated glycans during liver and lung cancer, whereas I2, I3 and I8 possessed an oligosaccharide profile resembling branching patterns seen by Hashimoto*, et al.,* (2004) in breast cancer associated with a poor prognosis as there was greater expression of tri- and tetra-sialylated structures. Samples I3 and I5 had peaks detected in the region prior to the bi-sialylated charge band again suggesting the unlikely presence of mono-sialylated glycans. However, I3 showed the presence of Fuc during monosaccharide analysis which can occasionally mask the negative charge of SA resulting earlier elution of glycans (Poland, *et al*., 2001). Therefore, the apparent mono-sialylated glycans could in fact have been early eluting bi-sialylated glycans. Yet, this would not explain the peak from sample I5 in the region before the bi-sialylated charge band as no Fuc was detected during monosaccharide analysis (see Table 5.2).

The majority of TNBC samples showed a preference for bi- and tri-sialylated branching as indicated by peaks in the corresponding region of chromatograms (see Figure 6.37). Ten TNBC samples showed the presence of tetra-sialylated branching but often only one peak was detected in this region compared to several peaks in bi- and tri-sialylated charged bands. Therefore, despite the presence of tetra-sialylated branching, there was an overall preference for less AGP glycan branching within TNBC samples contradicting oligosaccharide profiles seen during previous research into AGP branching in liver, lung and breast cancer (Fujii*, et al.*, 1988; Hansen*, et al.*, 1984; Smith*, et al.*, 2012). Fujii, *et al*., (1988) and Hansen, *et al*., (1984) observed increased binding of AGP from patients with liver and lung cancer respectively compared to inflammatory conditions of these organs since AGP from cancer patients showed less binding affinity in a Con A column which binds bi-sialylated glycans. Similarly, Smith, *et al*., (2012) showed increased branching of AGP through HPAEC-PAD analysis in patient with breast cancer. However, Hashimoto, *et al*., (2004) and Asao, *et al*., (2013) found that increased AGP glycan branching correlated with breast cancer progression. Similarly, a link between expression of tri-sialylated glycans and BC progression was discovered by Hamid, *et al*., (2008). Therefore, it is possible that the lack of predominant tetra-sialylated glycan branching in favour of tri-sialylated branching in TNBC samples could be due to early TNM stage of tumours, with most tumours in this sample cohort being classed as stage 1 or 2. Indeed, a shift towards increased branching in the only stage 3 TNBC sample (MT3352; see Figure 6.17) was observed as this sample had an increased number of peaks detected in the tri- and tetra-sialylated time frames.

Assessment of oligosaccharide profiles of TNBC samples by age, showed women <35 had much higher proportions of tri-sialylated branching. The majority of samples in this group had a low number bi-sialylated glycans present with these peaks eluting almost at the bi-sialylated time range cut off (see Figures 6.4 to 6.9). Looking at these results in conjunction with monosaccharide analysis of these samples showed that high levels of Fuc were detected in F5412 and MT2621. This may have meant that the late eluting bi-sialylated glycans were in fact early eluting tri-sialylated glycans as Fuc presence has been shown to reduce the elution time of oligosaccharide chains (Poland, *et al*., 2001). However, this does not explain the late eluting bi-sialylated peak in

MT3625 as no Fuc was detected during monosaccharide analysis. This preference for tri-sialylated branching corresponds with previous research from Hamid, *et al*., (2008) who observed an increased presence of tri-sialylated branching in BC denoted progression. As TNBC in younger women is notoriously aggressive, shows rapid growth and is associated with high tumour grades as well as poor prognosis (Blows, *et al*., 2010; Dent, *et al*., 2007; Schnieder, *et al*., 2008) the detection of increased branching could correlate with these features and indicate that these women's tumours are progressing to a higher TNM stage.

Additionally, the majority of samples had peaks present in the tetra-sialylated charge band. The detection of tetra-sialylated glycans in the TNBC samples from young women further indicates that increased glycan branching observed in these samples could be related to cancer progression as increased branching corresponds with previous observations by other research groups during cancer development (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012). These studies also highlighted an association between increased branching and poor prognosis (Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012). Therefore, the increased branching in TNBC samples from women <35 is consistent with the known poor prognosis associated with TNBC at a young age (Blows, *et al*., 2010; Dent, *et al*., 2007; Schnieder, *et al*., 2008).

While the majority of glycans were detected in the tri-sialylated charge band, CT79 had no bi-sialylated glycans present; instead it showed a tendency for tri- and tetra-sialylated glycans unlike other samples in this age group. Increased AGP glycan branching is associated with poorer prognosis in cancer patients after surgery (Hashimoto*, et al.*, 2004). Data provided by the Alberta Tumour Bank revealed that participant CT79 had undergone surgery for a stage 1 TNBC one day prior to blood draw. Therefore, if AGP glycan branching remained increased post-surgery this may indicate that participant CT79 had a poor prognosis for TNBC if Hashimoto and colleagues (2004) results and compared to this sample. In comparison, MT2305 had surgery 15 days before blood draw yet showed no presence of tetra-sialylated glycans thus may have had a better prognosis.

TNBC samples from participants aged >35-60 (see Figures 6.10 to 6.15) produced an oligosaccharide profile that contradicted previous studies investigating AGP glycan branching during cancer (Fujii*, et al.*, 1988; Hansen*, et al.*, 1984; Smith*, et al.*, 2012) which have shown increased branching during various cancer types. Five of the six samples in this age bracket favoured bi-sialylated branching while MT1177 illustrated similar numbers of peaks in the bi- and tri-sialylated charge bands with one particularly large peak towards the end of the tri-sialylated elution time. MT1177 had no treatment prior to blood draw but did have surgery 3 days prior to blood draw, and was TNM stage 2. Again this increase in branching could indicate a poorer post-surgery prognosis (Hashimoto, *et al*., 2004). MT1177's oligosaccharide profile corresponded with monosaccharide data collected from the same sample, in that MT1177 had increased levels of GlcNAc and Gal. Increases in the levels of GlcNAc and Gal are indicative of increased *N*-linked glycan branching (Behan, *et al*., 2013). The remaining five samples in this age category showed one or more peaks in the tetra-sialylated charge band, however the majority of peaks were still visible in the bi-sialylated charge band which does not conform with previous research findings (Fujii*, et al.*, 1988; Hansen*, et al.*, 1984; Smith*, et al.*, 2012) illustrating AGP oligosaccharide structures during cancer possess highly branched glycans. As several studies have linked increased branching with cancer progression and poor survival (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012) the similarity of this TNBC age group's oligosaccharide profile to the healthy control group could suggest better outcomes for these patients with less risk of cancer progression and metastasis. The oligosaccharide profiles from these samples fit the equivalent monosaccharide data which shows lower levels of GlcNAc and Gal than MT1177.

Finally, AGP oligosaccharide profiles analysed from TNBC samples of women <60 years old, indicated an increase in bi-sialylated glycan expression in line with the previous age category and contradiction of preceding research. As previously stated, sample MT3959's oligosaccharide chromatogram was discarded as an erroneous result due to erratic and closely eluted peaks being detected. Unfortunately due to small sample volume repeat oligosaccharide

analysis could not be performed. Four of the five remaining samples in this age category had a peak detected in the tetra-sialylated charge band but overall the majority of peaks were present in the bi-sialylated charge band. As discussed for the previous age category (aged >35-60), the preference for bi-sialylated glycans and resemblance to the healthy control population's oligosaccharide profiles could suggest that these patients will have a better outcome given studies have shown that increased glycan branching is associated with poor prognosis during various cancers (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012). These results match the monosaccharide data obtained from these samples which had the lowest GlcNAc and Gal content of the three age categories.

6.4.3 Trends in Oligosaccharide Branching Observed in Dataset 2.

All "normal" healthy control samples showed lower levels of AGP glycans branching as most peaks were detected in the bi- and tri-sialylated chromatogram regions (see Figure 6.37). While certain healthy control samples may have had peaks in the tetra-sialylated chromatogram region, these were few in number and small in size thus, still giving an overall preference for a lesser degree of AGP branching in healthy control samples. This matches the monosaccharide data obtained for these samples which displayed lower levels of branching associated monosaccharides, GlcNAc and Gal. Additionally no GalNAc was found in any healthy control samples. This corresponds with oligosaccharide analysis of "normal" healthy control samples from previous studies that demonstrated lower levels of glycan branching in healthy samples (Asao, *et al*., 2013; Behan, 2010; Kimura*, et al.*, 2006; Maresca, *et al*., 2012; Smith, *et al*., 2012) as well as the glycan branching observed in healthy samples from dataset 1.

