Analysing the Glycosylation Profile of Triple Negative Breast Cancer

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A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Master by Research

October 2019

Declaration:

I certify that:

a) That this thesis is a result of my own independent work.

b) This work has not been submitted for any other degree or professional qualification.

Shannon Sutherland.

Abstract

Protein glycosylation is a sequential process that involves many enzymes and aids in protein stability and cell signalling. Protein glycosylation is altered in cancer and can increase its invasive and metastatic potential. Altered glycosylation may be due to the hypoxic tumour microenvironment. Hypoxia inducible transcription factors (HIFs) orchestrate a shift in cellular metabolism towards glycolysis. As glycolytic intermediates are used in protein glycosylation, this shift can alter nucleotide sugar availability and potentially alters glycan structures. Furthermore, changes in gene transcription in hypoxia may also alter glycosylating gene and promote atypical protein glycosylation. Ultimately these may alter glycosylated protein function and confer aggressive tumour growth.

This project aimed to profile changes in glycogene expression in triple negative breast cancer (TNBC) where glycogene expression was analysed in tissue matched TNBC samples by qPCR using a profiler array. Thirty-two glycogenes were upregulated by 2-fold or more. Through qRT-PCR, altered glycogene expression was validated in glycogenes of the: *GALNT, B3GNT, B4GALT* families in hypoxic MDA-MB-468 TNBC cells, which replicated the tumour environment. Altered glycogene expression was also found in these hypoxic cells. Lectin binding analysis on MDA-MB468 cells elucidated that hypoxia significantly alters the proportions of sugar residues on the cells.

Furthermore, it was identified that HIF-1α expression correlated with the expression of glycogenes: *GALNT3, GALNT6, GALNT7, GALNT12, B3GNT2, B4GALT2, B4GALT3, NEU1* and *NEU3.* SiRNA knockdown of HIF-1α and HIF-2α in hypoxic MDA-MB-468 cells elucidated suppressed HIF-1α significantly altered the expression of *B3GNT2, B4GALT3, GALNT6* and *GALNT12.*

Acknowledgments.

I'd like to thank my director of studies Dr. Jenny Fraser for giving me this opportunity and for all her help and support throughout this process and Dr. Sharron Vass for her valuable input. Secondly, I'd like to thank my Family and Jamie for without them, I would not have completed this research.

I'd also like to thank my Uni friends and work friends, for putting up with me throughout the last two years.

This thesis is dedicated to Adeline Milne Sutherland.

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Abbreviations.

ATP	Adenosine Triphosphate
bHLH	Basic helix-loop-helix.
BP	Base Pair
BrCa	Breast Cancer
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic acid
C_T	Cycle Threshold
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor
ERK	Extracellular signal-related kinase
FSC-H	Forward scatter
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GBPs	Glycan Binding Proteins
GlcNAc	*N*-acetylglucosamine
HER2	Human Epidermal Growth Factor Receptor 2
HRE	Hypoxia Response Element
IHC	Immunohistochemistry
kDa	Kilodaltons
LacNAc	*N*-acetyllactosamine
Man	Mannose
MMP	Matrix Metalloproteinase
NTC	No Template Control
ODD:	Oxygen-dependent Degradation.
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline - Tween
PCR	Polymerase Chain Reaction
PE-Strep	Phycoerythrin-conjugated Streptavidin
PFFE	Paraffin-fixed Formalin Embedded.
PHD	Prolyl Hydroxylases

PR	Progesterone Receptor.
PTM	Posttranslational Modification.
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RT	Reverse Transcription
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SIA	Sialic Acid
siRNA	Small-interfering Ribonucleic acid
SSC-H	Side Scatter
TAD-C.	Transacting Domain C-terminal.
TAD-N:	Transacting Domain N-terminal
TCA	Tricarboxylic Acid Cycle
TGF	Transforming Growth Factor
TNBC	Triple Negative Breast Cancer
TR	Transfection Reagent
VHL	von Hippel-Lindau Protein

Chapter 1: Introduction

1.1: Breast Cancer.

Breast cancer (BrCa) is the most common form of cancer found in women in the UK and it is a leading cause of cancer mortality (Breast Cancer Now, 2019). BrCa is a heterogeneous group of neoplasms with different subtypes and morphologies. This heterogeneity is well established and serves as the basis for clinical outcomes and disease classification (Polyak, 2011). BrCa is broadly subcategorised into *in situ* carcinoma and invasive/infiltrating carcinoma (Stingl and Caldas, 2007). Additionally, BrCa subtypes can be distinguished based on the presence or absence of hormone receptors.

Extensive gene expression profiling classifies breast tumours into three subtypes: human epidermal growth factor 2 positive (HER2+), luminal and basal like (Sørlie, 2001). Luminal tumours are positive for oestrogen (ER) and progesterone receptors (PR) whereas HER2+ tumours show overexpression of *ERBB2*, an oncogene that can be controlled by anti-HER2 therapies (Hynes, 2016). Triple negative breast cancer tumours lack hormone receptors, making them very difficult to treat (Lehmann, 2011).

1.1.2: Triple Negative Breast Cancer.

TNBC accounts for approximately 15% of breast cancer and it typically affects younger women and those of African American/Hispanic decent (*Triple negative breast cancer* | *Cancer Research UK*, 2017). TNBC is characteristically a more

aggressive form of BrCa compared to other subtypes (Gonçalves *et al.*, 2018) and is associated with a worse patient prognosis. TNBC has a characteristic recurrence pattern with the majority of deaths occurring in the first 3 and 5 years after initial treatment (Ovcaricek *et al.*, 2011). TNBCs are highly invasive (Ovcaricek *et al.*, 2011), have an accelerated tumour proliferation rate and are of high nuclear grade (Jitariu *et al.*, 2017). In general, the higher the nuclear grade the more aggressive the tumour cells are due to abnormal cellular nuclei (Jitariu *et al.*, 2017). These factors collectively make TNBC particularly aggressive and difficult to treat with options limited to surgery in combination with radiation and chemotherapy (Park, Ahn and Kim, 2018).

1.2: Protein Glycosylation

Protein glycosylation is post translational modification (PTM) that produces glycosidic linkages of saccharides to other saccharides, lipids and proteins (Fuster and Esko, 2005). Glycosylation is one of the most complex and prevalent PTM that occurs in eukaryotes, eubacteria and archaea (Lechner and Wieland, 1989; Messner, 1997). PTMs are covalent and enzymatic proteins modifications that occur during or after protein biosynthesis and aid in protein folding, stability and cell signalling (Bah and Forman-Kay, 2016).

1.2.1: Monosaccharides: the basic structural units of glycans.

Monosaccharides are attached to other monosaccharides *via* glycosidic linkages to form linear or branched structures through an enzymatic process (Varki and Sharon, 2009). Glycosidic linkages bond monosaccharides to other residues typically through their hydroxyl group; forming α or β linkages (Varki and Sharon, 2009). When one or more monosaccharide or oligosaccharide units are covalently linked to a non-carbohydrate moiety, a glycoconjugate is formed (Varki, Freeze and Manzi, 2009). Glycans (polysaccharides) are simple or complex carbohydrate molecules that vary significantly and often encompass a substantial amount of the mass of a glycoconjugate molecule (Dwek, 1996). All cell types are heavily decorated with varying sugar molecules giving rise to the term cell-glycocalyx (Reitsma *et al.*, 2007).

1.2.2: The major glycan classes.

In eukaryotes, the most common classes of glycoconjugates found are defined based on the nature of their linkage to the non-glycosyl (aglycone) region (Varki *et al.*, 2015). The two main glycan types are described as *N*- or *O*- linked-glycans in which *O*-glycosylation is the covalent modification of serine or threonine residues in mammalian glycoproteins (Brockhausen, Schachter, & Stanley, 2009). Currently a consensus sequence for GalNAc addition to polypeptides has not been determined (Bapu *et al.*, 2016). *N*-linked glycosylations occur when a sugar moiety, most often *N*-acetylglucosamine (GlcNAc), is attached to an

asparagine residue of a polypeptide, in the consensus sequence Asn-x-ser/Thr, where X is any amino acid except for proline (Varki and Sharon, 2009).

1.3: *O*-Linked Glycosylation.

O-glycosylation is initiated when an α-linked *N*-acetylgalactosamine (*O*-GalNAc) residue becomes linked to a serine or threonine amino acid in the Golgi apparatus (Brockhausen, Schachter, & Stanley, 2009). This structure can be extended with a number of sugars including galactose, *N*-acetylglucosamine (GlcNAc), fucose or sialic acid through a highly regulated and sequential process (Yamashita *et al.*, 1995) (Figure 1.1).

There are four common forms of *O*-glycans: cores 1-4 (Brockhausen, Schachter and Stanley, 2009) (Figure 1.1). These structures are often branched, resulting in a highly variable population of sugars (Brockhausen, Schachter and Stanley, 2009). Many of the sugar molecules on mucin type *O*-glycans are antigenic, for example: the T antigen (also known as Core 1) (Brockhausen, Schachter and Stanley, 2009). T antigen is cancer specific and is made up of short *O*-GalNAc residues but can also carry sialic acid and form sialyl-T antigens (Tian and Ten Hagen, 2009) (Figure 1.1).

Core 2 structures are formed by the addition of β1-6 GalNac to core 1 structures; core 3 structures are less common and are synthesised when a β1-3 GlcNAc is added to *O*-GalNAc (Brockhausen *et al.*, 2009) (Figure 1.1). Core 4 is a simple extension of core 3 *O*-Glycans in which another GlcNAc residue is connected *via* a β1-6 linkage (Corfield and Berry, 2015) (Figure 1.1).

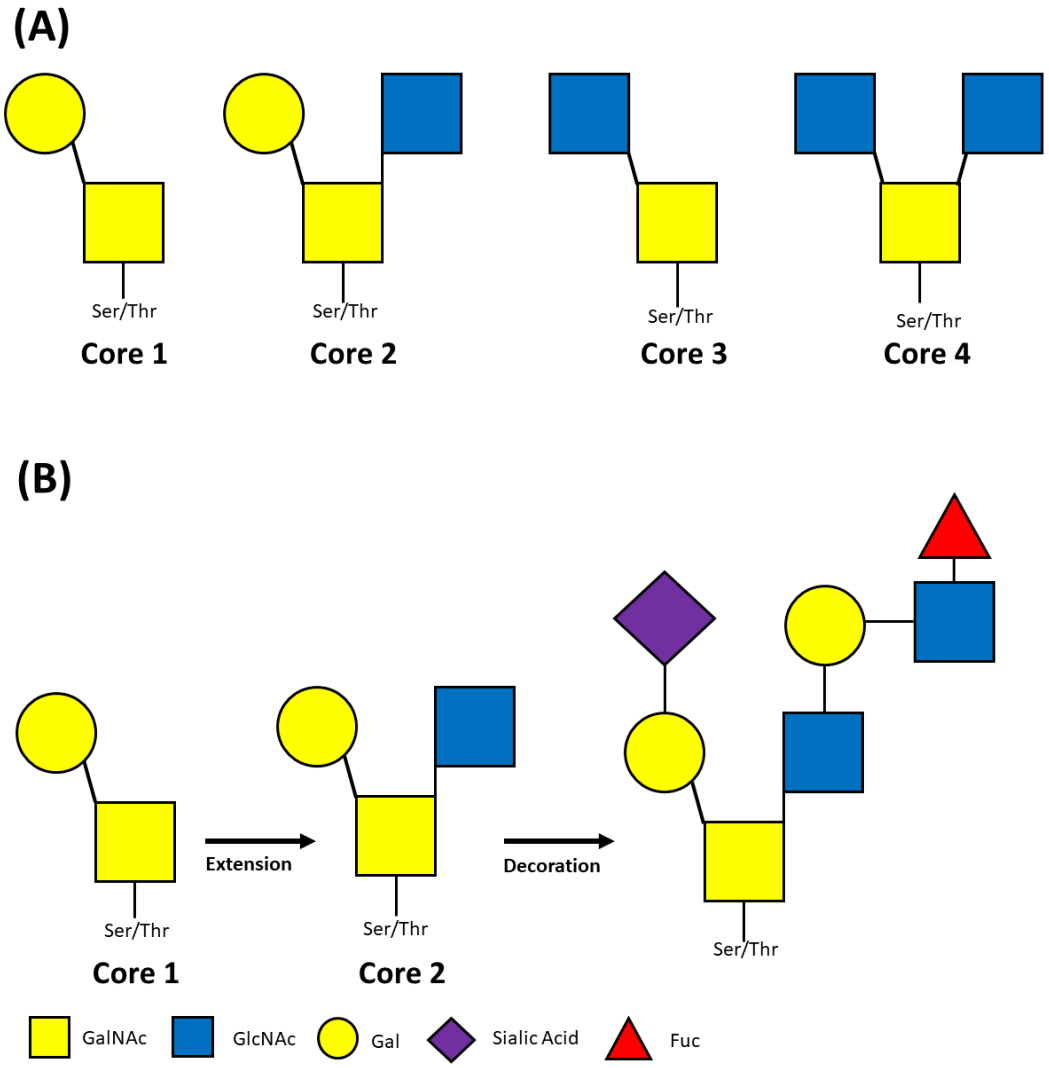

Figure 1.1: Mucin-type core structures and their decoration. Representative examples of core 1, 2, 3 and 4 extended *O*-glycans composed of *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) and galactose (Gal) residues. All four cores can be subject to branching, extension and can be terminated by sialic acid and fucose (Fuc) residues (Brockhausen, Schachter and Stanley, 2009).

1.3.1: *O*-linked glycosylation initiation: The GALNT family.

O-glycosylation is initiated by a group of polypeptide glycosyltransferases with the prefix GALNT. The GALNT family is made up of approximately 21 enzymes encoded by different genes (Brockhausen *et al.*, 2009). Little is known about these enzymes however, it has been proposed that glycosylated mucin domains are created by the action of several of the GALNT enzymes, in a hierarchical and successive process (Pratt *et al.*, 2004). Twelve mammalian isoforms for GALNT enzymes exist including GALNT1-13 (Ten Hagen, Fritz and Tabak, 2003). The distribution of these enzymes in adult tissues is discrete. Some isoforms are found in a number of tissues i.e. GALNT1 and GALNT2 and act upon a large range of substrates (Ten Hagen, Fritz and Tabak, 2003) while others are more specific in their substrate specificity and expression (GALNT5, GALNT7, GALNT10 and GALNT11) (Hagen *et al.*, 2003).

Pratt *et al*., (2004) proposed that GALNT1, 2 and 5 represent the first level of *O*-linked glycosylation as their enzymatic activity occurs early in the *O*-glycosylation process. These enzymes prefer non- and -monoglycosylated peptide substrates which coincides with the fact that their activity is inhibited by steric inhibition due to glycosylation of the neighbouring Ser/Thr residue (Gerken *et al.*, 2002). It was suggested that GALNT3 and GALNT4 represent the intermediate level of *O*-linked glycosylation and prefer di-glycosylated peptides (Pratt *et al.*, 2004). Furthermore, GALNT10 may facilitate the completion of mucin-domain assembly and contribute to the highly glycosylated glycans found *in vivo.*

1.4: *N*-linked glycosylation

N-linked glycosylation is one of the most important and chemically complex PTMs in all eukaryotes (Stanley, Taniguchi and Aebi, 2015). *N*-glycan synthesis occurs in a two-phased process in the endoplasmic reticulum and Golgi apparatus (Aebi, 2013). *N*-linked oligosaccharides share a common core $GlcNAc_2Man_3$ structure and synthesis begins on the cytoplasmic side of the ER membrane. Transfer of GlcNAc-P from UDP-GlcNAc to dolichol phosphate, a lipid like precursor, generates dolichol pyrophosphate *N*-acetylglucosamine (Dol-P-P-GlcNAc) (Chang *et al.*, 2015). Fourteen sugar residues are added to Dol-P and the entire glycan is then transferred “en bloc” to an Asn-x-Ser/Thr sequon in a protein (Stanley, Schachter, & Taniguchi, 2009). The *N*-glycan is modified further *via* hydrolytic removal of sugar residues by highly specific α-glycosidases and α-mannosidases that remove terminal glucose and mannose, respectively (Liebminger *et al.*, 2011). EDEMs (endoplasmic reticulum degradation-enhancing α-mannosidases like proteins) are also involved in early *N*-glycan processing, where they recognise and target misfolded proteins for ER degradation (Olivari and Molinari, 2007). These initial trimming steps are conserved among eukaryotes and regulate glycoprotein folding though endoplasmic reticulum chaperone interactions that recognise specific features of the shortened glycan (Stanley, Schachter and Taniguchi, 2009).

After trimming, the glycan is re-glycosylated with other sugars including galactose, fucose and sialic acid (Bieberich, 2014) (Figure 1.2). The final product of early *N*-glycan processing is $Man_5GlcNAc_2$, which is used as an acceptor substrate by GNT1 for the transfer of a single GlcNAc residue (Strasser *et al.*, 1999). Without the activity of *GNT1,* enzymes such as GM2, GNT2, XYLT, FUT11

and FUT12 cannot function and formation of complex glycans is inhibited. In the event of premature truncation, the *N*-glycan cannot undergo further remodelling to form a complex or hybrid structure.

In the *trans*-Golgi, the final *N*-glycan modifications occur. GALT1 and FUT13 synthesise Lewis-α-trisaccharide complexes on *N*-glycans (Strasser *et al.*, 2007). This Lewis epitope is highly expressed in the developing brain and is involved in cell-cell interactions (Yaji *et al.*, 2015).

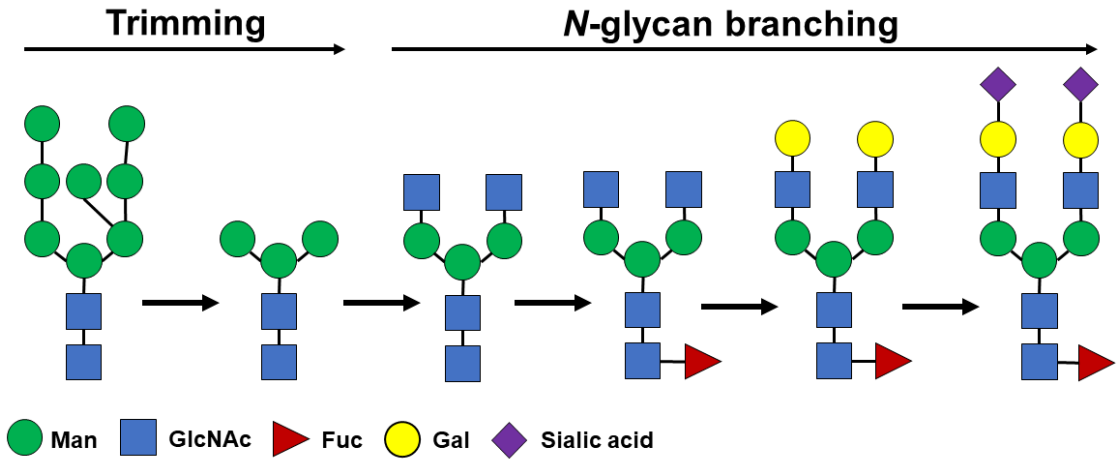

Figure 1.2: Biosynthesis of N-glycans within the Golgi Apparatus. Glycans are trimmed and extended in the Golgi apparatus. Glycotransferases use nucleotide sugars as extension reaction donors (adapted from Bieberich, 2014). Symbols used to represent sugar molecules are indicated in the key. Man: Mannose, GlcNAc: N-acetylglucosamine, Fuc: Fucose, Gal: Galactose.

1.4.1: *N*-glycan subtypes.

N-glycans are divided into three types: complex, hybrid and high mannose (Figure 1.3). In general, *N*-glycans are heterogeneous and this structural diversity is called glycoform (Nagasaki *et al.*, 2016). High mannose glycans comprise a glycan in which only mannose residues are attached to the core (Figure 1.3). Complex *N*-glycans contain outer chains with sialic acid, galactose, GalNAc and α-linked mannose residues (Tabas, Schlesinger and Kornfeld, 1978) (Figure 1.3). Hybrid *N*-glycans have two branches from the core, one of which terminates in mannose and the other which terminates with a complex sugar (Yamashita, Tachibana and Kobata, 1978) (Figure 1.3).

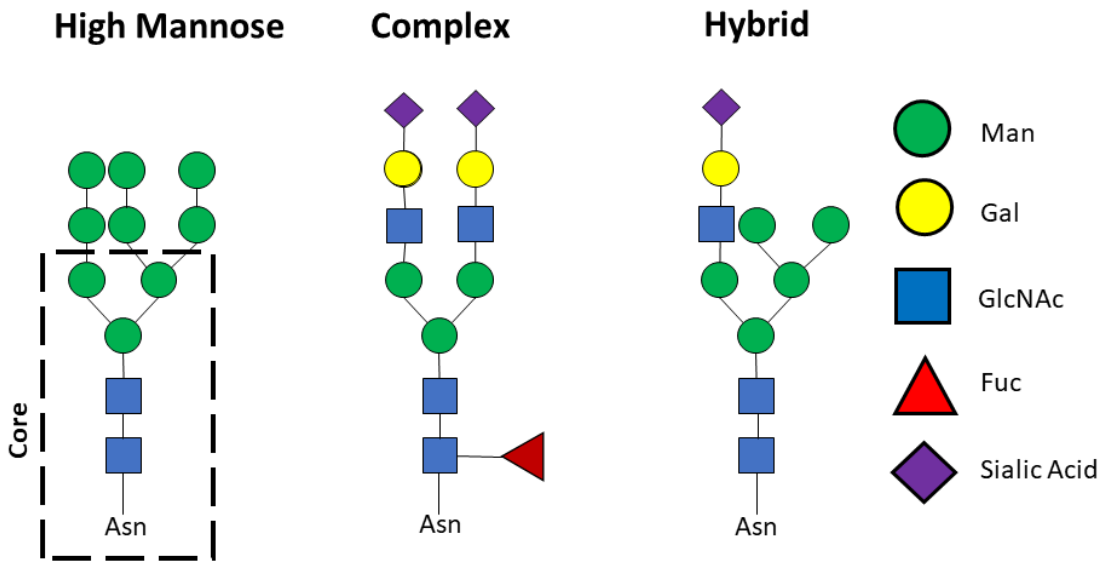

Figure 1.3: N-glycan structural subtypes. Three forms of N-glycans exist including high mannose (man), hybrid and complex type N-glycans. All three structures share a common core structure of di-N-acetylglucosamine tri-mannose linked to an asparagine. Sugars are indicated in the key. Adapted from (Higel *et al*., 2016).

1.5: The biological role of glycans.

The functional role of biological glycans is divided into three comprehensive categories: modulatory and structural properties, extrinsic recognition and intrinsic recognition (Varki and Lowe, 2009). Extrinsic glycan binding proteins (GBPs) are composed of pathogenic microbial adhesins, agglutinins or toxins, whereas intrinsic GBPs mediate cell-cell interactions or facilitate in the recognition of extracellular molecules (Varki and Lowe, 2009). Processes including cell development, immunity, and cancer invasion are regulated by glycans (Imai *et al.*, 1990; Solá and Griebenow, 2009; Zhou *et al.*, 2014). For example, the ability to swallow food is facilitated by lubricating glycans (Maytin, 2016), and the signalling properties of proteins attached to glycans can affect transforming growth factor (TGF) signalling - which aids in normal lung development (Wang *et al.*, 2005). In terms of immune recognition, heavily glycosylated cells can evade loading onto the major histocompatibility complex of immune B cells, as peptide loading is disrupted. This mechanism of immune evasion is often used by enveloped viruses, which are heavily glycosylated (Wang *et al.*, 2009). *N*-glycan structure can also influence the function and dynamics of glycoproteins. Removal of non-reducing end sugars and linking of sialic acid to the galactose 3-OH or 6-OH position of a complex *N*-glycans significantly affects glycoprotein distribution (Ogura *et al.*, 2016). One of the major modification of complex *N*-glycans includes the linkage of a fucose residue to the reducing end GlcNAc (Becker and Lowe, 2003). The transfer of fucose to complex *N*-glycans is facilitated by fucosyl transferase 8 (*FUT8*) which forms an α1-6 fucosyl linkage and has been shown to play an important role in cell growth, cell survival and cell mediated cytotoxicity (Wang *et al.*, 2005; Tomita *et al.*, 2012). Moreover, core

fucosylation has been implicated in the pathophysiological steps involved in carcinogenesis and tumour progression (Moriwaki and Miyoshi, 2010).

1.6: Cancer and glycosylation.

Altered glycosylation is a hallmark feature of cancer cells and the expression of specific glycans are known to be distinguished markers of tumour progression (Varki *et al.*, 2015). Tumour cells show numerous glycosylation alterations compared to healthy tissue, which results in a heterogeneous cell population (Pinho & Reis, 2015). The most common cancer-associated changes to cell glycosylation include sialylation, fucosylation, *O*-glycan truncation, and *N*- and *O*-linked glycan branching (Hakomori, 2002; Pinho & Reis, 2015). According to the hallmarks of cancer' proposed by Hanahan and Weinberg in 2011, avoiding immune surveillance, activating invasion, metastasis and deregulated cellular energetics are classical strategies of cancer progression (Hanahan and Weinberg, 2011). The ways which altered protein glycosylation facilitates these mechanisms under malignancy has been well-documented. Changes to protein glycosylation in cancer can lead to steric inhibition of adhesion molecules such as cadherins and integrins, which can promote cell displacement and initiation of metastasis (Varki *et al.*, 2015).

The Tn antigen is highly expressed in 90% of breast carcinomas (Prokop and Uhlenbruck, 1969), and in 10-90% of bladder, cervix, colon, lung, stomach, ovary and prostate carcinomas (Desai, 2000). Despite its abundant presence in cancer, the role of the Tn antigen in cancer development remains unclear (Fu *et al.*, 2016). However, the Tn antigen has been strongly tied to the metastatic potential

of a number of cancers including breast, gastric, and cervical (Fu *et al.*, 2016) and furthermore, its expression has been implicated in tumour immunosurveillance (Ju *et al.*, 2013).

Hakomori & Kannagi (1983) first described the two main mechanisms which promote tumour-associated alterations of carbohydrate structures. They postulated that incomplete synthesis of glycans occurs more often in early carcinogenesis, and subsequently inhibits the normal synthesis of complex glycans that are found in healthy tissues (Hakomori and Kannagi, 1983). By contrast, the theory of neo-synthesis was proposed, in which in advanced stages of cancer, sialyl Lewis X and sialyl Lewis A (cancer associated glycans) are induced by the malignancy itself. A shift from the classical glycosylation pathway occurs in cancer cells and inevitably leads to altered glycan expression. This change in glycan structures and expression is owed to a number of factors including altered expression (increase or decrease) of glycosylation enzymes including glycosyltransferases and the availability of nucleotide sugars (Pinho & Reis, 2015). The expression and activity of glycosidase's and glycosyltransferases have a large impact on the distribution and abundance of glycans (Doherty *et al.*, 2018). Interestingly, it has also been suggested that the intra-environment of the Golgi apparatus may promote aberrant glycosylation through changes in pH and structural organisation of the apparatus itself (Kellokumpu, Sormunen and Kellokumpu, 2002).

1.6.1: Altered glycosylation and TNBC.

Altered protein glycosylation is common is a number of cancers including 90% of BrCa (Hanahan and Weinberg, 2011; Zhou *et al.*, 2014; Chen *et al.*, 2016).

Aberrant expression of glycans has been seen in a high percentage of breast cancers and frequently presents as the loss of core glycan structures and the expression of tumour-associated antigen markers (Springer, 1984; Burchell, Mungul and Taylor-Papadimitriou, 2001). In TNBC, 72 differentially expressed glycoproteins have been identified, most notably: Vascular endothelial growth factor receptor 1, Insulin receptor and Tissue factor pathway inhibitor (Chen *et al.*, 2016). However, little is known about how altered glycosylation is influenced by the hypoxic tumour environment and how this promotes aggressive tumour growth in TNBC.

1.7: Hypoxia.

Hypoxia (pO_2 < 10 mmHg) is defined as the failure of oxygenation at the tissue level (Samuel and Franklin, 2008) and it is found in pathophysiological conditions including: atherosclerosis, sleep apnoea, mountain sickness, ischemic disease and many cancers (Li *et al.*, 2006; Brahimi-Horn and Pouysségur, 2007; Brahimi-Horn, Chiche and Pouysségur, 2007). In solid tumours, hypoxia is common due to uncontrolled cell proliferation in the absence of efficient vascularisation (Semenza, 2013). Due to unlimited proliferation of tumour cells, increased oxygen consumption is observed as Intratumoural hypoxia develops and the cells outstrip their blood supply (Koh, Spivak-Kroizman and Powis, 2010).

Hypoxia promotes an imbalance between pro- and anti-angiogenic factor production, which induces rapid and enhanced chaotic blood vessel formation (Conway, Collen and Carmeliet, 2001). In tumours, new blood vessels are often abnormal and prone to leakage and collapse which causes local hypoxia. The

formation of new blood vessels in tumour tissue increases blood and oxygen supply for continuous tumour growth (Carmeliet and Jain, 2000). However, the ever-growing tumour ultimately outgrows its blood supply and causes further tumoral hypoxia which stimulates additional angiogenesis, in a vicious cycle. Enhanced angiogenesis is linked to cancer metastasis due to improved extravasation, circulation and the movement of tumour cells to better vascularised normoxic tissues (Carmeliet and Jain, 2011).

The hypoxic tumour environment creates a particularly hostile environment which is subject to decreased respiration, increased glycolysis, low pH due to increased lactate production and compromised ATP production (Eales, Hollinshead and Tennant, 2016; San-Millán and Brooks, 2016; Sgarbi *et al.*, 2018). In healthy cells, oxidative phosphorylation is the mechanism for adenosine triphosphate (ATP) production however in tumours, pyruvate is converted into lactate as opposed to entering the tricarboxylic acid cycle (TCA), through aerobic glycolysis. This is called the Warburg effect (Figure 1.4) (Warburg, Wind and Negelein, 1927). This lactate acid production occurs at a rate 40 times higher than normal cells (Holm *et al.*, 1995) and results in a highly acidic tumour microenvironment. This promotes tumour metastasis and treatment resistance, partly because of increased cell detachment (Neri and Supuran, 2011) (Figure 1.4). As a result, tumour cells promote adaptive responses to match O_2 supply with bioenergetic, metabolic and redox demands.

Intratumoural hypoxia is commonly found in breast cancer and is strongly associated with a significantly increased rate of metastasis and patient mortality (Semenza, 2016). TNBC is inherently hypoxic due to poor vascularisation as shown by overexpression of hypoxia-inducible factor 1-α (HIF-1α) (Semenza,

2013) and a median partial oxygen pressure of 10 mmHg compared to 65 mmHg found in healthy breast tissue. The activation of hypoxia inducible factors (HIFs) drives expression of a battery of genes and promotes a toxic tumour microenvironment which facilitates aggressive tumour growth (Gilkes and Semenza, 2013).

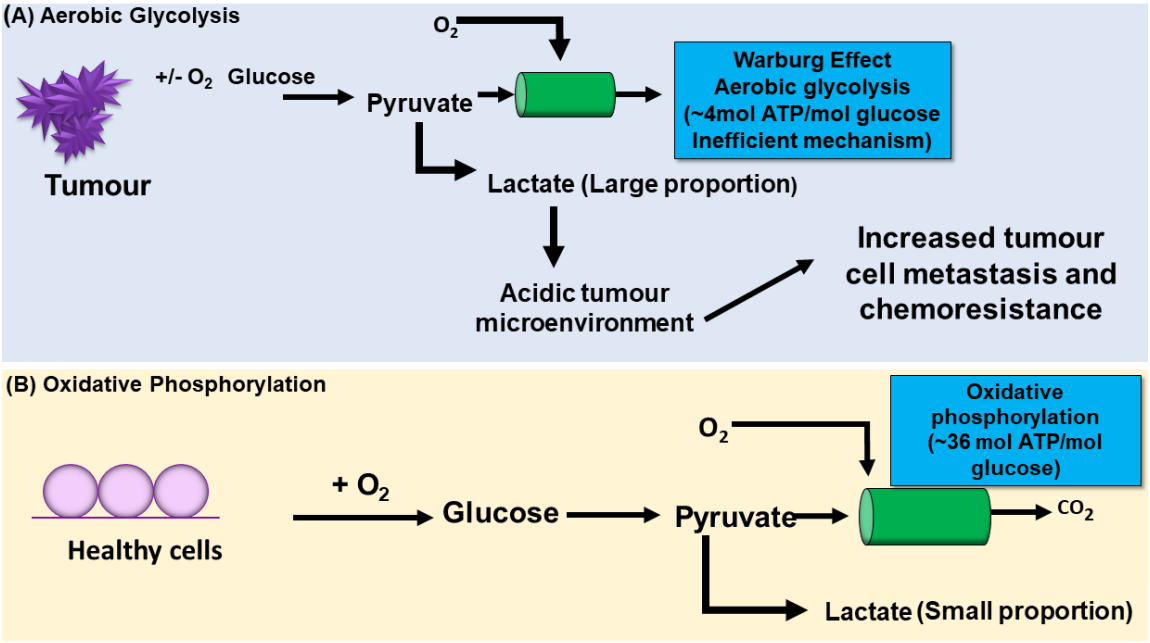

Figure 1.4: The metabolic shift in cancer. (A) To facilitate tumour growth, cancer cells promote uptake of glucose and increase lactate production (aerobic glycolysis) compared to healthy cells. This creates an acidic tumour environment which increases tumour metastasis and invasion. **(B)** Healthy cells favour the energy efficient oxidative phosphorylation mechanism for ATP production which creates a small proportion of lactate.

1.7.1: Hypoxia inducible factors (HIFs).

Hypoxia-inducible factors' (HIFs) play a crucial role in the adaptive response to reduction in oxygen availability and drive the expression of many genes involved

in glucose metabolism, cell proliferation and angiogenesis (Annibaldi and Widmann, 2010). HIF transcription factors are composed of a stable constitutively expressed β-subunit and oxygen-liable HIF-1α, HIF-2α or HIF-3α subunit (Dengler, Galbraith and Espinosa, 2014) (Figure 1.5). The stability of the oxygen-liable subunit is controlled through hydroxylation by prolyl hydroxylases (PHDs) and subsequent ubiquitination (Maxwell, 1999).

Under normal physiological conditions, prolines 402 and 564 are hydroxylated by prolyl hydroxylases (PHD) (Kanaya and Kamitani, 2003) which targets HIF-α for degradation by the proteasome through binding of the E3 ligase, the von Hippel-Lindau protein (VHL) (Semenza, 2001). In hypoxic conditions, the HIF-α subunit becomes stabilised (Henze and Acker, 2017) due to the low affinity of oxygen to the PHDs (Chan *et al.*, 2005) (Figure 1.5). Decreased PHD activity decreases hydroxylation which inhibits the binding of the VHL to the HIF-α subunit. This causes stabilisation of the HIF complex (Figure 1.5) (Chan *et al.*, 2005).

Once stable, HIF complex translocates to the nucleus, where hypoxia-responsive genes are transactivated though binding to hypoxia response elements located in the promoter or enhancer regions of hypoxia-inducible genes (Dengler, Galbraith and Espinosa, 2014) (Figure 1.5). The stabilized HIF-1α subunit translocates to the nucleus, dimerizes with ARNT, and transactivates hypoxia-responsive genes through binding to hypoxia response elements (HREs) located in the promoter or enhancer regions of hypoxia-inducible genes

In excessively proliferating tumour tissue, oxygen demand is outstripped by supply which creates a toxic hypoxic milieu (Thomlinson and Gray, 1955). As a result of the adaptation to the hypoxic tumour environment, HIFs can promote

several avenues of aggressive tumour growth including metastasis, invasiveness and chemoresistance (Kim and Lee, 2017).

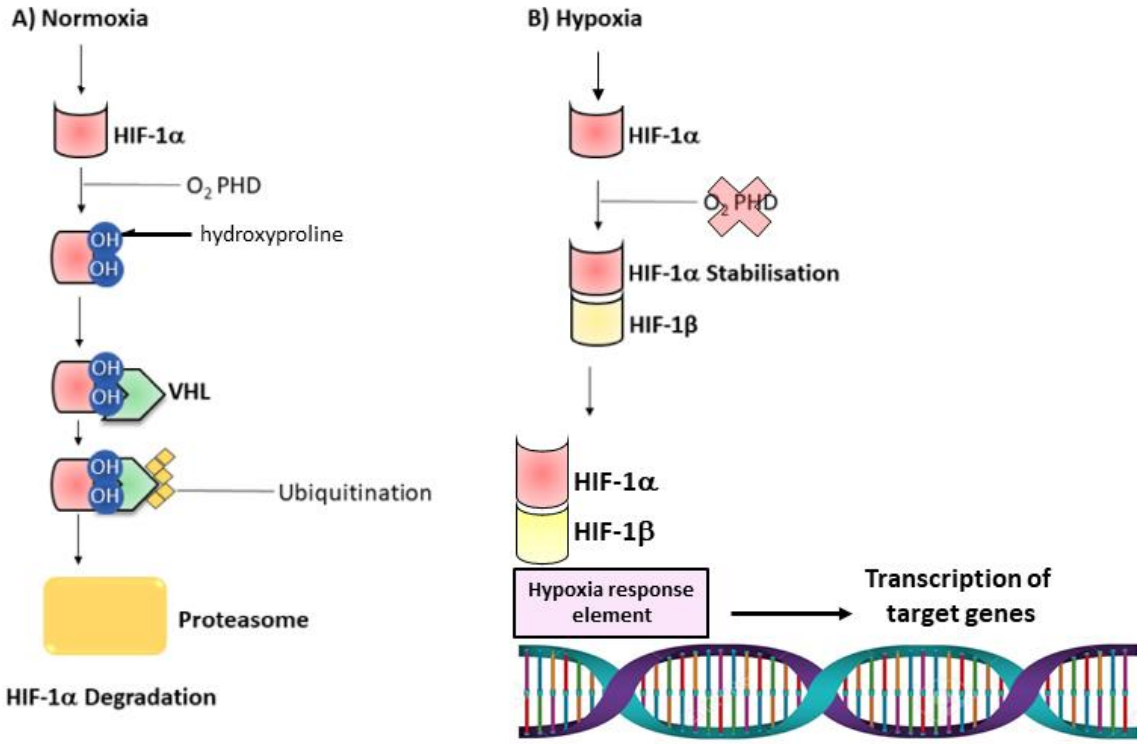

Figure 1.5: Representative schematic diagram of the impact of hypoxia on HIF-α subunit stability. The mechanism by which the transcription factors HIF-1α and HIF-2α respond to normoxia **(A)** and hypoxia **(B).** Under normoxic conditions HIF is hydroxylated and targeted for degradation by the proteasome. In hypoxic conditions HIF accumulates, binds to HIF-1β, which binds to the hypoxia response unit and leads to overexpression of HIF proteins. PHD: Prolyl-hydroxylase. VHL: von Hippel-Lindau factor.

1.7.2: HIF-1α.

HIF-1α plays a vital role in tumour adaptation to changes in O_2 concentration through the transcriptional regulation of over 100 downstream genes (Semenza, 2001), including glucose metabolism (*GLUT1* and *GLUT3*), cell proliferation (*C-MYC*), migration and angiogenesis (*VEGF*) genes (Masoud and Li, 2015). In growing tumour tissue, HIF-1α facilitates the shift from oxidative phosphorylation to aerobic glycolysis in order to maintain energy production (Warburg Effect) (Warburg *et al.*, 1956) (Figure 1.4). As a result, hypoxic cells metabolise an increased quantity of glucose in order to meet their energy needs. HIF-1α mediates this metabolic conversion partly through the expression of glucose transporters *GLUT1* and *GLUT3* as well as the upregulation of glycolytic enzymes (Denko, 2008). These transporters promote the influx of glucose into the hypoxic cell driving glycolysis and anaerobic metabolism (Seeber *et al.*, 2010).

HIF-1α mRNA is produced relatively constantly in TNBC cells and shows a marked increase in expression under hypoxia (Galbán and Gorospe, 2009). In TNBC, increased expression of HIF-1α is thought to contribute to tumour progression through a number of factors (Gilkes and Semenza, 2013). HIF-1α promotes the expression of *CA9*; a gene widely expressed in response to hypoxia (van den Beucken *et al.*, 2009). CA9 regulates intracellular pH balance and facilitates the adaptation of malignant cells to extracellular milieu toxicity (Benej, Pastorekova and Pastorek, 2014). In the hypoxic tumour regions, CA9 participates in bicarbonate transport (Ditte *et al.*, 2011) by catalysing hydration of pericellular CO_2, and therefore local production of bicarbonate ions are readily available for influx by transporters (Ditte *et al.*, 2011). As a result, protons as a

by-product of CO_2 hydration remain in the pericellular space and reduce the pH of the environment. This promotes tumour cell invasion (Ditte *et al.*, 2011) through stimulation of migratory pathways (Benej, Pastorekova and Pastorek, 2014).

1.7.3: HIF-2α.

HIF-1α and HIF-2α subunits are structurally similar, sharing 83 and 70% sequence identities in their DNA binding and dimerization domains, respectively (Hu *et al.*, 2007). However, they differ in their transactivation domains and may regulate different batteries of genes (Hu *et al.*, 2007). Interestingly, target gene selectivity is not determined by the bHLH DNA-binding domains. Alternatively, the N-TADs appear to contribute to target gene selectivity. HIF-1α and HIF-2α bind to the same hypoxia response element (HRE) consensus sequence: '5-(A/G)CGTG-3' (Mole *et al*., 2009).

HIF-2α expression has been reported to be restricted to endothelial cells, lung type II pneumocytes, liver parenchyma, interstitial cells of the kidney (Wiesener *et al.*, 2003; Keith, Johnson and Simon, 2012) and breast cancer cell lines. HIF-2α expression is a part of the normal physiological response to hypoxia (Loboda, Jozkowicz and Dulak, 2010) however, cancer cells show increased expression of HIF-2α to promote tumour growth and metastasis. HIF-2α activation of target genes such as *VEGF*, promotes tumour growth through mechanisms such as increased angiogenesis. In later stages of BrCa, HIF-2α expression is associated with a more aggressive BrCa phenotype (Holmquist-Mengelbier *et al.*, 2006), poor patient outcome and an increased rate of distant tumour reoccurrence (Helczynska *et al.*, 2008).