Similar to monosaccharide results obtained from dataset 2, two trends were apparent upon analysis of oligosaccharides (see Table 6.1).

Samples	
Trend 1	**Trend 2**
↑ Oligosaccharide Branching	**↓ or ↔ Oligosaccharide Branching**
W1	W6
W2	W7
W3	W8
W4	W10
W5	W12
W9	W13
W11	W15
W14	
W16	

Table 6.1 Oligosaccharide Branching Trends Observed in At-Risk Samples. Entries in the table highlight sample oligosaccharide variation (↔ = comparable; ↑ = increased; ↓ = decreased levels compared to age-matched healthy controls) pertaining to trend 1 (↑ increased branching compared to age-matched healthy controls) and trend 2 (↓ lower or ↔ similar branching to age-matched healthy controls).

At risk samples W1, W2, W3, W4, W5, W9, W11, W14 and W16 had an oligosaccharide profile featuring increased branching compared to healthy control samples (Trend 1 and Figures 6.21, 6.22, 6.23, 6.24. 6.25, 6.29, 6.31, 6.34, 6.36 and 6.37). Comparison with monosaccharide data collected for these samples previously (see Table 5.3), highlights a link between increased GlcNAc and Gal content and increased branching of glycan structures. All of these samples, bar W1 and W11 followed the initial monosaccharide trend identified in Chapter 5. W1 did not fit either of the monosaccharide trends found as W1 presented with lower GlcNAc, similar Gal to its counterpart control (C1) and no GalNAc presence which may have accounted for any apparent increase in AGP glycan branching. Therefore, it is unclear why W1 displays large peaks in the tetra-sialylated and tri-sialylated charge bands as this data contradicts this monosaccharide data gathered for this sample. W11 also did not fit the first monosaccharide profile as it was expected that W11 would reveal a preference for less highly branched AGP glycans. However, several large peaks in the tri-sialylated charge band and a number of smaller peaks in the tetra-sialylated band indicated a predominance of highly branched structures compared to low numbers of small peaks present in the bi-sialylated charge band. Yet, W11 possessed comparable GalNAc and Gal levels. The unusual presence of GalNAc in such quantities could explain the appearance of more highly branched structures as it may be present as part of a terminal glycan structure.

Earlier research has linked increases in glycan branching with a number of unfavourable features in cancer (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012). A link between increased glycan branching of AGP in cancer samples and higher cancer stage was found by Hashimoto, *et al*., (2004) and Asao, *et al*., (2013) using crossed affinity immunoelectrophoresis and mass spectrometry respectively. Similarly, increased presence of *N*-linked β1-6 branching was associated with increased mortality by Majid, *et al*., (2005) and Cui, *et al*., (2011); Smith, *et al*'s (2012) research also demonstrated increased glycan branching during BC. Moreover, studies have shown a relationship between lymph node metastasis and tumour growth with increased *N*-linked β1-6 branching (Guo, *et al*., 2003; Handerson, *et al*., 2005; Pinho, *et al*., 2009; Takahashi, *et al*., 2009)

Therefore, the results observed in Trend 1 samples are unexpected because at the time of sample collection these women were considered to be cancer free. It was expected that no differences in AGP glycan branching between age-matched healthy controls and at risk women would be identified. However, if previous literature is considered alongside these results, the increased branching detected in these samples could indicated that women displaying this trend are in the early stages of BC development. The results correlate well with glycan branching results from BC of unknown type and TNBC <35 which also showed a preference for increased branching which further supports the notion that samples from women displaying trend 1 (increased branching) may be developing BC. Additionally, this shift towards increased branching denoting BC development may be further supported by studies relating increases in *N*-linked β1-6 branching with tumour growth and metastasis.

Seven at-risk samples – W6, W7, W8, W10, W12, W13 and W15 – had similar oligosaccharide profiles to those found in "normal" healthy control samples (Trend 2 and see Table 6.1 as well as Figures 6.26, 6.27, 6.28, 6.30, 6.32, 6.33 and 6.35). All showed a predominance of bi- and tri-sialylated AGP glycans with little or no peaks detected in tetra-sialylated charge bands (see Figure 6.37). Samples W6, W8 and W12 oligosaccharide profiles correlated with the monosaccharide results collected for these sample whereby lower levels of Gal were detected compared to their counterpart healthy controls thus suggesting lower levels of glycan branching. This was particularly true for W8 as very low

levels of GlcNAc and Gal levels were present as well as no GalNAc being detected meaning it was highly likely that low levels of oligosaccharide branching would be present in the sample. However, W7, W10, W13 and W15 displayed oligosaccharide profiles that contradicted their monosaccharide data as they all displayed increased levels of GlcNAc and Gal. Thus a lesser degree of branching was not expected in these samples and it is unclear why this has occurred. One possible reason may be that lower levels of GalNAc were present in these samples compared to others fitting this monosaccharide trend thus less branching occurred in W7, W10, W13 and W15.

The results described for Trend 2 are more in-keeping with results expected from dataset 2 as a whole. As all participants of this cohort as considered at increased risk of BC development but as yet to develop the disease it was predicted that glycan branching of AGP within these samples would resemble that seen in age-matched healthy controls. Therefore, the AGP glycan branching following Trend 2 (similar or decreased branching) observed in seven at risk samples links well with the original hypothesis. Several studies have shown predominance of bi-sialylated glycans as opposed to more highly branched tri- and tetra-sialylated glycans (structures in Figure 6.37) in healthy volunteers (Asao, *et al*., 2013; Behan, 2010; Kimura*, et al.*, 2006; Maresca, *et al*., 2012; Smith, *et al*., 2012), including healthy controls in dataset 1 of this study. Therefore, a link between low levels of glycan branching and a disease-free state has been established by previous literature. In contrast to this, numerous research groups have shown a correlation between increased glycan branching and cancer (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012), which further suggests that samples displaying Trend 2 glycan branching in this study are indeed from participants who have not developed BC despite being at increased risk.

However, Fuc was detected at low levels in all at-risk and healthy control samples. As explained in section 6.4.2 this could lead to early elution of peaks during oligosaccharide analysis due to Fuc masking the negative charge of SA residues (Poland, *et al*., 2001). Therefore, it may be that Fuc presence was responsible for peaks appearing prior to the bi-sialylated charge band in certain samples which were in fact early eluting bi-sialylated glycans as opposed to the more unlikely presence of mono-sialylated bi-antennary glycans. However, as

Fuc was present in all dataset 2 samples analysed this left-ward shift to shorter retention times may have occurred in all oligosaccharide chromatogram still enabling variations in branching to be identified.

6.4.4 Profiles of Glycosylation Enzymes.

The results of the current study and of prior research discussed previously in sections 6.4.2 and 6.4.3 demonstrate aberrant glycan branching as a hallmark of breast cancer. Given that building and alteration of glycans is controlled enzymatically, research has been undertaken to demonstrate alterations of glycosyltransferases and glycosidases that may be deregulated during cancer. Therefore, the results of this study will now also be discussed briefly in the context of known alterations in glycan related enzymes.

The results of this study indicate a potential link between BC development and increased branching which corresponds well with similar results obtain by other research groups (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005; Smith, *et al*., 2012).These observed increases in glycan branching could be due to increased expression of *N*-acetylglucosaminetransferase V and α2-3 or α2-6 sialyltransferases.

N-acetylglucosaminetransferase V (GlcNAcT-V) is responsible for *N*-linked β1-6 branching and increased levels of this enzyme have been reported to be involved in cancer metastasis and progression (Guo, *et al*., 2003; Handerson, *et al*., 2005; Pinho, *et al*., 2009; Takahashi, *et al*., 2009). Lymph node metastasis of BC has been shown to occur with overexpression of GlcNAcT-V (Handerson, *et al*., 2005) and target proteins of GlcNAcT-V (such as integrin and cadherin) with increased *N*-linked β1-6 branching appear to play a role in metastasis and promoting malignant cell growth (Guo, *et al*., 2003; Pinho, *et al*., 2009; Takahashi, *et al*., 2009). Additionally, research by Granovsky, *et al*., (2000) revealed mice without GlcNAcT-V were able to suppress tumour growth and metastasis. Thus, increased branching of AGP found in the majority of at-risk samples and TNBC samples <35 could suggest enhancement of tumour growth and metastatic risk.