In TNBC, HIF-2α is thought to contribute to tumour angiogenesis, growth and cell proliferation through expression of genes such as erythropoietin (*EPO*). *EPO* has been shown to activate extracellular signal-related kinase (ERK) and promote migration in MCF-7 breast cancer cells (Lester *et al.*, 2005). Similarly, positive immunohistochemical expression of the HIF-2α target, vimentin, is associated with high-grade tumours and increased tumour proliferation in breast cancer (Hemalatha, Suresh and Harendra Kumar, 2013).

1.8: Hypoxia and altered glycosylation in cancer.

Hypoxia in tumours has been linked to aggressive phenotypes and treatment failure (Generali *et al.*, 2006; Bos *et al.*, 2009). HIF transcription factors activate genes involved in the tumour response to hypoxia, including those involved in angiogenesis (*VEGF*) and glucose transport (*GLUT1/3*). Hypoxia causes altered metabolism (López-Barneo, Pardal and Ortega-Sáenz, 2001), in which *GLUT1* and *GLUT3* caused increased glucose consumption to compensate for the high metabolic demand of tumour growth (Cheng *et al.*, 2006). The shift in metabolism due to hypoxia also alters the expression of glycolytic enzymes which suggests that hypoxia plays a critical role in regulation of nucleotide sugar biosynthesis pathways (Stanley, Taniguchi and Aebi, 2015).

1.9: Overall aims and research questions.

The overall aim of this project was to uncover changes that occur to the glycosylation profile of TNBC as a result of hypoxic adaptation. This included

alterations to glycogene expression and sugar residues proportions on the cell surface. Furthermore, it was aimed to assess the role of hypoxia inducible factors HIF-1α and HIF-2α in regulating glycogene expression in TNBC. In doing so, a greater understanding of the cellular glycome of TNBC has been gathered. Understanding the mechanisms involved in altered glycogene expression in TNBC and how this affects cell surface glycans is crucial to the development of novel drug targets to treat this particularly aggressive form of breast cancer.

Chapter 2: Materials and Methods

2.1: Reagents and chemicals.

All reagents and chemicals were obtained from Sigma-Aldrich (Gillinham, UK), ThermoFisher Scientific (Paisley, UK), VWR Chemicals (Leicestershire, UK) or Qiagen (Manchester, UK) unless otherwise stated.

2.2: TNBC RNA samples and Tissue Matched Controls.

Three TNBC samples (T) and three tissue matched controls (N) were obtained the Tayside Biorepository (Dundee University): Tumour 11004T, matched control 11004N, Tumour 10046T, matched control 10046N, Tumour 10076T and matched control 10076N. The RNA integrity number (RIN) and concentration of RNA in each sample was pre-determined by Dr Susan Bray at the Tayside Tissue Bank (Table 2.1).

Table 2.1: The integrity (RIN) and concentration of TNBC RNA obtained from the Tayside Biorepository.

Sample	RIN Value	Concentration (ng/µL)
11004T	6.6	458
11004N	5.5	26
10046T	3.1	54
10046N	5.5	36
10076T*	5.0	408
10076N*	2.7	47

*Samples not used for RT^2 Human Glycosylation Array.

Table 2.1.1: Pathological examination of tumour samples.

Tumour Identifier	11004	10046	10076
Tumour grade	3	2	3
Greatest tumour dimension	5cm	5cm	Extension to the chest wall
Number of lymph node metastasis	1-3	1-3	10+
qRT-PCR array profiler analysis? (Y/N)	Y	Y	N
Additional comments	N/A	N\A	*peau d'orange*

2.3: MDA-MB-468 cells.

MDA-MB-468 triple negative breast cancer cells were gifted from Dr Lisa Pang, The Roslin Institute, The University of Edinburgh at passage number 6. MDA-

MB-468 cells were cultured in 75 mL flasks (T75) in complete medium: Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermofisher Scientific, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invivogen) and 1% Penicillin-Streptomycin (P/S) (Invivogen) at 37°C, with 5% CO_2 in high humidity. All cell culture work was performed in sterile laminar flow hood.

2.3.1: Passaging cells.

Media was aspirated and cells were washed with 0.9% sterile saline solution (Baxter). Approximately 2mL of 1 X Trypsin (Bioline)was added to cover the monolayer of cells. Cells were incubated at 37°C for approximately 5 minutes. The trypsin was neutralised by the addition of 8 mL of complete media and the cells were detached by gentle pipetting. Cell suspension was transferred to a 15 mL falcon tube and the cells collected by centrifugation at 238*xg* for 5 minutes at room temperature in a Universal 320 centrifuge (Hettich Zentrifugen). The pellet was resuspended in 10 mL of complete media. Cells were seeded at dilutions ranging between 1:10 and 1:4 into a new T75 flask (Corning, Wiesbaden, Germany).

2.3.2: Cryopreservation of cell lines.

Cells were grown to ~80% confluency (as described in section 2.3) in a T75 flask and trypsinised (see section 2.3.1). They were transferred into a 15 mL falcon tube and collected by centrifugation at room temperature at 238*xg* for 5 minutes. The pellet was resuspended in 10 mL of complete media. Freezing media (80%

FBS, 20% DMSO) (Invivogen, VWR Chemicals) was added to the cells at a ratio of 1:1 mL and 0.5 mL of the cells transferred to a cryotube (Simport, Beloeil, Canada, PK/100). After 24 hours in an isopropanol bath (Mr. Frosty™ Freezing Container, Thermofisher Scientific, UK) at −80°C, the vials were transferred to liquid nitrogen (−196°C) for long term storage.

To recover cells after freezing, cells were resuspended in 10 mL of warm fresh complete medium before centrifugation at 238*x*g for 5 minutes. The cell pellet was resuspended in 10 mL fresh media, transferred to a new flask and incubated at 37°C in 5% CO_2.

2.4: Cell Counting.

Cell number was estimated using a Neubauer haemocytometer (Marienfeld, Lauda, Konigshofen, Germany). After passaging the cells (section 2.3.1), 10 µL of cell suspension was added onto the haemocytometer. One 5x5 grid on the haemocytometer was counted in duplicate and the number of cells averaged. Non-viable cells were not included in this count. This number was multiplied by 10^4, giving the total number of cells/mL.

2.5: Experimental hypoxia.

Acute hypoxia was induced in MDA-MB-468 TNBC cells at approximately 60% confluency. Immediately before induction of hypoxia, cells were supplemented with fresh complete media. The flasks were placed in a modular hypoxia chamber

(Billups-Rothenberg, Inc. Del Mar, CA, USA) and hypoxia was induced following methods of Wu & Yotnda (2011). The chamber was sealed and flushed with 1% O_2 gas mixture (1% O_2, 5% CO_2 and 94% N_2 gas: Cat: 2052055, BOC), at a flow rate of 20L/ min for 10 minutes. The sealed chamber was incubated at 37°C with 5% CO_2 in a standard incubator. After 1 hour, the chamber was removed from the incubator and flushed 1% O_2 as above and placed back into the incubator for a further 21 hours, totalling 22 hours of hypoxic exposure.

2.6: Lectin staining.

Biotinylated lectins and phycoerythrin-conjugated streptavidin (PE-Strep) were obtained from Vector Laboratories (Burlingame, CA, USA) and were used to stain cells (Table 2.2). Lectins (5 µg /mL) were added to each fluorescence-activated cell sorting (FACS) tube containing approximately $2.5x10^5$ cells and incubated on ice for 45 minutes. The cells were centrifuged to collect at 238*xg* for 5 minutes at 4°C and resuspended in 2 mL of ice-cold PBS. This was repeated three times. PE-Steptavadin was diluted to 1 µg/mL in PBS and 100 µL added to each tube which was incubated in the dark for 15 minutes. The cells were washed as above and resuspended in 150 µL PBS and analysed by flow cytometry counting 10000 gated events (FACSCalibur, Becton Dickinson Bio-sciences).

Table 2.2: Lectins used in this study.

Lectin Name	Abbreviation	Binding target
Dolichos Biflorus Agglutinin	*DBA*	*N*-acetyl-galactosamine (Ogino *et al.*, 1999)
Lens Culinaris Agglutinin	*LCA*	α-mannose on *N*-linked glycans (Kornfeld *et al.,* 1981)
Peanut Agglutinin	*PNA*	*N*-acetyl-galactosamine, galactose (Mérant *et al.*, 2005)
Phaseolus Vulgaris Erythroagglutinin	*PHA-E*	Complex type *N*-glycans (Cummings and Etzler, 2009)
Ricinius Communis Agglutinin	*RCA*	Terminal β-D-galactose (Itakura *et al.*, 2007)
Wheat Germ Agglutinin	*WGA*	*N*-acetyl-neuraminic acid (Kuno *et al.*, 2005)

2.7: siRNA Transfection

Known-target sequence control small interfering RNAs (siRNA) (1027310), HIF-1α (SI02664053) and *HIF-2α* (EPAS1) (SI00380212) FlexiTube siRNAs (Qiagen) were resuspended in 1 mL RNase free dH_2O to obtain a 20 µM stock concentration.

MDA-MB-468 cells were seeded at a density of $3.5x10^5$/ mL in 6 well dishes. This achieved 60% confluency after 24 hours of incubation at 37°C in high humidity. SiRNAs (50nM) were diluted in 100µL OptiMEM media (Gibco) and 12 µL

HighPerfect transfection reagent before vortexing to mix and incubating for 10 minutes at room temperature. Controls included FlexiTube non-targeting siRNAs and a HighPerfect Tranfection Reagent only. The transfected cells were incubated for 24 hours at 37°C in high humidity. After incubation, the media was aspirated and replaced with complete medium and cells were exposed to acute hypoxia (1% O_2) for 22 hours (as in section 2.5). Cells were harvested and stored at -80°C for later analysis.

The degree of knockdown following siRNA transfection was assessed using ImageJ software (v. 1.8.0_112). ImageJ was used to analyse the jpeg image of individual immunoblots which were exported from the LI-COR Odyssey imaging system (Odyssey-3074, LI-COR, Cambridge, UK) and Odyssey Image Studio v2.0 software (ThermoFisher Scientific, UK). The pixel density of the background, control and siRNA were determined dividing the image density of the loading control versus the protein of interest where the control was taken to equal 1.

Table 2.3: SiRNA molecules used in this study.

Target Gene	Cat.No	Sense/Anti-Sense Sequences
HIF-1α	SI02664053	**S**:GAAGAACUAUGAACAUAAATT **AS**:UUUAUGUUCAUAGUUCUUCCT
HIF-2α	SI00380212	**S**:CGGCGUCUGAACGUCUCAATT **AS**:UUGAGACGUUCAGACGCCGAG
Non-targeting scrambled	1027310	**S**:UUCUCCGAACGUGUCACG **AS**:ACGUGACACGUUCGGACA

2.8: RNA extraction and analysis.

Cells were washed with PBS and then harvested on ice by scraping into ice cold PBS solution. MDA-MB-468 cells were collected by centrifugation (5415R, Eppendorf, Stevenage, UK) at 3000*x*g at 4°C for 5 minutes, before discarding the supernatant. RNA was extracted from pelleted cells with TRIsure reagent (Bioline) following the manufacturer's instructions. Briefly, 1 mL of TRIsure was added per cell pellet which was disrupted by pipetting. RNA was separated from the organic phase and interphase by addition of 200 µL chloroform, vigorous shaking by hand for 15 seconds and centrifuging at 12000*xg* (5415R, Eppendorf) for 15 minutes at 4°C. RNA was precipitated by adding 0.5 mL ice cold isopropanol incubating for 10 minutes on ice and centrifuging for 10 minutes at 12000*xg* at 4°C. Cell pellets were washed with 1 mL 75% ethanol air-dried at room temperature and resuspended in RNase-free water.

2.9: Determining the integrity and purity of extracted RNA.

The concentration of RNA was estimated by diluting samples with dH_2O (1:5) and 1 µL was estimated via Nanodrop (Nanodrop 2000, Thermofisher). The purity and integrity of extracted RNA was assessed by fluorescence using the Agilent 2100 Bioanalyser (Aglient Technologies Ltd., Stockport, UK) and the Bioanalyser 2100 Expert Software. All samples were prepared according to the manufacturer's instructions using the Agilent RNA600 Nano Kit (Agilent Technologies Ltd, 5067-1522). Each sample was analysed on an RNA chip which contains an interconnected set of microchannel used for separation of nucleic acid fragments

based on size. Samples with an RNA integrity number (RIN) of >8 were used for glycogene expression analysis.

2.10: Complementary DNA (cDNA) Synthesis.

RNA samples were reverse transcribed in order to synthesise complementary DNA (cDNA) for quantification of gene expression. The High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, 4387406) was used according to the manufacturer's instructions. The reaction contained 1X RT buffer mix with 1µL of RT enzyme and 2 µg of total RNA. RNase-free water was added to the reaction to 20 µL. Negative control cDNA reactions lacked reverse transcriptase enzyme. Reactions were incubated at 37°C for 60 minutes followed by 5 minutes at 95°C in a thermal cycler (2720 Thermal Cycler Applied Biosystems, UK) and cDNA was stored at -20°C.

2.11: Oligonucleotide design.

Oligonucleotides were either designed in accordance to the criteria of Thornton and Basu (2011), obtained from the Harvard University PrimerBank (https://pga.mgh.harvard.edu/primerbank/index.html) or taken from published sources. All forward and reverse sequences were subject to BLAST analysis to assess self-complementarity and specificity (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All oligonucleotides were purchased from MWG Eurofins (Ebersberg, Germany), diluted to a stock concentration of 100µM in nuclease free water (Ambion) and stored at -20°C.

Table 2.4: Oligonucleotides used in this study.

Target Gene	Accession number	Sequence 5'-3'. Forward (F) and reverse (R).	Reference/ Harvard Database ID
β-Actin	NM_001101.3	**F**:GGGAAATCGTGCGTGACATT **R**:CCACAGGACTCCATGCCC	
β2M	NM_004048.2	**F**: GAGGCTATCCAGCGTACTCCA **R**: CGGCAGGCATACTCATCTTTT	37704380c1
B3GNT2	NM_006577	**F**: TCCAAAAGCAGTAGCCAAGAAA **R**: CGGTTCCAGTATGCCTCGG	92091578c1
B4GALT1	NM_001497	**F**: GGCGTCACCCTCGTTTACTAC **R**: GGGCCAGAATCCACGACTG	193211604c1
B4GALT2	NM_001005417	**F**: CTGCTTAACGTGGGCTTCCTA **R**: GTCCACATCGCTGAAGATGAA	339276011c3
B4GALT3	NM_001199874	**F:** CGAGATCAGGGACCGACATTT **R**: GATCGTTCTGGACAGTAGGGC	315467843c1
CA9	NM_001216	**F:** AGGGTGTCATCTGGACTGTG **R:**TGTGTGGCTCGGAAGTTCAG	Martín-Aragón Baudel *et al.*, (2017)
EPO	NG_021471.2	**F:**GAATATCACTGTCCCAGACACC **R:**CCCTGCCAGACTTCTACGG	Martín-Aragón Baudel *et al.*, (2017)
GALNT3	NM_004482	**F**: CAGCAGAATTGAAGCCTGTCC **R**: CTTCCCCACGTTCCTTTTCCT	153266877c1

Table 2.4 continued: Oligonucleotides used in this study.

GALNT6	NM_007210.3	**F**:ACTCTGTTCTCCATAAACCAGTC **R**: CTGGCAAAGGCATTGAAACA	
GALNT7	NM_017423.2	**F**: TCACCTCACACTACCCTTTGC **R**: CTGATTCCCTCCCATCCTGT	Munkley *et al.*, (2016)
GALNT12	NM_024642	**F**: GAGGGGCGATGTTCTGACC **R**: TGCCGACTCCTCTTCATGGA	325651866c3
GALNT14	NM_001329097.1	**F**: TAGCATCATCATCACCTTCCAC **R**: TTACAGTCATCAGGGTCATTGC	Huanna *et al.*, (2015)
GALNTL5	NM_145292.3	**F**: GAAGCTTGGGCATCGAAA **R**: GCGGGCTGGGTAATGTT	Takasaki *et al.*, (2014)
HIF-1α	NM_001243084	**F:**GTACCCTAACTAGCCGAGGAAGAA **R:**GTGAATGTGGCCTGTGCAGT	Martín-Aragón Baudel *et al.*, (2017)
HIF-2α	NM_001430	**F:**ACCTGGAAGGTCTTGCACTGC **R:**TCACACATGATGATGAGGCAGG	Martín-Aragón Baudel *et al.*, (2017)
RPLP0	NM_001002.3	**F**: CCTCATATCCGGGGGAATGTG **R**: GCAGCAGCTGGCACCTTATTG	Untersmayr *et al.*, (2010)
Vimentin	NM_003380.4	**F:** AGATTCAGGAACAGCATGTCC **R:** AGCCTCAGAGAGGTCAGC	Martín-Aragón Baudel *et al.*, (2017)

2.12: End-point PCR and gel electrophoresis.

End-point PCR amplification was performed in a 10 µL reaction containing approximately 100 ng of genomic template DNA, 10 µM of each primer (forward and reverse), 2 x Mangomix (Bioline) and 2 µL nuclease free H_2O. Control reactions included a negative RT control and a nuclease free H_2O primer only control. Cycling conditions included a 5-minute denaturing at 94°C, 40 cycles of amplification and annealing entailing 15 seconds at 94°C, 15 seconds at 50°C and 30 seconds at 72°C. All reactions were completed at 4°C. PCR products were analysed by gel electrophoresis.

Gel electrophoresis was performed on 1% agarose gels in 50 x Tris-acetate-EDTA (TAE buffer). Gels were supplemented with 0.005% Safeview for RNA visualisation. Samples were loaded on the gels and electrophoresed at 120V until the dye front travelled approximately ¾ of the way down the gel. Electrophoresed gels were visualised on a UV-trans-illuminator.

2.13: RT^2 Human Glycosylation Array.

To determine the glycogene expression profile of Tumours 11004 and 10046 and respective tissue matched samples, a commercially available glycosylation array was used (RT^2 Human Glycosylation Array, PAHS-046Z, Qiagen). Tumour 10076 and tissue matched samples were not used in this analysis. Equal concentrations of RNA (250 ng) were reverse transcribed to cDNA using the RT^2 First Strand Kit. The genomic DNA elimination mix was prepared by adding 250 ng of RNA to 2 µL GE buffer. The genomic DNA elimination mix was incubated for 5 minutes at 42°C, then immediately placed on ice. Reverse transcription mix was prepared

by combining Buffer BC3, control P2, RE3 Reverse Transcriptase Mix and RNase-free water to total 10 µL for each reaction. The Reverse Transcription Mix was mixed by pipetting before 10 µL genomic DNA elimination mix was added. Reactions were incubated at 42°C for 15 minutes and the reaction stopped by incubating at 95°C for 5 minutes. RNase-free water (91 µL) (Ambion) was added to each reaction. Quantitative-PCR (qPCR) master-mix was prepared by mixing 1X RT2 SYBR Green Master-mix, cDNA synthesis reaction and RNase-free water to a reaction total of 2.7 mL and 25µL of this mixture was pipetted into each well of the RT^2 PCR array. Gene expression levels were quantified by Q-PCR (StepOne™ Real Time PCR system (ThermoFisher Scientific, UK): 1 cycle of 95°C for 10 minutes, 40 cycles of 15s seconds at 95°C and 1 minute at 60°C. PCR array data analysis software (Sabiosciences RT^2 PCR profiler array v3.5) was used to normalise the data and calculate the fold change in gene expression. The C_T thresholds were adjusted based on positive PCR controls (PPC), which monitor for PCR inhibitors, so that they fell between the advised 20 +/- (2) stated in the manufacturer's instructions. Housekeeper genes Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*) and beta-2-microglobulin (*β2M*) were used to normalise the gene expression data of Tumours 11004 and 10046, respectively. Selection of housekeeper genes were based on stable C_T values across both the tumour and matched tissue samples as indicated by Sabiosciences RT^2 PCR profiler array v3.5 software.

2.14: qRT-PCR.

Independent qRT-PCR reactions (20 µL) contained 1 µL of forward and reverse oligonucleotides at a final concentration of 150 nM, 10 µL PrimerDesign Precision

qRT-PCR Mastermix and either 11.7 ng or 12.5 ng of template cDNA from tumour samples or MDA-MB-468 TNBC cells, respectively, in nuclease-free H_2O (Ambion). Controls included RNA from a reaction without reverse transcriptase (negative control) and cDNA replaced with nuclease-free water (template negative). Reactions were performed in triplicate using a StepOne™ Real Time PCR system (ThermoFisher Scientific). Instrument settings were as follows: 10 minutes at 95°C plus 40 cycles at 95°C for 15 seconds followed by 1 minute at 60°C. Melt curve analysis, which measures fluorescence at increasing temperature intervals (60°C to 95°C at 0.3°C intervals) was used for the identification of a single amplified product. Data was normalised to the geometric mean of *β-actin*, *RPLP0* and *β2M* housekeeper genes and fold change in gene expression was calculated using the delta-delta C_T method (Livak and Schmittgen, 2001). For siRNA analysis, gene expression was normalised to *β2M.*

2.15: Protein Extraction and quantification by Bradford analysis.

Cell pellets were lysed by resuspending the pellet in 3x the pellet volume with NP40 lysis buffer (50mM Tris, 150mM NaCl, 5mM EDTA, 1% NP40,) supplemented with 1x protease inhibitor (Thermofisher Scientific, UK). Cells were incubated on ice for 30 minutes and centrifuged at 16000*xg* (5415R, Eppendorf) for 5 minutes at 4°C. Protein concentration from the clarified supernatant was determined by Bradford analysis where a stock of 2 mg/ mL bovine serum albumin (BSA) prepared in dH_2O was used to generate a standard curve of known protein concentrations (1.5 – 0.125 mg/ mL). Protein samples were diluted 1:5 in dH_2O and 1 µL dilution was mixed with 200 µL Bradford reagent (100mg Coomassie blue G-250, 95% ethanol, 85% (w/v) phosphoric acid) in a 96 well

plate. A blank control was generated by mixing 1 µL of dH_2O with 200 µL Bradford reagent. Absorbance at 550nm was determined after 5 minutes of incubation at room temperature by plate reader (ELISA reader LT-5000MS, LabTech International) using Manta Lite software (LabTach International). A standard curve was generated from the known standards and the unknown protein concentrations were determined by their absorbance. Protein samples were diluted in 4X Loading Sample Buffer (LSB) (20% glycerol, 200mM Tris pH 6.8, 4% SDS, 10mM EDTA, and bromophenol blue) to give a final concentration of 1 mg/ mL and boiled at 100 °C for 2 minutes. Samples were then stored at -20°C before use.

2.16.1: Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Proteins were resolved *via* SDS-PAGE based on methods by Laemmli, (1970). Resolving acrylamide gels were composed of 8-12% (w/v) acrylamide (30% w/v stock acrylamide); resolving Tris solution (0.375 mM Tris-HCl, 0.1 (w/v) SDS adjusted to pH 8.8 and 10% (w/v) ammonium persulphate (AmPs). To polymerise the mixture 0.1% N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich) was added. After polymerisation the stacking gel was prepared and (5% (w/v) polyacrylamide, 1M Tris-HCL (pH = 6.8), 10% SDS, 10% AmPs) and 0.1% TEMED was added to promote polymerisation.

2.16.2: Resolving SDS-PAGE gels

Gels were removed from their casting chambers and placed into an electrophoresis tank (BioRad) and submerged in 1x running buffer (25 mM Tris-HCL, 192 mM Glycine, 35 mM SDS). Typically, for HIF-1α and HIF-2α protein expression 30 µg of cell lysate was resolved on an 8% SDS-polyacrylamide gel, for β-actin expression 10 µg was resolved on an 12% gel. A pre-stained broad range protein marker (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, ThermoFisher Scientific) was used to determine the size of the proteins of interest. The gels were electrophoresed at 185V for approximately 60 minutes or until the blue loading buffer dye front reached the bottom of the SDS gel.

2.16.3: Immunoblotting

Following the separation of proteins *via* SDS-PAGE they were transferred onto a nitrocellulose membrane following the methods of Towbin *et al.,* (1979). The nitrocellulose membrane (0.2 µm, Optiran BA-S 83) was sandwiched between paper (2mm, Whatman International Ltd., UK) and a transfer sponge soaked in 1 x transfer buffer (0.25M Tris pH 8.5, 0.2 M Glycine, 20% Methanol). The sandwich was assembled under 1x transfer buffer, compressed with a plastic tube to remove air bubbles. The sandwiches were inserted into the apparatus and submerged with 1x transfer buffer. An ice pack was added to the chamber then samples were electrophoresed 100V for 1 hour or at 30mA for 16 hours at 4°C. The nitrocellulose membrane was stained with Ponceau S solution (0.1% Ponceau S, 5% acetic acid) to ensure equal transfer of proteins, washed three

times in PBS with 1% tween (PBS-T) and blocked with 5% (w/v) non-fat milk (Marvel) or 5% BSA in PBS-T for one hour at room temperature with shaking.

2.16.4: Immunostaining

Primary antibodies were prepared at a dilution of 1:1000-1:750. The nitrocellulose membrane was incubated with specific primary monoclonal antibody (Table 2.5) in 5 % non-fat milk in 1x PBS-T overnight at 4°C. The membrane was washed three times in PBS-T before probing with the secondary antibody for 45 minutes (Table 2.5.1) at a dilution of 1:10000 in 5 % non-fat milk containing 0.01 % SDS. The membrane was then washed three times in PBS-T. Membranes were analysed by LI-COR Odyssey imaging system (Odyssey-3074, LI-COR, Cambridge, UK) and Odyssey Image Studio v2.0 software (ThermoFisher Scientific, UK).

Table 2.5: Primary antibodies used in this study.

Antigen	Antibody	Species	Company	Cat.No	Dilution
β-actin	actin C-11	Goat	Santa Cruz	sc-1615	1:1000
HIF-1α	anti-HIF-1α	Human	BD Biosciences	610958	1:1000
HIF-2α	anti-HIF-2α	Goat	R&D Systems	AF2886	1:750

Table 2.5.1: Secondary antibodies used in this study.

Species	Conjugate	Company	Cat.No	Dilution
Anti-goat	680LT	LI-COR	925-68024	1:10000
Anti-mouse	800CW	LI-COR	926-32210	1:10000

2.17: Statistical Analysis

All statistical analysis was performed using GraphPad Prism v.7 (GraphPad Software). Results are shown as the mean ± standard error of the mean (SEM), where n=3. To determine significance of fold change in gene expression data between tumour samples and cell replicates an unpaired Students *t*-test and Wilcoxon signed-rank test was used. For Lectin binding analysis two-way ANOVA with Šidàk's multiple comparisons was used. For siRNA analysis two-way ANOVA was used with Tukeys multiple comparison test was used where $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

Chapter 3: Determining the glycogene expression profile of triple negative breast cancer.

3.1: Introduction

3.1.1: Altered glycans in breast cancer.

Aberrant protein glycosylation is a hallmark of many human cancers including breast cancer (Hanahan and Weinberg, 2011). Glycans, which can be detected by lectins, are formed by *N*- or *O*- glycosylation and their function spans from relatively subtle to crucial for life, development, growth and functioning (Varki and Lowe, 2009). Under neoplasia, there are changes promoting specific alterations to *N*- and *O*-linked core structures, glycogene expression and the degree of core glycan branching (Song *et al.*, 2001; Stowell, Ju and Cummings, 2015); thus potentially altering glycan structure and function.

Aberrant *O*-linked glycosylation has been observed in over 90% of breast cancer, (Burchell *et al.*, 2018) most prevalently through the loss of core-2 glycans and expression of Tn antigen (Au *et al.*, 2014) (Figure 3.1). Sialylation of the Tn antigen involves the addition of a terminal sialic acid which averts further elongation of the structure resulting in premature truncation and potentially loss of glycan function (Figure 3.1) (Brockhausen, Schachter and Stanley, 2009). Core 2 structures however, can be cleaved to more simple sugars which results in breast cancers overexpressing Tn antigen (Core 1) (Springer, 1984).

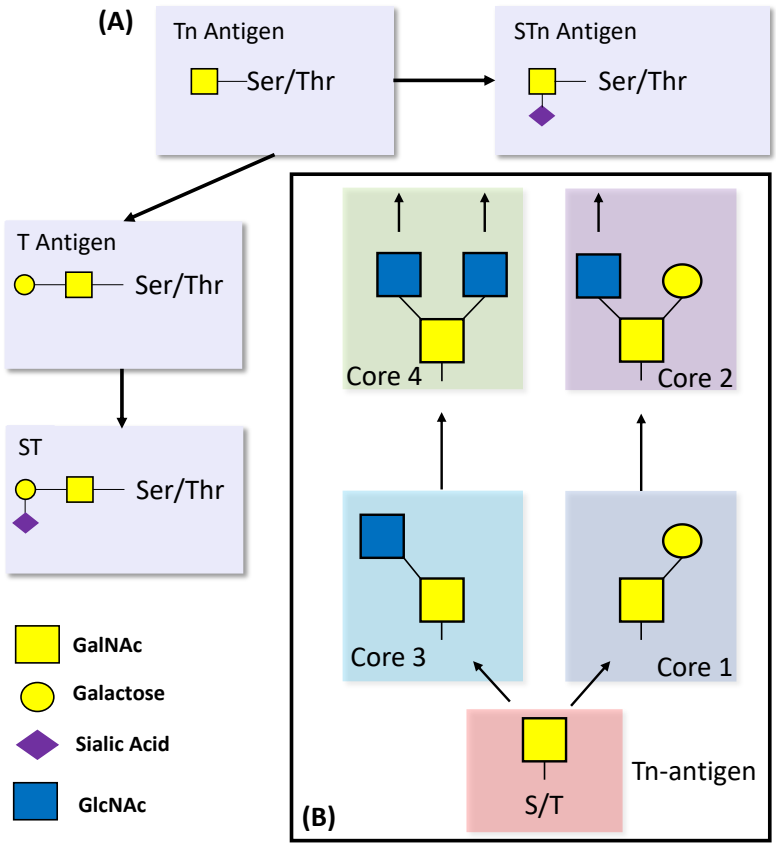

Figure 3.1: Antigens and core structures from the initiation of mucin-type *O*-linked glycosylation. (A) Incomplete glycosylation results in the carcinoma hallmark expression of T, Tn, ST and STn- antigens (Varki *et al.*, 2015). Core 1 *O*-GalNAc is found in many glycoproteins and it is termed the T antigen. The first sugar (GalNAc) added to this molecule creates the Tn antigen. Both the T and Tn antigen can be further modified by sialic acid to form sialylated-T or Tn antigens (Beatson *et al*., 2015; Brockhausen *et al*., 2009). **(B)** The formation of linear and branched mucin-type *O*-glycans showing the steps leading to the synthesis of core 1-4 di- and tri-saccharides (Corfield and Berry, 2015).

3.1.2: Aberrant glycogene expression in BrCa.

Glycogene expression in breast cancer cells compared to adjacent tissues have demonstrated significant differences in expression, with increased levels of sialyltransferases including *ST8SIA4* (Ma *et al.*, 2016). The highly metastatic TNBC cell line MDA-MB-231 likewise displays increased expression of *ST8SIA4,* which may promote aggressive tumour growth (Ma *et al.*, 2016). BrCa growth has furthermore been attributed to overexpression of *C1GALT1* through altered *O*-glycan structures on MUC1 (Chou *et al.*, 2015); a transmembrane protein that participates in intracellular signalling transduction pathways (Nath and Mukherjee, 2014). Breast cancer cell metastasis can be facilitated by altered glycosylation gene expression. For example, *ST6GalNAc5* enables BrCa metastasis to the brain through cancer cell adherence to the brain endothelium and blood-brain barrier permeability (Bos *et al.*, 2009).

3.1.3: Hypoxia and altered glycosylation in breast cancer.

Tumour hypoxia has been linked to aggressive phenotypes, chemoresistance and treatment failures of breast cancer (Generali *et al.*, 2006; Bos *et al.*, 2009). Hypoxia inducible factor 1-α (HIF-1α) regulates the transcription of various genes controlling tumour responses to hypoxia, including angiogenesis (López-Barneo, Pardal and Ortega-Sáenz, 2001). One of the many alterations in cancer is altered energy metabolism (Hanahan and Weinberg, 2011). HIF activates genes involved in glucose transport, such as *GLUT1* and *GLUT3,* which causes increased glucose consumption to meet the high metabolic demand for

nucleotide sugar biosynthesis (Cheng *et al.*, 2006). *GLUT1* and *GLUT3* also promote cell replication (Goda, Dozier and Johnson, 2003). Due to the dramatic shift in cell metabolism caused by hypoxia, the expression of glucose transporters and glycolytic enzymes; glucose metabolic patterns may be altered (Shirato *et al.*, 2011). This suggests that hypoxia has an important role in the modulation of nucleotide sugar biosynthetic pathways (Taniguchi, 2007). Furthermore, glycosylation is kinetically regulated by the dynamic portfolio of glycosylation genes, nucleotide sugars and nucleotide transporters (Shirato *et al.*, 2011). A deficiency or excess in the expression of glycosylation genes has been linked to aberrant glycosylation, which may contribute to the development and progression of breast cancer (Taniguchi, 2007). Nucleotide sugar availability can also modulate glycosylation due to their importance in glycan synthesis (Taniguchi, 2007). The absence or surplus of nucleotide sugars may result in a number of glycan alterations including excessive elongation or premature truncation (Potapenko *et al.*, 2010) Collectively, these modifications may then lead to an aggressive breast cancer phenotype and facilitate metastasis (Asada *et al.*, 1997; Kölbl, Andergassen and Jeschke, 2015).

By investigating the altered expression of glycosyltransferase genes, a better understanding of the cellular glycome in malignancy will be gathered; leading to identification of potential biomarkers of disease and better treatment options. In this investigation, TNBC samples and tissues matched controls were used to assess the glycosylation profile of TNBC. By comparing gene expression of tumour tissue and the patient matched samples, interpatient variability was eliminated resulting in highly proficient data. Glycosylation gene profiling was validated in hypoxic MDA-MB-468 TNBC cells, which effectively replicated the

tumour microenvironment *in vitro.* MDA-MB-468 cells are a well-established model of TNBC have been used extensively in BrCa research (Reilly, 2007).

3.2: Aims

Aim 1: To profile the change in expression of key glycogenes in human TNBC tumour samples and validate these changes hypoxic MDA-MB-468 cells as an *in vitro* model of TNBC.

Aim 2: To determine the lectin binding profile of hypoxic MDA-MB-468 cells.

3.3: Research Questions

RQ1: Does the glycosylation gene expression profile in TNBC differ to tissue matched control samples?

RQ2: Do hypoxic MDA-MB-468 cells share similarities in the glycosylation gene expression profile of TNBC tumour samples and have altered expression of key glycogenes?

RQ3: Is the glycan profile of MDA-MB-468 cells altered in response to acute hypoxia?

3.4: Results

3.4.1: Profiling the differences in glycogene expression between TNBC tumours and their tissue matched controls.

To identify glycosylation genes with altered expression in TNBC, a commercially available glycosylation qRT-PCR array was employed to assess the expression profile in TNBC tumours (11004 and 10046) and their patient-matched non-cancerous breast tissue controls. The RT^2 Human Glycosylation Array profiled eighty-four genes encoding glycosylating enzymes. The array included housekeeper genes (β-actin*, β2M, GAPDH, HPRT1* and *RPLP0*), as well as controls for reverse transcription, reaction success and human genomic DNA contamination. C_T values were compared between the tumour and its respective tissue-matched control. Genes with greater than 2-fold change in expression were taken to have altered expression.

Gene expression for each sample tumour was expressed as a heat map (Figure 3.2). In tumour 11004, sixty glycosylation enzymes were upregulated, including those from the 5 major glycosylation families: glycan extension, *O*-glycan initiating, *N*-glycan branching, sialidases and sialyltransferases. Eleven glycogenes were downregulated (Figure 3.2), including: *A4GNT, GALNT9, GALNT12, GALNT16, GALNTL5, MGAT4C, NEU2, NEU4, ST6GALNAC1, ST8SIA3* and *ST8SIA6* whilst thirteen glycosylation genes remained unchanged between tumour 11004 and its tissue matched control. In tumour 10046, forty genes were upregulated, three glycogenes were downregulated including *GALNT13, GCNT1* and *MAN2A2* and forty-one glycogenes remained unchanged compared to the tissue matched control (Figure 3.2).

Altered gene expression was observed in tumours 11004 and 10046 compared to their respective tissue matched controls – indicating that TNBC glycogene expression does indeed differ to tissue matched control samples. However, inter-individual variation was observed with each woman presenting with a different glycosylation profile - although upregulated glycogene expression in TNBC was the general trend.

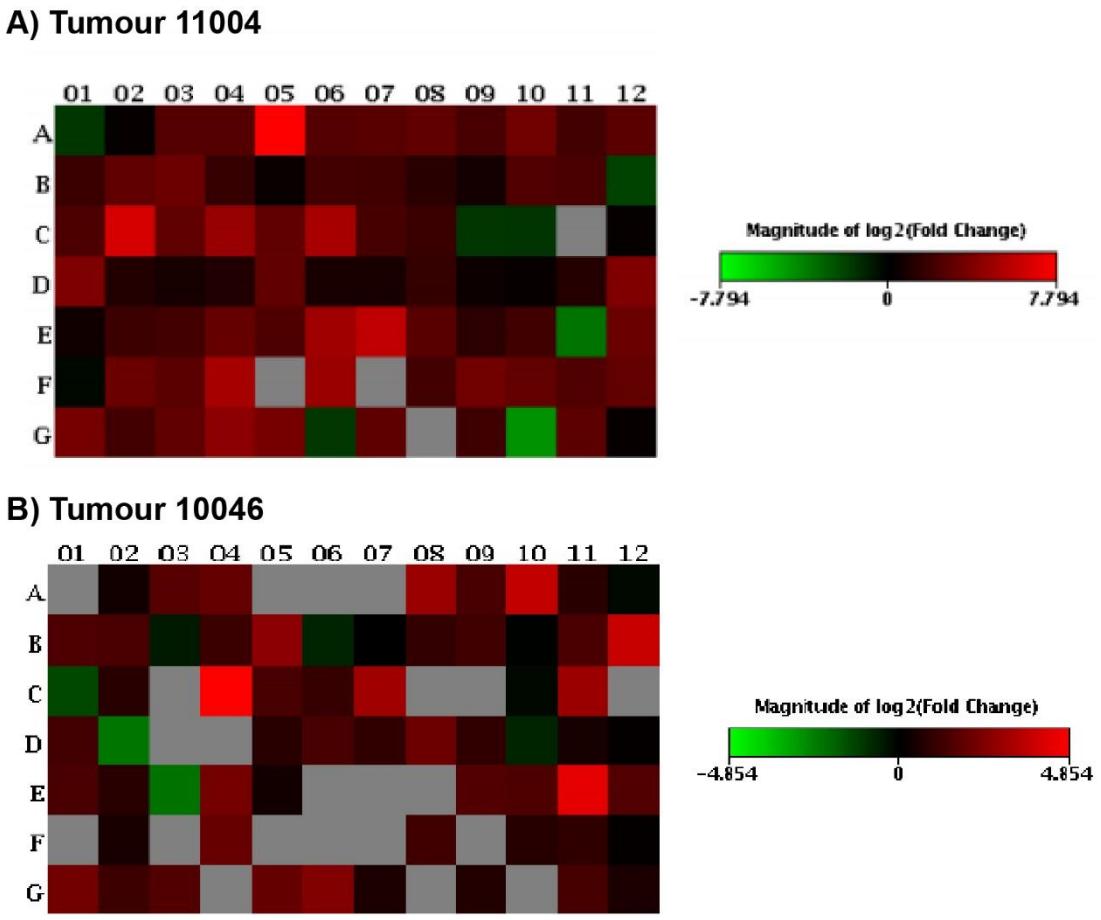

Figure 3.2: Profiling the change in glycosylation gene expression in TNBC. Heat map visualisation of the fold changes in gene expression between tumour 11004 **(A)** and tumour 10046 **(B)** and their respective tissue matched controls**.** Each pixel represents a gene and its colour indicates the magnitude of change in gene expression compared to its respective matched control; with green indicating upregulation, red indicating downregulation and grey showing no change: Scale bars show the magnitude of fold change in gene expression. Rows A-G and 01-12 represent the 84 key glycosylation genes profiled in this array. Samples were assessed by qRT-PCR and analysed with Sabiosciences data analysis software v2.3

3.4.2: Glycan-extension genes.

The glycosyltransferase family of genes encode extension enzymes which are responsible for the transfer of mucin-type *O*-glycans to glycoproteins as they cross the Golgi apparatus (Freeze & Ng, 2011). In tumour 11004, seventeen glycan-extending genes were upregulated including: *B3GLCT, B3GNT2-4*, *B3GNT8, B4GALT1-3, B4GALT5, C1GALT1, C1GALT1C1, GCNT1, OGT, POMGNT1, POMT1-2* and *UGGT. A4GNT* was the only downregulated extension gene in 11004, whilst *GCNT3, GCNT4* and *UGGT2* expression remained unchanged (Figure 3.3). In comparison, 8 glycan-extension genes were upregulated in 10046 including: *B3GLCT, B3GNT2, B4GALT1-3, C1GALT1C1, OGT* and *POMT2* (Figure 3.4). Expression of *A4GNT, B3GNT3, B3GNT4, B3GNT8, B4GALT5, C1GALT1, GCNT3, GCNT4, POMGNT1, POMT1,* and *UGGT2* remained unchanged between tumour 10046 and its matched control (Figure 3.4).