Moreover, the sialyltransferase family catalyse the addition of terminal sialic acid to glycan branches and are overexpressed during malignancy and involved in glycan branching, progression, metastasis and carcinogenesis (Burchell, *et al*., 1999; Julien, *et al*., 2006; Ogawa, *et al*., 1997; Perez-Garay,*et al*., 2010; Petretti, *et al*., 2000; Picco, *et al*., 2010; Recchi, *et al*., 1998). Breast tumour formation was shown to occur in mice who over expressed α2-3 sialyltransferase I (Picco, *et al*., 2010) which could indicate increased glycan branching due to increased addition of terminal sialic acid residues. A study investigating glycosylation in pancreatic cancer cell lines also established the involvement of α2-3 sialyltransferase III in tumour progression (Perez-Garay, *et al*., 2010). Similarly, Julien, *et al*., (2006) revealed enhanced breast tumour formation in the presence of increased α2-6 sialyltransferase III.

Additionally, elevation of sialyltransferase levels have been associated with poor prognosis in various cancers (Gretschel, *et al*., 2003; Patani, *et al*., 2008; Sato, *et al*., 2002; Schneider, *et al*., 2001). For example, increased metastasis and poor prognosis were linked to increased α2-3 sialyltransferase III and α2-6 sialyltransferase I in gastric cancer (Gretschel., *et al*., 2003) while elevated levels of α2-6 sialyltransferase I also correlated with poor prognosis in colorectal cancer (Schneider, *et al*., 2001). However, Patani, *et al*., (2008) revealed increases in α2-6 sialyltransferase I may infer a better prognosis in BC patients. Therefore, it may be the mode of terminal sialylation rather than increased levels of branching that may define prognosis in certain cancers as breast tumour growth was associated with increased α2-6 sialyltransferase III by Julien, *et al*., (2006).

The results of the previous literature further support that increased branching observed in the majority of at risk samples and TNBC <35 could suggest development, progression and risk of metastasis. However, details regarding the mode of terminal sialic acid linkages could provide further prognostic indications.

6.4.5 Benefits and Shortcomings in the Methodology.

Analysis of the extent of branching of AGP oligosaccharides first required the removal of *N*-linked glycans which was performed using PNGase F which cleaves high mannose, complex and hybrid *N*-linked glycan at the innermost GlcNAc to remove the oligosaccharide in its entirety (Plummer, *et al*., 1984). The successful use of PNGase F for the cleavage of *N*-linked glycans has been widely documented to remove all *N*-linked glycans from the protein backbone (Plummer, *et al*., 1984). This is often confirmed by SDS-PAGE to demonstrate the lower molecular weight of AGP post-glycan removal (Laemmmli, 1970). However, due to the limited funding for this study, it was not possible to confirm complete removal of all *N*-linked glycans from samples in this study. Additionally, using enzymatic cleavage to remove *N*-linked glycans avoids the desialylation observed when harsh chemical methodologies are used (Chan and Yu, 1991). Unfortunately, this study did not use controls to ensure the activity of PNGase F which was an oversight of the investigator. PNGase F minus isolated AGP and HPLC water could have been used alongside dataset 1 and dataset 2 samples as positive and negative controls respectively and analysed using HPAEC-PAD (Semaan, *et al*., 2012).

Furthermore, the use of HPAEC-PAD is a highly reliable and sensitive method for the analysis of oligosaccharide branching (Behan and Smith, 2011; Hardy and Townsend, 1988; Harazono, *et al*., 2011; Smith, *et al*., 2012). Another benefit of HPAEC-PAD is that samples to not require derivitisation prior to analysis which would alter their structure in an effort to aid detection (Lee, 1990). However, analysis of oligosaccharide chain branching by HPAEC-PAD is not quantitative (Smith *et al*., 2012) unlike analysis by mass spectrometry (Morelle and Michalski, 2007).

Inadequate sample storage may have affected the oligosaccharide branching of AGP detected in samples as the samples were purchased prior to the study commencing and post-translational modifications such as glycosylation may degrade if stored at -20°C instead of -80°C (Davies, 1968). Similarly, other studies have shown that post-translational modifications are sensitive to pH and heat (McCurdy, *et al*., 2011). As dataset 2 samples were processed

immediately and not stored for long periods of time, storage is unlikely to have affected monosaccharide content in these samples.

6.4.6 Additional Methodologies to Study Branching.

Removal of oligosaccharide chains could have been analysed on an SDS-PAGE gel pre and post incubation with PNGase F to detect the reduction of molecular mass that would have indicated successful removal of glycans from the protein (Laemmmli, 1970). Furthermore, a periodic acid-Schiff stain, could have been used in conjunction with SDS-PAGE and a nitrocellulose blot to confirm deglycosylation of AGP pre and post enzymatic digestion (Hart, *et al*., 2003).

Techniques to release and *O*-glycans present within samples could have been utilised with HPAEC-PAD for identification of *O*-glycosylation. However, there is no universal enzyme available to cleave *O*-linked oligosaccharide as there is for *N*-linked glycans using PNGase F (Jensen, *et al*., 2009). Therefore chemical removal must be used such as β-elimination or hydrazine treatment prior to analysis using HPAEC-PAD (Patel, *et al*., 1993; Xia, *et al*., 2005).

Additionally, commercially available ELISA's utilising antibodies against terminal α2-3 or α2-6 sialylation could have been used support the levels of branching detect by HPAEC-PAD through measurement of these terminal residues (Shah, *et al*., 2008). Increases or decreases of α2-3 or α2-6 sialylation are thought to relate directly to the degree of branching (Behan, 2010; Picco, *et al*., 2010; Smith, *et al*., 2012).

Finally, use of mass spectrometry has been widely used to measure oligosaccharide branching in conjunction with separation techniques such as gas chromatography, HPLC and HPAEC-PAD (Morelle and Michalski, 2007). However small sample sizes available to this study would preclude using these techniques which mostly rely on further chemical derivatisation and purification steps prior to analysis (Morelle and Michalski, 2007).

6.5 Summary Statement.

Overall, this chapter achieved its aim to investigate variation in the extent of branching of oligosaccharide glycan chains of isolated AGP.

In dataset 1, TNBC samples from women aged <35 had increased AGP glycan branching compared to women with TNBC above this age. Oligosaccharide profiles of women with TNBC aged >35 resemble those found in other cancer studies involving AGP and BC of unknown type (positive control) samples where bi-sialylated branching was shown as dominant. This therefore, highlights a difference between TNBC in young women compared to older women as well as BC of unknown type. Furthermore, a difference in branching was revealed between TNBC and BC of unknown type in comparison to healthy controls which showed increased glycan branching.

Additionally, results from dataset 2 could imply that lower levels of branching occurring in “at-risk” samples correlating with “normal” healthy control oligosaccharide profiles, may indicate that these individuals have no signs of BC development at present. However, deviation from this oligosaccharide profile with an increase in AGP glycan branching was shown in 9 of the at-risk samples and could suggest the beginnings of BC development in light of results obtained in other studies. Again the healthy control population for dataset 2 showed a preference for lower levels of branching.

Finally, use of PNGase F and HPAEC-PAD to facilitate analysis of AGP oligosaccharide branching had numerous benefits including, high sensitivity, ability to release and detect all *N*-linked glycans. However, the methodology used for this study could have been improved through the use of improved sample storage, and potentially, ELISA to support HPAEC-PAD results, SDS-PAGE and periodic acid-Schiff staining to confirm deglycosylation of proteins by enzymatic digestion; removal of any *O*-glycans using β-elimination with analysis by HPAEC-PAD and quantification of all glycan structures by mass spectrometry. However, due to issues with funding and low sample volumes, these additional techniques could not be employed for this study.

Chapter 7 – Conclusion and Future Work

7.1 Conclusions.

The main aim of this study was to determine whether disease-specific alterations in the glycosylation pattern of AGP occurred in TNBC and if this differed from BC of an unknown subtype or healthy individuals (termed Dataset 1) as well as examining any variations in AGP glycosylation patterns in women at increased risk of BC development for onset of BC compared to age-matched healthy controls (termed Dataset 2).