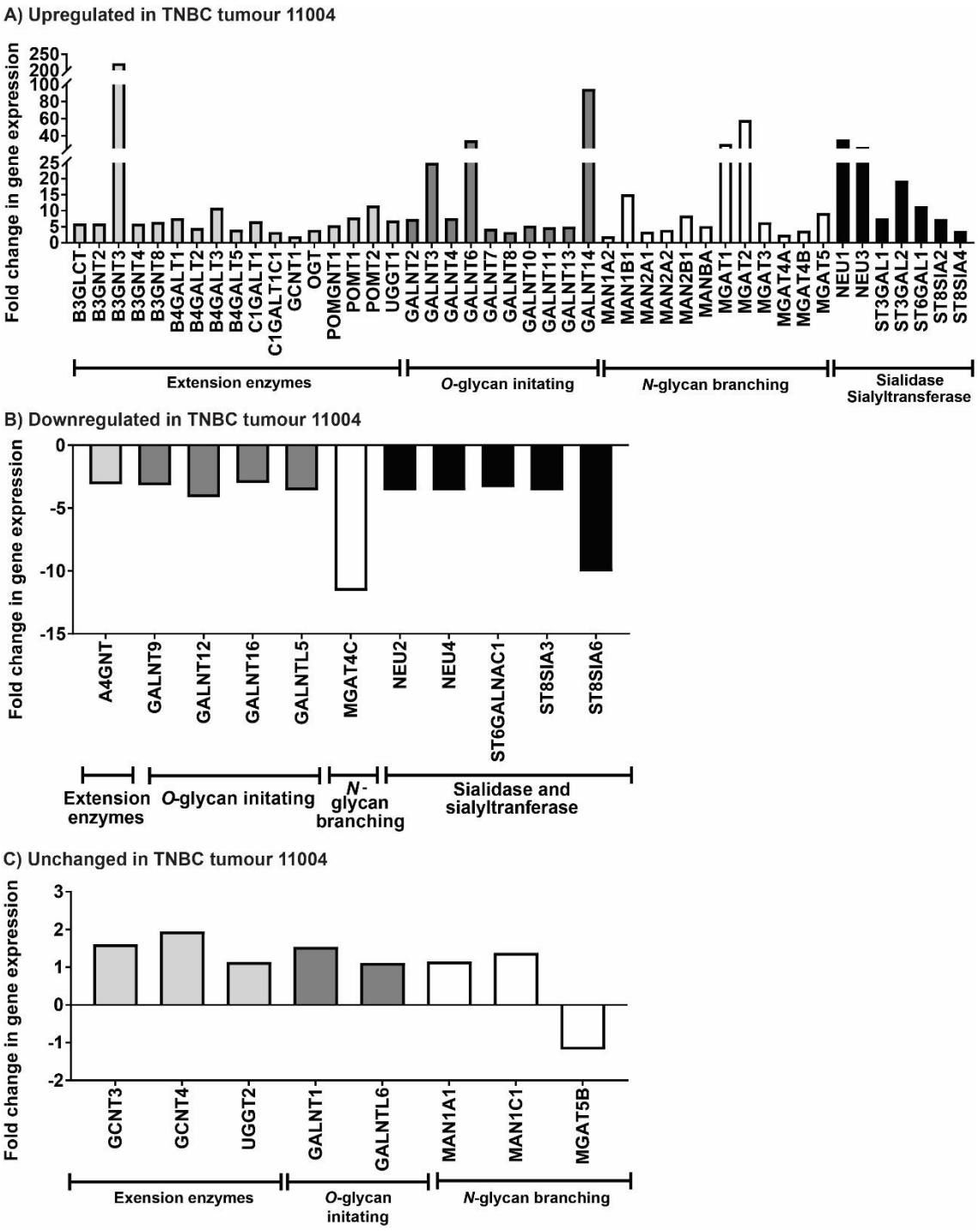

Figure 3.3: Analysis of the glycogene expression profile in TNBC tumour 11004.

Figure 3.3 Continued: Analysis of the glycogene expression profile in TNBC tumour 11004. Expression of key glycosylation enzymes were profiled by qRT-PCR. qRT-PCR values were normalised to housekeeper gene *RPLP0* expression and analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

3.4.3: *O*-glycan initiating genes.

O-Glycan initiating genes (*GALNT*s) are involved in the addition of *N*-acetylgalactosamine residues from the nucleotide sugar donor UDP-GalnNac to a serine or threonine residues. (Ju *et al.,* 2014). Ten *O*-glycan initiating genes were upregulated in tumour 11004 including: *GALNT2, 3, 4, 6, 7, 8, 10, 11, 13* and *14.* Four *O*-glycan initiating enzymes were downregulated including: *GALNT9, 12, 16* and *L5,* whilst *GALNT1* and *GALNTL6* remained unchanged (Figure 3.3).

Nine *O*-glycan initiating genes were upregulated in tumour 10046 including: *GALNT1, 3, 4, 6, 7, 11, 12, 14* and *L5*. The expression of six *O*-glycan initiating genes remained unchanged including: *GALNT2, 8, 9, 10, 16* and *L6* whilst *GALNT13* was downregulated (Figure 3.4). This data may indicate that altered *O*-glycan initiating gene expression is common in TNBC and these alterations may impact the subsequent glycosylation reactions occurring after initiation.

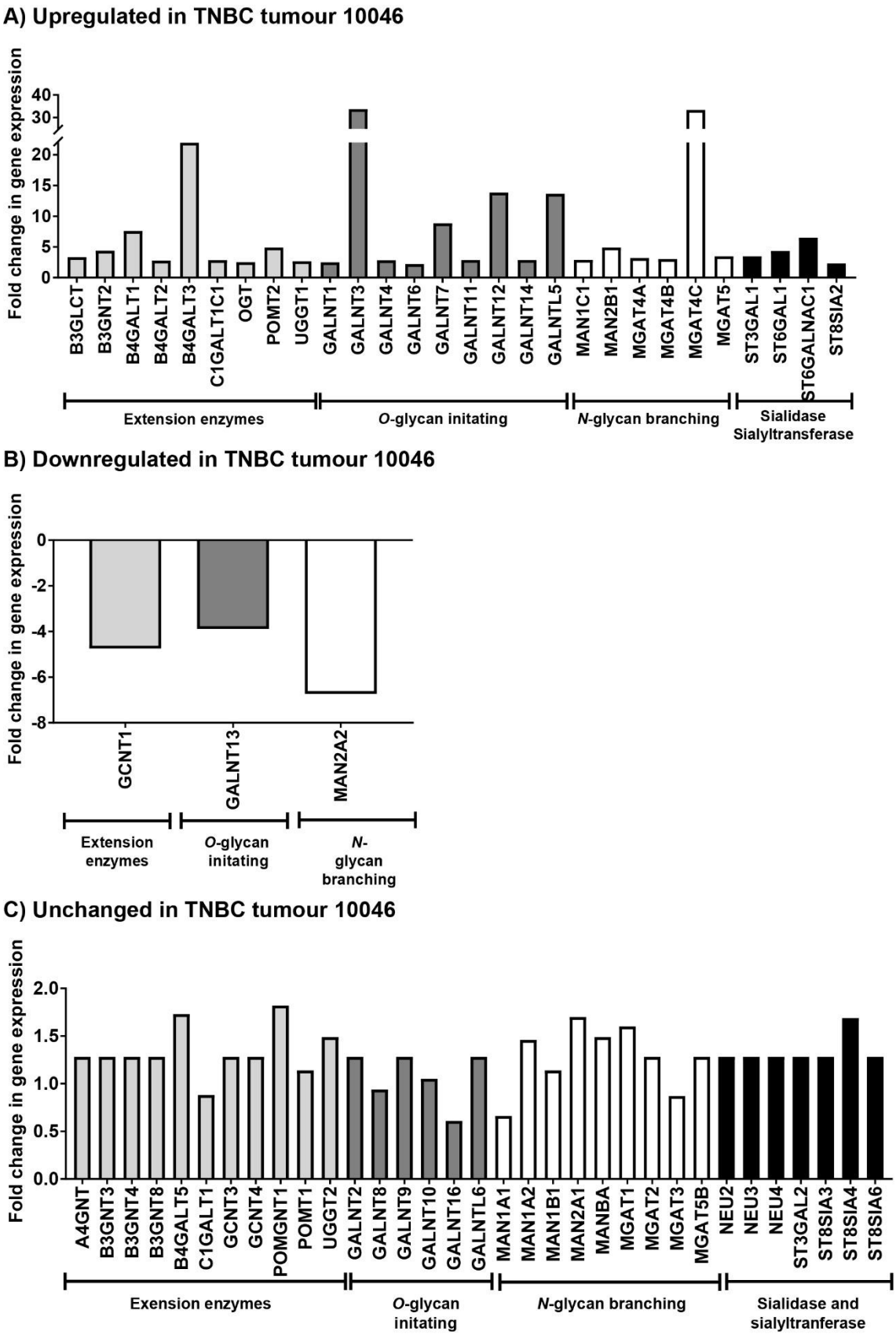

Figure 3.4: Analysis of the glycogene expression profile in TNBC tumour 10046.

Figure 3.4 continued: Analysis of the glycogene expression profile in TNBC tumour 10046. Expression of key glycosylation enzymes were profiled by qRT-PCR. qRT-PCR values were normalised to housekeeper gene *β2M* expression and analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

3.4.4: *N*-glycan branching genes.

N-Glycans are subject to a number of branching modifications including the addition and removal of mannose, *D*-glucose, *N*-acetylglucosamine, fucose and sialic acids (Legler *et al.*, 2018). Twelve *N*-glycan branching enzymes were upregulated in tumour 11004 including: *MAN1A2, MAN1B1, MAN2A1-2, MAN2B1, MANBA, MGAT1-3, MGAT4A, MGAT4B* and *MGAT5*. The expression of *MAN1A1, MAN1C1* and *MGAT5B* remained unchanged whilst *MGAT4C* was downregulated (Figure 3.3).

By contrast, *N*-glycan branching genes: *MAN1C1, MAN2B1, MGAT4A, MGAT4B, MGAT4C* and *MGAT5* were upregulated in tumour 10046 whilst *MAN2A2* was downregulated. *MAN1A1, MAN1A2, MAN1B1, MAN2A1, MANBA, MGAT1-3* and *MGAT5B* gene expression remained unchanged (Figure 3.4). This data suggests that *N*-glycan branching and cleaving may be altered leading to aberrant glycosylation's on the surface of TNBC cells.

3.4.5: Sialidase and Sialyltransferase genes

The sialidases/sialyltransferase enzymes are responsible for the addition or removal of *N*-acetylneuraminic acid residues on glycans. *NEU1, NEU3, ST3GAL1, ST3GALT2, ST6GAL1, ST8SIA2* and *ST8SIA4* were dramatically upregulated in tumour 11004 whilst: *NEU2, NEU4, ST6GALNAC1, ST8SIA3* and *ST8SIA6* expression was downregulated (Figure 3.3).

Sialyltransferase enzymes: *ST3GAL1, ST6GAL1, ST6GALNAC1* and *ST8SIA2* were upregulated in tumour 10046 whilst *NEU2, 3, 4, ST3GAL2, ST8SIA3, ST8SIA4* and *ST9SIA6* expression remained unchanged (Figure 3.4). Due to the upregultion of both sialic acid transferases and cleaving enzymes, it is highly likely that the sialic acid containing residues are altered on the surface of TNBC cells, leading to altered cell function.

3.4.6 Endoplasmic reticulum, decoration and trimming genes.

In addition to the main families of glycogenes several other important glycogenes were profiled including endoplasmic reticulum-degradation (ERAD), decoration (fucosyltransferase) and trimming (fucosidase, hexosaminodase) genes. *EDEM1, EDEM3, FUT8, GABAB, GLB1, MOGS, PRKCSH, HEXA* and *HEXB* were upregulated in both tumours 11004 and 10046 whilst, *NAGPA, EDEM2, FUT11, FUCA2, POFUT1* and *POFUT2* were only upregulated in tumour 11004. *FUCA1* and *GNPTAB* were upregulated in tumour 10046 but expression remained unchanged in tumour 11004 (Figure 3.5). This suggests that the altered glycosylation profile of TNBC may extend to all aspects of glycosylation including trimming, initiating and decoration.

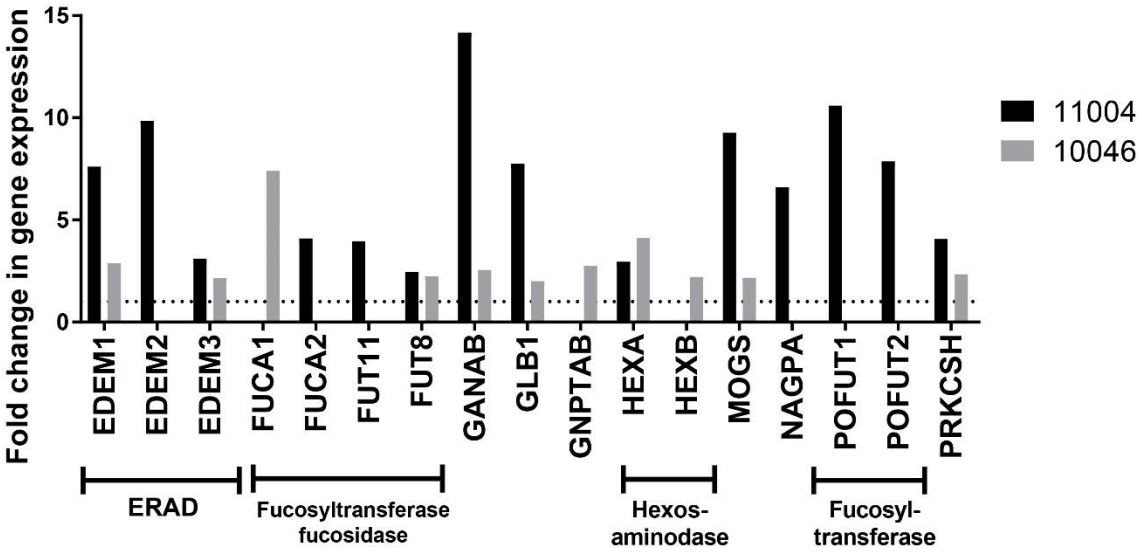

Figure 3.5: ERAD, decoration and trimming genes. Using a RT^2 profiler array, expression of key glycosylation enzymes were profiled by qRT-PCR. The fold change in gene expression is shown in tumours 11004 and 10046 for each gene. Unchanged gene expression is not shown. Data was normalised to housekeeper genes *β2M and RPLP0* for tumour 10046 and 11004 respectively and analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

3.4.7: Pilot analysis of glycogene expression suggests altered expression in TNBC.

Extensive qRT-PCR profiling of two TNBC tumours shows there are marked changes in glycogene expression in TNBC compared to matched tissue control samples. Furthermore, the glycogene expression alterations in TNBC was not limited to one family of enzymes, but to several groups including glycan extension enzymes, *O*-glycan initiating genes, *N*-glycan branching genes, sialidases and sialyltransferase genes.

As a result of the widespread upregulation of glycogenes in TNBC samples, it would be expected that glycan profiles would be altered on TNBC cells and therefore, glycan function may be changed. From the two TNBC tumours analysed, differences in glycogene expression were found which could be representative of multiple aberrant glycosylation's present in TNBC.

3.4.8: QRT-PCR validation of selected glycosylation gene expression in TNBC tumour samples.

The genes showing the most substantial changes in gene expression between tumours 11004 and 10046 (figure 3.3-3.5) were validated by independent qRT-PCR. Originally, tumour 10076 was not used in array profiling due to available array resources however, it could be included in QRT-PCR analysis to provide a more well-rounded understanding into changes in glycosylation gene expression in TNBC. RNA from the tumours and tissue matched controls was reverse

transcribed to cDNA and successful reverse transcription was assessed by end-point PCR amplifying the housekeeping gene β-actin as a positive control (Figure 3.6). The resulting gel electrophoresis showed clear bands present at 202bp in the +RT lanes of 11004, 10046 and 10076 in both the matched tissue control (C) and tumour (T) samples. The cDNA was therefore acceptable to use in qRT-PCR analysis. Gene expression was normalised to the geometric mean of housekeeper gene expression, β-actin*, β2M* and *RPLP0,* as they were stable references for normalisation.

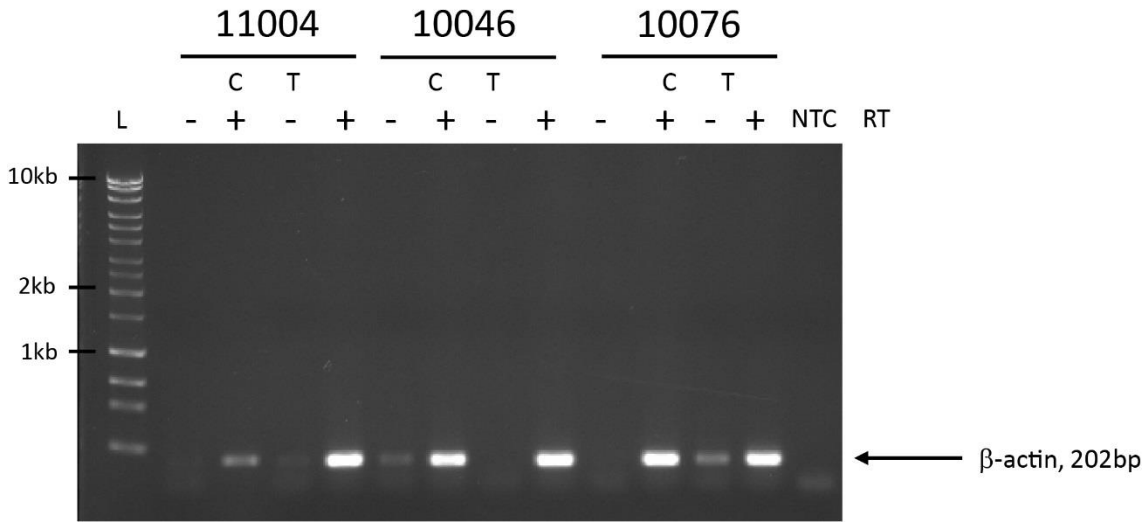

Figure 3.6: Verifying cDNA synthesis from tumour RNA. End point PCR was used to analyse cDNA synthesis reactions from RNA of TNBC tumours 11004, 10046 and 10076 and their respective tissue matched controls. Gel electrophoresis was used to resolve cDNA in 1x TAE buffer. Reactions contained DNA prepared with (+) or without (-) reverse transcriptase or with cDNA replaced with dH_2O (NTC). (L) Ladder. (C) Control. (T) Tumour. RT: Reverse transcriptase.

3.4.9: Validating altered expression the *O*-glycan initiating genes in TNBC.

Initially, *O*-glycan initiating genes (*GALNT3, GALNT6, GALNT7, GALNT12, GALNT14* and *GALNTL5*) were chosen for validation due to their intense upregulation in both TNBC samples and expression was assessed in tumours;

11004, 10046 and 10076 and their respective tissue matched controls (Figure 3.7).

In keeping with the array data *GALNT3, 6,* and *7* expression was upregulated in tumours 11004 and 10046 compared to control tissue. *GALNT12* and *L5* were upregulated in tumours 11004 and 10046 in the qRT-PCR validation, however these genes were only upregulated in tumour 10046, in the array profiling. *GALNT3* was significantly upregulated ($p>0.05$). *GALNT14* was upregulated in tumours 11004 and 10076 yet was downregulated in tumour 10046. *GALNT6, 7, 12,* and *L5* expression was downregulated in tumour 10076 (Figure 3.7).

This data proposes that *O*-glycan initiating gene expression is altered in TNBC. This would suggest that there are alterations in *O*-glycan structures in TNBC which may result in prematurely truncated or highly branched glycan structures.

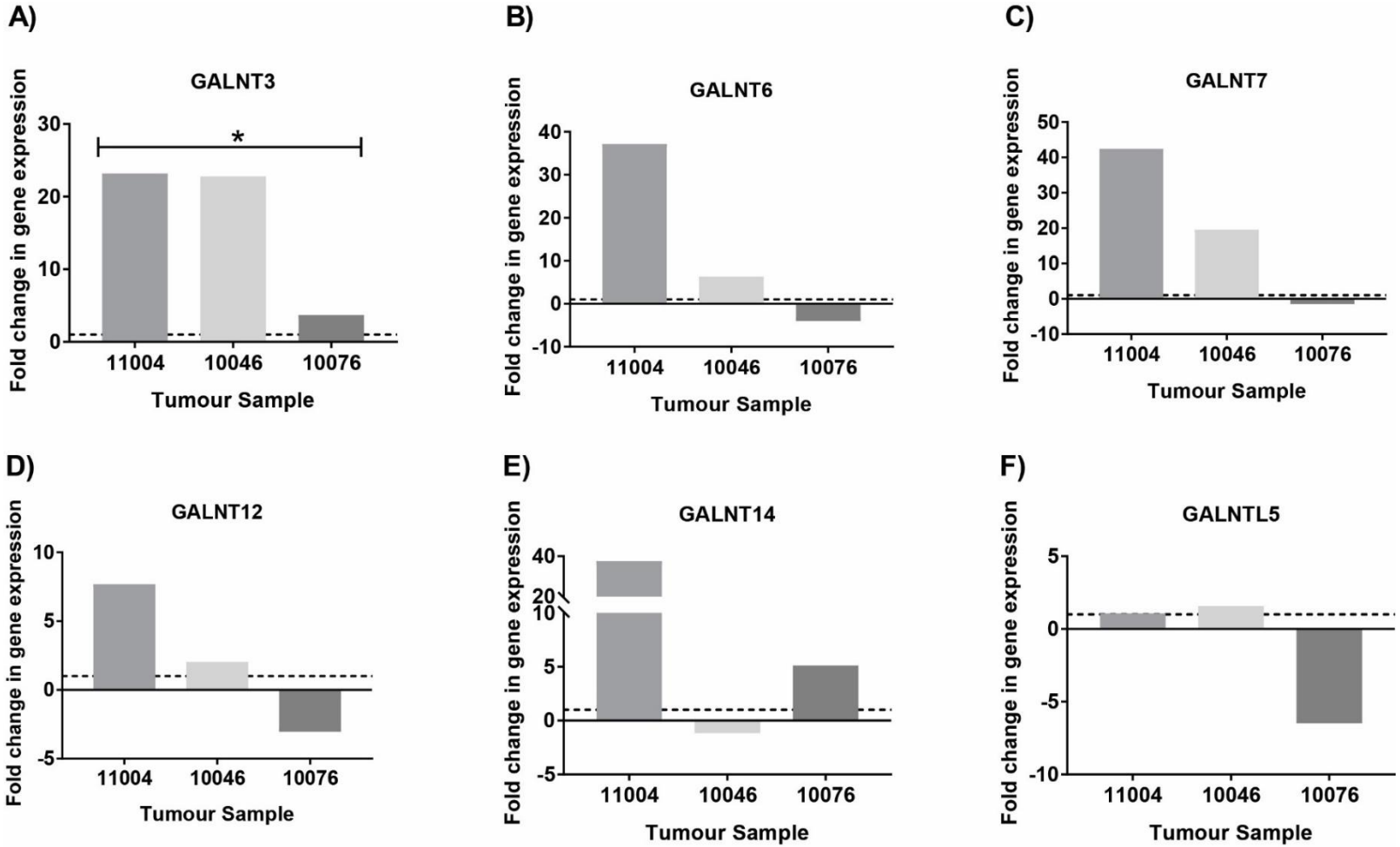

Figure 3.7: Comparing the expression of selected *O*-glycan initiating enzymes in TNBC.

Figure 3.7 continued: Comparing the expression of selected *O*-glycan initiating enzymes in TNBC: qRT-PCR values were normalised to the geometric mean of housekeeper genes *(*β-actin, *β2M* and *RPLP0)* and the fold change in gene expression was calculated by the [delta][delta]C_T method (Livak and Schmittgen, 2001). Shown is the fold change in gene expression from two technical replicates. Statistical analysis was performed on [delta]C_T values with the Student's t-test *p>0.05. The dotted line represents basal (i.e unperturbed) gene expression

3.4.10: Validating the glycosyltransferases, sialidases and sialyltransferase in TNBC.

The expression of a further five genes (*B3GNT2, B4GALT2, B4GALT3, NEU1* and *NEU3*) were chosen for validation tumours 11004, 10046 and 10076 due to their intense upregulation (Figure 3.8). *B3GTN2, B4GALT2, B4GALT3, NEU1* and *NEU3* expression was upregulated in tumours 11004 and 10046. In tumour 10076, *B4GALT2, B4GALT3* and *NEU1* expression was downregulated whilst B3GNT2 and *NEU3* were upregulated (Figure 3.8).

This data suggests that glycan-extension enzymes and sialidase enzymes are altered in TNBC which may result in aberrant cell glycosylation with abnormal glycan structures, facilitating aggressive tumour growth.

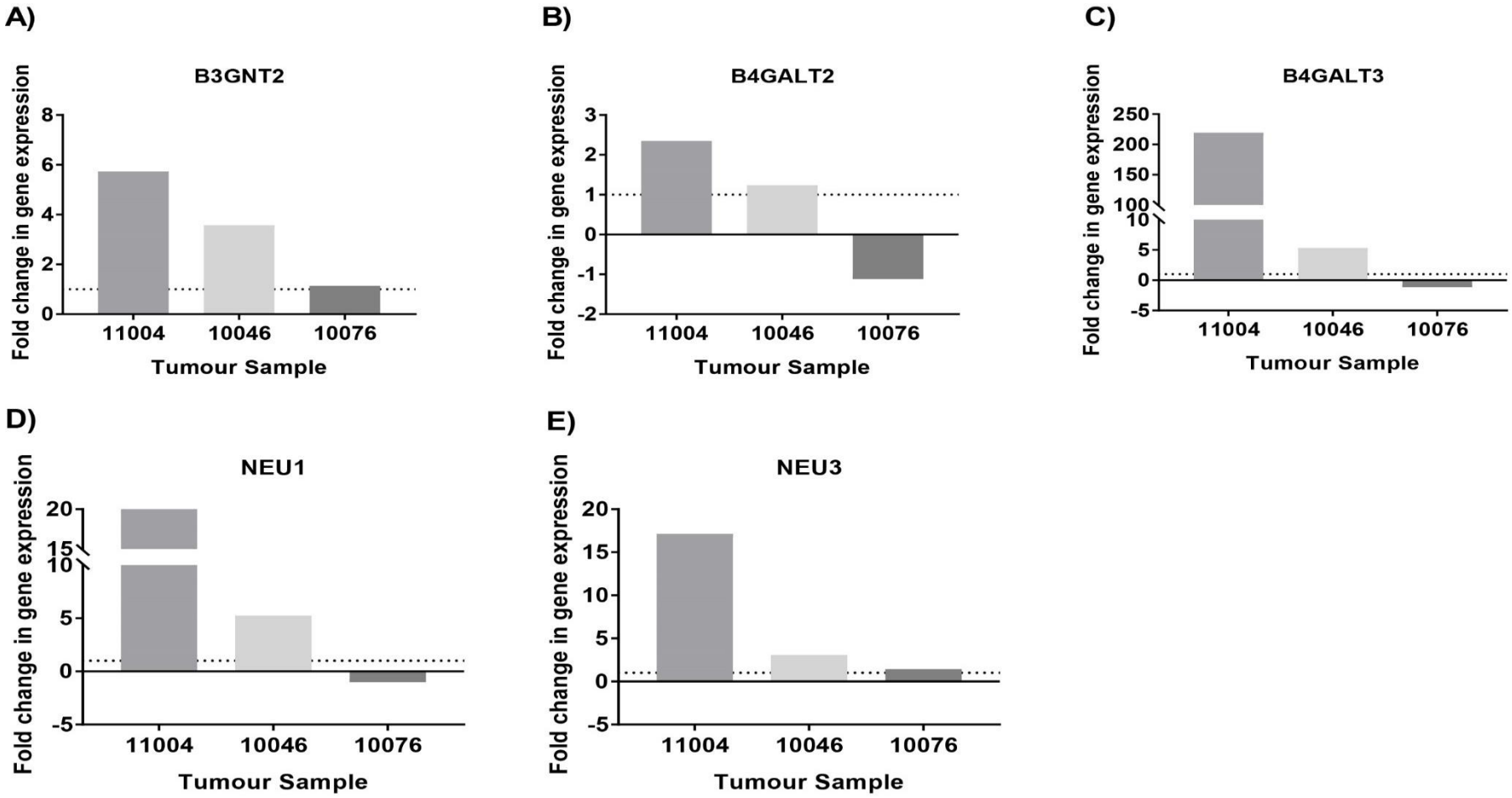

Figure 3.8: Analysis of glycosyltransferases and sialidases in TNBC.

Figure 3.8 continued: Analysis of glycosyltransferases and sialidases in TNBC (A-C) The relative mRNA expression of glycosyltransferase enzymes *B3GNT2, B4GALT2* and *B4GALT3* and sialidase enzymes *NEU1* and *NEU3* **(D&E)** were analysed by qRT-PCR in TNBC tumour samples (11004, 10046 and 10076) compared to their respective tissue matched controls. Shown is the fold change in gene expression from two technical replicates. qRT-PCR values were normalised to the geometric mean of housekeeper genes (β-actin, *β2M* and *RPLP0*). Fold change in gene expression was calculated by the [delta][delta]C_T method (Livak and Schmittgen, 2001). The dotted line represents basal (i.e unperturbed) gene expression

3.4.11: Correlating glycogene expression with hypoxia in TNBC.

TNBC is an inherently hypoxic tumour (Bernardi and Gianni, 2014). Therefore, expression of *HIF-1α* and *HIF-2α* was analysed in tumour samples 11004, 10046 and 10076 by qRT-PCR to assess the degree of hypoxia (Figure 3.9). Interestingly, *HIF-1α* was upregulated in all three TNBC samples whilst *HIF-2α* expression was downregulated (Figure 3.9). Expression of the HIF downstream target gene *CA9* (Semenza, 2000) was used to assess HIF-1α activation. *CA9* expression in all 3 TNBC samples was dramatically upregulated and correlated with *HIF-1α* expression in each sample. *HIF-2α* downstream targets were not analysed due to insufficient quantities of RNA. This data confirms TNBC tumours 11004, 10046 and 10076 were highly hypoxic and activation of HIF signalling was evident (Figure 3.9).

The degree of *HIF-1α* and *HIF-2α* expression was found to correlate with the trend in expression of glycogenes (Figures 3.7 - 3.9). Expression of *GALNT3, GALNT6, GANT7, GALNT12, B3GNT2, B4GALT3, NEU1* and *NEU3* was greatest in tumour 11004, was moderately expressed in tumour 10046 and was least expressed in tumour 10076, following the trend of *HIF-1α* expression in these samples (Figures 3.7 – 3.9). This trend suggests a possible correlation between *HIF-1α* induction and glycogene expression.

By contrast, expression of *GALNTL5* correlated with *HIF-2α* expression in tumours 11004, 10046 and 10076 (Figures 3.7 and 3.9), suggesting expression of certain glycogenes such as *GALNTL5* may correlate with or be regulated by *HIF-2α* signalling.

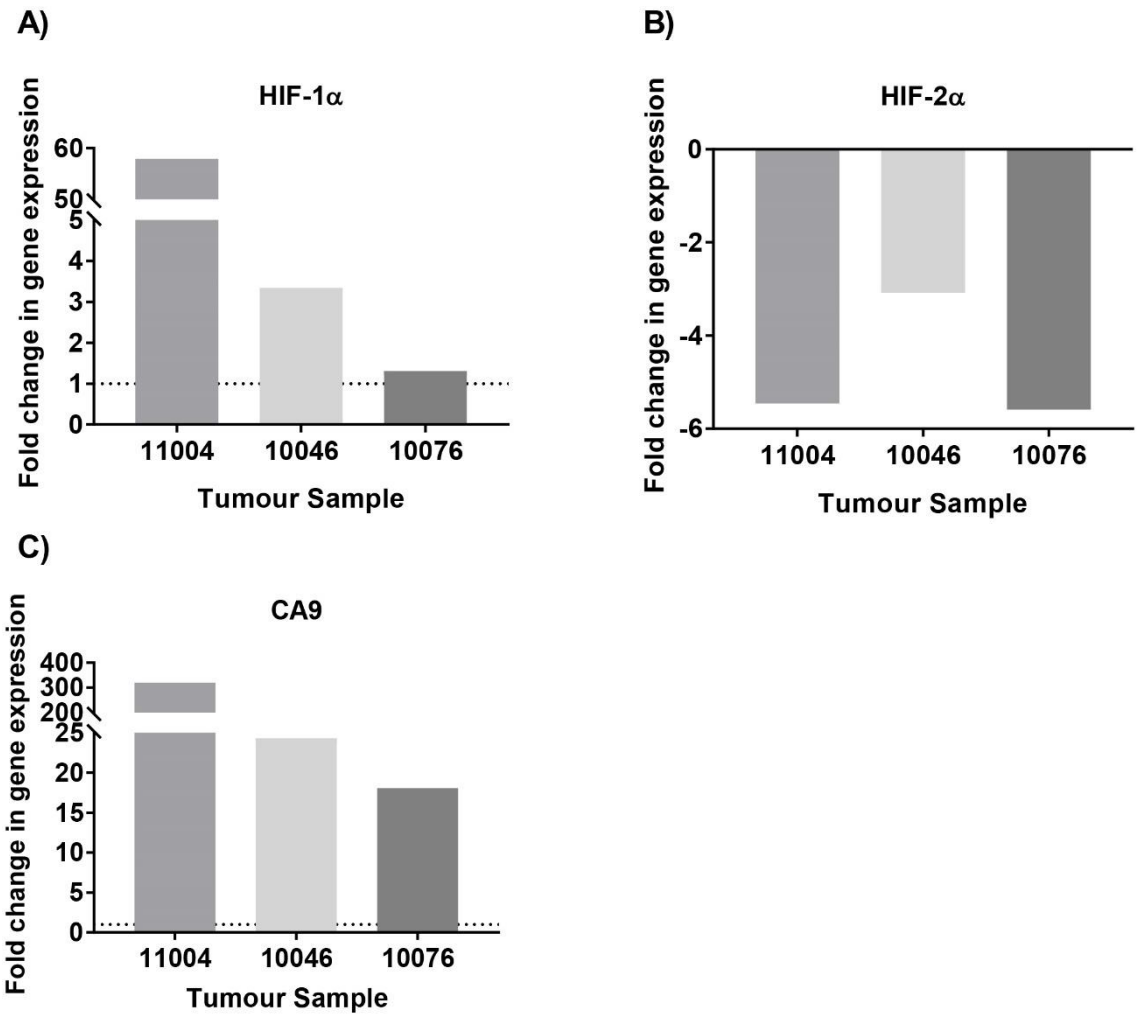

Figure 3.9: Expression of Hypoxia Inducible Factors and downstream target in TNBC. The fold change in gene expression of *HIF-1α*, *HIF-2α* and *CA9*, was analysed by qRT-PCR in TNBC tumours 11004, 10046 and 10076 compared to their respective tissue matched controls. Shown is the fold change in gene expression from two technical replicates. qRT-PCR values were normalised to the geometric mean of housekeeper genes β-actin*, β2M* and *RPLP0.* The dotted line represents basal (i.e unperturbed) gene expression.

3.4.12: Using hypoxic MDA-MB-468 cells as a model of TNBC.

Hypoxic MDA-MB-468 cells were used as an *in vitro* model of TNBC. Cells were exposed to acute hypoxia (1% O_2) for 22 hours and protein expression of HIF-1α and HIF-2α was assessed by immunoblotting (Figure 3.10). Clear bands were observed in hypoxic cells at approximately 116kDa for HIF-1α and 118kDa for HIF-2α indicating that these conditions successfully induced hypoxia and HIF stabilisation (Figure 3.10).

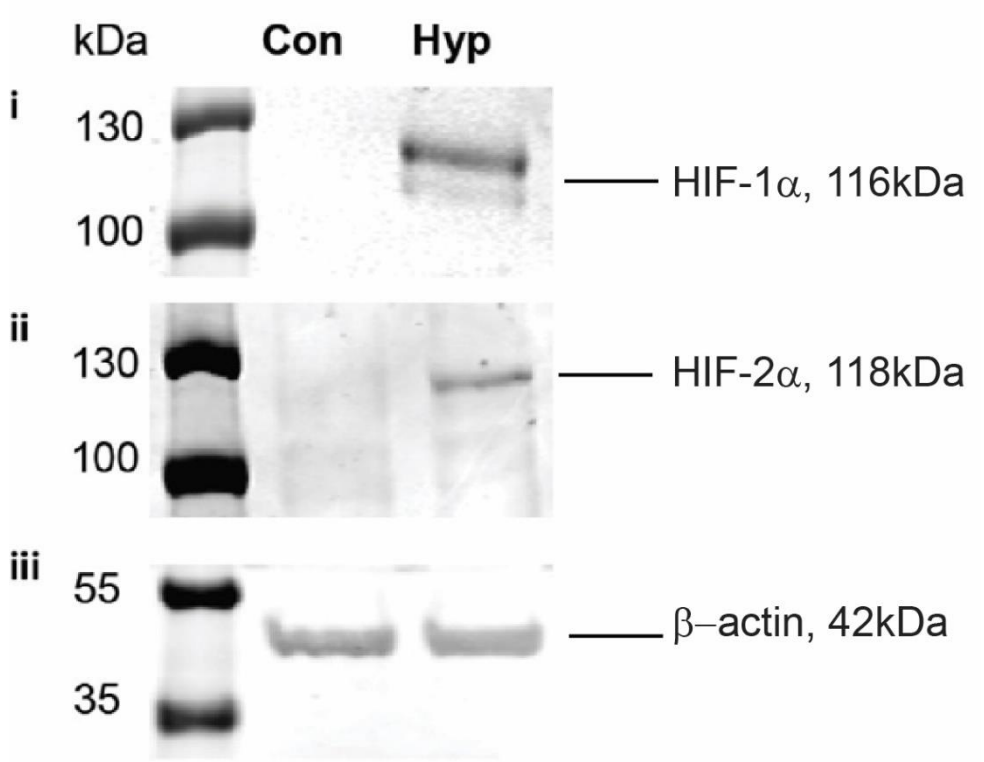

Figure 3.10: Analysis of HIF-1α and HIF-2α protein expression in hypoxic MDA-MB-468. Representative immunoblots of HIF-1α **(i)** and HIF-2α **(ii)** protein expression after 22 hours of acute hypoxia (1% O_2). Protein (30µg) was resolved on SDS polyacrylamide gels**,** transferred to nitrocellulose and probed with anti-HIF-1α, anti-HIF-2α, anti-β-actin antibodies and IR-conjugated secondary antibodies. Immunoblotting with β-actin was used to check equal protein loading **(iii).** The staining was visualised by LI-COR. Con: Control. Hyp: Hypoxic.

In order to investigate glycogene expression in hypoxia TNBC cells; RNA was extracted from control and hypoxic MDA-MB-468 cells and reverse transcribed to cDNA. Successful reverse transcription was shown by end-point PCR amplification of β-actin (Figure 3.11). The NTC lane did not show amplification of β-actin (Figure 3.11) confirming that there was not contamination.

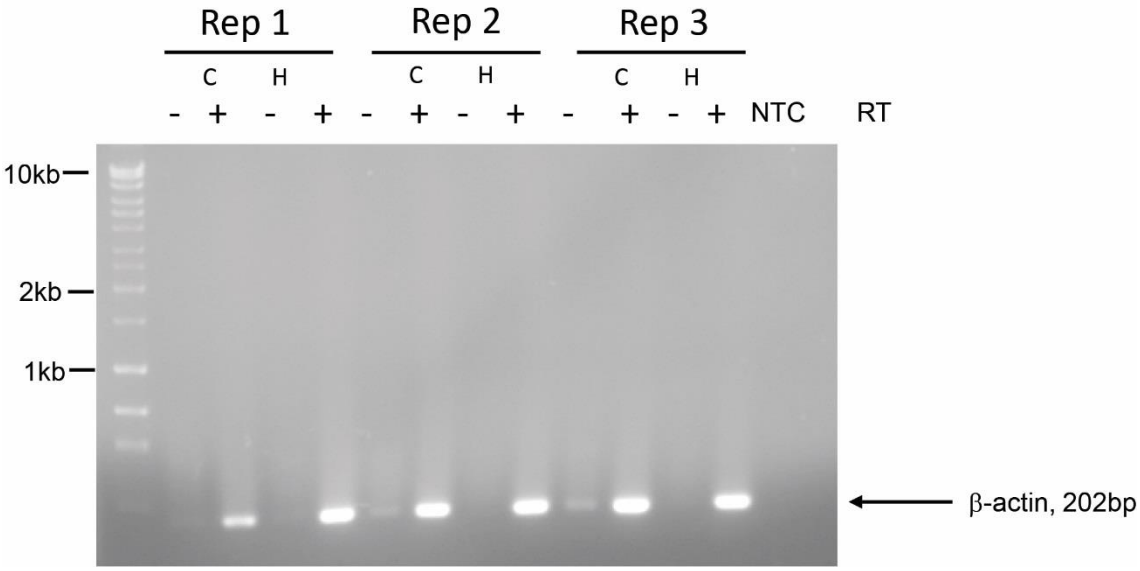

Figure 3.11: Verifying cDNA synthesis from tumour RNA. End point PCR was used to analyse cDNA synthesis reactions from MDA-MB-468 cells following hypoxia (1% O_2). Gel electrophoresis was used to resolve cDNA in (1x) TAE buffer. Reactions contained cDNA with (+) reverse transcriptase, RNA without reverse transcriptase (-) and with cDNA replaced with dH_2O (NTC). (L) Ladder. (C) Control. (H) Hypoxic. (RT) Reverse transcriptase. Rep: biological replicate.

3.4.13: Optimising qRT-PCR conditions in normoxic MDA-MB-468 cells.

Prior to assessing gene expression, qRT-PCR conditions were optimised to minimise use of materials yet maintain generation of reliable high-quality qRT-PCR data. A standard qRT-PCR contains 25ng of cDNA template and 10µM oligonucleotides. The qRT-PCR conditions were optimised by reducing the concentration of oligonucleotides from 3µM to 1.5µM (Figure 3.12). Under these conditions the C_T value of *β2M* reduced from 15.45 to 13.77 (Figure 3.12). A similar trend was observed where the C_T value decreased from 17.9 to 12.8 for *RPLP0*. While there was a reduction in the C_T value with decreasing oligonucleotide concentration, the C_T value still indicated strong expression without compromising results.

In order to optimise the concentration of cDNA used per qRT-PCR, the concentration of cDNA was reduced from 25.0ng per reaction to 6.25ng (Figure 3.12). As the concentration of cDNA template decreased, the C_T value of each gene tested increased as expected (Figure 3.12). For example, in *GALNT3,* the C_T value at 25ng of cDNA increased from 22.01 to 23.46 with 6.25ng cDNA. Although an increase in C_T value was observed, gene expression remained strong and therefore would not compromise the data analysis. Therefore, all qRT-PCR reactions contained 1.5µM oligonucleotides with 12.5ng of cDNA as this would minimise material use but still generate high-quality qRT-PCR data.