Currently, there is no reliable serum biomarker for BC onset and in Scotland detection of BC is reliant on self-examination and mammography. While screening of women over 50 using mammography has greatly increased the detection rate of BC, mammography is not a full proof detection method and exposes recipients to radiation (Berrington de Gonzalez., 2011). Also with screening only recommended for women over 50, unless determined at an increased risk of BC development due to family history, a BRCA1 or 2 mutation or previous treatment for Hodgkin's lymphoma, women unaware of their increased risk may miss out on early detection of BC occurrence. Furthermore, with recent research unveiling that BC may need to be classified as several separate diseases in the future dependent on molecular subtype of a tumour and links between TNBC occurrence at a young age and BRCA1/2 mutation carriers, investigating a potential serum biomarker for BC is essential. Particularly because a blood-based BC biomarker may negate the need for costly mammography screening and its associated radiation exposure as well as enabling screening to occur more frequently in those at higher risk of BC development.

The study acquired two sample populations termed dataset 1 and dataset 2. Dataset 1 sought to fulfil the aim of assessing whether AGP glycosylation patterns differed between TNBC, BC of an unknown subtype and "normal" healthy controls. Dataset 2 collected samples from women at increased risk of BC in comparison to age-matched healthy controls to assess any changes to AGP glycosylation which may have indicated BC development. Both datasets were subjected to the same laboratory methods for isolation and analysis of AGP which have been shown to preserve the *in vivo* state of AGP thus providing a true depiction of AGP glycosylation within the body. AGP isolation

was conducted using low pressure affinity chromatography and the isolated levels calculated using spectrophotometry. AGP glycans were then separated from the polypeptide backbone using acid hydrolysis (monosaccharide analysis) or enzyme digestion (oligosaccharide analysis) and analysis was performed using HPAEC-PAD.

Differences in AGP levels were found between various sample groups during this study. As expected, in dataset 1, levels of AGP in BC of an unknown subtype were greatly increased above the healthy population. However, TNBC samples had increased AGP levels above the healthy population yet levels were far lower than the BC of an unknown subtype. Thus highlighting an alteration in AGP between the TNBC and BC of an unknown type populations, providing further evidence of the differences between BC subtypes. Dataset 2 also produced unexpected results when examining AGP levels because despite both populations technically being considered healthy, isolated AGP levels were significantly raised in the at-risk population compared healthy controls. Two particular at-risk samples (W10 and W12) had levels more than double that of their controls which may have indicated BC development.

Variation of AGP concentration observed in this study could have been due to increases in F1*S and A variant forms of AGP. These variants are known to exist in a 3:1 ratio in healthy individuals (Dente *et al*., 1987) and alterations have been shown to have increased expression of F1*S and A variants in breast, ovarian and lung cancer, however the overall ratio tends to remain the same (Duche *et al*., 1998 and Duche *et al*., 2000). However, the study by Budai, *et al*., (2009) showed a change in F1*S to A variant ratios of up to 8:1 in lymphoma, melanoma and ovarian cancer but these differences were not dependent on cancer type. Additionally, an early study by Treuheit, *et al*., (1992) revealed that AGP variants had differing preferences for bi-, tri- and tetra-sialylated glycans at each glycosylation site. Yet, there is little evidence of these preferences differing during disease. Thus analysis of AGP variants would be of little use as a potential biomarker specific for breast cancer.

Analysis of AGP glycan monosaccharide content highlighted quantitative differences between samples groups in both datasets. In dataset 1, healthy samples had relatively low levels of all monosaccharides whereas BC of an unknown type possessed far higher levels of Gal. Most unusually, as AGP

solely expresses complex *N*-linked glycans, GalNAc was present in over 88% of TNBC samples. GalNAc is a component of *O*-glycans and previous literature investigating other proteins has shown a reversion to foetal glycosylation during cancer. Foetal AGP expresses *O*-linked glycans (Shiyan and Bovin, 1997) and there is a known link between TNBC and *O*-glycosylation of other proteins (Milde-Langosch, *et al*., 2014; Siroy, *et al*., 2013). Thus GalNAc presence could indicated *O*-glycosylation of AGP and could be a marker of TNBC as GalNAc was not present in the healthy control or BC of unknown type populations. TNBC samples were divided into several categories for comparison with one another as well as controls. When categorised by age (<35, 35-60 and >60) a similar trend was displayed throughout each group with Gal showing highest levels, followed by GlcNAc, then GalNAc and then Fuc. Interestingly with GalNAc presence was inversely correlated with age and TNBC in women >60 had lower levels of Gal compared to the other two age categories. Similar monosaccharide levels were detected in TNBC samples when comparing family history to no family history or unknown family history. However, the no family history group had lower levels of GalNAc compared to those with family history and those with unknown family history. Little difference was seen in monosaccharides between treated and untreated TNBC indicating that treatment may not be working as previous research within this group has shown AGP glycosylation patterns returning to normal upon treatment. This would fit with TNBC's notoriously aggressive nature. Separation by stage of TNBC also revealed few differences between stage 1 and stage 2 TNBC other than marginally lower levels of GalNAc and Gal in stage 2. Therefore, as with AGP levels, alterations in AGP monosaccharide content are present not only between healthy and BC populations but between TNBC and both controls as well.

Mean levels of monosaccharides in at-risk samples and healthy control samples in dataset 2 showed similar levels of Fuc and Gal. However GlcNAc was significantly elevated in the at-risk population and GalNAc was detected in 81% of at-risk samples but none was present in the healthy population. Two trends emerge upon analysis of each at-risk samples with their age-matched healthy control with most samples fitting one or the other. The first trend identified was at-risk samples possessing comparable Fuc levels to their control but having

GlcNAc and Gal levels above that of the control. The second trend found was at-risk samples containing similar Fuc levels to their control and increased GlcNAc levels but having lower Gal levels than their control. Only one sample did not fit either of these trends (W1). Therefore, it would appear that a difference in AGP monosaccharide content lies between women at risk of increased BC development and healthy controls despite both technically being healthy. Furthermore, GalNAc presence in the at-risk population indicates that it is not disease specific as it is unlikely that all at-risk samples analysed were in the early stages of BC development.

Oligosaccharide analysis demonstrated structural differences in AGP from the various sample populations studied. In dataset 1 there was a shift from bi- and tri-sialylated glycans in healthy samples to tri- and tetra-sialylated glycans in BC of unknown type samples which contradicted previous research from this group who had found truncation of AGP glycan branches in BC samples. However, when examining oligosaccharide profiles from TNBC samples from women aged 35-60 and >60 showed a preference for bi-sialylated branching in line with previous studies. Yet, AGP from women with TNBC <35 possessed more highly branched glycans, similar to BC of unknown type. Therefore indicating a difference between TNBC at a young age and TNBC in older women.

Oligosaccharide analysis of dataset 2 highlighted a division of oligosaccharide profiles in the at-risk group. Healthy control samples in dataset 2 showed a similar oligosaccharide profile to those from dataset 1 where bi-sialylated and tri-sialylated glycans were predominant. Seven of the at-risk samples showed glycan profiles with similar low levels of branching to the healthy controls. However, nine samples featured oligosaccharide profiles with increased branching through preference for tri- and tetra-sialylated glycans compared to the healthy controls. With samples featuring in either trend, the majority fitted well with the corresponding monosaccharide collected for each sample for instance an increase in GlcNAc or Gal was matched with an increase in branching and vice versa. Thus, these differences in glycan branching may further indicate the early development of BC in the at-risk samples that have glycan profiles similar to those of the TNBC <35 and BC of unknown type samples from dataset 1.

Additionally, concentration determination, monosaccharide and oligosaccharide analysis of healthy populations in both datasets unveiled base-line variability within the group. This could be attributed to inter-individual variation as AGP glycan content and concentration are known to change during inflammation (Higai, *et al*., 2005) as well as healthy volunteers being unaware of or untruthful about possessing inflammatory conditions that may have affected AGP glycan content and concentration.

Thus far no other research groups have examined AGP glycosylation in TNBC or performed a case-control style study to investigate AGP glycosylation using individuals confirmed to be at increased risk of BC development. Earlier studies have shown AGP concentration to increase dramatically during cancer and inflammation while remaining low in healthy populations (Rudman, *et al*., 1974; Duche, *et al*., 2000; Routledge, 1989; Twining and Brecher, 1977). The current study also revealed this pattern in BC of unknown type and healthy samples, yet TNBC samples showed a minimal increase compared to healthy samples and also showed lower concentrations of AGP compared to BC of unknown type. Hence AGP concentrations during TNBC appear to contradict previous research. Previous studies have also established that adult AGP expresses 5 complex *N*-linked glycans (Yoshima, *et al*., 1981) while foetal AGP displays 3 *N*-linked glycans and 3 *O*-linked glycans with the *O*-glycans being lost during development (Shiyan and Bovin., 1997) However results obtained in this study are suggestive of AGP expressing *O*-glycans in adults during TNBC and possible BC development in women at risk of BC development. This reversion to *O*-glycosylation could be due to expression of foetal antigens which has been shown to occur on other proteins during cancer (Farinati, *et al*., 2006 and Amri, *et al*., 2013) and may be caused by rapid cell proliferation (Sapra, *et al*., 2012; Farinati, *et al*., 2006). The results of this study support previous research linking increased glycan branching with cancer development and progression (Asao, *et al*., 2013; Cui, *et al*., 2011; Hashimoto, *et al*., 2004; Majid, *et al*., 2005) as TNBC samples <35 and at risk samples showed a higher degree of branching compared to other samples in this study. However, TNBC from women >35 had lower levels of branching detected which aligns with research by Fujii*, et al.*, (1988), Hansen*, et al.*, (1984) and Smith*, et al.*, (2012) investigating AGP

glycosylation during cancer. Therefore, the current study clearly contributes new knowledge and builds upon existing information regarding AGP.

The alterations of AGP glycosylation observed in this study may be linked to several key hallmarks of cancer such as sustaining proliferative signalling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis; resisting cell death (Hanahan and Weinberg, 2000); avoiding immune destruction; genome instability and mutation; tumour-promoting inflammation and deregulating cellular energetics such as the Warburg effect (Hanahan and Weinberg, 2011; Warburg, *et al*., 1928). Aberrant glycosylation of proteins such as sialylation, fucosylation, truncated *O*-glycans, increased branching (Stowell, *et al*., 2015) and expression of oncofoetal antigens (Sapra, *et al*., 2012) may aid cancer progression and metastasis (Chen, *et al*., 2013; Julien, *et al*., 2011; Guo,*et al*., 2003; Handerson, *et al*., 2005; Pinho, *et al*., 2009; Takahashi, *et al*., 2009). Presence of Fuc in samples may enhance cell proliferation as shown by Chen, *et al*., (2013) who observed increased levels of FUT 8. Similarly, Fuc could be present as a component of SLe^x which has a role in cell adhesion thus its expression could be aiding the binding of cancer cells to secondary organs and facilitating metastasis (Julien, *et al*., 2011).Thus, Fuc content of AGP could play a role in metastasis and malignant cell proliferation in BC. Furthermore, increased GlcNAc and Gal could be linked to increases in glycan branching (Behan, *et al*., 2013), specifically *N*-linked β1-6 branching which has be shown to rise in cancer (Stowell, *et al*., 2015). Increases in glycan branching have been shown to contribute to lymph node metastasis and tumour growth (Guo, *et al*., 2003; Handerson, *et al*., 2005; Pinho, *et al*., 2009; Takahashi, *et al*., 2009). Therefore the increased branching of AGP and corresponding increase in GlcNAc and Gal observed in TNBC samples <35 and several at risk samples could indicate that AGP is potentially contributing to BC development and metastasis. Additionally, the unexpected detection of GalNAc in samples of isolated AGP in this study could indicate two further hallmarks of cancer – truncated *O*-glycans and regression to expressing foetal antigens (Stowell, *et al*., 2015). But, clearly more work is necessary beyond the scope of the present study. GalNAc is a constituent of *O*-glycans and not present in *N*-linked glycans (Varki, *et al*., 2009). Thus as AGP has been shown to only express complex type *N*-linked

glycans, GalNAc presence suggests *O*-glycosylation of AGP. As mentioned previously, AGP does express *O*-linked glycans during foetal development but not in adult AGP (Shiyan and Bovin, 1997), suggesting that AGP in some samples from this study may have reverted back to glycan expression observed in foetuses. Expression of *O*-linked glycans during cancer is associated with enhancement of the malignant properties of cells such as increased proliferation, invasion of surrounding tissues and metastasis (Radhakrishnan, *et al*., 2014). Thus, if AGP is displaying O-linked glycosylation during BC, AGP could be functioning to promote BC development, progression and metastasis. Expression of GalNAc, potentially from *O*-linked glycans, may be due to Warburg effect which causes increased uptake and anaerobic metabolism of glucose by cancer cells for energy to enhance proliferation (Warburg, *et al*., 1928). Increased *O*-GlcNAc transferase which catalyses the addition of *N*-acetylglucosamine to proteins and lipids has been linked to the Warburg effect (Jozwiak, *et al*., 2014) and it has been hypothesised that *O*-GlcNAcylation acts as a nutrient sensor (Butkinaree, *et al*., 2010; Hanover, *et al*., 2010) resulting in enhanced proliferation, growth, angiogenesis and metastasis (Khan, *et al*., 2013; Porta, *et al*., 2014).

In summary, this study has fulfilled its aims of investigating variations in AGP glycosylation in TNBC and women at increased risk of BC development in the hope of identifying disease specific changes that could be utilised as a diagnostic biomarker. The data collected indicates that AGP glycosylation in TNBC varies compared to healthy controls and BC of unknown type mainly due to the unusual presence of GalNAc. Furthermore, differences in AGP glycosylation within an at-risk population that are similar to that of TNBC<35 and BC of unknown type samples could indicate early BC development. The results of this study suggest that increased levels of GlcNAc and Gal correlating to increased glycan branching could indicate BC development. However, decreased GlcNAc and Gal in combination with decreased glycan branching implies individuals are disease free. Based on the results, it would appear that AGP glycosylation is a good biomarker of BC as examination of glycan content and branching allowed the investigator to differentiate between healthy, BC of unknown type and TNBC in dataset 1 as well as suggesting BC development of a number of at-risk samples in dataset 2.

7.2 Future Work.

The study presented within has provided evidence of AGP's potential as a biomarker of BC in at risk individuals. However, the study was limited by the type of samples that could be acquired for analysis as well as the sample size that was feasible for the author to process and analyse. Yet the current study has provided useful preliminary data that could lead on to subsequent research to confirm the results and expand on this study's findings. The following are suggestions that could improve investigation into AGP's potential as a BC biomarker in at risk individuals.

Firstly, repetition of the method used in this study with larger sample cohorts to assess whether the current study's findings are supported. While the sample sizes used in the current study were suitable for this preliminary investigation, a secondary study using more participants could enhance the reliability of the data.

Repetition of this study using only untreated samples would allow glycosylation patterns that were uninfluenced by treatment to be obtained. Thus the accuracy of the monosaccharide and oligosaccharide data would be improved as changes detected in glycosylation would be a true reflection of those at the beginning of BC development.

Recruitment of women at increased risk of BC development who have developed BC to draw direct comparisons between women at risk without BC and a healthy population. Therefore, it would be possible to assess whether glycosylation patterns observed in at risk samples that differed from age-matched healthy controls did indicate BC.

Conducting a study running over several years, following women at risk of BC and analysing their AGP glycosylation at various points to detect onset of BC if it occurs. Performing a study in this manner would allow monitoring of glycosylation patterns in at risk individuals, with the potential to have BC confirmed by a conventional method, such as mammography, assess aberrant AGP glycosylation as a biomarker of BC.

Moreover, analysis of AGP glycosylation in other cancer types could be utilised in a future study. This would enable comparison of AGP glycosylation patterns

in different cancer types to determine whether BC can be distinguished from other cancer types.

Furthermore, additional methods could have been employed to complement and enhance the validity of results in this study, outlined in part in sections 4.5.3, 5.5.4, 6.4.6.

Investigation of AGP concentration in the various sample populations employed in this study could have been improved by assessing the efficiency of the low pressure chromatography columns used known concentrations of AGP to spike plasma samples to measure AGP recovery. Removal of unwanted plasma proteins using low pressure chromatography to purify AGP could have been evidence by SDS-PAGE of all fractions after application to each low pressure chromatography column. Western Blotting of SDS-PAGE gels using AGP specific antibodies could have confirmed purification of AGP (Behan, 2010) and a Bradford assay (Bradford, 1976) or an antihuman AGP ELISA would have enabled measurement of protein concentration (Hashimoto, 2004; McCurdy *et al*., 2011). Use of these techniques could have complemented the determination of AGP concentrations solely on absorbance methods.