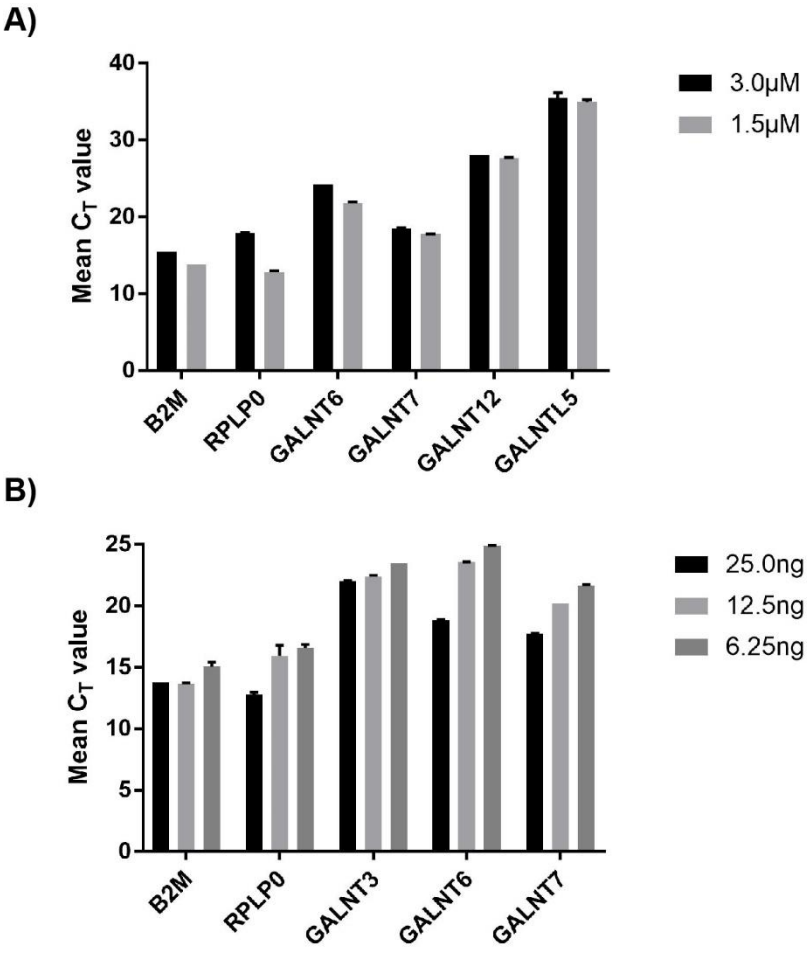

Figure 3.12: Optimisation of qRT-PCR reactions to assess glycogene expression in normoxic MDA-MB-468. **(A)** The effect of altering oligonucleotide concentration. qRT-PCR containing 3.0 μM or 1.5 μM oligonucleotides and 25ng cDNA were prepared and the expression of *β2M, RPLP0, GALNT6, GALNT7, GALNT12* and *GALNTL5* analysed. **(B)** The effect of altering cDNA template concentration. qRT-PCR reactions contained 1.5 μM oligonucleotides and 25-6.25 ng of cDNA template and gene expression of *β2M, RPLP0, GALNT3, GALNT6* and *GALNT7* was analysed. **(A+B)** C_T values are shown as the mean ± SD where n=2 technical replicates.

3.4.14: Validating HIF induction in hypoxic MDA-MB-468 cells.

MDA-MB-468 cells exposed to acute hypoxia (1% O_2) for 22 hours or grown under normoxia were used to assess the expression of *HIF-1*α and its downstream target carbonic anhydrase 9 (*CA9*) (Kuijper *et al.*, 2005). Expression of *HIF-2*α and its downstream targets erythropoietin (*EPO*) and vimentin (*VIM*) (Semenza and Wang, 1992) were also analysed in hypoxic MDA-MB-468 cells (Figure 3.13). Surprisingly, *HIF-1*α and *EPO* expression was downregulated under these conditions, whilst *HIF-2*α, *CA9* and vimentin expression were upregulated (Figure 3.13). Despite the decreased expression of *HIF-1*α, the marked increase in *CA9* expression suggests that the hypoxic conditions initiated a hypoxic response (Figure 3.13).

Successful induction of hypoxia within the cells was confirmed by the induction of *HIF-1*α and *HIF-2*α by immunoblotting (Figure 3.10). This is in keeping with the literature which suggests induction of HIF-1α occurs at the protein level because *HIF-1*α mRNA expression is variable (Greijer *et al.*, 2005). As can be seen from the large error bars there was a degree of variation between the degree of hypoxic adaptation within the MDA-MB-468 biological replicates (Figure 3.13). *HIF-1*α ranged from -7.76 to -1.55-fold and *HIF-2*α expression varied from -3.12 to 3.39-fold (Figure 3.13). This shows that although hypoxia was induced, the hypoxic conditions may not have been identical between biological replicates, therefore the downstream response may not have been as intense and therefore variation in intensity is present. Variability in the hypoxic conditions may have been introduced by means including that biological replicates were not exposed to hypoxia simultaneously.

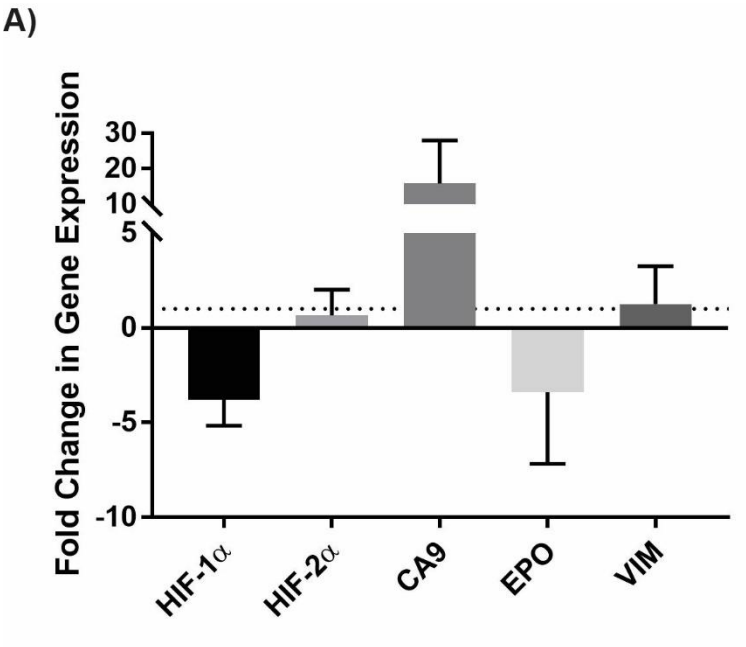

Figure 3.13: Analysing HIF induction in hypoxic MDA-MB-468. (A) The fold change in gene expression of hypoxia inducible factors *HIF-1*α and *HIF-2*α and downstream targets *CA9*, *EPO* and *VIM* were analysed in hypoxic MDA-MB-468 cells by qRT-PCR. Data was normalised to the geometric mean of β-actin*, β2M* and *RPLP0*. Fold change in gene expression is shown as the mean ± SEM where n=4 biological replicates. The dotted line represents basal (i.e unperturbed) gene expression.

3.4.15: Validating altered glycogene expression in hypoxic MDA-MB-468 cells.

Following the successful induction of hypoxia in MDA-MB-468 cells, the expression of 11 glycogenes (*GALNT3, 6, 7, 12, 14, L5, B3GNT2, B4GALT2-3, NEU1* and *NEU3*) was assessed in response to hypoxia as these genes showed the most intense induction in the tumour samples analysed. Surprisingly, *GALNTL5* was the only glycogene that was upregulated in hypoxic MDA-MB-468 cells whilst expression of *GALNT6, GALNT14* and *NEU3* was downregulated. *B3GTN2, B4GALT2, B4GALT3, GALNT7, GALNT12* and *NEU1* expression remained unchanged (Figure 3.14). Results of these analyses suggest that mRNA levels of *GALNTL5, GALNT6, GALNT14* and *NEU3* differ in hypoxic MDA-MB-468 cells compared to control cells and furthermore, that hypoxia and possibly *HIF-1*α*/HIF-2*α may be regulating expression of glycosylating enzymes.

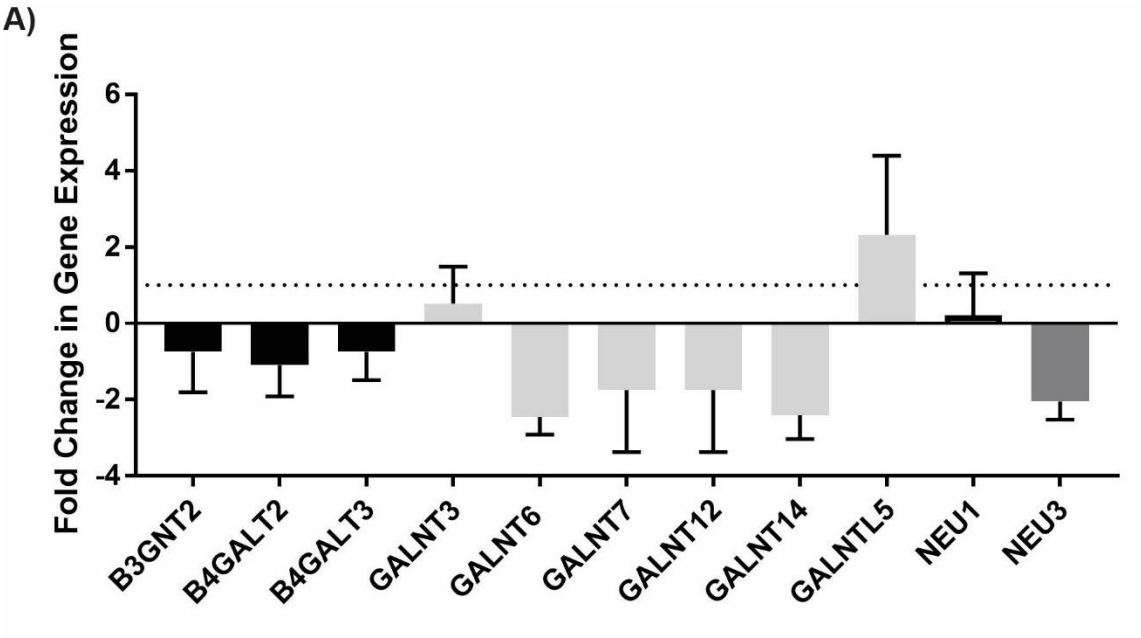

Figure 3.14: Analysing glycogene expression in hypoxic MDA-MB-468 cells. **(A)** The fold change in gene expression of glycogenes *B3GNT2, B4GALT2, B4GALT3, GALNT3, 6, 7, 12, 14 L5, NEU1* and *NEU3* was analysed in hypoxic MDA-MB-468 cells by qRT-PCR. Data was normalised to the geometric mean of β-actin, *β2M* and *RPLP0.* Fold change in gene expression is shown as the mean ± SEM where n=4 biological replicates. The dotted line represents basal (i.e unperturbed) gene expression.

3.4.16: Correlating HIF induction with glycogene expression under hypoxia in MDA-MB-468 cells.

As a result of the glycogene and HIF expression analyses in hypoxic MDA-MB-468 cells, a correlation in expression trends was observed. Furthermore, variation in the induction of hypoxia seemed to correlate with the induction of target downstream expression (Figure 3.15). Interestingly, the fold change in gene

expression of *B4GALT2, GALNTL5* and *NEU1* seemed to correlate with the level of *HIF-1*α and *HIF-2*α induction (Figures 3.15 and 3.16). The replicates which demonstrated strong *HIF-1*α or HIF-2α induction also had strong *B4GALT2, GALNTL5* and *NEU1* expression. Where *HIF-1*α or *HIF-2*α expression was not as intense, induction of *B4GALT2, GALNTL5* and *NEU1* was decreased. Currently there are no links between *B4GALT2, GALNTL5, NEU1* and either HIF-1α or HIF-2α established in the literature.

The correlation between the level of HIF induction and glycogene expression in TNBC tumours 11004, 10046, 10076 and hypoxic MDA-MB-468 cells suggests a possible link between transcription of these glycosylation genes and induction of HIF-1α and HIF-2α. Extrapolating from this, it would be expected that there would be an impact on the glycosylation profile and tumour aggression in hypoxic TNBC.

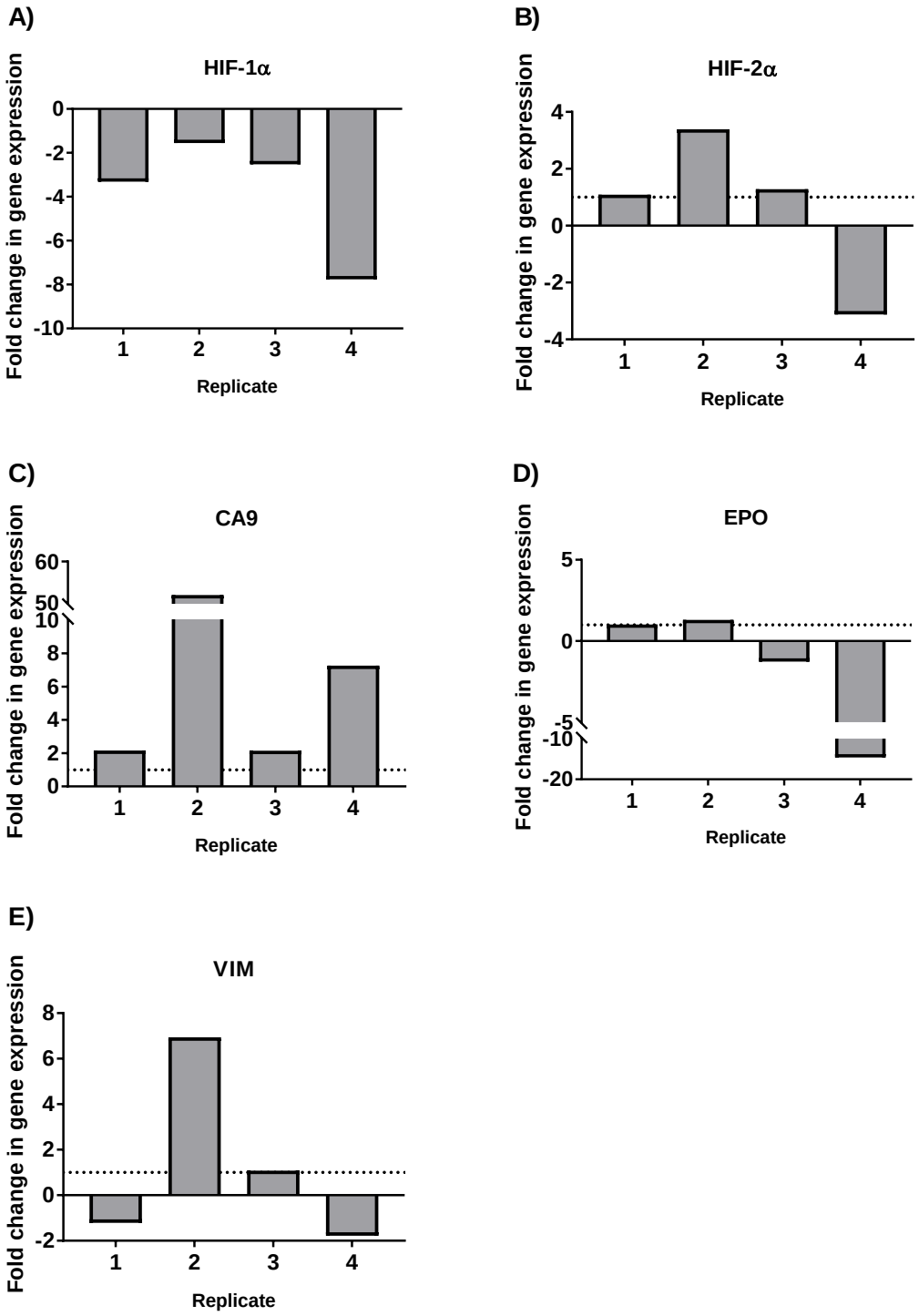

Figure 3.15: The variability of HIF induction in hypoxic MDA-MB-468 cells. The fold change in gene expression of *HIF-1α*, *HIF-2α* and downstream targets genes *CA9*, *EPO* and *VIM* was analysed in hypoxic MDA-MB-468 cells by qRT-PCR. Fold change in gene expression of each biological replicate was normalised to the geometric mean of β-actin, *β2M* and *RPLP0.* The dotted line represents basal (i.e unperturbed) gene expression.

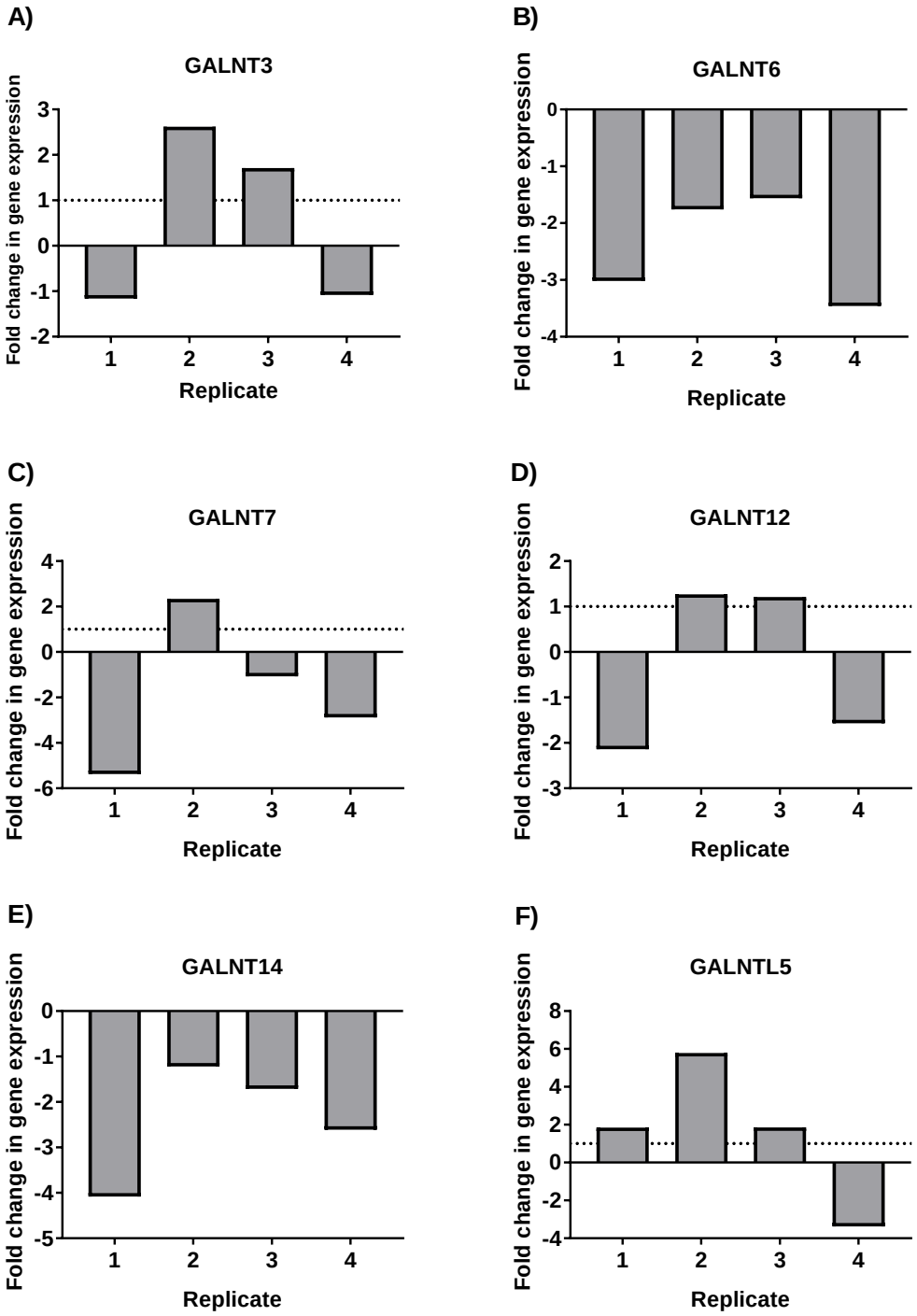

Figure 3.16: The variability of glycogene expression in hypoxic MDA-MB-468 cells.

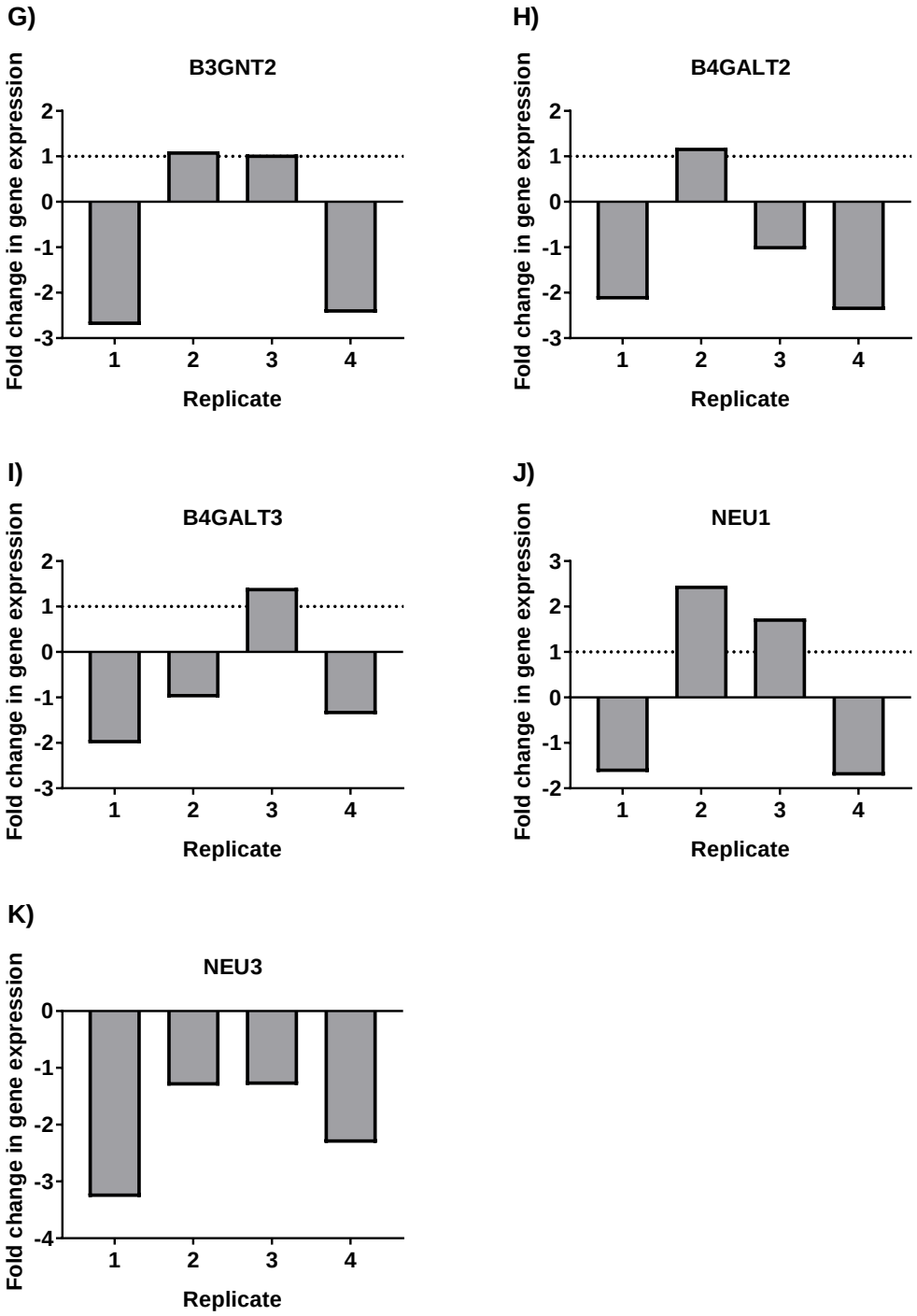

Figure 3.16 continued: The variability of glycogene expression in hypoxic MDA-MB-468 cells. The fold change in gene expression of glycogenes *B3GNT2, B4GALT2, B4GALT3, GALNT3, 6, 7, 12, 14, L5, NEU1* and *NEU3* was analysed in hypoxic MDA-MB-468 cells by qRT-PCR. Fold change in gene expression of each biological replicate was normalised to the geometric mean *of* β-actin, *β2M* and *RPLP0*. The dotted line represents basal (i.e unperturbed) gene expression.

3.4.17: Determining the glycan profile of hypoxic MDA-MB-468.

The glycosylation profile of TNBC is altered compared to control and in response to hypoxia and the above data suggests that the hypoxic tumour environment may alter glycogene expression. Furthermore, this may have an impact or corresponding change to the glycosylation profile of hypoxic TNBC cells. To assess this, lectins were used to detect different sugar moieties. Lectins were chosen based on their sugar specificities, covering a wide range of sugars. Hypoxic MDA-MB-468 cells were stained with biotinylated lectins (*PNA, DBA, PHA-E, RCA, LCA and WGA*) and analysed by flow cytometry.

3.4.17: The impact of lectin staining on cell size and granularity in hypoxic MDA-MB-468.

Forward scattering indicates cell size whilst side scatter indicates the cell's granularity (Tzur *et al.,* 2011). Hypoxia did not alter cell size or granularity in unstained control or hypoxic MDA-MB-468 cells. Similarly, the addition of PE-streptavidin to control and hypoxic cells did not significantly affect cell size or granularity (Figure 3.17).

Staining of cells with *PNA* increased cell size from 309 to 376 under hypoxic conditions. Cells stained with *DBA* also exhibited an increase in cell size under hypoxia (313 to 392). This increase in cell size in hypoxic cells was observed when staining with lectins *PHA-E, LCA and RCA* where cell sizes increased from 403 to 416, 425 to 439 and 412 to 419, respectively (Figure 3.17A).

Hypoxic cells stained with *PNA, PHA-E* and *LCA*, significantly increased cell granularity compared to control cells. Cell granularity increased from 343 to 428 when stained with *PNA* under hypoxia ($p<0.05$) and increased upon staining with *PHA-E* from 334 to 424 between control and hypoxic cells ($p<0.05$). Similarly, cell granularity increased from 324 to 424 when staining with LCA between control and hypoxic cells ($p<0.01$) (Figure 3.17B).

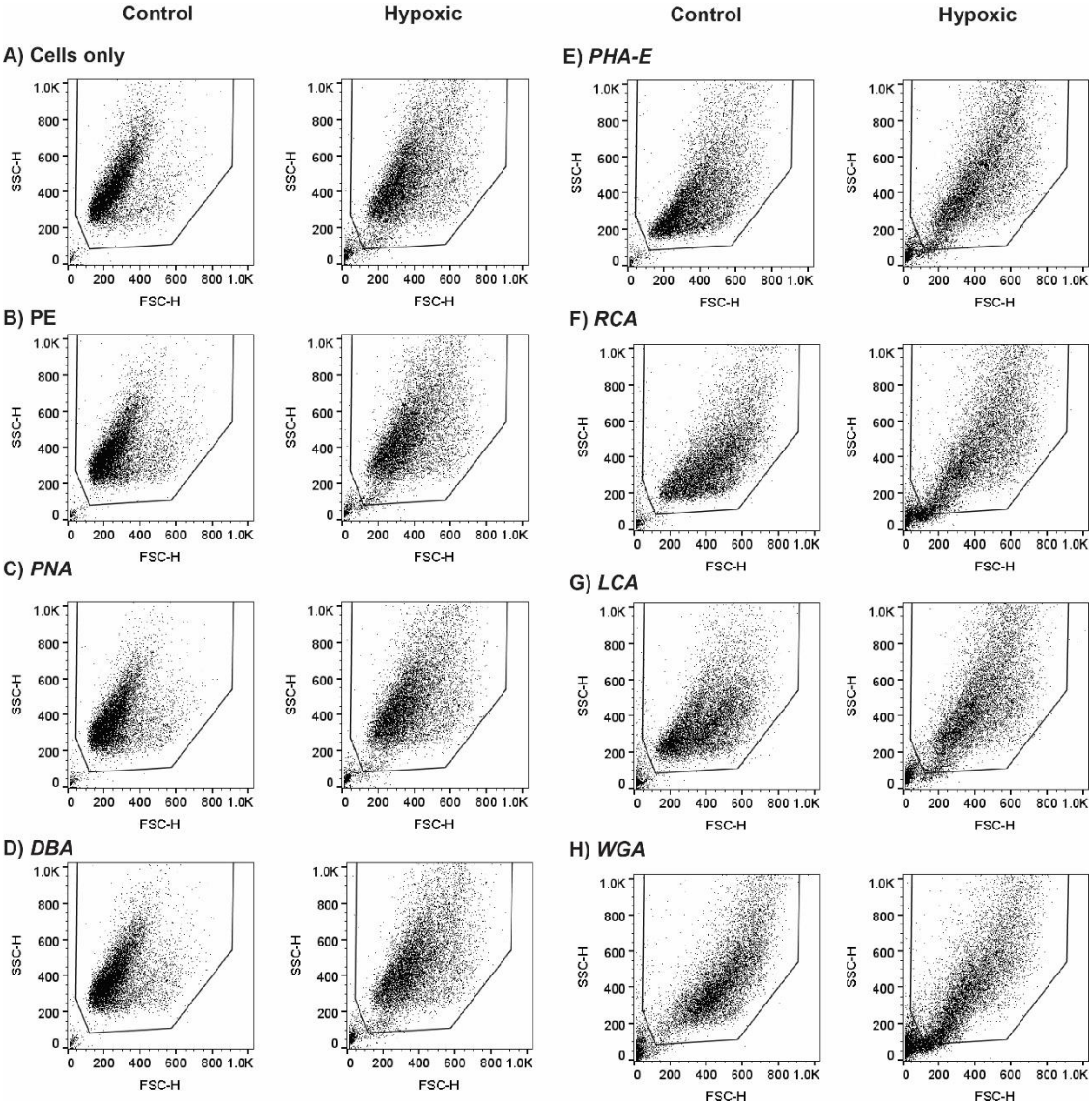

Figure 3.17A: Investigating the lectin staining profile of hypoxic MDA-MB-468 cells stained with lectins.

Figure 3.17A continued: Investigating the lectin staining profile of hypoxic MDA-MB-468 cells stained with lectins.MDA-MB-468 cells were exposed to hypoxia (1% O_2) for 22 hours and stained with biotinylated lectins: *PNA, DBA, PHA-E, RCA, LCA, WGA.* Staining was detected with phycoerythrin (PE) – streptavidin and was analysed by flow cytometry counting 10000 gated events. Forward scatter (FSC-H) and side scatter (SSC-H) profiles are shown: **(A)** cells only; **(B)** cells and Phycoerythrin (PE); **(C)** Peanut agglutinin *(PNA)*; **(D)** *Dolichos biflorus* agglutinin *(DBA);* **(E)** *Phaseolus vulgaris* erythroagglutinin (*PHA-E)*; **(F)** *Ricinis communis* agglutinin (*RCA)*; **(G)** *Lens culinaris* agglutinin *(LCA)*; **(H)** *Wheat germ* agglutinin (*WGA)*.

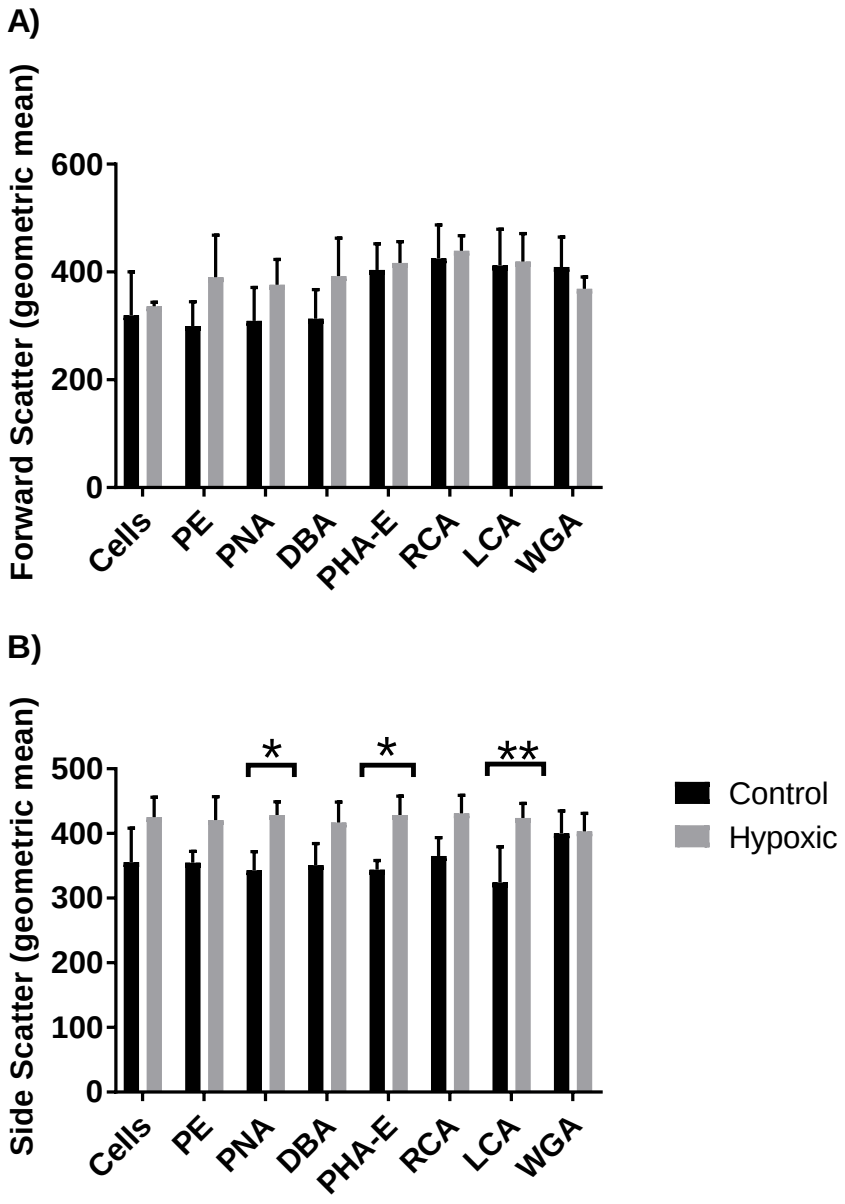

Figure 3.17B: The effect on lectin staining on cell size and granularity in hypoxic MDA-MB-468 cells. MDA-MB-468 cells were exposed to hypoxia (1% O_2) for 22 hours and stained with biotinylated lectins: *PNA, DBA, PHA-E, RCA, LCA, WGA.* Staining was detected with phycoerythrin (PE) – streptavidin and was analysed by flow cytometry counting 10000 gated events. Panels **(A)** and **(B)** show the geometric mean of forward and side scatter (n=3 biological replicates, mean ± SEM). Statistical analysis was performed by 2-way ANOVA with Sidak's multiple comparisons where *p<0.05, **p<0.01.

3.4.18: The impact of lectin staining on PE-streptavidin fluorescence in hypoxic MDA-MB-468.

In order to investigate the potential change in glycosylation profile in hypoxic TNBC cells to glycan structures on the cell surface, control and hypoxic MDA-MB-468 cells were stained with lectins *PNA*, *DBA*, *PHA-E*, *RCA, LCA* and *WGA* (Figure 3.18). Fluorescence was measured by streptavidin- conjugated-phycoerythrin (PE-streptavidin) by flow cytometry.

In unstained control and hypoxic cells, only background fluorescence was observed. This was also observed in cells stained with PE only, indicating that PE staining was specific for the presence of lectins. Similarly, in control cells stained with *PNA* and *DBA* only background fluorescence was present indicating little binding by these lectin sugars to the surface of TNBC MDA-MB-468 cells (Figure 3.18). However, there was a slight rightward shift in hypoxic cells stained with *PNA* and *DBA,* which suggests a higher proportion of bound lectins compared to control cells. Cells stained with *PHA-E*, *RCA* and *LCA* had intense lectin binding compared to control cells indicating strong binding by these lectin sugars to hypoxic MDA-MB-468 cells.

This data shows strong binding of *PHA-E, RCA, LCA* and *WGA* to MDA-MB-468 cells, indicating a high proportion of complex *N*-type glycans, non-reducing terminal β-*D*-galactose, core fucosylated glycans and *N*-acetyl-neuraminidase residues on the surface of MDA-MB-468 cells. This would suggest that there is a trend for increased *N*-type glycans, non-reducing terminal β-D-galactose, core fucosylated glycans and *N*-acetyl-neuraminidase glycans in hypoxic MDA-MB-468 cells.

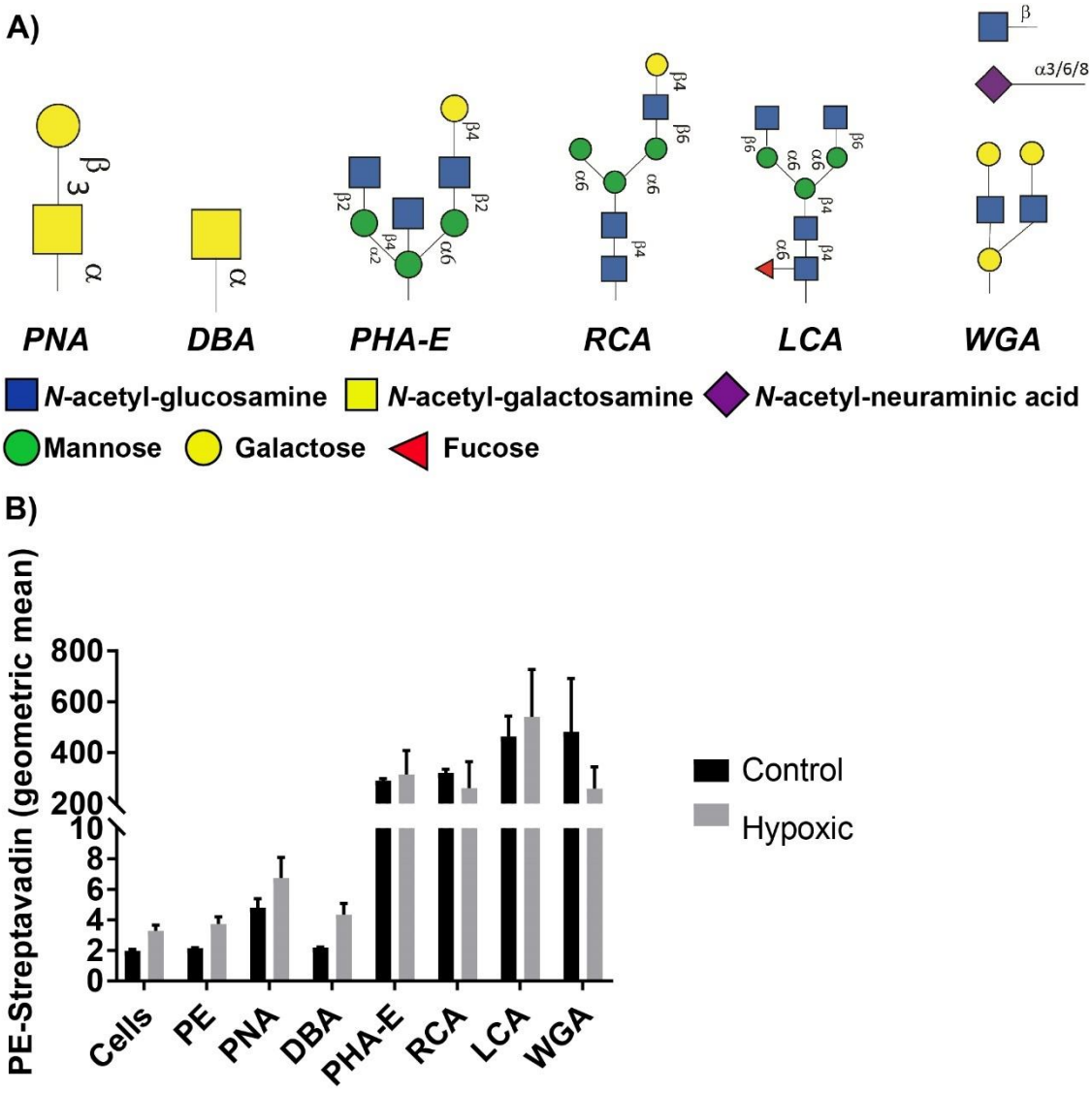

Figure 3.18: The change in lectin binding profile in response to acute hypoxia. (A) Representative target glycans for lectin binding. **(B)** MDA-MB-468 cells were exposed to hypoxia (1% O_2) for 22 hours and stained with biotinylated lectins: Peanut agglutinin (*PNA), Dolichos biflorus* agglutinin *(DBA), Phaseolus vulgaris* erythroagglutinin *(PHA-E), Ricinis communis* agglutinin *(RCA), Lens culinaris* agglutinin *(LCA), Wheat germ* agglutinin *(WGA).* Staining was detected with phycoerythrin (PE) – streptavidin and was analysed by flow cytometry counting 10000 gated events. The geometric mean of PE-streptavidin fluorescence is shown (n=3 biological replicates, mean ± SEM). The sugar residues detected by each lectin are shown in the key.

3.4.19: Conclusions.

The above data suggests that glycogene expression in TNBC is altered compared to tissue matched controls. qRT-PCR analysis indicated that the expression of glycan-initiating genes, glycan-extension genes and sialidases was changed in tumour tissue compared to control tissue. These alterations were assessed in hypoxic TNBC MDA-MB-468 cells to replicate the tumour environment - which elucidated abnormal expression of glycogenes: *GALNTL5, GALNT6, GALNT14* and *NEU3* in hypoxic cells compared to control cells. A correlation between hypoxia gene expression and glycogene expression was observed; potentially indicating regulation. Lectin binding analysis indicated hypoxia significantly increased cell granularity when stained with lectins: *PHA-E, RCA, LCA* and *WGA*. Furthermore, a trend between hypoxia and alterations in terminal galactose residues, fucosylated glycans, *N*-type complex glycans and sialylated glycans in MDA-MB-468 cells was identified.

3.5: Discussion

3.5.1: Aberrant glycosylation in TNBC.

TNBC is an aggressive subtype of breast cancer which lacks common receptors for drug targets (ER-, PR-, HER2-) (Hon *et al.*, 2016). Due to its insensitivity to current clinical therapies, TNBC is currently the most difficult type of breast cancer to treat (Shao, Sun and Deng, 2017). This underpins the urgent need to identify therapeutic targets and develop effective treatments. In many solid tumours, areas of moderate to severe hypoxia (<60 mm Hg) arise through uncontrolled cell proliferation with insufficient vascularisation (Muz *et al.*, 2015).

Adaptations mediated though HIFs cause changes to cell proliferation, angiogenesis and glucose metabolism (Annibaldi and Widmann, 2010). HIF-1α changes the primary metabolic substrate to glucose and glutamine which are the basis of DNA, protein and lipid synthesis (Liberti and Locasale, 2016). This causes a substantial change in the levels of intracellular nucleotide sugars which may alter cellular functions and modify glycan structures on the cell surface (Shirato *et al.*, 2011). Aberrant protein glycosylation is common in many cancers, including breast cancer (Hanahan and Weinberg, 2011) however, the underlying molecular mechanisms which facilitate these changes are poorly understood and therefore require further study (Venkitachalam *et al.*, 2016).

This chapter aimed to profile changes to glycogene expression in TNBC through profiler array and qRT-PCR. qRT-PCR provided a quantitative measure of changed gene expression in TNBC and hypoxic MDA-MB-468 cells whilst lectin staining, and flow cytometry assessed the impact of hypoxia on glycan structures in TNBC MDA-MB-468 cells.

The data collected indicates that the glycosylation gene expression profile is altered in TNBC compared to tissue matched controls, that hypoxic MDA-MB-468 cells share altered expression in some glycogenes and that the glycan profile of MDA-MB-468 cells is altered in some respects in response to acute hypoxia. It is important to acknowledge that the *in vivo* data present here only represents the changes to glycogene expression in three individual TNBC patients. To understand the global changes to the glycan profile of TNBC, a larger sample size of TNBC samples should be considered.

3.5.2: MDA-MB-468 cells as a model of TNBC.

MDA-MB-468 cells are one of the six distinct molecular subtypes of TNBC (Lehmann *et al.*, 2011). MDA-MB-468 cells are of the Basal-like 1 type and are enriched in cell cycle and cell division pathways and components (Lehmann *et al.*, 2011).

Since TNBCs are largely considered basal-like (Hu *et al.*, 2006), MDA-MB-468 cells are an adequate representation of the TNBC cell population; having been used in numerous TNBC studies (Goode *et al.*, 2017; Lanning *et al.*, 2017; Jung *et al.*, 2018). Therefore, the hypoxic MDA-MB-468 cells used in this study provided an adequate model to study the changes to glycosylation profile of hypoxic TNBC.

3.5.3: Glycogene expression is altered in TNBC.

Glycosylation aberrations are found in breast cancer and give rise to phenotypic changes which aid in tumour formation and aggression (Dall'Olio and Trinchera, 2017). The glycan structures present in breast cancer differ to those found in healthy tissue which are facilitated by enzymes such as *GALNT, B4GALT, FUT, ST3GAL* and *MGATs* (Figure 3.19). Previously, altered glycogene expression has been screened in cohorts of breast cancer patients (Potapenko *et al.,* 2010). Such analysis indicated changes to expression in a number of genes including those involved in *N*-glycan branching, sialylation, *O*-glycan initiation and glycan extension genes including *B4GALT3* (Potapenko *et al.,* 2010). Here, 84 key glycogenes were profiled in TNBC and a marked change in the glycosylation

profile between TNBC and control tissue was identified. For example, glycan extension genes: *B3GLCT, B3GNT2, B4GALT1-3, C1GALT1C1, OGT* and *POMT2* were upregulated in tumour 11004 and 10046. qRT-PCR analysis of tumour 10076 also indicated altered expression of *B3GNT2, B4GALT2* and *B4GALT3*.

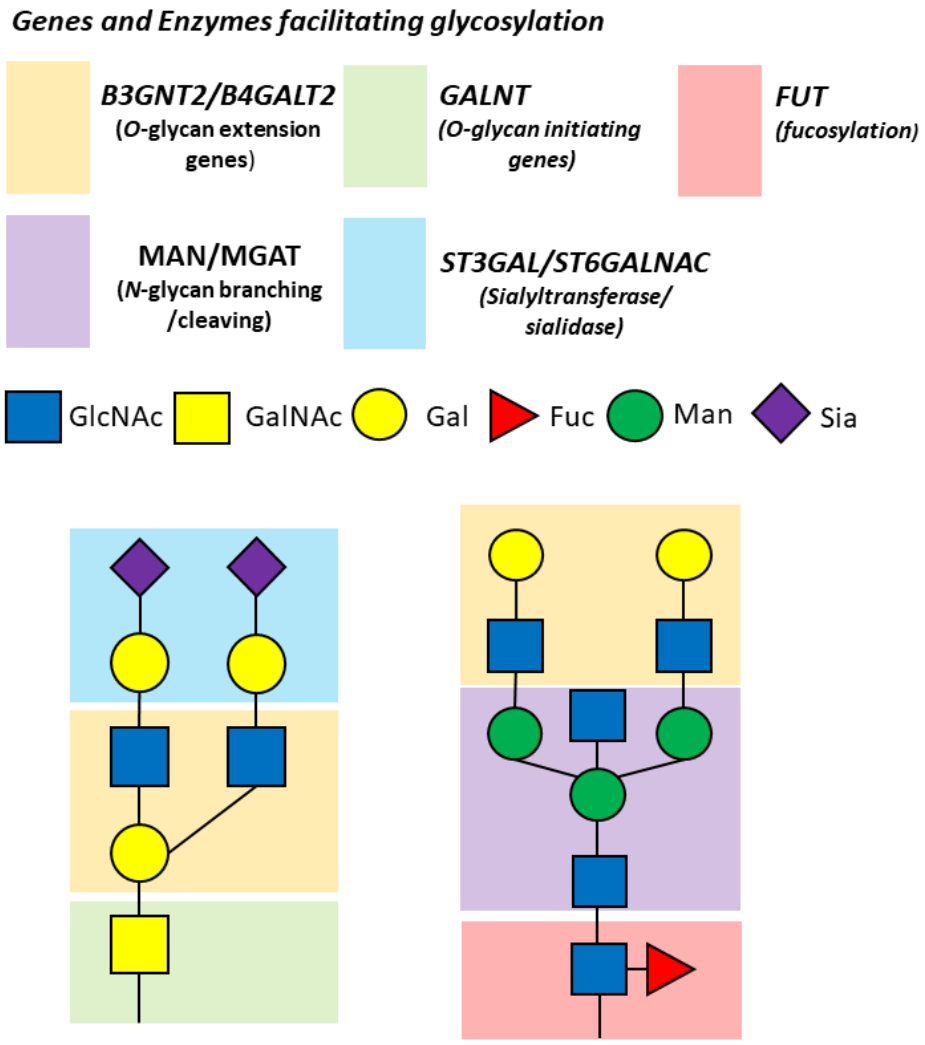

Figure 3.19: Sugar residues transferred to glycan structures by various glycogenes. Colour coding indicates which genes are responsible for the enzymatic addition of sugar residues. GlcNAc: *N*-acetylglucosamine. GalNAc: *N*-acetylgalactosamine. Gal: Galactose. Fuc: Fucose. Man: Mannose. Sia: Sialic acid.

3.5.3.: *O*-glycan initiating gene expression and sialidase is altered in TNBC.

The *GALNT* enzymes and sialidase enzymes are critical for the commencement and decoration of O-glycans (Brochhausen 2009). In this study, upregulation of *GALNT3, GALNT4, GALNT6, GALNT7, GALNT11* and *GALNT14* was observed in TNBC tumours 11004 and 10046 (Figures 3.5 and 3.6) and their upregulation has been identified in other BrCa studies (Potapenko 2010). Similarly, the upregulation of sialidase gene *ST3GAL1* has been noted in this study as well as others (Albequerque 2018).

GALNT14 specifically promotes breast cancer metastasis to the lung through the mediation of metastatic colonies, self-augmentation renewal of breast cancer cells and by overcoming the inhibitory effect of lung-derived bone morphogenetic proteins (Song et al., 2016). As *GALNT14* was upregulated in both tumours analysed *via* array profiling - lung metastasis in the patients from tumours 11004 and 10076 may have been observed. However, patient reports did not detail metastasis other than that of the auxiliary lymph node, so a direct link is unable to be established (Table 2.1.1).

Due to the observed alterations in sialidase genes it is likely that there is altered ST antigen expression on TNBC cells (Figure 3.1) which may contribute to mammary carcinogenesis and promotion of truncated O-glycan structures (Picco et al., 2010). In addition, NEU1, the lysosomal sialidase was upregulated in tumours 11004 and 10046. NEU1 forms a complex with MUC1, which is high glycosylated, and has been shown to facilitate metabolic reprogramming in triple negative breast cancer, most notably affecting glutamine dependency of the cells (Goode et al., 2017). Therefore, upregultion of NEU1 could lead to altered cell signalling, adhesion and facilitate an aggressive tumour phenotype in TNBC.

3.5.4: TNBC and hypoxic MDA-MB-468 do not preferentially express the same HIF-subunit.

Triple negative breast cancer is inherently hypoxic and these hypoxic conditions trigger a particularly aggressive tumour phenotype (Vaupel, 2008; Gregg L. Semenza, 2016). Hypoxia has the potential to significantly alter cell metabolism and gene expression (Semenza, 2012); therefore, it has the potential to significantly alter TNBC tumour glycosylation. In TNBC tumour samples the degree of *HIF1α* and *HIF-2α* induction was assessed by qRT-PCR. *HIF-1α* was preferentially expressed whereas *HIF-2α* exhibited downregulation unanimously in all three TNBC tumours (Figure 3.9). This suggests that in TNBC tumours *HIF-1α* was the key regulator of adaptations to hypoxia and therefore, changes to glycogene expression and glycan structures may have been a result of *HIF-1α.* However, it is important to note that *HIF-1α* and *HIF-2α* may be expressed for short periods of time in response to varying oxygen concentrations, and as a result may have different timeframes (Ratcliffe, 2007). This would suggest that HIF-1α and HIF-2α are perhaps involved at different stages of tumourigenesis.

In this study, hypoxic MDA-MB-468 cells were used to replicate the tumour environment. The cells were exposed to acute hypoxia (1% O_2) for 22 hours and protein expression was analysed by western blotting. Both HIF-1α and HIF-2α protein expression was observed (Figure 3.10), confirming *HIF-1α* and *HIF-2α* pathways are activated by acute hypoxia in TNBC (Koh *et al.*, 2011). Following the induction of acute hypoxia in MDA-MB-468 cells, the expression of *HIF-1α, HIF-2α, CA9, EPO* and *VIM* was analysed by qRT-PCR. Surprisingly, *HIF-1α* and *EPO* expression was downregulated following hypoxia. As *CA9* is a downstream

target of *HIF-1α* (Wykoff *et al.*, 2000), and was upregulated in hypoxic MDA-MB-468 cells, this is indicative of HIF-1α transcription and induction of hypoxia and activation of HIF-1α signalling in these cells (Kaluz *et al.*, 2009). Due to the short-lived transcript of *HIF-1α* and the fact that *HIF-1α* is downregulated following prolonged periods of hypoxia (Huang *et al.*, 1996), it is entirely possible that *HIF-1α* mRNA was present at one point, but was not detected at this time. *HIF-1α* and *HIF-2α* gene expression and protein expression were only analysed following 22 hours of 1% O_2 and therefore, the data here represents one timepoint. Hypoxic exposure for differing lengths of time and oxygen concentrations may have reported alternative levels of HIF induction.

In hypoxic MDA-MB-468, *HIF-2α* was preferentially expressed (Figure 3.15). HIF-1α and HIF-2α drive hypoxia response element (HRE) dependant gene transcription (Wenger, 2002), but have different functions; perhaps due differences in tissue specific and temporal patterns in induction of each HIF-subunit (Rosenberger, 2002; Wiesener *et al.*, 2003). It has been suggested that *HIF-1α* and *HIF-2α* have distinct transcriptional targets and therefore transcriptional responses of either may support a particular physiological adaptation to hypoxia (Rankin *et al.*, 2007). Knockout of *HIF-1α* or *HIF-2α* in mice causes distinctly altered phenotypes, with knockdown of HIF-2α resulting in reduced growth of neuroblastoma tumours in athymic mice (Holmquist-Mengelbier *et al.*, 2006). HIF-1α is the dominant mediator of the acute hypoxic response at 1% O_2, and it known to promote tumour growth, invasion, metastasis and therapy resistance (Lu and Kang, 2010; Semenza, 2012). Whereas HIF-2α is the dominant HIF regulating *VEGF* and other angiogenic factors in cancer (Rankin *et al.*, 2007).

It is unclear as to why *HIF-1α* was predominantly expressed in TNBC tumour tissue whereas *HIF-2α* expression dominated in hypoxic MDA-MB-468 cells. It is possible that acute hypoxia induced (24hrs exposure to 1% O_2) in this study may have favoured the gene expression of *HIF-2α* over *HIF-1α* in MDA-MB-468 cells. Furthermore, hypoxic MDA-MB-468 cells were bathed in high glucose media which provides the necessary nutrients for cellular metabolism however, the TNBC samples were likely poorly perfused due to lack of functioning vasculature; promoting a more hostile cellular environment which may have favoured *HIF-1α* expression.

In a number of tumours including prostate, pancreas, colorectal and clear cell renal carcinoma a switch between HIF-1α and HIF-2α-dependent transcription has been reported and is thought to be due to the hypoxia associated factor (HAF) activity (Koh *et al.*, 2011). HAF is an E3 ubiquitin ligase that binds to and ubiquitinates HIF-1α through a p-VHL independent mechanism, sending HIF-1α for proteasomal degradation (Ohh *et al.*, 2000). This system may have influenced the switch from *HIF-1α* to *HIF-2α* expression in the hypoxic MDA-MB-468 cell line if *HIF-1α* is the initially expressed subunit. Lastly, the tumour variation and grade must be considered regarding the preferential expression of HIF-1α in the TNBC samples. As documented in Table 2.1.1, tumours used in this study were of grade 2 and 3. It is unknown if the induction of hypoxia is altered throughout tumour stages, but it could be suggested that as the tumour growth progressed, a switch in *HIF-2α* to *HIF-1α* induction may have occurred.

3.5.5: Inter-individual tumour variation.

Despite tumours 11004, 10046 and 10076 being classified as TNBC, interindividual variation still exists between these patients. For example, the ethnicities of each women are unknown, and may be a factor in the discrepancies in gene expression identified between each tumour. TNBC is more common in women with African ancestry than women of other ethnic origins (Bauer *et al.*, 2007). Black women with late-stage TNBC present with the poorest 5 year relative survival of only 14% compared to approximately 37% for non-Hispanic white and Hispanic women (Bauer *et al.*, 2007). It could be suggested that tumours 11004 and 10046, which share a high proportion of the altered glycogene expression identified here, could be of the same race and therefore; share common gene aberrations.

In addition, the depth at which the tumour biopsy was taken at for each individual is not established. This factor could influence the degree of hypoxia found within each tumour sample and therefore the induction of HIF and subsequent changes to glycogene expression. This is due to the decrease in oxygen perfusion to each cancer cell at increasing distance from the nearest capillary (Tannock, 2001).

3.5.6: HIFs may drive glycogene expression in TNBC and hypoxic MDA-MD-468 cells.

Through qRT-PCR analysis of TNBC tumour samples, it was identified that *HIF-1*α expression correlated with the expression of glycogenes*: GALNT3, GALNT6, GALNT7, GALNT12, B3GNT2, B4GALT2, B4GALT3*, *NEU1* and *NEU3* (Figures 3.7 to 3.9). In addition to this, the expression of *GALNTL5* was found to correlate

with *HIF-2α* induction in tumours 11004, 10046 and 10076 (Figures 3.7 and 3.9). This suggests a possible correlation between *HIF-1α* and *HIF-2α* expression and glycogene expression in TNBC - which has not been shown before. These trends were also observed in hypoxic MDA-MB-468 cells where the expression of *B4GALT2, GALNTL5 and NEU1* correlated with the induction of *HIF-1α* and *HIF-2α* (though *HIF-2α* was predominantly expressed) (Figures 3.15 and 3.16). Although *HIF-1α* and *HIF-2α* share some target genes, they do have separate functions and target genes (Menrad *et al.*, 2010) so, this was interesting that they mediated expression of shared glycogenes. This could be linked to the different timeframes of *HIF-1α* and *HIF-2α* expression (Ratcliffe, 2007) and that glycosylation is a sequential process that does not occur at one single timepoint (Bieberich, 2014). Changes in glycogene expression may correlate with the induction of *HIF-1α* or *HIF-2α* when they become activated, and subsequently, prevent or promote the expression of selected glycogenes and therefore; glycan structures.

In both TNBC tumour tissue and the hypoxic MDA-MB-468 cells, *B4GALT2* and *NEU1* expression correlated with the predominantly expressed HIF subunit (i.e to HIF-1α in TNBC, but HIF-2α in hypoxic MDA-MB-468 cells) - suggesting that *B4GALT2* and *NEU1* may be mediated by either or both HIFs. Conversely, only *O*-glycan initiating genes *GALNT3, 6, 7,* and *12* expression correlated with *HIF-1α* in the TNBC tumour tissue. Similarly, the glycan-extension enzymes *B3GNT2* and *B4GALT3* and the sialidase *NEU3* also only correlated with *HIF-1α* in the TNBC tumour tissue. This indicates that *GALNT3, 6, 7, 12, B4GALT3 and NEU3* may facilitate traits attributable to the aggressive role of HIF-1α in breast cancer

including: altered metabolism, metastasis and radiotherapy-resistance (Gilkes and Semenza, 2013). This link has not been proposed before in the literature.

GALNTL5 was the only gene which was found to correlate with *HIF-2α* expression in both the tumour tissue and hypoxic MDA-MB-468 cells. This may indicate that *GALNTL5* may play more of a role in tumour angiogenesis, growth or cell proliferation – factors more characteristic to the *HIF-2α* subunit. Currently, there is no evidence linking expression of *GALNTL5* to breast cancer, or TNBC so these findings are novel.

These results taken together indicate that the hypoxic tumour microenvironment may significantly impact on protein glycosylation due to metabolic changes, availability of nucleotide sugars and glycogene expression to alter the glycosylation profile of TNBC cells. In turn, these hypoxia-driven changes to the TNBC glycosylation profile may impact cell-cell interactions, cell adherence, invasion and growth (Varki and Lowe, 2009); thus leading to a highly aggressive tumour phenotype.

3.5.7: Lectin staining affects cell granularity in hypoxic MDA-MB-468 cells.

Altered glycosylation of glycoproteins and glycolipids is a common occurrence in malignant transformation. In some cancers changes to cell surface glycans have been observed and correlate with poor patient prognosis (Dennis, Granovsky and Warren, 1999). This is particularly prevalent with the expression of the STn antigen in breast cancer (Figure 3.1) which is an indicator of resistance to adjuvant chemotherapy (Miles *et al.*, 1994)

Lectins have previously been used to investigate the relationship between membrane *RCA* binding and the metastatic capability of TNBC cells (Zhou *et al.*, 2015) where membrane-positive staining of *RCA* was most observed in late-stage rather than early-stage cancers (Zhou *et al.*, 2015).In this study, hypoxic MDA-MB-468 cells were stained with biotinylated lectins to elucidate changes to lectin-glycan binding upon hypoxic exposure. Analysis of flow-cytometry results indicated that hypoxia increased cell size and cell granularity. After hypoxic exposure, it was evident that the morphology of hypoxic cells was altered. Cells exposed to acute hypoxia appeared to be flat, larger and displayed vacuolization (data not shown). Vacuolization is an indicator of cellular senescence and autophagy and therefore staining with β-galactosidase would serve as a positive indicator of senescence (Chen *et al.*, 2018) while cell autophagy can be detected by LC3 immunostaining (Rosenfeldt *et al.*, 2012). Interestingly, there are many genes involved in the cell cycle and glycolysis that are regulated by HIFs and are associated with cellular senescence (Welford and Giaccia, 2011).

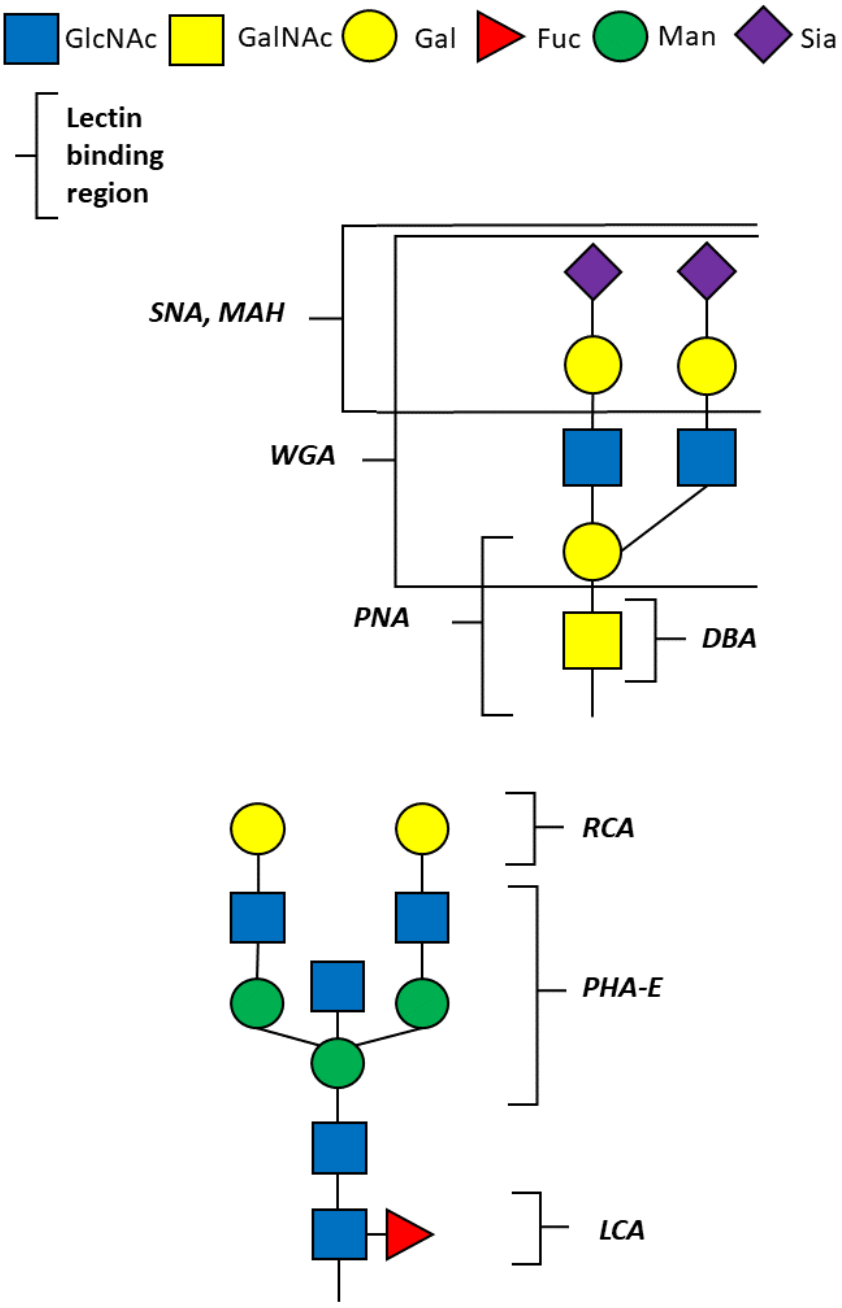

Figure 3.20: Examples of glycan synthesis indicating lectin binding regions. Square brackets indicate lectin binding regions to sugar residues. GlcNAc: *N*-acetylglucosamine. GalNAc: *N*-acetylgalactosamine. Gal: Galactose. Fuc: Fucose. Man: Mannose. Sia: Sialic acid.

3.5.8: Hypoxic MDA-MB-468 cells show differential lectin binding.

GALNT enzymes catalyse the addition of an *N*-acetylgalactosamine (GalNAc) to a serine or threonine residue during *O*-glycan synthesis (Fu *et al.*, 2016). qRT-PCR analysis indicated an increase in expression of *GALNT3* and *GALNTL5*, but a decrease in expression of *GALNT6, GALNT7, GALNT12* and *GALNT14* in hypoxic MDA-MB-468 cells (Figure 3.14). This may account for minimal binding of the lectin *DBA* as it binds to terminal *N*-acetylgalactosamine residues (Figure 3.20) (Ogino *et al.*, 1999). However, it must be noted that *N*-acetylgalactosamine is not usually a terminated monosaccharide and is more typical of a core structural element of *O*-glycans (Mulloy, Hart and Stanley, 2009). Characteristically, *N*-acetylgalactosamine is capped by sialic acid residues (Brooks and Carter, 2001) or is extended by glycosyltransferases: B3GNT2, B4GALT1, 2, and 3 which add galactose residues to GalNAc or GlcNac (Figure 3.19). This may justify the lack of binding of *DBA* to control and hypoxic cells as *N*-acetylgalactosamine residues simply may not exist in high numbers in the terminal form in MDA-MB-468 cells. Furthermore, the decrease in expression of *B4GALT2* and *B4GALT3,* which catalyse the addition of galactose and GlcNAc residues to glycans, may have influenced the lack of binding of *PNA* (which binds to galactose) (Figure 3.20) to hypoxic cells (Mérant *et al.*, 2005).

3.5.9: Hypoxic MDA-MB-468 cells have a high proportion of complex *N*-glycans.

Staining of MDA-MB-468 cells with *PHA-E* showed strong binding in both control and hypoxic cells. *PHA-E* binds to complex *N*-type glycans and specifically has a

high affinity for galactosylated *N*-glycans which bisect with *N*-acetylglucosamine (Figure 3.20) (Nagae *et al.*, 2014). The formation of this type of glycan is catalysed by the MGAT enzymes, most commonly by MGAT3. Unfortunately, the *MGAT* genes were not analysed in MDA-MB-468 cells due to time limitations, however these genes were examined in tumour samples 11004 and 10046. Array profiling indicated that *MGAT3* was upregulated in tumour 11004 compared to its tissue matched control. However, gene expression of *MGAT3* remained unchanged in tumour 10046 possibly because tumour 10046 was only of grade 2 as opposed to the grade 3 tumour 11004 (Table 2.1.1). In order to correlate the high level of binding of *PHA-E* to MDA-MB-468 cells, qRT-PCR analysis of *MGAT3* in both control and hypoxic cells should be investigated. Extrapolating from the *PHA-E* lectin binding observed here, high expression of *MGAT3* in hypoxic cells would be expected upon qRT-PCR analysis.

3.5.10: Downregulated B*4GALT2* and *B4GALT3* gene expression may correlate with decreased *RCA* binding in hypoxic MDA-MB-468 cells.

The lectin *RCA* showed intense binding in both control and hypoxic cells, with a slightly weaker PE-streptavidin signal in hypoxic cells (Figure 3.18). *RCA* binds to reducing terminal β-*D*-galactose residues (Figure 3.20) (Wang *et al.*, 2011) suggesting a decrease in galactose in hypoxic cells. During the formation of type-2 glycan LacNAc (*N*-acetyllactosamine) chains, terminal *N*-acetylglucosamine residues are usually galactosylated (Stanley and Cummings, 2015). This reaction is catalysed by glycan-extension enzymes B4GALT1, 2, 3, 4, 5, and 6 which form *N*-acetyllactosmaine units (Figure 3.19) (Stanley and Cummings, 2015). qRT-PCR analysis of *B4GALT2* and *B4GALT3* in hypoxic MDA-MB-468 cells indicated

a slight decrease in expression which would correlate with the *RCA* binding found here (Figure 3.18). Alternatively, the galactose residue may have been capped by sialic acid, therefore inhibiting *RCA* binding. Cell surface glycan-sialylation has been shown to block the binding of pro-apoptotic galectins in colon cancer; promoting tumour cell survival (Zhuo, Chammas and Bellis, 2008). It could be suggested that a parallel mechanism would occur TNBC cells, having similar effects.

3.5.11: Hypoxia may increase core fucosylated glycans in MDA-MB-468 cells.

The lectin *LCA* has a strong binding affinity to core fucosylated glycans and is a well-documented marker for the diagnosis and prognosis of some cancers (Tateno, Nakamura-Tsuruta and Hirabayashi, 2009). Results of *LCA* staining demonstrated a trend for increased binding to hypoxic MDA-MB-468 cells, suggesting that hypoxia may increase the number of core-fucosylated glycans in MDA-MB-468 cells (Figure 3.18). Core fucosylation is achieved through the enzymatic activity of FUT8 (Yang *et al.*, 2017). Expression of *FUT8* in hypoxic MDA-MB-468 cells was not determined - however, *FUT8* expression was analysed in TNBC tumours 11004 and 10046 where expression was upregulated in both tumours compared to tissue matched controls (Figure 3.7). To accurately determine if the stronger binding of *LCA* to hypoxic cells was a result of upregulation of *FUT8*, control and hypoxic MDA-MB-468 cells should be analysed by qRT-PCR. However, from the lectin-binding analysis results, upregulation of FUT8 in hypoxic MDA-MB-468 cells would be expected. *FUT8* has previously been investigated in breast cancer, where it was shown to increase migratory and

invasive abilities of several breast carcinoma cell lines, including MDA-MB-231; this was determined through Transwell assay with and without Matrigel coating (Tu *et al.*, 2017). With consideration to the increase in core fucosylated glycans observed here, the invasiveness of TNBC cells may be augmented.

3.5.12: Sialylation is decreased in Hypoxic MDA-MB-468 cells.

WGA has an affinity for *N*-acetylglucosamine and sialic acid residues (N-acetylneuraminic) (Goldstein, Winter and Poretz, 1997) (Figure 3.18). qRT-PCR analysis of the sialic acid cleaving neuraminidases *NEU1* and *NEU3* (Juge, Tailford and Owen, 2016) in MDA-MB-468 cells, found that these genes were not upregulated by hypoxia (Figure 3.14). *WGA* binding to hypoxic MDA-MB-468 cells was less intense than in control cells, suggesting that there are a lower proportion of sialic acid residues in hypoxic cells. This does not correlate with the general downregulation of sialidase enzymes *NEU1* and *NEU3*, as we would expect there to be a larger proportion of sialic acid residues in hypoxic cells, due to a lack of cleavage, and therefore an increase in *WGA* binding. As *WGA* has affinities to galactose and GlcNAc residues and does not exclusively bind to sialic acid residues (Cummings and Etzler, 2009) (Figure 3.20); further assessment of the degree of sialyation of hypoxic MDA-MB-468 cells should be conducted with lectins *SNA* and *MAH*, which are more specific (Shibuya *et al.*, 1987; Wang and Cummings, 1988). However, increased WGA binding may be observed at other timepoints and lengths of hypoxic exposure.

3.5.13: Conclusions and future work.

The work undertaken in this study aimed to elucidate changes to the glycosylation profile of TNBC, as a result of altered expression of glycosylation genes. In doing so, a greater understanding of the cellular glycome of TNBC has been gathered. Here, identification of changed glycogene expression has been illuminated in TNBC and will aid in the identification of disease biomarkers for better treatment options. Understanding the mechanisms involved in altered glycogene expression in TNBC and how this affects cell surface glycans is crucial. The results found here provides evidence that TNBC and hypoxic MDA-MB-468 cells do in fact show differentially expressed glycogenes and that hypoxia potentially influences the binding of some lectins to cell surface glycans. This indicates that altered glycogene expression due to hypoxia causes aberrant glycosylation and provokes a change to the cellular glycocalyx in TNBC.

This study has uncovered that TNBC preferentially expresses *HIF-1α* whereas hypoxic MDA-MB-468 cells mainly express *HIF-2α.* A correlation between glycogene expression and the induction of *HIF-1α* and *HIF-2α* was found, where the expression of glycogenes from the *O*-glycan initiating, glycan-extension and sialidase family correlate with *HIF-1α* in TNBC or with *HIF-2α* in hypoxic MDA-MB-468 cells.

Future work should include the validation of several other genes in both TNBC samples 11004, 10046 and 10076, depending on the remaining resources, and hypoxic MDA-MB-468 cells. These genes including *MGAT3, MGAT5, ST3GAL1* and *FUT8,* for example. These aforementioned genes play crucial roles in synthesis of galactosylated *N*-glycans, maturation of *N*-glycans, the expression

of the ST antigen (Figure 3.1) on TNBC cells and core fucosylation of *N*-glycans. These findings would add the growing body of evidence presented here and establish their importance in the altered glycosylation profile of TNBC.

In addition, it would advantageous to gauge changes to cell glycosylation at different exposure timepoints to hypoxia including 2, 4, 8 and 48 hours. This will allow the mapping of changes in glycogene expression with increasing hypoxic exposure and degree of HIF induction. Furthermore, it may be of interest to modulate the degree of hypoxia by selecting various gas mixtures including <0.01% and 5% oxygen to replicate anoxic tumour tissue and adjacent tumour tissues (Favaro *et al.*, 2011) and the effect on glycogene expression.

Chapter 4: The influence of hypoxia inducible factors on glycogene expression in hypoxic TNBC MDA-MB-468 cells.

4.1: Introduction.

4.1.1: Oxygenation of tumour tissue.

In rapidly proliferating and expanding tumours, oxygen demand increases and surpasses supply, resulting in an progressively hypoxic milieu (Semenza, 2000). In breast tissue, the normal tissue oxygen concentration is approximately 8% however, the oxygen concentration of breast cancer tissue is dramatically reduced to approximately 1.5% (Vaupel, Höckel and Mayer, 2007). Adaptation to hypoxia drives the upregulation of angiogenic factors (*VEGF*) from hypoxic areas of the tumour which promotes vascularisation (Semenza, 2012). However, these new blood vessels are poorly formed and are prone to leakage and collapse causing local tissue oedema. Consequently, tumours are filled with areas of mild to severe hypoxia and necrosis (Semenza, 2012).

4.1.2: Hypoxia inducible factors.

Hypoxia inducible factors (HIFs) are master regulators of oxygen homeostasis and play a critical role in hypoxic adaptation (Semenza, 2000). There are three HIF-α subunits: HIF-1α, HIF-2α and the lesser documented HIF-3α (Hu *et al.*, 2003). Regulation of HIF activity occurs on multiple levels (Semenza, 2000). Under normal oxygen concentrations, hypoxia inducible factor-α (HIF-α) is bound

tightly by the von Hippel-Lindau (VHL) protein which facilitates ubiquitination of HIF-α and its subsequent proteasomal degradation (See Figure 1.5) (Kaelin and Ratcliffe, 2008). Binding of VHL is dependent upon the hydroxylation of proline residues in HIF-α by the prolyl hydroxylases (PHD) (Epstein *et al.*, 2001). Three HIF prolyl hydrolases have been identified (PHD1-3), all three isoforms operate in a non-redundant manner to the regulation of both HIF-1α and HIF-2α and contribution of each isoform is dependent on the abundance of the enzyme (Appelhoff *et al.*, 2004).

This HIF-α/VHL complex uses oxygen and α-ketoglutarate as a substrate and therefore, its activity is inhibited under hypoxic conditions (Epstein *et al.*, 2001). Henceforth, under hypoxic conditions, hydroxylation and ubiquitination of HIF does not occur and the subunit becomes stabilised for transcription of target genes (See Figure 1.5). HIF-1α and HIF-2α share a common structures and target genes such as the angiogenic factor, *VEGF* (Keith, Johnson and Simon, 2011) (Figure 4.1).

4.1.3: HIF-1α and HIF-2α interplay.

In breast cancer cell lines, HIF-1α and HIF-2α are differentially expressed however, both proteins regulate arms of the hypoxic pathway (Blancher *et al.*, 2000) (Figure 4.1). HIF-1α plays a crucial role in tumour adaptation to changes in O_2 concentration through the transcriptional regulation of over 100 downstream genes (Semenza, 2001). Genes involved in glucose metabolism (*GLUT1 and GLUT3*) and pH maintenance (*CA9*) are all regulated by HIF-1α. HIF-2α activation of target genes promotes growth and metastasis in later stages of BrCa

and is associated with a more aggressive BrCa phenotype (Holmquist-Mengelbier *et al.*, 2006). HIF-2α promotes the expression of transcription of target genes including vimentin, which is associated with poor prognosis (Hemalatha, Suresh and Harendra Kumar, 2013) (Figure 4.1).

EPO is less selective with regards to its transcriptional upregulation, with reports of regulation by both HIF-1α and HIF-2α (Semenza and Wang, 1992; Rankin *et al.*, 2007). However, *EPO* is thought to be dominantly dependent on HIF-2α rather than HIF-1α (Rankin *et al.*, 2007) (Figure 4.1). Furthermore, the angiogenic factor *VEGF* is under the transcriptional control of both HIF-1α an HIF-2α which aids in adaptation to hypoxia *via* the formation of new blood vessels (Shweiki *et al.*, 1992).

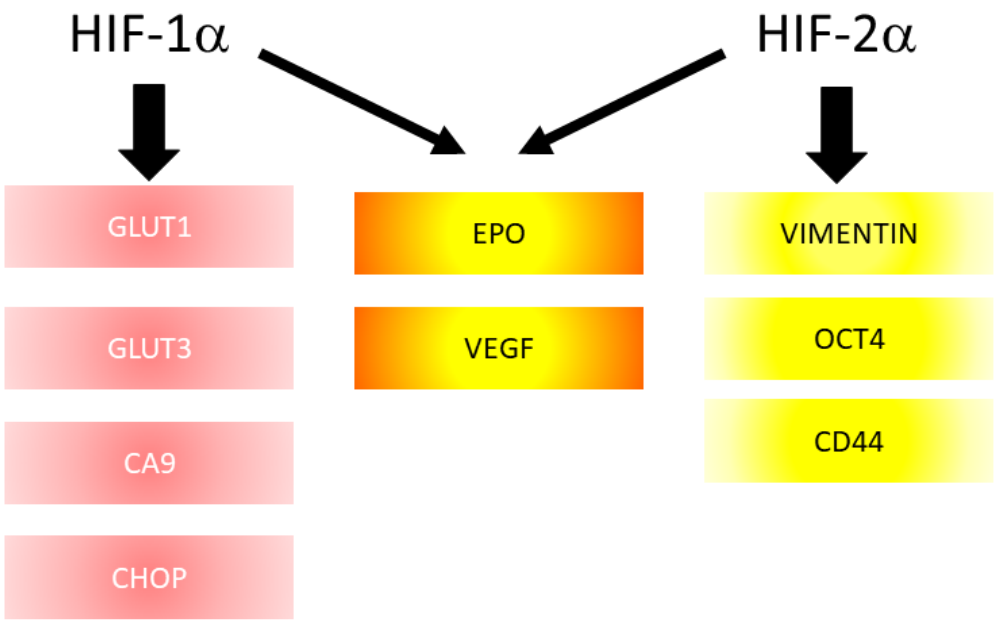

Figure 4.1: HIF-1α and HIF-2α regulate distinct batteries of target genes. Downstream regulation of target genes by HIF-1α and HIF-2α under hypoxia. HIF-1α and HIF-2α share regulation of several genes and also control the regulation of distinct subsets of target genes (Hu *et al.*, 2003; Koh and Powis, 2012). Figure adapted from (Martín-Aragón Baudel *et al.*, 2017).

4.1.4: Hypoxic regulation of glycosylation.

The mechanism through which hypoxic tumour microenvironments alter cell glycosylation patterns is not clear. HIF-1α controls the transcription of batteries of genes which aid in the adaptation to hypoxic stress including the altered metabolism which is observed in breast cancer (López-Barneo, Pardal and Ortega-Sáenz, 2001). Energy metabolism is one of the many alterations found in breast cancer, due to *HIF-1α* expression (Semenza, 2010). In growing tumour tissue, HIF-1α facilitates the shift from oxidative phosphorylation to aerobic

glycolysis in order to maintain energy production (aka Warburg effect) (Warburg *et al.*, 1956). As a result, hypoxic cells metabolise more glucose in order to meet their energy needs. HIF-1α mediates this metabolic conversion partly through the expression of glucose transporters GLUT1 and GLUT3 (Denko, 2008) as well up the upregulation of glycolytic enzymes. Therefore, expression of glucose transporters, glycolytic enzymes is altered causing a dramatic shift in the cell metabolism (Shirato *et al.*, 2011). In addition, altered glucose metabolism impacts some glycosylation genes, nucleotide sugars and transporters (Shirato *et al.*, 2011). An excess or surplus of nucleotide sugars can result in altered glycosylation due to their importance in glycan synthesis (Taniguchi, 2007).

It remains unknown if there is a link between the expression of the transcription factors *HIF-1*α and *HIF-2*α, and the regulation of glycosylation. In Chapter 3, *HIF-1*α expression correlate with expression of several glycogenes from the *O*-glycan-initiating, glycan extension and sialidase families in TNBC. Furthermore, expression of *GALNTL5* was found to correlate with *HIF-2*α induction in TNBC. In hypoxic MDA-MB-468 cells *B4GALT2, GALNTL5 and NEU1* expression correlated with induction of *HIF-1*α and *HIF-2*α. This suggests a relationship between *HIF-1*α and *HIF-2*α and glycogene expression in hypoxic TNBC – which has not previously been investigated.

In this chapter, the role of *HIF-1*α and *HIF-2*α in regulating glycogene expression was investigate by suppressed using siRNAs in hypoxic TNBC MDA-MB-468 cells. Attenuation of HIF protein expression was determined by immunoblotting and the relative expression of *HIF-1, HIF-2*α, their respective target genes: *CA9* and *EPO* and glycogenes *GALNT3, 6, 7, 12, 14, L5, B3GNT2, B4GALT2-3, NEU1* and *NEU3* were analysed by qRT-PCR.

4.2: Aims

Aim 1: To suppress the expression of hypoxia inducible factors, HIF-1α and HIF-2α in hypoxic MDA-MB-468 cells using siRNA and assess the impact of suppression on glycogene expression.

4.3: Research Questions

RQ1: Is glycogene expression in hypoxic TNBC correlated with specific arms of the hypoxic adaptation machinery?

RQ2: Does the knockdown of HIF-1α or HIF-2α in hypoxic MDA-MB-468 cells alter the expression of *O*-glycan initiating, glycan-extension and sialidases gene families?