Again, SDS-PAGE could have been utilised to prove deglycosylation of AGP by acid hydrolysis and PNGase F through a reduction in molecular weight while incorporation of a periodic acid-Schiff stain for glycosylation with a nitrocellulose blot could have also been used to assess deglycosylation of AGP; although acid-mediated chemical hydrolysis is generally accepted as a quantitative process. Moreover, analysis of monosaccharide and oligosaccharide content using ELISA's (to measure Fuc and SA) and mass spectrometry could have supported the HPAEC-PAD results. Lastly, possible *O*-linked glycosylation of AGP would have been possible if β-elimination or hydrazine treatment had been employed to release *O*-glycans to allow their analysis by HPAEC-PAD.

Additionally due to limited funding and sample volume it was not possible to use the techniques listed above to confirm the findings of the current study. Unfortunately, it was not possible to recruit women at increased risk of BC who had developed BC for this study. This is because once women have BC detected at the screening clinic, they are moved to a different department for

treatment and contact between the patient and the screening clinic is lost. No follow-up is carried out.

This study found alterations to AGP glycosylation in TNBC samples and samples from women at increased risk of BC development that could identify early BC development. While this data is preliminary, follow up studies carried out using the recommendations listed above could aid the development of a blood-based biomarker for diagnosis of BC which could revolutionise the screening of women for BC.

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Appendix

Appendix 1 – NHS Lothian REC Approval.

Lothian NHS Board

South East Scotland Research Ethics Committee 02

Waverley Gate
2-4 Waterloo Place
Edinburgh
EH1 3EG
Telephone 0131 536 9000
Fax 0131 465 5789

www.nhslothian.scot.nhs.uk

Date 19 April 2013
Your Ref
Our Ref

Enquiries to: Joyce Clearie
Extension: 35674
Direct Line: 0131 465 5674
Email: Joyce.Clearie@nhslothian.scot.nhs.uk

19 April 2013

MISS EMMA DEWAR
PHD STUDENTS
EDINBURGH NAPIER UNIVERSITY
SIGHTHILL CAMPUS
SIGHTHILL COURT
EH114BN

Dear MISS DEWAR

Study title:	**Alpha-1-Acid Glycoprotein (AGP) as a Biomarker of Breast Cancer in at Risk Individuals.**
REC reference:	**13/SS/0053**
IRAS project ID:	**115389**

The Research Ethics Committee reviewed the above application at the meeting held on 17 April 2013. Thank you for attending to discuss the application.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Ms Joyce Clearie, joyce.clearie@nhslothian.scot.nhs.uk.

Ethical opinion

The Committee reviewed the above study. This was a resubmission of a previous study given an unfavourable opinion by SESREC02 earlier in the year. The research team had now taken the comments made on that study on board and resubmitted. This research aims to identify a biomarker which may help identify breast cancer at a very early stage. The researchers aim to collect information on the sugar chain pattern of Alpha-1-Acid Glycoprotein (AGP) present in blood. The pattern of these sugar chains can change during diseases such as breast cancer. Researchers wish to investigate whether there are specific sugar chain patterns in women who are at risk of breast cancer and compare them with women who have a family history of breast cancer and have developed breast cancer. This may be used to detect breast cancer onset earlier in the future. Participants to be posted an information pack by the consultant and then consented by the geneticist. In discussion, the Committee noted the following ethical issues. The Committee discussed with the researcher the clarification provided over the pilot study objectives and they provided further details on this. The researcher was asked about the sample size. This was currently given as 12 but the researcher confirmed that additional samples have been collected already. The researcher explained the nature of these additional samples and what approval they had been obtained under. The researcher agreed these should

NHS
Lothian

be included in the figures. The researcher confirmed that the sample size was therefore larger more in region of 30. The Committee asked for further clarification about the consent process as currently conflicting information given in submission over this i.e. IRAS still mentions the student as being involved in ' the consenting process'. A number of issues relating to whether or not the results of the study should be conveyed to participants were discussed. The Committee agreed that to resolve this the best approach would be for all tissue to be irreversably anonymised and the researcher agreed to this approach i.e. study to be completely anonymised. Currently throughout the submission mention made that can link patient samples to marker so with full anonymisation all documentation to be amended accordingly. The researcher was asked if a participant says no to the bullet point in the ICF to allow for informing their GP would they still be able to take part. The researcher confirmed that, yes still able to participate. The researcher and her supervisor confirmed that process to obtain a research passport for the student was well underway.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

- Please confirm final sample size
- Please be consistent over Identification of participants A27.2 now answered yes and states only Miss Elaine Anderson however answer to A27.1 still mentions ... maybe undertaken by one of research team.
- A27.4 needs to be answered
- As tissue to be anonymised please amend <u>all</u> documentation to reflect this approach e.g. invitation letter at present suggests may get results etc
- Please confirm consent process and ensure all documentation is amended appropriately to reflect that the student will not be involved.
- GP letter suggest attach copy of PIS for their information

<u>Information sheet</u>

- Information sheet page 1 last paragraph relating to clinical data sheet make clearer i.e. reword to read (e.g. height, weight, **questions relating to your medical history)**
- Please make clear that alternative transport arrangements will be made available if this is deemed necessary
- Add' Do I have to take part section' and start paragraph with the word 'No'.
- Please do final typographical check e.g missing the words ...increased **risk**
- Option of receiving summary of the final study report should be made available to all participants.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

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Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		12 March 2013
Evidence of insurance or indemnity		04 February 2013
GP/Consultant Information Sheets	1	08 March 2013
Investigator CV	Dewar	05 February 2013
Letter of invitation to participant	1	04 February 2013
Other: CV Smith		04 February 2013
Other: CV Cetnarskyj		04 February 2013
Other: CV Matthews Smith		04 February 2013
Other: CV Anderson		01 December 2012
Other: Data Collection Sheet		08 April 2013
Other: Reply Slip	2	08 March 2013
Participant Consent Form: PCF	2	08 March 2013
Participant Information Sheet: PIS AGP	2	08 March 2013
Protocol	2	28 February 2013
REC application		20 March 2013

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in

the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/SS/0053	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Mr Thomas Russell
Chair

Email: joyce.clearie@nhslothian.scot.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers" [SL-AR2]

Copy to: *Ms Nina Hakanpaa*
N/A. R&D contact not specified in database.

Appendix 2 – NHS Lothian REC Amendment Approval.

University Hospitals Division

NHS Lothian

Queen's Medical Research Institute
47 Little France Crescent, Edinburgh, EH16 4TJ

RESEARCH & DEVELOPMENT
Room E1.12
Tel: 0131 242 3330
Fax: 0131 242 3343
Email:
R&DOffice@nhslothian.scot.nhs.uk

Director:
Professor David E Newby

KM/LAG

13th November 2013

Ms Elaine DC Anderson,
Edinburgh Breast Unit
Western General Hospital
Crewe Road
EDINBURGH
EH4 2XU

Dear Ms Anderson,

REC No:	13/SS/0053
R&D Project ID No:	2013/W/ON/06
Amendment:	Minor amendment dated 1st November 2013
Title of Research	Alpha1AcidGlycoprotein (AGP) as a Biomarker of Breast Cancer in at Risk Individuals.

I am writing in reply to recent correspondence in relation to an amendment(s) to the above project and the subsequent updated documents as follows.

- Participant consent form Version 4 dated 1st November 2013
- Reply Slip Version 3 dated 1st November 2013
- Participant Information sheet Version 4 dated 1st November 2013

We have now assessed any consequential changes and can confirm that NHS Lothian management approval is extended to cover the specific changes intimated.

Yours sincerely

Mrs Karen Maitland
Research Governance Manager

CC Emma Dewar, Chief Investigator

Appendix 3 – Case Invitation Letter.

University Hospitals Division

NHS Lothian

Edinburgh Breast Unit
Surgical & Associated Services
Western General Hospital, Crewe Road, Edinburgh, EH4 2XU

Clinical Lead: Miss Elaine Anderson

Personal Assistant: Louise Fraser
Telephone: 0131 537 3620

Dear

I am writing to inform you of a pilot research study which has gained ethical approval from the Ethics committee (13/SS/0053).