4.4: Results

4.4.1: Optimising transfection conditions for HIF-1α and HIF-2α siRNA knockdown

In order to investigate the trend in expression between *HIF-1α, HIF-2α* and a selection of glycogenes, targeted siRNAs were used to knockdown *HIF-1α, HIF-2α* mRNA expression in hypoxic MDA-MB-468 cells. Published siRNA sequences, concentrations and knockdown conditions were employed (Pawlus *et al.*, 2012; Pawlus, Wang and Hu, 2014).

The lipid transfection reagent used in this study has been show to suppress HIF-1α and HIF-2α in TNBC cells lines, including MDA-MB-231 cells, without cell toxicity (Li *et al.*, 2011; Pawlus *et al.*, 2012). It was important to confirm this was the case in this model and optimise the volume of transfection reagent used to achieve maximum gene knockdown efficiency (Montoya and Azorsa, 2016). Cells were therefore treated with 6 µL HiPerfect Transfection reagent and exposed to acute (1% O_2) hypoxia for 22 hours and HIF1/2 expression was assessed by immunoblotting. Immunoblotting confirmed that 6µL of HiPerfect transfection reagent did not cause adverse cell death or affect stabilisation of HIF-1α, HIF-2α expression in hypoxic MDA-MB-468 cells (Figure 4.2) and therefore, would be acceptable to use for further experiments.

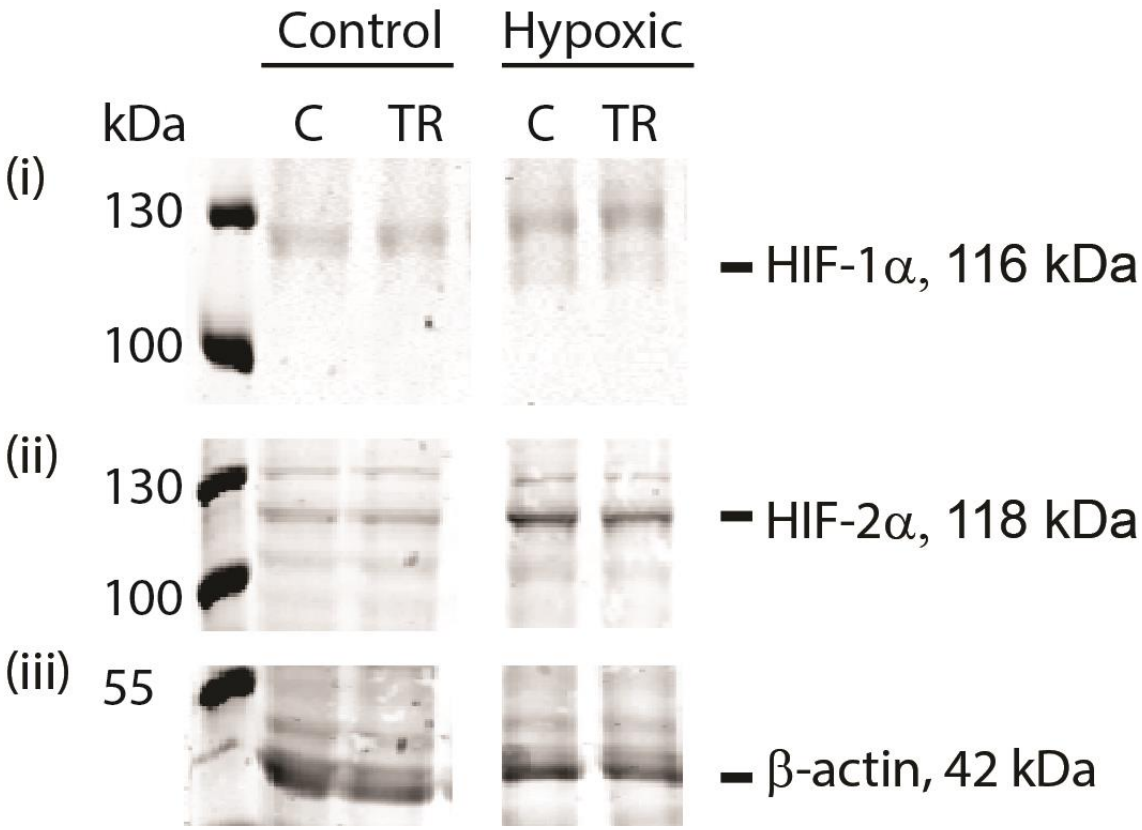

Figure 4.2: Transfection reagent does not prevent HIF stabilisation in hypoxic MDA-MB-468 cells. Representative Immunoblots (N=4 biological replicates) of HIF-1α and HIF-2α protein expression following 22 hours of acute hypoxia (1% O_2) and addition of transfection reagent. Protein (30 µg) was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with anti-HIF-1α **(i)** and anti-HIF-2α **(ii),** anti-β-actin and IR conjugated secondary antibodies. Immunoblotting with β-actin was used to assess equal protein loading **(iii)**. Staining was visualised by LI-COR. C: Control. TR: Transfection Reagent.

4.4.2: Optimising siRNA concentration conditions for HIF-1α and HIF-2α knockdown.

siRNAs towards HIF-1α and HIF-2α has been shown to create effective knockdown of HIF at 10 nM (Pawlus, Wang and Hu, 2014). Here MDA-MB-468 TNBC cells were treated with 10nM siRNA towards either HIF-1α or HIF-2α then exposed to acute hypoxia (1% O_2, for 22 hours). siRNA knockdown, and specificity of knockdown, was determined by immunoblotting for HIF-1α and HIF-2α (Figure 4.3). Optimisation was required because the siRNAs used by others were in MDA-MB-231 cells and therefore, it was unknown is the concentrations used by others would cause sufficient knockdown here (Pawlus, Wang and Hu, 2014). It was important to determine the siRNA specificity in order to attribute each treatment to its effect on glycogene expression, which was later determined.

Following establishment of transfection conditions, knockdown was examined by immunoblotting. Control reactions included normoxic cells which did not show expression of HIF proteins and hypoxic cells without siRNAs treatment which HIF-1α and HIF-2α were expressed, as expected. A non-targeting scrambled control siRNA was implemented which did not supress the expression of either HIF-1α or HIF-2α in the hypoxic MDA-MB-468 cells. Finally, a transfection reagent only control confirmed that the transfection reagent itself did not affect protein expression or induce cell toxicity (Figure 4.3).

HIF-1α showed weak suppression with 10nM HIF-1α siRNA treatment, however complete specificity could not be determined as the protein was not entirely supressed (Figure 4.3). HIF-2α protein expression remained intact with 10nM HIF-2α siRNA treatment prompting further optimisation.

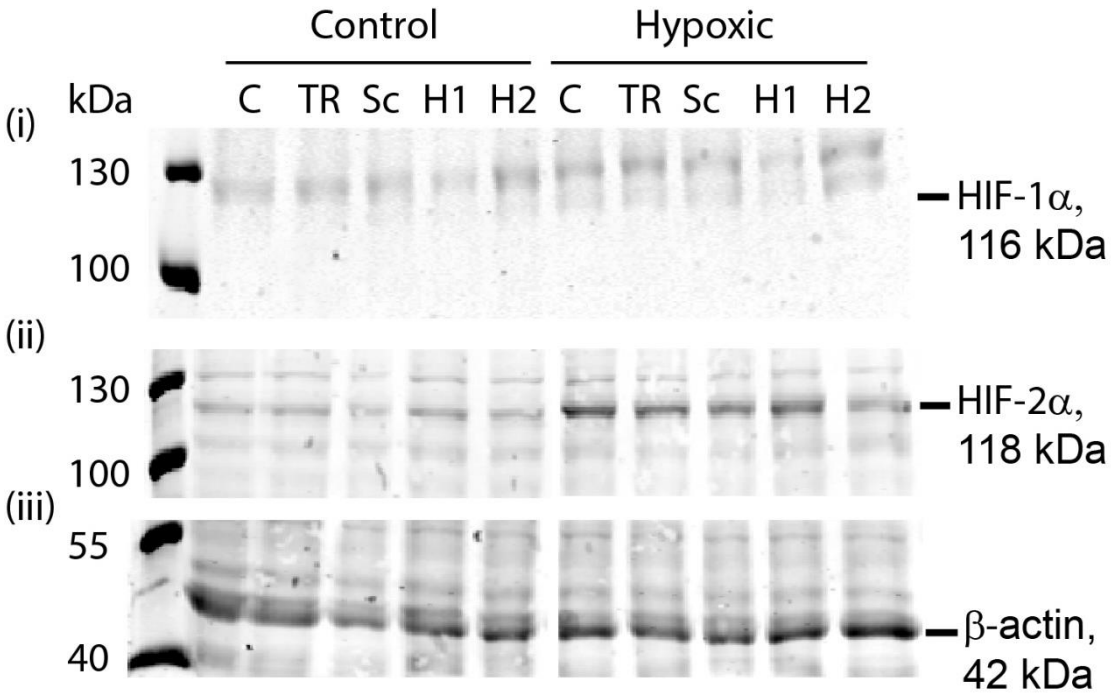

Figure 4.3: Assessing HIF-1α and HIF-2α protein expression following siRNA knockdown in hypoxic MDA-MB-468 cells. Representative immunoblots (n=2 biological replicates) of **(i)** HIF-1α and **(ii)** HIF-2α protein expression after *HIF-1α* and *HIF-2α* knockdown with 10nM siRNAs following exposure to 22 hours of acute hypoxia (1% O_2). Protein (30 µg) was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with anti-HIF-1α and anti-HIF-2α, anti-β-actin and IR conjugated secondary antibodies. Immunoblotting with β-actin was used to assess equal protein loading **(iii)**. Staining was visualised by LI-COR. C: Control. TR: Transfection Reagent. Sc: Scrambled non-targeting siRNAs. H1: HIF-1α siRNA. H2: HIF-2α siRNA.

The concentration of siRNAs used was increased 25nM and 50nM to cover a range of concentrations. siRNAs at a the highest concentration of 50nM were used as this proved sufficient in the suppression of HIF-1α in MDA-MB-231 breast cancer cells (Soleymani Abyaneh *et al.*, 2017).

HIF-1α protein expression showed a 3.3-fold knockdown with 10nM HIF-1α siRNA treatment whilst with 25nM siRNAs, HIF-1α protein expression was suppressed by 3.4-fold compared to the loading control. Treatment with 50nM HIF-1α siRNAs resulted in a 3.7-fold reduction in HIF-1α protein expression (Figure 4.4). HIF-2α protein expression was reduced by 1.3-fold with 10nM siRNAs which increased to a 3.9-fold reduction with 25nM siRNA treatment. The use of 50nM siRNAs on HIF-2α only produced a 2-fold reduction in the protein expression of HIF-2α, compared to the loading control (Figure 4.4). This prompted further optimisation including an increase in amount of transfection reagent used from 6 µL to 12 µL.

During siRNA optimisation, increasing the volume of transfection reagent promoted adequate HIF-2α protein knockdown in the presence of 50 nM siRNAs and expression was suppressed by 1.9-fold compared to the loading control (figure 4.5). Surprisingly an increase in HIF-1α protein expression was observed with HIF-2α siRNAs (Figure 4.5). The β-actin loading control showed that protein was loaded equally meaning protein expression of HIF-1α and HIF-2α was comparable. Furthermore, complete suppression of HIF-1α with 50nM HIF-1α siRNA treatment was observed here (Figure 4.5).

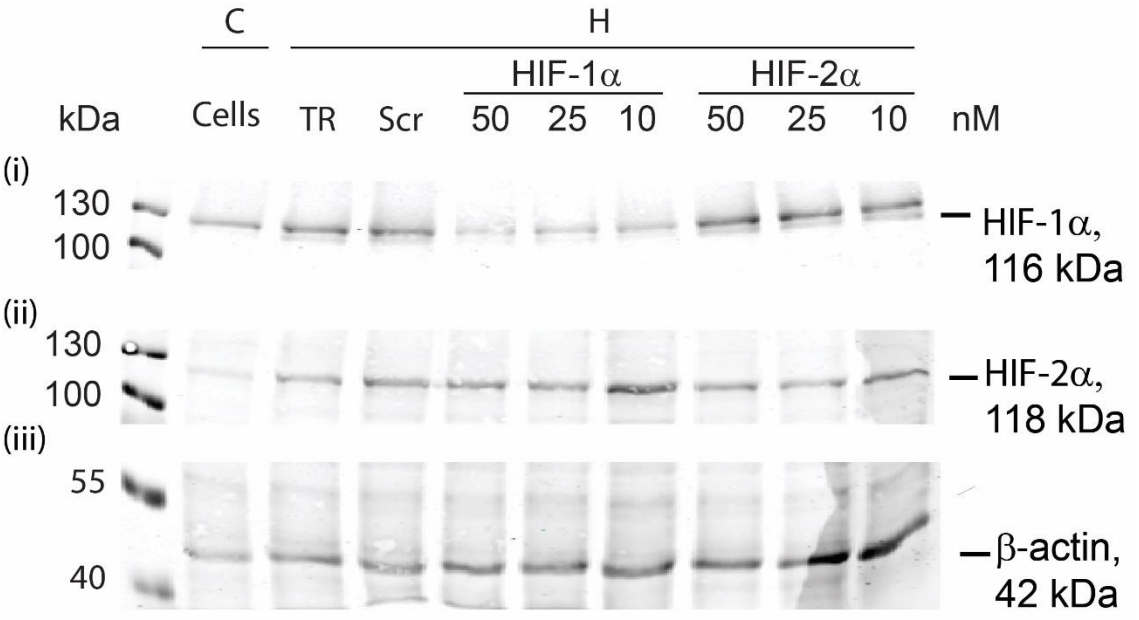

Figure 4.4: Optimising siRNA treatment in hypoxic MDA-MB-468 cells. Immunoblots of **(i)** HIF-1α and **(ii)** HIF-2α protein expression after HIF-1α and HIF-2α knockdown with siRNAs (10nM, 25nM, 50nM) following exposure acute hypoxia (1% O_2) where n=1. Protein (30µg) was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with anti-HIF-1α and anti-HIF-2α, anti-β-actin and IR conjugated secondary antibodies. Immunoblotting with β-actin was used to assess equal protein loading **(iii)**. Staining was visualised by LI-COR and densitometry was analysed with ImageJ software. C: Control. TR: Transfection Reagent. Scr: Scrambled non-targeting siRNAs.

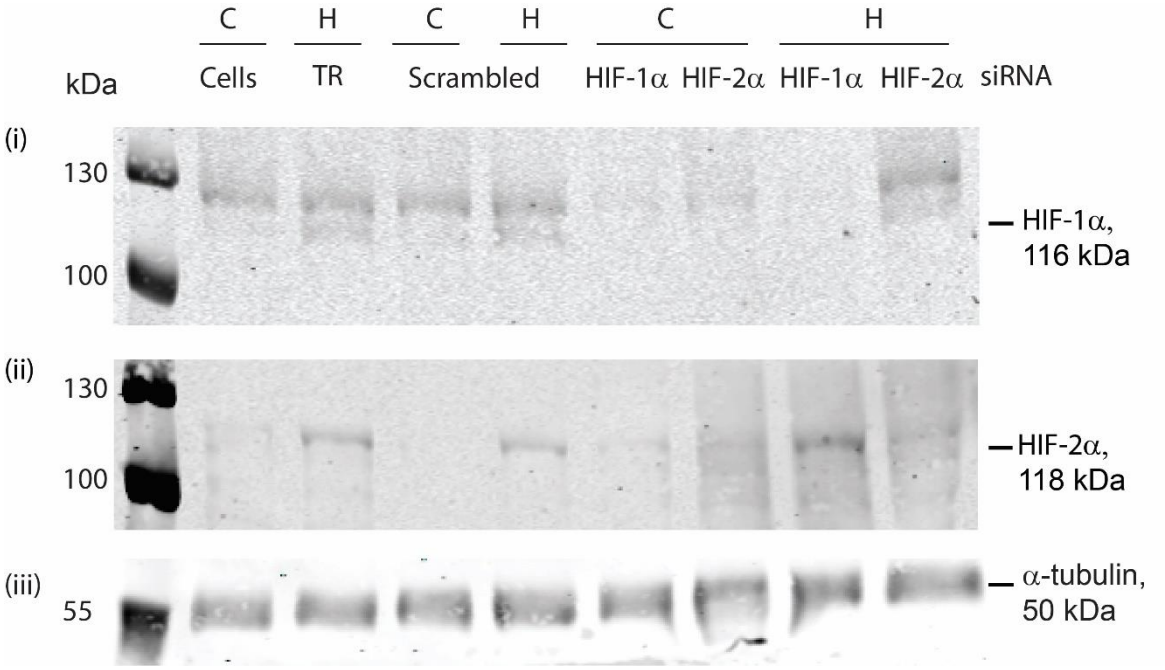

Figure 4.5: Analysis of HIF-1α and HIF-2α protein expression in hypoxic MDA-MB-468 cells following 50nM siRNA treatment. Immunoblots of **(i)** HIF-1α and **(ii)** HIF-2α protein expression after HIF-1α and HIF-2α knockdown with 50nM siRNAs following 22 hours exposure acute hypoxia (1% O_2) (representative of n=1 immunoblots). Protein (30µg) was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with anti-HIF-1α and anti-HIF-2α, anti-α-tubulin and IR conjugated secondary antibodies. Immunoblotting with α-tubulin was used to assess equal protein loading **(iii)**. Staining was visualised by LI-COR. C: Control. H: Hypoxic. TR: Transfection Reagent.

4.4.3: qRT-PCR optimisation following HIF-1α and HIF-2α knockdown.

qRT-PCR was used in order to assess the impact of HIF-1α and HIF-2α suppression on glycogene expression in hypoxic MDA-MB-468 cells. Gene expression of housekeeping genes β-actin, *β2M* and *RPLP0* were measured by qRT-PCR following 22 hours of acute hypoxia (1% O_2). This indicated the most

stable reference gene for qRT-PCR normalisation (Figure 4.6). Housekeeper genes were reanalysed in this section to account for any impact on gene expression following siRNA targeting of HIF-1α and HIF-2α. Statistical analysis indicated that between control and hypoxic cells, *β2M* was the most stable gene, with an average difference in C_T values between control and hypoxic cells of 0.1 ± 1.44. This is opposed to the average difference between control and hypoxic C_T values in β-actin (0.59 ± 0.83) and *RPLP0* (0.23 ± 1.38) (Figure 4.6). Therefore, all gene expression in siRNA treated cells was normalised to *β2M*.

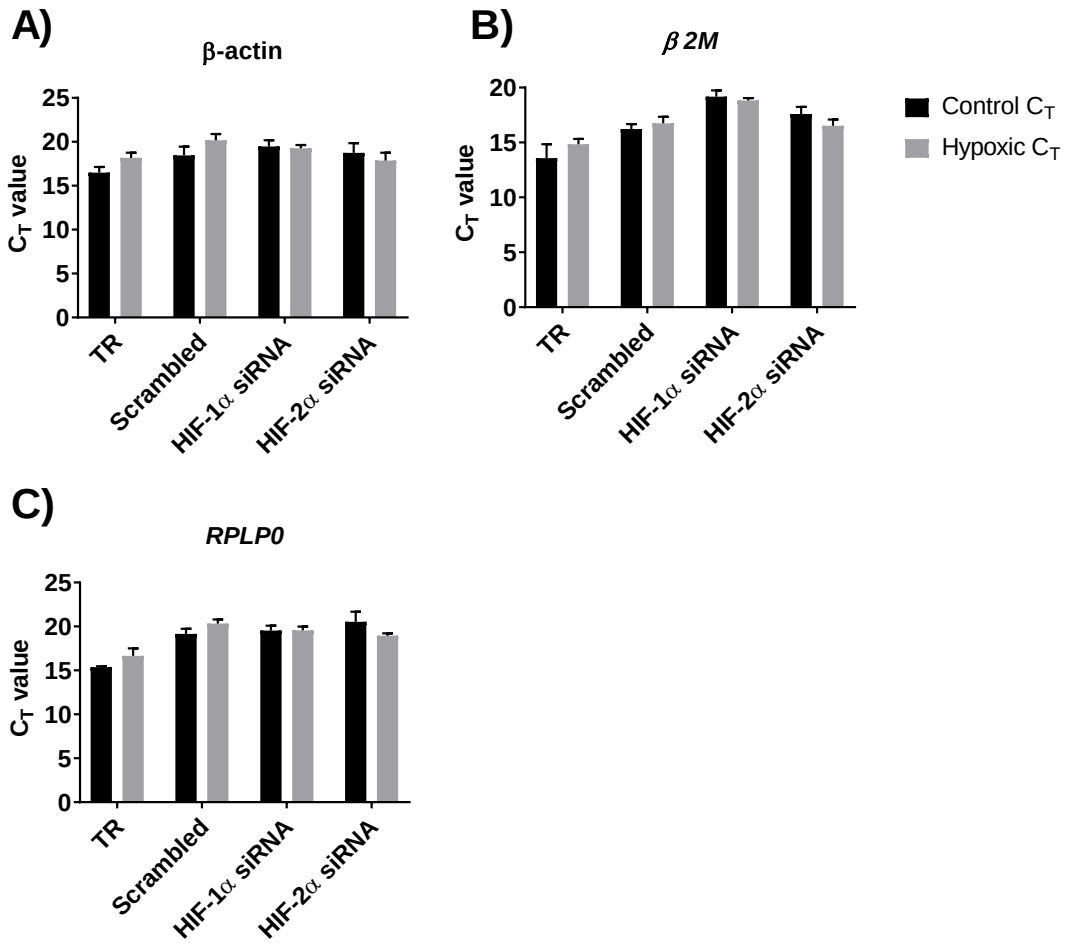

Figure 4.6: Assessing the stability of housekeeper genes in MDA-MB-468 cells following siRNA targeting of HIF-1α and HIF-2α. Cells were transfected with 50nM scrambled siRNA, HIF-1α siRNA, HIF-2α siRNA or supplemented with transfection reagent (TR) for a total of 46 hours. Cells were exposed to acute hypoxia (1% O2) for 22 hours and harvested on ice. C_T values of housekeeping genes β-actin, *β2M* and *RPLP0* were determined by qRT-PCR and indicated the stability of each gene when subject to different siRNA and oxygen treatments (n=3 biological replicates).

4.4.4: HIF-1α siRNAs inhibit *HIF-1α* mRNA expression.

Following transfection with HIF-1α and HIF-2α siRNAs in hypoxic MDA-MB-468 cells, qRT-PCR was used to establish the effects of HIF-1α and HIF-2α siRNA treatment on the mRNA expression of *HIF-1α, HIF-2α* and their respective downstream targets *CA9* and *EPO* (Figure 4.7A).

A reduction in HIF-1α and HIF-2α mRNA expression was observed when cells were treated with a non-targeting scrambled siRNA compared to untreated cells, but this was not significant (p=0.1688 and 0.8803 respectively). Analysis indicated that *HIF-1α* expression was significantly reduced with HIF-1α siRNA treatment (*p*=0.0005) (Figure 4.7A). Although HIF-1α siRNAs did successfully reduce the mRNA expression of HIF-1α, it may not have been specific. This is due to observation that HIF-2α expression was also significantly (*p*<0.0001) suppressed with HIF-1α siRNAs (Figure 4.7A).

Surprisingly, HIF-2α siRNAs did not supress the expression of *HIF-2α* mRNA (*p*=0.9909) but did significantly reduce *HIF-1α* mRNA (*p*<0.0001) – indicating that the published HIF-2α siRNAs may not have been entirely selective to HIF-2α mRNA (Figure 4.7A). The HIF-2α siRNAs used in this study have previously been used to suppress HIF-2α expression in normoxic clear cell renal cell carcinoma cells, and have demonstrated specificity to HIF-2α, however, HIF-2α siRNA treatment was not performed on a TNBC cell line (Pawlus, Wang and Hu, 2014). Furthermore, it is important to note that mRNA stability and protein turnover are different in both transcription factors and therefore, it is likely that the molecules are controlled in other manners (Marxsen *et al.*, 2004; Galbán and

Gorospe, 2009). This may account for the selective suppression of the HIF proteins but the non-selective nature of the siRNA molecules on mRNA expression.

CA9 contains a single HRE element that is essential for its transcriptional increase during hypoxia and it is one the best characterised targets of HIF-1α (Pastorekova, Parkkila and Zavada, 2006). Evidence in the literature is not present to indicate CA9 is under the transcriptional regulation of HIF-2α. As indicated in figure 4.7A, *CA9* was knocked down, yet whilst marked it was not statistically significant (2-way ANOVA with Tukey's multiple comparisons) with HIF-1α siRNAs (p=0.5058), and unexpectedly, was significantly downregulated by HIF-2α siRNAs (*p*=0.0148). This may indicate that HIF-2α siRNAs were not exactly specific to HIF-2α, as HIF2 siRNA treatment also impacted on HIF-1α target gene expression. *EPO* is less selective with regards to its transcriptional upregulation, with reports of regulation by both HIF-1α and HIF-2α (Semenza and Wang, 1992; Rankin *et al.*, 2007). However, *EPO* is thought to be dominantly dependent on HIF-2α rather than HIF-1α (Rankin *et al.*, 2007). This correlates with the data found here which indicates a significant reduction in *EPO* expression (*p*<0.0001) when treated with HIF-2α siRNAs (Figure 4.7A).

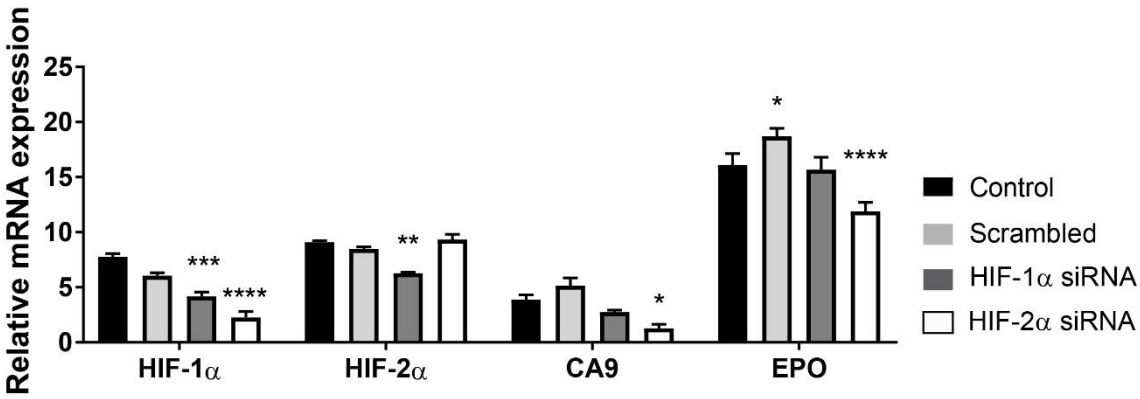

Figure 4.7A: Specificity of siRNA treatment on HIF-1α, HIF-2α and downstream target gene expression. The relative mRNA expression of *HIF-1α, HIF-2α* and their respective downstream targets: *CA9 and EPO following* transfection with non-targeting scrambled, HIF-1α or HIF-2α siRNAs. qRT-PCR values are shown as mean ± SEM (n=3 biological replicates) and were normalised to the housekeeper gene *β2M* and was analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

4.4.5: *O*-glycan initiating genes *GALNT6* and *GALNT12* may be regulated by HIF-1α in hypoxic MDA-MB-468 cells.

In Chapter 3, HIF-1α and HIF-2α expression correlated with *O*-glycan initiating, glycan extension and sialidase genes. To establish this link, expression of the selected glycogenes in hypoxic MDA-MB-468 cells treated with HIF-1α and HIF-2α siRNA and measured by qRT-PCR.

Unexpectedly, the mRNA expression of O-glycan initiating genes: *GALNT3, GALNT7* and *GALNT14* was significantly altered by non-targeting scrambled siRNAs (p=0.0164, 0.0021 and <0.0001, respectively) (Figure 4.7B). As a result,

the significant reduction in expression of *GALNT3 and GALNT14* following HIF-1α siRNA treatment was unable to be attenuated directly due to HIF-1α suppression (Figure 4.7B).

Knockdown of HIF-1α did however, result in a significant reduction in *GALNT6* (p=0.0007) and *GALNT12* expression (p=0.0269) which were unaffected by the scrambled control. This suggests that *GALNT6* and *GALNT12* expression may be regulated by HIF-1α. Knockdown of HIF-2α did not alter the expression of any of the GALNT *O*-glycan initiating genes tested and expression was comparable to the control cells (Figure 4.7B). *GALNT14* did show significantly suppression with HIF-2α siRNAs (p=0.0430), however expression was also significantly suppressed by the scrambled control (p<0.0001).

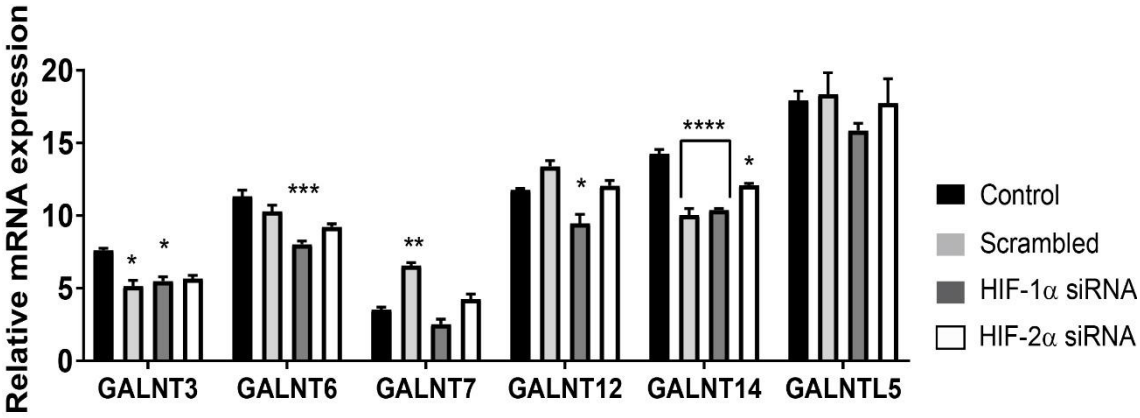

Figure 4.7B: Analysis of *O*-glycan initiating gene expression following siRNA suppression of HIF-1α and HIF-2α. The relative mRNA expression of *O*-glycan initiating genes: *GALNT3, 6, 7, 12, 14* and *L5* following transfection with non-targeting scrambled, HIF-1α or HIF-2α siRNAs. qRT-PCR values are shown as mean ± SEM (n=3 biological replicates) and were normalised to the housekeeper gene *β2M* and was analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

4.4.6: Expression of glycan extension genes B3GNT2 and B4GALT3 are significantly altered by HIF-1α suppression.

The mRNA expression of glycan-extension genes: *B3GNT2, B4GALT2, B4GALT3* and sialidase genes *NEU1* and *NEU3* were analysed by qRT-PCR in hypoxic MDA-MB-468 cells. This was in order to determine the effects of HIF-1α and HIF-2α suppression on varying arms of the glycosylation process.

Unexpectedly, the non-targeting scrambled siRNA treatment significantly suppressed the expression of *B4GALT2, NEU1* and *NEU3* in hypoxic cells; indicating that the siRNA transfection process itself caused altered glycogene expression (Figure 4.7C). However, the expression of *B3GNT2* and *B4GALT3* was unaffected by the scrambled siRNAs suggesting that the results presented here are valid.

Suppression of HIF-1α resulted in a significant reduction in expression of glycan extension genes *B3GNT2* (p=0.0388) and *B4GALT3* (p=0.0044). A reduction in the relative expression of *B4GALT2* was also observed compared to control cells, however due to the significant reduction in mRNA expression caused by the scrambled control, it cannot be confirmed that this suppression of *B4GALT2* was entirely due to HIF-1α siRNA treatment. HIF-2α suppression did not significantly alter the expression of *B3GNT2, B4GALT2* or *B4GALT3.*

qRT-PCR analysis of sialidase genes *NEU1* and *NEU3* indicated that HIF-1α suppression significantly reduced the expression of *NEU3* (p=0.0004). This was also observed with HIF-2α siRNA treatment (p=0.0225). However, due to the

effects of the scrambled control, this cannot be reported as exclusively the result of HIF-1α or HIF-2α suppression (Figure 4.7C).

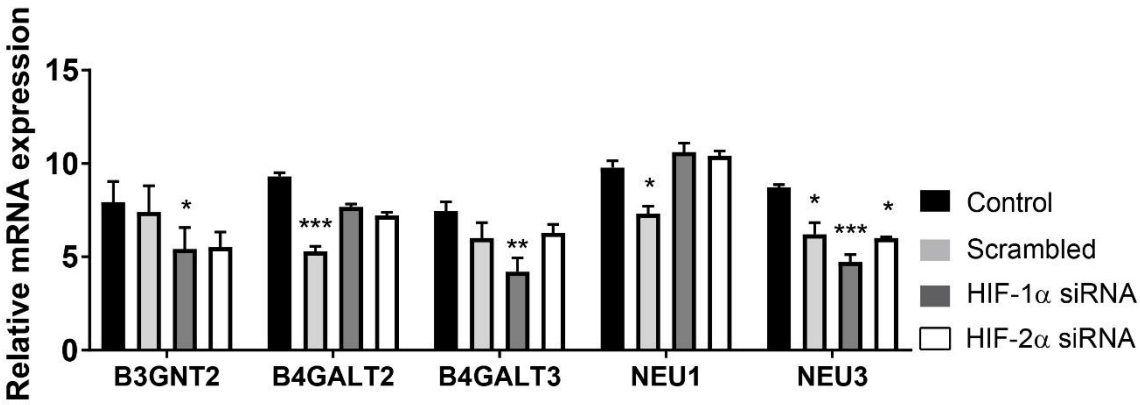

Figure 4.7C: Analysis of Glycan-extension and sialidase gene expression following siRNA suppression of HIF-1α and HIF-2α. The relative mRNA expression of *glycan extension genes: B3GNT2, B4GALT2, B4GALT3* and sialidase genes *NEU1* and *NEU3* following transfection with non-targeting scrambled, HIF-1α or HIF-2α siRNAs. qRT-PCR values are shown as mean ± SEM (n=3 biological replicates) and were normalised to the housekeeper gene *β2M* and was analysed with Sabiosciences software v2.3 and Graphpad Prism 7.

These results taken together indicate HIF-1α has a significant impact on the gene expression of glycan-extension genes: *B3GNT2 and B4GALT3,* and that these genes may be regulated by HIF-1α expression. This further implies that these glycan-extension genes may aid in the HIF-1α dominant adaptions to hypoxia in TNBC cells.

4.5: Discussion

4.5.1: Correlation between HIF and glycogene expression.

Aberrant protein glycosylation is common in TNBC (Hanahan and Weinberg, 2011) and the work undertaken in this study has elucidated numerous glycogenes with altered expression in hypoxic MDA-MB-468 cells. In TNBC, the primary metabolic substrate is shifted from glutamine to glucose (Liberti and Locasale, 2016) due to the adaptations to intra-tumoural hypoxia (Semenza, 2000a). This has an impact on DNA, protein and lipid synthesis, ultimately affecting the availability of intracellular nucleotide sugars (Liberti and Locasale, 2016). Nucleotide sugar availability, coupled with altered glycogene expression has the potential to modify the glycan structures on the cell surface (Shirato *et al.*, 2011) which can affect cellular attachment, invasion and promote a more aggressive tumour phenotype (Varki, 2009).

Triple negative breast cancer is inherently hypoxic (Vaupel, 2008; Semenza, 2016) and in this study, TNBC has been shown to preferentially express *HIF-1α in vivo,* but *HIF-2α in vitro*. Others have shown that MDA-MB-468 cells both express HIF-1α and HIF-2α protein (Christine Blancher *et al.*, 2000). HIF-1α and HIF-2α activity impacts cellular metabolism and the glycolytic cycle (Masoud and Li, 2015). However, evidence is not present to suggest that either HIF subunit may correlate with the expression of genes involved in glycosylation, in TNBC.

4.5.2: siRNA treatment effects on HIF-1α and HIF-2α protein expression.

In order to assess the impact of suppressed HIF-1α and HIF-2α on glycogene expression, expression of HIF-α subunits were inhibited with siRNA treatment. HIF-1α and HIF-2α siRNAs moderately suppressed HIF-1α and HIF-2α protein expression at a concentration of 50nM in hypoxic MDA-MB-468 cells (Figure 4.5). Absolute knockdown of either HIF-α protein was not achieved and is perhaps a result of either inefficient transfection, which would be overcome *via* the use of a positive siRNA transfection control, or due to saturation of the endogenous RNAi pathway.

Interestingly, where HIF-1α was suppressed by HIF-1α siRNA treatment, HIF-2α protein expression in hypoxic cells appeared to increase (Figure 4.5). Studies employing the siRNA used here have assessed HIF1-α and HIF-2α expression however, cross-regulation was not found at the protein level (Pawlus, Wang and Hu, 2014). Although, others have analysed HIF-1α/HIF-2α cross-regulation at the mRNA level in neuroblastoma cell lines SK-N-BE(2)c and KCN-69n, where knockdown of HIF-1α enhanced the expression of HIF-2α and vice versa (Hamidian *et al.*, 2015). Alternatively, the apparent cross-regulatory mechanism identified here may be as a result of the endogenous HIF-1α antisense transcript in which during prolonged (>12h) periods of hypoxia, HIF-1α can become regulated by autonegative feedback either by itself, or through trans-regulatory mechanisms between HIF-1α and HIF-2α (Uchida *et al.*, 2004).

4.5.3: HIF-2α suppression significantly decreased mRNA expression of the HIF-1α target gene, *CA9*.

It was surprising that potential cross regulation was observed, however this may have been a result of off-targeting effects. siRNAs bind several target mRNAs and share full mRNA sequence complementarity with their intended targets (Elbashir, Lendeckel and Tuschl, 2001). siRNAs trigger enzymatic cleavage of their matched mRNA however, off-target activity can occur; often due to the technology they are delivered by or by certain properties of the siRNA itself (Chi *et al.*, 2003; Semizarov *et al.*, 2003). It could be suggested that off-targeting effects occurred which resulted in the knockdown of HIF-2α mRNA with HIF-1α siRNAs because HIF-1α and HIF-2α share similar domain structure (Figure 4.8A) (Holmquist-Mengelbier *et al.*, 2006). siRNA treatment used here consisted of one siRNA sequence which may account for potential inefficiency of HIF-2α knockdown this targeted the HIF-2α coding sequence from approximately 600bp to 1200bp (Figure 4.8). This opposes now conventional methods of 4 siRNA sequences pooled together.

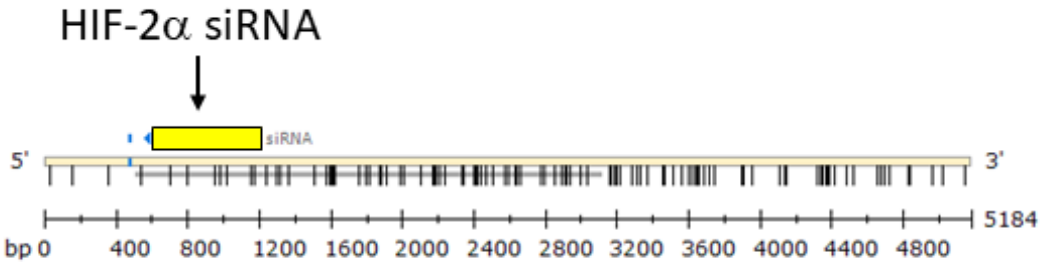

Figure 4.8: Targeting region of HIF-2α siRNAs used in this study. siRNAs targeted the coding region of the HIF-2α transcript at approximately 600bp to 1200bp which is indicated in yellow. Figure adapted from Qiagen GeneGlobe Specification (2019).

One of the best characterized targets of HIF-1 is carbonic anhydrase 9 (CA9). Interestingly, *CA9* expression was not significantly affected by the partial knockdown of HIF-1α, but HIF-2α. In other studies, *CA9* expression has remained unaffected by HIF-2α siRNA treatment, in MDA-MB-231 cells (Pawlus, Wang and Hu, 2014). Current literature does not describe HIF-2α mediated transcriptional regulation of *CA9;* therefore, it remains unclear as to why a decrease in HIF-2α significantly decreased *CA9* mRNA expression. In order to better investigate and determine if HIF-2α suppression also impacted HIF-1α downstream genes; the expression of additional HIF-1α downstream target genes should be assessed, such as *LDHA, GLUT1* and *GLUT3* (Chen *et al.*, 2001). Furthermore, it would be of interest to assess the protein expression of CA9 following HIF-1α and HIF-2α siRNA treatment, thus, determining if suppression of CA9 by HIF-2α siRNAs only occurred at the mRNA level.

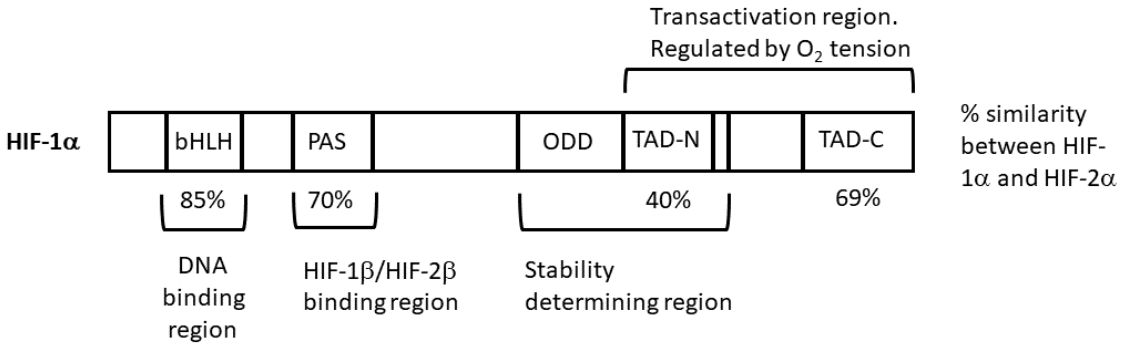

Figure 4.8A: Domain structure of HIF-1α and its mRNA percentage homology with HIF-1α. Potential function of the domains is indicated outside of brackets. bHLH: basic helix-loop-helix. PAS: PER-ARNT-SIM. ODD: oxygen-dependent degradation. TAD-N: Transacting domain N-terminal. TAD-C. transacting domain C-terminal. Figure adapted from (Hong, Lee and Kim, 2004; Koh and Powis, 2009).

Unexpectedly, HIF-2α siRNA treatment did not significantly decrease *HIF-2α* mRNA expression. However, *EPO* (a downstream gene predominantly regulated by HIF-2α) was significantly downregulated which suggests that *HIF-2α* transcription was in fact reduced in this model under the conditions employed here.

4.5.4: HIF-1α promotes expression of *B3GNT3, B4GALT3, GALNT6* and *GALNT12* in hypoxic MDA-MB-468 cells.

Protein glycosylation is recognised to be one of the most important post translational modifications in mammalian cells, however the process is one of the most complex to study (Varki, 2009). Changes to glycan structures is believed to occur early during malignant transformation (Munkley and Elliott, 2016) and it has

been shown (Chapter 3, and by others), that expression of glycosylation genes are significantly altered in TNBC compared to non-cancerous tissue (Potapenko *et al.*, 2010). In addition to altered glycogene expression (Albuquerque *et al.*, 2018), TNBCs are known to be inherently hypoxic and express HIF-1α and HIF-2α (Semenza, 2016).

Partial knockdown of HIF-1α significantly reduced the relative mRNA expression of glycan extension genes: *B3GNT2, B4GALT3,* and *O*-glycan initiating genes: *GALNT6* and *GALNT12. B4GALT1* is closely related to *B4GALT3* with a similar glycosylation function and has been linked to increased aggression in clear cell renal carcinoma (Xie *et al.*, 2016). However, there is little evidence in the literature to suggest that *B3GNT2* or *B4GALT3* promote aggressive tumour growth in TNBC (Potapenko *et al.*, 2010). From the evidence presented here, it is suggested that *B3GNT2* and *B4GALT3* may be under the transcriptional control of HIF-1α in hypoxic MDA-MB-468 cells. Therefore, it may be involved in facilitating the aggressive tumour phenotype associated with overexpression of HIF-1α (Jin *et al.*, 2016). It could be hypothesised that overexpression of glycan extension genes *B3GNT2* and *B4GALT3* due to HIF-1α may result in a high proportion of poly-*N*-acetyllactosamine chains. Tumour cells that are decorated with newly synthesised poly-N-acetyllactosamine chains are known to facilitate cell-cell interaction and increase metastasis through lectins such as P- and E-selectins (Nonaka and Fukuda, 2016).

O-glycan initiating genes *GALNT6* and *GALNT12* have been shown to be upregulated in breast cancer, but not specifically TNBC (Potapenko *et al.*, 2010). GALNT6 suppression in T47D BrCa cells resulted in enhanced cell adhesion and reduced growth of BrCa cells, thus implying GALNT6 promotes cell mobility (Park

et al., 2010). However, T47D is not a TNBC BrCa cell line, so it is not possible to directly relate GALNT6 with increased cell metastasis in TNBC. Similarly, evidence that directly relates HIF-1α and GALNT6 expression is not present in the literature; therefore, the data presented here is the first time that decreased *GALNT6* and *GALNT12* expression has been reported due to knockdown of HIF-1α in TNBC cells. These findings would imply that in TNBC, a high proportion of Tn antigen is expected to be expressed, due to the overexpression of *GALNT6* and *GALNT12.* Therefore, the suppression of HIF-1α should result in decreased expression of *GALNT6* and *GALNT12* which will in turn, decrease the expression of Tn antigen. As the Tn antigen is known to facilitate tumour metastasis (Ju *et al.*, 2013) due to alterations in cell-cell interactions, signal transduction and increased immune evasion (Bapu *et al.*, 2016), decreasing the expression of Tn antigen should improve TNBC patient outcomes.

These results taken together suggest: *B3GNT2, B4GALT4, GALNT6* and *GALNT12* may be regulated by HIF-1α in TNBC. This is the first time this has been reported to our knowledge.

4.5.5: HIF-1α/HIF-2α cross-regulatory mechanism.

In the literature, there are reports of HIF-1α/HIF-2α redundancy. HIF-1α is the main coordinator of genes encoding enzymes involved in glycolysis such as phosphofructokinase (*PFK*) and lactate dehydrogenase (*LDHA*), carbonic anhydrase 9 (*CA9*) and BCL2/adenovirus E1B interacting protein 3 (*BNIP3*) (Keith, Johnson and Simon, 2011). These genes are involved in various processes including pH regulation and apoptosis (Keith, Johnson and Simon,

2011). HIF-2α contrastingly prompts the induction of genes such as matrix metalloproteinases (*MMP*) *2*, and *13*; genes that are involved in cellular invasion (Warnecke *et al.*, 2004). However, the regulation of glycolytic enzymes has also attributed to HIF-2α in the absence of HIF-1α (Warnecke *et al.*, 2004; Scortegagna *et al.*, 2005). Moreover, HIF-1α has shown abilities in activating selected MMPs (Fujiwara *et al.*, 2007).

In murine models, targeted deletion of HIF-1α or HIF-2α subunits resulted in lethal phenotypes that die in utero (Franke *et al.*, 2013). However, in HeLa cells, HIF-1α and HIF-2α target gene specificity has been overcome by forced expression of HIFα subunits (Warnecke *et al.*, 2004). Overexpression of the HIFα subunits can also occur through tumorigenic events which affect the HIF/pVHL (von-Hippel Lindau tumour suppressor) in which it has been suggested that the two genes were performing similar functionals with little or no effect on the biological phenotype (Figure 4.9) (Warnecke *et al.*, 2004). This implies that both HIF subunits can substitute the other subunits specific functions under some circumstances.

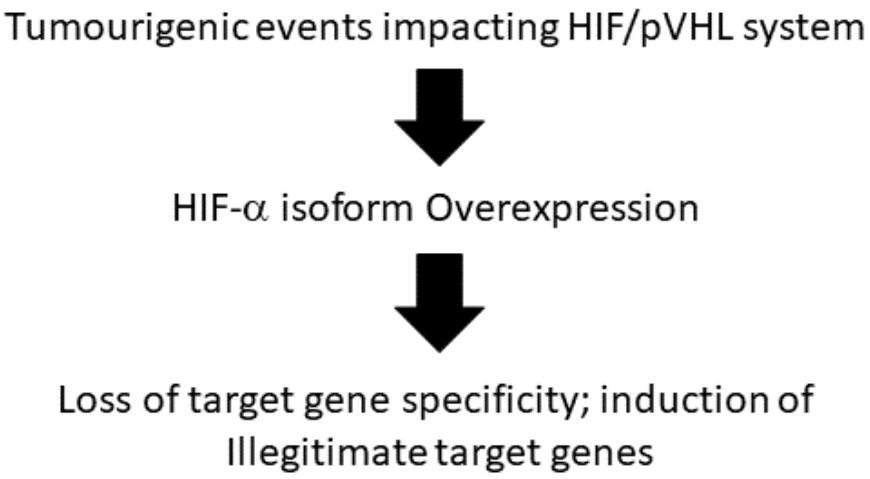

Figure 4.9: Schematic illustrating loss of HIF target gene due to altered HIF/pVHL system in cancer. Each HIF-α subunit shows high affinity to hypoxia response elements of its intended target genes in mammalian cells with functional HIF/pVHL. Overexpression of HIF-α subunits though tumourigenic events may affect the HIF/pVHL system leading to loss of target gene specificity (Warnecke *et al.*, 2004).

In this study, knockdown of *HIF-2α* appeared to increase the expression of HIF-1α protein (Figure 4.4) and furthermore, knockdown of *HIF-1α* mRNA appeared to decrease *HIF-2α* expression (Figure 4.8). This proposes that *HIF-2α* expression may rely on HIF-1α and in the absence of HIF-2α: *HIF-1α* may compensate. This evidence adds to the suggestion that on occasion, there may be cross-regulatory mechanisms between the HIF transcription factors. This may be due to the above-mentioned overexpression of either subunit due to tumourigenesis, or due to several other factors.

HIF-1α is predominantly regulated by VHL-mediates proteasomal degradation, however several studies have elucidated how it can be regulated by other post-transcriptional mechanisms (Galban *et al.*, 2008). RMB38 is an RNA-binding protein and target of the p53 family, which regulates gene expression through mRNA stability and translation (Zhang *et al.*, 2011). RMB38 has been shown to regulate HIF-1α expression through mRNA translation under hypoxia (Cho *et al.*, 2015). HCT116 cells treated with $CoCl_2$, a chemical inducer of hypoxia (Piret *et al.*, 2002), demonstrated that RMB38 overexpression decreased HIF-1α protein and knockdown of RMB38 resulted in increased expression of HIF-1α protein (Cho *et al.*, 2015), thus, suggesting that RMB38 is a transcriptional regulator of HIF-1α under hypoxia. In this study, HIF-α mediated mRNA regulation through mechanisms such as RMB38 may have factored into the cross-regulation observed between HIF-1α and HIF-2α.

4.5.6: Conclusions and future work

The work undertaken in this chapter aimed to knockdown the expression of HIF-1α and HIF-2α in hypoxic MDA-MB-468 cells and elucidate if the suppression of HIF-1α/HIF-2α results in altered expression of genes involved in protein glycosylation. In doing so, a greater understanding of the mechanisms by which hypoxia inducible factors regulate cell glycosylation has been gathered and potential biomarkers of disease have been identified. The possible identification of genes that are altered in expression as a result of the loss of HIF-1α or HIF-2α in hypoxic TNBC cells found here is novel and adds to the growing body of evidence that inter-tumoral hypoxia influences many aspects of cell glycosylation.

This provides evidence that hypoxic MDA-MB-468 cells may show differentially expressed glycogenes including: *B3GNT2, B4GALT3, GALNT6* and *GALNT12* as a result of the suppression of HIF-1α. This indicates that there may be signalling link between HIF-1α and these genes, which potentially aid in the tumour phenotype associated with HIF-1α expression in TNBC. However, further work is required to elucidate exactly which aggressive tumour traits are attributed to which glycogene due to HIF-1α.

4.5.6.1: siRNA treatment considerations: Cell passage number.

In this investigation cells were at approximately passage 30-35. Cells of this age may have been subject to cell senescence and therefore may not have responded to siRNA treatment with the same intensity of younger cells (Rubin, 1997). The manufacture's recommendations state that cells with high passage number are prone to change their growth behaviour and morphology and can be less susceptible to transfection (HiPerfect Transfection Handbook, 2010). Unfortunately, due to time restraints the passage number of the cells used in the siRNA experiments were unable to be performed at a lower passage.

4.5.6.2: siRNA treatment considerations: Investigating potential off-targeting siRNAs.

The HIF-1α downstream target *CA9* was significantly suppressed by HIF-2α siRNA treatment. In order to understand if this was an off-target effect, future experiments should include alternative HIF-1α downstream targets, such as *GLUT1*, *GLUT3* or *LDHA* (Semenza, 2000). This will indicate whether *HIF-2α* siRNA is indeed affecting *HIF-1α* expression and subsequent HIF-1α downstream targets, which was thought to occur in the results documented in section 4.4.4.

It is also important to acknowledge that the siRNA knockdown implemented throughout this study did not result in absolute knockdown of the target proteins. This is assumed to be as a result of poor siRNA specificity or suboptimal transfection efficiency. In future experiments, it is recommended that further transfection titrations are performed to identify optimal doses of siRNA and transfection reagent

4.5.7: Future experiments; HIF suppression and the binding of biotinylated lectins.

As documented in Chapter 3, biotinylated lectins can be used to establish the effect of hypoxia on cell surface glycans and the proportion of several sugar residues on the surface of TNBC cells. It would of great interest to repeat these investigations with cells transfected with HIF-1α and HIF-2α siRNAs to compared to sugar residues of HIF-1α/HIF-2α positive cells and those treated with siRNAs.

This would indicate whether the suppression of HIFs influence the expression of glycans on the cell surface and ultimately; cell glycosylation. Insight would be gathered into the cell glycocalyx of hypoxia MDA-MB-468 cells and determine the impact of HIF and HIF suppression on TNBC aggression due to the potential alterations observed ie. truncated and over-extended glycans.

Chapter 5: General discussion, study limitations and future directions.

5.1: Glycogene expression is altered in TNBC and these changes are also found in hypoxic TNBC cells.

In Chapter 3, array profiling of tumour samples 11004 and 10046 elucidated a marked change in the glycosylation profile between TNBC and tissue matched controls. Similar trends of altered gene expression among glycan-extension, *O*-glycan initiating, *N*-glycan branching, sialidase and sialyltransferase families was observed between three TNBC tumours. This provided an answer to the first research question proposed: the glycogene expression profile of TNBC does in fact differ to tissue matched samples. These findings add to the growing body of literature and provide further evidence that glycosylation gene expression is altered in TNBC malignancies (Potapenko *et al.*, 2010; Chen *et al.*, 2016).

5.2: The glycan profile of MDA-MB-468 cells is altered in response to acute hypoxia.

Hypoxia has the potential to significantly alter cell metabolism and gene expression (Semenza, 2012); therefore, it has the potential to alter hypoxic TNBC tumour glycosylation. In Chapter 3, the degree of hypoxia inducible factor induction was measured via qRT-PCR in TNBC tumour samples. HIF-1α was preferentially expressed in all three TNBC tumours, whereas HIF-2α was

unanimously downregulated. Hypoxic MDA-MB-468 cells were used as a cellular model of TNBC, both HIF-1α and HIF-2α protein expression was observed, confirming both HIF-α pathways are activated by acute hypoxia in TNBC (Koh *et al.*, 2011).

Following TNBC tumour glycogene expression profiling and validation in hypoxic TNBC cells, lectin analysis of hypoxic MDA-MB-468 cells indicated that hypoxia potentially influences the binding of some lectins to cell surface glycans. This was uncovered through differential binding of lectins: *PNA, PHA-E* and *LCA,* which suggested hypoxia increases the proportion of complex *N*-type glycans, non-reducing terminal β-*D*-galactose, core fucosylated glycans and *N*-acetyl-neuraminidase residues on the surface of MDA-MB-468 cells. These findings answered the third question proposed in this chapter: that the glycan profile of hypoxia MDA-MB-468 cells is altered in response to acute hypoxia. These results were significant as this type of lectin analysis provided novel information to the alterations of sugar moieties found on the surface of TNBC cells following hypoxic exposure. Furthermore, indicating that hypoxia does cause significant changes to cell surface glycosylation. This has wider implication and demonstrates that the hypoxic tumour environment has the ability to significantly impact cell-cell interactions and potentially facilitate cell metastasis, invasion and promote a more aggressive tumour phenotype.

5.3: HIF-1α and HIF-2α protein expression was suppressed by siRNA treatment.

In Chapter 3, qRT-PCR validation identified that HIF-1α expression correlated with the expression of glycogenes: *GALNT3, GALNT6, GALNT7, GALNT12, B3GNT2, B4GALT2, B4GALT3, NEU1* and *NEU3*. In addition, the expression of GALNTL5 correlated with HIF-2α induction in TNBC tumours.

The correlation between HIF-induction and glycogene expression was investigated in Chapter 4, where siRNA gene knockdown was employed targeting HIF-1α and HIF-2α in hypoxic MDA-MB-468 cells. Immunoblotting confirmed the successful suppression of HIF-1α and HIF-2α protein by their respective siRNA treatment. Interestingly, where HIF-1α was suppressed by HIF-1α siRNA treatment, HIF-2α protein expression in hypoxic cells appeared to increase. This cross-regulation was not found by others using identical siRNAs in TNBC cells (Pawlus, Wang and Hu, 2014). Henceforth, this may be the first time that cross-regulation between HIF-1α and HIF-2α has been observed in hypoxic MDA-MB-468 cells. These results may signify that both HIF subunits can substitute each other's specific functions under some circumstances.

5.3.1: B3GNT2, B4GALT3, GALNT6 and GALNT12 are downregulated when HIF-1α is suppressed in hypoxic TNBC cells.

Knockdown of HIF-1α significantly reduced the relative mRNA expression of glycan extension genes: *B3GNT2, B4GALT3*, and *O*-glycan initiating genes:

GALNT6 and *GALNT12.* This indicated that there is a link between HIF-1α signalling and these genes, which potentially aids in the tumour phenotype associated with HIF-1α expression in TNBC. Additionally, these findings answered to the second research question of this chapter: the knockdown of HIF-1α in hypoxic MDA-MB-468 cells alters expression of *O*-glycan initiating and glycan-extension gene expression. This is the first time that this has been reported in the literature and signifies a significant insight into the cellular glycome alterations in TNBC due to hypoxic adaptation. However, further work is required to elucidate exactly which aggressive tumour traits are associated with altered glycogene expression.

The role of HIF-2α in regulating glycogene expression in TNBC is still unclear as glycogene expression was also affected by control siRNA. However, based on initial observations, HIF-1α seemed to predominantly have a greater impact on glycogene expression in TNBC. This implies glycogene expression in hypoxic TNBC cells seems to correlate with the HIF-1α arm of the hypoxic adaptation machinery. Furthermore, altered glycosylation in hypoxic tumours may aid in promoting the key hallmarks of cancer including evading the immune response, invasion and metastasis (Hanahan and Weinberg, 2011).

5.4: Future directions.

Initially, this thesis broadly explored altered glycosylation gene expression in TNBC samples, compared to adjacent non-cancerous tissues. Several glycogenes were altered compared to healthy breast tissue, thus providing novel information regarding altered glycogene expression in TNBC. Future work should

include increasing the sample size of tumours profiled compared to patent matched, control tissue for greater statistical significance. Obtaining greater information on tumour stage, patient race, age and body mass index (BMI) would also be essential to correlate changes in gene expression with tumour stage and grade. This would indicate changes in glycogene expression with patient race and therefore susceptibility to TNBC. Interestingly, a higher proportion of obese patients are likely to suffer from TNBC, gathering data on patient BMI would allow for correlation of this data (Trivers *et al.*, 2009).

It would also be of interest to investigate other groups of glycogenes as this thesis focused mainly on the validation of altered expression of *O*-glycan initiating and glycan-extension genes. Analysing altered glycogene expression from *N*-glycan branching genes, sialyltransferases, fucosylases and other glycan decorating genes would provide a well-rounded dataset regarding how breast tissue becomes altered during malignancy due to the hypoxic tumour environment. Gathering further data would allow for identification of potential biomarkers of tumour progression and therefore allow for the development of new targeted treatments for this devastating subtype of breast cancer.

5.4.1: Does altered glycogene expression translate into altered tumour glycosylation profile?

The original aim of the project was to confirm that the observed changes in gene expression in TNBC correlated with altered glycan expression profile in hypoxic TNBC. This was to be achieved via immunohistochemistry (IHC) using paraffin-embedded formalin-fixed (PPFE) tissue sections. Altered biotinylated lectin

staining would be correlated with markers of hypoxia (*CA9*) to confirm the change was associated with hypoxia. This would have helped to distinguish subtle alterations in cellular glycosylation, as lectin IHC can be used to identify glycosylation changes that can aid cellular transformation to malignancy and tumour progression (Zhou *et al.*, 2015).

This knowledge that would aid the understanding of how sugar attachment occurs in response to the hypoxic tumour microenvironment. However, availability of the samples from the tissue bank meant sections could not be analysed. Undertaking this analysis would be essential so that a stronger biochemical understanding of how cancerous glycosylation occurs in TNBC. This would be achieved correlating positive staining of hypoxia markers in PFFE sections with altered glycogene expression.

5.4.2: Investigating the effects of suppressed HIF-1α and HIF-2α on sugar residue proportions on hypoxic MDA-MB-468 cells.

Future work will also address some of the shortcomings of this research due to time restraints of the MRes including analysis of the lectin binding profile in hypoxic MDA-MB-468 cells with suppressed HIF-1α or HIF-2α expression (by siRNA treatment). Therefore, the impact of HIF-1α or HIF-2α suppression on the expression of complex *N*-type glycans, core fucosylated glycans, non-reducing terminal β-*D*-galactose and *N*-acetyl-neuraminidase residues on the cell surface of hypoxic TNBC cells will be determined. This research would confirm observed changes in gene expression with physical changes in the glycosylation profile of TNBC.

In Chapter 3, lectin binding analysis showed minimal *DBA* binding to hypoxic MDA-MB-468 cells compared to control cells. *DBA* binds terminal N-acetylgalactosamine (GalNAc) residues and the lack of *DBA* binding was hypothesised to be a result of decreased expression of: *GALNT6, GALNT7, GALNT12* and *GALNT14*, genes involved in adding the first GalNAc residue to initiate *O*-glycans. Furthermore, decreased *B4GALT2* and *B4GALT3* expression in hypoxic MDA-MB-468 cells may have influenced the lack of *PNA* lectin binding. *PNA* binds to galactose and *B4GALT2* and *B4GALT3* enzymes catalyse the addition of galactose residues to glycans (Mérant *et al.*, 2005). Therefore, if galactose addition is diminished during glycan synthesis due to decreased *B4GALT2/3* expression - a lack of *PNA* binding in TNBC would be expected.

In Chapter 4, suppression of HIF-1α significantly decreased *GALNT6, GALNT12, B3GNT2* and *B4GALT3* expression following hypoxia. In hypoxic MDA-MB-468 cells with suppressed HIF-1α, it would be expected to see a significant decrease in *PNA* and *DNA* binding. This is due to the decreased enzymatic addition of GalNAc and galactose. This implies HIF-1α plays a role in increasing GalNAc and galactose glycosylations on the cell surface of hypoxic MDA-MB-468 cells. An increase in GalNAc residues is associated with expression of the Tn antigen. Tn antigen expression is strongly associated with poor prognosis and tumour metastasis in BrCa (Ju *et al.*, 2013) due to altered cell-cell interactions, signal transduction and immune evasion (Bapu *et al.*, 2016) (Figure 5.1). Numerous alterations to protein glycosylations have been tied to correlations in tumour grade, increased metastasis and invasion. Often, altered glycan expression is correlated with poor patient prognosis (Kim and Varki, 1997). Identifying the glycogenes which have a direct impact on tumour cell invasion, metastasis and immune system invasion could be used as a potential drug target to improve

patient prognosis through new treatment options. If successful, targeting altered glycans could be used in other types of breast cancers and hypoxic tumours. Potentially, similar examples of aberrant glycosylation may be found in other hypoxic tumours and may correlate with tumour stage.

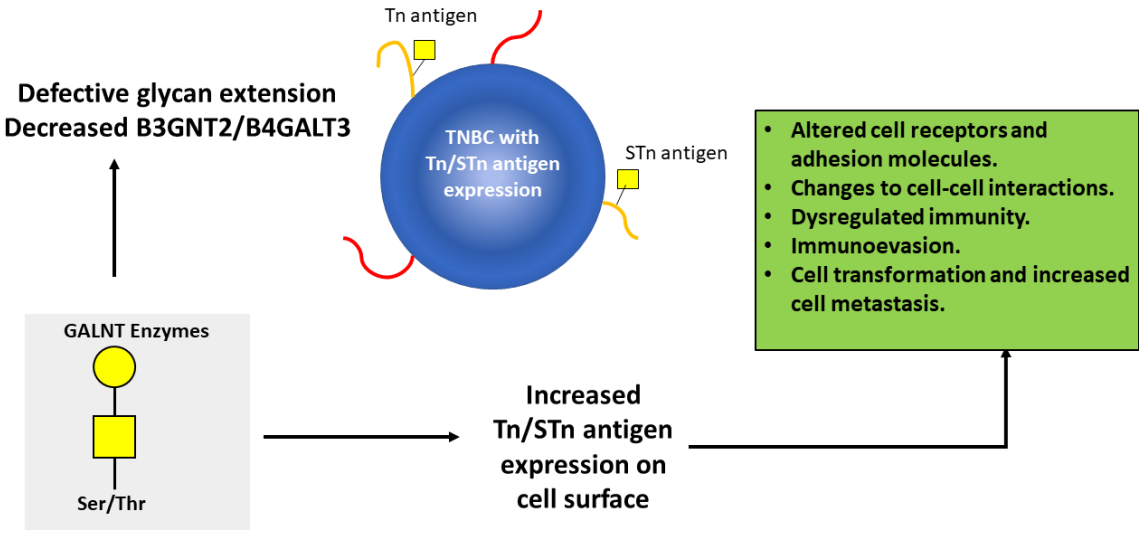

Figure 5.1: Altered cell adhesion, immune-evasion and increased cell metastasis in TNBC due to Tn/STn-antigen expression. STn/Tn antigens are tumour-associated markers that are absent in normal, non-malignant tissues. As a result of defective glycan extension by enzymes such as *B3GNT2* and *B4GALT3*; Tn antigen or STn antigen is expressed resulting in a more aggressive tumour phenotype. Figure adapted from (Ju *et al.*, 2013).

5.4.3: siRNA experiments.

During experiments where siRNAs were used, siRNA scrambled controls caused expression of several genes to change. Upon repetition of this experiment, it

would be suggested to use the cells at a lower passage; as recommended by the manufacturer's instructions. If siRNA controls persisted to cause unexpected changes in gene expression, a different siRNA scrambled control should be used. As the control siRNAs used in these experiments were designed to have no known mRNA targets in the cell used, it is unlikely that they caused knockdown of the genes in question and it was the addition of the siRNA itself that caused knockdown by other means. To improve the reliability of the results, fresh aliquots of siRNAs, transfection reagents and newly thawed cells should be used after they have been passaged at a minimum of three times to recover from thawing.

5.4.4: Is glycosylation impacted by the degree hypoxia at different timepoints?

This research focused on the changes to protein glycosylation in TNBC when exposed to hypoxia (1% O2) after 22 hours of exposure. Several studies have investigated the effects of hypoxia at different lengths of exposure and oxygen percentage and have found varying results (Shirato *et al.*, 2011). It has been well-established that differences in the hypoxia response are due to the severity of hypoxia, the timeframe of hypoxic exposure and that HIF-1α and HIF-2α may be involved at different points of the adaptive response (Keith, Johnson and Simon, 2011). As glycosylation is a successive and hierarchal process (Burchell *et al.*, 2018) it may be possible that varying degrees of hypoxia and the length of hypoxia duration may affect how protein glycosylation is impacted. It would be of interest to repeat the experiments in this thesis with varying hypoxic conditions including <0.1% O2 and timepoints of 12, 48 and 72 hours of exposure.

References

Aebi, M. (2013) ‘N-linked protein glycosylation in the ER’, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. Elsevier, 1833(11), pp. 2430–2437. doi: 10.1016/J.BBAMCR.2013.04.001.

Albuquerque, A. P. B. *et al.* (2018) ‘Hypoxia and serum deprivation induces glycan alterations in triple negative breast cancer cells’, *Biological Chemistry*, 399(7), pp. 661–672. doi: 10.1515/hsz-2018-0121.

Annibaldi, A. and Widmann, C. (2010) ‘Glucose metabolism in cancer cells’, *Current Opinion in Clinical Nutrition and Metabolic Care*, 13(4), pp. 466–470. doi: 10.1097/MCO.0b013e32833a5577.

Appelhoff, R. J. *et al.* (2004) ‘Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(37), pp. 38458–65. doi: 10.1074/jbc.M406026200.

Asada, M. *et al.* (1997) ‘Increased expression of highly branched N-glycans at cell surface is correlated with the malignant phenotypes of mouse tumor cells.’, *Cancer research*, 57(6), pp. 1073–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9067274 (Accessed: 3 October 2018).

Au, G. H. T. *et al.* (2014) ‘Quantitative assessment of Tn antigen in breast tissue micro-arrays using CdSe aqueous quantum dots’, *Biomaterials*. Elsevier, 35(9), pp. 2971–2980. doi: 10.1016/J.BIOMATERIALS.2013.12.034.

Bah, A. and Forman-Kay, J. D. (2016) ‘Modulation of Intrinsically Disordered Protein Function by Post-translational Modifications.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 291(13), pp. 6696–705. doi: 10.1074/jbc.R115.695056.

Bapu, D. *et al.* (2016) ‘N-acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: A role for truncated O-glycans in metastatic mechanisms’, *Cancer Letters*. Elsevier, 375(2), pp. 367–374. doi: 10.1016/J.CANLET.2016.03.019.

Bauer, K. R. *et al.* (2007) ‘Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype’, *Cancer*. Wiley-Blackwell, 109(9), pp. 1721–1728. doi: 10.1002/cncr.22618.

Beatson, R. *et al.* (2015) ‘The Breast Cancer-Associated Glycoforms of MUC1, MUC1-Tn and sialyl-Tn, Are Expressed in COSMC Wild-Type Cells and Bind the C-Type Lectin MGL’, *PLOS ONE*. Edited by L.-G. Yu. Public Library of Science, 10(5), p. e0125994. doi: 10.1371/journal.pone.0125994.

Becker, D. J. and Lowe, J. B. (2003) ‘Fucose: biosynthesis and biological function in mammals’, *Glycobiology*, 13(7), p. 41R–53R. doi: 10.1093/glycob/cwg054.

Benej, M., Pastorekova, S. and Pastorek, J. (2014) ‘Carbonic Anhydrase IX: Regulation and Role in Cancer’, in *Sub-cellular biochemistry*, pp. 199–219. doi: 10.1007/978-94-007-7359-2_11.

Bernardi, R. and Gianni, L. (2014) ‘Hallmarks of triple negative breast cancer emerging at last?’, *Cell research*. Nature Publishing Group, 24(8), pp. 904–5. doi: 10.1038/cr.2014.61.

van den Beucken, T. *et al.* (2009) ‘Hypoxia-induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 284(36), pp. 24204–12. doi: 10.1074/jbc.M109.006510.

Bieberich, E. (2014) ‘Synthesis, Processing, and Function of N-glycans in N-glycoproteins.’, *Advances in neurobiology*. NIH Public Access, 9, pp. 47–70. doi: 10.1007/978-1-4939-1154-7_3.

Blancher, C. *et al.* (2000) *Relationship of Hypoxia-inducible Factor (HIF)-1 and HIF-2 Expression to Vascular Endothelial Growth Factor Induction and Hypoxia Survival in Human Breast Cancer Cell Lines*, *CANCER RESEARCH*. Available at: http://cancerres.aacrjournals.org/content/canres/60/24/7106.full.pdf (Accessed: 4 December 2018).

Blancher, C. *et al.* (2000) ‘Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines.’, *Cancer research*, 60(24), pp. 7106–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11156418 (Accessed: 13 August 2018).

Bos, P. D. *et al.* (2009) ‘Genes that mediate breast cancer metastasis to the brain’, *Nature*, 459(7249), pp. 1005–1009. doi: 10.1038/nature08021.

Brahimi-Horn, M. C., Chiche, J. and Pouysségur, J. (2007) ‘Hypoxia signalling controls metabolic demand’, *Current Opinion in Cell Biology*, 19(2), pp. 223–229. doi: 10.1016/j.ceb.2007.02.003.

Brahimi-Horn, M. C. and Pouysségur, J. (2007) ‘Oxygen, a source of life and stress’, *FEBS Letters*, 581(19), pp. 3582–3591. doi: 10.1016/j.febslet.2007.06.018.

Breast cancer facts| Breast Cancer Now (no date). Available at: https://breastcancernow.org/about-breast-cancer/want-to-know-about-breast-cancer/breast-cancer-facts (Accessed: 10 March 2019).

Brockhausen, I., Schachter, H. and Stanley, P. (2009) ‘O-GalNAc Glycans’. Cold Spring Harbor Laboratory Press. Available at: https://www.ncbi.nlm.nih.gov/books/NBK1896/ (Accessed: 23 August 2018).

Brooks, S. A. and Carter, T. M. (2001) ‘N-acetylgalactosamine, N-acetylglucosamine and sialic acid expression in primary breast cancers’, *Acta Histochemica*, 103(1), pp. 37–51. doi: 10.1078/0065-1281-00576.

Burchell, J. M. *et al.* (2018) ‘O-linked mucin-type glycosylation in breast cancer’, *Biochemical Society Transactions*, p. BST20170483. doi: 10.1042/BST20170483.

Burchell, J. M., Mungul, A. and Taylor-Papadimitriou, J. (2001) ‘O-linked glycosylation in the mammary gland: changes that occur during malignancy.’, *Journal of mammary gland biology and neoplasia*, 6(3), pp. 355–64. Available

at: http://www.ncbi.nlm.nih.gov/pubmed/11547903 (Accessed: 6 August 2018).

Carmeliet, P. and Jain, R. K. (2000) 'Angiogenesis in cancer and other diseases.', *Nature*, 407(6801), pp. 249–257. doi: 10.1038/35025220.

Carmeliet, P. and Jain, R. K. (2011) 'Molecular mechanisms and clinical applications of angiogenesis', *Nature*, 473(7347), pp. 298–307. doi: 10.1038/nature10144.

Chan, D. A. *et al.* (2005) 'Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha.', *Molecular and cellular biology*. American Society for Microbiology Journals, 25(15), pp. 6415–26. doi: 10.1128/MCB.25.15.6415-6426.2005.

Chang, M. M. *et al.* (2015) 'N-Linked Glycans Are Assembled on Highly Reduced Dolichol Phosphate Carriers in the Hyperthermophilic Archaea Pyrococcus furiosus.', *PloS one*. Public Library of Science, 10(6), p. e0130482. doi: 10.1371/journal.pone.0130482.

Chen, A. *et al.* (2018) 'Intermittent hypoxia induces a metastatic phenotype in breast cancer', *Oncogene*, 37, pp. 4214–4225. doi: 10.1038/s41388-018-0259-3.

Chen, C. *et al.* (2001) 'Regulation of *glut1* mRNA by Hypoxia-inducible Factor-1', *Journal of Biological Chemistry*, 276(12), pp. 9519–9525. doi: 10.1074/jbc.M010144200.

Chen, L. *et al.* (2016) 'Body mass index and risk of luminal, HER2-overexpressing, and triple negative breast cancer.', *Breast cancer research and treatment*. NIH Public Access, 157(3), pp. 545–54. doi: 10.1007/s10549-016-3825-9.

Chen, X. *et al.* (2016) 'Comparative Profiling of Triple-Negative Breast Carcinomas Tissue Glycoproteome by Sequential Purification of Glycoproteins and Stable Isotope Labeling', *Cellular Physiology and Biochemistry*. Karger Publishers, 38(1), pp. 110–121. doi: 10.1159/000438613.

Cheng, D. W. *et al.* (2006) 'An analysis of high glucose and glucosamine-induced gene expression and oxidative stress in renal mesangial cells', *Archives of Physiology and Biochemistry*, 112(4–5), pp. 189–218. doi: 10.1080/13813450601093518.

Chi, J.-T. *et al.* (2003) 'Genomewide view of gene silencing by small interfering RNAs.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(11), pp. 6343–6. doi: 10.1073/pnas.1037853100.

Cho, S.-J. *et al.* (2015) 'Hypoxia-inducible factor 1 alpha is regulated by RBM38, a RNA-binding protein and a p53 family target, via mRNA translation.', *Oncotarget*. Impact Journals, LLC, 6(1), pp. 305–16. doi: 10.18632/oncotarget.2786.

Chou, C.-H. *et al.* (2015) 'Up-regulation of C1GALT1 promotes breast cancer cell growth through MUC1-C signaling pathway.', *Oncotarget*. Impact Journals, LLC, 6(8), pp. 6123–35. doi: 10.18632/oncotarget.3045.

Conway, E. M., Collen, D. and Carmeliet, P. (2001) ‘Molecular mechanisms of blood vessel growth.’, *Cardiovascular research*, 49(3), pp. 507–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11166264 (Accessed: 13 March 2019).

Corfield, A. P. and Berry, M. (2015) ‘Glycan variation and evolution in the eukaryotes’, *Trends in Biochemical Sciences*, 40(7), pp. 351–359. doi: 10.1016/j.tibs.2015.04.004.

Cummings, R. D. and Etzler, M. E. (2009) *Antibodies and Lectins in Glycan Analysis*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301245 (Accessed: 29 June 2018).

Dall’Olio, F. and Trinchera, M. (2017) ‘Epigenetic Bases of Aberrant Glycosylation in Cancer.’, *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 18(5). doi: 10.3390/ijms18050998.

Dengler, V. L., Galbraith, M. and Espinosa, J. M. (2014) ‘Transcriptional regulation by hypoxia inducible factors.’, *Critical reviews in biochemistry and molecular biology*. NIH Public Access, 49(1), pp. 1–15. doi: 10.3109/10409238.2013.838205.

Denko, N. C. (2008) ‘Hypoxia, HIF1 and glucose metabolism in the solid tumour’, *Nature Reviews Cancer*, 8(9), pp. 705–713. doi: 10.1038/nrc2468.

Dennis, J. W., Granovsky, M. and Warren, C. E. (1999) ‘Glycoprotein glycosylation and cancer progression’, *Biochimica et Biophysica Acta (BBA) - General Subjects*. Elsevier, 1473(1), pp. 21–34. doi: 10.1016/S0304-4165(99)00167-1.

Desai, P. R. (2000) ‘Immunoreactive T and Tn antigens in malignancy: role in carcinoma diagnosis, prognosis, and immunotherapy’, *Transfusion Medicine Reviews*, 14(4), pp. 312–325. doi: 10.1053/tmrv.2000.16229.

Ditte, P. *et al.* (2011) ‘Phosphorylation of Carbonic Anhydrase IX Controls Its Ability to Mediate Extracellular Acidification in Hypoxic Tumors’, *Cancer Research*. American Association for Cancer Research, 71(24), pp. 7558–7567. doi: 10.1158/0008-5472.CAN-11-2520.

Doherty, M. *et al.* (2018) ‘Plasma N-glycans in colorectal cancer risk’, *Scientific Reports*. Nature Publishing Group, 8(1), p. 8655. doi: 10.1038/s41598-018-26805-7.

Dwek, R. A. (1996) ‘Glycobiology: Toward Understanding the Function of Sugars’, *Chemical Reviews*. American Chemical Society , 96(2), pp. 683–720. doi: 10.1021/cr940283b.

Eales, K. L., Hollinshead, K. E. R. and Tennant, D. A. (2016) ‘Hypoxia and metabolic adaptation of cancer cells’, *Oncogenesis*, 5(1), p. e190. doi: 10.1038/oncsis.2015.50.

Elbashir, S. M., Lendeckel, W. and Tuschl, T. (2001) ‘RNA interference is mediated by 21- and 22-nucleotide RNAs.’, *Genes & development*, 15(2), pp. 188–200. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11157775

(Accessed: 5 December 2018).

Epstein, A. C. *et al.* (2001) 'C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.', *Cell*, 107(1), pp. 43–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11595184 (Accessed: 13 August 2018).

Favaro, E. *et al.* (2011) 'Gene expression and hypoxia in breast cancer.', *Genome medicine*. BioMed Central, 3(8), p. 55. doi: 10.1186/gm271.

Franke, K. *et al.* (2013) 'HIF-1α is a protective factor in conditional PHD2-deficient mice suffering from severe HIF-2α-induced excessive erythropoiesis.', *Blood*. The American Society of Hematology, 121(8), pp. 1436–45. doi: 10.1182/blood-2012-08-449181.

Freeze, H. H. and Ng, B. G. (2011) 'Golgi glycosylation and human inherited diseases.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 3(9), p. a005371. doi: 10.1101/cshperspect.a005371.

Fu, C. *et al.* (2016) 'Tumor-associated antigens: Tn antigen, sTn antigen, and T antigen', *HLA*, 88(6), pp. 275–286. doi: 10.1111/tan.12900.

Fujiwara, S. *et al.* (2007) 'Silencing hypoxia-inducible factor-1alpha inhibits cell migration and invasion under hypoxic environment in malignant gliomas.', *International journal of oncology*, 30(4), pp. 793–802. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17332917 (Accessed: 3 December 2018).

Fuster, M. M. and Esko, J. D. (2005) 'The sweet and sour of cancer: glycans as novel therapeutic targets', *Nature Reviews Cancer*, 5(7), pp. 526–542. doi: 10.1038/nrc1649.

Galban, S. *et al.* (2008) 'RNA-Binding Proteins HuR and PTB Promote the Translation of Hypoxia-Inducible Factor 1', *Molecular and Cellular Biology*, 28(1), pp. 93–107. doi: 10.1128/MCB.00973-07.

Galbán, S. and Gorospe, M. (2009) 'Factors interacting with HIF-1alpha mRNA: novel therapeutic targets.', *Current pharmaceutical design*. NIH Public Access, 15(33), pp. 3853–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19671045 (Accessed: 13 August 2018).

Generali, D. *et al.* (2006) 'Hypoxia-inducible factor-1alpha expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer.', *Clinical cancer research : an official journal of the American Association for Cancer Research*. American Association for Cancer Research, 12(15), pp. 4562–8. doi: 10.1158/1078-0432.CCR-05-2690.

Gerken, T. A. *et al.* (2002) 'Mucin core O-glycosylation is modulated by neighboring residue glycosylation status. Kinetic modeling of the site-specific glycosylation of the apo-porcine submaxillary mucin tandem repeat by UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases T1 and T2.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 277(51), pp. 49850–62. doi: 10.1074/jbc.M205851200.

Gilkes, D. M. and Semenza, G. L. (2013) 'Role of hypoxia-inducible factors in breast cancer metastasis', *Future Oncology*, 9(11), pp. 1623–1636. doi:

10.2217/fon.13.92.

Goda, N., Dozier, S. J. and Johnson, R. S. (2003) ‘HIF-1 in Cell Cycle Regulation, Apoptosis, and Tumor Progression’, *Antioxidants & Redox Signaling*, 5(4), pp. 467–473. doi: 10.1089/152308603768295212.

Goldstein, I. J., Winter, H. C. and Poretz, R. D. (1997) ‘Plant lectins: tools for the study of complex carbohydrates’, *New Comprehensive Biochemistry*. Elsevier, 29, pp. 403–474. doi: 10.1016/S0167-7306(08)60625-0.

Gonçalves, H. *et al.* (2018) ‘Survival Study of Triple-Negative and Non-Triple-Negative Breast Cancer in a Brazilian Cohort.’, *Clinical Medicine Insights. Oncology*. SAGE Publications, 12, p. 1179554918790563. doi: 10.1177/1179554918790563.

Goode, G. *et al.* (2017) ‘MUC1 facilitates metabolomic reprogramming in triple-negative breast cancer.’, *PloS one*. Public Library of Science, 12(5), p. e0176820. doi: 10.1371/journal.pone.0176820.

Greijer, A. *et al.* (2005) ‘Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1)’, *The Journal of Pathology*. Wiley-Blackwell, 206(3), pp. 291–304. doi: 10.1002/path.1778.

Ten Hagen, K. G., Fritz, T. A. and Tabak, L. A. (2003) ‘All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases’, *Glycobiology*. Oxford University Press, 13(1), p. 1R–16. doi: 10.1093/glycob/cwg007.