The research is aiming to identify a biomarker which may help identify breast cancer at a very early stage. The researchers would like to undertake this pilot study in women at increased risk of developing breast cancer due to their family history.

You are not under any obligation to take part in the study and, enclosed with this letter, is an information pack with full details of the study and contact details should you wish further information.

If you do wish to take part in the study, please complete the reply slip and you will be contacted to discuss an appointment.

Thank you for taking the time to read this information.

Yours sincerely,

Elaine D C Anderson
Clinical Director
NHS Lothian Breast and Plastic Surgery

Appendix 4 – Case Participant Information Sheet.

you will be reminded that you can withdraw from the study at any time with out consequence. You will be given a copy of the signed consent form to take away with you as well as a reminder of contact details for the research team should you wish to withdraw from the study at a later date. A member of the research team will give a you a data collection sheet to complete to gather clinically relevant information (e.g. height, weight and questions relating to your medical history) that may affect the results of this study. You are not obligated to give a response to any questions that you do not wish to answer, You will then be given the option to sit or lie down to allow Dr Roseanne Cetnarskyj to take a blood sample of approximately 1 teaspoon of blood. We estimate that this will take no more than 30 minutes. Your blood samples will be given a code so that you cannot be identified from your sample.

The research team will isolate AGP from your blood and analyse the sugar chain patterns to compare them with AGP sugar chain patterns of women who have a family history of breast cancer and have developed breast cancer. If any similarities are found this could be used to develop a new, faster method of breast cancer detection for women at an increased of breast cancer development.

What are the possible benefits of taking part?

There is no direct benefit for taking part in this study although long term the study may discover a mechanism that could help to diagnose breast cancer earlier.

What are the possible disadvantages and risks of taking part?

It is not thought that there are many disadvantages; however, you may feel mild discomfort when the blood sample is being taken and there is a minimal risk of infection but every effort will be made to avoid both of these events.

What happens when the study is finished?

At the end of the research we will destroy any remaining blood samples and the data collected from samples will be used to form the Chief Investigator's PhD thesis and help them achieve a Doctoral degree. Further to this, the data collected may also be used in publications such as journal articles, verbal presentations and poster presentations. To allow for publication, data collected will be kept for 2 years after the Chief Investigators achievement of a Doctoral degree,

Will my taking part in the study be kept confidential?

All the information we collect during the course of the research will be kept confidential and there are strict laws which safeguard your privacy at every stage. Your name will be removed from the data so that you cannot be recognised from it.

What will happen with the results of the study?

The study will be written up as part of the Chief Investigator's PhD thesis and may be published in scientific journals and/or presented at a conference. It is important to understand that you will not receive any results from this study. This is because the results will be of no benefit or disadvantage to participants.

However, you will have the option to receive a summary of the final report (PhD thesis) by leaving your address, **without your name**, in a sealed box at the end of your appointment allowing the summary to be posted out to you. Any addresses will be destroyed once summaries have been posted. Additionally, a summary poster will be sent to Miss Elaine Anderson for participants to view.

Who is organising the research and why?

This study has been organised and funded by Edinburgh Napier University.

Who has reviewed the study?

The study proposal has been reviewed by Edinburgh Napier University. A favourable ethical opinion has been obtained from South East Scotland REC. NHS management approval has also been obtained.

If you have any further questions about the study please contact the Chief Investigator (Miss Emma Dewar) on : 0131 455 2365 or email: e.dewar@napier.ac.uk

If you would like to discuss this study with someone independent of the study please contact Ms Nina Hakanpaa on: 0131 455 6256 or email: n.hakanpaa@napier.ac.uk

If you wish to make a complaint about the study please contact NHS Lothian:

NHS Lothian Complaints Team,
2nd Floor,
Waverley Gate,
2-4 Waterloo Place,
Edinburgh,
EH1 3EG.

Tel: 0131 465 5708

Thank you for taking the time to read this participant information sheet.

Appendix 5 – Case Consent Form.

Version 4, (01/11/2013)

Participant Consent Form

Project Title: Alpha-1-Acid Glycoprotein as a Biomarker of Breast Cancer in at Risk Individuals.

Name of Researcher: Miss Emma Dewar
Edinburgh Napier University Sighthill Campus,
Sighthill Court, Edinburgh, EH11 4BN.
Tel: 0131 455 2365

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Participant's Name: _____ Date of Birth: _____

Site of Research: EDINBURGH NAPIER UNIVERSITY, SIGHTHILL CAMPUS.

		Please Initial
1.	I have read and understand the information sheet dated 01/11/2013 (Version 4) and have had the opportunity to ask questions.	
2.	I understand that participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected.	
3.	I understand that I cannot be identified from my blood sample or data collection sheet.	
4.	I understand that if I withdraw from the study that my blood sample and any data collected will be destroyed.	
5.	I understand that sections of my medical notes and data of the study may be examined by responsible individuals from the sponsor, regulatory authorities or from the NHS organisation, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my medical records.	
6.	I give permission for my GP to be informed of my participation and given any relevant information.	
7.	I understand that I will receive no results from this study.	
8.	I agree to take part in the above study.	

_____	_____	_____
Name of participant	Signature	Date
_____	_____	_____
Name of Person taking consent	Signature	Date
_____	_____	_____
Researcher	Signature	Date

Thank you for agreeing to participate in this research.

Appendix 6 – Case Data Collection Sheet.

Version 2, (08/03/2013)

NHS Lothian Edinburgh Napier UNIVERSITY

Data Collection Sheet

Alpha-1-Acid Glycoprotein as a Biomarker for Breast Cancer in at Risk Individuals.

In order to determine other factors that may influence the results of this study, we would like to ask you to fill in this data collection sheet as much as you are able to. If there are any answers you are unsure how to answer or do not wish to answer, please leave them blank.

Age: _____

Height: _____ Weight::_____ (to calculate BMI)

Do you currently smoke? _____

Are you pre or post menopausal (if applicable)? _____

Breast cancer risk status i.e. Moderate or High: _____

Are you a carrier of a mutation that increases your risk of breast cancer development i.e. BRCA1 or BRCA2? If yes, please specify. _____

Have you ever under gone treatment for Hodgkin's Lymphoma? _____

Have you had any surgery to aid prevention of breast cancer development? _____

Are you on any medication. If yes please specify?

_____

_____

_____

If you would like us to inform your GP and have consented for us to do so, please leave contact details below so an information letter can be posted to your GP.

GP Name: _____

GP Address: _____

_____

_____

_____

Appendix 7 – Age-matched Control Recruitment Poster.

Version 2, (06/12/2013)

Edinburgh Napier UNIVERSITY

Alpha-1-Acid Glycoprotein as a Biomarker for Breast Cancer in at Risk Individuals.

A research study taking place at Edinburgh Napier University (Sighthill Campus) is looking for female participants to act as a control group for the above study.

What is the purpose of the study?

We are aiming to collect information on the sugar chain pattern of Alpha-1-Acid Glycoprotein (AGP). AGP is a protein present in everybody's blood which has sugar chains attached to it's surface. The pattern of these sugar chains can change during diseases such as breast cancer. We wish to investigate whether there are specific sugar chain patterns in women who are at increased risk of breast cancer development. In order to do this we need to have an age matched control for each person we recruit at increased risk. The findings may enable us to detect earlier onset of breast cancer in the future.

What would I be asked to do?

Participants would be required to donate approximately two teaspoons of blood (in one tube) taken by an experienced nurse. Complete a data collection sheet with information e.g. height, weight, age and any medical conditions that may affect the results of the study and have your family history taken by a genetic counsellor to assess your breast cancer risk, to ensure you are not at increased risk which would exclude you from the study. All data would be anonymised and you would be able to withdraw at any time.

Inclusion Criteria:

NOTE TO ETHICS COMIMITTEE: WHEN FINAL POSTERS ARE DISTRIBUTED WE WILL SPECIFY THE NUMBER AND AGE OF PARTICIPANTS WE REQUIRE FOR AGE MATCHING. E.G. Five people aged 30, Two people aged 27 etc.

Exclusion Criteria:

Individuals cannot participate if:

- They already know they have an increased risk of breast cancer.
- Have an inflammatory condition such as arthritis or Crohn's disease.
- Are diabetic or pregnant,.
- Have had recent surgery.