Hakomori, S. (2002) ‘Glycosylation defining cancer malignancy: New wine in an old bottle’, *Proceedings of the National Academy of Sciences*, 99(16), pp. 10231–10233. doi: 10.1073/pnas.172380699.

Hakomori, S. and Kannagi, R. (1983) ‘Glycosphingolipids as tumor-associated and differentiation markers.’, *Journal of the National Cancer Institute*, 71(2), pp. 231–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6576183 (Accessed: 27 August 2018).

Hamidian, A. *et al.* (2015) ‘Differential regulation of HIF-1α and HIF-2α in neuroblastoma: Estrogen-related receptor alpha (ERRα) regulates HIF2A transcription and correlates to poor outcome’, *Biochemical and Biophysical Research Communications*. Academic Press, 461(3), pp. 560–567. doi: 10.1016/J.BBRC.2015.04.083.

Hanahan, D. and Weinberg, R. A. (2011) ‘Hallmarks of cancer: the next generation.’, *Cell*. Elsevier, 144(5), pp. 646–74. doi: 10.1016/j.cell.2011.02.013.

Helczynska, K. *et al.* (2008) ‘Hypoxia-inducible factor-2alpha correlates to distant recurrence and poor outcome in invasive breast cancer.’, *Cancer research*. American Association for Cancer Research, 68(22), pp. 9212–20. doi: 10.1158/0008-5472.CAN-08-1135.

Hemalatha, A., Suresh, T. and Harendra Kumar, M. (2013) ‘Expression of vimentin in breast carcinoma, its correlation with Ki67 and other histopathological parameters’, *Indian Journal of Cancer*, 50(3), p. 189. doi: 10.4103/0019-509X.118724.

Henze, A.-T. and Acker, T. (2017) ‘Cell Cycle Feedback regulators of hypoxia-inducible factors and their role in cancer biology Feedback regulators of hypoxia-inducible factors and their role in cancer biology’. doi: 10.4161/cc.9.14.12249.

Higel, F. *et al.* (2016) ‘N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins’, *European Journal of Pharmaceutics and Biopharmaceutics*. Elsevier, 100, pp. 94–100. doi: 10.1016/J.EJPB.2016.01.005.

Holm, E. *et al.* (1995) ‘Substrate balances across colonic carcinomas in humans.’, *Cancer research*, 55(6), pp. 1373–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7882338 (Accessed: 15 March 2019).

Holmquist-Mengelbier, L. *et al.* (2006) ‘Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype.’, *Cancer cell*. Elsevier, 10(5), pp. 413–23. doi: 10.1016/j.ccr.2006.08.026.

Hon, J. D. C. *et al.* (2016) ‘Breast cancer molecular subtypes: from TNBC to QNBC.’, *American journal of cancer research*. e-Century Publishing Corporation, 6(9), pp. 1864–1872. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27725895 (Accessed: 3 August 2018).

Hong, S.-S., Lee, H. and Kim, K.-W. (2004) ‘HIF-1α: a Valid Therapeutic Target for Tumor Therapy’, *Cancer Research and Treatment*, 36(6), p. 343. doi: 10.4143/crt.2004.36.6.343.

Hu, C.-J. *et al.* (2003) ‘Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation.’, *Molecular and cellular biology*, 23(24), pp. 9361–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14645546 (Accessed: 18 December 2017).

Hu, C.-J. *et al.* (2007) ‘The N-Terminal Transactivation Domain Confers Target Gene Specificity of Hypoxia-inducible Factors HIF-1α and HIF-2α’, *Molecular Biology of the Cell*. Edited by W. Tansey, 18(11), pp. 4528–4542. doi: 10.1091/mbc.e06-05-0419.

Hu, Z. *et al.* (2006) ‘The molecular portraits of breast tumors are conserved across microarray platforms.’, *BMC genomics*. BioMed Central, 7, p. 96. doi: 10.1186/1471-2164-7-96.

Huang, L. E. *et al.* (1996) ‘Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit.’, *The Journal of biological chemistry*, 271(50), pp. 32253–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8943284 (Accessed: 7 August 2018).

Huanna, T. *et al.* (2015) ‘GALNT14 mediates tumor invasion and migration in breast cancer cell MCF-7’, *Molecular Carcinogenesis*, 54(10), pp. 1159–1171. doi: 10.1002/mc.22186.

Hynes, N. E. (2016) ‘ErbB2: From an EGFR Relative to a Central Target for Cancer Therapy.’, *Cancer research*. American Association for Cancer Research, 76(13), pp. 3659–62. doi: 10.1158/0008-5472.CAN-16-1356.

‘Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy’ (2012) *Trends in Pharmacological Sciences*. Elsevier Current Trends, 33(4), pp. 207–214. doi: 10.1016/J.TIPS.2012.01.005.

Imai, N. *et al.* (1990) ‘Physicochemical and biological characterization of asialoerythropoietin. Suppressive effects of sialic acid in the expression of biological activity of human erythropoietin in vitro.’, *European journal of biochemistry*, 194(2), pp. 457–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2269277 (Accessed: 27 August 2018).

Itakura, Y. *et al.* (2007) ‘Systematic Comparison of Oligosaccharide Specificity of Ricinus communis Agglutinin I and Erythrina Lectins: a Search by Frontal Affinity Chromatography’, *Journal of Biochemistry*. Oxford University Press, 142(4), pp. 459–469. doi: 10.1093/jb/mvm153.

Jin, M.-S. *et al.* (2016) ‘Overexpression of HIF1α and CAXI predicts poor outcome in early-stage triple negative breast cancer’, *Virchows Archiv*, 469(2), pp. 183–190. doi: 10.1007/s00428-016-1953-6.

Jitariu, A.-A. *et al.* (2017) ‘Triple negative breast cancer: the kiss of death.’, *Oncotarget*. Impact Journals, LLC, 8(28), pp. 46652–46662. doi: 10.18632/oncotarget.16938.

Ju, T. *et al.* (2013) ‘Tn and sialyl-Tn antigens, aberrant O-glycomics as human disease markers.’, *Proteomics. Clinical applications*. NIH Public Access, 7(9–10), pp. 618–31. doi: 10.1002/prca.201300024.

Ju, T. *et al.* (2014) ‘The Cosmc connection to the Tn antigen in cancer’, *Cancer Biomarkers*. Edited by P. A. Marino. IOS Press, 14(1), pp. 63–81. doi: 10.3233/CBM-130375.

Juge, N., Tailford, L. and Owen, C. D. (2016) ‘Sialidases from gut bacteria: a mini-review.’, *Biochemical Society transactions*. Portland Press Ltd, 44(1), pp. 166–75. doi: 10.1042/BST20150226.

Jung, K.-H. *et al.* (2018) ‘Targeted therapy of triple negative MDA-MB-468 breast cancer with curcumin delivered by epidermal growth factor-conjugated phospholipid nanoparticles.’, *Oncology letters*. Spandidos Publications, 15(6), pp. 9093–9100. doi: 10.3892/ol.2018.8471.

Kaelin, W. G. and Ratcliffe, P. J. (2008) ‘Oxygen Sensing by Metazoans: The Central Role of the HIF Hydroxylase Pathway’, *Molecular Cell*, 30(4), pp. 393–402. doi: 10.1016/j.molcel.2008.04.009.

Kaluz, S. *et al.* (2009) ‘Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show?’, *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1795(2), pp. 162–172. doi: 10.1016/j.bbcan.2009.01.001.

Kanaya, K. and Kamitani, T. (2003) ‘pVHL-independent ubiquitination of HIF1α and its stabilization by cobalt ion’, *Biochemical and Biophysical Research Communications*. Academic Press, 306(3), pp. 750–755. doi: 10.1016/S0006-291X(03)01041-6.

Kannagi, R. *et al.* (2008) ‘Current relevance of incomplete synthesis and neo-

synthesis for cancer-associated alteration of carbohydrate determinants—Hakomori's concepts revisited', *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1780(3), pp. 525–531. doi: 10.1016/j.bbagen.2007.10.007.

Keith, B., Johnson, R. S. and Simon, M. C. (2011) 'HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression.', *Nature reviews. Cancer*. Howard Hughes Medical Institute, 12(1), pp. 9–22. doi: 10.1038/nrc3183.

Keith, B., Johnson, R. S. and Simon, M. C. (2012) 'HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression', *Nature Reviews Cancer*. Nature Publishing Group, 12(1), pp. 9–22. doi: 10.1038/nrc3183.

Kellokumpu, S., Sormunen, R. and Kellokumpu, I. (2002) 'Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH.', *FEBS letters*, 516(1–3), pp. 217–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11959136 (Accessed: 27 August 2018).

Kim, J.-Y. and Lee, J.-Y. (2017) 'Targeting Tumor Adaption to Chronic Hypoxia: Implications for Drug Resistance, and How It Can Be Overcome.', *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 18(9). doi: 10.3390/ijms18091854.

Kim, Y. J. and Varki, A. (1997) 'Perspectives on the significance of altered glycosylation of glycoproteins in cancer.', *Glycoconjugate journal*, 14(5), pp. 569–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9298689 (Accessed: 16 March 2019).

Koh, M. Y. *et al.* (2011) 'The Hypoxia-Associated Factor Switches Cells from HIF-1 - to HIF-2 -Dependent Signaling Promoting Stem Cell Characteristics, Aggressive Tumor Growth and Invasion', *Cancer Research*, 71(11), pp. 4015–4027. doi: 10.1158/0008-5472.CAN-10-4142.

Koh, M. Y. and Powis, G. (2009) 'HAF: The new player in oxygen-independent HIF-1α degradation', *Cell Cycle*, 8(9), pp. 1359–1366. doi: 10.4161/cc.8.9.8303.

Koh, M. Y. and Powis, G. (2012) 'Passing the baton: the HIF switch.', *Trends in biochemical sciences*. Elsevier, 37(9), pp. 364–72. doi: 10.1016/j.tibs.2012.06.004.

Koh, M. Y., Spivak-Kroizman, T. R. and Powis, G. (2010) 'HIF-1α and Cancer Therapy', in. Springer, Berlin, Heidelberg, pp. 15–34. doi: 10.1007/978-3-540-78281-0_3.

Kölbl, A. C., Andergassen, U. and Jeschke, U. (2015) 'The Role of Glycosylation in Breast Cancer Metastasis and Cancer Control.', *Frontiers in oncology*. Frontiers Media SA, 5, p. 219. doi: 10.3389/fonc.2015.00219.

Kornfeld, K., Reitman, M. L. and Kornfeld, R. (1981) 'The carbohydrate-binding specificity of pea and lentil lectins. Fucose is an important determinant.', *The Journal of biological chemistry*, 256(13), pp. 6633–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7240233 (Accessed: 2 July 2018).

Kuijper, A. *et al.* (2005) 'Expression of hypoxia-inducible factor 1 alpha and its downstream targets in fibroepithelial tumors of the breast', *Breast Cancer Research*. BioMed Central, 7(5), p. R808. doi: 10.1186/bcr1296.

Kuno, A. *et al.* (2005) 'Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling', *Nature Methods*, 2(11), pp. 851–856. doi: 10.1038/nmeth803.

Laemmli, U. K. (1970) 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', *Nature*, 227(5259), pp. 680–685. doi: 10.1038/227680a0.

Lanning, N. J. *et al.* (2017) 'Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities.', *Cancer & metabolism*. BioMed Central, 5, p. 6. doi: 10.1186/s40170-017-0168-x.

Lechner, J. and Wieland, F. (1989) 'Structure and Biosynthesis of Prokaryotic Glycoproteins', *Annual Review of Biochemistry*, 58(1), pp. 173–194. doi: 10.1146/annurev.bi.58.070189.001133.

Legler, K. *et al.* (2018) 'Reduced mannosidase MAN1A1 expression leads to aberrant N-glycosylation and impaired survival in breast cancer', *British Journal of Cancer*, 118(6), pp. 847–856. doi: 10.1038/bjc.2017.472.

Lehmann, B. D. *et al.* (2011) 'Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 121(7), pp. 2750–67. doi: 10.1172/JCI45014.

Lester, R. D. *et al.* (2005) 'Erythropoietin Promotes MCF-7 Breast Cancer Cell Migration by an ERK/Mitogen-activated Protein Kinase-dependent Pathway and Is Primarily Responsible for the Increase in Migration Observed in Hypoxia *'. JBC Papers in Press. doi: 10.1074/jbc.M509446200.

Li, B.-X. *et al.* (2011) 'Effects of RNA interference-mediated gene silencing of JMJD2A on human breast cancer cell line MDA-MB-231 in vitro.', *Journal of experimental & clinical cancer research : CR*. BioMed Central, 30(1), p. 90. doi: 10.1186/1756-9966-30-90.

Li, J. *et al.* (2006) 'Altered metabolic responses to intermittent hypoxia in mice with partial deficiency of hypoxia-inducible factor-1α', *Physiological Genomics*, 25(3), pp. 450–457. doi: 10.1152/physiolgenomics.00293.2005.

Liberti, M. V. and Locasale, J. W. (2016) 'The Warburg Effect: How Does it Benefit Cancer Cells?', *Trends in Biochemical Sciences*. Elsevier, 41(3), pp. 211–218. doi: 10.1016/j.tibs.2015.12.001.

Liebminger, E. *et al.* (2011) 'Beta-N-acetylhexosaminidases HEXO1 and HEXO3 are responsible for the formation of paucimannosidic N-glycans in Arabidopsis thaliana.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 286(12), pp. 10793–802. doi: 10.1074/jbc.M110.178020.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method', *Methods*, 25(4), pp. 402–408. doi: 10.1006/meth.2001.1262.

Loboda, A., Jozkowicz, A. and Dulak, J. (2010) 'HIF-1 and HIF-2 transcription factors — Similar but not identical', *Molecules and Cells*, 29(5), pp. 435–442.

doi: 10.1007/s10059-010-0067-2.

López-Barneo, J., Pardal, R. and Ortega-Sáenz, P. (2001) 'Cellular Mechanism of Oxygen Sensing', *Annual Review of Physiology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 63(1), pp. 259–287. doi: 10.1146/annurev.physiol.63.1.259.

Lu, X. and Kang, Y. (2010) 'Hypoxia and Hypoxia-Inducible Factors: Master Regulators of Metastasis', *Clinical Cancer Research*, 16(24), pp. 5928–5935. doi: 10.1158/1078-0432.CCR-10-1360.

Ma, X. *et al.* (2016) 'Functional roles of sialylation in breast cancer progression through miR-26a/26b targeting ST8SIA4', *Cell Death & Disease*. Nature Publishing Group, 7(12), pp. e2561–e2561. doi: 10.1038/cddis.2016.427.

Martín-Aragón Baudel, M. A. S. *et al.* (2017) 'Preferential activation of HIF-2α adaptive signalling in neuronal-like cells in response to acute hypoxia', *PLOS ONE*. Edited by G. Simos. Public Library of Science, 12(10), p. e0185664. doi: 10.1371/journal.pone.0185664.

Marxsen, J. H. *et al.* (2004) 'Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases.', *The Biochemical journal*. Portland Press Ltd, 381(Pt 3), pp. 761–7. doi: 10.1042/BJ20040620.

Masoud, G. N. and Li, W. (2015) 'HIF-1α pathway: role, regulation and intervention for cancer therapy', *Acta Pharmaceutica Sinica B*. Elsevier, 5(5), pp. 378–389. doi: 10.1016/J.APSB.2015.05.007.

Maxwell, P. H. *et al.* (1999) 'The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis', *Nature*, 399(6733), pp. 271–275. doi: 10.1038/20459.

Maytin, E. V (2016) 'Hyaluronan: More than just a wrinkle filler', *Glycobiology*, 26(6), pp. 553–559. doi: 10.1093/glycob/cww033.

Menrad, H. *et al.* (2010) 'Roles of hypoxia-inducible factor-1α (HIF-1α) versus HIF-2α in the survival of hepatocellular tumor spheroids', *Hepatology*. Wiley-Blackwell, 51(6), pp. 2183–2192. doi: 10.1002/hep.23597.

Mérant, C. *et al.* (2005) 'PNA-binding glycans are expressed at high levels on horse mature and immature T lymphocytes and a subpopulation of B lymphocytes', *Glycoconjugate Journal*, 22(1–2), pp. 27–34. doi: 10.1007/s10719-005-0228-2.

Messner, P. (1997) 'Bacterial glycoproteins', *Glycoconjugate Journal*. Kluwer Academic Publishers, 14(1), pp. 3–11. doi: 10.1023/A:1018551228663.

Miles, D. W. *et al.* (1994) 'Expression of sialyl-Tn predicts the effect of adjuvant chemotherapy in node-positive breast cancer.', *British journal of cancer*, 70(6), pp. 1272–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7981088 (Accessed: 25 November 2018).

Mole, D. R. *et al.* (2009) 'Genome-wide Association of Hypoxia-inducible Factor (HIF)-1α and HIF-2α DNA Binding with Expression Profiling of Hypoxia-inducible Transcripts', *Journal of Biological Chemistry*, 284(25), pp. 16767–

16775. doi: 10.1074/jbc.M901790200.

Montoya, J. J. and Azorsa, D. O. (2016) 'Optimization of Transfection Conditions for siRNA Screening', in, pp. 15–24. doi: 10.1007/978-1-4939-6337-9_2.

Moriwaki, K. and Miyoshi, E. (2010) 'Fucosylation and gastrointestinal cancer.', *World journal of hepatology*. Baishideng Publishing Group Inc, 2(4), pp. 151–61. doi: 10.4254/wjh.v2.i4.151.

Mulloy, B., Hart, G. W. and Stanley, P. (2009) *Structural Analysis of Glycans*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301234 (Accessed: 8 August 2018).

Munkley, J. *et al.* (2016) 'Glycosylation is an Androgen-Regulated Process Essential for Prostate Cancer Cell Viability ☆'. doi: 10.1016/j.ebiom.2016.04.018.

Munkley, J. and Elliott, D. J. (2016) 'Hallmarks of glycosylation in cancer.', *Oncotarget*. Impact Journals, LLC, 7(23), pp. 35478–89. doi: 10.18632/oncotarget.8155.

Muz, B. *et al.* (2015) 'The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy.', *Hypoxia (Auckland, N.Z.)*. Dove Press, 3, pp. 83–92. doi: 10.2147/HP.S93413.

Nagae, M. *et al.* (2014) 'Phytohemagglutinin from Phaseolus vulgaris (PHA-E) displays a novel glycan recognition mode using a common legume lectin fold', *Glycobiology*, 24(4), pp. 368–378. doi: 10.1093/glycob/cwu004.

Nagasaki, M. *et al.* (2016) 'Chemical Synthesis of a Complex-Type *N* -Glycan Containing a Core Fucose', *The Journal of Organic Chemistry*, 81(22), pp. 10600–10616. doi: 10.1021/acs.joc.6b02106.

Nath, S. and Mukherjee, P. (2014) 'MUC1: a multifaceted oncoprotein with a key role in cancer progression.', *Trends in molecular medicine*. Elsevier, 20(6), pp. 332–42. doi: 10.1016/j.molmed.2014.02.007.

Neri, D. and Supuran, C. T. (2011) 'Interfering with pH regulation in tumours as a therapeutic strategy', *Nature Reviews Drug Discovery*, 10(10), pp. 767–777. doi: 10.1038/nrd3554.

Nonaka, M. and Fukuda, M. (2016) 'Expression and Function of Poly-N-Acetyllactosamine Type Glycans in Cancer', in *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*. Tokyo: Springer Japan, pp. 141–161. doi: 10.1007/978-4-431-55939-9_9.

Ogino, M. *et al.* (1999) 'N-acetylgalactosamine (GalNAc)-specific lectins mediate enhancement of Hantaan virus infection.', *Archives of virology*, 144(9), pp. 1765–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10542025 (Accessed: 28 June 2018).

Ogura, A. *et al.* (2016) 'Visualizing Trimming Dependence of Biodistribution and Kinetics with Homo- and Heterogeneous N-Glycoclusters on Fluorescent Albumin.', *Scientific reports*. Nature Publishing Group, 6, p. 21797. doi:

10.1038/srep21797.

Ohh, M. *et al.* (2000) ‘Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel–Lindau protein’, *Nature Cell Biology*, 2(7), pp. 423–427. doi: 10.1038/35017054.

Olivari, S. and Molinari, M. (2007) ‘Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins’, *FEBS Letters*. No longer published by Elsevier, 581(19), pp. 3658–3664. doi: 10.1016/J.FEBSLET.2007.04.070.

Ovcaricek, T. *et al.* (2011) ‘Triple negative breast cancer - prognostic factors and survival.’, *Radiology and oncology*. De Gruyter Open, 45(1), pp. 46–52. doi: 10.2478/v10019-010-0054-4.

Park, J.-H. *et al.* (2010) ‘Critical Roles of Mucin 1 Glycosylation by Transactivated Polypeptide N-Acetylgalactosaminyltransferase 6 in Mammary Carcinogenesis’, *Cancer Research*. American Association for Cancer Research, 70(7), pp. 2759–2769. doi: 10.1158/0008-5472.CAN-09-3911.

Park, J. H., Ahn, J.-H. and Kim, S.-B. (2018) ‘How shall we treat early triple-negative breast cancer (TNBC): from the current standard to upcoming immuno-molecular strategies’, *ESMO Open*, 3(Suppl 1), p. e000357. doi: 10.1136/esmoopen-2018-000357.

Pastorekova, S., Parkkila, S. and Zavada, J. (2006) ‘Tumor-associated carbonic anhydrases and their clinical significance.’, *Advances in clinical chemistry*, 42, pp. 167–216. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17131627 (Accessed: 19 January 2019).

Pawlus, M. R. *et al.* (2012) ‘Upstream stimulatory factor 2 and hypoxia-inducible factor 2α (HIF2α) cooperatively activate HIF2 target genes during hypoxia.’, *Molecular and cellular biology*. American Society for Microbiology (ASM), 32(22), pp. 4595–610. doi: 10.1128/MCB.00724-12.

Pawlus, M. R., Wang, L. and Hu, C.-J. (2014) ‘STAT3 and HIF1α cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells.’, *Oncogene*. NIH Public Access, 33(13), pp. 1670–9. doi: 10.1038/onc.2013.115.

Pinho, S. S. and Reis, C. A. (2015) ‘Glycosylation in cancer: mechanisms and clinical implications’, *Nature Reviews Cancer*, 15(9), pp. 540–555. doi: 10.1038/nrc3982.

Piret, J.-P. *et al.* (2002) ‘CoCl 2 , a Chemical Inducer of Hypoxia-Inducible Factor-1, and Hypoxia Reduce Apoptotic Cell Death in Hepatoma Cell Line HepG2’, *Annals of the New York Academy of Sciences*, 973(1), pp. 443–447. doi: 10.1111/j.1749-6632.2002.tb04680.x.

Polyak, K. (2011) ‘Heterogeneity in breast cancer.’, *The Journal of clinical investigation*. American Society for Clinical Investigation, 121(10), pp. 3786–8. doi: 10.1172/JCI60534.

Potapenko, I. O. *et al.* (2010) ‘Glycan gene expression signatures in normal and malignant breast tissue; possible role in diagnosis and progression’, *Molecular Oncology*. No longer published by Elsevier, 4(2), pp. 98–118. doi:

10.1016/J.MOLONC.2009.12.001.

Pratt, M. R. *et al.* (2004) 'Deconvoluting the Functions of Polypeptide N-α-Acetylgalactosaminyltransferase Family Members by Glycopeptide Substrate Profiling', *Chemistry & Biology*. Cell Press, 11(7), pp. 1009–1016. doi: 10.1016/J.CHEMBIOL.2004.05.009.

Prokop, O. and Uhlenbruck, G. (1969) '[N-acetyl-D-galactosamine in tumor cell membranes: demonstration by means of Helix agglutinins].', *Die Medizinische Welt*, 46, pp. 2515–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4195639 (Accessed: 24 August 2018).

Rankin, E. B. *et al.* (2007) 'Hypoxia-inducible factor–2 (HIF-2) regulates hepatic erythropoietin in vivo', *Journal of Clinical Investigation*, 117(4), pp. 1068–1077. doi: 10.1172/JCI30117.

Ratcliffe, P. J. (2007) 'HIF-1 and HIF-2: working alone or together in hypoxia?', *The Journal of clinical investigation*. American Society for Clinical Investigation, 117(4), pp. 862–5. doi: 10.1172/JCI31750.

Reilly, R. (2007) 'Breast Cancer', *xPharm: The Comprehensive Pharmacology Reference*. Elsevier, pp. 1–9. doi: 10.1016/B978-008055232-3.60809-8.

Reitsma, S. *et al.* (2007) 'The endothelial glycocalyx: composition, functions, and visualization.', *Pflugers Archiv : European journal of physiology*. Springer, 454(3), pp. 345–59. doi: 10.1007/s00424-007-0212-8.

Rosenberger, C. (2002) 'Expression of Hypoxia-Inducible Factor-1 and -2 in Hypoxic and Ischemic Rat Kidneys', *Journal of the American Society of Nephrology*, 13(7), pp. 1721–1732. doi: 10.1097/01.ASN.0000017223.49823.2A.

Rosenfeldt, M. T. *et al.* (2012) 'Analysis of macroautophagy by immunohistochemistry.', *Autophagy*. Taylor & Francis, 8(6), pp. 963–9. doi: 10.4161/auto.20186.

Rubin, H. (1997) 'Cell aging in vivo and in vitro', *Mechanisms of Ageing and Development*. Elsevier, 98(1), pp. 1–35. doi: 10.1016/S0047-6374(97)00067-5.

Samuel, J. and Franklin, C. (2008) 'Hypoxemia and Hypoxia', in *Common Surgical Diseases*. New York, NY: Springer New York, pp. 391–394. doi: 10.1007/978-0-387-75246-4_97.

San-Millán, I. and Brooks, G. A. (2016) 'Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect', *Carcinogenesis*. Oxford University Press, 38(2), p. bgw127. doi: 10.1093/carcin/bgw127.

Scortegagna, M. *et al.* (2005) 'HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner.', *Blood*. American Society of Hematology, 105(8), pp. 3133–40. doi: 10.1182/blood-2004-05-1695.

Seeber, L. M. S. *et al.* (2010) 'Hypoxia-inducible factor-1 as a therapeutic target in endometrial cancer management.', *Obstetrics and gynecology international*. Hindawi Limited, 2010, p. 580971. doi: 10.1155/2010/580971.

Semenza, G. L. (2000a) ‘HIF-1 and human disease: one highly involved factor’, *Genes & Development*. Cold Spring Harbor Laboratory Press, 14(16), pp. 1983–1991. doi: 10.1101/GAD.14.16.1983.

Semenza, G. L. (2000b) ‘Hypoxia, Clonal Selection, and the Role of HIF-1 in Tumor Progression’, *Critical Reviews in Biochemistry and Molecular Biology*, 35(2), pp. 71–103. doi: 10.1080/10409230091169186.

Semenza, G. L. (2001) ‘HIF-1 and mechanisms of hypoxia sensing’, *Current Opinion in Cell Biology*. Elsevier Current Trends, 13(2), pp. 167–171. doi: 10.1016/S0955-0674(00)00194-0.

Semenza, G. L. (2010) ‘Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics’, *Oncogene*, 29(5), pp. 625–634. doi: 10.1038/onc.2009.441.

Semenza, G. L. (2012) ‘Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy’, *Trends in Pharmacological Sciences*, 33, pp. 207–214. doi: 10.1016/j.tips.2012.01.005.

Semenza, G. L. (2013) ‘HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations.’, *The Journal of clinical investigation*. American Society for Clinical Investigation, 123(9), pp. 3664–71. doi: 10.1172/JCI67230.

Semenza, G. L. (2016) ‘The hypoxic tumor microenvironment: A driving force for breast cancer progression.’, *Biochimica et biophysica acta*. NIH Public Access, 1863(3), pp. 382–391. doi: 10.1016/j.bbamcr.2015.05.036.

Semenza, G. L. (2016) ‘The hypoxic tumor microenvironment: A driving force for breast cancer progression’, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1863(3), pp. 382–391. doi: 10.1016/j.bbamcr.2015.05.036.

Semenza, G. L. and Wang, G. L. (1992) ‘A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.’, *Molecular and cellular biology*, 12(12), pp. 5447–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1448077 (Accessed: 19 January 2019).

Semizarov, D. *et al.* (2003) ‘Specificity of short interfering RNA determined through gene expression signatures’, *Proceedings of the National Academy of Sciences*, 100(11), pp. 6347–6352. doi: 10.1073/pnas.1131959100.

Sgarbi, G. *et al.* (2018) ‘Hypoxia and IF_1 Expression Promote ROS Decrease in Cancer Cells.’, *Cells*. Multidisciplinary Digital Publishing Institute (MDPI), 7(7). doi: 10.3390/cells7070064.

Shao, F., Sun, H. and Deng, C.-X. (2017) ‘Potential therapeutic targets of triple-negative breast cancer based on its intrinsic subtype.’, *Oncotarget*. Impact Journals, LLC, 8(42), pp. 73329–73344. doi: 10.18632/oncotarget.20274.

Shibuya, N. *et al.* (1987) ‘Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry (Sambucus nigra L.) bark lectin.’, *Archives of biochemistry and biophysics*, 254(1), pp. 1–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3579290 (Accessed: 22

October 2018).

Shirato, K. *et al.* (2011) 'Hypoxic regulation of glycosylation via the N-acetylglucosamine cycle.', *Journal of clinical biochemistry and nutrition*. The Society for Free Radical Research Japan, 48(1), pp. 20–5. doi: 10.3164/jcbn.11-015FR.

Shweiki, D. *et al.* (1992) 'Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis', *Nature*, 359(6398), pp. 843–845. doi: 10.1038/359843a0.

Solá, R. J. and Griebenow, K. (2009) 'Effects of glycosylation on the stability of protein pharmaceuticals', *Journal of Pharmaceutical Sciences*, 98(4), pp. 1223–1245. doi: 10.1002/jps.21504.

Soleymani Abyaneh, H. *et al.* (2017) 'STAT3 but Not HIF-1α Is Important in Mediating Hypoxia-Induced Chemoresistance in MDA-MB-231, a Triple Negative Breast Cancer Cell Line', *Cancers*, 9(12), p. 137. doi: 10.3390/cancers9100137.

Song, E. Y. *et al.* (2001) 'Expression of bisecting N-acetylglucosaminyltransferase-III in human hepatocarcinoma tissues, fetal liver tissues, and hepatoma cell lines of Hep3B and HepG2.', *Cancer investigation*, 19(8), pp. 799–807. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11768033 (Accessed: 1 October 2018).

Sørlie, T. *et al.* (2001) 'Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(19), pp. 10869–74. doi: 10.1073/pnas.191367098.

Springer, G. F. (1984) 'T and Tn, general carcinoma autoantigens.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 224(4654), pp. 1198–206. doi: 10.1126/SCIENCE.6729450.

Stanley, P. and Cummings, R. D. (2015) *Structures Common to Different Glycans*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. doi: 10.1101/GLYCOBIOLOGY.3E.014.

Stanley, P., Schachter, H. and Taniguchi, N. (2009) *N-Glycans*, *Essentials of Glycobiology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301244 (Accessed: 1 August 2018).

Stanley, P., Taniguchi, N. and Aebi, M. (2015) *N-Glycans*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. doi: 10.1101/GLYCOBIOLOGY.3E.009.

Stingl, J. and Caldas, C. (2007) 'Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis', *Nature Reviews Cancer*, 7(10), pp. 791–799. doi: 10.1038/nrc2212.

Stowell, S. R., Ju, T. and Cummings, R. D. (2015) 'Protein glycosylation in cancer.', *Annual review of pathology*. NIH Public Access, 10, pp. 473–510. doi: 10.1146/annurev-pathol-012414-040438.

Strasser, R. *et al.* (1999) ‘Molecular cloning and characterization of cDNA coding for beta1, 2N-acetylglucosaminyltransferase I (GlcNAc-TI) from Nicotiana tabacum.’, *Glycobiology*, 9(8), pp. 779–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10406843 (Accessed: 25 August 2018).

Strasser, R. *et al.* (2007) ‘A unique beta1,3-galactosyltransferase is indispensable for the biosynthesis of N-glycans containing Lewis a structures in Arabidopsis thaliana.’, *The Plant cell*. American Society of Plant Biologists, 19(7), pp. 2278–92. doi: 10.1105/tpc.107.052985.

Tabas, I., Schlesinger, S. and Kornfeld, S. (1978) ‘Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain.’, *The Journal of biological chemistry*, 253(3), pp. 716–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/202596 (Accessed: 27 August 2018).

Takasaki, N. *et al.* (2014) ‘A heterozygous mutation of GALNTL5 affects male infertility with impairment of sperm motility.’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(3), pp. 1120–5. doi: 10.1073/pnas.1310777111.

Taniguchi, N. (2007) ‘A sugar-coated switch for cellular growth and arrest’, *Nature Chemical Biology*, 3(6), pp. 307–309. doi: 10.1038/nchembio0607-307.

Tannock, I. F. (2001) ‘Tumor Physiology and Drug Resistance’, *Cancer and Metastasis Reviews*. Kluwer Academic Publishers, 20(1/2), pp. 123–132. doi: 10.1023/A:1013125027697.

Tateno, H., Nakamura-Tsuruta, S. and Hirabayashi, J. (2009) ‘Comparative analysis of core-fucose-binding lectins from Lens culinaris and Pisum sativum using frontal affinity chromatography’, *Glycobiology*, 19(5), pp. 527–536. doi: 10.1093/glycob/cwp016.

‘The hypoxic tumor microenvironment: A driving force for breast cancer progression’ (2016) *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. Elsevier, 1863(3), pp. 382–391. doi: 10.1016/J.BBAMCR.2015.05.036.

Thomlinson, R. H. and Gray, L. H. (1955) ‘The histological structure of some human lung cancers and the possible implications for radiotherapy.’, *British journal of cancer*, 9(4), pp. 539–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13304213 (Accessed: 14 March 2019).

Tian, E. and Ten Hagen, K. G. (2009) ‘Recent insights into the biological roles of mucin-type O-glycosylation.’, *Glycoconjugate journal*. NIH Public Access, 26(3), pp. 325–34. doi: 10.1007/s10719-008-9162-4.

Tomita, U. *et al.* (2012) ‘Poly(ethylene glycol)-Lipid-Conjugated Antibodies Enhance Dendritic Cell Phagocytosis of Apoptotic Cancer Cells.’, *Pharmaceuticals (Basel, Switzerland)*. Multidisciplinary Digital Publishing Institute (MDPI), 5(5), pp. 405–16. doi: 10.3390/ph5050405.

Triple negative breast cancer | Cancer Research UK (2017). Available at: http://www.cancerresearchuk.org/about-cancer/breast-cancer/stages-types-grades/types/triple-negative-breast-cancer (Accessed: 21 November 2017).

Trivers, K. F. *et al.* (2009) 'The epidemiology of triple-negative breast cancer, including race', *Cancer Causes & Control*, 20(7), pp. 1071–1082. doi: 10.1007/s10552-009-9331-1.

Tu, C.-F. *et al.* (2017) 'FUT8 promotes breast cancer cell invasiveness by remodeling TGF-β receptor core fucosylation.', *Breast cancer research : BCR*. BioMed Central, 19(1), p. 111. doi: 10.1186/s13058-017-0904-8.

Tzur, A. *et al.* (2011) 'Optimizing optical flow cytometry for cell volume-based sorting and analysis.', *PloS one*. Public Library of Science, 6(1), p. e16053. doi: 10.1371/journal.pone.0016053.

Uchida, T. *et al.* (2004) 'Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(15), pp. 14871–8. doi: 10.1074/jbc.M400461200.

Untersmayr, E. *et al.* (2010) 'The High Affinity IgE Receptor FcεRI Is Expressed by Human Intestinal Epithelial Cells', *PLoS ONE*. Edited by S. Bereswill. Public Library of Science, 5(2), p. e9023. doi: 10.1371/journal.pone.0009023.

Varki, A. (2009) *Essentials of glycobiology*. Cold Spring Harbor Laboratory Press.

Varki, A. *et al.* (2015) *Glycosylation Changes in Cancer*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. doi: 10.1101/GLYCOBIOLOGY.3E.047.

Varki, A., Freeze, H. H. and Manzi, A. E. (2009) 'Overview of glycoconjugate analysis.', *Current protocols in protein science*. NIH Public Access, Chapter 12, p. Unit 12.1 12.1.1-8. doi: 10.1002/0471140864.ps1201s57.

Varki, A. and Lowe, J. B. (2009) *Biological Roles of Glycans*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301233 (Accessed: 12 March 2019).

Varki, A. and Sharon, N. (2009) *Historical Background and Overview*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301255 (Accessed: 23 August 2018).

Vaupel, P. (2008) 'Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis.', *The oncologist*. AlphaMed Press, 13 Suppl 3(Supplement 3), pp. 21–6. doi: 10.1634/theoncologist.13-S3-21.

Vaupel, P., Höckel, M. and Mayer, A. (2007) 'Detection and Characterization of Tumor Hypoxia Using pO 2 Histography', *Antioxidants & Redox Signaling*, 9(8), pp. 1221–1236. doi: 10.1089/ars.2007.1628.

Venkitachalam, S. *et al.* (2016) 'Biochemical and functional characterization of glycosylation-associated mutational landscapes in colon cancer.', *Scientific reports*. Nature Publishing Group, 6, p. 23642. doi: 10.1038/srep23642.

Wang, C.-C. *et al.* (2009) 'Glycans on influenza hemagglutinin affect receptor binding and immune response', *Proceedings of the National Academy of*

Sciences, 106(43), pp. 18137–18142. doi: 10.1073/pnas.0909696106.

Wang, W. C. and Cummings, R. D. (1988) 'The immobilized leukoagglutinin from the seeds of Maackia amurensis binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked alpha-2,3 to penultimate galactose residues.', *The Journal of biological chemistry*, 263(10), pp. 4576–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3350806 (Accessed: 22 October 2018).

Wang, X. *et al.* (2005) 'From The Cover: Dysregulation of TGF- 1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice', *Proceedings of the National Academy of Sciences*, 102(44), pp. 15791–15796. doi: 10.1073/pnas.0507375102.

Wang, Y. *et al.* (2011) 'Specificities of *Ricinus communis* agglutinin 120 interaction with sulfated galactose', *FEBS Letters*, 585(24), pp. 3927–3934. doi: 10.1016/j.febslet.2011.10.035.

Warburg, O. *et al.* (1956) 'On the origin of cancer cells.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 123(3191), pp. 309–14. doi: 10.1126/science.123.3191.309.

Warburg, O., Wind, F. and Negelein, E. (1927) 'THE METABOLISM OF TUMORS IN THE BODY.', *The Journal of general physiology*, 8(6), pp. 519–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19872213 (Accessed: 15 March 2019).

Warnecke, C. *et al.* (2004) 'Differentiating the functional role of hypoxia-inducible factor (HIF)-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells', *The FASEB Journal*, 18(12), pp. 1462–1464. doi: 10.1096/fj.04-1640fje.

Welford, S. M. and Giaccia, A. J. (2011) 'Hypoxia and senescence: the impact of oxygenation on tumor suppression.', *Molecular cancer research : MCR*. NIH Public Access, 9(5), pp. 538–44. doi: 10.1158/1541-7786.MCR-11-0065.

WENGER, R. H. (2002) 'Cellular adaptation to hypoxia: O_2 -sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2 -regulated gene expression', *The FASEB Journal*, 16(10), pp. 1151–1162. doi: 10.1096/fj.01-0944rev.

Wiesener, M. S. *et al.* (2003) 'Widespread hypoxia-inducible expression of HIF-2α in distinct cell populations of different organs', *The FASEB Journal*, 17(2), pp. 271–273. doi: 10.1096/fj.02-0445fje.

Wu, D. and Yotnda, P. (2011) 'Induction and Testing of Hypoxia in Cell Culture', *Journal of Visualized Experiments*, (54). doi: 10.3791/2899.

Wykoff, C. C. *et al.* (2000) 'Hypoxia-inducible expression of tumor-associated carbonic anhydrases.', *Cancer research*, 60(24), pp. 7075–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11156414 (Accessed: 13 December 2018).

Xie, H. *et al.* (2016) 'Increased B4GALT1 expression associates with adverse outcome in patients with non-metastatic clear cell renal cell carcinoma.', *Oncotarget*. Impact Journals, LLC, 7(22), pp. 32723–30. doi:

10.18632/oncotarget.8737.

Yaji, S. *et al.* (2015) ‘Major glycan structure underlying expression of the Lewis X epitope in the developing brain is O-mannose-linked glycans on phosphacan/RPTPβ’, *Glycobiology*, 25(4), pp. 376–385. doi: 10.1093/glycob/cwu118.

Yamashita, K., Tachibana, Y. and Kobata, A. (1978) ‘The structures of the galactose-containing sugar chains of ovalbumin.’, *The Journal of biological chemistry*, 253(11), pp. 3862–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/649611 (Accessed: 27 August 2018).

Yamashita, Y. *et al.* (1995) ‘Alterations in Gastric Mucin With Malignant Transformation: Novel Pathway for Mucin Synthesis’, *JNCI: Journal of the National Cancer Institute*. Oxford University Press, 87(6), pp. 441–446. doi: 10.1093/jnci/87.6.441.

Yang, Q. *et al.* (2017) ‘Revisiting the substrate specificity of mammalian α1,6-fucosyltransferase reveals that it catalyzes core fucosylation of *N* -glycans lacking α1,3-arm GlcNAc’, *Journal of Biological Chemistry*, 292(36), pp. 14796–14803. doi: 10.1074/jbc.M117.804070.

Zhang, J. *et al.* (2011) ‘Translational repression of p53 by RNPC1, a p53 target overexpressed in lymphomas’, *Genes & Development*, 25(14), pp. 1528–1543. doi: 10.1101/gad.2069311.

Zhou, R. W. *et al.* (2014) ‘N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca2+ signaling’, *Nature Immunology*, 15(11), pp. 1038–1045. doi: 10.1038/ni.3007.

Zhou, S.-M. *et al.* (2015) ‘Lectin RCA-I specifically binds to metastasis-associated cell surface glycans in triple-negative breast cancer’, *Breast Cancer Research*. BioMed Central, 17(1), p. 36. doi: 10.1186/s13058-015-0544-9.

Zhuo, Y., Chammas, R. and Bellis, S. L. (2008) ‘Sialylation of beta1 integrins blocks cell adhesion to galectin-3 and protects cells against galectin-3-induced apoptosis.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 283(32), pp. 22177–85. doi: 10.1074/jbc.M8000015200.