For more information please contact

About the study:

Miss Emma Dewar

e.dewar@napier.ac.uk

0131 445 2365

About having family history taken:

Dr Roseanne Cetnarskyj

r.cetnarskyj@napier.ac.uk

0131 455 5713

Appendix 8 – Age-matched Control Participant Information Sheet.

Version 2, (06/12/2013)

Edinburgh Napier UNIVERSITY

Control Participant Information Sheet

Alpha-1-Acid Glycoprotein as a Biomarker for Breast Cancer in at Risk Individuals.

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being undertaken and what it will involve. Please take time to read the following information carefully. Talk to other people about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We are aiming to collect information on the sugar chain pattern of Alpha-1-Acid Glycoprotein (AGP). AGP is a protein present in everybody's blood which has sugar chains attached to it's surface. The pattern of these sugar chains can change during diseases such as breast cancer. We wish to investigate whether there are specific sugar chain patterns in women who are at increased risk of breast cancer development. In order to do this we need to have an age matched control for each person we recruit at increased risk. The findings may enable us to detect earlier onset of breast cancer in the future.

Why have I been asked to take part?

You have been asked to take part as you are an individual, who could be age matched to one of our increased risk participants, thus acting as a control population for this study.
You cannot participate if you know you have an increased risk of breast cancer, have diabetes, are pregnant, have had recent surgery or have an inflammatory condition such as arthritis or Crohn's disease.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Deciding not to take part or withdrawing from the study will not affect the healthcare that you receive.

What will happen if I take part?

If you agree to participate, by contacting Miss Emma Dewar, you will be contacted by a member of the research team to arrange a suitable time for you to attend the Clinical Skills laboratory at Edinburgh Napier University's Sighthill Campus at your convenience. Your written consent will be taken using a consent form (draft copy enclosed) by Dr Roseanne Cetnarskyj and you will be reminded that you can withdraw from the study at any time with out consequence. You will be given a copy of the signed consent form to take away with you as well as a reminder of contact details for the research team should you wish to withdraw from the study at a later date. A member of the research team will give a you a data collection sheet to complete to gather clinically relevant

information (e.g. height, weight and questions relating to your medical history) that may affect the results of this study. You are not obligated to give a response to any questions that you do not wish to answer. You will then have your family history taken and risk assessed by Dr Roseanne Cetnarskyj. If you are assessed at increased risk by Dr Roseanne Cetnarskyj, she is a genetic counsellor and will be able to give you advice and discuss any anxieties. Unfortunately, you will not be able to proceed if you are at increased risk. If you are assessed at low risk we would like your blood sample. You will then be given the option to sit or lie down to allow a trained nurse to take a blood sample of approximately 2 teaspoons of blood in one tube. We estimate that this will take no more than 40 minutes. Your blood samples will be given a code so that you cannot be identified from your sample.

The research team will isolate AGP from your blood and analyse the sugar chain patterns to compare them with AGP sugar chain patterns of women who have a family history of breast cancer and have/have not developed breast cancer. If any differences that are found this could be used to develop a new, faster method of breast cancer detection for women at an increased of breast cancer development.

What are the possible benefits of taking part?

There is no direct benefit for taking part in this study although long term the study may discover a mechanism that could help to diagnose breast cancer at an earlier stage.

What are the possible disadvantages and risks of taking part?

You may feel mild discomfort when the blood sample is being taken and there is a minimal risk of infection but every effort will be made to avoid both of these events. If you are assessed as being at increased risk of developing breast cancer this may make you anxious but you will be referred to the appropriate clinical service for on-going support, with your permission.

What happens when the study is finished?

At the end of the research we will destroy any remaining blood samples and the data collected from samples will be used to form the Chief Investigator's PhD thesis and help them achieve a Doctoral degree. Further to this, the data collected may also be used in publications such as journal articles, verbal presentations and poster presentations. To allow for publication, data collected will be kept for 2 years after the Chief Investigators achievement of a Doctoral degree,

Will my taking part in the study be kept confidential?

All the information we collect during the course of the research will be kept confidential and there are strict laws which safeguard your privacy at every stage. Your name will be removed from the data so that you cannot be recognised from it.

Your family history will be shredded after your risk is assessed or you can take it home.

What will happen with the results of the study?

The study will be written up as part of the Chief Investigator's PhD thesis and may be published in scientific journals and/or presented at a conference. It is important to understand that you will

not receive any results from this study. This is because the results will be of no benefit or disadvantage to participants. However, a summary poster will be placed around the university campuses, detailing the results of the study.

Who is organising the research and why?

This study has been organised and funded by Edinburgh Napier University.

Who has reviewed the study?

The study proposal has been reviewed by Edinburgh Napier University and obtained favourable ethical opinion.

If you have any further questions about the study please contact the Chief Investigator (Miss Emma Dewar) on : 0131 455 2365 or email: e.dewar@napier.ac.uk

If you have questions regarding having your family history taken please contact Dr Roseanne Cetnarskyj on: 0131 455 5713 or email: r.cetnarskyj@napier.ac.uk

If you would like to discuss this study with someone independent of the study please contact Miss Jay MacKinnon on: 0131 455 5720 or email: j.mackinnon@napier.ac.uk

Thank you for taking the time to read this participant information sheet.

Appendix 9 – Age-matched Control Consent Form.

Version 2, (06/12/13)

Edinburgh Napier UNIVERSITY

Control Participant Consent Form

Project Title: Alpha-1-Acid Glycoprotein as a Biomarker of Breast Cancer in at Risk Individuals.

Name of Researcher: Miss Emma Dewar
Edinburgh Napier University Sighthill Campus,
Sighthill Court, Edinburgh, EH11 4BN.
Tel: 0131 455 2365

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Participant's Name: _____ Date of Birth: _____

Site of Research: EDINBURGH NAPIER UNIVERSITY, SIGHTHILL CAMPUS.

		Please Initial Box
1.	I have read and understand the information sheet dated 02/012/2013 (Version 1) and have had the opportunity to ask questions.	☐
2.	I understand that participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected.	☐
3.	I understand that I cannot be identified from my blood sample or data collection sheet.	☐
4.	I understand that I will have my family history taken and a risk assessment given.	☐
5.	I understand that my family history information will be shredded after risk is assessed or I am free to take it away from the appointment.	☐
6.	I understand that if I withdraw from the study that my blood sample and any data collected will be destroyed.	☐
5.	I understand that I will receive no results from this study.	☐
6.	I agree to take part in the above study.	☐

_____	_____	_____
Name of participant	Signature	Date
_____	_____	_____
Name of Person taking consent	Signature	Date
_____	_____	_____
Researcher	Signature	Date

Thank you for agreeing to participate in this research.

Appendix 10 – Age-matched Control Data Collection Sheet.

Version 2, (06/12/2013)

Edinburgh Napier UNIVERSITY

Control Data Collection Sheet

Alpha-1-Acid Glycoprotein as a Biomarker for Breast Cancer in at Risk Individuals.

In order to determine other factors that may influence the results of this study, we would like to ask you to fill in this data collection sheet, as much as you are able to. If there are any answers you are unsure how to answer please discuss with the researcher. If you do not wish to answer any questions, please leave them blank.

Age: _____

Height: _____ Weight::_____ (to calculate BMI)

Have you ever been a smoker? _____

Are you pre or post-menopausal (if applicable)? _____

Are you on any medication or receiving any medical treatments? If yes please specify.

_____

_____

_____

Appendix 11 – Case Reply Slip.

Version 2, (08/03/2013)

Reply Slip.

Alpha-1-Acid Glycoprotein as a Biomarker for Breast Cancer in at Risk Individuals.

Thank you for taking the time to read the patient information sheet (version 2). Once you have considered your options, please reply below. We will use this information to contact you to arrange an appointment at the Clinical Skills Centre located at Edinburgh Napier University's Sighthill Campus. This information will also be used to either provide a Lothian Bus Day Ticket or book a taxi for you to attend your appointment.

Full Name: _____

Address: _____

_____

_____

Post Code: _____

Telephone/Mobile Number (that you are happy to be contacted on): _____

When is the best time of day to contact you to arrange an appointment?:

_____

Would it be acceptable to leave a message on an answer phone to say we had called? _____

Please tick (✓) as appropriate.

I would like to participate in this study. ☐

I do not wish to participate in this study. ☐

PLEASE RETURN IN THE PRE-PAID ENVELOPE PROVIDED, THANK YOU.